The Dual PI3K/mTOR Inhibitor BEZ235 Is Effective in Lung Cancer Cell Lines

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Abstract. *Background: BEZ235 is a dual phosphatidylinositol 3-kinase (PI_3K)/mammalian target of rapamycin (mTOR) inhibitor that is orally available and that has been shown to be effective in several malignancies in vitro. Recently, BEZ235 entered clinical trials for solid tumors. We aimed at investigating if BEZ235 is effective in lung cancer cell lines. Materials and Methods: The human lung cancer cell lines EPLC, HCC and H1339 were analysed by fluorescence in situ hybridization, gene sequencing and Western blot analysis. Cells were exposed to BEZ235 and/or cisplatin and the survival fraction was quantified. Results: In all cell lines, BEZ235 reduced pAkt and pS6 expression indicating interference with the epidermal growth factor (EGF) pathway. Furthermore, BEZ235 inhibited tumor cell growth and added to the effects of cisplatin. This was independent of EGFR amplification and EGFR, KRAS, PI3K and AKT mutation. Conclusion: The dual PI3K/mTOR inhibitor BEZ235 is effective in lung cancer cell lines and a promising compound to be tested in clinical phase I studies.*

Lung cancer is the leading cause of cancer death in the industrialized nations (1). Therapeutical regimens including chemotherapy support quality of life but frequently fail to increase long-term survival. Recently, the receptor tyrosine kinase (RTK) inhibitors erlotinib and gefitinib targeting epidermal growth factor receptor (EGFR) activation have been introduced in the treatment of lung cancer (2). Predominantly in patients with activating mutations of *EGFR* this new treatment option has proven be effective. However, primary or acquired resistance considerably limits its clinical effectiveness (3). Therefore, novel therapeutics targeting RTK pathways are currently being developed.

After binding of EGF to its receptor dimerization activates RTKs. This leads to increased proliferation involving the downstream effectors phosphatidylinositol 3-kinase (PI3K), Akt and mammalian target of rapamycin (mTOR) (4) (Figure 1). BEZ235 is a dual PI3K/mTOR inhibitor that is orally available (5). A dual approach targeting more than one downstream effector is a promising approach because it may delay or even prevent therapy resistance. BEZ235 elicited antitumor activity in mouse lung cancer models (6, 7), in cells of human glioma (8, 9), neuroendocrine tumor (10), breast cancer (11), melanoma (12), pancreatic cancer (13), and sarcoma (14), and hemato-oncological malignancies (15-17). Clinical trials in solid tumors have recently been initiated but not yet for lung cancer.

In our study, we aimed at investigating if BEZ235 is effective in lung cancer cell lines. We further investigated if *EGFR* expression and mutation status alters BEZ235 effectiveness and if combination of cisplatin with BEZ235 provides additional antitumor effects.

Materials and Methods

Reagents. Cell culture reagents were purchased from Life Technologies (Eggenstein, Germany). Other reagents were bought from Sigma-Aldrich (Deisenhofen, Germany) unless stated otherwise. The human lung cancer cell lines HCC (derived from an adeno lung carcinoma) and H1339 (derived from a small cell lung carcinoma) were from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The human cell line EPLC M1 (derived from a squamous cell carcinoma) was kindly provided by Dr. G. Jaques, Philipps University, Marburg, Germany.

Amplification and mutation anlaysis. Cell lines were analyzed for EGFR amplification by fluorescence *in situ* hybridization and for *EGFR, KRAS, PI3K* and *AKT* mutation by gene sequencing using routine procedures.

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Table I. *The lung cancer cell lines EPLC, HCC and H-1339 were characterized in terms of EGFR amplification, as well as of EGFR, KRAS, PI3K and AKT mutation. EGFR mutation was observed in HCC and KRAS mutation in H-1339 cells.*

Parameter	Method	Cell line		
		EPLC	HCC	H-1339
EGFR amplification	FISH	Polysomy low; quotient 1.22	Polysomy low; quotient 1.15	Polysomy high; quotient 1.06
EGFR mutation	Sequencing of exons 18, 19, 21	WT	Exon 19 deletion: c.2236_2250del15 (p.E746-A750)del	WT
KRAS mutation	Sequencing of exon 2	WT	WT	c.34G>C(p.G12R)
PI3K mutation	Sequencing of exons 9, 20	WT	WT	WT
AKT mutation	Sequencing of exon 3	WT	WT	WT

WT: Wild-type.

