Effects of PI3K inhibitor NVP-BKM120 on overcoming drug resistance and eliminating cancer stem cells in human breast cancer cells

Y Hu, R Guo, J Wei, Y Zhou, W Ji, J Liu, X Zhi and J Zhang

The multidrug resistance (MDR) phenotype often accompanies activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which renders a survival signal to withstand cytotoxic anticancer drugs and enhances cancer stem cell (CSC) characteristics. As a result, PI3K/AKT-blocking approaches have been proposed as antineoplastic strategies, and inhibitors of PI3K/AKT are currently being trailed clinically in breast cancer patients. However, the effects of PI3K inhibitors on MDR breast cancers have not yet been elucidated. In the present study, the tumorigenic properties of three MDR breast cancer cell lines to a selective inhibitor of PI3K, NVP-BKM120 (BKM120), were assessed. We found that BKM120 showed a significant cytotoxic activity on MDR breast cancer cells both in vitro and in vivo. When doxorubicin (DOX) was combined with BKM120, strong synergistic antiproliferative effect was observed. BKM120 activity induced the blockage of PI3K/AKT signaling and NF-κB expression, which in turn led to activate caspase-3/7 and caspase-9 and changed the expression of several apoptosis-related gene expression. Furthermore, BKM120 effectively eliminated CSC subpopulation and reduced sphere formation of these drug-resistant cells. Our findings indicate that BKM120 partially overcomes the MDR phenotype in chemoresistant breast cancer through cell apoptosis induction and CSC abolishing, which appears to be mediated by the inhibition of the PI3K/AKT/NF-κB axis. This offers a strong rationale to explore the therapeutic strategy of using BKM120 alone or in combination for chemotherapy-nonresponsive breast cancer patients.

Cell Death and Disease (2015) 6, e2020; doi:10.1038/cddis.2015.363; published online 17 December 2015
NVP-BKM120 overcomes MDR and eliminates CSC

Y Hu et al

Cell Death and Disease
emphasized the enhanced antitumor effects in mouse models when BKM120 was co-treated with inhibitors of other signaling pathways.18–20

In this study, we analyzed, for the first time, the efficacy of BKM120 in several MDR breast cancer cell lines with which the MDR phenotype is induced by different molecular mechanisms. BKM120 exerted potent efficacy of apoptosis promoting as well as CSCs eliminating through inhibiting the PI3K/AKT/NF-κB cascade in vitro and in vivo. In addition, BKM120 synergized with DOX, a common chemotherapeutic agent of breast cancer. Here we demonstrate the potential of BKM120 in overcoming chemotherapy resistance in breast cancer.

Results

PI3K inhibitor BKM120 shows potent cytotoxicity against both sensitive and MDR breast cancer cell lines. The MDR breast cancer cell lines MCF-7/A02 and CALDOX were derived from chemosensitive cell lines MCF-7 and Cal51, respectively. The MDR phenotype of these derived cell lines was manifested by their cross-resistance to a wide range of structurally and functionally unrelated drugs (Supplementary Tables S1 and S2). BKM120 inhibited growth of chemosensitive and chemoresistant breast cancer cells in a dose-dependent manner (Supplementary Figure S1A); the IC50 assay results revealed that two drug-resistant cell lines exhibited only 4.6- and 1.56-fold resistance to BKM120, respectively, compared with their parental chemosensitive counterparts (Figure 1a). Similar results were also observed in another pair of breast cancer cell lines, the relatively sensitive MTMEC cell line and its DOX-resistant derivative MD60 cell line (Supplementary Figures S2A and B). BKM120 appeared to be more effective in eliminating drug-resistant cells than chemotherapeutic drugs. To further evaluate the cytotoxic effect of BKM120 on chemosensitive and chemoresistant breast cancer cells, the cells were treated with either DOX or BKM120. Crystal violet staining was used to determine the cell mass 1 week after treatment. As expected, the chemoresistant cells robustly defined DOX, whereas the same concentration of DOX eradicated most drug-naïve cells. However, BKM120 significantly decreased the capacity of MDR cells to survive after 1-week treatment (reduction ranging from 72.4 to 77.7%, Figure 1b and Supplementary Figure S1B). BKM120 is cytotoxic to MDR breast cancer cells by activating apoptosis. (Figure 1)

