# Targeting the Akt/mammalian target of rapamycin pathway for radiosensitization of breast cancer

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## Abstract

**The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is known to be activated by radiation. The mammalian target of rapamycin (mTOR) is downstream of Akt, and we investigated the effects of radiation on Akt/mTOR signaling in breast cancer cell models. RAD001 (everolimus), a potent derivative of the mTOR inhibitor rapamycin, was used to study the effects of mTOR inhibition, as the role of mTOR inhibition in enhancing radiation remains unexplored. RAD001 decreased clonogenic cell survival in both breast cancer cell lines MDA-MB-231 and MCF-7, although the effect is greater in MDA-MB-231 cells. Irradiation induced Akt and mTOR signaling, and this signaling is attenuated by RAD001. The radiation-induced signaling activation is mediated by PI3K because inhibition of PI3K with LY294002 inhibited the increase in downstream mTOR signaling. Additionally, caspase-dependent apoptosis is an important mechanism of cell death when RAD001 is combined with 3 Gy radiation, as shown by induction of caspase-3 cleavage. An increase in $G_2$-M cell cycle arrest was seen in the combination treatment group when compared with controls, suggesting that cell cycle arrest may have been a contributing factor in the increased radiosensitization seen in this study. We conclude that RAD001 attenuates radiation-induced prosurvival Akt/mTOR signaling and enhances the cytotoxic effects of radiation in breast cancer cell models, showing promise as a method of radiosensitization of breast cancer. [Mol Cancer Ther 2006;5(5):1183–9]**

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## Introduction

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is a cell survival pathway that is important for normal cell growth and proliferation (1). This pathway has also been implicated in tumorigenesis (2) and is becoming an important target for cancer treatment (3, 4). The PI3K/Akt pathway has also been specifically shown to promote breast cancer cell survival and resistance to tamoxifen (5) and many other chemotherapeutic agents (6). Clinically, increased Akt activity has been associated with decreased survival in tamoxifen-treated breast cancer patients, supporting the role of Akt in tamoxifen resistance (7). Additionally, patients who test positive for activated phospho-Akt are more likely to relapse with distant metastasis (8).

Mammalian target of rapamycin (mTOR) is a 289-kDa serine/threonine kinase that is a downstream target of Akt (9). It has been shown that mTOR is important for the oncogenic transformation induced specifically by PI3K and Akt, and that the mTOR inhibitor rapamycin inhibits cellular transformation (10). Recently, mTOR has been explored as a target for cancer therapy (3). mTOR presents an attractive target in the pathway because it is downstream of PI3K and Akt. Thus, mTOR inhibition could avoid possible side effects from inhibiting these broader function upstream proteins (3, 11). The normal activation of mTOR results in an increase in protein translation because mTOR phosphorylates and activates the translation regulators eukaryotic initiation factor 4E-binding protein 1 and ribosomal p70 S6 kinase (12, 13). Therefore, by inhibiting mTOR, rapamycin causes a decrease in phosphorylation of these effectors, and a decrease in protein synthesis, effectively blocking the progrowth, pro-proliferative, and prosurvival actions of mTOR (14).

The rapamycin analogue CCI-779 has been shown to be an effective chemotherapeutic agent against breast cancer in phase II clinical trials (15), and the rapamycin derivative RAD001 is also currently in clinical trials. It has been recently suggested that combining RAD001 with DNA-damaging chemotherapeutic agents may be an effective means of enhancing cancer treatment (16). However, the role of mTOR inhibition in enhancing radiation-induced DNA damage remains unexplored. We aim to investigate the combination of the mTOR inhibitor RAD001 with radiation.

Breast cancer is the most common type of cancer in women, excluding nonmelanoma skin cancer, and is the second leading cause of cancer deaths in women. Based on current data, 13.2% of all women will be diagnosed with invasive breast cancer (17). Although early detection is leading to declining mortality rates, the high incidence of breast cancer requires continued use of different treatment modalities, including combinations of surgery,

radiotherapy, and chemotherapy. In particular, radiotherapy plays a crucial role in achieving local control following surgery. Accordingly, development of radioresistance in breast cancer cells presents a difficult problem in the course of treatment (18). Therefore, a breast cancer cell model is an important and useful model when studying radiosensitization and cancer cell survival pathways. The purpose of the present study was to determine the effects of radiation on mTOR signaling and determine whether mTOR inhibition with RAD001 enhances the cytotoxic effects of radiation in breast cancer cells. We found that radiation induces prosurvival Akt/mTOR signaling, and that RAD001 attenuates this induction and enhances the cytotoxic effects of radiation. Additionally, caspase-dependent apoptosis and cell cycle arrests seemed to contribute to the increased levels of cell death.

