In Vitro and In Vivo Synergistic Antitumor Activity of the Combination of BKM120 and Erlotinib in Head and Neck Cancer: Mechanism of Apoptosis and Resistance

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Abstract

We previously reported that the EGFR-targeted inhibitor erlotinib induces G\textsubscript{1} arrest of squamous cell carcinoma of the head and neck (SCCHN) cell lines without inducing significant apoptosis. Large-scale genomic studies suggest that >50% of SCCHN cases have activation of PI3K pathways. This study investigated whether cotargeting of EGFR and PI3K has synergistic antitumor effects and apoptosis induction. We examined growth suppression, apoptosis, and signaling pathway modulation resulting from single and combined targeting of EGFR and PI3K with erlotinib and BKM120, respectively, in a panel of SCCHN cell lines and a xenograft model of SCCHN. In a panel of 12 cell lines, single targeting of EGFR with erlotinib or PI3K with BKM120 suppressed cellular growth without inducing significant apoptosis. Cotargeting of EGFR and PI3K synergistically inhibited SCCHN cell line and xenograft tumor growth, but induced variable apoptosis; some lines were highly sensitive, others were resistant. Mechanistic studies revealed that the combination inhibited both axes of the mTORC1 (S6 and 4EBP1) pathway in apoptosis-sensitive cell lines along with translational inhibition of Bcl-2, Bcl-xl, and Mcl-1, but failed to inhibit p-4EBP1, Bcl-2, Bcl-xl, and Mcl-1 in an apoptosis-resistant cell line. siRNA-mediated knockdown of eIF4E inhibited Bcl-2 and Mcl-1 and sensitized this cell line to apoptosis. Our results strongly suggest that cotargeting of EGFR and PI3K is synergistic and induces apoptosis of SCCHN cell lines by inhibiting both axes of the AKT–mTOR pathway and translational regulation of anti-apoptotic Bcl-2 proteins. These findings may guide the development of clinical trials using this combination of agents.

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Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer worldwide, with more than 59,000 new cases and approximately 12,000 deaths annually in the United States alone \cite{1}. Although the next generation of cancer treatments will be guided by personalized approaches in which molecularly targeted agents will be the key players, the current status of personalized therapy for SCCHN patients is disappointing due to the lack of well-defined molecular drivers associated with carcinogenesis of this disease \cite{2, 3}. Unlike other solid tumors, SCCHN involves multiple anatomic sites and risk factors (sometimes a combination of risk factors), contributing to intra- and intertumor heterogeneity. Following the development of next-generation sequencing, several large-scale genomic profiling studies of SCCHN tumors have been reported, which further demonstrate the molecular diversity of these tumors \cite{3–5}.

Over 90% of SCCHN tissues have overexpression of EGFR mRNA \cite{6} and 38%–47% have overexpression of EGFR protein \cite{7, 8}. High expression of EGFR has been associated with reduced survival \cite{9} and poor prognosis \cite{9–11}. Until the very recent approval of immunotherapeutics for SCCHN, the EGFR-targeted mAb cetuximab was the only FDA-approved targeted agent for SCCHN with a response rate of less than 15% \cite{12}. Unlike non-small cell lung cancers (NSCLC), SCCHN rarely have the activating mutation in EGFR that determines EGFR-TKI sensitivity; thus, EGFR-targeting small-molecule tyrosine kinase inhibitors (TKI) have an even lower response rate than cetuximab in SCCHN \cite{13–15}. Studies in NSCLC suggest that EGFR-TKI induced apoptosis of cell lines containing EGFR-activating mutation at a very low concentration, but failed to do so in other cell lines \cite{16, 17}. We have previously reported that erlotinib induced G\textsubscript{1} cell-cycle arrest without significant apoptosis in SCCHN cell lines although erlotinib also induced Bim \cite{18, 19}, which might explain the low response rate of this agent in SCCHN. However, the induction of apoptosis is critical for successfully eliminating cancer cells from the human body.