Western blot analysis. Cells were washed twice with ice-cold phosphate-buffered saline (PBS; 10 mM, pH 7.4). The cells were lysed and cell suspensions treated according to the complete Lysis-M (Roche, Germany). The extracts were collected, aliquoted and then stored at –20˚C until use with Western blot analysis. The protein concentrations of the extracts were determined with a Non-Interfering Protein Assay Kit according to the manufacturer's protocol (Calbiochem, Germany). The extracts were treated with NuPAGE LDS Sample Buffer and NuPAGE Reducing Agent at 70˚C for 10 min and separated by SDS-PAGE on a 10% Bis-Tris gel (Invitrogen, Canada). Staining was performed using specific antibodies (phospho-S6 ribosomal protein (Ser 240/244), dilution 1:2,000; S6 ribosomal protein (54D2), dilution 1:1,000; phospho-AKT (Ser 473), dilution 1:1,000; AKT antibody, dilution 1:1000; secondary antibodies anti-mouse IgG (horseradish peroxidase (HRP)), dilution 1:2,000 and anti-rabbit IgG (HRP), dilution 1:2,000; all Cell Signaling, USA).

β-Actin staining served as loading control (mouse anti-β-actin HRP, dilution 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibody complexes were visualized using Hyperfilm ECL chemiluminescence (Amersham Biosciences, UK) and evaluated using the Image-J analysis software (National Institute of Health, open source software).

Survival curves. Cells were seeded in 25 cm^2 cell culture flask and cultured for 24 h. Cell viability was evaluated by trypan blue exclusion cell counting 24 h to 96 h after exposure to BEZ235 (100 nM and 1000 nM). For cisplatin treatment, cells were exposed for 3 h to 2 μM cisplatin, analogous to the plasma concentration of unbound cisplatin in humans (18), before the addition of BEZ235.

Statistics. Students *t*-test, one-way ANOVA repeated measurements and two-way ANOVA (combined with pairwise multiple comparisons) were performed using Sigma Stat software (Jandel Scientific, Chicago, USA). A *p*-value of less than 0.05 was considered statistically significant. All values are expressed as the mean±SEM.

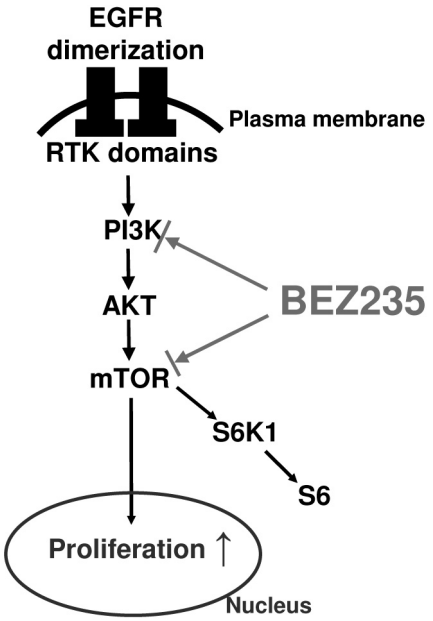

Figure 1. *Simplified schematic of PI3K/mTOR pathway. After binding of EGF to its receptor dimerization leads to activation of RTKs. Via RAS, PI3K, AKT and mTOR, an increase in proliferation is induced. BEZ235 inhibits PI3K and mTOR, making it a dual antagonist.*

Results

Firstly, the lung cancer cell lines HCC, EPLC and H13393 were characterized in terms of *EGFR* amplification as well as *EGFR, KRAS*, *PI3K*- and *AKT* mutation. The results are summarized in Table I.

To test if BEZ235 inhibits PI3K and mTOR in lung cancer cell lines, the expression of the downstream proteins

