Cancer Therapy: Preclinical

Novel Phosphoinositide 3-Kinase/mTOR Dual Inhibitor, NVP-BGT226, Displays Potent Growth-Inhibitory Activity against Human Head and Neck Cancer Cells In Vitro and In Vivo

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Abstract

Purpose: Dysregulation of the phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway frequently accounts for the tumorigenesis in head and neck cancer. To develop a new treatment, we investigated the effect of a novel dual PI3K/mTOR inhibitor, NVP-BGT226 (BGT226), in head and neck cancer cells.

Experimental Design: The in vitro antitumor effect of BGT226 was determined in various cancer cell lines. Animal models were also applied to examine drug potency. The inhibitory ability of BGT226 on the PI3K/AKT/mTOR signaling pathway was analyzed.

Results: The growth inhibition assay revealed that BGT226 was active against all tested cancer cell lines. Cross-resistance was not observed in the cisplatin-resistant cell line. The activation of the AKT/mTOR signal cascade was suppressed by BGT226 in a concentration- and time-dependent manner. Flow cytometric analysis revealed an accumulation of cells in the G0–G1 phase with concomitant loss in the S-phase. Results of the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay and the analysis of caspase 3/7 and PARP indicated that BGT226 induced cancer cell death through an apoptosis-independent pathway. BGT226 induced autophagy as indicated by the aggregation and upregulation of the microtubule-associated protein light chain 3B-II, and p62 degradation. Gene silencing of Beclin1 or cotreatment of the autophagosome inhibitor, 3-methyladenine, inhibited the BGT226-induced autophagy and led to the retrieval of colony survival. In a xenografted animal model, BGT226 significantly delayed tumor growth in a dose-dependent manner, along with suppressed cytoplasmic expression of p-p70 S6 kinase and the presence of autophagosome formation.


Introduction

Head and neck squamous cell carcinomas (HNSCC) are a group of cancers that originate from epithelial cells of the oral cavity, pharyngeal, and laryngeal regions, sharing alcohol, tobacco, and betel nut as their common carcinogen. Although the incidence of HNSCC is relatively low in developed countries, the age-adjusted standardized rate for the global incidence of cancer in the oral cavity and larynx in males still accounts for 6.3 and 5.1 per 100,000, respectively (1). This disease frequently occurs in Taiwan, where HNSCC ranks fourth in the incidence of all male cancers and 7.3% of all malignancies (2). This unique feature is a consequence of prevalent betel quid consumption, which is a major national health issue in the country (3). Despite the well-known etiology, many unsolved problems of the disease remain. With respect to the cancer treatment, HNSCC has a moderately good survival rate, which is achieved by the multimodality strategy that includes surgical intervention, radiotherapy, and chemotherapy. However, the disease often recurs locally and/or as distant metastases, leading to a poor prognostic disease course and tends to fail in treatment (4). In addition, physicians may find themselves in a clinical predicament due to the lack of efficient drugs. Therefore, investigations for novel agents against head and neck cancer are urgently needed.

Modern cancer treatment focuses on the molecular defects of intracellular signals transduction that have pivotal roles in cancer development. One of the successful
This study shows the antitumoral activity of the novel PI3K/mTOR dual inhibitor, NVP-BGT226, against head and neck cancer cell growth in vitro and in vivo. BGT226 inhibits cancer cell growth in association with the induction of autophagy. In addition, our data show that the antiproliferative activity of BGT226 is not affected by the status of aberration of the PI3K/AKT/mTOR signaling pathway. Notably, BGT226 is also active toward cancer cells with acquired resistance to cisplatin, which is a standard drug for the first-line treatment of head and neck cancer in patients. These data indicate the potential use of BGT226 for clinical investigation of patients with head and neck cancer in the future.

**Materials and Methods**

**Chemicals**

NVP-BGT226, a novel dual inhibitor against class I PI3K and mTOR (16), was provided by Novartis. The full chemical name of the compound is 8-(6-methoxy-pyridin-3-yl)-3-methyl-1-(4-piperazin-1-yl-3-trifluoromethyl-phenyl)-1,3-dihydroimidazo[4,5-c]quinolin-2-one maleate. For preparation of the in vitro study, the compound was dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C before use.