Recently, the Cancer Genome Atlas (TCGA) data from SCCHN patients have demonstrated that more than 50% of patient tumors have activation of the PI3K and related pathways due to mutation in PI3KCA, loss of PTEN, or activation of RTKs,
suggesting PI3K as an excellent target in SCCHN (3). A number of small-molecule inhibitors targeting PI3K are currently under different stages of clinical development. Although cell lines harboring PI3KCA mutations are clearly more sensitive to PI3K inhibition than those having wild-type PI3KCA, there are differences in sensitivity based on the specific PI3KCA mutations (20, 21). Unfortunately, targeting PI3K with the PI3K-α–specific inhibitor BYL719 failed to induce significant apoptosis even at a very high concentration in head and neck cancer (22). Several clinical studies using PI3K inhibitors in squamous cell lung cancers, which are molecularly similar to SCCHN, suggest that PI3K inhibition alone might not be sufficient to induce successful tumor regression (23). Combination of BKM120 with cetuximab showed synergistic antiproliferative effects against SCCHN cell lines in vitro and in vivo irrespective of their PI3KCA mutation status (24, 25).

The purpose of this study was to examine whether the combination of EGFRTKI with small-molecule PI3K inhibitors has synergistic antitumor effects and induces efficient apoptosis. We found that combination of the EGFRTKI erlotinib and pan-PI3K inhibitor BKM120 synergistically inhibited the growth of nine out of ten SCCHN cell lines in vitro. Erlotinib is an oral EGFRTKI, approved in the United States as therapy for advanced pancreatic and NSCLC. NVP-BKM120 (referred to hereafter as BKM120) is a potent and highly selective pan-class I PI3K inhibitor, which belongs to the 2,6-dimorpholino pyrimidine derivatives (26). It selectively inhibits wild-type and mutant PI3K p110α, β, δ, γ isoforms and exerts a strong antiproliferative effect to induce apoptosis in several cancer types by specifically inhibiting the PI3K–AKT signaling pathway (27–29). Phase I clinical trials show that BKM120 is well tolerated overall in patients with several types of solid tumors (23, 29). We found that the combination of erlotinib and BKM120 induced variable levels of apoptosis. Some cell lines were very sensitive, some were moderately sensitive, and others were apoptosis resistant. We further demonstrated that inhibition of both axes of the mTORC1 pathway (i.e., S6 and 4E-BP1) is important for apoptosis induction. Finally, we found that the combination of the two agents more efficiently inhibited xenografted SCCHN tumor growth in vivo. Therefore, our study will guide the further clinical development of this combination against SCCHN.

Materials and Methods

Cell lines
The primary source and information about MDA686TU, MDA686LN, MSK-Leuk1, JHU022, SqCCy1, 1483, MSK-Leuk1, and FaDu cell lines were described previously (30). The head and neck premalignant cell line MSK-Leuk1 (MSK) was established from a dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue and maintained in keratinocyte basal media (31). MDA686TU, MDA686LN, and MSK-Leuk1 cell lines were obtained in 2014 and JHU022 in 2011. Cal27 and FaDu cell lines were purchased from the ATCC in 2014. SqCCy1, 1483 were obtained in 2007. The authenticity of all cell lines was verified through genomic short tandem repeat (STR) profiling by the Research Animal Diagnostic Laboratory, University of Missouri (Columbia, MO) in September 2009, and by the Emory University Integrated Genomics Core (EIGC) in October 2013. HPV-positive SCCHN cell lines UD-SCC2, UM-SCC47, 93-VLI-147T, and UPCI:SCC090 were obtained from the laboratories of Drs. Ferris and Gollin, respectively (University of Pittsburgh, Pittsburgh, PA) in 2011. The HPV status of these cell lines was confirmed by Western blotting (expression of p16). The mutation status of p53, PI3KCA, PTEN, and HRAS in MDA686TU, MDA686LN, 93-VLI-147T, UDSCC2, UM-SCC47, UPCI:SCC090, FaDu, JHU022, and SqCCy1 cell lines was confirmed in our laboratory (data not shown). Information for other cell lines was gathered from PubMed and summarized in Supplementary Table S1. All SCCHN cell lines were maintained in DMEM/F12 (1:1) medium supplemented with 10% heat-inactivated FBS in a 37°C, 5% CO2 humidified incubator.

