mTOR inhibitor RAD001 (everolimus) induces apoptotic, not autophagic cell death, in human nasopharyngeal carcinoma cells

YUCHEN CAI1,2*, QING XIA1,3*, QUANGUAN SU1,3, RONGZHEN LUO1,3, YUELI SUN1,2, YANXIA SHI1,3 and WENQI JIANG1,3

1State Key Laboratory of Oncology in South China, Guangzhou, Guangdong; Departments of 2Experimental Research, 3Medical Oncology, Sun Yat-sen University Cancer Center, Guangzhou, Guangdong, P.R. China

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Abstract. Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase and a key element in the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway. Moreover, it is a negative regulator of autophagy and acts as a central regulator in cell growth. For the treatment of cancer, mTOR is a novel and validated therapeutic target. Previous studies have shown that Akt is frequently activated in nasopharyngeal carcinoma (NPC) tissues; thus, the inhibition of mTOR may be a treatment strategy for this tumor type. To evaluate the effect of the mTOR inhibitor RAD001 on NPC cell lines, we performed 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assays, lactate dehydrogenase (LDH) assays, western blotting and flow cytometry to evaluate the mechanisms of cell death. The growth of both CNE-1 and HONE-1 cells was inhibited in a time- and dose-dependent manner. CNE-1 was more sensitive, with a 50% growth inhibition (GI50) of 30.0±1.0 µM compared to HONE-1, cells which had a GI50 of 56.9±13.1 µM. RAD001 induced apoptosis and autophagy in both cell lines. RAD001 induced a significant increase in growth inhibition in the two cell lines when used in combination with the autophagy inhibitor, 3-methyladenine; however, the percentages of apoptotic cells decreased when RAD001 was combined with the caspase inhibitor, z-VAD-fmk. In conclusion, the main mechanism of the mTOR inhibitor RAD001 in these two NPC cells was apoptotic, not autophagic cell death. The combination of RAD001 with autophagy inhibitors may be a useful therapeutic strategy for nasopharyngeal carcinoma.

Introduction

Nasopharyngeal carcinoma (NPC) is a significant health burden in Southeast Asia and Southern China. Radiotherapy is the primary treatment modality, and the use of radiation therapy in combination with chemotherapy is recommended for the treatment of locoregionally advanced tumors. However, in patients who develop distant recurrence following radiotherapy, the median survival time is ~12-15 months (1,2); thus, new treatments are needed. Targeted therapy using specific inhibitors is currently in development and has demonstrated promising antitumor efficacy. The increasing knowledge of growth factor signal transduction pathways has led to speculation that proteins in these pathways could offer crucial targets for cancer therapy.

Cell death is the result of an unsuccessful cytoprotective mechanism against intracellular and extracellular stressors, which includes apoptosis, autophagy, necrosis and mitotic catastrophe. Autophagic cell death is morphologically characterized by a cell with an intact nucleus and an accumulation of cytoplasmic double-membrane autophagic vacuoles called autophagosomes. Apoptosis is characterized by chromatin condensation and DNA fragmentation. Anticancer drugs have been shown to induce not only apoptosis but also autophagy in cancer cells (3-5). However, the relationship between autophagy and apoptosis is complex as the molecular regulators of both pathways are interconnected. The crosstalk between autophagy and apoptosis can be in unison or in an opposing fashion, and it varies depending on cell type and the type and duration of the stimulus. Some published data suggest that tumor cell autophagy induced by anticancer therapy inhibits tumor cell killing. However, it has also been proposed that autophagy is a cell death mechanism that could function as a backup mode of cell death when apoptosis is disabled.