## Materials and Methods

### Cell Culture

MDA-MB-231 cells and MCF-7 cells (obtained from the American Type Culture Collection, Rockville, MD) were cultured in BMEM (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were incubated at 37°C and humidified 5% CO2.

### Western Immunoblots

Cells were treated with various radiation doses and drugs and collected at different time points, according to the individual study. The cells were harvested and then washed with ice-cold PBS twice before the addition of lysis buffer. Protein concentration was quantified using the Bio-Rad method. Equal amounts of protein were loaded into each well and separated by 10% SDS-PAGE gel followed by transfer onto polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA). Membranes were blocked using 5% nonfat dry milk in PBS-T. The blots were then incubated with total caspase-3, cleaved caspase-3, mTOR, phospho-mTOR ($Ser^{2448}$; Cell Signaling, Beverly, MA), Akt, phospho-Akt ($Ser^{473}$; Cell Signaling), S6 ribosomal protein, or phospho-S6 ribosomal protein ($Ser^{240}/Ser^{244}$; Cell Signaling) antibodies overnight at 4°C. Goat anti-rabbit IgG secondary antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) was incubated for 1 hour at room temperature. Immunoblots were developed using the chemiluminescence detection system (Perkin-Elmer, Wellesley, MA) according to the manufacturer's protocol and autoradiography. Image analysis was used to quantify the intensity of the bands.

### *In vitro* Clonogenic Assay

Cells were treated with RAD001 (10 nmol/L) or DMSO control for 1 hour. Various doses of radiation were given, and the medium was changed. After irradiation, cells were returned to the 37°C incubator and maintained for 10 days. Cells were fixed for 15 minutes with 3:1 methanol/acetic acid and stained for 15 minutes with 0.5% crystal violet (Sigma, St. Louis, MO) in methanol. After staining, colonies were counted with a cutoff of 50 viable cells. Surviving fraction was calculated as (mean colonies counted) / (cells plated × plating efficiency), where plating efficiency was defined as (mean colonies counted) / (cells plated) for nonirradiated controls. Mean, SD, and $P$ (for RAD001-treated cells versus DMSO control, using a Student's $t$ test) were calculated.

### Cell Cycle Analysis

Cells ($10^6$) were seeded in 10-$cm^2$ dishes 24 hours before treatment with 5 Gy radiation and/or 10 nmol/L RAD001. The cells were collected at 8 and 24 hours by trypsinization. They were fixed with 70% ethanol and stored overnight at −20°C. After thawing, cells were collected by centrifugation, and the cell pellet was resuspended in 1 mL of PBS with propidium iodide (50 μg/mL). Cell number in each phase of the cell cycle was determined and calculated as a percentage of the total cell population. The analysis was repeated thrice, and the mean value and SD were calculated and graphed.

## Results

### Radiation Induces mTOR Signaling in Breast Cancer Cells

Western analysis was used to determine the effects of radiation and mTOR inhibition on mTOR signaling in breast cancer cells. The relative levels of the probed proteins were estimated by the densitometer and compared among the samples collected at different time points. After 15 minutes, phospho-mTOR was increased in both breast cancer cell lines used, and this increase persisted through 180 minutes (Fig. 1A and B). The phospho-mTOR was increased 1.4-fold from control in MDA-MB-231 cells and increased 2.0-fold in MCF-7 cells. Additionally, phospho-Akt was increased between 1.7- and 1.8-fold in both cell lines, and phospho-S6 ribosomal protein (a downstream marker of mTOR signaling) was increased 1.2- to 1.3-fold in both cell lines, showing increased signaling both upstream and downstream from mTOR. Notably, phospho-S6 levels began to decrease after 60 minutes in both cell lines despite phospho-mTOR levels remaining elevated and were reduced to control levels by 180 minutes (Fig. 1). To establish whether activation of mTOR signaling was mediated by PI3K, cells were treated with either the known PI3K inhibitor LY294002 (10 μmol/L) or RAD001 (20 nmol/L). A slightly higher concentration of RAD001 was used for Western immunoblots than for the other assays to emphasize cell signaling inhibition and protein level changes. After 1 hour of treatment, cells were treated with 3 Gy radiation. Cell lysates were then collected and analyzed. As shown in Fig. 2, MDA-MB-231 cells treated with 3 Gy showed dramatically increased levels of S6 phosphorylation, from negligible levels to strong induction. A moderate increase in S6 phosphorylation (~1.2-fold) was seen in MCF-7 cells (Fig. 2). Cells treated with LY294002 or RAD001 showed very little S6 phosphorylation with or without irradiation, similar to nonirradiated controls.

