Abstract. Deregulation of the phosphatidylinositol-3-kinase (PI3K)/Akt signalling pathway is common in breast cancer and is frequently associated with resistance to both traditional chemotherapy and targeted drugs. There is a growing body of evidence indicating that a small subpopulation of self-renewing cells, the so-called cancer stem cells (CSC), are responsible for the growth of drug-resistant secondary tumors. As many CSCs have upregulated the PI3K/Akt signalling pathway, preclinical and clinical studies are addressing the inhibition of this axis to target drug resistance. We evaluated the susceptibility of breast CSCs to NVP-BKM120 (BKM120), a new generation of PI3K-specific inhibitor, when used individually or in combination with trastuzumab or RAD001 both in vitro and in vivo. For this, a stem-like cell population (SC) was enriched from breast cancer cell lines after mammosphere cultures. We demonstrated that BKM120 inhibits growth, generation of drug-resistant derivatives and SC formation in a panel of four breast cancer cell lines: MCF-7, MDA-MB-231, SK-BR-3 and CAL51. Importantly, BKM120 inhibits the PI3K/Akt signalling pathway in SCs from these cell lines. When BKM120 was used in combination with trastuzumab, a targeted therapy to treat HER2-positive breast cancer, we found synergistic cell growth inhibition, generation of drug-resistant cells as well as SC formation from SK-BR-3 cells. Importantly, SK-BR-3 xenograft-derived tumors showed marginal growth when the drug combination was used. We also found a similar synergistic anticancer effect of BKM120 in combination with RAD001, an mTOR inhibitor, when treating triple-negative breast cancer cells in vitro and in both MDA-MB-231 and CAL51 mouse xenografts. Moreover, mouse data indicate that these drug combinations are well tolerated and provide the proof-of-concept and rationale to initiate clinical trials in both HER2-positive and triple-negative breast cancer.

Introduction

Breast cancer is the most frequent tumor in women and is the second cause of death from malignant diseases among women worldwide (1). Early diagnosis and development of targeted therapies have contributed to a reduction in breast cancer mortality. However, after an initial response to treatment, a high proportion of breast cancer patients become non-responsive to therapy. This drug resistance phenomenon has been observed with both traditional and targeted therapies and is the main cause of breast cancer mortality (2,3). Therefore, an understanding of the mechanisms of drug resistance is crucial for patient stratification and to develop novel targeted therapies. Cancer stem cells (CSCs), a small subpopulation of cancer cells with self-renewal, differentiation, and tumorigenic capabilities (4), have been suggested to explain many of the features of drug-resistant tumors (5). CSCs have an increased resistance to a variety of chemotherapeutics in comparison to non-CSCs and are thought to drive tumor growth after an initial response to therapy (5). Although targeting CSCs is...
The phosphatidylinositol-3-kinase (PI3K) pathway plays a key role in the regulation of cell survival, growth, migration and proliferation of normal cells. Importantly, it is the most frequently misregulated signalling pathway in cancer (7). It has been implicated in the development, progression, and therapy resistance of breast cancer (8), mainly due to the activation of Akt (9). PI3K resistance of breast cancer (8), mainly due to the activation of its major downstream effector Akt (9). PI3Kα is a heterodimer with adaptor function, made up of a regulatory subunit of 85 kDa (p85) and one of three possible catalytic subunits of 110 kDa (p110) encoded by the PIK3CA (p110α), PIK3CB (p110β) and PIK3CD (p110γ) genes (10). Activation of PI3K stimulates phosphorylation of phosphatidylinositol-4,5-diphosphate (PIP2), a phospholipid component of the cell membrane, and generation of phosphatidylinositol-3,4,5-triphosphate (PIP3), which bind pleckstrin homology domains of various signalling proteins. An inhibitory effect is exerted by the tumor suppressor PTEN (phosphatase and tensin homologue) which hydrolyses and thus inactivates PIP3 (10). Importantly, activating mutations in the catalytic subunit of PI3K (p110α) and inactivating mutations in PTEN are frequently found in cancer (7).

