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ORIGINAL ARTICLE

Selective PI3K inhibition by BKM120 and BEZ235 alone or in combination with chemotherapy in wild-type and mutated human gastrointestinal cancer cell lines

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Abstract
Purpose New targeted agents like antibodies or small molecules against tyrosine and lipid kinases clearly expand the standard therapy options in oncology. However, tumour resistance is still a challenge, often induced by mutations in growth-related signalling cascades. Twenty and ten percentage of all patients with colorectal and gastric cancers, respectively, carry phosphatidyl-3-kinase (PI3K) mutations and do not respond to receptor-blocking therapies. Recently, selective kinase inhibitors have been generated, which block the PI3K signalling pathway in tumour cells. So far, their therapeutic role for the treatment of mutated versus wild-type human gastrointestinal cancers has not been clarified in detail.

Methods To define the inhibitory and pro-apoptotic effects of the two PI3K inhibitors BEZ235 and BKM120 in three human colon cancer (HT-29, HCT-116 and DLD-1) and three gastric cancer (NCI-n87, AGS and MKN-45), cell lines with different *PIK3CA* gene mutation status were used. Firstly, viability, apoptosis and caspase assays were performed during incubation with either the inhibitors alone or combined with different cytotoxic agents. Secondly, the molecular consequences for the cell cycle and signalling pathways were analysed by defining the protein levels by FACS and Western blot analysis.
Results Both the PI3K inhibitors BEZ235 and BKM120 induced a clear concentration-dependent reduction in cell viability and an increase in apoptotic cell death, with the mutated cells being more sensitive to treatment. However, single-agent BEZ235 caused a G1 arrest in tumour cells, whilst BKM120 induced a G2 shift in a half of the gastrointestinal cancer cell lines. There was a clear downregulation in the protein levels of the PI3K–AKT pathway at the concentrations of 100nM for both agents and for BEZ235 the additional inhibition of the mTOR pathway. Furthermore, BEZ235 caused synergistic induction of apoptosis when combined with irinotecan in colon cancer cell lines. Human gastric cancer cells were less sensitive to both BEZ235 and BKM120.
Conclusions BEZ235 and BKM120 induced pro-apoptotic effects in all cell lines and especially with an increased response in the *PI3KCA* mutated cells. Our data support the clinical development of these PI3K inhibitors for patients with wild-type or mutated colon cancers.

Keywords Inhibition · PI3K · Cancer cell lines · Chemotherapy · Wild type

Abbreviations
PI3K Phosphatidylinositol 3-kinase

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EGFR	Epidermal growth factor receptor
VEGF(R)	Vascular endothelial growth factor (receptor)
mTOR	Mammalian target of rapamycin

Background

Recently, the concept of the 'Hallmarks of Cancer' has been expanded by a further general process: tumours contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the 'tumour microenvironment' [1]. The PI3K (phosphatidylinositol 3-kinase)–AKT signalling pathway plays a central role in a large array of diverse biological processes in normal and malignant cells and is therefore often deregulated in human cancer and implicated in pathological changes [2]. The activated pathway is involved in multiple cellular functions such as cell proliferation, differentiation, metabolism and survival, all of which might critically influence cancer progression [3, 4].

Cancers of the gastrointestinal tract, especially of the colon and stomach, are one of the most common causes of malignancy in Western countries [5]. Combinations of standard chemotherapy with targeted therapies have recently been introduced into clinical routine as they were found to improve response and overall survival [6, 7]. These new targeted agents, such as monoclonal antibodies, have definitely widened the range of standard therapies. The currently approved monoclonal antibodies for the treatment of colorectal cancer are active against growth factors (e.g. vascular endothelial growth factor, VEGF), such as bevacizumab, or the tyrosine kinase receptor (epidermal growth factor receptor, EGFR), such as cetuximab and panitumumab [8]. They inhibit the signalling cascades, which are responsible for angiogenesis, inhibition of apoptosis and proliferation of the tumour [9]. The development of tumour resistance is, however, a continuous challenge, which often occurs after initial remission under chemotherapy or in combination with antibodies. The resistance is frequently based on mutations in the signalling molecules, such as mutations in the genes of the GTPase KRAS, the protein kinase BRAF or the lipid kinase PI3K [10].

PI3K consists of a regulatory and a catalytic subunit and is directly activated by growth factor stimulation via the intracellular domain of a receptor tyrosine kinase [11, 12]. It may also be activated via stimulated GTPase RAS or by G-protein-coupled receptors [13] and is indirectly activated by loss of the tumour suppressor PTEN [14]. The absence of the antagonist PTEN leads to uncontrolled AKT kinase activity. AKT, the central downstream effector of PI3K, is phosphorylated at two residues, Thr308, which is activated by PI3K-PDK1, and Ser473, which is phosphorylated by mTOR (mammalian target of rapamycin) [15, 16].

Deregulation of the PI3K–AKT pathway is triggered by loss of tumour suppressors, for example, PTEN, and activation of cross-talk signalling, for example, via RAS, and deregulation is promoted by mutations in the *PIK3CA* gene, which encodes the catalytic subunit 110α of PI3K [17]. There are two hotspot regions on exons 9 (E542K and E545K) and 20 (H1047R), where oncogenic substitution leads to constitutive activation [18]. The *PIK3CA* gene is known to be mutated in a range of human cancers [19]. For example, 20 % of patients with colorectal cancer and 10 % of patients with gastric cancer carry a PIK3CA mutation [20]. As a consequence, these patients do not respond to tyrosine kinase receptor-blocking therapies [8, 21].

The constellation of features of PI3K/AKT signalling—critical cellular functions, prevalent oncogenic genetic aberrations, consequent therapeutic resistance and its potential reversal—have made the inhibition of this pathway an attractive target for anticancer strategies [22]. A new generation of small molecules directly blocks signalling pathway proteins inside the cell via their ATP binding site. If these substances specifically inhibit the protooncogenic signalling molecules, the induced resistance might be overcome.

The dual PI3K and mTOR inhibitor BEZ235 and the next generation PI3K inhibitor BKM120 are both synthetic small molecules of the class of imidazoquinolones and inhibit the catalytic subunit p110α of PI3K by competing at its ATP binding site. BEZ235 also inhibits the catalytic activity of mTOR [23]. The therapeutic potential of both substances is under investigation in different solid tumour types in phase I and II clinical studies (http://clinicaltrials.gov).

Because preclinical and clinical data suggest that mutations in the *PIK3CA* gene influence the response to PI3K/AKT/mTOR inhibitors and that *KRAS* or *BRAF* gene mutations may mediate resistance to these inhibitors, we first characterised the *PI3KCA, KRAS* and *BRAF* mutational status of our human gastrointestinal cancer cell lines. We then examined the therapeutic effects of the PI3K inhibitor BKM120 and the dual PI3K/mTOR inhibitor BEZ235 alone and in combination with standard chemotherapeutic agents in the different gastric and colon cancer cell lines according to their *KRAS, BRAF* and *PI3K* mutational status.

Methods

Cell culture

The human colorectal cancer cell lines HT29, HCT-116 and DLD-1 were obtained from the German Resource

Centre for Biological Material (DSMZ, Braunschweig, Germany). All cells were cultured in RPMI 1640 (Invitrogen, Germany) supplemented with 10 % foetal calf serum (FCS; PAA, Cölbe, Germany), 100 units/mL penicillin and 100 μg/mL streptomycin (1 %, Cambrex, Germany). The three gastric cancer cells MKN-45, NCI-n87 and AGS were obtained from the DSMZ and from the European Collection of Cell Cultures (ECACC, Salisbury, United Kingdom). MKN-45 was cultured in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 20 % FCS and 1 % penicillin/streptomycin. The AGS cell line was cultured in Ham's F12 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10 % FCS and 2 mM glutamine (Invitrogen, Karlsruhe, Germany) containing 1 % penicillin and streptomycin. NCI-n87 cells were maintained in RPMI 1640 medium with 2 mM glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1 mM sodium pyruvate (all additives from Invitrogen, Karlsruhe, Germany), plus 10 % FCS and 1 % penicillin and streptomycin. Cultures were maintained in a 5 % CO_2 humidified incubator at 37 °C. All experiments were performed with exponentially growing cells.

