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ORIGINAL PAPER

Targeting PI3K in neuroblastoma

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Abstract
Purpose This work employs pharmacological targeting of phosphoinositide 3-kinases (PI3K) in selected neuroblastoma (NB) tumors with the inhibitor AS605240, which has been shown to express low toxicity and relative specificity for the PI3K species γ.
Methods The expression pattern of PI3K isoforms in 7 NB cell lines and 14 tumor patient samples was determined by Western blotting and immunocytochemistry. The effect of AS605240 on the growth of four selected tumor cell lines was assessed. Two cell lines exhibiting (SK-N-LO) or lacking (SK-N-AS) PI3Kγ expression were chosen for further in vitro analysis, which involved propidium iodide (PI)-based cell cycle staining, terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL-staining) of apoptotic cells and analysis of PI3K/Akt-related signaling pathways via Western blotting and translocation experiments. The action of AS605240 in vivo was addressed by xenograft experiments in severe combined immunodeficiency (SCID) mice, thereby comparing SK-N-LO and SK-N-AS derived tumors. Apoptosis induced in SK-N-LO tumors was shown by immunohistochemical TUNEL-staining.
Results Significant expression of PI3Kγ in neuroblastoma patient biopsies and tumor cell lines was detected. AS605240 induced apoptosis in NB cell lines proportional to this expression and suppressed growth of PI3Kγ positive, but not negative, tumors in a xenograft mouse model. No adverse effects of the inhibitor treatment were observed.
Conclusions Our observations hint to an oncogenic function of PI3Kγ in distinct neuroblastoma entities and reveal PI3K targeting by AS605240 as a promising molecular therapy of these tumors.

Keywords Neuroblastoma · PI3K · AS605240 · Apoptosis · Tumor · Target

Introduction

Neuroblastoma is a malignant neoplasia of the sympathetic nervous system. The solid tumor occurs mostly in children younger than 2 years but also older children and adults are affected. Malignancies usually arise in the adrenal gland, the sympathetic trunk or ganglia, but are also found in the chest and peritoneum. Neuroblastoma tumors are graded into 4 stages: Stages 1 and 2 are local tumors, which (in stage 2) metastasize to the adjacent lymph nodes. Both have a good prognosis and are treated surgically and chemotherapeutically. Stages 3 and 4 comprise tumors crossing the body's midline and metastasize to distant locations (e.g. lymph nodes, bone, and liver). Here, prognosis is poor with a 5-year survival of only about 30% (Maris et al. 2007).

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Recent investigations of the molecular characteristics of neuroblastoma increasingly reveal potential targets for pharmacological treatment of these tumors. As a significant event during neuroblastoma tumorigenesis mutations of MYCN have been identified. Amplification of this gene in about 15% of neuroblastoma tumors appears to be related to an increased invasive potential and poor clinical outcome (Ambros et al. 2009; Cohn et al. 2009). Promoting the tumorigenic phenotype of neuroblastoma, the transcription factor MYCN seems to cooperate with signaling proteins of the phosphoinositide 3-kinase type 1 family (PI3K). Inhibition of these proteins was shown to destabilize MYCN-protein and to inhibit malignant progression in an in vivo mouse model of neuroblastoma. Additional evidence for the involvement of PI3K in neuroblastoma oncogenic signaling has been provided by comparative investigations of the PI3K target Akt/PKB in primary tumor samples (Opel et al. 2007). This study revealed increased Akt phosphorylation status as a marker for poor prognosis in neuroblastoma. In addition, reversal of IGF-1-induced protection of neuroblastoma cells from apoptosis was observed after admixture of the PI3K inhibitor LY294002.

Pro-oncogenic characteristics of the four PI3K species have been shown in many cellular models, but constitutive stimulatory mutations so far were only demonstrated for the catalytic subunit p110 of the PI3Kα isotype (Horn et al. 2008; Samuels et al. 2004; Zhao and Vogt 2008). Cancer-specific mutations in the other isoforms of class I PI3K have not yet been identified. However, overexpression of wild-type p110β, p110γ or p110δ is sufficient to induce cellular transformation in fibroblasts (Denley et al. 2008). Oncogenic signaling mediated by PI3K in these cells correlates with the activation of the Akt/mTOR pathway and consequent stimulation of S6K, 4-EBP1 and inactivation of GSK3β and FOXO1.

Initial efforts to use PI3K as targets for tumor treatment have been hampered by vital physiological functions of PI3K and serious adverse effects of PI3K-inhibitors. Thus, the pan-PI3K-inhibitor LY294002 mostly used in these studies was shown to express significant toxicity (Billottet et al. 2006; Wetzker and Rommel 2004). To solve this dilemma, ongoing efforts treat selected downstream mediators of PI3K including Akt and mTOR in order to suppress the oncogenic phenotype (Hirsch et al. 2008; Marone et al. 2008).

The present study suggests targeting of PI3K in neuroblastoma with the low-toxicity inhibitor AS605240, which has been developed for the treatment of inflammatory diseases. AS605240 was shown to induce preferential inhibition of PI3Kγ a master mediator of inflammatory processes. Significant expression of the PI3Kγ catalytic subunit could be shown in neuroblastoma patient biopsies and in the tumor cell lines SK-N-LO and SK-N-MC. Treatment of these cell lines with AS605240 restricts cell proliferation and induces apoptosis. The inhibitor suppresses tumor growth of SK-N-LO cells in a xenograft mouse model suggesting therapeutic value of definite PI3K inhibition in selected neuroblastoma entities.

Materials and methods

Permanent cell lines

The human cell lines SK-N-LO, SK-N-AS and SK-N-MC characterized by a neuroblastoma cell type were obtained from the Children's University Hospital Tübingen, Germany. Because of the detection of FLI1-EWS gene fusion in SK-N-MC cells, which is typical for the Ewing's sarcoma family, this cell line is now mostly designated as neuronal epithelial cell line (Dunn et al. 1994). NB-1, NB-9 and NB-19 cells were purchased at the Riken Cell Bank (Tsukuba, Japan). SH-SY5Y cells are from DSMZ collection (Braunschweig, Germany).