NVP-BKM120 (BKM120) is a 2,6-dimorpholino pyrimidine derivative that is a potent pan-class I PI3K inhibitor, highly selective against other kinases including mammalian target of rapamycin (mTOR) (11). It shows anti-proliferative activity and induces apoptosis in cancer cell lines through inhibiting the PI3K/Akt signalling pathway (12,13). Phase I clinical trials indicate that BKM120 is safe at the maximum-tolerated dose with a favourable pharmacokinetic profile in several solid tumors (14) and has been reported to override trastuzumab resistance in several breast cancer cell lines (15). Importantly, BKM120 has shown enhanced antitumor effect in mouse models when combined with inhibitors of other signalling pathways (16,17).

An important downstream effector of PI3K/Akt is mTOR, a key activator of protein synthesis, a process which is frequently enhanced in cancer cells (18). Thus, the rapamycin analogue, and mTOR inhibitor, RAD001 (Everolimus) has gained attention as an anticancer agent and has been used in advanced renal cancer after failure of therapy to target vascular endothelial growth factor (19). Importantly, in addition to mTOR signalling inhibition, rapamycin analogues cause Akt activation and attenuation of their therapeutic efficacy (20,21). Thus, it has been suggested that the combination of BKM120 and RAD001 may overcome these effects and has shown positive results in lung cancer mouse models (16).

In this study we demonstrate the efficacy of BKM120 combined with either trastuzumab or RAD001 targeting breast cancer stem cells. BKM120 displays antitumor activity by inhibiting the PI3K/Akt signalling pathway. The combination of BKM120 with either trastuzumab or RAD001 leads also to a decrease in the generation of drug resistant derivatives in vitro and excellent tumor response in xenograft mouse models.

Materials and methods

Cell lines and chemicals. Luminal A group MCF-7, claudin-low group triple-negative MDA-MB-231, triple-negative CAL51 (22), and HER2 group trastuzumab-responsive SK-BR-3 breast cancer cell lines were used (23). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 g/l glucose, 10% foetal calf serum and 4 mM L-glutamine (Life Technologies). NVB-BKM120 and RAD001 were a kind gift of Novartis. The HER2-inhibitor trastuzumab was obtained from Roche.

Flow cytometry. For stem cell markers, FITC-conjugated anti-CD44 and phycoerythrin-conjugated anti-CD24 antibodies, or their respective isotype controls, all from BD Biosciences were used essentially as described (24). An Aldefluor assay kit (StemCell Technologies) was used for the determination of aldehyde dehydrogenase (ALDH) activity by flow cytometry essentially as described (24). Briefly, cells were resuspended in assay buffer (10^6 cells/ml) and activated aldefluor substrate (5 µl) was added to samples and incubated at 37°C for 45 min to allow substrate conversion. A sample with the ALDH inhibitor diethylaminobenzaldehyde was used as a negative control.

Mammosphere formation. Mammospheres were grown as described (24). In brief, cells (1x10^5) were plated in each well of an ultralow attachment 6-well plate (Corning) with 3 ml serum-free mammary epithelial growth medium (MEGM; BioWhittaker), supplemented with 2% B27 (Invitrogen), 20 ng/ml EGF and 20 ng/ml bFGF (BD Biosciences). Mammospheres were grown for 10 days and phase contrast images were obtained using Nikon TS100 microscope (Nikon, Shanghai, China). Where indicated, mammospheres were collected by centrifugation at 150 x g for 10 min at room temperature, trypsinized, counted and used in further experiments (25).

Cell viability analysis. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were performed to evaluate the cell growth inhibitory effect in response to drug treatments and were used to determine the concentration of drug that inhibited cell growth by 50% (IC50) after 3 days of treatment (26). For drug combination experiments, a combination index (CI) was calculated using the CalcuSyn software (Biosoft) based on the Chou and Talalay method (27). CI values between 0.9 and 1.1 being additive, whereas values >1.1 are antagonistic.

