Survivin, an antiapoptotic protein, is elevated in most malignancies and attributes to radiation resistance in tumors including glioblastoma multiforme. The downregulation of survivin could sensitize glioblastoma cells to radiation therapy. In this study, we investigated the effect of rapamycin, an inhibitor of mammalian target of rapamycin (mTOR), in attenuating survivin and enhancing the therapeutic efficacy for glioblastoma cells, and elucidated the underlying mechanisms. Here we tested various concentrations of rapamycin (1–8 nM) in combination with radiation dose 4 Gy. Rapamycin effectively modulated the protein kinase B (Akt)/mTOR pathway by inhibiting the phosphorylation of Akt and mTOR proteins, and this inhibition was further enhanced by radiation. The expression level of survivin was decreased in rapamycin pre-treatment glioblastoma cells followed by radiation; meanwhile, the phosphorylation of H2A histone family member X (H2AX) at serine-139 (γ-H2AX) was increased. p21 protein was also induced on radiation with rapamycin pre-treatment, which enhanced G1 arrest and the accumulation of cells at G0/subG1 phase. Furthermore, the clonogenic cell survival assay revealed a significant dose-dependent decrease in the surviving fraction for all three cell lines pre-treated with rapamycin. Our studies demonstrated that targeting survivin may be an effective approach for radiosensitization of malignant glioblastoma.

Keywords survivin; rapamycin; radiation; mTOR; glioblastoma

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Introduction

Mammalian target of rapamycin (mTOR) is a 289 kDa serine/threonine kinase that acts as a downstream target of protein kinase B (Akt) [1]. It has been shown that mTOR is important for the oncogenic transformation specifically induced by phosphoinositide-3-kinase (PI3K) and Akt proteins [2]. On the contrary, a new function for mTOR in regulating the Akt was described by Sarbassov et al. [3]. Deregulation of mTOR pathway occurs in many human diseases [4], and then the molecules that inhibit mTOR pathway are attractive targets of clinical interest. In this context, mTOR gains importance in the signal transduction pathways and molecular pathology of cancer [5].

Rapamycin has been reported to cause myriad effects, suggesting that it can be a useful antitumor agent [4]. Rapamycin is a macrolide antibiotic produced by Streptomyces hygroscopicus. It binds to FK506 binding protein 12 (FKBP-12), which is a member of the immunophilin protein family and plays a role in immunoregulation, basic cellular processes involving protein folding and trafficking. FKBP-12 protein is a cis–trans prolyl isomerase that binds to immunosuppressants FK506 and rapamycin. The rapamycin–FKBP12 complex inhibits mTOR and prevents further phosphorylation of proteins involved in the transcription, translation, and cell cycle control. Furthermore, prolonged rapamycin treatment reduces the levels of cAMP responsive element binding (CREB)-regulated transcription coactivator 2 (mTORC2) that is essential to maintain Akt/PKB signaling in many cell types [6]. Rapamycin has been reported to induce apoptosis in cancer cell lines and also been proved as a potential antiangiogenic agent [7]. However, recent works have indicated that tumor cells lacking phosphatase and tensin homolog (PTEN) or von Hippel–Lindau tumor suppressors are sensitive to rapamycin [8–10].

Glioblastoma multiforme is a malignant primary brain tumor associated with poor survival rate. mTOR activity is required not only for the survival but also for maintaining the astrocytic characteristics of glioblastoma cells [11]. Although the mortality rate is decreased with the introduction of temozolomide, a triazene analog of dacarbazine with antineoplastic activity, more effective treatment modalities are still required. Radiotherapy plays a crucial role in achieving local control following surgery for the management of glioblastoma that are usually radiation resistant.
Accordingly, radioresistant cells present a problem in the course of treatment [12]. The mechanism of radiation sensitization needs to be explored. Our aim was to determine the effect of rapamycin treatment combined with radiation on mTOR pathway in PTEN-null glioblastoma cells A172, T98, and U87. Here, we report an in vitro study on the radiosensitizing effects of rapamycin with respect to PI3-K and mTOR pathway. Our results demonstrated that when combined with radiation, rapamycin inhibited the inhibitor of apoptosis protein (IAP) family protein survivin in glioblastoma cell lines through the repression of phospho-Akt. Targeting Akt through mTOR with rapamycin increased the radiation sensitivity.