Reagents

BEZ235 and BKM120 were provided by Novartis (Novartis, Basel, Switzerland), dissolved in dimethylsulphoxide (DMSO, Invitrogen, Karlsruhe, Germany) to a stock concentration of 10 mM and stored at −80 °C. Untreated control cell lines (MTT, caspase and apoptosis assays, and western blot) were treated with the appropriate volume of DMSO in all experiments, to ensure that there were no possible side effects of DMSO.

Mutational analysis

Mutational analyses of the *KRAS, BRAF* and *PIK3CA* genes were carried out. For each cell line, DNA was extracted from 1×10^6 cells using the peqGold Tissue DNA Mini Kit (PeqLab, Erlangen, Germany), according to the manufacturer's instructions. DNA (10 ng) was amplified using oligonucleotide primers specific for human *KRAS* (G12V and G13D), *BRAF* (V600E) and *PIK3CA* (E542K and E545K, and H1047R) genes (supplementary Table 1, online). High-resolution melting PCRs were run on a LightCycler 480 (LC480) with the High Resolution Melting Master (Roche Diagnostic Spa, Indianapolis, IN), according to the manufacturer's instructions. The data were analysed with the LightCycler 480 Gene Scanning Software Module for deletion and mutation identification.

Measurement of cell viability using the MTT assay

Cells (1×10^4 per well) were seeded into 96-well plates and incubated for 24 h. They were then treated with different concentrations (0–10 μM) of BEZ235 or BKM120 either as single agents or in combination with cytostatic drugs (as appropriate), for 96 h. After this, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added per well and the cultures incubated for 4 h at 37 °C. Ten per cent SDS in 0.01 M HCl (Roth, Karlsruhe, Germany) were added after incubation (100 μL) to each well, and cells were left overnight at 37 °C. The following day, the absorbance of each well was measured with a multi-well scanning spectrophotometer (Appliskan, Thermo Fisher Scientific, Schwerte, Germany) at 550 nm. Growth inhibition was expressed as the ratio of the mean absorbance of treated cells to that of control cells. Each experiment was performed in triplicate.

Measurement of cell cycle changes and apoptosis by propidium iodide labelling

Cell cycle changes and apoptosis were quantified by detecting the fraction of, G1/G0, S, G2/M and sub G1 cells by nuclear staining with propidium iodide (PI). Cells were seeded in 12-well plates in 1 mL of medium and incubated with BEZ235 or BKM120 at the required dose alone and in combination with cytostatic drugs such as irinotecan (1 μg/mL), 5-fluorouracil (5-FU, 1 μg/mL) and oxaliplatin (1–1.5 μg/mL) for 24–96 h.

For the PI flow cytometric assay, 2×10^6 tumour cells were seeded in 12-well plates and were treated for 72/96 h. The cells were then collected and incubated on ice for 30 min. Afterwards, cells were resuspended in fluorochrome solution (50 μg/mL PI, 0.1 % Na citrate, 0.1 % Triton X-100) and placed in the dark for at least 1 h. Stained cells were measured by flow cytometric analysis (FACS) by a BD FACS Calibure. Each experiment was performed in triplicate.

Caspase assay

Cells were treated with placebo (medium, 10 % FCS, appropriate volume DMSO), 5 μM BEZ235 or 5 μM BKM120. After incubation for 24 h, cells were lysed in buffer containing 20 mM Tris/HCl pH 8.0, 5 mM EDTA, 0.5 % Triton X-100 and onefold complete protease inhibitor cocktail (Roche, Germany). The protein concentration was determined by Bradford assay (Sigma, Germany). A volume of 60 μg protein was incubated in reaction buffer (25 mM HEPES pH 7.5, 50 mM NaCl, 10 % glycerol, 0.05 % CHAPS, and 5 mM DTT) in the presence of 50 μM fluorogenic substrate (Biomol, Germany), which was

specific for caspase-3 (DEVD-AMC), caspase-6 -8 10 (Ac-IETD-AFC) or caspase-9 (Ac-LEHD-AFC). Analyses were performed in triplicate.

Assays were performed in black microtitre plates (Nunc, Germany), and after 1 h incubation at 37 °C, the generation of free AMC or AFC was measured using a fluorometer plate reader (Appliscan, Thermo Fisher, Germany) at an excitation wavelength of 380 nm (AMC and AFC) and an emission wavelength of 460 nm (AMC) or 505 nm (AFC).

Western blot analysis

2×10^6 HT29, HCT-116, DLD-1, MKN-45, NCI-n87 and AGS cells were harvested after 1-h exposure to placebo, a dual PI3K/mTOR inhibitor (BGT226, 10 nM, Novartis) as positive control, BEZ235 and BKM120 at different concentrations (10 nM, 100 nM, 1 μM). Cells were washed twice with PBS (1×) and lysed in 2 × RIPA solution. For Western blot analysis, 60 μg protein was loaded on 8 % SDS-PAGE gels. After separation, the gel was transferred to nitrocellulose transfer membranes (Schleicher & Schuell, Dassel, Germany). Proteins were detected with specific primary antibodies (supplementary Table 2; 4 °C, overnight) in 5 % BSA. The specific secondary antibodies diluted 1:2,000 in 5 % non-fat dried milk were exposed for 1 h at room temperature. The ECL chemiluminescence detection kit (Perkin Elmer, Waltham, USA) was used for visualisation.

Statistical analysis

Significant effects between treatment groups or between treatment groups and control were accomplished by using the two-sample Student's t test using SPSS statistical analysis software. For more than two independent samples, the total significance level $a = 0.05$ was Bonferroni adjusted for each pairwise test. Results are expressed as mean standard deviation (SD). p values <0.05 were considered to indicate significant differences.