### mTOR Inhibition Sensitizes Breast Cancer Cells to Radiation by Decreasing Cell Survival

Using clonogenic assays, the PI3K inhibitor LY294002 has been previously shown to sensitize cancer cells to

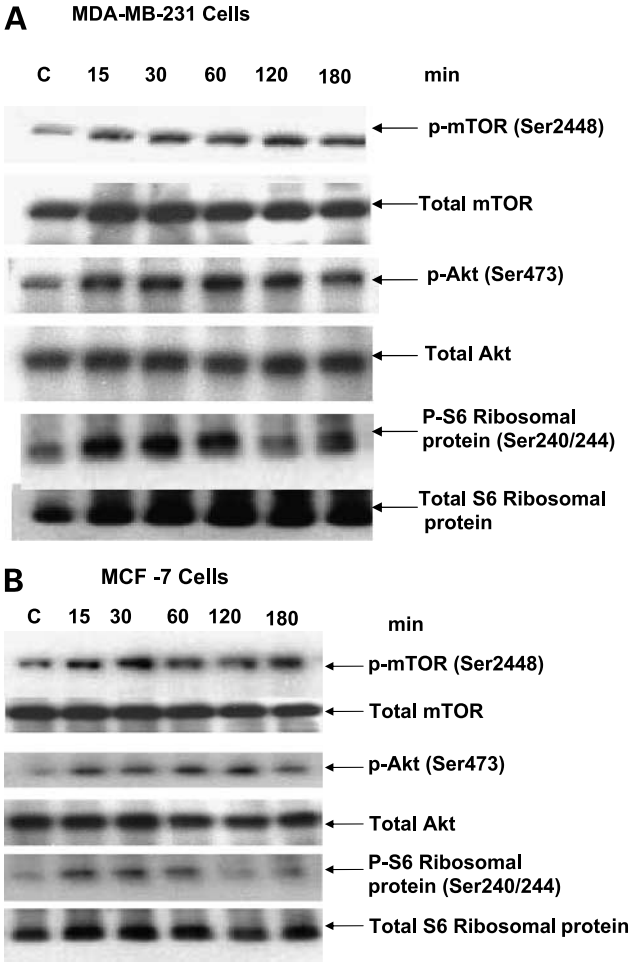


**Figure 1.** Radiation induces mTOR signaling in breast cancer cells. MDA-MB-231 (**A**) and MCF-7 (**B**) cells were incubated without serum for 7 h and irradiated with 3 Gy. Total cell lysates were extracted at indicated time points after irradiation. C indicates nonirradiated control in both **A** and **B**; 50 μg total protein per lane were probed with phospho-mTOR (*p-mTOR*), phospho-Akt (*p-Akt*), and phospho-S6 (*P-S6*) ribosomal protein antibodies. mTOR, Akt, and S6 ribosomal protein were also probed to show equal loading.

radiation (19–21). In the present study, clonogenic assays were used to determine whether downstream mTOR inhibition with RAD001 radiosensitizes MDA-MB-231 and MCF-7 breast cancer cells. Cells were treated with RAD001 (10 nmol/L) or DMSO control for 1 hour before irradiation, based on previous studies (22, 23). One hour is long enough for mTOR inhibition to occur but not long enough for RAD001 to show a significant cytotoxic effect. Radiation doses from 0 to 6 Gy were then given, and the medium was changed. The colonies grew for 10 days and were then stained, scored, and graphed. A decrease in the survival curve of irradiated cells was seen for both breast cancer cell lines (Fig. 3). The effect is greater in MDA-MB-231 cells (*A*) than in MCF-7 cells (*B*).