BKM120 promotes cell apoptosis, induces caspase activity and modulates apoptosis-related genes. Next, we asked whether the cytotoxic effect of BKM120 on chemoresistant breast cancer cells is mediated by promoting cell apoptosis. As DOX can be excited at 488 nm by blue excitation laser, with emission at ~595 nm,21 thereby interfering with the results of the Annexin V/propidium iodide (PI) staining assay, we used etoposide, another widely used drug known to induce cell apoptosis22,23 as a positive control. As illustrated in Figure 1c, 10 μM etoposide showed similar apoptosis-inducing effect as 2 μM BKM120 did in MCF-7 cells, but failed to do so in MCF-7/A02 cells. After 2 μM BKM120 treatment, the percentage of apoptotic MCF-7/A02 cells increased remarkably. A similar effect of BKM120 treatment on promoting apoptosis was also observed in CALDOX cells. In accordance with the Annexin V/PI analysis, BKM120 strongly induced caspase-3/7 and caspase-9 activities in MCF-7/A02 and CALDOX cells, respectively (Figure 1d), which was also confirmed in MD60 cells (Supplementary Figure S2C). As Supplementary Figure S1D shows, the pancaspase inhibitor z-VAD-fmk was able to abrogate Caspase-3/7 and Caspase 9 activities mediated by BKM120 intensively in MCF-7/A02 and CALDOX cells, confirming the specificity of the observed effects. Cells were untreated or pretreated with z-VAD-fmk for 4 h, followed by treatment with BKM120, and were subsequently analyzed for cell viability by MTT assay and cell apoptosis by Annexin V/PI staining assays. z-VAD-fmk reduced the percentage of Annexin V-positive cells (both PI low, early apoptotic and PI high, late apoptotic/necrotic) upon BKM120 treatment. In addition, there was almost a 2.6-fold shift in the IC50 for MCF-7/A02 cells and a 3.9-fold shift in the IC50 for CALDOX. These data demonstrate the specific induction of cell death by BKM120 through the activation of the apoptotic cascade (Supplementary Figure S1E and F). As the induction of apoptotic cell death could be partly due to alteration of apoptosis-related genes, we evaluated the mRNA levels of several survival genes, including Bcl-2, Bcl-xl, Survivin and Mcl-1, as well as apoptotic genes, Bim and Bax. Reverse transcription quantitative real-time-qPCR (RT-qPCR) and western blot results revealed that BKM120 repressed pro-survival gene Survivin expression and upregulated pro-apoptotic genes Bim and Bax expression in MDR cells (Figure 1e), although the expressions of Bcl-2, Bcl-xl and Mcl-1 were not changed (Supplementary Figure S1C).

To further confirm that promoting effect of BKM120 on apoptosis is specifically mediated by PI3K/AKT inhibition, chemoresistant breast cancer cells were treated with LY294002, another well-characterized selective PI3K/Akt inhibitor. Similar to BKM120, the IC50 values of LY294002 in MCF-7/A02 and CALDOX cells are only 7.38 and 2.18 times greater than those in MCF-7 and Cal51 cells, respectively (Figure 2a). LY294002 significantly induced cell apoptosis and
activated caspases in MCF-7/A02 and CALDOX cells (Figure 2b and c). In addition, LY294002 treatment also enhanced Bax and Bim expression and reduced Survivin mRNA and protein levels (Figure 2d). Thus, attenuating PI3K/AKT signaling appears to be an important pathway to induce chemoresistant breast cancer cell apoptosis.

**Figure 2**  Blocking the PI3K/Akt pathway by LY294002 induces apoptosis in MDR breast cancer cells. (a) IC50 value of LY294002 in MCF-7 and MCF-7/A02 (upper panel), Cal51 and CALDOX (lower panel). (b) Cells were treated with LY294002 (10 μM for MCF-7 and MCF-7/A02, 2 μM for Cal51 and CALDOX) for 48 h. Annexin V/PI staining was detected using flow cytometry. Representative plots of three independent experiments are shown. Quantitative data show the average percentage of annexin V-positive cells (both in early apoptosis, lower right quadrant and late apoptosis, upper right quadrant) of three independent experiments (right panel). (c) Caspase-3/7 and caspase-9 activities of MCF-7/A02 (upper histograms) and CALDOX (lower histograms) after LY294002 treatment. (d) Fold changes of Bax, Bim and Survivin expression levels determined using RT-qPCR (left panel) and western blot (right panel) in MCF-7/A02 and CALDOX after LY294002 treatment with various concentrations for 48 h. Numerical data are presented as mean ± S.D. of three independent replicates. *P<0.05

**BKM120 induces cytotoxicity through blocking the PI3K/AKT/NF-κB signaling pathway.** The PI3K/AKT signaling pathway is frequently dysregulated in human cancer and has been implicated in the development of resistance to standard anticancer therapies. It is known that chemoresistant MCF-7/A02 cells are mainly mediated by increased P-gp
NVP-BKM120 overcomes MDR and eliminates CSC

Y Hu et al.

Cell Death and Disease
expression, whereas the chemoresistance in CALDOX cells is due to the loss of expression of TOP2A, rather than overexpression of membrane transporters.27,28 Although the leading causes of MDR phenotype are quite different, the PI3K/AKT signaling cascade in these two MDR cell lines was found to be overactivated compared with their parental cell lines (Figure 3a). Our data indicated that induction of cell apoptosis by BKM120 in both MDR cancer cell lines was associated with a significant reduction in AKT phosphorylation, suggesting a decrease in PI3K activity (Figure 3a).

As PI3K/AKT signaling has a pivotal role through regulating diverse downstream effectors, we sought to determine the potential downstream effector that involves in BKM120-induced apoptosis in MDR cells. The crosstalk between NF-κB activity and PI3K/AKT pathway has been shown in various cancers, and constitutive activation of NF-κB induced by the PI3K/AKT pathway may have a major role in the development of chemoresistance.29,30 Our western blot results revealed that MDR cells contained higher NF-κB p65 protein levels in whole-cell extracts as well as in the nuclear compartment, where NF-κB p65 exerts its transcriptional activity (Figure 3a). Therefore, we speculated that BKM120 exerts its apoptotic induction through downregulating the PI3K/AKT/NF-κB cascade in MDR breast cancer cells. To address this issue, we examined mRNA and protein levels (Figure 3a and Supplementary Figure S3A), cellular localization (Figure 3a and c) and DNA-binding activity (Figure 3b) of the NF-κB p65 subunit in MCF-7/A02 and CALDOX cells. The results showed that BKM120 significantly decreased NF-κB p65 expression and its DNA-binding activity (Figure 3a–c and Supplementary Figure S3A).