Results

Genetic background of human colon and gastric cancer cells

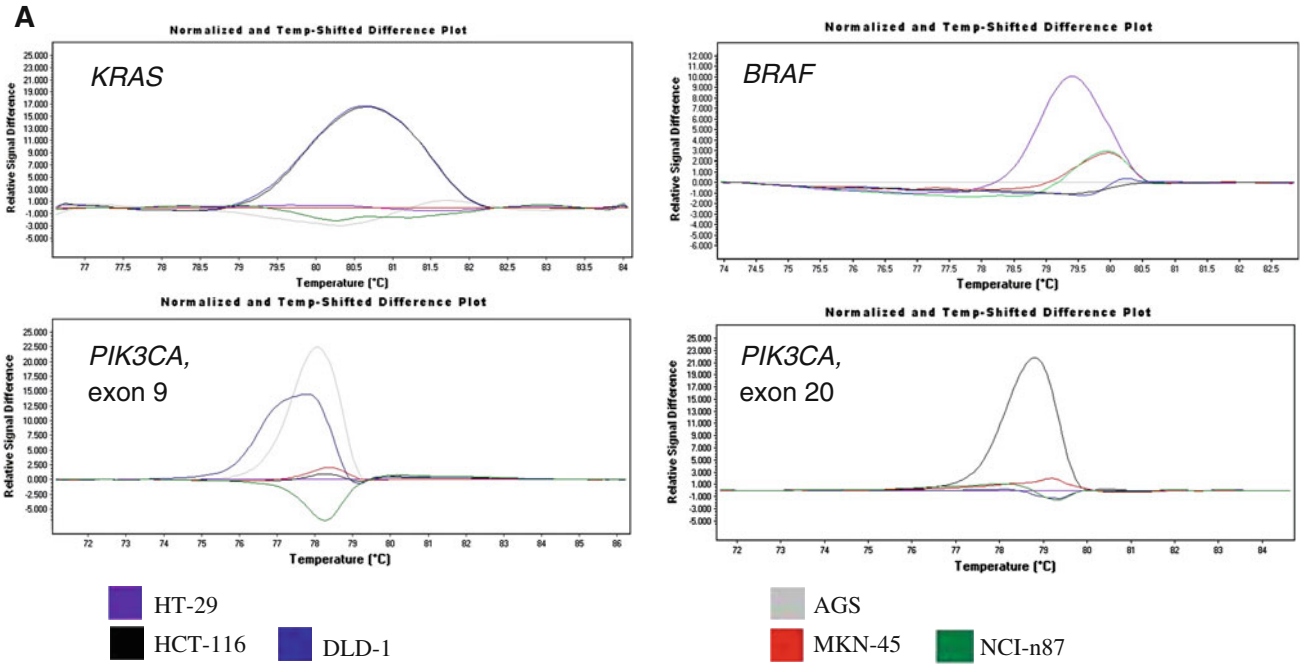
Before analysing the targeted agents in cell cultures, we genetically characterised the human colon (HT-29, HCT-116 and DLD-1) and gastric cancer (MKN-45, AGS, NCI-n87) cell lines for their mutation status at the previously described mutational hotspots, using high-resolution melting PCR. The detailed mutational spectra can be confirmed at http://www.sanger.ac.uk [24]. The known hotspots in *KRAS* (exon2-codon 12/13) were verified for the HCT-116, DLD-1 and AGS cell lines, which also showed mutations in *PIK3CA* at exon20-codon1047 (HCT-116), exon9-codon 542/545 (DLD-1 and AGS), respectively. Only the HT-29 cell line carried the *BRAF* mutation at exon 15-codon 600 and showed *PIK3CA* wild-type (wt) status for the mutation hotspots in exons 9 and 20. None of these mutations were detectable in the human gastric MKN-45 and NCI-n87 cells (Fig. 1a, b). The expression status of the typical target proteins of BEZ235 and BKM120 was then determined in the gastric and colon cancer cell lines by Western blotting. These assays confirmed the full presence of mTOR, its downstream target S6K1, PI3K with its catalytic subunit p110α and AKT (Fig. 1c).

BEZ235 and BKM120 decrease cell viability in all the gastrointestinal cell lines tested

Cell viability was measured on the basis of NADPH production in the cells by MTT assay after 3 days incubation. BEZ235 and BKM120 showed a concentration-dependent decrease in cell viability in all treated *PIK3CA* wt cell lines (HT-29, NCI-n87 and MKN-45) and *PIK3CA* mutated cell lines (HCT-116, DLD-1 and AGS). The colon cancer cells were more sensitive to the effects of BKM120 with an IC_{50} of 1 μM, whilst the gastric cancer cell lines required at least 2–5 μM. BKM120 induced a reduction of viability at higher concentrations than BEZ235. During treatment with BEZ235, a 50 % reduction in viability was reached at concentrations up to 100 nM in the colon cancer cell lines (Fig. 2a). BEZ235 had the greatest effect on the *PIK3CA* mutant DLD-1 cell line with an IC_{50} value of between 10 and 50 nM. For the gastric cancer model, the IC_{50} was reached only with a dose of BEZ235 above 250 nM (Fig. 2b). Again, BEZ235 reduced cell viability more effectively (at lower concentrations) than BKM120. Thus, the gastric cancer cells were less sensitive than the colon cells, and the mutated colon cells reacted more sensitively to the treatment than the wt cells. Combinations with cytotoxic agents such as irinotecan, oxaliplatin and 5-FU did not augment the effects of the inhibitors on cell viability (data not shown).

BEZ235 induces G1 arrest; BKM120 leads to a G2 shift

Propidium iodide-stained nuclei were detected by FACS for changes in cell cycle. A G1 arrest was observed after 2 days of treatment with 2 μM BEZ235. The increase in the number of cells in the G1 phase of the cell cycle was detectable in all colon and two gastric cancer cell lines, MKN-45 and NCI-n87 (Fig. 3a). AGS, the gastric cancer cell line with a *KRAS* and a *PI3KCA* mutation, reacted



B

mutation / cell line	*KRAS*	*BRAF*	*PIK3CA* exon 9	*PIK3CA* exon 20
HT-29	-	x	-	-
HCT-116	x	-	-	x
DLD-1	x	-	x	-
MKN-45	-	-	-	-
AGS	x	-	x	-
NCI-n87	-	-	-	-

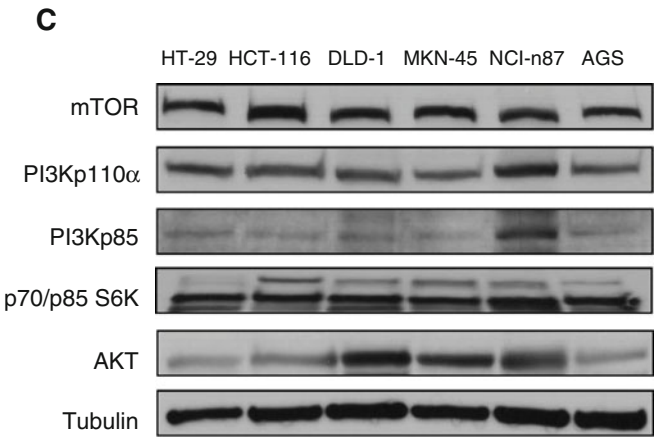


Fig. 1 Characterisation of human colon and gastric cancer cells on DNA and protein levels. **a** HRM-PCR data are presented as relative differences in fluorescence of the melting curves of mutated versus wild-type amplification products for *KRAS*, *BRAF* and *PIK3CA* (exon 9 and 20). **b** Overview of the mutations detected in human colon (HT-29, HCT-116 and DLD-1) and gastric cancer cells (MKN-45, AGS and NCI-n87). **c** Western blot of colon and gastric cancer cells to detect the total amount of proteins involved in the AKT signalling pathway (mTOR, PI3K, S6K1 and AKT PI3K p110) and targets of BEZ235 and BKM120. Tubulin was used as control for equal loading

only marginally to treatment. Overall, colon cancer cells reacted less sensitively than gastric cells (MKN-45 and NCI-n87).

In contrast, 2 μM BKM120 caused an S/G2 shift in one gastric and two colon cell lines (Fig. 3b). Within 2 days, BKM120 had forced the cells into the G2 phase, with more intense reactions from the colon cancer cell lines HCT-116 and HT-29 as well as the gastric line MKN-45. In general, the gastrointestinal cancer cells, especially the colon cancer model, responded better to BEZ235 than to BKM120 treatment.

Induction of caspase-3 after treatment with BEZ235 and BKM120 leads to a concentration-dependent increase in apoptosis

To examine whether BEZ235 and BKM120 have pro-apoptotic effects in gastric and colon cancer cell lines, we examined the induction of caspase activity using a fluorescence-based assay. Induction of caspase-3 after treatment with BEZ235 was found in all the cell lines, except the DLD-1 colon cancer cell line. The increase in enzyme activity was significant in the colon cancer cell lines

