Dual PI3K/mTOR Inhibitors, GSK2126458 and PKI-587, Suppress Tumor Progression and Increase Radiosensitivity in Nasopharyngeal Carcinoma

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Abstract

Although combined chemoradiotherapy has provided considerable improvements for nasopharyngeal carcinoma (NPC), recurrence and metastasis are still frequent. The PI3K/Akt/mTOR pathway plays a critical role in tumor formation and tumor cell survival after radiation-induced DNA damage. In the present study, we evaluated whether inhibition of PI3K/mTOR by two novel dual inhibitors, GSK2126458 and PKI-587, could suppress tumor progression and sensitize NPC cells to radiation. Four NPC cell lines (CNE-1, CNE-2, 5-8F, and 6-10B) were used to analyze the effects of GSK2126458 and PKI-587 on cell proliferation, migration, invasion, clonogenic survival, amount of residual γ-H2AX foci, cell cycle, and apoptosis after radiation. A 5-8F xenograft model was used to evaluate the in vivo effects of the two compounds in combination with ionizing radiation (IR).

Both GSK2126458 and PKI-587 effectively inhibited cell proliferation and motility in NPC cells and suppressed phosphorylation of Akt, mTOR, S6, and 4EBP1 proteins in a concentration- and time-dependent manner. Moreover, both compounds sensitized NPC cells to IR by increasing DNA damage, enhancing G2–M cell-cycle delay, and inducing apoptosis. In vivo, the combination of IR with GSK2126458 or PKI-587 significantly inhibited tumor growth. Antitumor effect was correlated with induction of apoptosis and suppression of the phosphorylation of mTOR, Akt, and 4EBP1. These new findings suggest the usefulness of PI3K/mTOR dual inhibition for antitumor and radiosensitizing. The combination of IR with a dual PI3K/mTOR inhibitor, GSK2126458 or PKI-587, might be a promising therapeutic strategy for NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is the most common cancer of the head and neck, and it is highly prevalent and endemic in Southern China and Southeast Asia (1). Currently, patients with NPC are mainly treated by ionizing radiation (IR) or chemoradiation, and many early-stage patients could be cured. However, there are still a substantial proportion of patients with NPC who fail to achieve long-term disease control and die from local recurrence and metastasis (2). The dismal prognosis of NPC may due to its radioresistance (3, 4). Therefore, finding a way of enhancing the radiosensitivity may provide a more effective therapeutic strategy and improve the prognosis of NPC.

The PI3K/Akt/mTOR pathway is an intracellular signaling pathway that plays a critical role in tumor formation, and these proteins are central to the regulation of tumor cell proliferation, metabolism, cell-cycle progression, apoptosis, and survival (5). The possible mechanisms by which this pathway regulates NPC proliferation include mutations in PI3K, PTEN deleted from chromosome 10 (PTEN), and Ras oncogene, as well as EGFR amplification (6–8). Previous studies showed that PI3K/Akt/mTOR signaling is frequently activated in many cancers, including lung, gastric, renal, and ovarian cancers (9–12), and inhibition of this pathway could increase radiosensitivity (8, 13–18). NPC cell lines and tissues also overexpressed phosphorylated Akt (p-Akt; refs. 19–21). Recently, we reported that Akt expression in NPC cells and biopsies from patients with NPC could be increased by IR, which is associated with metastasis, suggesting that the PI3K/mTOR pathway might be an ideal therapeutic target in NPC (22). Preclinical studies have demonstrated that targeting the PI3K/mTOR pathway by a dual PI3K/mTOR inhibitor, NVP-BEZ235, sensitized the antitumor effect of cisplatin in NPC (23). However, there is little data about the effect of inhibition of PI3K/Akt/mTOR signaling on tumor progression and radiosensitivity in NPC.

GSK2126458 and PKI-587 are highly selective and potent small-molecule inhibitors which could effectively suppress both multiple class I PI3K isoforms and mTOR kinase activity (24, 25).
GSK2126458 has been identified as a highly potent, orally bioavailable inhibitor of p110α, p110β, p110γ, p110δ, mTORC1, and mTORC2. It can induce a significant reduction in the levels of p-Akt, inhibit growth, and induce G1 phase arrest in breast cancer (24). Furthermore, GSK2126458 is currently tested in a phase I clinical trial in patients with solid tumors or lymphoma (NCT00972686). PKI-587 is a highly potent dual inhibitor of PI3Kα, PI3Kγ, and mTOR. Preclinical studies have demonstrated potent inhibitory effects of PKI-587 on a variety of human cancer cell lines, such as breast, glioma, lung, melanoma, colon, and liver cancer (25, 26). Because PKI-587 has strong antitumor activity in vitro and in xenograft models, it is currently being evaluated in a phase I clinical trial in patients with solid tumors (NCT00940948).

