Preclinical Development

Characterization of the Mechanism of Action of the Pan Class I PI3K Inhibitor NVP-BKM120 across a Broad Range of Concentrations

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Abstract

The pan-phosphoinositide 3-kinase (PI3K) inhibitor BKM120 was found, at high concentrations, to cause cell death in various cellular systems, irrespective of their level of PI3K addiction. Transcriptional and biochemical profiling studies were used to identify the origin of these unexpected and apparently PI3K-independent effects. At 5- to 10-fold, the concentration needed to half-maximally inhibit PI3K signaling, BKM120 treatment caused changes in expression of mitotic genes and the induction of a robust G2–M arrest. Tubulin polymerization assays and nuclear magnetic resonance-binding studies revealed that BKM120 inhibited microtubule dynamics upon direct binding to tubulin. To assess the contribution of this off-target activity vis-à-vis the antitumor activity of BKM120 in PI3K-dependent tumors, we used a mechanistic PI3K-α-dependent model. We observed that, in vivo, daily treatment of mice with doses of BKM120 up to 40 mg/kg led to tumor regressions with no increase in the mitotic index. Thus, strong antitumor activity can be achieved in PI3K-dependent models at exposures that are below those necessary to engage the off-target activity. In comparison, the clinical data indicate that it is unlikely that BKM120 will achieve exposures sufficient to significantly engage the off-target activity at tolerated doses and schedules. However, in preclinical settings, the consequences of the off-target activity start to manifest themselves at concentrations above 1 μmol/L in vitro and doses above 50 mg/kg in efficacy studies using subcutaneous tumor-bearing mice. Hence, careful concentration and dose range selection is required to ensure that any observation can be correctly attributed to BKM120 inhibition of PI3K.

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Introduction

The phosphoinositide 3-kinase (PI3K) pathway plays a pivotal role in cell growth, proliferation, survival, and metabolism (1, 2). Lesions in key pathway components can lead to gain-of-function, pathway hyperactivation, aberrant cell proliferation, and subsequently to the pro-
motion and maintenance of cancer. For example, the PIK3CA gene encoding the p110α catalytic subunit has been found to be amplified and frequently mutated in a variety of human cancers (3). Furthermore, the antagonistic dual lipid/protein phosphatase PTEN is often inactivated by copy number loss, mutation, or epigenetic silencing (4). In addition, the downstream target Akt is often engaged by PI3K inhibitors (5). Over the last years, evidence of oncogenic mutations in the gene coding for the regulatory subunit of PI3K, p85, has also been accumulating (6, 7). Last but not least, constitutively activated receptor tyrosine kinases, such as for example, amplified HER2 (breast) can cause hyperactivation of the PI3K pathway (8).

The pharmaceutical industry heavily invested in the last decade to develop PI3K inhibitors with various profiles, such as dual mTOR/PI3K, pan-PI3K, and even isoform-specific PI3K inhibitors for clinical application. From this plethora of molecules (9), efficacy and safety data from phase I clinical trials have recently become available (10).

NVP-BKM120 (referred herein as BKM120) is a pan-
PI3K inhibitor, which has recently entered clinical phase II
for treatment of PI3K-dependent cancers (11). In mechanistic cellular systems, BKM120 inhibits all class IA PI3K paralogs (p110α, β, and δ) that are generally activated by receptor tyrosine signaling. In contrast, BKM120 does not significantly inhibit class II and IV PI3K homologs or protein kinases.

When tested in proliferation assays across a large panel of cell lines (the Cell Line Encyclopedia or CLE) encompassing different lineages and oncogenic addictions, BKM120 behaved differently compared with other PI3K inhibitors, at concentrations above 2 μmol/L. Specifically, the compound was efficacious against tumor lines that did not display PI3K addiction. Hence, despite the fact that its biochemical profile is very specific, we suspected that at concentrations 5- to 10-fold of those necessary to half-maximally modulate PI3K signaling, other properties were acquired. Here, we show that BKM120, at high concentrations, can act as a microtubule destabilizer via direct tubulin binding. The consequences of these findings for the interpretation of in vitro and in vivo data are presented and discussed.