Drug resistance clonogenic assay. Cells (2x10^5/well of a 6-well plate) were treated with a single drug or a combination of drugs as indicated for 1 week. Drug resistant proliferating clones were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. Crystal violet retained within the cells was quantified by solubilization with 0.5% acetic acid and measurement of optical density at 592 nm (28).

Protein extraction and western blotting. A modified RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.25% SDS, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) with protease inhibitor cocktail (Sigma) was used for protein isolation from cells. Protein concentrations were determined using the BCA Protein Assay kit (Pierce). Cell lysates containing 50 µg of protein were...
resolved on 12% (w/v) polyacrylamide gels, transferred to nitrocellulose membranes (Millipore) and blocked with 5% blotting grade milk (Bio-Rad) in PBST (0.1% Tween-20 in PBS). Membranes were then incubated with primary antibodies to phospho-Akt (D9E), Akt1 (C73H10), phospho-S6 (D57.2.2E), S6 (54D2) and β-actin (13E5) (Cell Signaling Technology) at 1:1,000 dilution at 4˚C overnight, followed by HRP-conjugated secondary antibodies (Cell Signaling Technology) at 1:2,000 dilution for 2 h at room temperature. Signals were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce) according to the manufacturer’s instructions.

In vivo assays. Cells (5x10^6) were trypsinized and resuspended in a total volume of 100 µl PBS containing 50% Matrigel (BD Biosciences) and were injected into the mammary fat pad of nude mice (5-6 weeks of age). Tumor sizes were measured every three days in two dimensions using callipers, and the tumor volume calculated [tumor volume (mm^3) = 0.5 x ab^2; a and b being the longest and shortest diameters of the tumor, respectively]. Fifteen days after cell injection, the tumor-bearing mice were randomly divided into four groups (five animals per group) and received: group 1, saline (control group); group 2, 50 mg/kg BKM120 (BKM120 group); group 3, either 5 mg/kg trastuzumab (trastuzumab group) or 2 mg/kg RAD001 (RAD001 group); and group 4, a combination of BKM120 and either trastuzumab or RAD001 (at the above doses; combination group). Drugs were injected intraperitoneally every three days and tumor volume and mouse weight monitored until mice were sacrificed in a humane manner. All mice were maintained as required under the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. The use of animals in this study was approved by the Animal Care and Use Committee of Tianjin Cancer Hospital.

Statistical analysis. Statistical evaluations were performed by Student’s t-test for paired data and by ANOVA for sets of data with multiple comparison points. Statistical significance was considered at p<0.05.

Results

BKM120 effectively inhibits the growth of breast cancer stem-like cells. Given the importance of the PI3K pathway in cancer, we asked whether BKM120 had a differential effect in the stem-like sub-populations from several breast cancer cell lines. Breast stem-like cells (SCs) exhibit the ability to survive and grow as mammospheres in low attachment plates (29,30), are characterized by a CD44^+CD24^-ALDH1^+ phenotype and show strong tumorigenicity in NOD/SCID...
mouse models (31,32). We isolated SCs from SK-BR-3, MDA-MB-231, MCF-7 and CAL51 cells after proliferation in low attachment plates. In all cases, the proportion of CD44+/CD24- and ALDH1+ cells was higher in SCs than in the original cell population (Figs. 1A and 5A). Next we tested the effect of BKM120 on cell survival using MTT assays. As expected, the pan-PI3K inhibitor had a dose-dependent effect on cell proliferation, both in the SC subpopulation and, to a greater extent, in the total cells (Figs. 1B and 5C). SCs resistance ratios varied between 4.26 in MCF-7 and 6.79 in CAL51 cells (Table I). Comparison with the IC50 obtained previously by us on similar cell sub-populations (33) indicates that BKM120 is more effective targeting SCs than docetaxel, a taxane type drug affecting cell proliferation by disruption of microtubules. As drug resistant cells have been proposed to arise from the selection of a small population of cells with stem-like properties (5), we asked whether BKM120 could inhibit the formation of drug resistant clones. For this, SCs were left to grow as monolayers up to one week with or without BMK120 and the cell mass determined by crystal violet staining. The pan-PI3K inhibitor decreased the proliferation of resistant cells in a dose-dependent manner in MDA-MB-231, MCF-7 and SK-BR-3 cells (Fig. 1C). As BKM120 inhibited growth on monolayer cultures, we also performed mammosphere forming assays to detect whether BKM120 could eliminate SC growth. Indeed, the mammosphere-forming efficiency (MFE) decreased in a dose-dependent manner in MDA-MB-231, SK-BR-3, MCF-7 (Fig. 1D) and CAL51 (Fig. 5E) cells. Thus, BKM120 inhibits the growth of breast cancer SCs.