Materials and Methods

Cell culture and chemicals
Rapamycin (R1018) was obtained from the AG Scientific Inc. (San Diego, USA) and dissolved in ethanol to yield 1 mg/ml stock solution. The stock solution was stored at −20°C. The drug was diluted in media immediately before treatment of cells. All the cells used in this study are PTEN defective and were obtained from KCLB/KCLRF Korea cell line bank (Seoul, Korea). A172 and U87 cell lines were maintained in RPMI media, whereas T98 cells in DMEM media (WelGENE, Daegu, Korea). Both media were supplemented with 10% fetal bovine serum (Serum Source International, Charlotte, USA), 100 U/ml penicillin and 100 µg/ml streptomycin, and cells were cultured in a humidified incubator at 37°C and 5% CO₂.

Radiation treatment
Irradiation was carried out when the cells reach 80% confluency and performed with a 6 MeV X-ray linear accelerator (Siemens Mevatron M6700, Concord, USA) at various fluency and performed with a 6 MeV X-ray linear accelerator (Siemens Mevatron M6700, Concord, USA) at various doses (0, 2, 4, 6, and 8 Gy) at a dose rate of 300 cGy/min in a single fraction.

Cell cycle analysis
Cells (1 × 10⁶) were seeded in 10 cm² dishes and allowed to grow until 80% confluency before treatment with radiation. For mTOR inhibition experiments, the cells were pre-treated with rapamycin for 10 h followed by radiation. Then the cells were collected at 24 h after the radiation by trypsinization, fixed with 70% ethanol, and stored over-night at −20°C. After thawing, cells were collected by centrifugation, and the cell pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) with propidium iodide (50 µg/ml). The cell number in each phase of the cell cycle was determined using a flow cytometer (FACScalibur™, Becton-Dickinson, San Jose, USA) and calculated as the percentage of total cell population.

Western blot analysis
The cells were pre-incubated with rapamycin 0, 1, 2, 4, and 8 nM for 10 h, before radiation exposure. After 4 h radiation exposure, the cells were washed with PBS and lysed with extraction buffer (Cell lysis buffer, Cell Signaling, Beverly, USA), along with a protease inhibitor cocktail tablet (Roche, Mannheim, Germany). Cellular debris were cleared by centrifugation at 11,270 g for 15 min at 4°C. The protein concentration was determined by BioRad reagent (BioRad, Hercules, USA). An equal amount of protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, USA). The membrane was then blocked with 5% milk in 0.2% Tris-buffered saline with Tween-20 (TBST) and washed in 0.2% TBST three times for 10 min each. The membrane was incubated overnight with primary antibodies against either survivin, AKT, Bcl2, Bax, and actin (Santa cruz Biotechnology, Santa Cruz, USA) or antiphospho-H2AX (Upstate, Billerica, USA) or pmTOR2481, pmTOR2448, mTOR, Pakt, and X-linked inhibitor of apoptosis (XIAP) (Cell signaling, Danvers, MA, USA). Subsequently, the membranes were washed in 0.2% TBST three times for 10 min each followed by an incubation with secondary antibody for 45 min. The proteins were visualized by Horseradish Peroxidase detection system West-Zol™ plus (iNtRON Biotechnology, Seongnam, Korea).

Clonogenic assay
Exponentially growing cells were treated with rapamycin for 10 h and irradiated. The cells were counted, diluted, and seeded in triplicate at 500 cells per culture dish (60 mm²). Thereafter, the cells were allowed to grow in culture dishes for 15 days. Then the culture dishes were stained with 0.4% crystal violet, and the colonies (≥50 cells) were counted. The surviving fraction was calculated as the mean number of colonies divided by the plating efficiency.