SK-N-LO, SK-N-AS, SK-N-MC and SH-SY5Y were cultivated in a 1:1 mixture of HAM'S F12 and IMDM containing 10% FCS. NB-1, NB-9 and NB-19 cells were cultivated according to the Riken Cell Bank database. Cells were grown at 37°C in a humidified incubator with 5% CO_2. Approaching confluence all cell lines were splitted in a ratio of 1:5 to 1:10 one or two times a week using 2 mM EDTA/PBS for cell detachment.

Tumor samples

Tumor specimens were obtained both from 12 solid tumors resected on operations at the Childrens Hospital, Jena and from two patients who presented tumor cell contaminated bone marrows. One patient was diagnosed with stage 1 neuroblastoma, three patients with stage 2, eight patients with stage 4 and two patients with stage 4S. Solid tumor samples were cut into small pieces and passed through a cell strainer with a pore size of 100 µm to obtain a single cell suspension, which was frozen on stock with 5% DMSO/RPMI-medium in liquid nitrogen.

Antibodies

Following antibodies were obtained from Cell Signaling (Danvers, USA): pAkt Thr308 (#9275) and pAkt Ser473 (#4058), panAkt (#9272), pGSK 3α/β (#9331), p-p70S6K Thr389 (#9205), p-p70S6K Thr421/Ser424 (#9204) and pan-p70S6K (#9202).

Antibodies purchased at Millipore (Billerica, USA): PTEN (#04-409), MYCN (#05-424) and p110β (#04-400). Further antibodies were obtained at: Abcam (Cambridge,

UK)—panGSK α/β (ab52179), Epitomics (Burlingame, USA)—p110α (#1683-1) and Santa Cruz (Santa Cruz, USA)—p110δ (sc-7176).

The monoclonal p110γ-antibody H1 was produced in our own facility (Patrucco et al. 2004; Stoyanov et al. 1995).

The p53 antibody was kindly provided by Bernhard Schlott from the Fritz Lipman Institute Jena, Germany.

Secondary, HRP-coupled antibodies were purchased at KPL (Gaithersburg, USA): goat-anti-mouse (#214-1806) and goat-anti-rabbit (#074-1506).

For cytochemical analysis, the rabbit anti-p110γ polyclonal antibody H-199 (#sc-7177) from Santa Cruz Biotechnology (Santa Cruz, USA) and mouse anti-GD2 monoclonal antibody 14G2a (#554272) from BD Biosciences Pharmingen (Franklin Lakes, USA) were utilized. Rabbit anti-IgG (Dako, Glostrup, Denmark) and mouse anti-IgG2a (Sigma, St. Louis, USA) were used as negative isotype controls.

Inhibitors

The PI3Kγ-inhibitor AS605240 was obtained from Alexis (Lausen, Switzerland). LY294002 was purchased from Jena Bioscience (Jena, Germany). Both inhibitors were dissolved in DMSO.

SDS–PAGE and Western blotting

For analysis of signaling effects, cells were seeded into 6-well plates, incubated at 37°C and 5% CO2 and inhibitor treated for 30 min when approaching confluency. Afterward cells were briefly washed with ice-cold PBS containing corresponding amounts of inhibitor and suspended in lysis buffer composed of 20 mM HEPES (pH 7,4), 10 mM EGTA, 40 mM β-glycerophosphate, 2.5 mM $MgCl_2$, 1 mM PMSF, 2 mM orthovanadate, Pepstatin A and Leupeptin (20 µg/ml each) and 1% NP40. For expression studies, cells were harvested as usual, washed twice with ice-cold PBS und suspended in lysis buffer. Samples were then centrifuged at 16.000×g for 15 min at 4°C. Supernatant was mixed with 6× protein sample buffer (6% SDS, 40% glycerol, 30% β-mercaptoethanol, and traces of bromphenol blue) and heated to 95°C for 6 min.

Protein samples were separated on a 10% PAGE-Gel at 120 V, transferred to a PVDF membrane (Millipore, Billerica, USA) and immunoblotted with indicated antibodies followed by Enhanced chemiluminescence reaction (PerkinElmer, Waltham, USA) for visualization according to the manufacturer's instructions.

If necessary, antibodies were stripped using 100 mM β-mercaptoethanol 62.5 mM Tris (pH 6.7) and 2% SDS for 30 min at 50°C.

Immunocytochemical detection of PI3Kγ and GD2

Immunocytochemical analysis was performed following standard methods. Isolated cells were obtained from tumor specimens or tumor cell–contaminated bone marrow. Using a Shandon-cytocentrifuge (Thermo Fisher Scientific, Waltham, USA), 1×10^5 cells per sample were attached to a slide. The slides were fixed in ice-cold methanol and acetone for 1 min, respectively, allowed to drain for some minutes, and stored at −21°C. After thawing, the samples were blocked with blocking solution (Candor Bioscience, Weißensberg, Germany). Then the cells were incubated overnight in the dark at 4°C with 3 µg/ml of rabbit anti-PI3Kγ polyclonal antibody H-199 and 2 µg/ml mouse anti-GD2 monoclonal antibody 14G2a, a recognized marker for NB cells. Rabbit anti-IgG and mouse anti-IgG2a were used as negative isotype controls, respectively.

The reactivities of appropriate antigens were detected with the Dako REAL™ Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse (Dako, Glostrup, Denmark) according to the manufacturer's instructions. The staining pattern of PI3Kγ and GD2 was semiquantitatively graded evaluating the number (0 = 0–3%; 1 = 4–20%; 2 = 21–50%; 3 = 51–75%; 4 = 76–100%) as well as the color intensity (0 = negative; 1 = weakly positive; 2 = moderately positive; 3 = strongly positive) of stained cells in comparison with the control. The values of both categories were multiplied by each other resulting in scores in the graph.

Cell proliferation

For each sample, 1×10^5 cells were seeded into 6-well plates and cultivated over night for adhesion. Afterwards, cells were treated as indicated. After 72 h, cells were detached using 1 ml 2 mM EDTA/PBS and counted using a CASY-1 cell counter.