Reagents

BKM120, BYL719, RAD001, rapamycin, and BEZ235 were purchased from Selleckchem and erlotinib from Genentech. These compounds were dissolved in DMSO as stock solutions and further diluted in DMEM/F12 medium immediately before use. The final concentration of DMSO was <0.1%.

Cell growth inhibition assay and measurement of IC50, combination index (CI), and dose reduction index (DRI)

To test the effects of single-agent BKM120 and erlotinib or their combination on the growth of SCCHN cells, sulforhodamine B (SRB) cytotoxicity assays were performed. Cells maintained in medium with 10% FBS were seeded in 96-well plates at a density of 4,000 cells/well overnight prior to drug treatment. Afterwards, drugs were added as single agents in various concentrations (0–4 μmol/L for BKM120 and erlotinib) or 1:1 combinations. After 72 hours, cells were fixed with 10% cold trichloroacetic acid for 1 hour, washed 5 times in water, air-dried, and then stained with 0.4% SRB for 10 minutes. After washing 4 times with 1% acetic acid and air-drying, bound SRB was dissolved in 10 mmol/L unbuffered Tris base (pH 10.5). Plates were read in a microplate reader by measuring absorbance at 492 nm. The percent survival was then calculated based upon the absorbance values relative to the untreated samples. The IC50 values, CI, and DRI were calculated by using CalcuSyn software (Biosoft). A CI value >1 is defined as antagonism, equal to 1 as additive and <1 as synergy. DRI represents the fold change of a focal effect when individual agents are used simultaneously relative to their separate effects, and their activity is synergistic if DRI >1. The experiment was repeated three times.

Annexin V–phycoerythrin staining for apoptosis

Cells were treated with the agents as indicated in the figure legends, trypsinized, and washed in cold 1 × PBS. The cells were then resuspended in 1 × Annexin binding buffer (BD Pharmingen), and stained with Annexin V–phycoerythrin (Annexin V–PE, BD Pharmingen) and 7-AAD (BD Pharmingen) for 15 minutes at room temperature. The stained samples were analyzed using a FACSCalibur bench-top flow cytometer (BD Biosciences). Data were analyzed for the apoptotic population using FlowJo Software (Tree Star). Cells stained with Annexin V–PE or double stained with Annexin V–PE and 7-AAD are considered apoptotic cells.

RNA extraction and qRT-PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). A total of 2 μg of RNA was reverse transcribed to
cDNA using a cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. Relative mRNA concentrations were quantified by qRT-PCR using the 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR green kit (Roche) for Bcl-2 and TaqMan kit (Applied Biosystems) for Bcl-xL and Mcl-1, respectively. The primers used for qRT-PCR of Bcl-2 were purchased from Sigma-Aldrich, and Bcl-xL and Mcl-1 from Life Sciences. Quantifications were always normalized using GAPDH as endogenous control and each reaction was performed three times. Relative quantification was performed according to the comparative $2^{-\Delta\Delta C_t}$ method as described previously (32).

Western blot analysis

Whole-cell lysates were extracted from cells using lysis buffer. The protein concentration of each sample was determined by protein assay kit (Bio-Rad). Equal amounts of protein (15 μg/lane) from each sample were separated on 12% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and incubated with appropriately diluted specific primary antibodies. Mouse anti-β-actin antibody was used as a sample loading control. Protein bands were detected with an enhanced chemiluminescence kit (Pierce). Sources and specific dilution factors for different antibodies are listed in Supplementary Table S2.