**Cell cultures**

Cell lines of human head and neck cancer, including FaDu, SCC4, SCC25, KB, and Detroit 562, were purchased from American Type Culture Collection. HSC3 was from Japanese Cancer Research Resources Bank. OECM1, provided by Dr. Ching-Liang Meng (Tri-Service General Hospital, Taipei, Taiwan), and UMSCC1, TU183, provided by Dr. Ming-Chi Hung (The University of Texas MD Anderson Cancer Center, Houston, TX) were also included in our studies. In addition, we used an epithelial tumor cell line from nasopharyngeal carcinoma (NPC), HONE-1 (17) and its derived cisplatin-resistant variant HONE-1-C15 for our experiment. In general, the cell lines were maintained at 37°C under 5% CO2 in 10-cm Petri dishes containing the indicated medium supplemented with 5% or 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin. The cisplatin-resistant cells were maintained in complete medium containing an additional 15 μmol/L cisplatin.

**Growth-inhibition assay**

Cells in the logarithmic growth phase were cultured at a density of 5,000 to 20,000 cells per milliliter per well in a 24-well plate. The cells were exposed to various concentrations of BGT226 for 3 generation times. At the end of the incubation period, cells were fixed and stained with 50% ethanol containing 0.5% methylene blue for 30 minutes. The resulting colored residues were dissolved in 1% N-lauroyl-sarcosine, and optical density was read at 550 nm using a microplate reader. The IC50 value resulting from 50% inhibition of cell growth was calculated graphically as a comparison with control groups. Each point represents the average of at least 3 independent experiments run in duplicate.

**Clonogenic assay**

Untreated or gene-silenced FaDu cells were plated at a density of 400 cells per well in a 6-well plate for 1 day. Pretreatment with or without 3-methyladenine (3MA) 1 hour prior to coincubation of BGT226 was then applied for study. Cells were then washed with PBS 3 times 24 hours after treatment and were subsequently cultured in drug-free medium for the following 10 days. Colonies were then counted after 30 minutes of methylene blue staining.

**Western blot analysis**

Cells were treated with various concentrations of BGT226, LY294002, and rapamycin (LY294002 and

**Translational Relevance**

This study shows the antitumoral activity of the novel PI3K/mTOR dual inhibitor, NVP-BGT226, against head and neck cancer cell growth in vitro and in vivo. BGT226 inhibits cancer cell growth in association with the induction of autophagy. In addition, our data show that the antiproliferative activity of BGT226 is not affected by the status of aberration of the PI3K/AKT/mTOR signaling pathway. Notably, BGT226 is also active toward cancer cells with acquired resistance to cisplatin, which is a standard drug for the first-line treatment of head and neck cancer in patients. These data indicate the potential use of BGT226 for clinical investigation of patients with head and neck cancer in the future.

examples is the application of cetuximab, an epidermal growth factor receptor (EGFR) inhibitor, in the treatment of the HNSCC (5, 6). The original concept of this therapy is derived from the observation that more than 80% to 90% HNSCCs overexpresses EGFR (7). In addition to EGFR, the aberrant expression of phosphoinositide 3-kinase (PI3K)/AKT/mTOR, which is one of its downstream pathways, is also known to play a critical role in cancer cell growth and proliferation (8, 9). Recent studies have focused on the disorder of this pathway in HNSCC with one of its key proteins, AKT, actively expressed in clinical tumor samples (10, 11). Moreover, aberration of the other cascade proteins was also emphasized in HNSCC, including PTEN and mTOR (12). These findings highlight the signals leading to tumorigenesis, and specific targeting agents are designed for the treatment of the disease.

LY294002 is one of the PI3K/AKT/mTor–specific inhibitors that had been under consideration for clinical use. However, this pan-PI3K inhibitor has been restricted to preclinical use because of the insolubility and the significant toxicity at the therapeutic index (13). To overcome the discouragement, modern pharmacology has provided structure-modified drugs that are analogues to LY294002 but possess higher solubility and lower toxicity at the therapeutic index (13). In addition, recent concern has made researchers aware of the coexistence of PI3K alteration that may add to the complexity of this pathway (14). One of the examples is from the experience with rapamycin, an mTOR inhibitor. The activated AKT from the compensation route resulted in resistance to the drug, emphasizing the necessity of coinhibition against the feedback loop (15). This led to the development of multitargeting compounds that simultaneously inhibit 2 or more cascade proteins in the PI3K/AKT/mTOR pathway (13).