### Induction of Caspase-3 Cleavage in MDA-MB-231 Breast Cancer Cells

Western analysis was done to determine whether mTOR inhibition with RAD001 induces caspase-dependent apoptosis in MDA-MB-231 cells. This cell line was chosen for closer mechanism examination because the clonogenic assay shown a stronger radiosensitizing effect than in the MCF-7 cells (Fig. 3), and Western analysis showed that radiation induced much more S6 phosphorylation than in MCF-7 cells (Fig. 2). Some caspase-3 cleavage was induced by 3 Gy or RAD001 alone. However, significantly more caspase-3 was cleaved using the RAD001/3 Gy combination than with either treatment alone (Fig. 4A). To further analyze the effects of combination treatment on caspase-3 activity, pan-caspase-inhibitor z-VAD-fmk was combined with 3 Gy and RAD001. As expected, z-VAD-fmk notably reduced the level of cleaved caspase-3 seen with combination treatment. To quantify the caspase-3 activity seen in the different treatment groups, the intensity of the cleaved caspase-3 bands was then measured and calculated relative to actin control (Fig. 4B). The control cells showed insignificant levels of caspase-3 cleavage; 3 Gy alone had a relative intensity of about 0.33, RAD001 alone was 0.19, but combination 3 Gy + RAD001 was 0.7. Adding z-VAD-fmk to the 3 Gy/RAD001 combination reduced the band intensity to 0.27.

### Cell Cycle Analysis of MDA-MB-231 Breast Cancer Cells

Changes in the cell cycle have been previously analyzed to explore radiosensitivity after mTOR inhibition (22). To determine whether changes in cell cycle distribution contributed to the increased radiosensitivity of MDA-MB-231 cells, propidium iodide staining and flow cytometry were used. The experiment was repeated thrice, and the mean cell cycle distribution for DMSO control, 5 Gy radiation, 10 nmol/L RAD001, and 5 Gy/RAD001 combination was graphed (Fig. 5). Eight hours after treatment, there was no notable change in cell cycle distribution seen after treatment with 5 Gy, RAD001, or combination 5 Gy/RAD001 (data not shown). At 24 hours, there was no significant change in cell cycle distribution after treatment with RAD001 alone. However, there was a moderate increase in $G_2$-M phase cells after treatment with 5 Gy

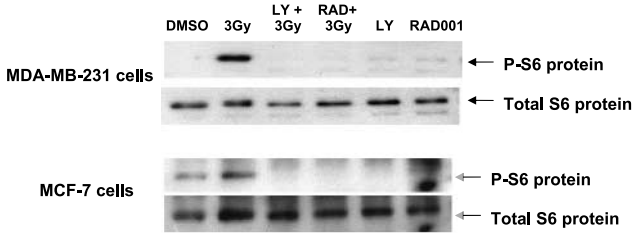


**Figure 2.** RAD001 inhibits radiation-induced mTOR signaling. MDA-MB-231 cells and MCF-7 cells were treated with DMSO, LY294002 (10 μmol/L), or RAD001 (20 nmol/L) for 1 h before irradiation (3 Gy). After 30 min of irradiation, total cell lysates were prepared in lysis buffer; 50 μg total protein per lane were immunoblotted for phospho-S6 (*P-S6*) ribosomal protein. S6 ribosomal protein was probed to show equal loading.