Results

Akt/mTOR activity is reduced synergistically after treatment with rapamycin and radiation
To investigate the involvement of Akt/mTOR pathway in conferring radiation resistance to PTEN defective glioblastoma, the cells were incubated with pre-determined concentrations of rapamycin for 10 h prior to radiation exposure. Western blotting analyses revealed that treatment of rapamycin diminished the levels of phosphorylated mTOR at Ser 2448 in all three glioblastoma cell lines [Fig. 1(A–C), Panel 1]. The attenuation of phosphorylation at Ser 2481 was observed only in A172 cells, whereas not in T98 and
Furthermore, to reassure that the rapamycin treatment was effective in blocking mTOR activity, we observed the expression of phospho-p70S6kinase (Thr 389), a direct downstream protein of mTOR. Rapamycin treatment dramatically downregulated the phosphorylation of p70S6kinase, suggesting that rapamycin specifically blocked the mTOR pathway [Fig. 1(A–C), Panel 3]. Additionally, rapamycin pre-treatment effectively inhibited the activation of Akt by blocking the phosphorylation at Ser473 and this phenomenon was observed in all three glioblastoma cell lines tested [Fig. 1(A–C), Panel 4]. However, rapamycin alone or in combination with radiation treatment did not cause any change in the protein levels of total Akt, whereas the combination treatment produced a slight reduction in total mTOR levels. The decreases in the phosphorylation revealed a potent inhibitory effect of rapamycin on the Akt/mTOR signaling pathway.

**Rapamycin results in specific attenuation of apoptotic inhibitory proteins**

To determine whether inhibition of mTOR affects survivin or XIAP in sensitizing glioblastoma cells to radiation therapy, the cells were pre-treated with rapamycin for 10 h followed by radiation at 0 or 4 Gy. As shown in Fig. 2, the rapamycin pre-treatment along with radiation significantly reduced the survivin levels in all three cell lines tested, whereas the reduction in survivin levels were not observed in rapamycin treatment alone [Fig. 2(A–C), Panel 1]. The XIAP levels were downregulated only in T98 cells [Fig. 2(B), Panel 2] but not in U87 [Fig. 2(A), Panel 2] and A172 [Fig. 2(C), Panel 2] cells. This phenomenon exhibited the potential synergism between rapamycin and radiation inhibiting mTOR signaling in glioblastoma cells.

![Figure 1](https://academic.oup.com/abbs/article-fig/43/4/292/1000)

**Figure 1** Radiation inhibits mTOR signaling in glioblastoma cells U87 (A), T98 (B), and A172 (C) cells were incubated without serum for 10 h and irradiated at 4 Gy. Total cell lysates were extracted at 4 h after irradiation. Control indicates no treatment. Thirty milligrams of total protein was loaded per lane and probed with respective antibody. Actin was probed to show equal loading.

![Figure 2](https://academic.oup.com/abbs/article-fig/43/4/292/1000)

**Figure 2** Rapamycin-mediated inhibition of survivin and expression of γ-H2AX sensitizes glioblastoma cells to radiation (A) U87 cells, (B) T98 cells, and (C) A172 cells. For all experiments XIAP, survivin, and histones were resolved by 12% SDS–PAGE followed by western analysis with antibodies against XIAP, survivin, or γ-H2AX.
radiation in growth suppression by inhibiting IAP proteins, especially survivin.

Rapamycin enhances the radiation-induced phosphorylation of H2A histone family member X (H2AX)

We first tested the formation of γ-H2AX at different time points in all three cell lines, and found the induction was maximum at 30 min post-radiation (Supplementary Fig. S1). Therefore, the following experiments were carried out at 30 min after the respective treatments. Rapamycin treatment induced γ-H2AX formation in U87 cells even with low concentration such as 1 nM and the combination treatment with 4 Gy radiation was also equally effective in this induction [Fig. 2(A), Panel 3]. In T98 and A172 [Fig. 2(B,C), Panel 3], the cells induced the formation of γ-H2AX at higher concentration of rapamycin. However, when the cells were subjected to radiation along with rapamycin pre-treatment, they exhibited increased γ-H2AX formation. The control treatments in all the cell lines were incapable of inducing γ-H2AX. This observation indicated that rapamycin induced γ-H2AX formation and promoted apoptosis in glioblastoma cells.

Cell cycle analysis of glioblastoma cells

Changes in the cell cycle have been previously analyzed to explore the radiosensitivity after mTOR inhibition [13]. To examine the effect of rapamycin pre-treatment followed by radiation, we performed cell cycle analysis. Notable changes in the cell cycle distribution were observed at 24 h after treatment. The cell cycle analysis showed that combination of rapamycin pre-treatment and radiation increased sub-G1 population (apoptotic fraction) as compared with either radiation or rapamycin alone (radiation and rapamycin: 57.09%, 49.90%, 7.02%; radiation: 17.69%, 14.59%, 17.69%; rapamycin: 9.91%, 12.48%, 6.77%; and control 11.43%, 13.54%, 13.89% for A172, T98, and U87 cells, respectively). We recorded profound G2-M phase arrest only in U87 cells subjected to 4 Gy radiation, whereas T98 and A172 cells displayed a moderate increase along with a considerable G1 arrest (Fig. 3, Panel-B).