Nude mouse xenograft model

The animal experiments were approved by the Animal Care and Use Committee of Emory University. Twenty-eight female nude mice (athymic nu/nu, Taconic, aged 4–6 weeks (about 20 g weight), were inoculated with $2 \times 10^6$ MDA686TU cells by subcutaneous injection into the right flank. After 7 days of tumor cell inoculation when tumors were visible (average tumor volume 26.8 mm$^3$), the animals were randomly divided into 4 groups, 7 in each group. Each group was orally gavaged for 22 days with vehicle control [(1% Tween 80), BKM 120 (20 mg/kg in 1% Tween 20), erlotinib (50 mg/kg in 1% Tween 20)] or the combination of BKM 120 (20 mg/kg) and erlotinib (50 mg/kg) 5 days a week using a blunt tipped 20G needle (Popper and Sons) followed by two-day rest for recovery from oral gavage. In the combination group, the drugs were administered separately. The tumor size was measured twice a week. The tumor volume was calculated using the formula: $V = \frac{1}{2} \times \pi \times d_1 \times d_2 \times d_3$ (smaller diameter)$^2$. Growth curves were plotted using average tumor volume ± SE within each experimental group at the set time points. The mice were sacrificed at the end of the experiment and fresh tumor tissues were collected for IHC analyses.

siRNA transfection

Nontargeting control siRNA and eIF4E- and PTEN-specific siRNA were purchased from GE Dharmacon. Cells were seeded in 6-cm plates, 24 hours before transfection in medium containing 5% FBS, so that they reached 30%–50% confluency. siRNA was complexed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and applied to each plate. Transfection media were removed and replaced with fresh media after 8 hours of transfection. Knockdown efficiency of each target gene was evaluated by Western blotting 48 hours after transfection.

IHC analysis

IHC analysis of paraffin-embedded nude mouse xenograft tissues was performed using a Tissue Staining Kit following the standard manufacturer’s protocol (R&D Systems). The immunohistochemical staining of Ki67, p-AKT, p-S6, p-EGFR, and p-4EBP1 was conducted as previously described in detail (10-33). The antibodies against Ki67, p-AKT, and p-S6 were described in our previous studies (34). Antibody against p-4EBP1 was the same as used in immunoblotting, and antibody against p-EGFR was from Cell Signaling Technology. The incubation time for the primary antibodies was overnight at 4°C for all except the Ki67 antibody, which was incubated for 2 hours at room temperature. The slides were stained with R&D 3,3-diaminobenzidine and counterstained with hematoxylin (Vector Laboratories). Staining and fluorescent signals from each assay were visualized by Olympus BX41 microscopy at 200× magnification.

Statistical analysis

Linear mixed models were employed to test whether there were any significant differences in tumor volume growth over the whole period between different treatments. The significance level was set at 0.05 in all tests. SAS 9.4 was used for data analyses and management.

Results

Synergistic inhibition of SCCHN cell growth by the combination of erlotinib and BKM120

Recent TCGA data suggests that more than 50% of SCCHN patient tumors have activation of PI3K pathways (3). We also confirmed that the majority of SCCHN cell lines we tested have high expression of EGFR and activation of p-AKT and its downstream p-S6 and p-4EBP1 pathways (Supplementary Fig. S1). Our laboratory has previously reported that targeting EGFR with the small-molecule TKI erlotinib strongly inhibited growth of SCCHN cell lines, but failed to induce apoptosis and only partially inhibited p-AKT (19, 35). To explore the sensitivity of SCCHN cell lines to the combination of EGFR and PI3K cotargeting, we treated the SCCHN cell line MDA686TU with erlotinib, BKM120, and their combination and found that cells remained viable even after four weeks of continuous treatment with a single agent (Supplementary Fig. S2A). Moreover, cells grew back after removal of the drugs (Supplementary Fig. S2B). On the other hand, the majority of the cells were killed by the combination treatment and no resistant colony was found after drug withdrawal. Next, we examined the sensitivity of a panel of SCCHN cell lines by determining IC$_{50}$ values, CI, and DRI after 72-hour treatment. The IC$_{50}$ values ranged from 0.48 to 2.16 μmol/L for BKM120 and from 0.07 to 7.13 μmol/L for erlotinib depending on the cell line (Supplementary Table S3). Combination of the two agents strongly increased cell sensitivity (Supplementary Fig. S3) and was highly synergistic as evidenced by CI values <1 (Table 1) and DRI values >1 (Supplementary Tables S4 and S5).