The mammalian target of rapamycin (mTOR), which is well-known as a major negative regulator of autophagy, is a serine/threonine protein kinase and is a key element of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway. Moreover, it is a negative regulator of autophagy and acts as a central regulator in cell growth. For the treatment of cancer, mTOR is a novel and validated therapeutic target. Previous studies have shown that Akt is frequently activated in nasopharyngeal carcinoma (NPC) tissues; thus, the inhibition of mTOR may be a treatment strategy for this tumor type. To evaluate the effect of the mTOR inhibitor RAD001 on NPC cell lines, we performed 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assays, lactate dehydrogenase (LDH) assays, western blotting and flow cytometry to evaluate the mechanisms of cell death. The growth of both CNE-1 and HONE-1 cells was inhibited in a time- and dose-dependent manner. CNE-1 was more sensitive, with a 50% growth inhibition (GI50) of 30.0±1.0 µM compared to HONE-1, cells which had a GI50 of 56.9±13.1 µM. RAD001 induced apoptosis and autophagy in both cell lines. RAD001 induced a significant increase in growth inhibition in the two cell lines when used in combination with the autophagy inhibitor, 3-methyladenine; however, the percentages of apoptotic cells decreased when RAD001 was combined with the caspase inhibitor, z-VAD-fmk. In conclusion, the main mechanism of the mTOR inhibitor RAD001 in these two NPC cells was apoptotic, not autophagic cell death. The combination of RAD001 with autophagy inhibitors may be a useful therapeutic strategy for nasopharyngeal carcinoma.
pathway that integrates signals that govern protein biosynthesis, cell division, motility, survival and angiogenesis (6). Therefore, mTOR acts as a central regulator of cell growth and cell cycle progression. The inhibition of mTOR prevents protein synthesis and cell proliferation. Recently, it has been suggested that dysregulation of mTOR contributes to oncogenesis in a broad range of cancers (7). Therefore, this protein is a novel and validated therapeutic target for the treatment of cancer (8-12).

RAD001 (everolimus) is the first oral mTOR inhibitor to reach oncology clinics (13). RAD001 inhibits mTOR complex 1 (mTORC1) and the phosphorylation of its downstream signaling mediators, ribosomal p70S6 kinase 1 (S6K) and 4E-binding protein 1 (4E-BP1). Treatment with RAD001 has been shown to induce autophagy in papillary thyroid cancer and enhance the therapeutic response to cytotoxic chemotheraphy and external beam radiation (14). RAD001 inhibits growth in HPV-associated oral and cervical squamous carcinomas, ovarian cancer, post-transplant Epstein-Barr virus-related lymphoproliferative disorders, breast cancer and pancreatic neuroendocrine tumors (15-19), as well as diminishes lymphangiogenesis in primary tumors and prevents the dissemination of head and neck squamous cell cancer cells to the cervical lymph nodes (20). At present, RAD001 is currently undergoing evaluation studies in phases I-III as an antitumor agent.

Previous studies have shown that Akt is frequently activated in NPC tissues (21); therefore, the inhibition of mTOR may be an appropriate treatment strategy for this tumor type. In this study, we evaluated the activity of the mTOR inhibitor RAD001 on NPC cell lines and revealed that the inhibitory effect of RAD001 was mainly due to apoptosis rather than autophagy. Our data demonstrated that the combination of RAD001 and autophagy inhibitors may be a useful therapeutic strategy for nasopharyngeal carcinoma.

Materials and methods

Cell lines and small-molecule inhibitors. The human NPC cell lines HONE-1 and CNE-1 were grown in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). All cells were cultured in a 5% CO2 incubator at 37°C. The working stock of RAD001 (Selleck Chemicals, USA) was diluted to 20 mM using dimethyl sulfoxide (DMSO) and stored at -20°C. The concentration of DMSO in the final solution did not exceed 1% (v/v). 3-Methyladenine (3-MA, #M9281) from Sigma-Aldrich was diluted to 20 mM using dimethyl sulfoxide (DMSO) and stored at -20°C. The concentration of DMSO in the final solution did not exceed 1% (v/v). 3-MA (Sigma-Aldrich) was dissolved in hot water (70˚C) to 30 mg/ml before use. Caspase 3-Methyladenine (3-MA, #M9281) from Sigma-Aldrich was diluted to 20 mM using dimethyl sulfoxide (DMSO) and stored at -20°C. The concentration of DMSO in the final solution did not exceed 1% (v/v). 3-Methyladenine (3-MA, #M9281) from Sigma-Aldrich was dissolved in hot water (70˚C) to 30 mg/ml before use. Caspase inhibitor 7,5-diaminofluorescein diacetate (DCF-DA) (Invitrogen) was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.

Western blot analysis. Lysates were prepared from 4x10^6 cells by dissolving the cell pellets in 100 μl of cell lysis buffer (#9803; Cell Signaling Technology) for 30 min on ice. The lysates were centrifuged at 12,000 x g for 20 min and the supernatant was collected. The protein content was determined using the Pierce BCA Protein Assay (#23225; Thermo Scientific). A total of 30 μg of protein was loaded into each well of an 8-15% SDS-PAGE gel. The resolved proteins were electrophoretically transferred to PVDF membranes and incubated sequentially with primary and secondary antibodies [anti-rabbit IgG, HRP-linked antibody (#7074P2) or anti-mouse IgG, HRP-linked antibody (#7076P2; both from Cell Signaling Technology)]. After washing, the bound antibody complex was detected using LumiGLO reagent (#7003; Cell Signaling Technology) and XAR film (XBT-1; Kodak) as described by the manufacturers. The following primary antibodies were used: caspase-3 antibody (#9662; Cell Signaling Technology); poly(ADP-ribose) polymerase (PARP) antibody (sc-7150; Santa Cruz Biotechnology, Inc.); LC3 antibody (NB100-2220; Novus Biologicals); and glyceraldehyde 3-phosphate dehydrogenase antibody (#23233; Santa Cruz Biotechnology, Inc.).