In this study, we showed the antitumor activity of a novel PI3K/mTOR dual inhibitor, NVP-BGT226 (BGT226), against head and neck cancer cells in vitro and in vivo. The detailed mechanism of antiproliferative activity for BGT226 against cancer cells is described.
rapamycin purchased from Selleck Chemicals) for indicated durations. For preparation of whole-cell lysate, ice-cold lysis buffer containing NP-40 was used and then was stored at −80°C until analysis. To conduct Western blot analysis, proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane by an electroblot apparatus. The membrane was subsequently blocked with 5% skimmed milk and incubated with primary antibodies overnight. The antibodies applied in this study are listed as follows: p-AKT (Thr308 and Ser473), phosphorylated mTOR (p-mTOR; Ser2481 and Ser2448), mTOR, p-p70 S6 kinase (p-p70S6K; Ser371 and Thr389), p70 S6 kinase (p70S6K), p-4E-BP1 (Thr36/42), 4E-BP1, p-GSK-3β (Ser9), GSK-3β, PARP, caspase-3, autophagy protein 5 (Atg5), Beclin1, and p62/SQSTM1 (p62) were purchased from the Cell Signaling Technology. AKT-1/2/3, cyclin-E, CDK4, β-actin, α-tubulin, and α-actinin were purchased from Santa Cruz; and microtubule-associated protein light chain 3B (LC3B) and cyclin-D1 were purchased from Sigma-Aldrich and Thermo Fisher Scientific, respectively. After the incubation with different antibodies, membranes were subsequently incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature. The labeled proteins were detected using Enhanced Chemiluminescence Kit (ECL; Pierce) and X-ray film according to the manufacturer’s instructions.

**Cell-cycle analysis**

After drug treatment, cells were trypsinized, washed with PBS, and fixed in 70% ethanol. The fixed cells were then treated with 50 μg/ml RNase and stained with 50 μg/ml propidium iodide at room temperature for 20 minutes. The DNA content was determined by BD FACSCalibur flow cytometry system (BD Biosciences). For each analysis, 10,000 events were collected, and the percentage of cells in each phase was calculated using ModFit LT software (Verity Software House, Inc.).

**Apoptosis assay**

Cells were grown in the chamber culture slide and treated with drugs. Two methods, including terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) and real-time caspase 3 and 7 activity detection assay, were applied for apoptosis detection. For the TUNEL assay, In Situ Cell Death Detection Kit and TMR red dye (Roche) were used. Cells were fixed with paraformaldehyde and subsequently permeabilized with PBS containing 0.1% Triton X-100. After incubation with the TUNEL reaction mixture and 4’,6-diamidino-2-phenylindole (DAPI) nuclear staining, cells were examined using fluorescence microscopy. For the real-time detection of both caspase 3 and 7 activity, drug-treated cells were incubated with real-time caspases 3 and 7 substrate MR-(DEVD)2 and counterstained with Hoechst dye as included in the Magic Red Caspase Detection Kit (Immunocytometry Technologies). The active caspase 3 and 7 as indicated by red fluorescence were detected and examined by fluorescence microscopy.

**Investigation of the autophagy in cells**

The autophagosome formation in cells was detected by the localization of exogenous LC3 fused to GFP (GFP-LC3), which was generously provided by Dr. Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan) and Dr. Li-Jin Hsu (National Cheng Kung University, Tainan, Taiwan). Briefly, the transfection of GFP-LC3 expression vector was mediated through Lipofectamine LX and PLUS reagent (Invitrogen) as per manufacturer’s protocol. After indicated treatment, the localization of GFP was then directly observed by fluorescence microscopy, and cells expressing multiple punctate GFP-LC3–labeled vacuoles were identified for quantification. For blockage of autophagy degradation, cells were pretreated with chloroquine (Sigma-Aldrich; ref. 18).

To study the formation of acidic vesicular organelles (AVO) in cells, acridine orange staining was applied for indirect indication (19). Trypsinized cells were incubated with 1 μg/ml acridine orange (Sigma-Aldrich). Stained cells were analyzed by flow cytometry using the BD FACSCalibur Flow Cytometry System. For each analysis, 10,000 events were recorded.

**Downregulation of gene expression by siRNA**

Cells were seeded on a 6-well plate and allowed to grow until the confluence reached 50%. For transfection, Atg5 or Beclin1-specific siRNA oligomers (SignaSilence; Cell Signaling Technology) and the transfection reagent (Lipofectamine 2000; Invitrogen) were mixed together for 20 minutes. The transfection mixture was then overlaid onto the cells. After 24 hours of incubation, the transfection complex was removed, and cells were treated with various drugs for the indicated period.