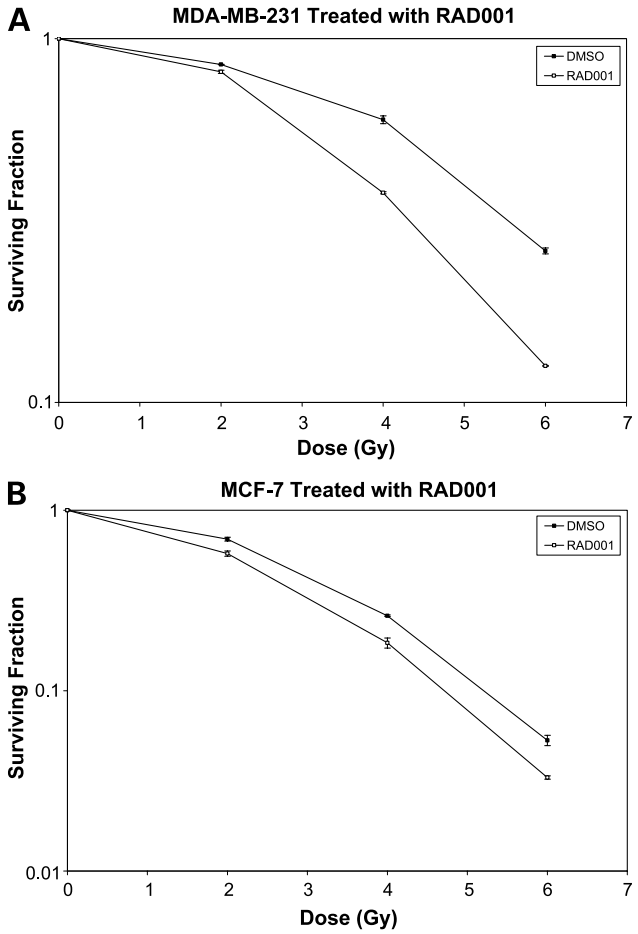


**Figure 3.** mTOR inhibition radiosensitizes MDA-MB-231 and MCF-7 breast cancer cells. **A**, MDA-MB-231 cells treated with radiation alone and radiation with RAD001 at indicated doses. **B**, MCF-7 cells that were similarly treated. Cells were treated with 10 nmol/L RAD001 or DMSO for 1 h. Cells were then irradiated with 0, 2, 4, or 6 Gy, and the medium was changed. After 2 wks, colonies were scored. *Points,* mean; *bars,* SD. $P < 0.003$, for RAD001 versus control in MDA-MB-231 cells; $P < 0.004$, for RAD001 versus control in MCF-7 cells.

alone, and a large increase in $G_2$-M phase cells was seen 24 hours after combination treatment (Fig. 5). At 24 hours, the percentage of cells in $G_2$-M phase was 15.0% (SD 1.2%) for control, 33.5% (SD 0.9%) for 5 Gy alone, 7.7% (SD 0.4%) for RAD001 alone, and 69.6% (SD 0.5%) for RAD001 + 5 Gy.

## Discussion

We have found that mTOR signaling is increased by radiation in breast cancer cell models, and that inhibition of mTOR obstructs this signaling activation. The mTOR inhibitor RAD001 radiosensitizes the two models used in this study, although the effect is greater in MDA-MB-231 cells than in MCF-7 cells. The study of mTOR as a therapeutic target is becoming increasingly important in cancer research. The role of mTOR as a downstream kinase in the PI3K/Akt pathway, a pathway crucial to cell growth and survival, makes it a clear target.

Rapamycin is well characterized as an mTOR inhibitor (24) and has led to the development of rapamycin derivatives with improved pharmaceutical properties (i.e., CCI-779 and RAD001; ref. 25). Both of these mTOR inhibitors are in clinical trials, and have shown significant chemotherapeutic antitumor activity in various models (14, 15).

In the present study, we showed that mTOR signaling is induced by ionizing radiation in the two breast cancer cell lines MDA-MB-231 and MCF-7, which is consistent with the PI3K/Akt pathway activation seen in breast cancer cells in previous studies (26, 27). Because radiotherapy is important as adjuvant therapy for breast cancer, it is undesirable that radiation can contribute to cancer cell survival. Therefore, blocking radiation-induced PI3K/Akt/mTOR pathway activation presents a method of enhancing the cytotoxic effects of radiation. To show this, we showed that treating breast cancer cells with the mTOR inhibitor RAD001 blocked the increase in phosphorylation of the downstream marker S6 ribosomal protein. This strongly suggests that mTOR inhibition is an effective means of blocking the prosurvival response of breast cancer cells to radiation and suggests a mechanism for how RAD001 may enhance the efficacy of radiotherapy.

One notable unexpected result was that phospho-S6 levels decreased after 60 minutes in both cell lines, despite no corresponding decrease in phospho-mTOR levels. This finding suggests that in addition to mTOR, other pathways may be regulating the phosphorylation of S6 protein, perhaps through feedback inhibition. For example,