Cell cycle and flow cytometric TUNEL assay

Analysis of both parameters was performed using the Apo-BrdU Apoptosis Detection Kit from Natutec (Frankfurt, Germany). In this method, 2×10^5 cells were seeded into 6-well plates and left over night for cell adhesion following treatment with indicated concentrations of inhibitors or only DMSO as a dilution control for 36 h. Cells were detached using 2 mM EDTA/PBS, washed twice in PBS and processed following kit instructions. A total of 1×10^4 cells of each sample were analyzed using a BD FACS Canto flow cytometer applying recommended gating strategies.

Animal experiments

All animal experiments were performed using SCID mice (Charles River Wiga, Sulzfeld, Germany) characterized by

lack of functional lymphocytes due to a gene mutation disrupting the differentiation of both B- and T-lymphocytes. These mice have normal numbers of natural killer cells, macrophages and granulocytes. The mice were maintained under sterile and standardized environmental conditions (22 ± 2°C room temperature, 55 ± 5% relative humidity, and 12 h light–dark rhythm) and fed ad libitum with water and food pellets. Animal weight and general appearance were recorded two times per week throughout the experiments. All experiments were approved by the regional ethics committee for animal research according to the German Animal Protection Law. In compliance with such regulations, mice had to be killed when tumors reached a mass >10% of body weight.

Neuroblastoma cells were taken from frozen stocks in liquid nitrogen, which were prepared from routine cell culture and already portioned with required cell number per mouse. After thawing and washing with isotonic NaCl, cells were aspirated with 300 µl NaCl by a syringe. Six weeks old female mice (18–22 g body weight) were implanted subcutaneously (s.c.) with 2×10^7 SK-N-LO cells or 1×10^7 SK-N-AS cells on the right lumbar region of the back. Tumors were measured with a caliper, starting when they were palpable, and tumor volume (cm^3) was calculated as length × width × width × 0.5 (Johnson et al. 2008). Tumors were allowed to grow for 28 days, if they did not reach the critical size to terminate. Mice were killed by cervical dislocation; the tumors were excised, weighted and proceeded for histological analysis.

Administration of the AS605240 inhibitor

Two approaches were used to investigate the influence of the PI3Kγ-inhibitor AS605240 on tumor growth in SCID-mice. For testing the influence of the inhibitor concentration, 2×10^7 tumor cells in 300 µl isotonic NaCl were incubated for 30 min with 100 µl of inhibitor at final concentrations of 5, 10 or 20 µg/g mouse dissolved at last in 5% DMSO/NaCl. Subsequently, this incubation mixture was injected. Reapplication was done on three following days with the corresponding inhibitor concentration, which was placed into the same site of each injection.

Furthermore, tumor growth was determined after retarding the inhibitor application time. Therefore, PI3Kγ-inhibitor (20 µg/g mouse) treatment was commenced one hour (treatment day 0–3) or 5 days after cell inoculation (treatment day 5–8). This treatment was repeated on the three consecutive days, respectively.

Control mice were injected with 100 µl sterile 5% DMSO/NaCl.

Immunohistochemical TUNEL-apoptosis assay

SK-N-LO tumors were grown in SCID mice as described earlier. When palpable, the tumors were treated with 20 µg/g AS605240 or vehicle control on four consecutive days. About 2 h after last application the mice were killed by cervical dislocation, tumors were isolated, fixed in 4% paraformaldehyde at 4°C over night and then kept in 30% sucrose/PBS at 4°C for 7 days. Afterward tumors were freezed in isopentane at −30°C and stored at −80°C.

Tumor cryosections with a diameter of 10 µm were used for apoptotic cell detection with TUNEL-staining by the ApopTag Plus Fluorescin in situ Apoptosis Detection Kit (Millipore, Billerica, USA). Cell nuclei were counterstained using DAPI. For each tumor section, five areas at 40-fold magnification were counted for apoptotic cells. Six sections per tumor were counted. The counted number of apoptotic cells was normalized against the DAPI intensity in each field of view as determined with ImageJ software.

Results

Expression of PI3K in neuroblastoma cell lines and patient biopsies

Initial screening of the expression pattern of PI3K species in different neuroblastoma cell lines revealed pronounced differences in the level of the p110γ catalytic subunit of PI3Kγ. Whereas the tumor cell lines SH-SY5Y, SK-N-AS, NB-1, NB-9 and NB-19 did not disclose considerable signals in Western blotting, SK-N-LO cells exhibit high PI3Kγ expression comparable to the level in the hematopoietic Jurkat cell line (Fig. 1a). In addition, low but significant expression of PI3Kγ was found in SK-N-MC cells. Notably, the distinct levels of PI3Kγ in SK-N-LO and SK-N-MC cells are accompanied by low expression of the PI3K antagonist and tumor suppressor protein PTEN (Fig. 1b). Additional analysis disclosed high expression of the tumor suppressor p53 in SK-N-LO cells. MCYN overexpression was detected in NB-1 and NB-19 cells.

To elucidate the incidence of PI3Kγ expression in human neuroblastoma, tumor samples from patients exhibiting different stages of neuroblastoma have been analyzed. Immunocytochemistry and co-staining of GD2 as an established marker for neuroblastoma cells were used to search for PI3Kγ positive tumor samples. As shown in Fig. 1c, d overall 6 of 14 neuroblastoma specimens (43%) exhibited significant expression level of this PI3K species. Five of these six PI3Kγ positive samples have been obtained from stage 4 or stage 4S neuroblastomas.

These data are supported by oncogenomics data, which reveal differential expression of PI3Kγ in neuroblastoma entities with a tendency of increased expression in patients with poor prognosis (http://pob.abcc.ncifcrf.gov/cgi-bin/JK) (Neuroblastoma Prognosis Database—Seeger Lab).