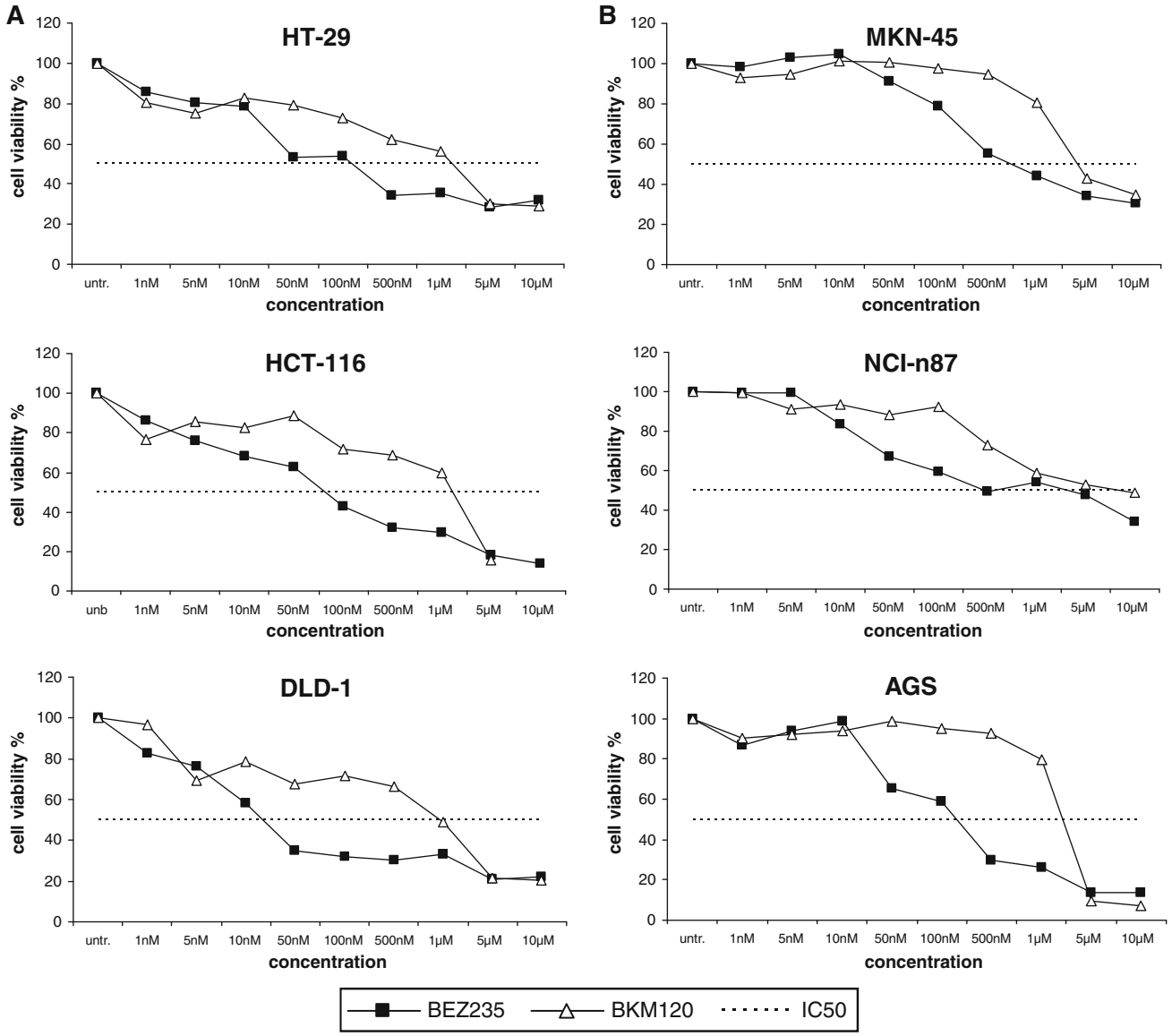


Fig. 2 Effects of BEZ235 and BKM120 on cell viability. **a** Human colon cancer cells HT-29, HCT-116 and DLD-1 showed an IC_{50} up to a concentration of 100 nM of BEZ235. **b** The gastric cancer cells MKN-45, NCI-n87 and AGS reacted less sensitively and did not reach the IC_{50} until a dose of 100 nM BEZ235 had been given. The viability of all 6 cell lines decreased to almost 50 % from 1 μM BKM120 upwards. *PIK3CA*-mutated cells, AGS, HCT-116 and DLD-1 reacted more sensitively to treatment than the HT-29, MKN-45 and NCI-n87 cell lines. Data are expressed as mean percentage of untreated controls. Experiments were done in three separate experiments, each in triplicate. All standard deviations were less than 10 % and have been ignored for clarity in the diagrams

HCT-116 and HT-29 after 24 h treatment with 5 μM BEZ235. The PIK3CA mutant DLD-1 showed no effects, so the caspase activation seems to be independent of the known mutations. Caspase-3 activity was significantly increased after incubation with the dual PI3K/mTOR inhibitor BEZ235 in all the gastric cancer cell lines. In particular, the wt NCI-n87 cell line showed a fourfold enhancement when compared with the untreated control. The pure PI3K inhibitor BKM120 also showed an induction of caspase in the HT-29 and HCT-116 colon cancer cell lines. Furthermore, the wt gastric cell lines, NCI-n87 and MKN-45, showed a fourfold and twofold increase in caspase activity, respectively. The relative caspase-3 activation of AGS was also slightly increased compared to the control (Fig. 4a). Induction of caspase-6, caspase-8 and caspase-10 was measured but showed no variations (data not shown).

A PI flow cytometry assay was used to detect apoptosis in all cells after treatment with BEZ235 and BKM120 and demonstrated distinct, dose-dependent induction of apoptosis. As a single agent, BEZ235 induced higher apoptosis rates at concentrations of more than 2.5 μM in colon

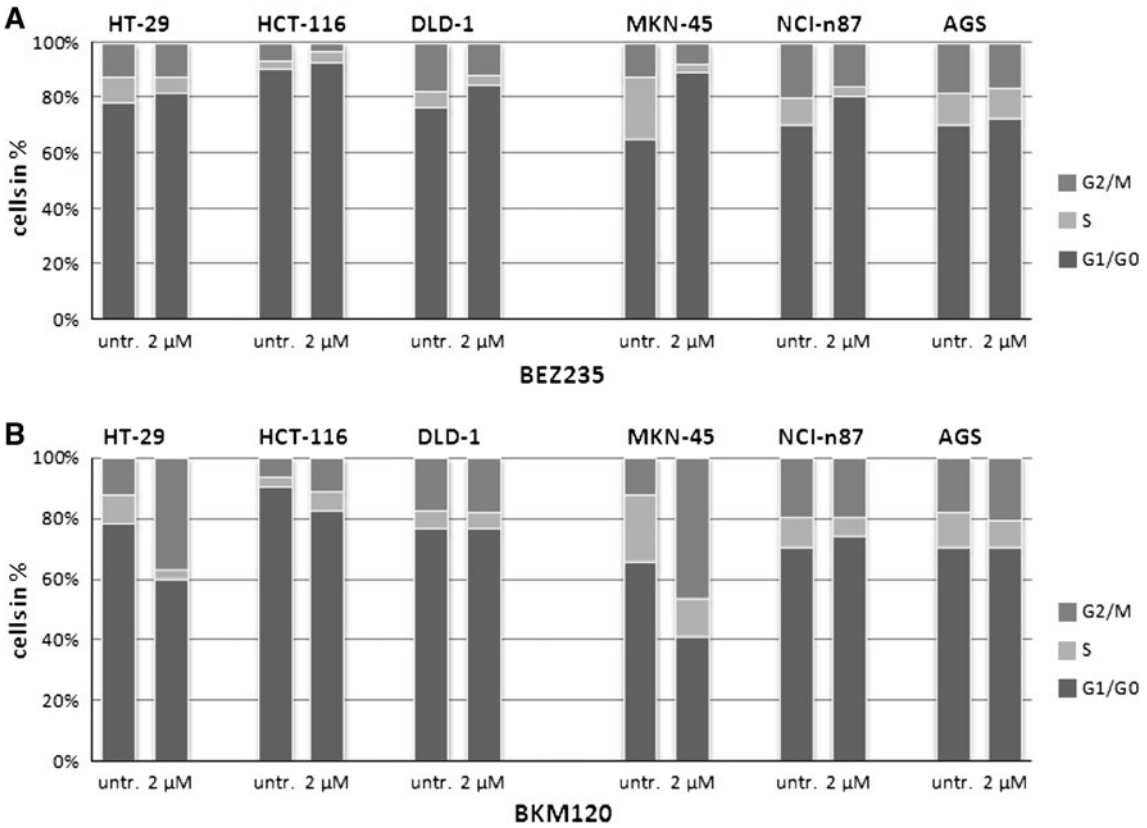


Fig. 3 Effects of BEZ235 and BKM120 on cell cycle. **a** Cells were treated with 2 µM BEZ235 for 2 days and incubated with Nicoletti buffer. G1/G0 cells were presented in the *black column*, in the *light grey,* the cells in the S phase and in *grey* the percentage of G2/M cells. Treatment with BEZ235 led to G1 arrest in all colon and gastric cancer cell lines. **b** Cells were treated with 2 µM BKM120 for 2 days. Two of three colon cell lines (HT-29 and HCT-116) and one gastric cancer cell line (MKN-45) showed a shift in the G2 phase after treatment with BKM120. Data are expressed as mean percentage of untreated control. Representative results from 3 separate experiments are shown