**In vivo antitumor activity of BGT226**

The antitumor effect of BGT226 was examined in the human FaDu xenografted mice. Subcutaneous inoculation of 5 × 10⁶ FaDu cells in specific pathogen-free male athymic mice (strain BALB/cAnN.Cg-Foxn1nu/CrlNarl) from National Laboratory Animal Center, Taiwan, was applied. The animals were subsequently fed under specific pathogen-free conditions and provided with sterile water and food. When the tumor volume reached 200 to 400 mm³, animals were assigned to groups randomly and given vehicle, BGT226 [dissolved in 90% N-methyl-2-pyrrolidone (NMP)/10% PEG300], rapamycin (prepared as in ref. 20) orally once a day, or LY294002 by intraperitoneal injection (prepared as in ref. 21) 3 times a week. The administrations were scheduled for 21 consecutive days, with tumor size and body weight measured and recorded. At the end of the experiments, animals were euthanized in a CO₂-containing chamber, and the tumors were collected and fixed in formaldehyde.

**Immunohistochemical stain of tumor tissue**

Formalin-fixed tissue sections were prepared in 5-μm slices and were deparaffinized before analysis. The
endogenous peroxidase within the tissue cells was inhibited by hydrogen peroxide for 15 minutes. After antigen retrieval in citrate buffer by autoclave boiling for 10 minutes, the sections were incubated with antibodies against p-p70S6K Thr389 (Abcam) with diluent at 4°C overnight. For the labeling and tag-staining procedure, we followed the manufacturer’s protocol (EnVision+ Kit; Dako). Finally, the sections were counterstained with hematoxylin.

Transmission electron microscopy
Mice under anesthesia were perfused through the heart with saline followed by 20 to 30 mL of fixative containing 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 mol/L PBS (pH, 7.2–7.4). The tumors were then removed and dissected into about 1-mm³ blocks. The specimens were fixed with 1% osmium tetroxide in double-distilled water at room temperature for 1 to 2 hours. They were then washed with double-distilled water for 10 minutes 3 times and were dehydrated in increasing grades of ethanol (from 50% to 100%) and pure propylene oxide. Then samples were embedded in Epon and polymerized in an oven at 55°C for 1 day. The 80-nm thin sections were dissected from blocks and collected onto the copper grids. Sections were counterstained with lead citrate and uranyl acetate and examined under an electron microscope (JEOL JEM-1200EX) at 80 kV.

Results
BGT226 displays potent antiproliferative activity against various human head and neck cancer cell lines in vitro
To evaluate the antiproliferative activity of BGT226 against head and neck cancer cells, 9 HNSCC cell lines from different sites of the oral cavity and 1 NPC cell line with its cisplatin-resistant variant were used. As shown in Table 1, BGT226 displayed potent growth-inhibitory activity against all tested cell lines, with the IC₅₀ ranging from 7.4 to 30.1 nmol/L. Notably, both Detroit 562 and HONE-1 cells, which express PIK3CA mutation H1047R (22, 23), were still sensitive to the growth-inhibitory effect of BGT226 treatment. In addition, the sensitivity to BGT226 between HONE-1 cells and its cisplatin-resistant variant was almost identical.

BGT226 interferes with the PI3K/mTOR signaling pathway
To further investigate the effect of BGT226 on the PI3K/AKT signaling pathways, all cell lines were treated with 200 nmol/L BGT226 for 24 hours. As shown in Fig. 1A, the expression levels of p-mTOR Ser2481 were decreased in all the drug-treated cell lines. The expression levels of both p-AKT Ser473 and p-mTOR Ser2448 were also decreased in most of the drug-treated cell lines except HSC3 and KB cells. FaDu cells were selected as a model to investigate the concentration and time effects of BGT226 on the PI3K/mTOR signaling pathway. As shown in Fig. 1B, the expression levels of p-AKT Ser473 and p-mTOR Ser2481, Ser2448 were downregulated in FaDu cells treated with BGT226 at a concentration as small as 30 nmol/L for 6 hours. In addition, the expression levels of p-AKT Thr308 and p-GSK-3β were significantly decreased in the BGT226-treated cells at the concentrations of 120 nmol/L and above. In the time-dependent study of BGT226, the results showed that the levels of p-AKT Ser473 were decreased in BGT226-treated cells as early as 30 minutes after treatment (Fig. 1C). Accordingly, BGT226 downregulated the expression levels of the downstream proteins p-p70S6K and p-4E-BP1 in cells. Moreover, the expression levels of both p-AKT and p-p70S6K were reduced to a similar degree between HONE-1 and its cisplatin-resistant cells (Fig. 1D).