As the induction of apoptosis is critical for effective tumor regression, we next measured apoptosis. As shown in Fig. 1A, BKM120 alone dose-dependently induced variable apoptosis ranging from 48.6 to 4.7 at a dose of 1.25 μmol/L. Over 50% cell lines displayed less than 25% apoptosis at the highest dose. To investigate the effect of the combination of BKM120 and erlotinib, we examined apoptosis in MDA686TU cells and found a
time-dependent increase in apoptosis (Supplementary Fig. S4). Next, we measured apoptosis in a panel of SCCHN cell lines at 96 hours. As shown in Fig. 1B, combination of the two agents increased apoptosis in most cell lines although some cell lines were resistant to apoptosis induced by the two agents. Among the tested cell lines, the greatest synergy/additivity in apoptotic effect was observed in the MDA686TU and Cal27 cell lines, while other cell lines such as 93-VU-147T and MDA686LN also showed synergistic/additive apoptosis. Some cell lines, notably UD-SCC2, MSK-LEUK1, and JHU022, were mostly resistant to apoptosis even after extended periods of treatment (Fig. 1B; Supplementary Fig. S5). To investigate whether apoptosis induction is limited to pan-PI3K inhibitors, we also measured apoptosis using erlotinib in combination with PI3K-α inhibitor

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<th>Cell line</th>
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<td>0.36</td>
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<tr>
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<td>0.42</td>
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<td>0.80</td>
<td>0.78</td>
<td>0.67</td>
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Table 1. Combination indices for 1:1 combination of BKM120 and erlotinib at different doses

Figure 1. Induction of apoptosis by BKM120, erlotinib and their combination. A, SCCHN cell lines were treated with different concentrations of BKM120 for 72 hours and apoptosis was measured by Annexin V-7AAD staining. B, SCCHN cell lines were treated with 1.25 µmol/L BKM120 (BKM), 1.0 µmol/L erlotinib (ER), or the combination of 1.25 µmol/L BKM120 and 1.0 µmol/L erlotinib (C) for 96 hours and apoptosis was measured as above. C, MDA686TU, 93-VU-147T, and UD-SCC2 cells were treated with 1.25 µmol/L BKM120, 1 µmol/L erlotinib, 1 nmol/L RAD001, 50 nmol/L BEZ235, 1 µmol/L BYL719, 1 µmol/L erlotinib plus 1 nmol/L RAD001, 1 µmol/L erlotinib plus 125 µmol/L BKM120, 1 µmol/L erlotinib plus 50 nmol/L BEZ235, and 1 µmol/L erlotinib plus 1 µmol/L BYL719 for 72 hours and apoptosis was measured as above. * and $, significant changes (P < 0.5). All experiments were conducted at least three times.
BYL719, dual pan-PI3K/mTOR inhibitor BEZ235, and mTORC1 allosteric inhibitor RAD001 (Fig. 1C). Among the different inhibitors, the pan-PI3K inhibitors (BKM120 and BEZ235) in combination with erlotinib induced significantly higher apoptosis than BYL719 and RAD001 in the sensitive cell lines MDA686TU and 93-VU-147T.

Combination of erlotinib and BKM120 more efficiently inhibits MDA686TU xenograft growth in nude mice

We next examined the effect of erlotinib, BKM120, and their combination in vivo using MDA686TU xenografts in nude mice. Mice bearing xenografts were treated with erlotinib, BKM120, and the combination of erlotinib and BKM120. Tumor volumes were plotted against time (Fig. 2A). Pairwise comparison between groups by linear mixed model suggested that erlotinib alone had a significant effect ($P < 0.05$) on the tumor volume during the whole experimental period (Supplementary Table S6). BKM120 had no significant effect on tumor volume, but the addition of BKM120 to erlotinib significantly improved the inhibitory effect of erlotinib on tumor volume. The combination was also significantly more effective than either single agent ($P < 0.05$). We also measured tumor weight at the end of the study and found similar results as with tumor volume (Fig. 2B). Of note, among the 7 mice in the combination group, tumors in 2 mice completely disappeared (Supplementary Fig. S6). As a measure of cell proliferation, we also analyzed and quantified the expression of Ki67 in tumor tissues collected at the end of the study. Although both erlotinib and BKM120 significantly inhibited tumor cell proliferation, the inhibition was much stronger by the combination treatment (Fig. 2C and D). No significant changes in mice body weights were observed throughout the study (Supplementary Fig. S7).