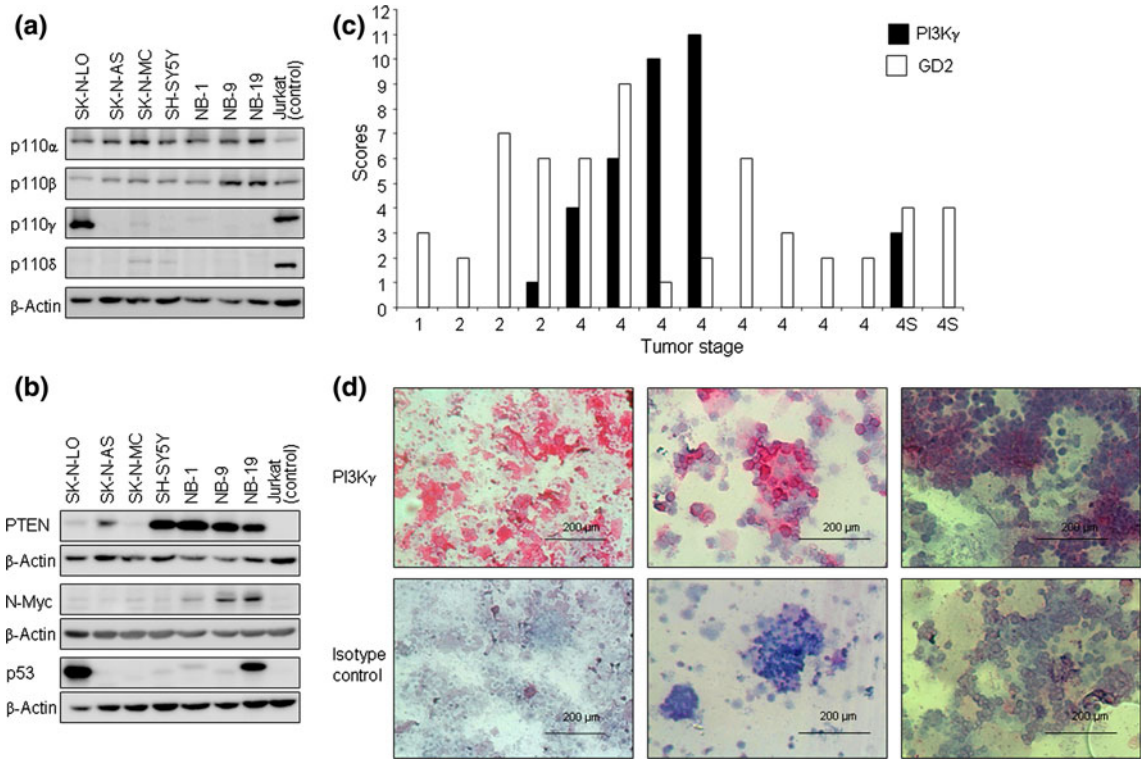

Fig. 1 Expression of PI3K in neuroblastoma. Lysates of different neuroblastoma cell lines containing equal amounts of total protein were analyzed by Western blotting with PI3K isoform–specific antibodies (**a**). Expression pattern of MYCN, PTEN and p53 in neuroblastoma cell lines obtained by Western blotting of cell lysates containing identical amounts of total protein (**b**). Immunocytochemical detection of PI3Kγ and GD2 in neuroblastoma cells derived from 14 patients in comparison with tumor stage. The scoring corresponds to the number of stained cells and the intensity of the staining as shown in Sect. "Materials and methods" (**c**). Representative positive staining (*red*) in neuroblastoma tumor cells of 3 patients after cytospin preparation labeling with the polyclonal rabbit anti-PI3Kγ-antibody H-199 (**d**)

Effects of PI3K inhibition on growth and viability of neuroblastoma cells

Next, we asked for potential contributions of PI3Kγ to the tumorigenic phenotype of four neuroblastoma cell lines. Cells were treated with two selected inhibitors of PI3K activity—LY294002 (IC_{50} of 720 nM for PI3Kα, 310 nM for PI3Kβ, 7.3 µM for PI3Kγ and 1.3 µM for PI3Kδ under in vitro conditions), commonly described as a pan PI3K inhibitor and AS605240 (IC_{50} of 60 nM for PI3Kα, 270 nM for PI3Kβ, 8 nM for PI3Kγ and 300 nM for PI3Kδ), a substance, which has been shown to preferentially inhibit PI3Kγ (Camps et al. 2005, Smith et al. 2007). Cell growth has been assayed after 72 h of cultivation (Fig. 2a).

Increasing concentrations of LY294002 induced significant decrease of the cell number of all cell lines under investigation suggesting an essential role of PI3K-dependent signaling paths for their viability. Notably, the SK-N-LO line exhibits higher sensitivity to the inhibitor treatment indicating a crucial role of PI3K for these cells. Especially at higher micromolar concentrations AS605240, the inhibitor with relatively high selectivity to PI3Kγ, caused stronger suppression of the cell growth of neuroblastoma lines SK-N-LO and SK-N-MC, the two cell lines expressing PI3Kγ.

The inhibitory effects of LY294002 and AS605240 on cell growth may be caused either by interference with the cell cycle or by induction of apoptosis. Corresponding comparative investigations have been performed using the SK-N-LO line, which expresses high level of PI3Kγ and the SK-N-AS line, which does not exhibit PI3Kγ expression. Propidium iodide-based cell cycle assay revealed unaltered relation of SK-N-LO and SK-N-AS cells in G1, G2 and S/M states of cell cycle after treatment with increasing concentrations of LY294002 or AS605240 (Fig. 2b). In contrast, both inhibitors induced significant apoptosis of both cell lines in the micromolar range (Fig. 2c). The pronounced cell death of SK-N-LO cells following treatment with the specific inhibitor AS605240 may indicate a distinct anti-apoptotic function of PI3Kγ in these tumor cells. The considerably less-expressed apoptosis upon LY294002 treatment may reflect the relatively low affinity of this inhibitor to PI3Kγ.