Materials and Methods

Compounds, reagents, and antibodies

The structures of the compounds used in the manuscript are shown in Fig. 5B. BKM120 (Novartis), BE2235 (Novartis), and GDC-0941 (BioDuro) were prepared as 10 mmol/L stock solutions in 100% dimethyl sulfoxide (DMSO). Working solutions were freshly prepared before addition to the cell media such that final DMSO concentrations were kept constant at 0.1% in both control and compound-treated cells. Nocodazole (#M1404) and poly-β-lysine (#P6407) were purchased from Sigma. MG-132 and N,N′,N″,N′′-tetramethyl-2-phenylindole (DAPI) were from Calbiochem (#474790) and Invitrogen (#D3571), respectively. The origin of the primary antibodies used were as follows: anti-S473P-Akt (#9271), anti-Histone 3-P (#9701), anti-caspase 7 (#9491), anti-alpha tubulin (#F2128) antibodies were from Sigma. The secondary Alexa Fluor 568–conjugated anti-mouse antibody (#11031) was purchased from Invitrogen.

In vitro assays

Tubulin polymerization assay. All assays were conducted with the porcine tubulin polymerization kit from Cytoskeleton (#BK006-P), according to the manufacturers’ protocol.

Nuclear magnetic resonance–binding studies. Before studies, lyophilized purified bovine brain tubulin (Cytoskeleton, # TL238) was dissolved in 50 mmol/L PBS (pH 7.0), without GTP and Mg2+ to prevent polymerization. BKM210 was freshly prepared as a 20 mmol/L stock solution in d6-DMSO (Armar Chemicals/# 015200.2940). The final concentration in nuclear magnetic resonance (NMR) samples was 0.2 mmol/L. The spectroscopy studies were conducted on a Bruker AV-III-600 spectrometer equipped with a QCI cryo-probe for sensitive detection of 1H and 19F. T1ρ experiments were recorded with a 6 kHz spinlock pulse of 10 to 200 ms and acquisition using excitation sculpting for water suppression. T2 experiments were measured with a CPMG pulse train of 200 ms. WaterLOGSY experiments were measured in sensitive mode as described before (12).

Cellular biology

Cell lines and cell culture. All human cell lines are part of the Cancer Cell Line Encyclopedia from the Broad Institute (Cambridge, MA) and have been authenticated by 46SNP fingerprinting and expression arrays. Accordingly, these cell lines were obtained from the Broad Institute. (13). Cells from the original purchased vials were expanded and a reserve stock of 12 vials created. Master and working stocks were prepared by the individual Novartis laboratories from 1 reserve vial and were used for the described studies. A2058, MDA-MB231, U87MG, MCF-7, and Rat1-myr-p110α cells (11) were cultured at 37°C in 5% CO2 and 80% relative humidity in either Dulbecco’s Modified Eagle’s Media (DMEM; MDA-MB231, A2058, MCF7, and Rat1-myr-p110α cells), EEMEM (U87MG) high glucose media (Gibco) supplemented with 10% FBS, 2 mmol/L glutamine, 1% penicillin/streptomycin, and 1% sodium pyruvate. MCF7 pools expressing MCF7-myr-Akt or not (MCF7-BP) a hemagglutinin (HA)-tagged version of a dominant-active form, myristoylated form of Akt, were generated upon infection of parental MCF7 cells with viral particles generated from a pBabe-puro–based retroviral expression vector (material and sequences are available on request).

Proliferation assays, cell lysis preparation for Western blotting, and S473P-Akt reverse-phase protein array phosphorylation assays. Antiproliferative activities (GI50) as well as cell death markers (LD50 and LD90) were quantified by methylene blue staining, as described (14). Biochemical characterization upon compound exposure was conducted on the mentioned cells seeded in 10-cm dishes at the indicated inoculum. Cells were exposed either for either 1 hour (for PI3K pathway markers) or 6 hours (for G2–M markers), before lysis for Western blotting, and S473P-Akt reverse-phase protein array (RPA) analysis as described (14).

Colony formation and fluorescence-activated cell-sorting assays. The fluorescence-activated cell-sorting (FACS) assays were conducted as described (15). Colony formation assays were conducted by seeding 5 × 103 MCF7-BP of MCF7-myr-Akt cells in 6-well clusters. Sixteen hours later, the medium was discarded and replaced with 2 mL of fresh medium containing the test items. The media were replaced every 3 days throughout the experiment. The experiment was stopped by adding 500 μL of 20% glutaraldehyde to the media. Ten minutes later, the wells were washed with water and exposed to a 0.05% methylene blue solution for 15 minutes. Wells were then washed with water and colonies photographed with a Canosan 4400F scanner.