The presence of S phase population and G1 phase population was observed in the control cells (Fig. 3, Panel-A), whereas the S phase population decreased in cells subjected to either radiation (Fig. 3, Panel-B) or rapamycin treatment (Fig. 3, Panel-C). In addition, we also observed G1 phase arrest for these treatments. In particular, increased subG1 phase accumulation was noticed along with G1 arrest in T98 and A172 cells, whereas U87 cells displayed higher levels of G1 arrest (Fig. 3, Panel-D). Taken together, our results revealed that the rapamycin pre-treatment on radiation produced an intense G1 arrest, which eventually led to apoptosis.

Rapamycin enhances radiation-induced p21cip1 and p27kip1 expression and downregulation of cyclin proteins

The role of p21cip1 and p27kip1 on rapamycin pre-treatment followed by radiation were examined by western blot analysis. p21cip1 and p27kip1 were synergistically upregulated [Fig. 4(A), Panels 3 and 4], simultaneously cyclin D1 and cyclin D3 were inhibited [Fig. 4(A), Panels 5 and 6] in the pre-treated U87 cells subjected to radiation. Cyclin D1 was significantly downregulated more than cyclin D3. T98 cells displayed the induction of p21cip1 only in the combination of rapamycin with radiation treatment [Fig. 4(B), Panel 3], whereas p27kip1 expression was altered moderately on rapamycin and the effect was enhanced on radiation treatment [Fig. 4(B), Panel 4]. This overexpression of p21cip1 influenced the downregulation of both cyclin D1 and cyclin D3. Particularly, cyclin D3 was completely inhibited on rapamycin treatment, as well as in combination with radiation treatment [Fig. 4(B), Panels 5 and 6]. In A172 cells, p21cip1 expression was noticed after radiation and this induction was maintained in the samples subjected to rapamycin pre-treatment, whereas an opposite effect was observed for p27kip1 protein. The induction of p27kip1 by radiation could not be maintained by rapamycin pre-treatment [Fig. 4(C), Panels 3 and 4]. Cyclin D3 [Fig. 4(A), Panel 6] was completely downregulated in the A172 cell line under the treatment of rapamycin, and this inhibition was maintained even after radiation [Fig. 4(C), Panel 5]. Collectively, these results indicated that the increase of p21cip1 protein level might be responsible for sensitizing glioblastoma cells to radiation.

mTOR inhibition sensitizes glioblastoma cells to radiation by decreasing cell survival

Using clonogenic survival assay, we determined whether the rapamycin radiosensitizes U87, T98, and A172 glioblastoma cells. Since high concentration of rapamycin yielded larger cytotoxic effects, we opted for lower concentration (1 nM) and pre-treated the cells for 10 h before radiation. The tested concentration 1 nM of rapamycin significantly inhibited mTOR and reduced the survival fraction by 0.178, 0.433, and 0.224 in log scale for U87, T98, and A172 cells, respectively. To study the effect of rapamycin pre-treatment on radiation, we normalized the decreased survival fraction to 1.00 in rapamycin pre-treated samples. Radiation decreased the proliferation capacity of the cells and further reduced the survival fraction to 0.103, 0.053, and 0.062 in log scale at the end of 8 Gy. Rapamycin pre-treatment produced an additive effect in sensitizing the glioblastoma cells to radiation. Among the three cell lines subjected to rapamycin pre-treatment, T98 cells were found to be more sensitive for radiation [Fig. 5(B)] followed by A172 cells [Fig. 5(C)] and U87
Figure 3 Cell cycle analysis of U87, T98, and A172 cells by flow cytometry. Comparison of G1 arrest in control, irradiated, and Rapamycin-treated cells as well as combination of rapamycin and radiation treatments. (A) U87, (B) T98, and (C) A172 cells pre-treated with 1 nM rapamycin and radiated at 4 Gy were stained with propidium iodide (PI). The percentage of G1, S, and G2/M population was calculated based on the results obtained from cell cycle analysis by FACS flow cytometry.
cells [Fig. 5(A)]. Quantification of colony numbers and statistical analysis showed significant ($P < 0.05$) radiosensitization effect due to rapamycin pre-treatment.