LY294002 also displayed a suppressive effect on NF-κB p65 expression similar to what was observed in BKM120 treatment in these MDR cells (Figure 3d and Supplementary Figure S3B).

To ascertain whether the downregulation of NF-κB activity influenced apoptosis-related gene expression, we used a small interfering RNA (siRNA) approach to knockdown NF-κB p65. Figure 3e and f showed that NF-κB p65 siRNA transfection significantly reduced NF-κB p65 and Survivin mRNA levels, increased Bim and Bax expression and concomitantly led to cell apoptosis when compared with scramble siRNA-transfected cells. Taken together, these results demonstrate that PI3K/AKT inhibitor BKM120 induces MDR breast cancer cell apoptosis through suppressing NF-κB activity.

**BKM120 eliminates stem cell subpopulation of MDR breast cancer cells.** Drug-resistant cells have been proposed to arise from the selection of a small population of cells with stem-like properties.5 BKM120 has been shown to have robust anticancer properties in MDR breast cancer cells; therefore, we then asked whether BKM120 could also eliminate stem cell population of MDR cells. First, we tested the effect of BKM120 on the proportion of stem-like cell (SC) population in chemoresistant breast cancer total cells (TCs). We analyzed the expression of CD44 and CD24, two cell surface markers whose expression in the CD44(N+)CD24(N) configuration is associated with breast SCs.31 Emergence of MDR was indeed accompanied by an increase in the percentage of CD44(N+)CD24(N) cells (Figure 4a). As expected, the percentage of CD44(N+)CD24(N) cells was reduced in MCF-7/A02 and CALDOX cells after BKM120 treatment in a range of doses lower than IC50 (Figure 4a). We then analyzed aldehyde dehydrogenase (ALDH) activity, another important marker of SCs.32 Comparing MCF-7 and Ca151 cells, both MCF-7/A02 and CALDOX cell lines were composed of a much higher proportion of cells exhibiting ALDH activity (Figure 4b), and the ALDH activity in these MDR cell lines were remarkably inhibited after exposure to BKM120 in the doses lower than IC50 (Figure 4b). Similar effect of BKM120 was observed in MD60 cells (Supplementary Figure S2E).

Breast SCs exhibit other stem-like properties, including the ability to survive and grow as spheres or colonies under certain conditions, such as low attachment plates and soft agar medium.33 First, we observed that MCF-7/A02 and CALDOX cells indeed formed higher numbers of spheres and clones than their parental cell lines (Figure 4c and d). Second, BKM120 eliminated MCF-7/A02 and CALDOX cells’ sphere-forming efficacy (SFE), as well as the ability to produce colonies (Figure 4c and d and Supplementary Figure S4A and B). Third, primary mammospheres from each cell line were enzymatically dispersed to single cells and assayed for their ability to form secondary mammospheres. BKM120 treatment maintained SFE inhibition in the second passage of both MCF-7/A02 and CALDOX cell lines (Figure 4c).

As the mammosphere technique has been proven for enriching the highly tumorigenic SCs,34,35 the mammosphere cells (MCs) from MCF-7/A02 and CALDOX cells were obtained through sphere-forming assay as mentioned above. Comparing with TCs, MC has more CD44(N+)CD24(N) subpopulation and ALDH1(N+) subpopulation analyzed using flow cytometry (Supplementary Figure S5). As the MTT assay results show, DOX and etoposide were more cytotoxic in chemoresistant TCs than in their MCs. MCF-7/A02 and CALDOX MCs exhibited 68- and 47-fold greater resistance to DOX than MCF-7/A02 and CALDOX TCs, respectively (Table 1). In addition, MCF-7/A02 and CALDOX MCs...
Figure 4  The inhibitory effects of BKM120 on SC in MDR breast cancer cells. (a) Flow cytometry plots for CD44 and CD24 of MDR and their parental cells. Cells in Q3 (CD44<sup>high</sup>CD24<sup>low</sup>) are associated with SC subpopulation. Quantitative data show the average percentage of CD44<sup>high</sup>CD24<sup>low</sup> cells (right panel).  

(b) Flow cytometry analysis of ALDH activity. Cells treated with or without BKM120 for 48 h were assayed with an Aldefluor assay kit in the presence and absence of the ALDH inhibitor diethylaminobenzaldehyde (DEAB). Gating in the control was set up to a maximum of 1% of cells. Representative plots of at least three independent experiments are shown. Quantitative data show the average percentage of ALDH<sup>high</sup> cells (right panel).  

(c) Primary and secondary mammosphere-forming efficacies of MDR and their parental cells. SFE was calculated as the number of spheres formed in 10 days divided by the original number of single cells seeded and expressed as percentage. Bars represent the mean percentage of mammospheres.  

(d) Anchorage independence was determined by the formation of clones in soft agar cultured for 3 weeks. Histogram data represent the average number of colonies counted in randomly chosen five visual fields under the microscope (magnification ×40).  

(e) Dose–response curves were used to calculate the IC<sub>50</sub> of BKM120 for MCs of MCF-7/A02 cells (upper) and CALDOX cells (lower).  

(f) Caspase-3/7 and caspase-9 activities of MCF-7/A02-MC (upper histograms) and CALDOX-MC (lower histograms) after BKM120 treatment. Numerical data are presented as mean ± S.D. of three independent experiments. *P<0.05, **P<0.01

NVP-BKM120 overcomes MDR and eliminates CSC

Y Hu et al
exhibited 42- and 18-fold greater resistance to etoposide than MCF-7/A02 and CALDOX TCs, respectively (Table 1). BKM120 also displayed a cytotoxic effect on these MCs in a dose-dependent manner and significantly induced caspase activities (Figure 4e and f). Compared with the TCs, the resistance of MCs to BKM120 only ranged from 4.61- to 5.73-folds (Table 1), which indicated that BKM120 could kill MCs more efficiently than DOX and etoposide.