Immunofluorescence of tubulin networks. Cells were seeded on 6-well dishes containing poly-β-lysine–treated...
coverslips. For investigating effects on the mitotic tubulin network, cells were treated with the indicated inhibitors either for 24, 6, or 6 hours followed by an 18-hour washout period. Cells were fixed for at least 15 minutes with ice-cold methanol (−20°C), washed 3× with PBS, and blocked for 10 minutes with 3% bovine serum albumin (BSA)/PBS at room temperature. The primary antibody of choice was incubated in a moist chamber for 3 hours at room temperature (diluted 1:500 in blocking solution), washed 3 times with PBS, and incubated for 1 hour at room temperature with the secondary antibody (diluted 1:400 in blocking solution) and DAPI (diluted 1:1,000) in a moist, light-protected chamber. The cells were washed 3 times with PBS and mounted with a drop of prolong gold-antifade (Invitrogen, Ref# P36930) on glass. The next day, coverslips were sealed with nail polish, and the cells were analyzed and photographed with a Zeiss Axioplan microscope. For tubulin network in interphase cells, the microtubule network of the cells was challenged by transferring the plates for 1 hour from 37°C to 4°C and switched back to 37°C for 1 hour in presence or absence of the indicated concentration of the test item. Cells were fixed, stained, and analyzed for the effects of the treatment conditions on the microtubule network as above.

**Gene expression analysis**

*mRNA extraction and microarray profiling.* mRNA was extracted with the QiShredder and RNeasy Mini Kit (Qiagen/#79656 and #74104, respectively), according to the manufacturer’s protocol. Synthesis of labeled cDNA, hybridization to HG-U133-plus2 arrays (Affymetrix Inc.), and quality control and processing using the MAS5 algorithm were done essentially as described previously (16). Microarray data are available at the Gene Expression Omnibus (GEO) database under the accession number GSE33643.

*Expression data analysis.* Analysis was restricted to Affymetrix probe sets mapping unambiguously to single Entrez gene IDs (NetAffx annotation version na29). Furthermore, when multiple probe sets were assigned to the same Entrez gene IDs, only those with highest values (percentile 90) in an internal reference data set of 5216 HG-U133-plus2 arrays were kept. Data were log2-transformed, and a subsequent filter (median > 2.25) was applied to exclude low expression genes, decreasing the total number of analyzed genes to 14,104. Principal component analysis was run using the Partek Genomics Suite 6.4 (Partek Inc.) using the default parameters (dispersion matrix, correlation; normalized eigenvectors). To generate the BKM120 off-target effect gene list, the loadings of the second and third components, which maximized the separation between the BKM120 IC50/max sample group and the rest of the samples, were used. Gene scores were derived from the loadings by taking the absolute values of the sum of the loadings for each gene. The gene list ranked according to this score was submitted to a gene set enrichment analysis (GSEA).

GSEA was conducted with an in-house implementation of Mootha’s method using the 2-sample Wilcoxon rank-sum test (17, 18) using the MetaCore database by GeneGO, Inc. The enrichment score was divided by the square root of the set size to adjust for the set size bias as suggested in the work of Newton and colleagues (19). Calculations were conducted with R (20), final results were plotted with Spotfire (TIBCO Spotfire Inc.).

**In vivo studies**

*Compound preparation.* BKM120 was formulated in NMP/PEG300 (10/90, v/v). Solutions were freshly prepared for each day of dosing by dissolving the powder, first in NMP with sonication and then by adding the remaining volume of PEG300.

*In-life experimentation, analytic, and immunohistochemistry.* All aspects of in-life experimentation, analytic, preparation of tumors for immunohistochemistry as well as section staining were described previously (14, 15). For pHistone H3 immunohistochemistry, tissue section samples were stained with the anti-phospho Histone H3 Ser10 antibody, coverslipped, and air-dried. Stained sections were scanned (×20 magnification) using an Aperio scanner and the ImageScope software (Aperio) for image acquisition and automatic exclusions of regions with dominant necrosis. Quantification of the staining used the Novartis in-house software ASTORIA and this was used to establish the mitotic index [(number of pHistone H3–positive nuclei/total number of nuclei) × 1,000].