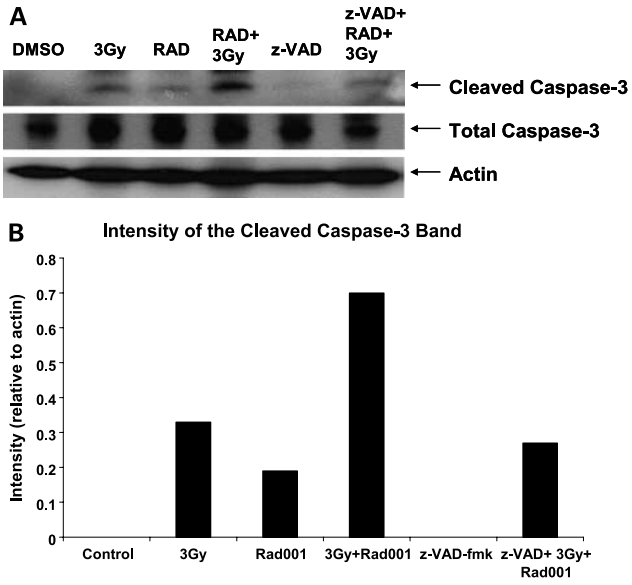


**Figure 4.** Induction of caspase-3 cleavage in treated MDA-MB-231 cells. MDA-MB-231 cells were treated with DMSO, 3 Gy, RAD001 (20 nmol/L), RAD001 + 3 Gy, z-VAD-fmk (50 μmol/L), or z-VAD-fmk + 3 Gy + RAD001. **A**, Western blots probed for cleaved caspase-3, total caspase-3, and actin. **B**, the intensity of the bands was then quantified and calculated as intensity relative to actin control.

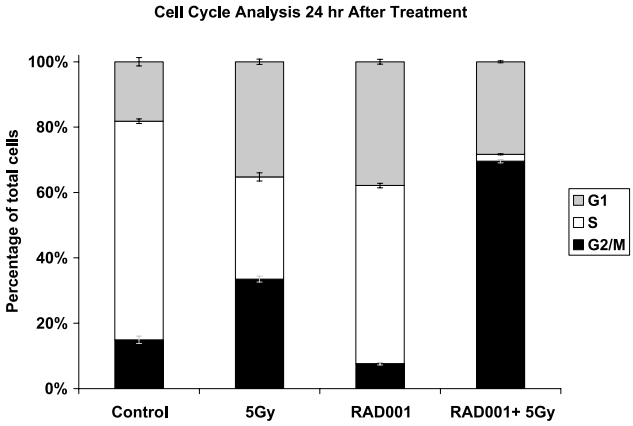


**Figure 5.** Cell cycle analysis of treated MDA-MB-231 cells. Cells were treated with DMSO control, 5 Gy radiation, 10 nmol/L RAD001, and 5 Gy/RAD001 combination. Cells were then fixed and resuspended in propidium iodide (50 μg/mL). Cell number in each phase of the cell cycle was determined and calculated as a percentage of the total cell population and then graphed. *Columns,* mean from three repeated studies; *bars,* SD.

phosphoinositide-dependent protein kinase 1 has been shown to activate ribosomal p70 S6 kinase, which in turn phosphorylates S6. Phosphoinositide-dependent protein kinase 1 can bypass the Akt/mTOR pathway and directly activate ribosomal p70 S6 kinase (28–30). It has also been suggested that the c-Raf/mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway can control activation of ribosomal p70 S6 kinase (31), providing another possible mechanism for the late decrease in phospho-S6 seen in the present study.

A recent study used a combination of RAD001 and the DNA-damaging agent cisplatin to show that RAD001 sensitizes cancer cells to DNA damage–induced apoptosis (16). We hypothesized that combining radiation, a DNA-damaging agent, with RAD001 would also be effective at sensitizing cancer cells to apoptosis. We, therefore, investigated apoptosis in MDA-MB-231 cells treated with RAD001 and radiation. Western analysis probing for cleaved caspase-3 showed that apoptosis was greatly increased in these cells after combination treatment with RAD001 and 3 Gy versus treatment with RAD001 or 3 Gy alone (Fig. 4A), indicating that caspase-dependent apoptosis contributes to the mechanism of the observed increase in cell death. When considering the relative intensities of the cleaved caspase-3 bands in the Western blot, 3 Gy alone was 0.33, RAD001 alone was 0.19, and 3 Gy/RAD001 combination was 0.7, showing that the combination treatment induced more than an additive amount of apoptosis (Fig. 4B). However, this does not account for the total observed increase in cell death, suggesting that RAD001 functions via both apoptosis-dependent and apoptosis-independent pathways. Additionally, cell cycle regulation is important in mediating radiosensitivity. It is known that cell cycle arrested cells can be radioresistant (32), and cell cycle distribution has been previously examined in glioma cells to assess radiosensitivity after treatment with rapamycin (22). It is also established that cells have varying radiosensitivity in different cell cycle phases. That is, cells are most sensitive to radiation during the $G_2$-M phase, less sensitive during $G_1$, and least sensitive near the end of S phase (33). We found a time-dependent effect on the cell cycle after treatment of MDA-MB-231 cells with radiation and/or RAD001. At 8 hours, there was a minimal change in cell cycle distribution compared with control for treatment with RAD001, 5 Gy, or combination. However, at 24 hours, there was a radiation-induced $G_2$-M arrest (33.5% $G_2$-M compared with 15.0% $G_2$-M in control), but no $G_2$-M arrest was seen with RAD001 alone.