**Discussion**

Therapeutic strategies that target mTOR are becoming increasingly important in cancer research. mTOR as a downstream kinase in the PI3K/Akt pathway contributes to cell growth and survival, which makes it a clear target. In the present study, we showed that mTOR signaling was induced by radiation in glioblastoma cell lines U87, T98, and A172, which was consistent with previous studies [8,13]. We showed that treating glioblastoma cells with mTOR inhibitor rapamycin blocked the increase of mTOR phosphorylation even at low concentration such as 1 nM rapamycin. Strikingly, the rapamycin treatment was also equally effective in reducing the Akt phosphorylation at ser 473. This reciprocal effect of mTOR on Akt was due to its association with rictor, a member protein of mTOR complex, and the reduction in rictor or mTOR expression inhibited the Akt effector. The rictor–mTOR complex directly phosphorylated Akt/PKB on Ser473 in vitro [3], and the disruption of this complex by rapamycin led to the inhibition of pAkt. This strongly suggests that mTOR inhibition by rapamycin is effective in blocking the prosurvival response of glioblastoma cells to radiation and suggests a possible mechanism for radiosensitization.
The apoptotic inhibitory proteins play a vital role in cell viability and proliferation. Recent studies have demonstrated that survivin expression is associated with glioma progression from low to high grade [14–17]. In addition, these studies also demonstrate that survivin expression is a marker for radiation resistance, with strongest expression in radiation-resistant glioma phenotypes. In our panel of cell lines, radiation treatment alone showed stronger survivin expression than other treatments, indicating the critical role of survivin in radiation resistance. The inhibition of mTOR with rapamycin abolished survivin increase by the downregulation of p70S6K [18] a downstream target of mTOR, which resulted in the glioma cell viability. Downregulation of survivin levels using a variety of approaches including siRNA or dominant negative mutants has been consistently associated with impaired cell proliferation, apoptosis, and sensitizes to cell death stimuli including cytotoxics and ionizing radiation [19]. Regulation of survivin expression has also been linked to increased Akt activity in experimental systems [20]. Our results clearly showed a concomitant decrease of survivin and Akt activity in glioma cells where PTEN is defective or mutated. In addition, a recent study indicated that Akt signaling involving mTOR and p70S6K activation increases the translation of a survivin mRNA pool and protein stability [21]. This circuitry involving Akt, mTOR, and p70S6K is centered on survivin [22] mRNA entry site sequence located in its 5’ untranslated region [23]. This phenomenon was convincing especially for U87 and A172 cells, where the XIAP levels were unaltered in spite of phosphorylation of AKT occurred rapidly upon radiation.

Cell cycle regulation is important in mediating radiosensitivity and it is known that cell cycle arrested cells are found to be radioresistant [24]. However, it is also established that cells have varying radiosensitivity in different cell cycle phases that involves sensor, transducer, and effector genes. In the present study, the G1 phase arrest was observed in either radiation or rapamycin treatment, whereas G2-M arrest was observed only in U87 cells on radiation treatment alone. The rapamycin pre-treatment with radiation produced sub-G1 phase accumulation in T98 and A172 cells, which was consistent with previous studies [13]. On the other hand, U87 cells produced an intense G1 arrest. At this point, it is important to consider the phenotypic characters of the cells used in this study may be responsible for the differences existed among them. U87 cells showed increased activation of mTOR signaling after radiation, which offered a greater potential for mTOR inhibition. These results suggest a possible mechanism for the observed differences in the cell cycle arrest between U87 and T98, A172 cells. This phenomenon was further analysed by CDKs (cyclin D1 and D3) and its inhibitors p21cip1 and p27kip1 protein levels.