In vitro and in vivo inhibition of EGFR and PI3K pathway markers by erlotinib, BKM120, and their combination

To examine effective inhibition of EGFR and PI3K pathway markers, we analyzed the expression of p-ERK (marker of EGFR), p-AKT, p-S6, p-4E-BP1, and p-PRAS40 (markers for PI3K) in whole-cell lysates of apoptosis-sensitive MDA686TU and 93-VU-147T cell lines and in one apoptosis-resistant (UD-SCC2) cell line. As shown in Fig. 3A; Supplementary Fig. S8, treatment with erlotinib almost completely inhibited p-ERK.
but only partially inhibited p-AKT and its downstream p-S6 and p-4E-BP1. On the other hand, BKM120 completely inhibited p-AKT, p-S6, p-4E-BP1, and p-PRAS40 in sensitive cell lines, but failed to inhibit p-4E-BP1 and p-PRAS40 in the apoptosis-resistant cell line. Finally, combination of the two agents completely inhibited p-ERK, p-AKT, and both axes of the mTOR pathway in the sensitive cell lines and again failed to inhibit p-4E-BP1 and p-PRAS40 in the apoptosis-resistant cell line. We also examined inhibition of p-AKT, p-S6, and p-4E-BP1 after treatment with BYL719, BEZ235, rapamycin, and RAD001. Although BEZ235 effectively inhibited both axes of the mTOR pathway, BYL719, rapamycin, and RAD001 only inhibited p-S6 without a major effect on p-4E-BP1 (Supplementary Fig. S9). We also confirmed our in vitro findings in xenograft tumor tissues by IHC (Fig. 3B; Supplementary Fig. S10). We detected partial inhibition of p-AKT in xenograft tumors treated with erlotinib, but strong inhibition of p-AKT, p-S6, and p-4E-BP1 in tumors exposed to either BKM120 or the combination of erlotinib and BKM120. As expected, erlotinib alone strongly inhibited p-EGFR.

Translational inhibition of antiapoptotic Bcl-2 proteins by the combination of BKM120 and erlotinib

We have previously reported that the Bcl-2 proteins play a pivotal role in regulating apoptosis in response to the combination of erlotinib and EGCG (35). To elucidate the mechanisms by which the combination of BKM120 and erlotinib synergistically induce apoptosis, we examined the expression of antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, and Mcl-1) in two apoptosis-sensitive (MDA686TU and UV-93-147T) cell lines and one apoptosis-resistant (UD-SCC2) cell line. As shown in Fig. 4A, combination of the two agents more strongly inhibited the expression of Bcl-2, Bcl-xL, and Mcl-1 proteins in sensitive cell lines, but failed to do so in the apoptosis-resistant cell line. To further understand the mechanism of inhibition of Bcl-2 proteins, we assessed the expression of their mRNA by qRT-PCR (Fig. 4B). Interestingly, the expression of Bcl-2 mRNA was significantly increased rather than decreased after drug treatment in the apoptosis-sensitive MDA686TU cell line, but remained unchanged in the apoptosis-resistant cell line, UD-SCC2. mRNA expression of the other members of the PI3K-Akt pathway and its downstream effectors were also analyzed by qRT-PCR (Supplementary Fig. S9).
antiapoptotic family, Bcl-xL and Mcl-1, remained unchanged in both cell lines. To examine increased posttranslational degradation of Bcl-2 proteins by BKM120, erlotinib, or their combination, we pretreated the cells with the proteasome inhibitor bortezomib and found that it failed to rescue Bcl-2 expression (Fig. 4C).