In this study, we investigated the antitumor and radiation-sensitizing effect of dual PI3K/mTOR inhibitors in NPC both in vitro and in vivo. To provide better insight into the effect of dual PI3K/mTOR inhibitors and test whether comparable results would be obtained, we investigated two dual PI3K/mTOR inhibitors, GSK2126458 and PKI-587. We propose that the combination of dual PI3K/mTOR inhibitors and IR may lead to important clinical benefits in NPC and provide the basis for further development of a targeted therapeutic strategy for NPC.

Materials and Methods

Cell cultures and reagents

Three NPC cell lines (CNE-2, 5-8F, and 6-10B) were kindly provided by Prof. Yunfei Xia (Sun Yat-sen University Cancer Center, Guangzhou, P.R. China) in 2013. The CNE-1 and NP69 cell lines were maintained in our laboratory. Human immortalized nasopharyngeal epithelial cell line NP69 was cultured in Keratinocyte/serum-free medium (Invitrogen) supplemented with bovine pituitary extract (BD Biosciences). Other human NPC cell lines were maintained in RPMI-1640, supplemented with 10% FBS, 100 U/mL penicillin, 50 μg/mL streptomycin, and 2 mmol/L glutamine in a humidified CO2 incubator at 37°C. All cells were passage for less than 3 months before renewal from frozen, early-passage stocks obtained from the indicated sources. Cells were tested and authenticated within 3 months before the start of this study by examining their karyotypes, images, and specific gene expression.

The dual PI3K and mTOR inhibitors, GSK2126458 and PKI-587, were obtained from Selleck Chemicals (Houston) and dissolved in DMSO (Sigma-Aldrich) at a concentration of 10 mmol/L.

Cell proliferation assay

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method. In brief, tumor cells (2 × 10^5/100 μL/well) were plated into each well of 96-well plates in RPMI-1640 with 10% FBS and incubated for 24 hours. Culture medium containing several concentrations of GSK2126458 (0–3 μmol/L) or PKI-587 (0–3 μmol/L) was added to each well, and the cells were incubated for 24 to 72 hours. Next, 50 μL of MTT (2 mg/mL; Sigma-Aldrich) was added to each well, and cells were incubated for 2 hours at 37°C. The medium containing MTT solution was removed, and the dark blue crystals were dissolved by adding 100 μL DMSO. The absorbance was measured with a microplate reader at test and reference wavelengths of 550 and 630 nm, respectively. The percentage growth was determined relative to untreated controls. Each experiment was performed at least three times, each with triplicate samples.

Antibodies and Western blotting

The primary antibodies used in this study were rabbit anti-mTOR (2972), phospho-mTOR (5536), Akt (9272), phospho-Akt (4060), S6 (2217), phospho-S6 (2211), 4EBP1 (9644), phospho-4EBP1 (2855), c-PARP (9544), p21 (2947), p27 Kip1 (3688), Cyclin D1 (2978; 1:1000 each; Cell Signaling Technology), mouse anti-γ-H2AX (1:1000, ab22551, Abcam), E-Cadherin (610181), and Vimentin (550513; 1:2000 each; BD Biosciences). Western blotting was performed as previously described (27).

Wound-healing assay

Cells were seeded on 6-well plates at 80% to 90% confluence. After 24-hour incubation, cells were wounded by using a 0.2-mm pipette tip to scratch the subconfluent cell monolayer. Then, the detached cells were removed by washing with PBS before GSK2126458 (0.003 μmol/L) or PKI-587 (0.03 μmol/L) was added to the plates. Cells were maintained in serum-free medium and allowed to migrate for 24 hours. An inverted microscope was used to photograph the cell migration images at different time points after wounding (0, 12, and 24 hours), and the speed of migration was acquired by dividing the length of the gap at different time points. Motic Images Advanced 3.2 software was used to analyze the relative surface distance traveled by the leading edge. Three replicates each of two independent experiments were performed.