Owing to the elevated levels of either p21cip1 or p27kip1 proteins in response to extracellular signals, the expression of G1 regulatory proteins cyclin D1/D3 was downregulated in T98 and U87 cells, whereas in A172 cells cyclin D3 alone was downregulated [25]. Cyclin D1 is a critical regulator involved in cell cycle progression through the G1 phase into the S phase, thereby contributing to cell proliferation. Earlier reports and evidences indicate that, among G1 phase cyclins, cyclin D1 is the most strongly implicated protein in tumorigenesis [26,27]. Our results showed a complete downregulation of cyclin D1 in T98 and U87 cells by rapamycin pre-treatment and radiation. Inappropriate overexpression of cyclin D1 has been recorded in malignant human glioblastoma in A172 cells [28–30]. Considerable studies have been published on the roles of p21cip1 and p27kip1 in carcinogenesis of tumors, including glioblastoma [31–33]. In accord with its molecular functions, both p21cip1 and p27kip1 are often increased in proliferation-arrested cells, indicating that these proteins are not only essential for growth arrest, but also for inducing apoptotic morphology in U87 cells [34]. Moreover, increased p27kip1 staining was observed for U251 intracranial xenografts treated with rapamycin [35]. The increase in p21cip1 and inhibition of cyclin D1 and cyclin D3 explained the accumulation of cells in G1 phase of cell cycle at least in part in our study. The overexpression of p21cip1 and p27kip1 might therefore be a pivotal candidate for the perturbed cell cycle progression induced by radiation under rapamycin pre-treatment.

Various apoptosis regulatory proteins such as Bcl2, Bax contribute to the rate of apoptosis in many cancers. We observed no significant change in the level of Bcl2 protein, whereas a slight increase in Bax protein was noticed in A172 and T98 cells [Fig. 4(A), Panels 1 and 2; Fig. 4(B), Panels 1 and 2]. In U87 cells Bax protein alone was downregulated on rapamycin treatment followed by radiation [Fig. 4(C), Panels 1 and 2]. The modulation in the Bax protein could directly be attributed to the increased level of p27kip1 [36]. This increase in the levels of these proteins by rapamycin was specific to Bax and p27kip1, since other apoptosis-related proteins Bcl2 and Bcl-xl (Bcl-xl data not shown) remained unchanged. The stability of Bcl2 and Bcl-xl is mediated by FK506 binding protein 38 (FKBP38) as reported by Choi et al. [37]. FKBP38 is an endogenous inhibitor of mTOR, which is associated with Bcl2 and Bcl-xl and co-localizes with these proteins in mitochondria [38]. These observations suggested that Bax and p27kip1 could be modulated by common pathways or a coordinated mechanism [36,39]. Therefore, cell cycle arrest and its effector proteins may be a contributing factor in the increased radiosensitivity of rapamycin observed in this study.
Ionizing radiation or radiomimetic drugs usually induces chromatin fragmentation followed by DNA double-strand break, where γ-H2AX is formed that serves as an indicator of cells undergoing apoptosis [40]. Radiosensitive tumor cells exposed to irradiation retained γ-H2AX longer than radioresistant cells and tumors [41]. The long retention of γ-H2AX apparently makes radiosensitive cells more sensitive to apoptosis. Our results showed that rapamycin could induce phosphorylation of H2AX and further enhanced on radiation. Similar study on gastrointestinal stromal tumor also reveals that excessive levels of H2AX cause chromatin aggregation, which leads to impaired transcription [42], and thereby sensitizes tumor cells to undergo apoptosis [43]. And the downregulation of γ-H2AX would allow the tumor cell to escape from cell death. Moreover, the downregulation of γ-H2AX pathway that involves PI3K, mTOR, and ubiquitin-proteasome machinery means that H2AX is a tumor suppressor. Hence, targeting mTOR through rapamycin might upregulate γ-H2AX formation and lead the cell to apoptosis.

In conclusion, we reported that survivin plays an important role in the rapamycin-mediated apoptosis. Rapamycin pre-treatment induced the expression of γ-H2AX as well as attenuated survivin and this effect was further enhanced by radiation. Moreover, clonogenicity of the glioblastoma cells were also greatly reduced on rapamycin pre-treatment followed by radiation. Our data provided additional evidence in supporting the crucial role of mTOR and its inhibition in cancer therapy. Understanding the mechanisms of these signal molecules in rapamycin-induced growth arrest and apoptosis may be useful to improve radiation therapy.

Supplementary Data
Supplementary data is available in ABBs online.

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Rapamycin attenuates survivin and radiosensitizes glioblastoma cells