Statistical analyses. All experiments were repeated three times. The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using the
SPSS 17.0 statistical software. The P-values were calculated using a one-way analysis of variance (ANOVA). A P-value of <0.05 was considered to indicate a statistically significant result.

Results

**RAD001 inhibits the proliferation of NPC cells.** We first examined the viability of the two NPC cell lines in the presence of different concentrations of RAD001 (0-100 µM) using the WST-1 assay. CNE-1 cells were more sensitive, with a 50% growth inhibition (GI$_{50}$) of 30.0±1.0 µM at 72 h compared to the GI$_{50}$ of HONE-1, which was 56.9±13.1 µM (Fig. 1A and Table I).

**Cell morphological change.** To examine the lethal effect of RAD001 in CNE-1 and HONE-1 cells, both cell lines were treated with different concentrations of RAD001 (0, 20 and 40 µM) for 24 h. Cellular apoptosis was determined using DAPI staining. Chromatin condensation and cell shrinkage were clearly observed after RAD001 treatment (Fig. 1B).

**RAD001 induces cellular apoptosis.** To identify whether mTOR inhibition induces apoptosis, the treated cells were stained with Annexin V-FITC/PI and the apoptotic cell population was analyzed using flow cytometry. RAD001 treatment significantly increased the proportion of apoptotic cells (Fig. 2). In the control group, 3.1±1.1% cells were positive for Annexin V-FITC staining, and the 20, 40 and 80 µM RAD001 treatments resulted in Annexin V-FITC-positive rates of 3.5±0.9, 15.0±4.7 and 27.0±15.3%, respectively, in the CNE-1 cells treated for 24 h. At 48 h post-treatment, the apoptotic rates increased to 3.8±0.8% for the control and to 9.5±6.3, 52.3±11.8 and 96.5±2.2%, respectively, for the above-mentioned treatment groups. Similar results were observed in HONE-1 cells (Fig. 2 and Table II).

To demonstrate whether RAD001 treatment results in increased activation of caspases, we analyzed both the cleavage of PARP-1, a substrate cleaved by caspases during apoptosis, and the activated cleavage of caspase-3. Our western blotting results determined that procaspase-3 was cleaved to yield a 17 kDa fragment and PARP-1 was cleaved to an 89 kDa fragment following treatment in both cell lines (Fig. 3). These results
Table II. Effect of RAD001 on the apoptosis of CNE-1 and HONE-1 cells.

<table>
<thead>
<tr>
<th>RAD001 (µM)</th>
<th>24 h CNE-1</th>
<th>48 h CNE-1</th>
<th>24 h HONE-1</th>
<th>48 h HONE-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.1±1.1</td>
<td>3.8±0.8</td>
<td>3.0±1.4</td>
<td>2.4±0.7</td>
</tr>
<tr>
<td>20</td>
<td>3.5±0.9</td>
<td>9.5±6.3</td>
<td>3.1±1.2</td>
<td>3.5±1.9</td>
</tr>
<tr>
<td>40</td>
<td>15.0±4.7</td>
<td>52.3±11.8</td>
<td>8.3±5.0</td>
<td>90.6±6.5</td>
</tr>
<tr>
<td>80</td>
<td>27.0±15.3</td>
<td>96.5±2.2</td>
<td>51.2±32.4</td>
<td>96.3±1.4</td>
</tr>
</tbody>
</table>

Data represent the means ± SDs from triplicate experiments.

Figure 2. Effect of RAD001 on the apoptosis in CNE-1 and HONE-1 cells. Cells were treated with 0, 20, 40 and 80 µM RAD001 for 24 and 48 h and then stained using the Annexin V-FITC/PI apoptosis detection kit. Data points represent the means ± SDs from triplicate experiments.
further confirmed that RAD001 induced caspase-3-dependent cell death in these two nasopharyngeal carcinoma cell lines. RAD001-treated NPC cells displayed typical signs of apoptotic cell death including chromatin condensation, cell shrinkage, cleavage of PARP and activation of caspase-3.