Table 1. The IC₅₀ of growth-inhibition assay using BGT226 or cisplatin in various head and neck cancer cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Origin</th>
<th>IC₅₀</th>
</tr>
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<tbody>
<tr>
<td>Squamous cell carcinoma</td>
<td></td>
<td>BGT226, nmol/L</td>
</tr>
<tr>
<td>SCC4</td>
<td>Tongue</td>
<td>13.2 ± 2.4</td>
</tr>
<tr>
<td>SCC25</td>
<td>Tongue</td>
<td>27.8 ± 11.6</td>
</tr>
<tr>
<td>HSC3</td>
<td>Tongue</td>
<td>12.5 ± 0.3</td>
</tr>
<tr>
<td>UMSCC1</td>
<td>Retromolar trigone</td>
<td>7.6 ± 6.4</td>
</tr>
<tr>
<td>OECM1</td>
<td>Gingiva</td>
<td>12.5 ± 5.1</td>
</tr>
<tr>
<td>KB</td>
<td>Oral</td>
<td>9.3 ± 6.7</td>
</tr>
<tr>
<td>TU183</td>
<td>Oral</td>
<td>7.4 ± 4.1</td>
</tr>
<tr>
<td>FaDu</td>
<td>Pharynx</td>
<td>23.1 ± 7.4</td>
</tr>
<tr>
<td>Detroit 562</td>
<td>Pharynx (H1047R)</td>
<td>16.7 ± 4.0</td>
</tr>
<tr>
<td>HONE-1</td>
<td>NPC (H1047R)</td>
<td>22.6 ± 6.0</td>
</tr>
<tr>
<td>HONE-1-C15</td>
<td>NPC²</td>
<td>30.1 ± 5.2</td>
</tr>
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</table>

²Cisplatin-resistant variant.
The potency and molecular functions of BGT226 were then compared with those of LY294002 and rapamycin, which are known as single specific PI3K and mTOR inhibitors, respectively. The result showed different growth inhibition (Supplementary Fig. S1A) or signal blockage profiles in each compound. Both BGT226 and LY294002 attenuated p-AKT and p-p70 Thr389 expression after short-term treatment, whereas rapamycin diminished the phosphorylation of p70S6K but not AKT (Fig. 1E for FaDu; Supplementary Fig. S1B for OECM1).

**BGT226 induces cell-cycle arrest at the G₀–G1 phase**

The effect of BGT226 on cell-cycle progression was examined by flow cytometry. Cells were treated with equal potency (4× IC₅₀) of BGT226 for one doubling time. In asynchronous-culturing conditions, the flow cytometric analysis clearly showed that BGT226 caused cell accumulation in the G₀–G₁ phase with the decrement of those in S-phase (Fig. 2A). Notably, the percentages of cells in sub-G₁ phase were not significantly increased with application of the compound. Furthermore, FaDu cells were treated with various concentrations of BGT226 for 24 hours to examine the dose effect of BGT226 on cell-cycle progression. The results revealed that BGT226 increased cells in G₀–G₁ phase at the concentration of 60 nmol/L and reached the plateau at the concentration of 120 nmol/L (Fig. 2B).

We further investigated the alteration of expression in the cell-cycle–associated proteins in cells treated with BGT226. As shown in Fig. 2C, treatment of FaDu with 120 nmol/L BGT226 resulted in a reduction of cyclin-D1 expression after 4 hours of treatment. The expression levels of cyclin-E, CDK4, and p21 were not altered.

**BGT226 induces cancer cell death through apoptosis-independent pathway**

A TUNEL assay was applied to determine whether BGT226 could induce DNA fragmentation, indirectly indicating the induction of cellular apoptosis. As shown in Fig. 3A, cells treated with DNase (positive control) resulted in the induction of DNA fragmentation. In contrast, DNA
fragmentation was not observed in cells treated with BGT226. In addition, no activated caspase 3 and 7 in cells treated with 120 and 240 nmol/L for 12 and 24 hours was observed in the real-time activity imaging assay (Fig. 3B). Furthermore, the results of the Western blot analysis showed that neither the cleavage form of PARP nor that of caspase 3 was detected in cells treated with 120 nmol/L BGT226 for up to 24 hours (Fig. 3C).