BKM120 effectively inhibits the PI3K/Akt/mTOR signalling pathway. As BKM120 is a pan-PI3K inhibitor, we next sought to determine its effect on the PI3K/Akt/mTOR axis in breast cancer cells. For this, we analyzed by western blotting the total and phosphorylated levels of Akt and ribosomal protein S6, both in SCs and the total cellular population. Both phosphorylated Akt and S6 levels were higher in SCs than in the whole cell population, although there were variations among cells. MCF-7 and SK-BR-3 SCs showed higher activation of Akt than MDA-MB-231 SCs. Ribosomal protein S6 was clearly activated in SK-BR-3 SCs, although the activation was less robust in the other two SC subpopulations (Fig. 2A).

Having confirmed an activation of the PI3K/Akt/mTOR pathway in SCs, we treated both SCs and the whole cell population with a range of BKM120 concentrations for 24 h and determined the extent of the above proteins by western blotting. As expected, higher doses of BKM120 were necessary to decrease the levels of phospho-Akt and phospho-S6 in SCs than in the whole cell population. For instance, 2 µM BKM120 completely inhibited the Akt pathway in MDA-MB-231 cells whereas in the SC subpopulation a partial inhibition was obtained after treatment with 4 µM and a total inhibition was achieved only with 10 µM BKM120. Similarly, phospho-S6 was absent in cells treated with up to 4 µM BKM120, whereas 10 µM BKM120 partly inhibited S6 activation in SCs (Fig. 2B).

Therefore, BKM120 exerts potent suppressive effects on PI3K/Akt/mTOR signalling in both total and SCs subpopulations of breast cancer cells.

The combination of BKM120 and trastuzumab synergistically inhibits the growth of SK-BR-3 cells and eliminates the SC subpopulation. Alterations in the PI3K/Akt signalling pathway have been associated with therapy-induced resistance in
breast cancer patients, including endocrine-based therapy and combined chemotherapy and HER2-targeted-therapy (34,35). Recent studies demonstrate that targeting the PI3K/Akt pathway in combination with trastuzumab, a monoclonal antibody that interferes with the HER2/neu receptor, is beneficial in trastuzumab-resistant breast cancer (15). As BKM120 has been shown to have robust anticancer properties in breast cancer SCs, we asked whether BKM120 could synergize with trastuzumab in SK-BR-3, a HER2+ breast cancer cell line, especially in its SC subpopulation. For this, SK-BR-3 total cells and SCs were treated with increasing concentrations of trastuzumab, either alone or in combination with BKM120. As expected, trastuzumab decreased cell survival, although the effect was more noticeable in the whole SK-BR-3 population (IC50 ~10 µg/ml) than in SCs (IC50 >100 µg/ml) (Fig. 3A). When used in combination, the CI values ranged from 0.3 to 0.6 (Fig. 3B), indicating that trastuzumab and BKM120 act synergistically both in total SK-BR-3 cells and SCs (27). Importantly, trastuzumab in combination with BKM120 had a greater effect suppressing the generation of resistant cells (Fig. 3C) and mammospheres (SCs; Fig. 3D) than the individual drugs acting alone. Western blot analyses also indicated a stronger effect on the PI3K/Akt/mTOR pathway when the drugs were combined. Both phospho-Akt and phospho-S6 levels decreased to a high extent in SCs, whilst, as expected, the effect was stronger in the whole cell population (Fig. 3E).