Failure to inhibit p-4E-BP1 confers resistance to BKM120 and erlotinib combination

As the combination of BKM120 and erlotinib failed to inhibit p-4E-BP1 in the apoptosis-resistant UD-SCC-2 cell line, we hypothesized that inhibition of the 4E-BP1–eIF4E pathway might sensitize this cell line to the combination of BKM120 and erlotinib. To examine our hypothesis, we used eIF4E siRNA to ablate the expression of eIF4E in UD-SCC-2 cells and then measured apoptosis. As shown in Fig. 5A, ablation of eIF4E in the UD-SCC-2 cell line significantly increased apoptosis in comparison with cells transfected with control siRNA. Moreover, ablation of eIF4E inhibited the expression of Bcl-2 and Mcl-1 with minimum effect on the expression of Bcl-xL (Fig. 5B). UD-SCC2 cell lines also do not have detectable expression of PTEN which is known to confer resistance to EGFR inhibition (36). It is possible that lack of PTEN expression might also contribute to apoptosis resistance. To test this, we ablated the expression of PTEN in the sensitive cell line MDA686TU by siRNA transfection (Fig. 5C) and measured apoptosis at 72 hours. As shown in Fig. 5D, ablation of PTEN did not have any significant effect on apoptosis induction.

Discussion

Developing effective strategies to improve clinical outcome and circumvent resistance is a challenging task for the treatment of cancers including SCCHN. The anti-EGFR antibody cetuximab in combination with platinum-based chemotherapy is approved for the first-line treatment of patients with recurrent locoregional disease and/or metastatic SCCHN. Nonetheless, cetuximab shows only limited efficacy, even though more than 90% of SCCHN tumors overexpress EGFR and EGFR overexpression correlates with poorer clinical outcomes (9–11, 37). Recent studies have focused on exploring alternative pathways that tumor cells can activate to escape EGFR blockade. The PI3K–AKT–mTOR pathway is frequently activated in SCCHN and may constitute a source of tumor escape during EGFR targeting (38–40). On this basis, we investigated whether the combination of the EGFR-TKI erlotinib with P13K–AKT–mTOR inhibitors has synergistic effects and induces apoptosis. In our study, the combination of pan P13K inhibitor BKM120 with erlotinib induced synergistic antitumor effect in SCCHN cell lines as evidenced by CI <1 and DRI >1, induced apoptosis, and more effectively inhibited tumor growth in a nude mouse xenograft model of SCCHN. Combination of erlotinib with the dual pan P13K/mTOR inhibitor BEZ235 also induced significantly higher apoptosis than with allosteric mTOR inhibitor or P13Kα–specific inhibitor, suggesting that synergistic/additive apoptosis induction is limited to pan-P13K inhibition. To our knowledge, this is the first study demonstrating the additive/
synergistic antitumor effects of the combination of erlotinib and BKM120 in vitro and in vivo in SCCHN. Induction of apoptosis is crucial for eliminating tumor cells from the body and for effective tumor regression. Although our in vitro cell growth inhibition assay showed highly synergistic effects in SCCHN cells except the JHU022 cell line, apoptosis was induced to varying degrees. The MDA686TU, Cal27, and 93-VU-147T cell lines underwent massive apoptosis upon combination treatment, while UD-SCC2, JHU022, and MSK-Leuk1 lines were almost completely resistant to apoptosis. Consequently, we explored the mechanism of apoptosis induced by the combination of erlotinib and BKM120. We found that inhibition of both axes of the mTOR pathway is important for apoptosis induction. Failure to inhibit the mTOR–4E-BP1 axis confers apoptosis resistance in the UD-SCC2 cell line. This is consistent with previous studies suggesting that inhibition of the mTOR pathway with allosteric mTOR inhibitor only partially inhibited mTORC1 (failed to inhibit p-4E-BP1) and had limited effect on apoptosis induction (41, 42). Although inhibition of eIF4E significantly increased apoptosis, the effect was not as dramatic as in MDA686TU or 93-VU-147T cells. Lack of PTEN expression is also associated with resistance to erlotinib. However, ablation of PTEN in apoptosis-sensitive cell line does not confer resistance to the combination of erlotinib and BKM120. As the resistance to targeted drugs is context specific, rescuing the expression of PTEN in UD-SCC2 cell line is required to confirm the effect of PTEN loss in this cell line.