Furthermore, a much larger increase in $G_2$-M arrest was seen after combination treatment (69.6% $G_2$-M; Fig. 5). Therefore, cell cycle arrest may be a contributing factor in the increased radiosensitivity seen in this study.

It is important to consider the differences between the two cell lines used in this study. Again, the MDA-MB-231 cells showed more radiation-induced mTOR signaling than MCF-7 cells and increased radiosensitivity after treatment with RAD001. There are several possible explanations for these differences. Our finding that mTOR signaling is less activated by radiation in MCF-7 cells implies that there is a smaller target for mTOR inhibition by RAD001. Because MDA-MB-231 cells have much more mTOR signaling activation after irradiation, there is a greater potential for mTOR inhibition. This suggests a possible mechanism for the observed difference in radiosensitization. It is also noteworthy that MCF-7 cells are deficient in caspase-3, an important effector in cellular apoptosis (34, 35). We also found that there were insignificant levels of caspase-3 present in MCF-7 cells (data not shown). Apoptosis resistance in MCF-7 cells treated with radiation has been shown to be caused by lack of caspase-3, and reexpression of caspase-3 in these cells caused an increase in apoptotic changes (36). Consistent with Zapata et al. (37), we found that MDA-MB-231 cells express high levels of caspase-3, and we also found that cleaved caspase-3 was increased after treatment with radiation and RAD001 (Fig. 4). This shows that the radiosensitization observed in MDA-MB-231 cells is at least partially attributable to caspase-dependent apoptotic cell death. Therefore, in the present study, the inability of MCF-7 cells to undergo caspase-dependent apoptosis may have been a contributing factor in their decreased radiosensitivity.

Our data provide additional evidence to the growing amount of research that indicates the effectiveness of mTOR inhibition in treating cancer. Specifically, we suggest that mTOR inhibition with rapamycin derivatives provides a means of radiosensitizing breast cancer cells. Of note, mTOR inhibitors are likely to be more effective at targeting tumor cells than normal cells. This is because transformed cells show increased activation of the Akt/mTOR pathway (10) and therefore present a better target with larger potential for the effects of mTOR inhibition. There are additional possible mechanisms of rapamycin derivatives

against tumorigenesis and tumor growth that were not investigated in the present study but propose interesting directions for future studies. For example, phosphate and tensin homologue on chromosome 10 (PTEN) normally opposes activation of the PI3K/Akt pathway (38). Although both cell lines used in this study are wild type for PTEN (39), mutations in at least one copy of the *PTEN* gene are found in ~50% of patients with breast cancer (40). Decreased PTEN expression in breast cancer cells has been shown to increase sensitivity to inhibition of the PI3K/Akt pathway (39, 41), emphasizing the expanded role that mTOR inhibitors can play in treating the large percentage of breast cancers with mutated PTEN.

It is possible that mTOR inhibition with rapamycin analogues would show a greater increase in radiosensitivity in breast cancer cells *in vivo*. Glioma models have previously shown no increased radiosensitivity in response to rapamycin derivatives *in vitro* (22, 23) but have shown significantly increased radiosensitivity *in vivo* (22). This suggests that rapamycins have an additional effect that is dependent on its action within whole tumors, as opposed to a tumor cell monolayer. Additionally, it has been shown that rapamycin inhibits angiogenesis (42), and tumor vasculature is significantly sensitized to radiotherapy by rapamycin and RAD001 both *in vitro* and *in vivo* (23). This suggests that combining mTOR inhibition with radiation may result in a dual mechanism of tumor inhibition, promoting both tumor cell cytotoxicity and inhibiting tumor angiogenesis. Therefore, further studies are indicated to examine the effects of mTOR inhibition combined with radiation in breast tumor models *in vivo*.

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