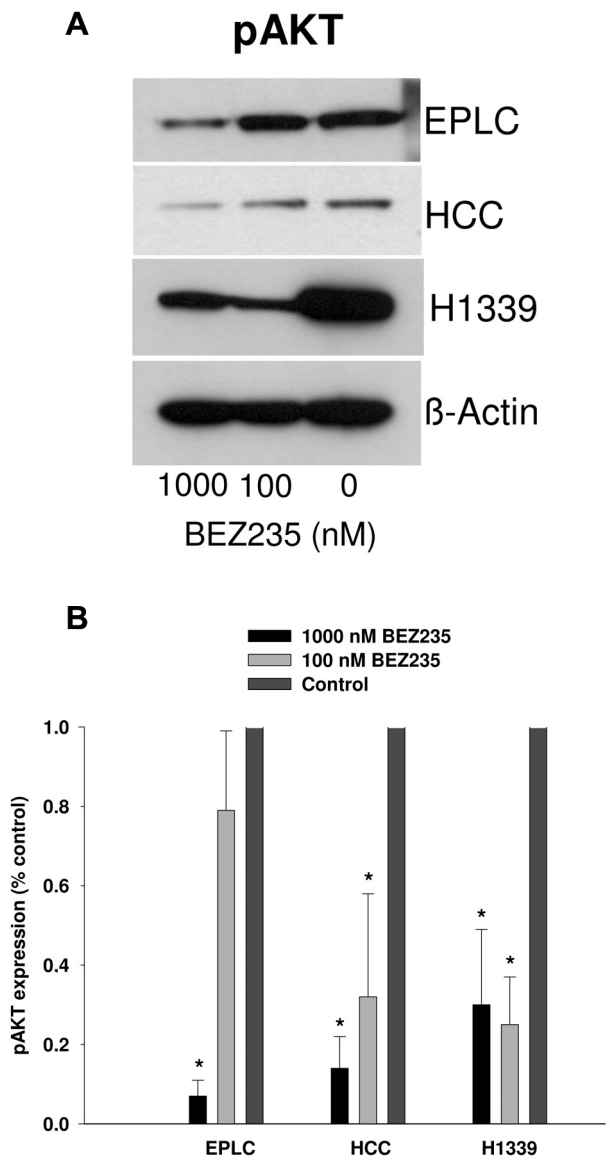

Figure 2. *Cells of the lung cancer cell lines HCC, EPLC and H1339 were treated with 100 nM or 1,000 nM BEZ235 for 5 days and the expression of the PI3K downstream phosphorylated protein pAKT (activated form) was quantified using Western blot analysis. A: In all cell lines, the expression of pAKT was reduced after treatment with BEZ235. B: However, no difference between treatments with 100 nM BEZ235 and 1,000 nM BEZ235 was observed (n=3 experiments, *=p<0.05 versus control). Treatment with BEZ235 had no effect on the expression of nonphosphorylated AKT (inactive form, data not shown).*