We also found that the combination of erlotinib and BKM120 strongly inhibited p-PRAS40 in sensitive cell lines, but failed to do so in the apoptosis-resistant cell line. PRAS40 plays a pivotal role in the regulation of mTOR signaling and in apoptosis. Binding of unphosphorylated PRAS40 to mTORC1 negatively regulates mTOR activity, while PRAS40 phosphorylation results in its dissociation from mTORC1, releasing its inhibition of mTORC1 (43, 44). A decrease in the level of p-PRAS40 promotes apoptosis and increases chemosensitivity (45). Failure to inhibit p-PRAS40 in the UD-SCC2 cell line might contribute to its apoptosis resistance. However, additional studies are required to further confirm the role of p-PRAS40 in apoptosis resistance.

The antiapoptotic Bcl-2 proteins play crucial roles as a gatekeeper of mitochondria and in promoting cell survival and apoptosis resistance. The mTOR signaling pathway regulates protein translation through two downstream pathways, S6K-S6 and 4E-BP1-eIF4E. Although effective inhibition of both of these pathways is important for apoptosis induction by the combination of erlotinib and BKM120, how these pathways link to apoptotic signaling or which proteins are translationally

Figure 5.
Inhibition of 4E-BP1–eIF4E pathway sensitizes resistant cells to apoptosis. A, Expression of eIF4E was ablated in UD-SCC2 cell line and apoptosis was measured after 72-hour treatment with the combination of BKM120 and erlotinib. P value was calculated using Student t test and P < 0.05 was considered statistically significant. B, Expression of eIF4E, Bcl-2, Bcl-xL, and Mcl-1 after ablation of eIF4E. C, Expression of PTEN in MDA686TU cells after siRNA transfection. D, Induction of apoptosis in MDA686TU cells by the combination of BKM120 and erlotinib at 72 hours after PTEN ablation.
inhibited was not clear. We found that the combination of erlotinib and BKM120 inhibited antipotopotic Bcl-2 proteins at the translational level through the 4E-BP1 axis, linking the mTOR protein translation pathway with the intrinsic apoptotic pathway.

Results of several phase I dose escalation clinical trials with the combination of pan-PI3K inhibitor and erlotinib have been published (46, 47). Although, the combination is well tolerated, some patients exhibited dose-related toxicities requiring dose reduction or treatment discontinuation. Two trials are ongoing testing the combination of BKM120 with erlotinib (NCT01487265) or gefitinib (NCT01570296). Our studies have explored the mechanism of apoptosis and apoptosis resistance induced by the combination of erlotinib and BKM120. Understanding these mechanisms will help to identify future drug combinations for the resistant population. In fact, a small-molecule inhibitor (4EGI-1) is available that inhibits the eIF4e–eIF4G interaction and shows in vitro and in vivo antitumor effects (48, 49). This compound may be combined with erlotinib and BKM120 to overcome apoptosis resistance in SCCHN, such as in U-D-SCC2 cells. Thus, the results from our study will guide further combinatorial approaches to improve the outcomes of EGFR-targeted therapy in SCCHN treatment with the combination of BKM120.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Correction: *In Vitro* and *In Vivo* Synergistic Antitumor Activity of the Combination of BKM120 and Erlotinib in Head and Neck Cancer: Mechanism of Apoptosis and Resistance

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In the original version of this article (1), an IHC image (expression of p-EGFR control) was mistakenly used twice in Fig. 3B. The appropriate BKM panel in the p-EGFR column has replaced the duplicated panel. The error has been corrected in the latest online HTML and PDF versions of the article. The authors regret this error.

Reference


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