**Results**

*Comparison of BKM120 with another pan-PI3K inhibitor, GDC-0941, across a large panel of cell lines*

We compared the sensitivity profile of BKM120 with another class I PI3K inhibitor, GDC-0941 (21, 22) in a panel of 381 cell lines from the Novartis/Broad Institute CLE. The results are represented using the density distribution of the Amax (efficacy) and the crossing point (potency) for both compounds (Fig. 1). We observed a shift to the right of the density distribution of the crossing point for BKM120, indicating that the compound is generally less potent than GDC-0941 but we also noted a significant shift of the Amax density distribution for GDC-0941 indicating, that BKM120 is overall more efficacious than GDC-0941. By setting thresholds of sensitivity using the median efficacy and potency of BKM120, 21 cell lines are defined as sensitive to GDC-0941, whereas 131 cell lines are sensitive to BKM120 (Supplementary Fig. S1). However, as GDC-0941 is more potent than BKM120 in inhibiting Akt phosphorylation and proliferation of PI3K-addicted cell lines (Figs. 2 and 3A), we speculated that BKM120 carries activities beyond targeting PI3K.

**BKM120 exhibits an off-target activity at high concentrations that is not related to PI3K inhibition**

To further characterize the potential off-target activities of BKM120, we determined the effects on pathway
inhibition and cell proliferation and viability in both PI3K (PIK3CA-mutant MCF7 cell line) and non–PI3K-addicted (PTEN-mutant/BRAF-mutant A2058 cell line) models. As expected, in MCF7 cells, both BKM120 (Fig. 2A, top left) and GDC-0941 (Fig. 2A, top right) displayed potent anti-proliferative activity (GI$_{50}$ = 160 ± 91 and 52 ± 8 nmol/L, respectively), as well as efficient cell killing, as judged by the reduction of the cell number below the initial seeding number (LD$_{50}$ = 415 ± 193 and 207 ± 78 nmol/L, respectively; LD$_{50}$ = 980 ± 273 and 678 ± 220 nmol/L, respectively). In contrast, BKM120 (Fig. 2A, bottom left) but not GDC-0941 (Fig. 2A, bottom right) was capable of inducing robust cell death in A2058 cells at high concentrations (LD$_{50}$ = 2,996 ± 187 and >20,000 nmol/L, respectively), despite the fact that GDC-0941 was more efficient than BKM120 in reducing Akt phosphorylation levels (IC$_{50}$ = 114 ± 3 and 636 ± 36 nmol/L, respectively).

To further elaborate on the hypothesis that additional properties besides PI3K inhibition were involved in the cell killing effects observed at high concentrations in non–PI3K-addicted models, similar studies were conducted in genetically engineered MCF7 cells overexpressing a dominant-active form (MCF7-myr-Akt) of the downstream PI3K effector Akt (Supplementary Fig. S2A). In contrast to the MCF7 control cell pool (MCF7-BP), both BKM120 and GDC-0941 were less efficient in inhibiting the pathway, showing that the exogenously expressed myr-Akt protein was bypassing PI3K dependence for its activation (Supplementary Fig. S2B). In proliferation assays (Fig. 2B), MCF7-myr-Akt cells were found to be less sensitive than MCF7-BP cells to GDC-0941 (GI$_{50}$ = 270 ± 18 and 29 ± 10 nmol/L, respectively) and BKM120 (GI$_{50}$ = 299 ± 68 and 76 ± 17 nmol/L, respectively) resulting in a 9- and 4-fold shift in GI$_{50}$, respectively. Moreover, while MCF7-myr-Akt cells were completely resistant to cell death when exposed to GDC-0941 (LD$_{50}$ and LD$_{90}$ > 10,000 nmol/L), BKM120 treatment still led to efficient cell killing (LD$_{50}$ = 1,535 ± 157 nmol/L). Similarly, the expression of myr-Akt caused a shift in sensitivity to both GDC-0941 (Fig. 2C, top) and BKM120 (Fig. 2C, bottom) in colony formation inhibition. However, while treatment with 2 μmol/L BKM120 completely inhibited colony formation, the same concentration of GDC-0941 was less efficacious in this PI3K-resistant model.