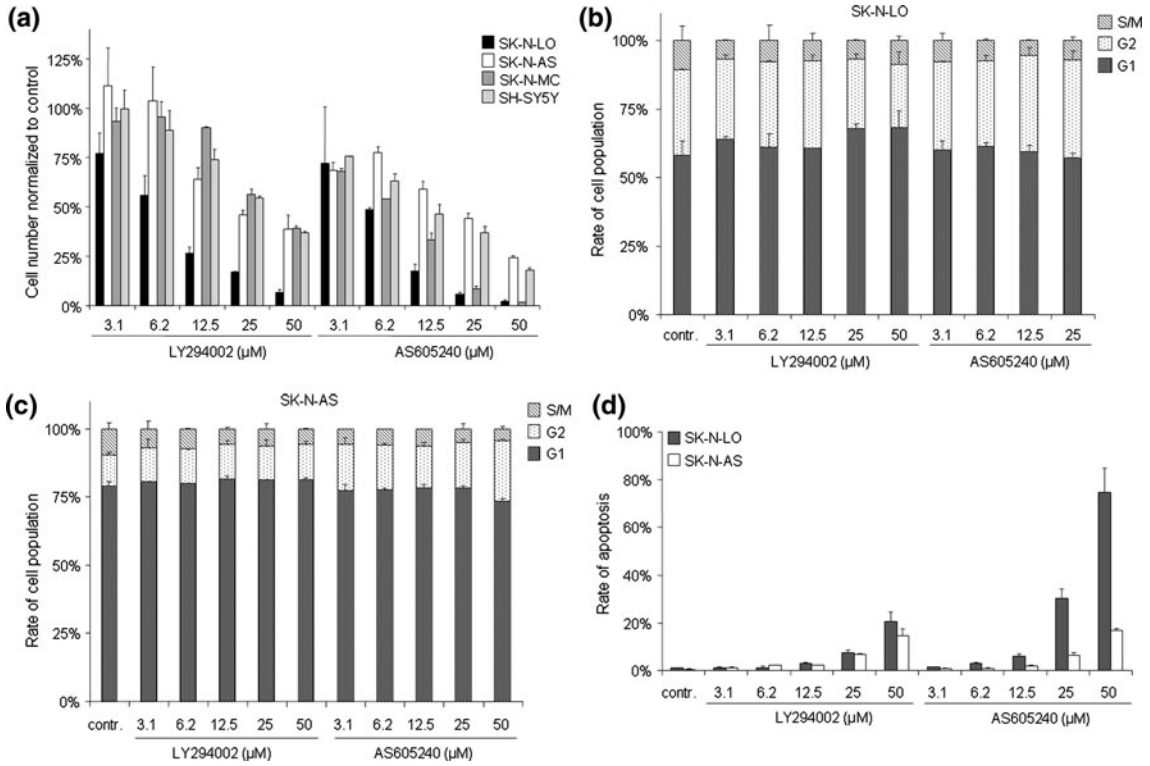

Fig. 2 Effects of PI3K inhibitors on SK-N-LO and SK-N-AS cells. Cell number assay of four different human neuroblastoma cell lines after 72 h treatment with the pan PI3K inhibitor LY294002 and the PI3Kγ specific inhibitor AS605240. Results are shown in relationship to DMSO-treated control cells (**a**). Flow cytometric determination of G1-, S/M- and G2-phase populations of SK-N-LO (**b**) and SK-N-AS (**c**) by cell fixation and PI-staining after 36 h of treatment with LY294002 or AS605240. Assay of apoptosis in the neuroblastoma cell lines SK-N-LO (*black*) and SK-N-AS (*white*) after 36 h of treatment with LY294002 or AS605240. Cells were fixed, TUNEL-staining was performed using BrdU and corresponding FITC-labeled antibody followed by flow cytometry (**d**)

Signaling effects of PI3K inhibitors

Next we analyzed the impact of PI3K inhibitors on Akt as the prototype mediator of PI3K-dependent signaling events (Fig. 3a). Both, the pan PI3K inhibitor LY294002 as well as the PI3Kγ specific inhibitor AS605240 decreased the phosphorylation of Akt at the main sites Ser473 and Thr308 in the lower micromolar range (Fig. 3a). Notably, Akt phosphorylation in SK-N-LO neuroblastoma cells is highly sensitive to the inhibitory effect of AS605240, which fits to the high efficacy of this inhibitor in the induction of apoptosis as shown in Fig. 2c. The inhibitory effect of AS605240 on Akt phosphorylation in PI3Kγ deficient SK-N-AS cells may reflect unspecific suppression of PI3Kα activity.

Then we looked for additional downstream mediators of PI3K/Akt affected by PI3K inhibitors in SK-N-LO and SK-N-AS neuroblastoma cell lines. Akt-dependent phosphorylation has been described for the protein kinases GSK3 and S6K, which are both mediators of stimulatory effects of Akt on cellular proliferation (Marone et al. 2008; Wymann and Schneiter 2008). As shown in Fig. 3a, limited sensitivity of these phosphorylation events emerged after treatment of SK-N-LO or SK-N-AS cells with LY294002 or AS605240. Akt-mediated phosphorylation of FOXO has been shown to induce the translocation of this transcription factor from the nucleus to the cytoplasm (Birkenkamp and Coffer 2003). This process was investigated in SK-N-LO and SK-N-AS cells transfected with GFP-labeled FOXO using fluorescence microscopy. Figure 3b shows cytoplasmic localization of FOXO in the absence of the inhibitors due to the basal phosphorylation of Akt. Increasing concentrations of LY294002 and AS605240 induce the translocation of FOXO from the cytoplasm to the nucleus in both cell lines. In remarkable agreement with the low sensitivity of PI3K dependent phosphorylation of GSK3 and S6K only high concentrations of AS605240 provoke significant move of FOXO from the cytosol to nucleus. Together these data are in line with a distinct antiapoptotic function of the PI3Kγ/Akt axis in SK-N-LO cells. Potential impact of GSK3, S6K and FOXO on the proliferation of these cells seems less important in the implementation of signaling effects mediated by PI3Kγ in SK-N-LO. These data also agree with recent observations, which indicate prognostic value of Akt phosphorylation but not of S6 phosphorylation in primary neuroblastoma tumor samples (Opel et al. 2007).