cells. In this setting, the DLD-1 cell line seemed to be less sensitive (<8 % sub G1 cells) to BEZ235 treatment, than the HT-29 and HCT-116 cell lines, which reacted with 22 and 34 % apoptotic cells under the same conditions. The gastric cancer cells MKN-45, NCI-n87 and AGS showed moderate induction of apoptosis with up to 35 % apoptotic cells after treatment with BEZ235 for 4 days at concentrations up to 5 µM (Fig. 4b). In contrast, at concentrations of 5 µM, BKM120 led to a higher induction of more than 65 % apoptotic cells in the mutant cell line HCT-116 and the wt HT-29, whilst the mutant DLD-1 reacted with only 20 % apoptotic cells. BKM120 also decreased cell survival in the gastric cancer cell lines. The NCI-n87 cell line associated with high caspase induction also underwent apoptosis at lower doses of BKM120 than the other gastric cancer lines. Here, concentrations of 1 µM BKM120 were sufficient to induce 40 % apoptotic cells for NCI-n87, compared with the 2.5 µM for MKN-45 and the 5 µM for AGS required to reach the same amount on sub G1 cells (Fig. 4b). The apoptosis induction by the different inhibitors on the gastric cancer lines was weaker than that seen for the colon cell lines. For the colon cell lines HT-29 and HCT-116, BKM120 was able to induce levels of apoptotic cells of more than 60 %.

BEZ235 and BKM120 enhancement of irinotecan cytotoxicity in colon cancer cells

Administration of BEZ235 in combination with irinotecan showed synergistic effects for the induction of apoptosis in colon cancer cells (Fig. 5a). Except for the HT-29 cell line, no effects were seen with 5-FU (Suppl. Fig 1a). Also no supportive effects were seen with oxaliplatin (data not shown). With BEZ235 in combination with irinotecan, the apoptotic effects were more than 20 % higher than for either agent alone. The greatest induction of apoptosis was found for the *KRAS* wt colon cells HT-29. The percentage of apoptotic cells increased from 5 % in untreated HT-29 control cells to 45 % and more than 60 %, following incubation with 1 and 2 µM BEZ235 combined with 1 µg/mL irinotecan, respectively. The percentages under monotherapy were less than 12 and 20 %, respectively. The mono application was associated with a twofold increase in

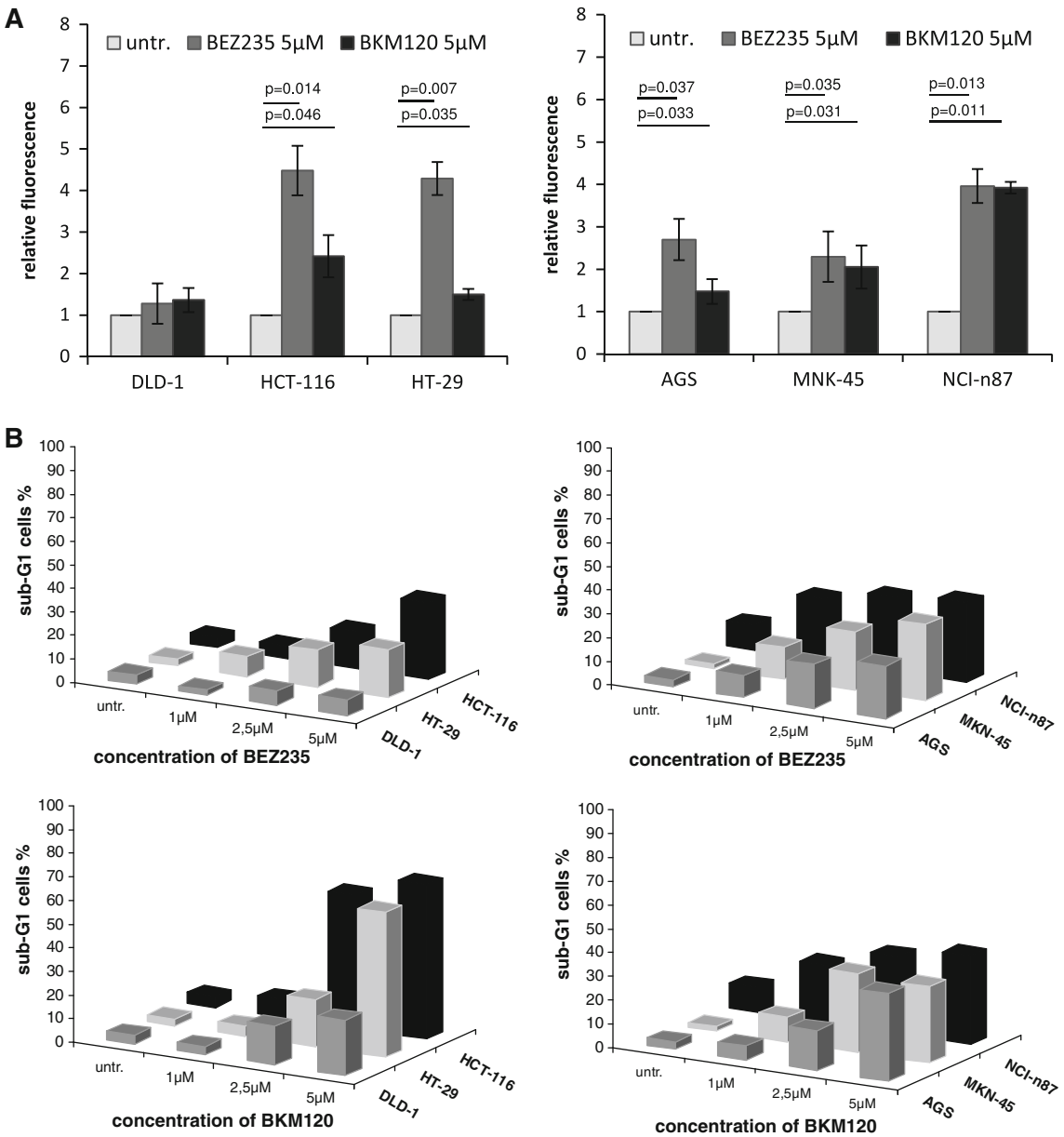


Fig. 4 Caspase and apoptosis induction by BEZ235 and BKM120. **a** Cells were treated with 5 μM BEZ235 or BKM120 for one day. The isolated proteins were incubated with fluorescence-linked substrate for the caspases. The increase in caspase-3 activity in the colon cancer cells (*left panel*) was significant for HT-29 and HCT-116 after treatment with both substances. In all gastric cell lines (*right panel*), treatment with the inhibitors led to a marked increase in caspase-3 activity. Findings as mean from at least three independent experiments with standard deviation are presented in relation to the untreated control. Statistically significant ($p < 0.05$) induction of caspase 3 is displayed with $p=$. **b** Cells were treated with increasing doses of the inhibitors for 4 days, and cells of the sub-G1 phase were detected by flow cytometry. The colon cancer cells HT-29, HCT-116 and DLD-1 (*left panel*) reacted in a concentration-dependent manner to treatment with BEZ235. Treatment with BKM120 led to broad induction of apoptosis in the colon cancer cell lines. HCT-116 reacted most sensitively to both substances (*lower panel*). The gastric cancer cells MKN-45, NCI-n87 and AGS (*right panel*) showed moderate induction of apoptosis after treatment with BEZ235 and BKM120. The standard deviation amounted to less than 10 % and has been ignored in the diagrams. Data are presented as mean from at least three independent experiments relative to the untreated control

caspase-3 activity in HT-29 (Fig. 4a). The PIK3CA mutated colon cells reacted similarly under combination therapy, with a maximum of 50 and 35 % of apoptotic cells for the HCT-116 and DLD-1 cell lines, respectively. The combination of cytotoxic agents with BKM120 lead to no clear augmentation of the pro-apoptotic effects on the colon cancer cell lines.