To ascertain that blockage of the PI3K/AKT/NF-κB cascade was functionally important for BKM120-induced stemness reduction in MDR cell lines, we used LY294002 and NF-κB p65 siRNA to specifically inhibit PI3K/AKT activity and NF-κB p65 expression. Figure 5 confirmed that abolishing PI3K/AKT signaling or NF-κB p65 silencing was sufficient to decrease ALDH<sup>high</sup> population as well as SFE in MCF-7/A02 and CALDOX cells.

**BKM120 is synergistic with chemotherapeutic agents in MDR breast cancer cells.** Alterations in the PI3K/AKT signaling pathway have been shown to track consistently with therapy-induced resistance in breast cancer patients, including endocrine-based therapy, chemotherapy and HER2-targeted therapy. Recent studies demonstrate that targeting the PI3K/AKT pathway in combination with trastuzumab or tamoxifen is beneficial in trastuzumab-resistant breast cancer or endocrine therapy-resistant breast cancer. Hence, we further investigated whether BKM120 could synergize with chemotherapeutic agents commonly
The inhibition of PI3K by BKM120 results in a dramatic decrease in the expression of the PI3K/AKT/NF-κB signaling pathway in chemoresistant breast cancer cells, which subsequently induces xenograft tumor regression in vivo.

Discussion

Most cytotoxic anticancer therapies are encumbered by the development of acquired resistance of cancer cells. Chemoresistance is a complex phenomenon involving multiple mechanisms. Reduction of drug accumulation, enhancement of DNA repair, impediment to apoptosis and alterations in cell cycle are believed to be the major causes of chemoresistance. Many of these factors are manifested in intracellular signaling pathways, and one of the most prominent is the PI3K/AKT pathway.

Here, we employed three drug-resistant human breast cancer cell lines for the experiments, MCF-7/A02, CALDOX and MD60, all of which possess the MDR phenotype but are caused by different mechanisms. The overexpression of P-gp has been recognized as the most significant factor conferring chemoresistance of MCF-7/A02 and MD60. In contrast, compared with Cal51, CALDOX cells have no increased P-gp expression or any other drug transporters, implying that mechanisms independent of drug transporters have a leading role in chemoresistance of CALDOX cells. Despite that the causes of drug resistance in these cell lines are different, the PI3K/AKT/NF-κB signaling is overactivated in all three cell lines, which is consistent with the findings reported by other groups.10,12 Indeed, this crucial survival pathway promotes acquired resistance in a wide range of cancers, such as breast cancer, leukemia, ovarian cancer and non-small-cell lung carcinoma.39–41 Hence, novel therapeutics against this PI3K/AKT/NF-κB pathway may offer new strategies to overcome drug resistance.

In this study, we examined the responses of three chemoresistant breast cancer cell lines to the PI3K inhibitor BKM120. Our studies were performed both in vitro and in vivo. The inhibition of PI3K by BKM120 results in a dramatic

---

**Table 1** Mammosphere cells of MCF-7/A02 and CALDOX sensitivity to different drugs

| Drug (μM) | IC50 of MCF-7/A02 | Mammosphere-cell resistance ratio |
|-----------|-------------------|---------------------------------|
|           | Total cells       | Mammosphere cells               |
| Doxorubicin | 128.3             | 8827                            | 68.80 |
| Etoposide   | 206.8             | 8747                            | 42.30 |
| BKM120     | 5.6               | 32.1                            | 5.73  |

| Drug (μM) | IC50 of CALDOX | Mammosphere-cell resistance ratio |
|-----------|----------------|---------------------------------|
|           | Total cells    | Mammosphere cells               |
| Doxorubicin | 5.73            | 272.7                           | 47.59 |
| Etoposide   | 26.8            | 487.8                           | 18.20 |
| BKM120     | 1.8             | 8.3                             | 4.61  |

**Table 2** Mammosphere cells of MCF-7/A02 and CALDOX sensitivity to different drugs

| Drug | IC50 of MCF-7/A02 | Mammosphere-cell resistance ratio |
|------|-------------------|---------------------------------|
| Etoposide | 206.8             | 8747                            | 42.30 |
| Doxorubicin | 5.73            | 272.7                           | 47.59 |
| BKM120 | 1.8              | 8.3                             | 4.61  |
decrease in AKT phosphorylation and subsequent down-regulation of NF-κB. Furthermore, activation of caspase-3,7,9, inhibition of survivin expression, as well as augment of Bax and Bim expression, were also detected after BKM120 treatment. These are consistent with recent studies that BKM120 elevated Bim expression in chronic lymphocytic leukemia cells42 and induced Bax expression and caspase-3/7 activation in glioma cell lines, T-cell acute lymphoblastic leukemia and ER-positive breast cancer cells.19,43,44 It is well known that AKT is a central node in the PI3K signaling pathway that activates a number of downstream pathways implicated in tumorigenesis. BKM120 has previously been shown to inhibit some other pathways, including the AKT/mTOR pathway in breast cancer and hepatocellular carcinoma, the AKT/GSK3/FBXW7 pathway in chronic lymphocytic leukemia cells and the AKT/FOXO3a axis in lung cancer.19,43,44