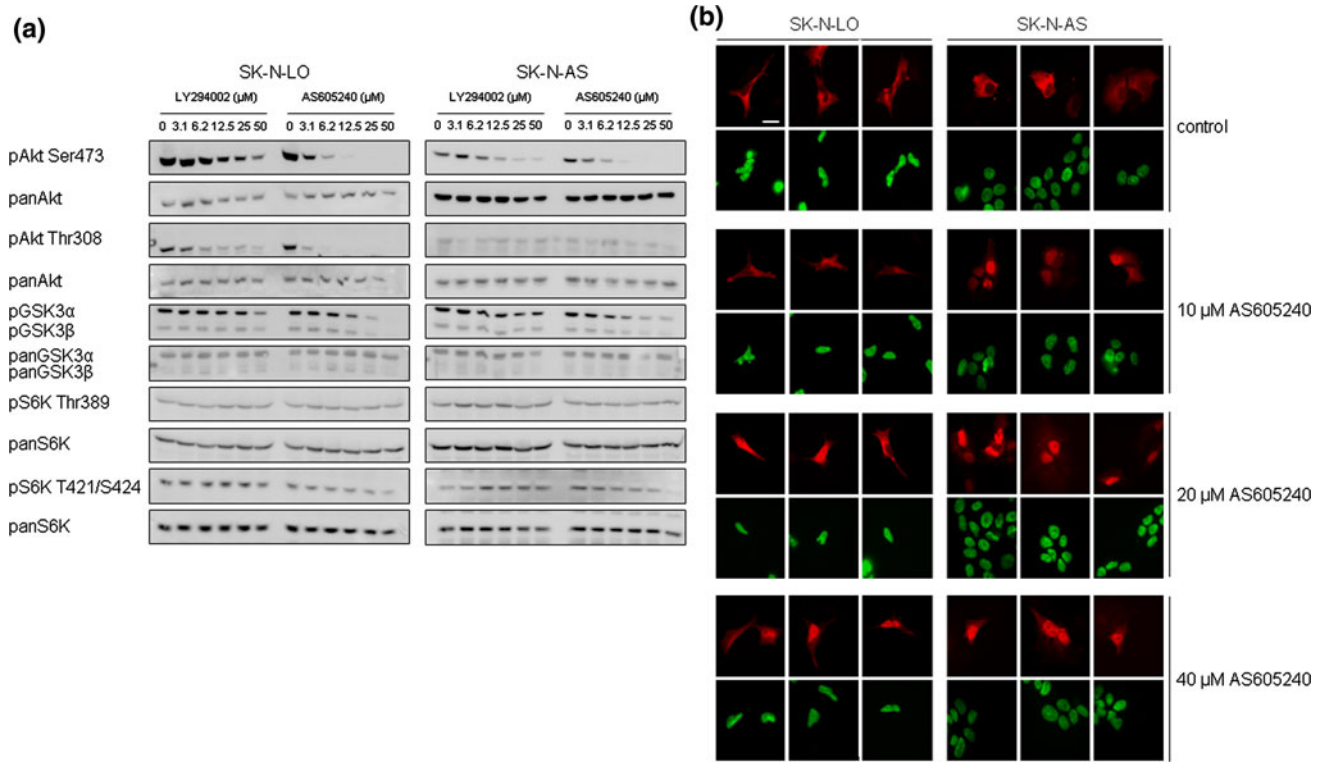

Fig. 3 Signaling effects of PI3K inhibitors in SK-N-LO and SK-N-AS cells. Phosphorylation status of different PI3K-effector proteins in the cell lines SK-N-LO and SK-N-AS after 30 min treatment with LY294002 or AS605240. Cells were treated in normal growth medium (**a**). Localization of transfected GFP-labeld FOXO (*red*) in SK-N-LO and SK-N-AS cells after treatment with indicated concentrations of LY294002 or AS605240. Cell nuclei were counterstained using Hoechst33342 (*green*) (**b**)

Effects of PI3Kγ inhibitor on a neuroblastoma model in SCID mice

The inhibitory effect of AS605240 on the growth of SK-N-LO cells prompted us to elucidate the activity of the PI3K inhibitor in a SCID mouse model of neuroblastoma. Besides the PI3Kγ positive cell line SK-N-LO, PI3Kγ deficient SK-N-AS cells have been used for these investigations. Neuroblastoma cells were implanted subcutaneously (s.c.) on the right lumbar region of the back. Solutions of AS605240 in 5% DMSO/NaCl were immediately injected at different concentrations and tumor size was measured when they were palpable. Twenty-eight days after initial injection of neuroblastoma cells, the mice were killed and the tumors were excised and proceeded to histological analysis.

As shown in Fig. 4, short-term incubation of inoculated tumor cells and consecutive s.c. injection of AS605240 for 3 days induced a concentration-dependent suppression of SK-N-LO tumor growth. At the highest dosage of 20 μg AS605240 per g mouse, the tumor growth was efficiently suppressed ($P < 0.05$). In contrast, inhibitor administration to SK-N-AS-induced solid tumors was without any significant effect on the tumor development.

Finally, we applied a therapeutic regime of AS605240 treatment on SK-N-LO-induced tumors. 20 μg/g of the inhibitor was injected s.c. starting at day 0 or day 5 after SK-N-LO cell inoculation, respectively. Treatment was proceeded for three consecutive days. Even under these conditions the inhibitor efficiently affected tumor growth suggesting significant contribution of PI3Kγ to the development of this tumor entity (Fig. 5a, b, $P < 0.05$).

Histological analysis of AS605240 treated tumor samples confirmed the in vitro data demonstrating initiation of apoptosis caused by the inhibitor in SK-N-LO cells. As shown in Fig. 5c, AS605240 provoked considerable incidence of apoptotic cells after treatment of SK-N-LO induced tumors ($P < 0.05$).