BEZ235 combined with irinotecan did not enhance the induction of apoptosis in the wt gastric cancer cell lines MKN-45 and NCI-n87 (Fig. 5b). Only mutated AGS

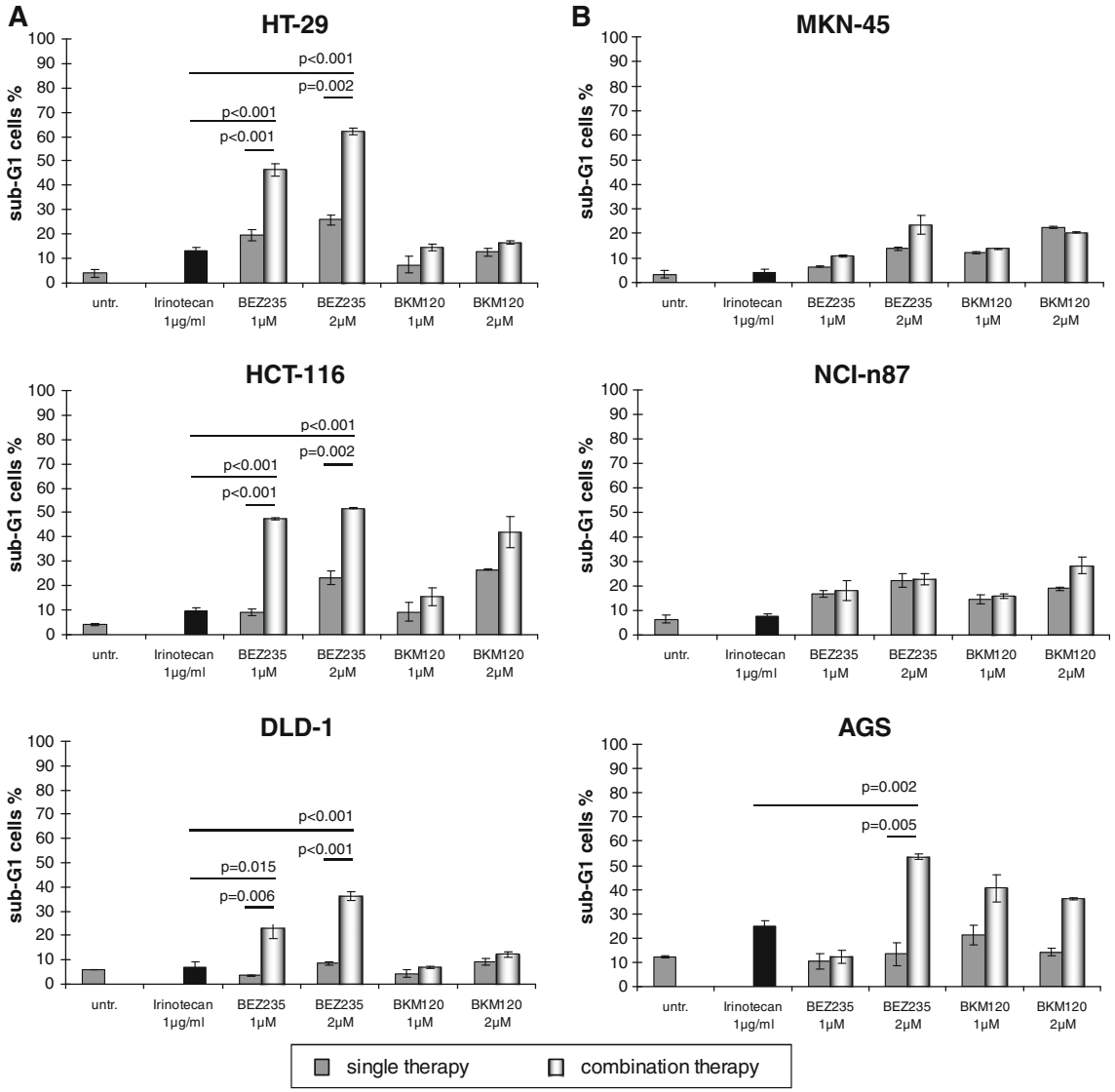


Fig. 5 Apoptosis induction by combination of BEZ235 or BKM120 with chemotherapeutic agent irinotecan. Cells were treated with 1 and 2 µM of BEZ235 and BKM120 or either agent combined with 1 µg/mL irinotecan for 3 days, harvested, and the sub-G1 phase was detected by flow cytometry. **a** The combination of irinotecan with BEZ235 led to a synergistic induction of apoptosis in the colon cancer cells, but only an additive effect could be seen for the combination with BKM120. B, the treatment of irinotecan combined with BEZ235 or BKM120 did not intensify the induction of apoptosis in the gastric cancer cells MKN-45 and NCI-n87. The mutated AGS reacted in the highest combination with a synergistic increase in apoptotic cells. Results are displayed as mean with standard deviation of three independent experiments. Statistically significant ($p < 0.05$) induction of apoptosis is displayed with p=, and in such cases, the sum of the mono applications (substance and irinotecan) are significantly lower as the achieved synergistic effect ($p < 0.05$)

responded to the combination of 2 µM BEZ235 and 1 µg/mL irinotecan with a synergistic increase in apoptotic cell death to 50 % from 12 (control), and 25 % when BEZ235 was given alone. The combination of 5-FU and BEZ235 had no additional effects on any of the cell gastric lines (Suppl. Fig 1b), nor did oxaliplatin combined with the inhibitors enhance the degree of apoptosis in any of the cell lines (data not shown). Again, the PIK3CA wt gastric cancer cells showed no synergistic effects during combined exposure with BEZ235 and any cytotoxic agent (5-FU, oxaliplatin or irinotecan). The PIK3CA mutated AGS was the only gastric cancer cell line with an additional increase in apoptosis in the combination with irinotecan. Apoptosis-inducing effects were not supported or enhanced by the PI3K inhibitor BKM120, similar to the colon cancer results.