Overall, these data suggest that in cells, BKM120 displays activities independent of PI3K inhibition at concentrations equal or higher than 2 μmol/L.

The off-target activity of BKM120 is linked to mitosis

To identify additional targets of BKM120, global gene expression profiles for BKM120, GDC-0941, and for the dual mTOR/PI3K inhibitor NVP-BEZ235 (BEZ235) were established upon exposure to concentrations corresponding to different degrees of pathway inhibition (50% or 90% inhibition, as judged by reduction of pAkt levels) in the A2058 cell line (Fig. 3A, left). Principal component analysis of the microarray data revealed that concentrations of BKM120 leading to 50% pathway modulation induced similar expression profiles as concentrations of GDC-0941 leading to either 50% or 90% pathway modulation. Treatment with BEZ235 caused similar (at IC$_{50}$) or even stronger changes (at IC$_{90}$) to those caused by GDC-0941 (at IC$_{90}$), but within the same directionality (Fig. 3A, middle). However, the maximal concentration of BKM120 tested (IC$_{90}$, dark red symbols) displayed a strong outlier behavior characterized by changes in gene expression not related to those observed with the 2 other inhibitors at any concentration (Fig. 3A, right). Thus, high concentrations of BKM120 elicit changes in additional sets of transcripts compared with other PI3K inhibitors.
To identify gene sets linked to the transcriptional effects induced upon exposure to high concentrations of BKM120, the most significantly changed transcripts (versus all other conditions) were identified and subjected to a GSEA (Fig. 3B). Interestingly, the gene sets with the highest score were found to be related to cell cycle, spindle assembly, and the metaphase checkpoint. Overall, these results suggest that at high concentrations, BKM120 displays activities that might have an impact on G2–M progression.

**BKM120 blocks the prometaphase to metaphase transition?**

To test whether BKM120 could cause a mitotic block, the effects on the cell cycle were analyzed in A2058 cells using BKM120 or GDC-0941 at concentrations sufficient to cause complete pathway inhibition (10-fold the IC50 for phospho-Akt inhibition). Treatment with GDC-0941 had no effect on the cell cycle, whereas treatment with BKM120 led to a significant increase in the G2–M population, in comparison with control untreated cells (Fig. 4A). The increase in G2–M occurred in a dose-dependent manner, but the concentration required to achieve half of this effect (EC50) was 8-fold higher than the concentration needed to reach the EC50 on PI3K pathway inhibition (measured by pAkt levels; Supplementary Fig. S3A). Furthermore, treatment with either BEZ235 or GDC-0941 at concentration as high as 5 μmol/L had no effect on the cell-cycle distribution (Supplementary Fig. S3B).

Phenotypic analysis of the A2058 cells using immunofluorescence analysis revealed that treatment with 5 μmol/L of BKM120 (but not with GDC-0941) induced the...
accumulation of mitotic cells. Most cells displayed duplicated centrosomes (determined by γ-tubulin staining), early bi- and multipolar spindles (determined by α-tubulin staining), and condensed but not fully aligned DNA, indicating early mitotic phases (Fig. 4B). Similar effects were also observed with BKM120 in the K-RAS mutant MDA-MB231 and PTEN null U87MG cell lines (Supplementary Fig. S4). Interestingly, treatment of these cells with the microtubule destabilizer nocodazole caused a remarkably similar phenotype. These results suggest that at high concentrations, BKM120 causes a prometaphase to metaphase arrest in a PI3K-independent manner.

**BKM120 inhibits tubulin polymerization**

To test whether BKM120 might influence microtubule dynamics, potential effects on tubulin polymerization were analyzed. Cells were preincubated at 4 °C to cause peripheral microtubule depolymerization followed by a switch back to 37 °C, either in presence or absence of inhibitors, to allow repolymerization of the microtubule network to the rim of the cells (Fig. 5A). In contrast to GDC-0941, incubation with BKM120 or nocodazole enhanced the loss of the microtubule network in the cell periphery, showing that BKM120 exhibits microtubule-destabilizing activity.