Next, we used a xenograft model to assess the efficacy of this drug combination against the growth of SK-BR-3-derived tumors. For this, SK-BR-3 SCs were injected into the mammary fat pad of female nude mice. Mice were then randomly divided into four groups 14 days after injection and treated with vehicle, BKM120, trastuzumab, or a combination of the two. As expected, tumor growth followed a steady progress during the following 15 days in the control group, whereas the tumor volume increased at lower rates in the BKM120 and trastuzumab groups. Importantly, the group receiving both BKM120 and trastuzumab showed a slight tumor growth
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with no significant mouse body weight loss, indicating that the drug combination is well tolerated (Fig. 3F).

In summary, BKM120 in combination with trastuzumab acts synergistically inhibiting the PI3K/Akt/mTOR pathway, the growth of HER2+ cells, the generation of drug-resistant SCs in vitro, and the formation of tumors in vivo.

The combination of BKM120 and RAD001 synergistically inhibits the growth of triple-negative breast cancer cells both in vitro and in vivo. We have previously demonstrated that the combination of RAD001 with docetaxel, latrozole or trastuzumab has enhanced growth-inhibitory effects against breast cancer SCs (33,36,37). As RAD001 has been reported to act synergistically with BKM120 in lung cancer models (16), we asked whether a similar effect would be observed in triple-negative breast cancer (TNBC). TNBC remains a challenging clinical problem due to a lack of targeted therapies and, consequently, a high mortality rate. For this purpose, total cells and SCs of MDA-MB-231 and CAL51 cell lines were treated with increasing concentrations of RAD001, either alone or in combination with a range of doses of BKM120. As expected, RAD001 decreased cell survival, although the effect was more noticeable in the total cell population than in SCs (Figs. 4A and 5B). When used in combination, RAD001 and BKM120 CI values ranged from 0.3 to 0.7 in MDA-MB-231 (Fig. 4B) and from 0.2 to 0.8 and CAL51 cells (Fig. 5C). This indicates that RAD001 and BKM120 act synergistically when used in combination, both in the total population and in the SC subpopulations of these two cell lines (27). Importantly, RAD001 in combination with BKM120 had a greater effect on the generation of resistant cells (Figs. 4C and 5D) and mammosphere formation (Figs. 4D and 5E) than the drugs individually.

Inhibition of mTOR leads to feedback reactivation of PI3K activity in a variety of systems (21,38). We confirmed that RAD001 treatment, as a single agent, increased Akt phosphorylation in MDA-MB-231 (Fig. 4E) and CAL51 cells (Fig. 5F). However, RAD001 failed to activate PI3K activity in the presence of BKM120, both in the total cell popu-
lation and SCs. The combination of BKM120 and RAD001 also showed more activity in reducing phospho-S6 levels than either single agent did when acting alone (Figs. 4E and 5F). Thus, the combination of BKM120 and RAD001 blocks RAD001-induced phosphorylation of Akt and exerts enhanced effects on suppression of phospho-S6.

Because of the growth-inhibitory effects of the BKM120 and RAD001 combination in TNBC SCs in vitro (Figs. 4B and 5C), we sought to determine whether the same effect could be found in vivo. For this, MDA-MB-231 SCs or CAL51 SCs were injected into the mammary fat pad of female nude mice. Mice were then randomly divided into four groups 14 days after injection and treated with vehicle, 1 µM BKM120, 0.5 µM RAD001 or a combination of the two drugs for seven days and stained with crystal violet. After dye solubilization, the optical density at 592 nm was determined. (E) Effect of a combination of RAD001 and BKM120 on the growth of mammospheres from CAL51 cells. Cells were treated with vehicle, 1 µM BKM120, 0.5 µM RAD001 or a combination of the two drugs for ten days. Mammosphere formation was visualized at x20 magnification (left panel) and MFE calculated (right panel). (F) Effect of BKM120 and RAD001 on Akt and S6 phosphorylation in CAL51 cells. Both TCs (left panel) and SCs (right panel) were treated with 1 µM RAD001 in the absence or presence of 1 µM BKM120 for 24 h and the levels of Akt, S6 and their phosphorylated forms analyzed by western blotting. β-actin was used as a loading control. (G) Antitumor activity of BKM120 combined with RAD001 in CAL51 xenograft tumors. Growth of CAL51 xenograft tumors (expressed as tumor volume) after treatment with PBS (control), RAD001, BKM120, or BKM120 plus RAD001 (left panel, see Materials and methods for experimental details). Data represent mean tumor size ± SD of five tumors per group. Right panel illustrates body weight of nude mice bearing CAL51 xenografts. Data indicate mean body weight ± SD of five mice per group. Other numerical data represent the mean ± SD of three independent experiments (*P<0.05; NS, not significant). Pictorial data were repeated at least in triplicate and a representative picture is shown.