**RAD001 induces cellular autophagy.** mTOR is well known as a major negative regulator of autophagy and acts as a central regulator of the PI3K/Akt pathway, which can induce autophagy when inhibited. The membrane-associated light chain 3 protein (LC3, Atg8) is a key marker of autophagy. After the induction of autophagy, LC3-I is converted to LC3-II, which is most likely conjugated to phosphatidylethanolamine (PE) and tightly bound to the autophagosomal membranes, forming ring-shaped structures in the cytoplasm. The amount of PE-conjugated LC3 (LC3-II) correlates well with the number of autophagosomes. Therefore, we examined LC3-I/II expression using western blotting. RAD001 treatment caused the levels of LC3-II to increase in a dose- and time-dependent manner in both cell lines (Fig. 3).

**Inhibition of autophagy enhances RAD001-induced cell growth inhibition and apoptosis.** Since RAD001 induced both apoptosis and autophagy in CNE-1 and HONE-1 cells, we aimed to identify which mechanism of cell death was dominant. We first inhibited autophagy by combining RAD001 with the autophagy inhibitor, 3-MA. 3-MA is used to inhibit and study the mechanism of autophagy (lysosomal self-degradation). It inhibits autophagy by blocking autophagosome formation via the inhibition of type III PI3K. RAD001 of 40 and 80 µM resulted in decreased survival rates of 34.9±2.4 and 19.9±0.9% compared with the control group (100.0%) in the CNE-1 cells (Fig. 4A). Furthermore, there was extensive growth inhibition in the combined treatment group. A survival rate of 17.3±0.5% (P<0.05) and 3.9±0.2% (P<0.05) was noted when 40 and 80 µM RAD001 was combined with 10 mM 3-MA in CNE-1 cells. Similar results were observed in the HONE-1 cells (Fig. 4A).

We next examined whether the combination was effective at inducing cellular apoptosis. As displayed in Fig. 4B, 3.4±1.3% of the cells were positive for Annexin V-FITC staining in the CNE-1 cell control group. Treatment with 40 or 80 µM RAD001 resulted in Annexin V-FITC-positive staining rates of 6.0±0.7 and 15.2±3.6% at 24 h. When 40 or 80 µM RAD001 was combined with 3-MA, the Annexin V-FITC-positive staining rates were 9.0±1.8% (P<0.05) and 34.5±4.7% (P<0.01), respectively. The combination treatment significantly increased the proportion of apoptotic cells compared to this proportion following RAD001 treatment (80 µM) alone (P=0.0024). Regarding the HONE-1 cells, RAD001 of 40 or 80 µM resulted in the Annexin V-FITC-positive staining rates of 4.6±2.0 and 28.6±3.8% compared with the control group (2.7±0.8%) at 24 h. When 40 or 80 µM RAD001 was combined with 10 mM 3-MA,
the Annexin V-FITC-positive staining rates were 11.4±3.7% (P<0.05) and 51.5±9.0% (P=0.0076), respectively (Fig. 4B).

**Inhibition of apoptosis decreases RAD001-induced cell death.** We next combined RAD001 with the caspase-3 inhibitor z-VAD-fmk to determine whether inhibition of apoptosis decreases RAD001-induced cell death. Using an LDH assay we determined that the growth inhibition effect was markedly reduced in both CNE-1 and HONE-1 cells. As shown in Fig. 5A, 40 and 80 µM RAD001 resulted in increased cytotoxicity rates of 37.1±4.3 and 51.8±1.7% compared with the control group (0.0%) in CNE-1 cells. When 40 or 80 µM RAD001 was combined with 20 µM z-VAD-fmk, the cytotoxicity rates were decreased to 12.5±5.9% (P<0.01) and 23.8±1.7% (P<0.01), respectively. Similar results were observed in HONE-1 cells (Fig. 5A).