To determine whether BKM120 would directly interfere with microtubule polymerization, *in vitro* polymerization assays using purified tubulin were conducted. As expected, the microtubule stabilizer paclitaxel significantly increased the tubulin polymerization kinetics, whereas nocodazole caused the opposite effects (Supplementary Fig. S5A). Interestingly, and in contrast to GDC-0941, BKM120 decreased the tubulin polymerization kinetics in a concentration-dependent manner (Fig. 5C).

To further show direct binding of BKM120 to tubulin, assessment of direct interactions upon changes in relaxation of resonances was conducted by NMR spectroscopy (Fig. 5D). $T_1$r (left) and $T_2$ ($^{19}$F, right) relaxation...
experiments upon addition of freshly prepared tubulin to BKM120 (20-fold excess) showed an enhancement of the relaxation. These effects were tubulin concentration–dependent (Supplementary Table S1) and were further confirmed by waterLOGSY relaxation experiments (Fig. 5C, left). The tubulin/BKM120 interaction was found to be in fast exchange as observed for other tubulin ligands (23). Furthermore, relaxation competition studies could not show binding to the colchicine site, when well-described tubulin colchicine site binders were used as competitors (Supplementary Fig. S5B).

The microtubule-stabilizing activity of BKM120 does not translate to antitumor activity in vivo

We previously showed that BKM120 was able to cause significant regressions in the mechanistic Rat1-myrt-p110α in vivo model, when dosed once per day at doses of 40 mg/kg and above (T/C of −25% and −48% at 40 and 50 mg/kg, respectively; ref. 11). To test whether at these dose levels, the exposure of BKM120 would have reached concentrations to engage its off-target (tubulin-binding) activity, tumors were fixed and stained for phospho-Histone H3 levels as a mitotic marker, and the mitotic index was calculated. In cellular assays, a strong increase in phospho-Histone H3 levels could be observed as early as 6 hours (Supplementary Fig. S6). In vivo, (Rat1-myrt-p110α tumor model), no mitotic index increase was evident at the 40 mg/kg dose [plasma area under the curve (AUC): 65 hμmol/L], up to 16 hours after last dose administration. This result suggests that the tumor regression (which is accompanied with a robust increase in caspase-7 cleavage) seen upon the exposure to BKM120 at the dose of 40 mg/kg (Fig. 6A, right) is due to the sole merit of PI3K inhibition. However, a 2.5-fold transient (6 hours but not anymore at 16-hour time point) and statistically significant increase in mitotic index to 5% could be observed for the 50 mg/kg dose (plasma AUC: 75 hμmol/L; Fig. 6A, left). To assess whether such a mild and transient increase in mitotic index at 50 mg/kg translates into efficacy, a similar study was repeated in the PTEN-null U87MG tumors. Daily treatment of BKM120 resulted in antitumor activity with T/C of 20% and 7% at 40 and 50 mg/kg, respectively, but these differences were not statistically different (P > 0.05; Supplementary
As in the Rat1-myr-p110α model, a similar transient and statistically significant increase in mitotic index to 3% was observed at the 50 mg/kg dose level (Fig. 6B, left). Importantly, no increase in caspase-7 cleavage was observed at the 40 and the 50 mg/kg dose levels (Fig. 6B, right). Altogether, these data suggest that the exposure of BKM120 at a dose of 50 mg/kg might reach sufficient blood/tumor levels to engage the off-target activity in the first 6 hours following administration, which then cause a mild and transient mitotic index (3% to 5%). In contrast to BKM120, other microtubule-binding agents such as paclitaxel cause peaks of mitotic index that range between 10% and 25% (Milas and colleagues; ref. 24). Furthermore, BKM120-induced mitotic block seemed to be reversible as soon as the compound gets cleared as no increase of mitotic index was observed 16 hours after compound administration. This reversibility was also observed in in vitro pulse chase studies where BKM120 was washed out (Supplementary Fig. S7).