BGT226 inhibits cell growth by the induction of autophagy

We then further determined whether BGT226 could induce autophagy. The enhancement of a microtubule-associated protein light chain 3 family member, LC3B, is widely used as an indicator for the activation of cell autophagy (24). As shown in Fig. 4A, BGT226 treatment increased the amount of LC3B-II in FaDu cells in both a time-dependent (left) and a concentration-dependent (right) manner. Notably, the result showed early increment of LC3B-II following the suppression of p-mTOR. In contrast, LC3B-II expression induced by BGT226 was alleviated by pretreatment with siRNA for Atg5 gene silencing (Fig. 4B). For the direct observation of autophagosome formation, plasmid carrying GFP-LC3 expression vector was transfected into cells before treatment with BGT226 (25). Punctate cells were then counted, showing an increasing expression of GFP aggregation in a concentration-dependent manner (Fig. 4C, top). Next, we used chloroquine to block the autophagic degradation (18). The combination of BGT226 and chloroquine led to more aggregated GFP spots in cells than BGT226 or chloroquine single treatment (Fig. 4C, bottom). Finally, we determined whether the autolysosome formed is functionally active by detecting the degradation of p62 (24). Results of the Western blot analysis revealed that the expression of p62 level was decreased in cells with BGT226 treatment (Fig. 4D).

The presence of autophagy in BGT226-treated cells was reconfirmed by staining of AVOs with acridine orange, representing another classical characteristic in autophagic process (19). The quantification of the acridine orange stained cells by flow cytometry showed an accumulation of AVOs in cells after treatment with the compound (5.6% in control and 37.2% in 120 nmol/L BGT226, Supplementary Fig. S2B). In addition, coincubation of BGT226 with 3MA, which is used to inhibit autophagosome formation (26), could reverse the accumulation of AVOs in cells in a concentration-dependent manner (23.7% and 19.1% with 1 mmol/L and 5 mmol/L 3MA, respectively). Clonogenic assay showed that cotreatment with 3MA in FaDu cells attenuated the cytotoxicity effect of BGT226 in 45- and 60-nmol/L doses, recovering the number of colonies formed to 1.38- and 1.54-fold, respectively (Fig. 4E).
addition, downregulation of Beclin1 by siRNA also showed significant retrieval of colony survival in those treated with BGT226 (Fig. 4F).

In vivo antitumor efficacy of BGT226 in xenograft experiments

Next, we determined whether oral administration of BGT226 was effective in vivo. As shown in Fig. 5A, BGT226 inhibited tumor growth in a dose-dependent manner in a FaDu cell xenografted mouse model. Oral administration of BGT226 at 2.5 and 5 mg/kg for 3 weeks caused 34.7% and 76.1% reduction of the tumor growth on day 21, respectively (compared with control). In addition, we arranged another animal study for BGT226, LY294002, and rapamycin. As the result, BGT226 displayed comparable inhibition against tumor growth to rapamycin ($P > 0.05$). The final volume of both groups was significantly smaller than those treated with LY294002 or the control ($P < 0.05$; Fig. 5B). Notably, we observed no significant morbidity and a body weight loss of less than 10% in all the treated mice (Supplementary Fig. S3).

We then collected the tumor samples from euthanized mice on day 21. Immunohistochemical staining was applied to determine the expression levels of p-p70S6K (Thr389) in tumors of both control and treatment groups. As shown in Fig. 5C, BGT226 caused a diminishment of the cytoplasmic immunoreactivity of p-p70S6K in a dose-dependent manner. Furthermore, results of the electronic microscopic analysis showed the formation of autophagosome as indicated by the formation of double membrane vacuoles and internal debris in mice treated with BGT226 (Fig. 5D).

Discussion

Cisplatin-based chemotherapy is the most active regimen for patients with locally advanced and/or metastatic head and neck cancers. There is almost no effective chemotherapeutic agent for patients who fail with cisplatin treatment. Although cetuximab has recently been introduced to treat chemotherapy-naïve patients with head and neck cancer, the response rate of cetuximab for cisplatin-resistant patients remains low (27). Thus, investigation for novel agents for head and neck cancer is urgently needed. Accumulating evidence shows a prevalence of 47% of head and neck cancer with at least one of the PI3K/AKT/PTEN molecular alterations (28). The defects, accompanied with the molecular aberrations, contribute to the high prevalence of p-AKT expression in head and neck cancer (11). The pivotal role of the PI3K/AKT cascade in tumorigenesis was further determined in the study of Lim and Counter because tumor maintenance relied on the pathway, as well as oncogenic addiction to Ras signaling for cancer initiation (29). Hence, development of a PI3K/mTOR dual inhibitor against head and neck cancer is worthwhile. In this study, our data show that BGT226 displays potent antiproliferative activity against all tested head and neck cancer cell lines. In particular, BGT226 is also active against NPC cells (HONE-1) with acquired cisplatin resistance, suggesting that BGT226 may be useful for treating patients with head and neck cancer who have previously failed the cisplatin treatment. Furthermore, because PI3K/mTOR is one of the most important cascades downstream of the EGFR, aberration of this pathway often gives rise to cetuximab resistance (30, 31). Our data show that BGT226 exhibits a similar potency against cell growth in OECM1, KB, and UMSCC1, which are all relatively resistant to cetuximab when compared with FaDu and SCC25 (data not shown). In addition, BGT226 inhibited Hep3B hepatocarcinoma-derived cetuximab-resistant variants (Hep3B-C1 and Hep3B-C7) as well as the...
parental cancer cells (Supplementary Fig. S4A). Taken together, these results suggest that the novel PI3K/mTOR dual inhibitor, BGT226, can be used in salvage setting for patients with head and neck cancer.