Cell migration and invasion assay

Cell migration and invasion assays were evaluated in Transwell chambers (Corning Costar), and cell invasion was evaluated by adding Matrigel (BD Bioscience) to the chambers. Before the assay, cells were serum-starved for 24 hours. The top and bottom Transwell chambers were separated by an 8-μm-pore filter that had been coated with collagen (30 μg/filter). For the migration assay, 1 × 10^5 tumor cells were plated in the top chamber with 0.1% BSA. For the invasion assay, cells were seeded on filters coated with 20 to 50 μg/cm^2 of reconstituted Matrigel basement membranes (BD Biosciences). Culture medium containing GSK2126458 (0.003 μmol/L) or PKI-587 (0.03 μmol/L) was added to the bottom chamber. After incubation for 24 hours at 37°C, cotton swabs were used to remove the cells that had not migrated or invaded from the top surface of the filters. The cells that had migrated or invaded to the bottom surface were fixed in 100% methanol and stained with 0.5% crystal violet. Migration or invasion cells were quantitated by counting cells in six randomly selected fields on each filter under a microscope at ×200 magnification and graphed as the mean of three independent experiments.

Clonogenic survival assays

Clonogenic survival assay were performed as previously described (27). Treatment with GSK2126458 (0.003 μmol/L) or PKI-587 (0.03 μmol/L) was 1 hour before cells exposed to different doses of IR (0–8 Gy) and washed away 24 hours after IR.

γ-H2AX assay

Cells were plated in chamber slides, incubated for 24 hours, and pretreated with either GSK2126458 (0.003 μmol/L) or PKI-587 (0.03 μmol/L) 1 hour before IR (4 Gy), and the number of residual foci was determined at 24 hours after IR. Cells were fixed in 4% paraformaldehyde and incubated with a primary antibody against γ-H2AX (Abcam). Then, the primary antibody was washed...
off, and a secondary antibody conjugated to FITC was applied to the slides. DNA damage was visualized with a fluorescence microscope (Olympus BX51). For each group, the γH2AX foci were counted in at least 50 cells.

**Cell cycle and apoptosis**

Cells were seeded in 6-well plates and treated with GSK2126458 (0.003 μmol/L) or PKI-587 (0.03 μmol/L) for 1 hour before IR (4 Gy) and were harvested at 24 hours after IR. Cell-cycle distribution was analyzed by propidium iodide (PI) staining and flow cytometry. After 24 hours of IR, cells were collected, gently washed with cold PBS containing 2% FBS, fixed in 70% cold ethanol, and stored at −20°C overnight. Then, cells were pelleted, washed, and stained with PI/ribonuclease staining buffer (BD Biosciences) for 15 minutes at room temperature. Apoptosis was measured with the Annexin V-FITC apoptosis detection kit (BD Biosciences) according to the manufacturer’s protocols. Analysis was performed on the FACS Calibur using Cell Quest software (BD FACS Array). All experiments were performed at least three times.

**Xenograft studies**

All the animal experiments were carried out in strict accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of Southern Medical University (Guangzhou, People’s Republic of China). Suspensions of 5 × 10⁶/0.2 mL 5-8F cells were injected subcutaneously into the right hindlimbs of 5- to 7-week-old female BALB/c-nu/nu nude mice. When tumor volumes reached 200 mm³, mice were randomly assigned to control and treated groups (5 mice per group). The treated groups received 300 μg/kg GSK2126458, 25 mg/kg PKI-587, IR, 300 μg/kg GSK2126458 combined with IR, or 25 mg/kg PKI-587 combined with IR. The doses of GSK2126458 and PKI-587 used in vivo were based on the instructions from Selleck Chemicals and the results from a preliminary experiment. GSK2126458 was administered by intragastric administration once daily for 5 consecutive days each week and PKI-587 was administered by intravenous injection via tail vein once per 5 days according to the instructions from Selleck Chemicals. Mice in the IR groups were irradiated with 2 Gy every other day for four treatments. In combination therapy, GSK2126458 or PKI-587 was administered 2 hours before IR exposure. Tumor sizes were calculated every 2 days using the formula: (length × width²)/2. Tumor regrowth delay was expressed as the time in days for untreated tumors to reach the same size.