Discussion

Our studies show that sustained exposure to BKM120 at concentrations above 1 μmol/L engages PI3K-independent activities, resulting in enhanced antiproliferative and cell killing effects. Biochemical and transcriptomic profiling studies with BKM120 and other PI3K inhibitors such as BEZ235 or GDC-0941 pointed to a unique role of BKM120 in regulating microtubule dynamics causing a prometaphase to metaphase block in cell lines without strong PI3K addiction where ontarget PI3K inhibition is not able to induce apoptosis.
Microtubule stabilizers (such as paclitaxel and derivatives) and destabilizers (such as vinca-alkaloids or nocodazole) are known to activate the spindle assembly checkpoint leading to an arrest of cells in mitosis and subsequent cell killing probably as a result of induction of mitotic catastrophe. These agents have been used for many years as antineoplastic therapy for various types of cancers (25). The phenotype detected upon BKM120 treatment at high concentration was highly reminiscent of that observed upon nocodazole treatment, suggesting that BKM120 also interferes with microtubule dynamics directly (i.e., by tubulin-binding capacities) or indirectly (i.e., by blocking the activities of factors associated to the functions of microtubules such as the kinesin Eg5).

Direct binding of BKM120 to pure tubulin was showed using in vitro tubulin polymerization assays and further confirmed by NMR studies. Different microtubule-targeting agents have distinct binding modes and mechanisms of actions. Taxanes preferentially bind to polymerized β-tubulin and more precisely at the inner surface of the microtubules (26). Vinca-alkaloids bind at the interface of a 2 α/β tubulin heterodimer, at the + end microtubules (27). A third category of tubulin interactors bind to the so-called colchicine domain, which mostly lies within the β-tubulin subunit (28, 29). Taxanes and vinca-alkaloids are both high-molecular-weight molecules and derivatives of natural products. In contrast, colchicine site binders are generally small molecules (30), hence, we hypothesized that BKM120 might share similar binding modalities. However, NMR competition studies did not confirm this hypothesis. Further structural studies will be needed to elucidate BKM120-binding mode to tubulin.

The pronounced spindle assembly defects in mitosis seen in vitro were not observed in vivo after multiple administrations of BKM120 at a dose of 40 mg/kg. Interestingly, at a dose of 50 mg/kg, a small and transient increase of the mitotic index was observed. These findings suggest that BKM120 displays tubulin-binding and microtubule-destabilizing activities only above a certain concentration/exposure threshold. Furthermore, the fast binding kinetics to tubulin as well as the

Figure 6. Treatment with BKM120 leads to a transient increase in mitotic markers. A and B, rat1-myr-p110a (A) or U87MG (B) tumor-bearing animals were treated orally with the indicated dose of BKM120, once per day for a period of 6 days. Upon last treatment, animals were sacrificed at the indicated time points for collection of plasma and tumor tissues. Compound concentration in plasma (left, left y-axis) as well as quantification of histone H3 levels and subsequent determination of the mitotic index (MI; left, right y-axis) were plotted; effects on apoptosis were also assessed by immunohistochemistry by staining of tumor sections with an anti-cleaved caspase-7 antibody (right).
intrinsic compound clearance probably result in reversibility of the mitotic effects, as no accumulation of G2–M arrested cells could be detected following chronic BKM120 administration. It therefore appears that BKM120 can cause regression in PI3K-dependent tumors when administered orally to animals bearing subcutaneous tumors without engaging the tubulin off-target activity to a sufficient level to contribute to the therapeutic effect.

It is interesting to observe that the plasma exposure in patients treated with BKM120 at the efficacious maximum tolerated dose (100 mg, AUC: 56 μmol/L; ref. 31), lies below the exposure necessary to transiently engage the off-target in a mouse model (AUC > 65 μmol/L). These findings strongly argue that in patients, the threshold for microtubule-destabilizing activity of this compound is not reached. Therefore, it is anticipated that efficacy in patients will solely stem from PI3K inhibition. Further analysis of clinical data, such as the assessment of mitotic markers in tumor biopsies from patients treated with BKM120, will be required to fully confirm that the compound off-target activity is not clinically relevant.

Disclosure of Potential Conflicts of Interest
W. R. Sellers is employed as VP/GLOBAL Head of Oncology and has ownership interest (including patents) in Novartis Institutes for BioMedical Research. All authors except J. Kleylein-Sohn and C. Garcia-Echeverria are Novartis employees. J. Kleylein-Sohn is now employed at the FMI and C. Garcia-Echeverria is now employed at Sanofi-Aventis.

References

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BKM120 Alters Microtubule Dynamics at High Concentrations


Molecular Cancer Therapeutics

Characterization of the Mechanism of Action of the Pan Class I PI3K Inhibitor NVP-BKM120 across a Broad Range of Concentrations

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