Figure 5 Inhibiting the PI3K/AKT/NF-κB signaling pathway diminishes breast cancer stem cell population. (a) Flow cytometry analysis of ALDH activity. Cells with LY294002 treatment for 48 h or after siRNA transfection for 72 h were assayed with an Aldefluor assay kit in the presence and absence of ALDH inhibitor DEAB. Gating in the control was set up to a maximum of 1% of cells. Representative plots of three independent experiments are shown. Quantitative data show the average percentage of ALDHhigh cells ± S.D. of three independent experiments (right panel). (b) Mammosphere-forming efficacy of cells with LY294002 treatment or siRNA transfection. SFE was calculated as the number of spheres formed in 10 days divided by the original number of single cells seeded and expressed as a percentage. Bars represent the mean percentage of mammospheres ± S.D. from three separate experiments. *P<0.05
NVP-BKM120 overcomes MDR and eliminates CSC
Y Hu et al

Table 2 Cytotoxicity of BKM120 and doxorubicin/etoposide to different breast cancer cell lines

|       | MCF-7/A02 | CALDOX |
|-------|-----------|--------|
| BKM120 (μM) | 1 2 4 8 16 | 0.5 1 2 4 8 |
| Doxorubicin (μM) | 0.368 0.279 0.184 0.241 0.144 | 0.625 1.25 2.5 5 10 |
| CI | 0.306 0.213 0.436 0.212 0.129 | 0.5 1 2 4 8 |
| Etoposide (μM) | 0.351 0.412 0.501 0.522 0.223 | 12.5 25 50 100 200 |

Cytotoxicity of BKM120 and doxorubicin/etoposide to different breast cancer cell lines

Our study uncovers for the first time that BKM120 can also modulate NF-κB, another critical substrate of AKT. Importantly, NF-κB signaling and cell apoptosis are frequently associated with many solid cancers, although the molecular mechanisms remain obscure. It is tempting to speculate that AKT/NF-κB represents a 'salvage pathway' that links BKM120 with apoptosis induction.

Comparing traditional chemotherapeutic agents such as doxorubicin and etoposide, BKM120 reduced cell viability more effectively in a concentration-dependent manner in all tested MDR breast cancer cells. Others also proved that therapy resistance could be partially overcome by down-regulating AKT/NF-κB. For instance, salinomycin induced apoptosis in cisplatin-resistant ovarian cancer cells through inhibiting the AKT/NF-κB pathway. PS1145 can overcome imatinib resistance in leukemia through blocking the NF-κB pathway. LY294002, another important selective inhibitor of PI3K, has been proven to overcome acquired resistance of 5-FU in gastric cancer by modulating NF-κB activity. Our study also demonstrated that the AKT/NF-κB pathway blocked by LY294002 or NF-κB p65 siRNA led to apoptosis of MDR breast cancer cells. Although some studies reported that several AKT/NF-κB inhibitors reverse the MDR phenotype by decreasing P-gp expression, our data indicated that BKM120 did not alter P-gp expression, suggesting that BKM120 overcame chemoresistance through inducing apoptosis, rather than increasing drug influx.

Accumulating evidence indicates that breast CSCs have a crucial role in therapy resistance and recurrence of breast cancers. Hence, breast CSCs are considered to be critical therapeutic targets, and elimination of breast CSCs may improve the outcomes of cancer chemotherapy. Recent studies indicate that the activation of the PI3K/AKT/NF-κB pathway is indispensable for maintaining the stemness and chemoresistance of breast CSCs. Present and other studies have shown that PI3K inhibition sensitizes CSCs to chemotherapy and molecular targeted therapy in several cancers including leukemia, hepatocellular carcinoma and breast cancer. Moreover, the blockage of NF-κB activity also provokes cytotoxic effects on CSCs in glioblastoma multiforme.

In line with these results, our findings revealed that, in contrast to highly resistant to traditional chemotherapeutic agents, the CSC population of the three MDR cell lines tested remained relatively sensitive to BKM120-induced cytotoxicity. Furthermore, BKM120 remarkably eliminated CSCs in drug-resistant cells. After exposure to BKM120, the clonogenicity of resistant cells was evidently eradicated in vitro, and the ALDHhigh and CD44high/CD24low population decreased obviously in the survival cells. In addition, LY294002 and NF-κB silencing also inhibited stemness significantly, indicating that targeting PI3K/AKT/NF-κB pathway is emerged as a promising approach to kill CSCs and consequently surmount MDR in breast cancers.

BKM120 exerted a synergistic effect with doxorubicin both in vitro and in vivo. It has recently been reported that BKM120 in combination with trastuzumab is beneficial in trastuzumab-resistant breast cancer. Other PI3K inhibitors, including LY294002 and buparlisib, have also been proven to have greater clinical efficacy combined with endocrine therapy. In some other solid tumors, BKM120 has been reported to synergistically work with an mTOR inhibitor RAD001 and a Bcl-2 inhibitor ABT-737 in suppression of lung cancer and glioblastoma cell growth, respectively. It is of note that these combined therapies were all well tolerated. Consistently, our study demonstrates that BKM120 sensitizes MDR breast cancer cells to cytotoxic drugs (doxorubicin and etoposide) using CI-isobologram analysis, suggesting that combination with BKM120 therapy can augment the cytotoxic activity of chemotherapies against MDR breast cancer growth but with no further toxicities.