In summary, BKM120 in combination with RAD001 acts synergistically inhibiting the PI3K/Akt/mTOR pathway, the growth of TNBC cells, the generation of drug-resistant derivatives in vitro, and the formation of tumors in vivo.

Discussion

The development of cancer targeted therapies has been of paramount importance for the increase in patient survival achieved during the last few decades (39,40). However, most targeted therapies, as well as traditional non-targeted cytotoxic and radiation therapies, are encumbered by the acquisition of resistance. Accumulating evidence indicates that CSCs play a crucial role in therapy resistance and recurrence of breast cancers (5,32). Therapy resistance is a complex phenomenon involving multiple mechanisms, including activation of signalling pathways such as the PI3K/Akt/mTOR axis (41), and the activation of this pathway is crucial for maintaining the stemness and chemoresistance of breast CSCs (42). Hence, breast CSCs are critical therapeutic targets and their elimination may improve the prognosis and outcome of cancer therapy (43). PI3K inhibition has
recently been shown to sensitize CSCs to chemotherapy and targeted therapy in several cancers including leukemia (44), hepatocellular carcinoma (45) and breast cancer (33). In line with these studies, we present data indicating that the PI3K inhibitor BKM120 is also effective in eliminating breast CSCs. After BKM120 treatment, the in vitro tumorigenicity of breast cancer cells is highly impaired. Moreover, BKM120 exerts tumor inhibiting effect on breast SCs-derived xenograft models in vivo, further confirming the potency of BKM120 in CSCs.

Aberrant activation of several signalling pathways downstream of HER2, including the MAPK (46), Notch (47) and PI3K/Akt pathways (48), leads to HER2-targeted therapy resistance. Since HER2 mediates signal transduction through the PI3K/Akt pathway, inhibition of components of this pathway is a reasonable approach to overcome resistance to HER2-targeted therapy (15,49). Indeed, the combination of PI3K inhibitor BAY 80-6946 with HER2-targeted therapy inhibits HER2-positive breast cancer cell growth more effectively than either therapy used alone (50), and the phase lb study of BKM120 plus trastuzumab in HER2-positive breast cancer patients has shown promising results (51). Here, we report that BKM120 has a synergistic effect with trastuzumab on HER2-positive breast SCs in vitro and, importantly, that the drug combination is well tolerated in mouse models. This adds weight to the design of future trials with a combination of BKM120 and trastuzumab in HER2-positive breast cancer patients.

TNBC is a heterogeneous disease comprised of several biologically distinct subtypes (52). In addition to our poor understanding of the molecular characteristics of each TNBC subtype, we lack effective targeting strategies, leading to a poor prognosis for TNBC patients. However, anti-angiogenic, EGFR-targeted, PARP inhibitors, PI3K/Akt/mTOR inhibitors and Src inhibiting therapies have demonstrated promising results (53). It is important to note that CSCs are highly resistant to these drugs (54). In this study, we used a combination of a pan-PI3K inhibitor BKM120, with either trastuzumab or RAD001, which is effective in targeting breast cancer SCs in vitro and offers the rationale to develop further clinical trials for HER2-positive and TNBC, respectively.

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