**IHC and TUNEL assays**

IHC staining was performed using tissues (5 mm thick) harvested from the 5-8F xenografts. Tissue sections were fixed overnight in 4% formaldehyde and then embedded in paraffin for IHC. After deparaffinization and hydration, paraffin-embedded sections were pretreated for 20 minutes with 10 mmol/L sodium citrate buffer in a microwave for antigen retrieval. Next, the tissue sections were incubated overnight at 4°C with antibodies against p-mTOR (2976, 1:100 dilution), p-Akt (3837, 1:100 dilution), p-4EBP1 (2855, 1:100 dilution), and Ki67 (9027, 1:600 dilution; Cell Signaling Technology). The sections were then incubated with horseradish peroxidase-conjugated secondary antibodies (SP-9000; Zhongshan Gold Bridge) for 1 hour. Finally, slides were counterstained with hematoxylin and eosin and observed and photographed with a microscope-mounted camera system. At least three random fields were examined in each section.

The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was performed using TdT-mediated TUNEL method, performed according to manufacturer’s instructions of Apoptosis Detection System (Promega).

**Statistical analysis**

All data are presented as the mean ± SDs of experiments repeated at least three times. Significant differences between the means were measured by two-tailed unpaired Student t test or one-way ANOVA. *P < 0.05* was considered statistically significant.

**Results**

GsK2126458 and PKI-587 inhibit NPC cell proliferation by blocking PI3K/mTOR signaling

We first examined the expression levels of total and phosphorylated mTOR, Akt, S6, and 4EBP1 in four NPC cell lines and an immortalized nasopharyngeal epithelial cell line (NP69). Western blotting showed strong expression of phosphorylated mTOR, Akt, S6, and 4EBP1 in NPC cells, whereas only weak expression of these proteins were detected in NP69 (Fig. 1A), indicating that a high level of activation of the PI3K/mTOR pathway is characteristic of NPC cells. Next, we evaluated the effect of GSK2126458 and PKI-587 on the proliferation of NPC cells. The structures of these two compounds were shown in Supplementary Fig. S1. As shown in Fig. 1B and C, GSK2126458 and PKI-587 decreased the viability of NPC cells at low nanomolar concentrations and in a concentration- and time-dependent manner, and all four NPC cell lines were sensitive to GSK2126458 and PKI-587, especially CNE-2 and 5-8F cells. Furthermore, we determined whether GSK2126458 and PKI-587 inhibited proliferation of NPC cells via modulating the PI3K/Akt signaling pathway. As shown in Fig. 1D and E, GSK2126458 and PKI-587 inhibited phosphorylation of mTOR, Akt, S6, and 4EBP1, leading to increased cleaved-PARP in NPC cell lines in a concentration- and time-dependent manner. These effects were shown at concentrations of 0.003 to 0.01 μmol/L and 0.03 to 0.1 μmol/L, respectively (Fig. 1E and Supplementary Fig. S2), and started within 1 hour of exposure and persisted for at least 24 hours (Fig. 1D). These results demonstrated that PI3K/mTOR inhibitors, both GSK2126458 and PKI-587, effectively inhibited proliferation of NPC cells by blocking the PI3K/mTOR/S6/4EBP1 signaling pathway. Because administration of 0.003 μmol/L GSK2126458 and 0.03 μmol/L PKI-587 could significantly inhibit the signaling pathway with little effect on cell viability after 24 hours, these concentrations of inhibitors were chosen for additional experiments.

GsK2126458 and PKI-587 inhibit migration and invasion of NPC cells

To determine whether these two inhibitors could affect migration and invasion of NPC cells, we performed wound-healing and migration assays with GSK2126458 and PKI-587 treatment. As shown in Fig. 2A and Supplementary Fig. S3A, these two compounds significantly decreased the speed of wound healing. Moreover, the number of cells migrating through the transwell chamber polycarbonate membrane in the GSK2126458 and PKI-587 treated groups was markedly less than that in the control.
Figure 1. GSK2126458 and PKI-587 inhibit NPC proliferation by blocking PI3K/mTOR signaling. A, basal expression of total and phosphorylated mTOR, Akt, S6, and 4EBP1 in NPC cell lines compared with an immortalized human nasopharyngeal epithelial cell line NP69. The indicated proteins were determined by Western blotting. B, NPC cells were incubated with various concentrations of GSK2126458 or PKI-587, and cell growth was determined after 72 hours using MTT assay. C, 5-8F cells were incubated with various concentrations of GSK2126458 or PKI-587, and cell growth was determined at 24, 48, and 72 hours using MTT assay. D, 5-8F cells were incubated with GSK2126458 (0.003 μmol/L) or PKI-587 (0.03 μmol/L) for different time points. The cell lysates were harvested and phosphorylation of indicated proteins was determined by Western blotting. E, CNE-2 and 5-8F cells were incubated with various concentrations of GSK2126458 or PKI-587 for 1 hour. The cell lysates were harvested and phosphorylation of indicated proteins was determined by Western blotting. Bars, SD.
group (Fig. 2B and Supplementary Fig. S3B). Treatment with GSK2126458 or PKI-587 also substantially impaired ability of cells to invade the filters (Fig. 2C and Supplementary Fig. S3C). For epithelial-to-mesenchymal transition (EMT) markers, the level of E-cadherin was increased and the level of Vimentin was reduced in CNE-2 and 5-8F cells 24 hours after treatment with GSK2126458 or PKI-587 (Fig. 2D). Collectively, these results suggest that the dual PI3K/mTOR inhibitors, GSK2126458 and PKI-587, could effectively inhibit the migration and invasion of NPC cells, which may be associated with reduced EMT phenotypic expression.