Inhibition of AKT and mTOR signalling pathway by BEZ235 and BKM120 in wt and mutated gastrointestinal cancer cell lines

We explored the regulation of protein levels by BEZ235 and BKM120 using Western blot analysis. As a dual

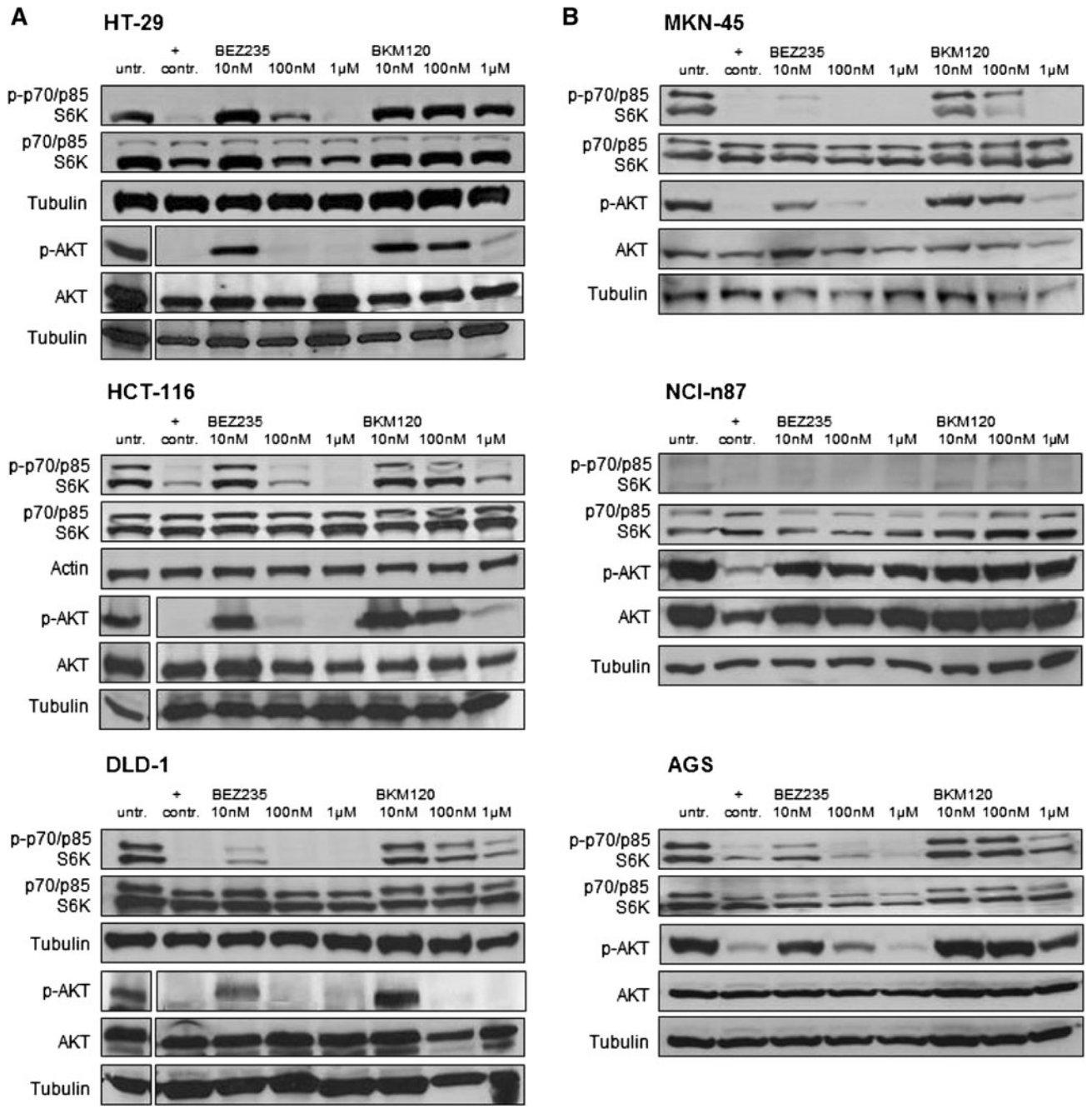


Fig. 6 Influence of BEZ235 and BKM120 on AKT and mTOR signalling pathways. Cells were treated with increasing doses of the inhibitors for 1 h, and 60 μg protein was analysed by Western blot. **a** The activation of AKT and S6K1 was inhibited in all colon cancer cells dependent on the concentration of the substances. The effect of BKM120 was lower, especially on S6K1 inhibition. DLD-1 reacted most sensitively. **b** pAKT and p70S6K were also suppressed in the gastric cancer cells MKN-45 and AGS, but p70S6K could not be detected in NCI-n87. AKT activation was not inhibited by BEZ235 or BKM120 at the concentrations used. Tubulin and actin were used as control for equal loading. Blots were repeated for three times

PI3K/mTOR inhibitor, BEZ235 totally blocked pAKT signalling at a concentration of 100 nM in all colon cancer cell lines (Fig. 6a). Notably, the mutant DLD-1 cell line already showed partial inhibition at a concentration of 10 nM. The downstream protein of mTOR analysed was S6K1. The phosphorylation of this protein was completely inhibited by BEZ235 in all colon cancer cell lines at a concentration of 1 μM and of 100 nM for the DLD-1 cell line. Only 10 nM BEZ235 was necessary to clearly reduce the p70S6K signal in this cell line. Thus DLD-1, a PIK3CA mutant, reacted was most sensitive to BEZ235.

BKM120 induced pAKT inhibition in the colon cancer cell lines HT-29 and HCT-116 at concentrations of only 1 μM. Only the mutant DLD-1 cells demonstrated total pAKT inhibition at lower dose levels of 100 nM. BKM120, a straight PI3K inhibitor, partial inhibited the p70S6K phosphorylation only at its highest concentration of 1 μM and only in mutated cells. The mutated colon cancer cell line DLD-1 showed clear downregulation of p70S6K, despite BKM120 not being a direct mTOR inhibitor.

In the wt gastric cancer cells line MKN-45 and the PIK3CA mutated cell line AGS, the phosphorylated PI3K effectors pAKT and p70S6K were partial blocked by treatment with BEZ235 and BKM120 (Fig. 6b). BEZ235 inhibited the phosphorylation of pAKT at concentrations of 0.1–1 μM and p70S6K at 10–100 nM in MKN-45. In AGS, a nearly full inhibition of these downstream targets could be reached at concentrations of 1 μM BEZ235. In these two lines, the mTOR downstream target p70S6K seemed to be more efficiently inhibited by BEZ235 than the AKT pathway in the range used. BKM120 had to be administered at concentrations of at least 1 μM to block the p70S6K signal and nearly the whole pAKT signal in the MKN-45 cell line and to start the reduction of the phosphorylation level of these proteins in the AGS cell line. In contrast, in the wt cell line NCI-n87, AKT activation was not inhibited by the concentrations of up to 1 μM of BEZ235 or BKM120 used, when compared with the untreated controls and the positive control. Activated S6K1 and p70S6K could not be detected at all in this cell line.

No additional blocking effects of other phosphorylated proteins involved in the AKT signalling pathway, such as PDK1, PTEN or mTOR itself, were observed (data not shown).

Discussion

Currently, clinical oncologists are struggling to optimise the treatment of cancer because new therapeutic options are accompanied by concomitant tumour-escape mechanisms. Recent improvements in the treatment of gastrointestinal cancers, such as anti-EGFR therapy, are limited by resistance due to oncogenic mutations in signalling receptors and pathways. In colorectal carcinoma, 40 % of patients carry a *KRAS* mutation; 15 %, a *BRAF* mutation; and up to 20 %, a *PI3KCA* mutation [25]. These patients do not respond to available anti-EGFR therapies. These mutations are also associated with more aggressive tumours with more marked tumorigenesis, as reflected in the shorter progression free survival, shorter overall survival, and higher rates of lung metastasis observed in metastatic colorectal cancer patients [26, 27]. Similarly, 20 % of gastric cancer patients carry a *KRAS*, <10 % a *BRAF* and 10–15 % a *PI3KCA* mutation [28]. In this setting, microsatellite instability (MSI) seems to correlate with the PI3K and MAPK pathway-associated mutations [29]. Therapy-induced resistance after development of mutations in tumour-associated signal pathways is and will be an increasingly serious challenge over the next few years [30]. Recently, it was shown that BEZ235 could override drug-induced resistance against the irreversible ErbB inhibitors in human breast cancer cells [31]. *PI3KCA* mutations are also prevalent in breast cancer patients and BEZ235 also seems to abrogate lapatinib resistance [32].