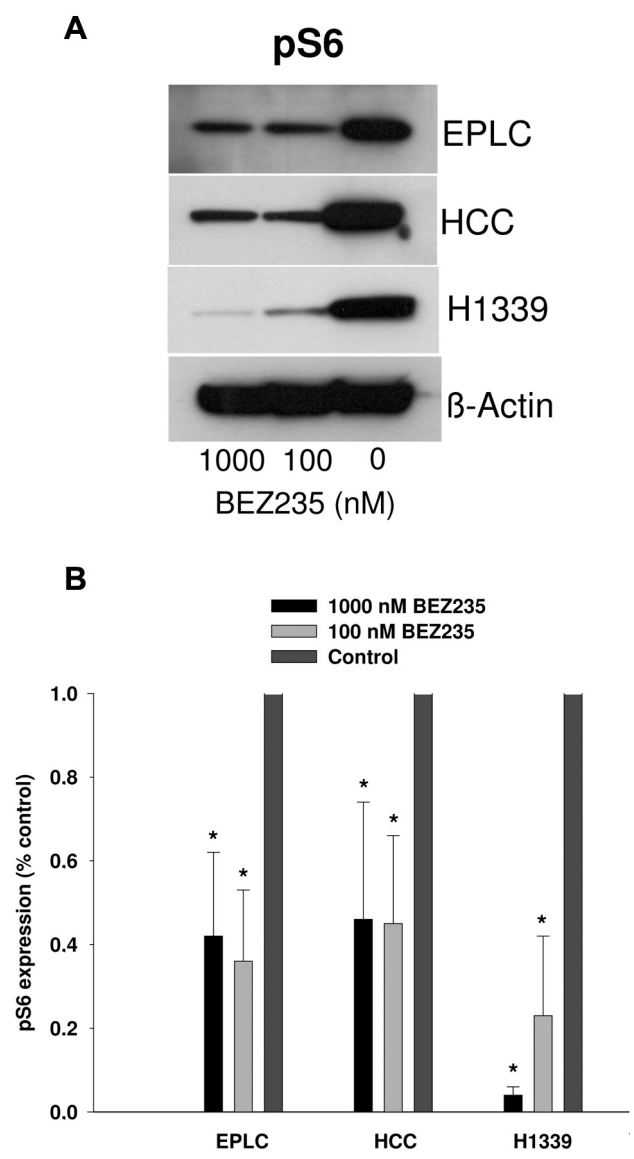

Figure 3. *HCC, EPLC and H1339 cells were treated with 100 nM or 1,000 nM BEZ235 for 5 days and the expression of the mTOR downstream phosphorylated protein pS6 (activated form) was quantified using Western blot analysis. A: In all cell lines, the expression of pS6 was reduced after treatment with BEZ235. B: However, no difference between treatments with 100 nM BEZ235 and 1,000 nM BEZ235 was observed (n=3 experiments, *p<0.05 versus control). Treatment with BEZ235 had no effect on the expression of nonphosphorylated S6 (inactive form, data not shown).*

AKT and S6 were quantified with Western blot analysis. In all cell lines, the expression of unphosphorylated AKT and S6 (not activated forms) was unchanged by treatment with BEZ235 (data not shown). However, the expression of phosphorylated AKT (pAKT, activated form) (Figure 2) and phosphorylated S6 (pS6, activated form) (Figure 3) was reduced after treatment with BEZ235 for 5 days. No differences between treatments with 100 nM BEZ235 and 1,000 nM BEZ235 were observed.

After treatment with 100 nM or 1,000 nM BEZ235 for 5 days, a concentration-dependent antitumor effect was found in all cell lines (Figure 4). Comparing the three cell lines,

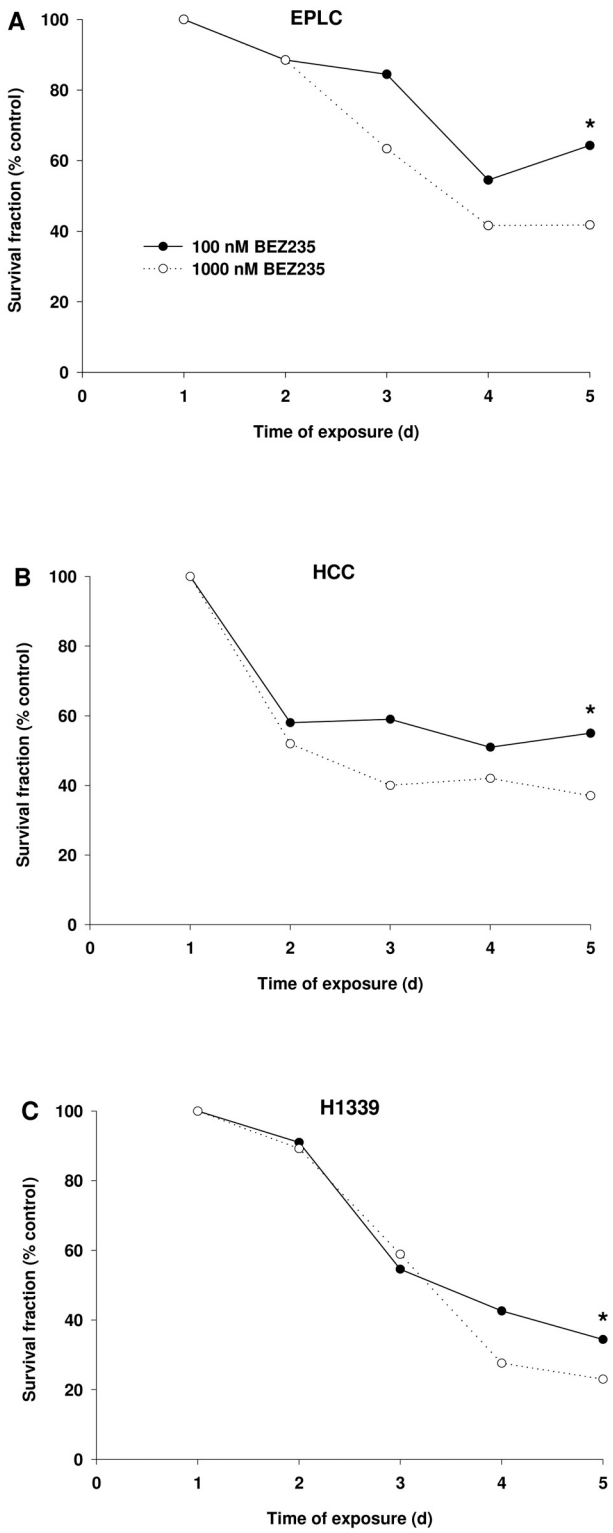

Figure 4. *Cells were treated with 100 nM or 1,000 nM BEZ235 for 5 days and the survival fraction as a percentage of the control (no incubation with BEZ235) was assessed. In EPLC (A), HCC (B) and H1339 (C) cells, a concentration-dependent effect was observed (n=3 experiments, *p<0.05).*