Targeting the PI3K/mTOR pathway with GSK2126458 or PKI-587 increases the radiosensitivity of NPC cells

Because Akt has been implicated in cell survival responses after IR, we hypothesized that the combination of IR with PI3K/mTOR blockade would increase the radiosensitivity of NPC cells (28–30). As expected, the combination of IR with either of these two compounds significantly reduced survival fraction post-IR in NPC cells (Fig. 3A and Supplementary Fig. S4A and Supplementary Table). We selected 0.003 μmol/L of GSK2126458 and 0.03 μmol/L of PKI-587 in combination with IR because these concentrations significantly inhibited the signaling pathway (Fig. 1E) but had little effect on cell viability after 24 hours (Fig. 1C). Further investigation indicated that GSK2126458 or PKI-587 could significantly suppress the activation of PI3K and mTOR, causing reduced phosphorylation of S6 and 4EBP1 in NPC cell lines (Fig. 3B and Supplementary Fig. S4B). These data strongly suggested that GSK2126458 and PKI-587 could increase the radiosensitivity of NPC cells by inhibiting the PI3K/mTOR signaling pathway.
Radiosensitization induced by the dual PI3K/mTOR inhibitors is accompanied by persistence of γ-H2AX foci

To better understand the molecular mechanism of the radiosensitizing role of GSK2126458 or PKI-587, we investigated the effects of GSK2126458 and PKI-587 on the DNA damage response by measuring the number of γ-H2AX foci post-IR. Treatment with GSK2126458 or PKI-587 alone has little effect on γ-H2AX foci. However, GSK2126458 or PKI-587 in combination with IR led to a dramatic persistence of γ-H2AX foci and protein at 24 hours post-IR compared with IR alone (Fig. 4 and Supplementary Fig. S5), indicating that radiosensitization of these inhibitors is accompanied by the persistence of γ-H2AX expression.

The combination of IR and dual PI3K/mTOR inhibitors induces cell-cycle arrest and apoptosis in NPC cells

To investigate whether these two PI3K/mTOR inhibitors radiosensitized NPC cells via redistribution of cell cycle, we performed cell-cycle assay on NPC cells exposed to IR and/or the inhibitors. Treatment with GSK2126458 or PKI-587 alone led to an increased percentage of cells in G1 phase, whereas IR alone led to a G2–M arrest. Strikingly, the combination of IR with GSK2126458 (0.003 μmol/L) or PKI-587 (0.03 μmol/L) induced a further arrest in the G2–M phase, which highlights the potential radiosensitizing capability of these reagents (Fig. 5A and Supplementary Fig. S6A). Further investigation indicated that the level of cyclin D1 was decreased 24 hours after treatment with GSK2126458 or PKI-587 (Supplementary Fig. S6B). Meanwhile the expression of p21 and p27 were increased 24 hours post-IR (Supplementary Fig. S6B). The combination of IR with GSK2126458 or PKI-587 further increased the level of p21 and p27, accompanied with dramatic decrease of cyclin D1 (Supplementary Fig. S6B).