Discussion

The expression pattern of PI3K and other proteins in patient samples and in different cell lines reflects the distinct clinical heterogeneity of neuroblastoma. Our study discloses PI3Kγ as a novel player in the tumorigenesis of selected neuroblastoma entities. In addition to the significant expression in primary patient biopsies, the SK-N-LO and SK-N-MC cell lines have been found to exhibit significant levels of this PI3K species. Either cell lines display low expression of the PI3K antagonist PTEN suggesting constitutively

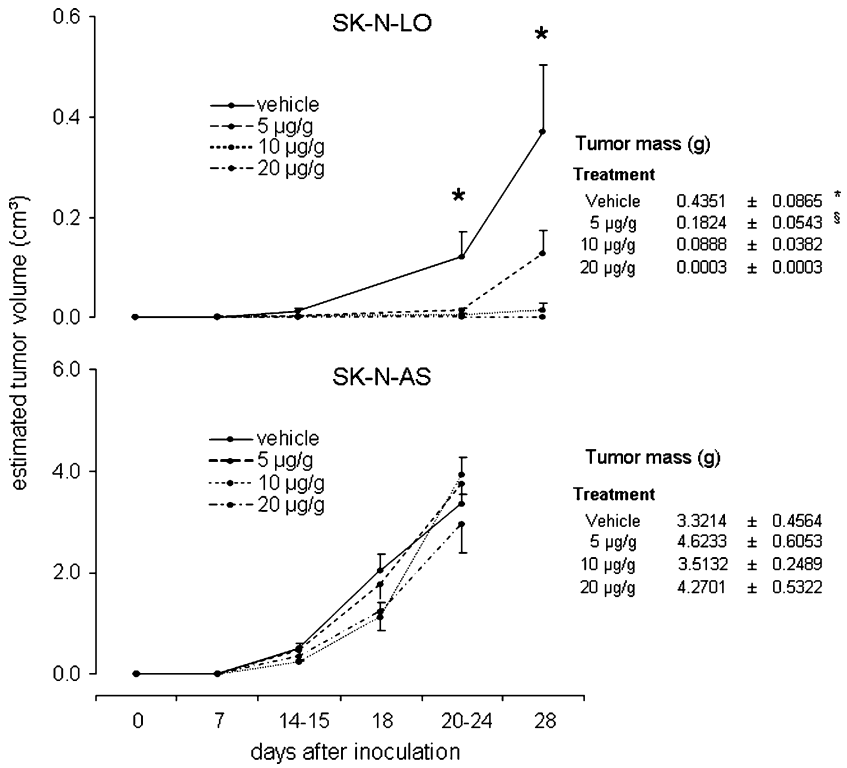

Fig. 4 Effect of PI3Kγ inhibitor on neuroblastoma xenograft growth in mice. SCID mice were randomly engrafted with 2×10^7 SK-N-LO cells (*upper panel*) or 1×10^7 SK-N-AS cells (*lower panel*) subcutaneously on the right lumbar region of the back. Cells were initially incubated for 30 min with either vehicle (5% DMSO/NaCl) or the PI3Kγ-inhibitor AS605240 (100 µl) at a final concentration of 5, 10 or 20 µg/g mouse, respectively. After inoculation, animals were treated with either vehicle or AS605240 (5, 10 or 20 µg/g mouse) for 3 days at the injection site. Tumors were allowed to grow till an estimated tumor mass of ~10% of its body weight, and then animals were killed by cervical dislocation. The tumors were excised for tumor mass measurement. Experiments were finished on day 28. Tumor volume was estimated during the experiments as described in "Materials and methods". (Values are mean ± SEM; *significant differences between AS605240-treated and vehicle-treated mice; §significant difference between mice treated with 5 or 20 µg/g AS605240; $P < .05$)

increased activity of the PI3K dependent signaling pathways in these cells.

Considerable contribution of PI3Kγ to the oncogenic pattern of SK-N-LO and SK-N-MC cell lines becomes apparent by inhibitory analysis using AS605240 for the suppression of PI3Kγ enzymatic activity. Growth of both cell lines exhibits pronounced sensitivity to this inhibitor and induction of apoptosis emerges as the main cause of the limited viability of SK-N-LO cells after treatment with AS605240. The data are in line with recent investigations showing that overexpression of wild type PI3Kγ is sufficient to induce oncogenic transformation of chicken fibroblasts (Denley et al. 2008; Kang et al. 2006). The oncogenic potential of PI3Kγ suggested by these results has been now validated by our results for neuroblastoma as a solid tumor entity. In addition, these data comply with the result of the previous controversy on a potential tumor suppressor function of PI3Kγ in colon cancer (Barbier et al. 2001; Sasaki et al. 2000, 2003).

Investigating the signaling effects of PI3Kγ overexpression in chicken fibroblasts Kang et al. (2006) did not notice significant activation of Akt. This result contrasts to our observation on constitutively active Akt in SK-N-LO cells even in the absence of added serum. In our hands Akt stimulation in SK-N-LO cells is highly sensitive to inhibition by the PI3Kγ specific inhibitor AS605240. These data point to an important regulatory function of the PI3Kγ/Akt axis for the viability of SK-N-LO tumor cells. Due to the limited specificity of AS605240 contributions of the other PI3K species to the oncogenic phenotype of these neuroblastoma cells cannot be excluded. In accordance with the absence of any significant effects of the PI3Kγ inhibitor on stages of the cell cycle, our data reveal only negligible impact of AS605240 on the downstream mediators of Akt, which have been shown to control cell cycle or metabolic functions. Neither FOXO, nor S6K or GSK3 are notably affected by the inhibitor under the conditions used. This specific signaling pattern contrasts to numerous reports demonstrating prominent effects of PI3K species on the cell cycle control in tumor cell lines (Horn et al. 2008; Zhao and Vogt 2008). Notwithstanding the in vitro experiments indicate PI3Kγ as a signaling protein contributing significantly to the tumorigenic pattern of SK-N-LO cells.