Furthermore, using flow cytometry we examined whether the combination of RAD001 and z-VAD-fmk was effective at reducing cellular apoptosis. As shown in Fig. 5B, 1.2±1.3% of the cells were positive for Annexin V-FITC staining in the CNE-1
cell control group. In regards to the CNE-1 cells, treatment with 40 or 80 µM RAD001 resulted in Annexin V-FITC-positive staining rates of 4.7±3.6 and 61.5±12.5% at 24 h. When 40 or 80 µM RAD001 was combined with 20 µM z-VAD-fmk, the Annexin V-FITC-positive staining rates were reduced to 2.9±2.0% (P>0.05) and 39.1±6.5% (P<0.05), respectively. For HONE-1 cells, RAD001 of 40 or 80 µM resulted in Annexin V-FITC-positive staining rates of 3.2±1.1 and 60.2±9.6% when compared to the rates in the control group (1.7±1.4%) at 24 h. When 40 or 80 µM RAD001 was combined with z-VAD-fmk, the Annexin V-FITC-positive staining rates were reduced to 2.3±0.6% (P>0.05) and 30.7±10.3% (P<0.05), respectively (Fig. 5B).

Discussion

Nasopharyngeal cancer was invariably lethal prior to the advent of radiation. With improved knowledge and technology, locoregional control of this disease exceeds 90%. However, further reduction of distant failure and major late toxicities remain as challenges, and the development of targeted therapy without cytotoxicity is urgently needed. There is a
Tumor samples will be used to determine whether the response changed. We determined that the growth inhibition was enhanced, which is consistent with the growth inhibition was enhanced, which is consistent with the study by Rosich et al. Because this growth inhibition was represented by additional cellular apoptosis, we evaluated the mechanisms of action, including the induction of autophagy in prostate cancer (32), hepatocellular carcinoma (33) and acute lymphoblastic leukemia (34).

Rosich et al (35) demonstrated that the selective triple knockdown of the autophagy genes ATG7, ATG5 and ATG3 and pretreatment with the autophagy inhibitor hydroxychloroquine effectively overcame RAD001 resistance in a mantle cell lymphoma cell line, leading to the activation of the mitochondrial apoptotic pathway. In this study, cells displayed high levels of autophagy when RAD001 was used alone. We then combined RAD001 with autophagy inhibitor 3-MA to determine whether the response changed. We determined that the growth inhibition was enhanced, which is consistent with the study by Rosich et al. Because this growth inhibition was represented by additional cellular apoptosis, we evaluated whether caspase-3 inhibition in combination with RAD001 impaired its effect. Using an LDH assay and flow cytometry, we determined that the growth inhibitory effect was markedly reduced when RAD001 was used in combination with a caspase-3 inhibitor in both CNE-1 and HONE-1 cells.

Numerous reports suggest that autophagy is a survival mechanism protecting cells from cell death due to DNA damage. Other studies indicate that autophagy may be the mechanism of cell death during tumor treatment or that autophagy may be involved in the induction of apoptosis. Currently, both rapamycin (an mTOR inhibitor) and chloroquine (an autophagosome-lysosome fusion step blocker) are being used in combination with chemotherapy in clinical trials for different cancer types. Whether autophagy is an apoptosis-promoting mechanism per se or whether it acts as a protective mechanism that reduces tumor cell death upon treatment is still a controversial and complicated issue that remains to be determined (36).

mTOR possesses both pro-apoptotic and anti-apoptotic effects. On one hand, mTOR is capable of translocating into the nucleus resulting in the phosphorylation and activation of p53. This in turn results in the transcription of the pro-apoptotic proteins BAX and others (37). On the other hand, activated S6K is capable of binding to mitochondrial membranes and phosphorylating BAD (38), which then becomes inactive and is unable to promote apoptosis. In the present study, we combined either an autophagy inhibitor or an apoptosis inhibitor with the mTOR inhibitor RAD001 and observed more cell death when autophagy was inhibited. This result suggests that mTOR possesses mainly anti-apoptotic effects in CNE-1 and HONE-1 cell lines. RAD001 combined with an autophagy inhibitor aggravated mTOR inhibition and thus enhanced the induction of apoptosis.

In conclusion, our results are the first to suggest that the inhibition of mTOR by RAD001 may result in a potential therapeutic benefit for NPC. The predominant mechanism involved was the induction of cellular apoptosis rather than cell cycle arrest or autophagy. The combination of RAD001 and the autophagy inhibitor 3-MA resulted in enhanced tumor inhibition by promoting tumor cell cytotoxicity. Therefore, our present observations may provide additional evidence to the growing amount of research that indicates the effectiveness of mTOR inhibition for treating cancer as well as a strong rationale for the clinical evaluation of RAD001 combined with autophagy inhibitors for the management of nasopharyngeal malignancies. Further experiments will need to be performed to verify our conclusion in vivo. Tumor samples will be collected and autophagy- and apoptosis-related proteins will be examined to confirm our conclusions.

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References