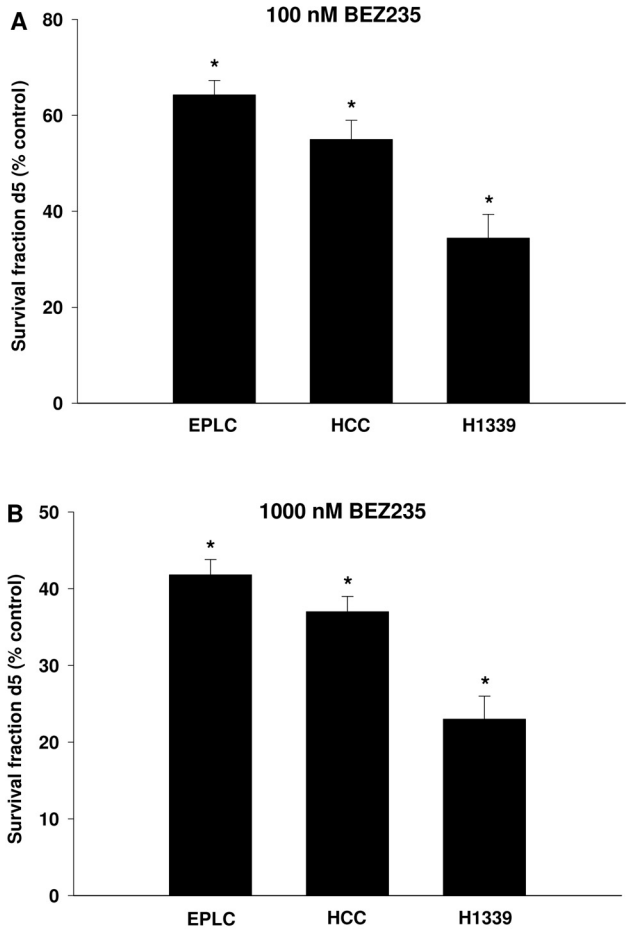

Figure 5. *Comparing the effects of 100 nM (A) and 1,000 nM BEZ235 (B) on the different cell lines, their sensitivity to BEZ235 was H1339>HCC>EPLC (n=3, *p<0.05).*

their sensitivity to BEZ235 at both concentrations used was H1339>HCC>EPLC (Figure 5).

Finally, cells were exposed to 2 μM cisplatin for 3 h and subsequently treated with BEZ235 for 3 days. BEZ235 significantly augmented the antitumor effects of cisplatin (Figure 6).

Discussion

In our study, we showed that the dual PI3K/mTOR inhibitor BEZ235 interfered with the EGF pathway in lung cancer cell lines, inhibited tumor cell growth and increased the effects of cisplatin.

Although the effects of BEZ235 on tumor cell growth were concentration dependent, the effects on pAKT and pS6 were not. This discrepancy may be caused by the fact that the differences between the survival after treatment with 100 and 1,000 nM BEZ235 were relatively small. On the other