In addition, treatment with IR (17.1%), GSK2126458 (10.6%), or PKI-587 (9.2%) alone could induce apoptosis in 5-8F cells when compared with untreated cells (7.7%). Strikingly, the combination of either GSK2126458 or PKI-587 with IR could induce apoptosis rate of up to 46.4% and 46.9%, respectively. Similar results were obtained in CNE-2 cells (Fig. 5B and C). These data indicate that GSK2126458- or PKI-587-mediated radiosensitization may be associated with increased cell apoptosis. Taken together, these results suggest that the radiosensitization effects of the dual PI3K/mTOR inhibitors GSK2126458 and PKI-587 were primarily due to inhibition of DNA repair or augmentation of damage by cycle arrest and induction of apoptosis.

Combination of dual inhibition of PI3K/mTOR and radiation therapy is an effective treatment for NPC in vivo

To assess the antitumor effects of dual PI3K/mTOR inhibitors in combination with radiation therapy in vivo, we used the 5-8F xenografted model, which is a well-established model of NPC using one of the NPC cell lines from our in vitro experiments. As shown in Fig. 6A, GSK2126458 or PKI-587 alone had a modest antitumor activity and that the IR showed better inhibition of tumor growth. The combination of IR with GSK2126458 or PKI-587 resulted in >50% reduction in xenograft volume and tumor regrowth delay when compared with IR alone (P < 0.05; Fig. 6A and Supplementary Fig. S7A and S7B).
To confirm that PI3K/mTOR inhibitor suppresses PI3K/Akt signaling in vivo, we assessed the expression of phosphorylated Akt, mTOR, and 4EBP1 in the xenograft tumors. Pathologic examination of sections stained with hematoxylin and eosin revealed no significant morphologic abnormalities in any group. However, GSK2126458 or PKI-587 inhibited phosphorylated Akt, mTOR, and 4EBP1. Consistent with in vitro findings, we observed dramatically lower levels of phosphorylated mTOR, Akt, and 4EBP1 in tumors from the inhibitor combined with IR treatment groups (Fig. 6B). Interestingly, IR alone slightly reduced the phosphorylation of these proteins in vivo. Western blotting revealed that high levels of phosphorylated Akt and 4EBP1 were detected in control groups and IR-treated groups, but lower levels of these two proteins were detected in tumors treated with either GSK2126458 or PKI-587. The lowest levels of these two proteins were observed in both inhibitors combined with IR-treated tumors (Fig. 6C). These data showed the efficacy of GSK2126458 and PKI-587 as dual PI3K/mTOR inhibitors in vivo.

Consistent with our in vitro findings, we detected significantly fewer proliferating cells (Ki67-positive) and more apoptotic cells (TUNEL-positive) in the inhibitor combined with IR-treated tumors when compared with either treatment alone (Fig. 6B and D).

Taken together, our in vivo results demonstrated that treatment with GSK2126458 or PKI-587 alone slightly inhibits tumor growth, whereas the combination of dual PI3K/mTOR inhibition and IR markedly suppressed tumor progression. This antitumor effect is associated with PI3K/Akt pathway inhibition and apoptosis.

**Discussion**

The PI3K/Akt pathway is an essential survival factor in the set of signal transduction cascades that regulate cell survival and apoptosis. Aberrant upregulation of the PI3K/Akt signaling pathway occurs commonly in NPC (31–33). In this study, our results showed that the dual PI3K/mTOR inhibitors, GSK2126458 and PKI-587, could suppress the PI3K/mTOR activity, thereby inhibiting NPC cell proliferation, migration, and invasion while inducing apoptosis and cell-cycle arrest. Furthermore, we demonstrated that the novel dual PI3K/mTOR inhibitors, GSK2126458 and PKI-587, could enhance the radiosensitivity of NPC both in vitro and in vivo.

Studies of the PI3K/Akt/mTOR pathway have mainly focused on its role in cell survival, and these proteins are generally described to work in parallel with the RAS/RAF/MAPK pathway,
which drives cell proliferation (12). However, this is an oversimplified division of functions because the PI3K/Akt/mTOR pathway has been involved in the most important mechanisms of radioresistance, including intrinsic radioresistance, tumor-cell proliferation, and hypoxia (34). We have reported that radiation-inducible PTEN expression radiosensitizes hepatocellular carcinoma cells, indicating the importance of the PTEN/PI3K/Akt pathway in radioresistance (35). In this study, our data further indicated that PI3K/mTOR inhibition could sensitize NPC cells to radiation by inducing apoptosis, DNA damage, and cell-cycle arrest. The radiation experiments in the current study were performed under fully oxygenated conditions. Recent studies have indicated that dual PI3K/mTOR inhibitors enhance the radiosensitivity of prostate (13) and breast (36) cancer cells under normoxic and hypoxic conditions. Future studies will address whether these two compounds have an effect on the radiosensitivity of NPC cells under hypoxic conditions.