Numerous factors contribute to the aberration of the PI3K/AKT/mTOR pathway in cancer cells. In head and neck cancers, factors including PTEN dysregulation and PIK3CA point mutation, which encodes the p110α catalytic subunit of PI3K (23, 28, 32), have both given important contributions in the alteration of the PI3K/AKT/mTOR pathway. In our tested cell lines, the efficacy of BGT226 was unaffected regardless of PIK3CA mutation or diverse PTEN expression in cells (Table 1 and Supplementary Fig. S4B). Wang and colleagues reported that colon cancer cells carrying PIK3CA mutation count on the aberrant pathway against apoptosis, and exposure to LY294002 led to more significant cell death (29).

**Figure 4.** Analysis of autophagy in BGT226-treated cell lines. A, FaDu cells were incubated with DMSO or BGT226. Left, the treatment of BGT226 was applied in different durations. Right, the treatment was given for 6 hours at various concentrations. Antibodies against p-mTOR and LC3B were used for the Western blot analysis. The top and bottom arrows indicate LC3B-I and LC3B-II, respectively. B, gene silencing of Atg5 using siRNA. FaDu cells pretreated with scramble siRNA (lanes 1 and 2) and siRNA (lanes 3 and 4) were followed by treatment with DMSO (lanes 1 and 3) and BGT226 (lanes 2 and 4). The band intensity of LC3B-II (arrow) and β-actin was quantified, and the result was compared with the first lane (control) for ratio. C, effect of BGT226 treatment in FaDu cells transfected with GFP-LC3. Cells were pretreated with or without 10 μmol/L of chloroquine (CQ) for 15 minutes and cotreated with BGT226 for 2 hours. Top, the bar chart shows quantification of cells with punctate GFP spots when treated with BGT226 in various concentrations. The individual bars represent mean percentage of the 2 repeated studies, with each counting to 200 cells. Bottom, representative images of punctate cells induced by BGT226, with or without pretreatment of CQ. D, analysis of p62 expression after BGT226 treatment in a dose-dependent manner. Cell lysate was collected 2 hours after administration of the compound. E and F, assessment of colony survival effect associated with autophagy by clonogenic assay. The ratio of colony numbers in the tested groups was compared with the control. E, each individual bar represents the mean percentage of the triplicate experiment using BGT226 at various concentrations, with or without cotreatment of 5 mmol/L 3MA. F, the individual bars indicate the ratio of BGT226-treated cells as compared with those without treatment. The gray bars represent the results of gene-silenced cells against Beclin1, and the white ones illustrate those cells transfected with scrambled (SC) siRNA. Top right, Western blot analysis for Beclin1 in the manipulated cells (t test; *, P < 0.05; **, P < 0.01; †††, P < 0.001). Rapa, rapamycin.
inhibition compared with the wild type (33). In our data, BGT226 exhibits potent antiproliferative activity against Detroit 562 and HONE-1, both carrying heterozygous A3140G, which is one of the frequent hotspot mutations in PIK3CA (22, 23). Similar results were also observed in the study using another PI3K/mTOR dual inhibitor, NVP-BEZ235, which exhibited identical antiproliferative activity against cancer cells carrying either wild-type or mutated p110α (34). These results suggest that BGT226 is not affected by the status of the aberration of the PI3K/AKT/mTOR pathway.