Overall, the present study discloses that the MDR phenotype of breast cancer cells is associated with an aberrant activation of the PI3K/AKT/NF-κB signaling pathway. Our findings establish that BKM120 effectively inhibits this signaling pathway, and provide strong evidence demonstrating that BKM120 potently induces cell apoptosis and aggressively eliminates breast CSCs in these MDR breast cancer cells. Moreover, the combination therapy of BKM120 and doxorubicin shows a synergistic effect both in vitro and in vivo. Our data suggest that targeting the PI3K/AKT/NF-κB signaling pathway using selective inhibitors such as BKM120 can be a potential strategy for treatment of relapsed MDR breast cancers.

Materials and Methods

Cell culture. Human breast cancer cell line MCF-7 and its MDR counterpart MCF-7/A02 were gifts from Professor Dongsheng Xiong (Institute of Hematology, PUMC, Tianjin, China) and were cultured as previously described. Human breast cancer cell line Cal51 and its MDR counterpart CALDOX were gifts from Dr. Ernesto Yague (Imperial College London, UK) and were cultured as previously described. Human breast cancer cell line Cal51 and its MDR counterpart CALDOX were gifts from Dr. Ernesto Yague (Imperial College London, UK) and were cultured as previously described. MTMEC and its doxorubicin-resistant derivative MD60 were also gifts from Dr. Ernesto Yague, and they were routinely maintained in a serum-free HuMEC medium (Life Technologies, Paisley, UK) as previously described. MTMEC and its doxorubicin-resistant derivative MD60 were also gifts from Dr. Ernesto Yague, and they were routinely maintained in a serum-free HuMEC medium (Life Technologies, Paisley, UK) as previously described. The immortalized human mammary epithelial cell line expressing TERT, SV40 large T antigen, and oncogenic RAS was also used in the combination experiments, a CI number was calculated using the CalcuSyn software.

Cell viability analysis. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazo- lium bromide) assays were performed to evaluate the cell viability in response to drug treatments and were also used to determine the concentration of drug that inhibited cell growth by 50% (IC50) after 3 days of treatment. For drug combination experiments, a CI number was calculated using the CalcuSyn software.
(Biosoft, Cambridge, UK) based on the Chou and Talalay method. CI values between 0.1 and 0.9 define different grades of synergism: values between 0.9 and 1.1 are additive, whereas values > 1.1 are antagonistic.

**Drug resistance clonogenic assay.** Cells at a density of $2 \times 10^5$ cells/well in six-well plates were treated with a single dose of doxorubicin (3 $\mu$M for MCF-7 and MCF-7/A02, 0.2 $\mu$M for Cal51 and CALDOX), or 2 $\mu$M BKM120 for 1 week.
Resistant clones were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet and counted. Crystal violet retained in the cells was solubilized with 0.5% acetic acid and quantified by measurement of optical density at 592 nm.

siRNA transfection. Cells at a density of 3 × 10^5 cells/well in six-well plates were used for siRNA transfection. Briefly, 50 nM siRNA against the NF-κB p65 subunit or scrambled siRNA control (GenePharma Company, Shanghai, China) was mixed with lipofectamine 3000 (Life Technologies, Grand Island, NY, USA) and then added to each well. The effects of siRNA on NF-κB p65 subunit mRNA and protein levels were examined 48 and 72 h after transfection, respectively.

Protein extraction and western blotting. A modified RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.25% SDS, 1% Triton X-100, 0.05% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol) with protease inhibitor cocktail (Sigma) was used for protein isolation from whole cells or nuclear fraction. Cell nuclei were isolated using a Nuclear EZ Prep Kit (Sigma) according to the instruction recommended by the manufacturer. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). An equal quantity (50 μg) of proteins was resolved on 12% polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA), and then blocked with 5% blotting grade milk (Bio-Rad, Hercules, CA, USA) in PBST (0.1% Tween 20 in phosphate-buffered saline (PBS)). The membranes were incubated with primary antibodies to phospho-AKT (D9E), AKT1 (C73H10), Bax (D2E11), Bim (C54S5), Cell Signaling Technology), Survivin (ab76424, Abcam, Cambridge, UK) and NF-κB p65 (sc372, Santa Cruz Biotechnology, Dallas, TX, USA) at 4 °C overnight. The peroxidase-conjugated anti-rabbit IgG secondary antibody (Cell Signaling Technology) was incubated with the membranes for 2 h at room temperature. Immunoblotting signals were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the instruction suggested by the manufacturer. All the membranes were re-probed with anti-β-actin (sc47778) or lamin B (sc6216) antibodies (Santa Cruz Biotechnology), which served as loading controls.

RNA isolation and RT-qPCR. Total RNA was isolated using Trizol (Roche, Basel, Switzerland) and served as loading controls. cDNA was generated using Oligo(dT) primers and SuperScript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer. The cDNA was then diluted in PBST to a final concentration of 500 μM. A standard curve for each gene (Supplementary Table S3) was designed using the Primer Express program (Applied Biosystems). The cDNA was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer recommendation. Briefly, cells were harvested 48 h after treatment and suspended in PBS at 1 × 10^6 cells/ml. Both APC-Cy544 and PE-Cy7 antibodies or their respective isotype controls APC-IgG1 and PE-IgG1 were added to the cells suspensions at concentrations recommended by the manufacturer and incubated for 40 min at 4 °C in the dark. The labeled cells were washed in PBS and then analyzed with a flow cytometer (BD FACSCanto II, Becton Dickinson). Gating was set to relative isotype control (APC-IgG1 and PE-IgG1)-labeled cells for each cell line.