Direct, intracellular, specific inhibition of single signalling pathway molecules such as PI3K is the next step in targeted tumour therapy. Both the novel PI3K inhibitor BKM120 and the dual PI3K/mTOR inhibitor BEZ235 specifically inhibit the PI3K/AKT pathway, independent of the mutational status of the catalytic subunit 110α of PI3K.

In the first steps, we characterised the mutational status in the currently known hotspot regions of the human colon and gastric cancer cells used in our laboratory. We did this for mutations in *PIK3CA* (E542K, E545K and H1047R), *KRAS* (G12V and G13D) and BRAF (V600E). A combination of *PIK3CA* and *KRAS* was detected in AGS, DLD-1 and in HCT-116; only BRAF mutations were detected in HT-29, and MKN-45 and NCI-n87 cell lines were wt for all measured polymorphisms. The missense mutation P449T in *PIK3CA* is reported for HT-29. So far, this gain of function mutation is not described in the clinic for constitutive activation or induction of resistance to inhibitors. Mutations in *BRAF* and *KRAS* belonging to the MAPK signalling pathway are mutually exclusive [33]. The two mutations in *PI3KCA* and *KRAS* can, however, occur together [20], whilst RAS can also cross talk to the AKT pathway by activating PI3K [13]. This arrangement of the mutation combinations was also seen in the colon and gastric cancer cells used.

Both compounds were effective in blocking the PI3K/AKT signalling pathway. BKM120 reduced the viability of the colon and gastric cancer cells only with higher IC_{50} values, that is, in micro molar concentrations, whilst BEZ235 was effective in the nano molar range. In general, colon cancer cell lines were more sensitive to a reduction in viability by blocking the PI3K pathway than the gastric cancer cell lines. Overall, cells with mutations, that is, all colorectal and the gastric carcinoma AGS cells responded more sensitively than the *PI3KCA* wt NCI-n87 and MKN-45 gastric cancer cell lines. Our results confirm that *PIK3CA* mutations in gastric tumours increase the response rate to PI3K/mTOR inhibitors. Moreover, colon cancer cell lines reacted independent of their mutational status of the *PI3KCA* to such inhibitors [34, 35]. Due to their oncogenic mutations, these cells may be more aggressive and intensively driven by the AKT-dependent pathways, which in

addition could be amplified by KRAS mutation [36]. These mutated cells therefore reacted more sensitively to AKT pathway inhibition.

In our analysis, BEZ235 induced G1 arrest in all cells, whereas in two colon and one gastric cancer cell lines, BKM120 tended to induce a G2 shift that was followed by apoptosis induction. This related G1-arrest phenomenon—which led to inhibition of cell proliferation which was not necessarily associated with marked cell-death induction—has also been seen with other PI3K inhibitors in a range of different carcinomas [37–39]. Here, modulation of the cycle regulatory proteins p21 and p27 by inhibition of the PI3K–AKT pathway may be a possible explanation, which has already been shown in breast, pancreatic and lung tumour cells [40, 41].

Apoptosis-related caspase-3 was induced by BEZ235 in all gastric cell lines and only in the HT-29 and HCT-116, colon cancer cell lines. BKM120 did so in the same cell lines. This caspase induction was followed by apoptosis, as has been observed in other human tumour cells with these agents [42].

BKM120 and BEZ235 directly induced apoptosis at only micro molar concentrations, with BKM120 appearing more toxic than BEZ235, as also seen for the G2 shift. BKM120, which has a greater effect when given alone, was also more effective in colon cancer cell lines, regardless of mutational status. In combination with cytotoxic agents such as irinotecan, synergistic effects have been observed in all colon cancer cells with BEZ235. Similar synergistic effects for the combination of BEZ235 with an MEK inhibitor were also detected in melanoma cells and for combinations with cytotoxic agents in different entities [43–45]. Different in vitro and in vivo analyses have shown that AKT inhibition sensitises the cells to apoptotic stimuli from chemotherapy [46, 47]. No such effects were seen for the gastric cell lines. Besides the *PI3KCA* mutant cell line AGS were slightly more responsive than the wt cells. Here, a synergistic induction of apoptosis was detected for 2 μM BEZ235 in combination with irinotecan. That gastric cells were not as sensitive to PI3K-inhibiting therapy as colon cells may be due to escape mechanisms and cross talk between pathways as well as the higher frequency of mutations in the signalling cascades in colon cancer [31].

BEZ235 and BKM120 have already been investigated in several different cancer cells. BEZ235 was seen not to induce apoptosis in *KRAS* mutant breast cancer cells but did induce apoptosis in *PI3KCA* mutants [42]. BEZ235 was shown to be effective in PI3KCA mutant lung carcinoma cells, not in *KRAS*-driven lung carcinoma cells. Here, a combination of the PI3K inhibitor with a MEK blockade was successful in overcoming *KRAS*-induced resistance [48].

Characterisation of the signalling cascades led to specific differences. Taken together, BEZ235 was more potent than BKM120, with distinct inhibition of pAKT in all colon cancer cells. AKT, as a key regulator, promotes cell growth and protein synthesis, especially via the serine/threonine protein kinase GSK3, and also reduces cell cycle inhibitors such as p27. As a consequence, production of cell cycle proteins such as c-myc is induced, which also promote the anti-apoptotic effect [49]. Furthermore, BEZ235, as a dual PI3K/mTOR inhibitor, also clearly reduced the phosphorylation of p70S6K. BKM120 was only effective in higher concentrations, and in this case, it also inhibited p70S6K as the downstream target of AKT/mTOR. In contrast, in the gastric cells, pAKT inhibition was more variable. The wt cell line MKN-45 showed a broadly similar response to those of the colon cell lines. In contrast, the wt NCI-n87 cells did not change the regulation of AKT. These cells may contain unknown mutations that activate the AKT pathway. The mutated AGS gastric cells responded to BEZ235, but only at higher concentrations. They did not respond to BKM120, although S6K1 was close to activation. In vivo BEZ235 has inhibited the phosphorylation of AKT and mTOR downstream signalling proteins, has reduced proliferation and has induced apoptosis in a range of breast, prostate or glioblastoma cells [23, 42, 50]. Furthermore, BEZ235 has also inhibited VEGF-induced angiogenesis, which is one important hallmark of solid tumours [51, 52]. There is also evidence that BEZ235 combined with docetaxel can inhibit cancer stem cells in prostate cancer as well as glioblastoma [53, 54]. Controlling the activity of this pathway by effective inhibitors might improve cancer therapy [30], and not only in gastrointestinal tumours.

BEZ235 and BKM120 have so far been investigated in different solid tumour entities in a number of clinical trials, including colorectal cancer, but not gastric cancer. Increasing resistance to treatment because of mutations in tumour-specific signalling has been observed. New substances are therefore needed urgently to enable patients to be offered effective, tailored treatment. The critical role of predictive and prognostic biomarkers in relation to the PI3K pathway must be thoroughly investigated [55, 56].

Conclusion

This study showed that the PI3K inhibitor BKM120 and the dual PI3K/mTOR inhibitor BEZ235 are active in human colon independent of the mutational status of *KRAS, BRAF* as well as *PIK3CA* and especially in mutated gastric carcinoma cells. Particularly, the PI3K 110α subunit mutant cells (HCT-116, DLD-1 and AGS) reacted more sensitively to treatment with small molecules. We further showed a strong synergy with the combination of BEZ235 and the cytotoxic agent irinotecan in different colon cancer cells,

but not for wt gastric cancer cells. Whilst BEZ235 specifically inhibited the AKT and S6K1 signals, BKM120 directly and efficiently blocked AKT as a downstream PI3K signal. Thus, our results clearly support the treatment of human colon cancers with dual PI3K/mTOR inhibitors.

The two highly active substances investigated here are now under clinical investigation in different solid tumours. We suggest that treatment with BEZ235 is a promising therapeutic strategy for colon cancer patients, especially after recurrence of tumour resistance.

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Conflict of interest All authors declared no conflict of interest. There are no other financial or non-financial competing interests.

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