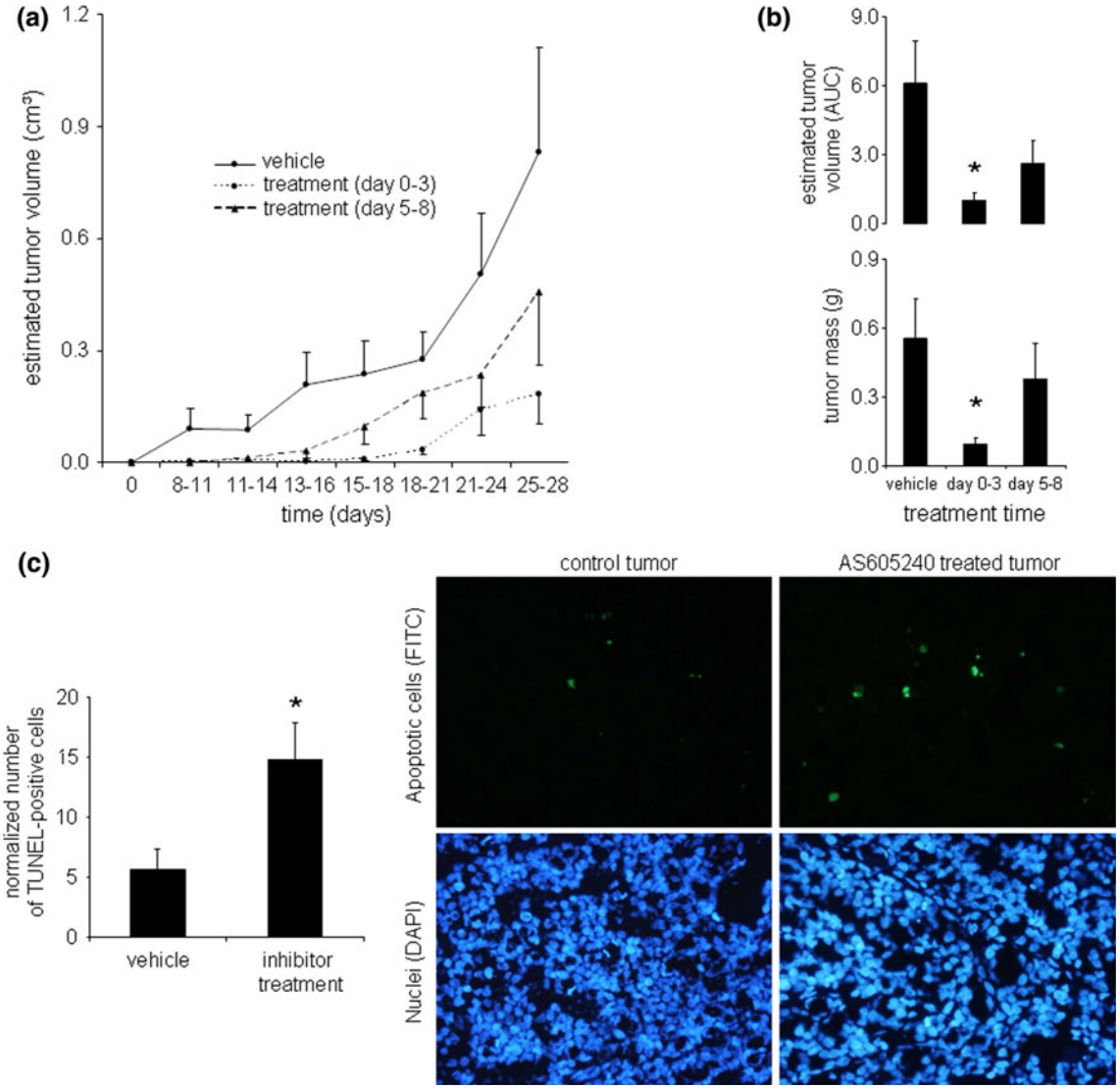

Fig. 5 Effect of retarded treatment of the PI3Kγ inhibitor on neuroblastoma xenograft growth in mice. SCID mice were randomly engrafted with 2×10^7 SK-N-LO cells subcutaneously on the right lumbar region of the animal's back. After inoculation, animals were treated once a day with either vehicle or AS605240 (20 µg/g mouse) at the injection site on day 0–3 and on day 5–8, respectively. Experiments were finished on day 28. Tumor volume was estimated during the experiments as described in Sect. "Materials and methods". Tumor mass was measured after excision of the tumor at the end of the experiment (**a**, **b**). Apoptosis rate in AS605240-treated SK-N-LO tumors xenotransplanted subcutaneously in SCID mice compared to NaCl-treated control tumors was determined by histological evaluation via TUNEL-staining (**c**). (Values are mean ± SEM; *significant differences between AS605240-treated and vehicle-treated mice; $P < .05$., AUC abbreviates area under the *curve*)

Immunosuppressed SCID mice have been frequently used for investigations of the in vivo pattern of xenotransplanted neuroblastoma cell lines (Sims et al. 2008; Wang et al. 2008). These xenograft models were also deployed to analyze the antitumoral activity of low molecular compounds (Parashar and Shafit-Zagardo 2006; Wang et al. 2008). Our comparative study used the PI3Kγ containing SK-N-LO cells and the PI3Kγ deficient cell line SK-N-AS for engraftment of neuroblastoma cells in mice. Tumor growth has been monitored over 28 days in the presence or absence of the PI3Kγ inhibitor AS605240. Whereas the inhibitor did not reveal any effects on the growth of SK-N-AS-induced tumors, a significant suppression of SK-N-LO tumors has been observed. The efficacy of inhibitor treatment decreased when the inhibitor application is temporally delayed in relation to the injection of tumor cells. The declining effect may be related to the pharmacokinetics of the inhibitor producing lower effective inhibitor concentrations in the up-growing tumor at later time points. Pharmacokinetics may also contribute to the reduced inhibitor effectiveness in SK-N-AS-induced tumors, because these tumors grow much faster compared to SK-N-LO tumors. Nevertheless, a specific inhibitory effect of tumor

growth in SK-N-LO tumors has been shown. Higher concentrations of AS605240 could be tested in order to increase the therapeutic effect of the inhibitor on tumor growth. According to recent estimations, no immediate toxic effects of AS605240 on mice have been observed even at concentrations up to 500 μg/g (C. Rommel, unpublished observations). Treatment of SK-N-LO cells in vitro and in the SCID mouse model with AS605240 clearly reveals induction of apoptosis as the main tumor-static effect of the inhibitor. These data add to the increasing efforts targeting signaling pathways of the cell death and survival control for the alleviation of selected types of cancer.

Taken together, these data propose PI3Kγ as a mediator in the tumorigenesis of selected neuroblastoma entities. In addition, this work expands the therapeutic potential of AS605240 and related compounds.

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Conflict of interest statement None.

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