Aldefluor assay. An Aldefluor assay kit (StemCell Technologies, Vancouver, BC, Canada) was used for the determination of ALDH activity with flow cytometry as described by Hu et al.5 Briefly, cells were harvested 48 h after treatment and suspended in Aldefluor assay buffer at 1 × 10^6 cells/ml. As a negative control, half the sample was transferred to a tube containing 5 μl of the ALDH inhibitor diethylamino benzaldehyde. Activated Aldefluor substrate (5 μl) was added to both samples and incubated at 37 °C for 45 min to allow substrate conversion. The cells were resuspended in Aldefluor assay buffer and analyzed using a flow cytometer (BD FACSCanto II, Becton Dickinson).

Figure 6 The antitumor activity of BKM120 in MCF-7/A02 and CALDOX xenograft tumors. (a and b) Tumor sizes of MCF-7/A02 (a) and CALDOX (b) xenografts after treatment with PBS (control), DOX, BKM120 or BKM120 plus DOX. Data are presented as the mean tumor size ± S.D. of six mice per group. (c and d) Body weight of nude mice bearing MCF-7/A02 (c) and CALDOX (d) xenografts treated with PBS (control), DOX, BKM120 or BKM120 plus DOX. Data are presented as the mean body weight ± S.D. of six mice per group. (e) Western blot analysis of pAKT, AKT, nuclear NF-κB p65 and total NF-κB p65 on MCF-7/A02 and CALDOX derived tumors treated with BKM120 or PBS. Tumors were obtained from two mice randomly chosen from six mice per group. Both lamin B and β-actin were used as loading controls. (f) Relative fold of Bax, Bim and Survivin gene expression levels in MCF-7/A02 and CALDOX derived tumors treated with BKM120 or PBS. * P < 0.05.
step of the staining procedures. The coverslips were covered with ProLong Gold antifade mounting medium (Invitrogen) and then were viewed and photographed with a Zeiss LSM 510 Meta fluorescence microscope (Zeiss, Jena, Germany).

**Soft agar colony formation assay.** Cells were disassociated and suspended in DME/DMEM supplemented with 3% agar and plated on the top of a solidified layer of 0.6% agar. The cells were plated at a density of 2 × 10³ cells/well in six-well plates, and the colonies were counted 21 days later.

**Mammmosphere culture.** Cells (1 × 10⁶) were plated in each well of an ultralow attachment plate (Corning Incorporated, Corning, NY, USA), supplemented with B27 (Invitrogen), 20 ng/ml EGF and 20 ng/ml basic FGF (BD Biosciences). Colony formation was assessed 10 days later.

**In vivo xenografts.** Cells (1 × 10⁶) were suspended in 100 μL PBS containing 50% Matrigel (BD Biosciences) and injected into the mammary fat pad of 4–5-week-old female nude mice (Vital River Company, Beijing, China). Tumor sizes were measured every 3 days in two dimensions using a caliper, and the tumor volume was calculated with the following formula: tumor volume (mm³) = 0.5 × a² × b (a and b being the longest and shortest diameters of the tumor, respectively). Fourteen days after cell injection, the tumor-bearing mice were randomly divided into four groups (six animals/group): (1) control group (normal saline), (2) BKM120 group (50 mg BKM120 per kg BW), (3) DOX group (2 mg doxorubicin per kg BW), and (4) BKM120+DOX group (50 mg BKM120 and 2 mg DOX per kg BW). Drugs were injected every 3 days, and tumor volumes were monitored till the mice were killed.

**Statistical analysis.** Comparisons of the means among more than two groups were performed by one-way analysis of variance. Student's t test was used when comparing the means of two groups. A P-value < 0.05 was considered statistically significant.

**Conflict of Interest.** The authors declare no conflict of interest.

**Acknowledgements.** We thank Professor Dongsheng Xiong (Institute of Hematology, PUMC, Tianjin, China) for a generous gift of human breast cancer cells MCF-7 and MCF-7/A02. We thank Dr. Ernesto Yagüe (Imperial College London, UK) for a generous gift of human breast cancer cells Cal51, CALDOX, MTMCE and MDM6. We also thank Professor Zhenmin Lei and Dr. Ernesto Yagüe for helpful comments to the manuscript. This work was supported by the Chinese National Natural Sciences Foundation (81402480 to YH), the Science and Technology Foundation of Tianjin Municipal Health Bureau (2014KZ078 to YH) and Tianjin municipal Major Scientific and Technological Special Project for Significant Anticancer Development (12ZCDZSY15700 to ZJ).

1. Hennessy M, Spiers JP. A primer on the mechanics of P-glycoprotein the multidrug transporter. Pharmacol Res 2007; 55: 1–15.
2. Steinbach D, Legrand O, ABC transporters and drug resistance in leukemia: was P-gp – the first? Leukemia 2007; 21: 1172–1176.
3. Niero EL, Rocha-Sales B, Lauand C, Cortez BA, de Souza MM, Rezende-T eixeira P et al. The multiple facets of drug resistance: one history, different approaches. Leukemia 2007; 21: 1172–1176.
4. Andre F, Nahta R, Conforti R, Boulet T, Aziz M, Yuan LX et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst 2008; 100: 672–679.
5. Cheng JD, Lindsey CW, Cheng GZ, Yang H, Nicolaov SV. The Akt/PKB pathway: molecular target for cancer drug discovery. Oncogene 2005; 24: 7482–7492.
6. Steinbach D, Legrand O. ABC transporters and drug resistance in leukemia: was P-gp – the first? Leukemia 2007; 21: 1172–1176.
7. Bendell JC, Rodon J, Burris HA, de Jonge M, Verweij J, Birle D et al. Phase I, dose-escalation study of BKM120, an oral pan-Class I PI3K inhibitor. J Clin Oncol 2012; 30: 292–300.
8. O'Brien NA, McDonald K, Tong L, von Eau E, Kalous O, Conklin D et al. Targeting PI3K/mTOR overcomes resistance to mTOR-targeted therapy independent of feedback inhibition of AKT. Cell Cancer Res 2012; 18: 3057–3062.
33. Thiery JP, Adoque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell 2009; 139: 871–890.