In this study, GSK2126458 or PKI-587 reduced the levels of phosphorylated Akt, mTOR, S6, and 4EBP1 in a time- and concentration-dependent manner, consistent with other reports in NPC (23, 37, 38). In addition, we found that very low concentrations of GSK2126458 and PKI-587 could effectively inhibit the migration and invasion of NPC cells, which may be associated with reduced EMT phenotypic expression, indicating the high efficacy of these compounds.

Our data showed that treatment with GSK2126458 or PKI-587 alone induced G1 arrest, IR alone resulted in growth arrest in the G2–M phase, and the combination of IR with either GSK2126458 or PKI-587 markedly prolonged G2–M arrest in NPC cells. This finding is consistent with a previous report on another PI3K/mTOR inhibitor, BEZ235 (13). Combining the dual PI3K/mTOR inhibitor and IR induced an elevated level of DNA damage, and this could explain the G2–M arrest because G2–M phase arrest is a hallmark of DNA damage (39). Furthermore, the level of cyclin D1, which is a vital protein, required for the G1–S transition, was decreased 24 hours after treatment with GSK2126458 or PKI-587, supporting the observations on cell-cycle changes. The expression of p21 and p27, both of which are associated with suppressing cell-cycle progression, were increased 24 hours post-IR. The combination of IR with GSK2126458 or PKI-587 further increased the level of p21 and p27, accompanied with dramatic decrease of cyclin D1. P21 is reported to be involved in DNA damage repair after exposure to irradiation. Studies indicated that p21 interacts with the proliferating cell nuclear antigen (PCNA). This p21/PCNA interaction could inhibit the process of DNA synthesis, which is a major process in DNA damage repair, leading to an arrest in the G2–M phase (40–42). Moreover, we found that lower concentration of dual PI3K/mTOR inhibitors alone slightly induced apoptosis. However, the combination of inhibitors and IR exerts potent proapoptotic properties. The apoptosis may be caused by increased DNA damage when IR is combined with GSK2126458 or PKI-587 (43).

We and other investigators have shown that IR increased the expression and activation of Akt in NPC (22, 44). Therefore, aberrantly activated Akt may decrease radiosensitivity as a self-protective feedback loop in tumor cells. In this study, our data showed that IR alone trends to enhance phosphorylation of Akt/mTOR signaling proteins in vitro, which was consistent with
previous reports and further indicated the usefulness of PI3K/mTOR inhibition to radiosensitivity. Interestingly, our in vivo data showed that the levels of Akt and mTOR phosphorylation in the tumors in the IR group were lower than those in control tumors. This is still reasonable because, although IR may increase Akt/mTOR activation and thereby decrease radiosensitivity, tumors were eventually inhibited, accompanied by Akt/mTOR inhibition. Therefore, we supposed that IR-induced Akt/mTOR activation could be maintained for a short period, after which Akt/mTOR activation decreased. This hypothesis will be examined in future studies. On the other hand, although GSK2126458 or PKI-587 alone could not markedly reduce tumor load in 5-8F xenografts in the doses used in this study, when combined with IR, they displayed dramatic antitumor effects, suggesting that the combination of dual PI3K/mTOR inhibitors and IR is an effective antitumor strategy for NPC. In addition, our results also confirmed that combination of dual PI3K/mTOR inhibitor and IR produced lower levels of phosphorylation of mTOR and Akt in the 5-8F xenografts compared with either treatment alone. These data suggested that if IR-induced Akt/mTOR activation was inhibited, radioresistance might be prevented.

In summary, our results show that targeting PI3K/mTOR signaling in NPC not only has antitumor effect, but also improves the efficacy of radiotherapy. The radiosensitizing effect of the dual PI3K/mTOR inhibitors, GSK2126458 or PKI-587, may be caused by persistent residual DNA damage and by inducing cell-cycle arrest and apoptosis. These findings suggest that a combination of IR and GSK2126458 or PKI-587 may be a useful therapeutic strategy against NPC. Further evaluation of IR in combination with GSK2126458 or PKI-587 in clinical trials is warranted to improve the outcomes of patients with NPC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: D. Xiao, Y. Yuan, L. Chen, W. Wang
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