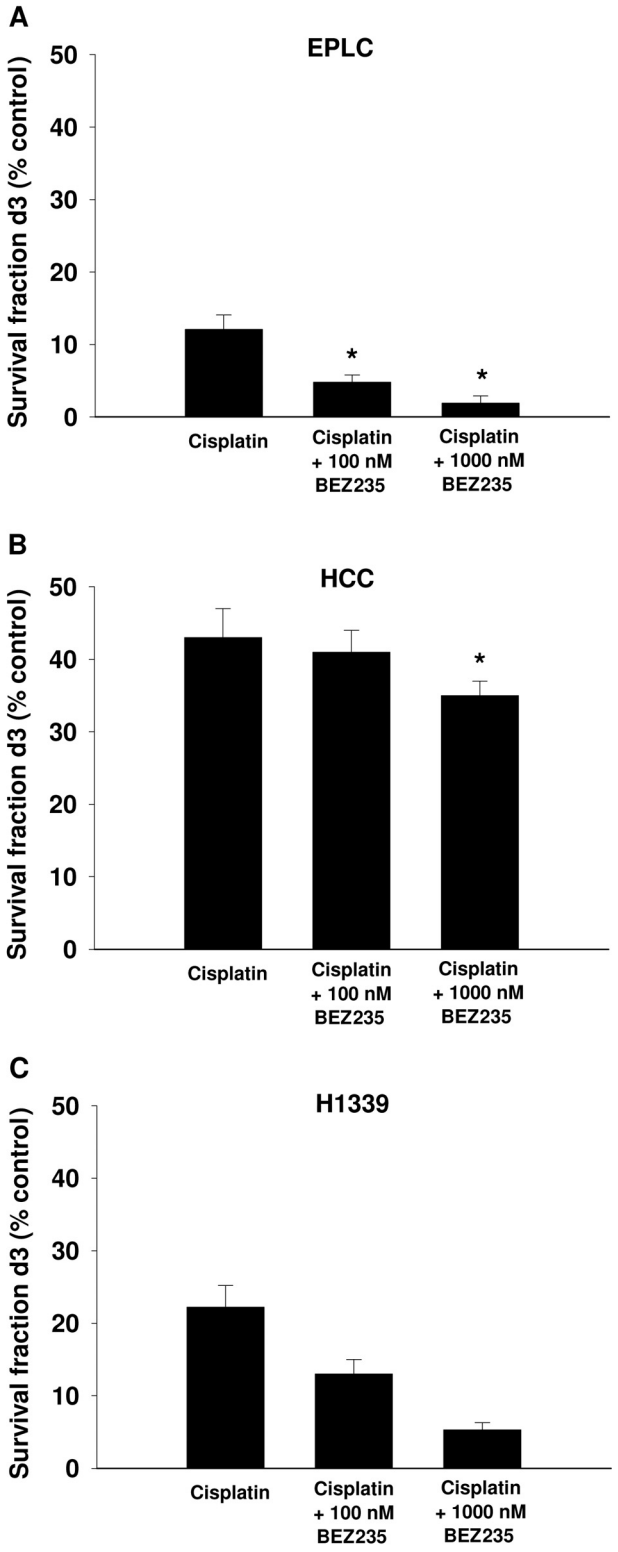

Figure 6. *Cells were exposed to 2 μM cisplatin for 3 h. Subsequently, the cells were treated with BEZ235 for 3 days and the survival fraction was assessed. BEZ235 significantly augmented the antitumor effect of cisplatin in EPLC (A), HCC (B) and H1339 (C) cells (n=3 experiments, *p<0.05 versus cisplatin alone).*

hand, the ability to discriminate small differences in expression using Western blot analysis is low. However, we aimed to show the principle effectiveness of BEZ235 in human lung cancer cell lines.

In clinical practice, the selection of patients who are more likely to benefit from a given therapy is of great importance. As BEZ235 targets the EGF pathway, mutations of genes coding for proteins involved in this signal transduction pathway may alter the effectiveness of BEZ235. This has been shown for TKIs such as erlotinib and gefitinib and is still under investigation (19). In our study, the effects of BEZ235 on tumor cell growth occurred in cell lines with and without *EGFR* amplification, *EGFR* mutation or *KRAS* mutation. However, H1339 cells, which were the most sensitive to BEZ235, showed both *EGFR* amplification and *KRAS* mutation in contrast to EPLC and HCC cells. In mice, Engelman *et al*. reported lung carcinoma regression in mutated PI3K but not in mutated *KRAS* tumors after treatment with BEZ235 (6). In contrast, Konstantinidou *et al*. reported striking antitumor effects of BEZ235 in oncogenic *KRAS* mouse tumors (7). These conflicting data render the importance of specific genetic alterations for the effectiveness of BEZ235 unclear. However, if BEZ235 proved to be effective regardless of mutation status, a greater number of patients may have the chance to benefit from this therapy.

For TKIs such as erlotinib and gefitinib, it has been shown that combination with chemotherapy resulted in no further benefit, which was a rather disappointing finding (20). We found that BEZ235 gave an additional effect on cisplatin-induced cell death in all cell lines investigated. If this could be shown to be the case in clinical trials, patients would greatly benefit, as the effectiveness of the first-line therapy predominantly predicts the patient's outcome.

In our study, we showed that the dual PI3K/mTOR inhibitor BEZ235 is effective in lung cancer cell lines. We believe that this compound, which is currently being tested for several malignancies, should be introduced into phase I studies in lung cancer.

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