34. Porti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradin D et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2006; 66: 5565–5572.

35. Grimshaw MJ, Cooper L, Papazisis K, Coleman JA, Bohnenkamp HR, Chiapero-Stanke L et al. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. Breast Cancer Res 2008; 10: R52.

36. Miller TW, Baiko JM, Artesega CL. Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. J Clin Oncol 2011; 29: 4452–4461.

37. Ghebeh H, Al-Khalidi S, Olati S, Al-Dhtyan A, Al-Mohanna F, Barnawi R et al. Fascon involved in the chemotherapeutic resistance of breast cancer cells predominantly via the PI3K/Akt pathway. Br J Cancer 2014; 111: 1552–1561.

38. Bunts HA 3rd. Overcoming acquired resistance to anticancer therapy: focus on the PI3K/AKT/MTOR pathway. Cancer Chemother Pharmacol 2013; 71: 829–842.

39. Fumara C, Bonelli MA, Petronini PG, Affifi RR. Targeting PI3K/AKT/MTOR pathway in non small cell lung cancer. Biochem Pharmacol 2014; 90: 197–207.

40. Polak R, Buitenhuys M. The PI3K/PKB signaling module as key regulator of hematopoiesis: implications for therapeutic strategies in leukemia. Blood 2012; 119: 911–923.

41. Kott M, Goering RJ, Naim P, Haehrel A, Crane C, Weberpal J et al. Identification of the IGF/PI3K/Akt inhibitor, perifosine, as gene signaling networks associated with chemotherapeutic resistance and treatment response in high-grade serous ovarian epithelial cancer. BMC Cancer 2013; 13: 549.

42. Rosich L, Sabato-Villarroya I, Lopez-Guerra M, Xargay-Torrent S, Montserrat A, Aymerich M et al. The phosphatidylinositol-3-kinase inhibitor NVP-BKM120 overcomes resistance signals derived from microenvironment by regulating the Akt/FoxO3a/Bim axis in chronic lymphocytic leukemia cells. Haematologica 2013; 98: 1739–1747.

43. Miller TW, Baiko JM, Fox EM, Ghazoui Z, Dunbier A, Anderson H et al. E2F transcription can mediate resistance to estrogen deprivation in human breast cancer. Biochem Pharmacol 2011; 81: 338–351.

44. Lonetti A, Antunes IL, Chiarini F, Orsini E, Buontempo F, Ricci F et al. Clinical response of disulfiram/copper on human glioblastoma cell lines and ALDH-positive cancer stem-like cells. Br J Cancer 2012; 107: 1488–1497.

45. O’Brien NA, McDonald K, Tong L, von Euw E, Kalous O, Conklin D et al. Targeting PI3K/mTOR overcomes resistance to HER2-targeted therapy independent of feedback activation of AKT. Clin Cancer Res 2014; 20: 3507–3520.

46. Chen X, Zhao M, Hao M, Sun X, Wang J, Mao Y et al. Dual inhibition of PI3K and mTOR mitigates compensatory AKT activation and improves tamoxifen response in breast cancer. Mol Cancer Res 2013; 11: 1269–1278.

47. Majer IA, Abramson VG, Iasikoff SJ, Forero A, Balko JM, Kuba MG et al. Stand up to cancer phase Ib study of pan-panthiostatin-3-kinase inhibitor buparlisib with letrozole in estrogen receptor-positive/human epidermal growth factor receptor 2-negative metastatic breast cancer. J Clin Oncol 2014; 32: 1202–1209.

48. Shi R, Li W, Zhang X, Yang H, Xie Y et al. A novel indirubin derivative PHII-L potentiates adriamycin cytotoxicity via inhibiting P-glycoprotein expression in human breast cancer MCF-7/ADR cells. Eur J Pharmacol 2011; 659: 38–44.

49. Zhou Y, Hu Y, Yang M, Jat P, Li K, Lombardo Y et al. The mR-106b–25 cluster promotes bypass of doxorubicin-induced senescence and increase in motility and invasion by targeting the E-cadherin transcriptional activator EP300. Cell Death Differ 2014; 21: 462–474.

50. Zhao JJ, Gjoerup OV, Subramanian RR, Cheng Y, Chen W, Roberts TM et al. Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. Cancer Cell 2003; 3: 483–495.

51. Hu Y, Li S, Yang M, Yan C, Fan D, Zhou Y et al. Sorcin silencing inhibits epithelial-to-mesenchymal transition and suppresses breast cancer metastasis in vivo. Breast Cancer Res Treat 2014; 143: 267–299.

52. Hu Y, Cheng X, Li S, Zhou Y, Wang J, Cheng T et al. Inhibition of sorcin reverses multidrug resistance of MCF7/A02 cells and MCF-7/A02 cells via regulating apoptosis-related proteins. Cancer Chemother Pharmacol 2013; 72: 769–786.

**NVP-BKM120 overcomes MDR and eliminates CSC**

Y Hu et al.