It has been reported that many oncogenes rely on intracellular signaling from the PI3K pathway (8, 9). Activation of this signaling pathway promotes cell division instead of cell-cycle/growth arrest. In an animal study, the constitutively vigorous AKT in pancreatic β cells led to the bypass of cell-cycle checkpoint through increased expression of G1 components such as cyclin-D and p21 in a CDK4-associated manner (35). It has also been suggested that GSK-3β and eIF-4E act as major determinants for the expression level of cyclin-D1 (36, 37). As a consequence, our data show that the blockage of both AKT and mTOR activation by BGT226 leads to the decreased expression levels of its downstream associated proteins, including p-GSK-3β, p-p70S6K, and p-4E-BP1 (Fig. 1B and C), resulting in the suppression of cyclin-D1 expression (Fig. 2C) and arresting cells in the G0–G1 phase (Fig. 2A and B). In accordance with the finding using another specific inhibitor, PI-103, to inhibit the PI3K/mTOR pathway in malignant peripheral nerve sheath tumor (38), no alteration of p21 expression levels was observed. It has been reported that despite the protein amount, AKT also regulates the translocation of p21 (39). This hypothesis warrants further study.

A previous study has shown that activation of AKT is commonly known to prevent apoptosis in cancer cells. It
has also been proven that suppression of AKT results in cell death through apoptosis (40). In addition to the lack of change in the sub-G1 populations (Fig. 2A), our data show that BGT226 inhibits cancer cell growth through apoptosis-independent pathway as neither DNA fragmentation, caspase 3/7 activation, nor the cleavage of PARP was observed in the drug-treated FaDu and HSC3 cancer cells (Fig. 3; Supplementary Fig. S2A). A recent study has shown that, in addition to apoptosis and necrosis, autophagy also plays an important role in deciding the fate of cells under various stresses (41). The lack of apoptosis prompted us to investigate whether autophagy plays an important role in inducing cell death in the BGT226-treated cancer cells. Our data show that BGT226 elicits autophagosome formation as evidenced by aggregation and increasing levels of LC3B-II (Fig. 4A–C). Furthermore, both the reduced degradation of BGT226-induced autophagosome in the presence of chloroquine and the depletion of p62 in BGT226-treated cells suggest the induction of autophagic flux (Fig. 4C and D; refs. 18, 24).

Stimulation of autophagy can either promote cell survival or induce cell death depending on the conditions and agents used (42). In this study, significant retrieval of colony numbers for cells cotreated with 3MA and BGT226 in 45- and 60-nmol/L doses was up to 1.38- and 1.54-fold, respectively, as compared with BGT226 monotherapy (both P < 0.05; Fig. 4E). In addition, pretreatment of siRNA targeting Beclin1, a key factor of autophagy, resulted in the recovery of colony formation from BGT226 treatment (Fig. 4F).

These results suggest that BGT226 induces cancer cell death through activation of autophagy instead of apoptosis. In fact, recent studies have focused on cell death that is unrelated to apoptosis or caspase activation. One route is through lysosomal dysfunction with the activation of cathepsin (43), and indeed, cathepsin is often associated with the autophagic process (44, 45). Our experimental results show that the activation of cathepsin B/L in FaDu is significantly elicited in the presence of BGT226 (Supplementary Fig. S2D), but not cathepsin D or calpains, a family of cytosolic proteases (data not shown). These results suggest that the compound diminishes tumor survival through regulatory cell autophagy and lysosomal dysfunction.

The antitumoral activity of BGT226 was further verified in xenografted animal models. BGT226 can significantly inhibit tumor growth by 34.7% and 76.1% at doses of 2.5 and 5 mg/kg, respectively (Fig. 5A). Consistent with in vitro findings, results of the immunohistochemical analysis show a decreased expression level of cytoplasmic p-p70S6K at residue Thr389 (Fig. 5C). Furthermore, autophagosome formation with inner debris in tumor samples treated with BGT226 was also detected by transmission electron microscopic examination (Fig. 5D). These data suggest the in vivo effect of the compound displaying tumor-inhibitory mechanism in accordance with the in vitro study. In the same xenograft model, BGT226 and rapamycin display significant tumor inhibitions compared with the vehicle or LY294002 (Fig. 5B), suggesting the necessity of mTOR inhibition despite its upstream PI3K blockage (46). Notably, no significant morbidity or death of mice was observed in the BGT226 treatment group when the doses were less than 10 mg/kg. Moreover, in the experiment using human lymphocytes from voluntary donors, no viability difference was found in the study or the control groups (data not shown). Nevertheless, the concern about safety should still be addressed when the compound is translated to human clinical study.

In summary, our data show that the novel PI3K/AKT/mTOR inhibitor, BGT226, is effective in targeting head and neck cancer in vitro and in vivo. BGT226 also exhibits potent antiproliferative activity against acquired cisplatin resistance in nasopharyngeal cancer cells. This information provides a rationale for further clinical study of this compound in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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