Published OnlineFirst September 22, 2009; DOI: 10.1158/1078-0432.CCR-09-0589
Published Online First on September 22, 2009 as 10.1158/1078-0432.CCR-09-0589

Combined Bcl-2/Mammalian Target of Rapamycin Inhibition Leads to Enhanced Radiosensitization via Induction of Apoptosis and Autophagy in Non–Small Cell Lung Tumor Xenograft Model

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Abstract

Purpose: Radiotherapy has a central role in the treatment of non–small cell lung cancer. Effectiveness of this modality, however, is often limited as resistance results from defects in cell death.

Experimental Design: We investigated whether simultaneous up-regulation of apoptosis, via Bcl-2 inhibitor ABT-737, and autophagy, via mammalian target of rapamycin inhibitor rapamycin, can be used to enhance radiosensitivity of H460 cells *in vitro* and growth delay in a xenograft model.

Results: *In vitro* studies confirmed that ABT-737 and rapamycin induce apoptosis and autophagy, respectively. ABT-737 induced cleaved caspase-3, a marker of apoptosis, and rapamycin correlated with an increase in punctate localization of green fluorescent protein-LC3, characteristic of autophagy. The combination ABT-737/rapamycin markedly enhanced sensitivity of H460 cells to radiation (dose enhancement ratio = 2.47; P = 0.002) in clonogenic assay. In addition, the combination ABT-737/rapamycin/radiation showed a dramatic tumor growth delay in a mouse xenograft model. *In vivo* immunohistochemistry staining showed that combination therapy yielded over a 100% increase in caspase-3 activity (apoptosis) and a 6-fold decrease in p62 protein level (indicative of autophagic flux) compared with radiation alone control group. Moreover, cell proliferation (Ki-67 staining) was reduced by 77% (P = 0.001) and vascular density (von Willebrand factor staining) by 67.5% (P = 0.09) compared with radiation alone. Additional *in vitro* studies in human umbilical vein endothelial cells indicated that combined therapy also significantly decreases tubule formation.

Conclusion: These results suggest that concurrent induction of apoptosis and autophagy enhances radiation therapy both *in vitro* and in lung cancer xenograft models. Further investigations are warranted to assess the clinical potential of such strategy in lung cancer patients. (Clin Cancer Res 2009;15(19):OF1–10)

In 2008, lung cancer remained the leading cause of cancer-related mortality in the United States, with an estimated 215,000 individuals diagnosed with lung cancer and a mortality exceeding 161,000 (1). Non–small cell lung cancer (NSCLC) accounts for 75% of these cases, and despite advances made in radiotherapy and chemotherapy, the median overall survival is only 15 months, indicating a need for new strategies to improve outcome.

In recent years, apoptosis has become an attractive target for cancer therapy. Apoptosis is a genetically programmed cell death pathway regulated by the complex interaction between two groups of Bcl-2 family proteins: antiapoptotic proteins such as Bcl-2 itself, as well as Bcl-xL, Bcl-w, and Mcl-1, and proapoptotic proteins Bax, Bak, Bad, and Bim (2). Defects in the apoptotic pathway correlate with cellular resistance to therapy and are frequently observed in NSCLC (3–5). Recently, ABT-737, a small-molecule BH3 domain mimetic that functions as a Bcl-2 inhibitor, has been shown to bind with high affinity (Ki-67 ≤ 1 nmol/L) to Bcl-2 and Bcl-xL, freeing Bax or Bak to trigger permeabilization of mitochondrial membrane and caspase-3 activation and subsequently cell death (6). Furthermore, it has been shown that ABT-737 potentiates anticancer treatments in lymphoma cell lines and small cell lung cancer xenograft models (7, 8).

Autophagy is a complex cellular process with a dual role. Under conditions of limited stress such as starvation, it promotes cell survival, degrading and recycling long-lived proteins and cellular components (9). However, when the cell is exposed to prolonged or excessive conditions of stress, autophagy has been shown to result in death by self-digestion. The mammalian target of rapamycin (mTOR), which regulates cell growth, proliferation, angiogenesis, and metabolism, is a major

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Received 3/10/09; revised 5/4/09; accepted 5/25/09; published OnlineFirst 9/22/09.

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Translational Relevance

Treatment of non–small cell lung cancer is characterized by intensive chemoradiation regimens, which, in the instance of locally advanced disease, succeed only to prolong patient survival by a few months. Treatment failure is partially attributed to the relative resistance of non–small cell lung cancer to apoptosis. We have shown previously that an alternate cell death type, autophagy, has the potential to enhance radiation. In this study, we simultaneously induce apoptosis, using ABT-737, and autophagy, via rapamycin to maximize radiation-induced cell death. As a result, the tri-modality treatment leads to radiosensitization of H460 lung cancer cells and extended tumor growth delay in an *in vivo* mouse xenograft model. Immunohistochemistry analyses (active caspase-3 and p62 stainings) confirm an increase in both processes and decreased vascular density (von Willebrand factor staining). We conclude that the simultaneous induction of apoptosis and autophagy in combination with radiation warrants further investigation and may one day offer a viable option for treatment of resistant tumors.

negative regulator of autophagy. In addition, the mTOR pathway has been shown to be constitutively activated in a variety of solid tumors (10), including an estimated 74% of resected NSCLC malignancies (11). Based on the frequent dysregulation of the mTOR signaling in cancer, inhibition has been proposed for cancer therapy (10). We and others have reported previously the inhibition of tumor proliferation by rapamycin and its analogues in several cancer xenograft models, including brain, breast, and NSCLC (12–14). Rapamycin is a natural macrolide antibiotic that cross-links with immunophilin FKBP-12, resulting in a complex that inhibits mTOR signaling and results in translation of RNA, cell cycle progression, and, importantly, induction of autophagy (15).

Although both ABT-737 and rapamycin have suggested promise as cancer treatment options, neither drug has proven completely successful. Certain cell lines, including NSCLC and small cell lung cancer expressing high levels of Mcl-1 or low levels of Bcl-2, remain resistant to apoptosis even following treatment with ABT-737 (16–18). Similarly, rapamycin may not be able to sensitize all cell lines to radiotherapy (13). In this study, we tested the triple-combination ABT-737/rapamycin and radiation to circumvent the defects of a single cell death pathway by simultaneously up-regulating both apoptosis and autophagy in lung cancer. Data on the efficacy of ABT-737 and rapamycin in combination with radiation in NSCLC cells and xenograft have direct implications for the clinical evaluation of Bcl-2 inhibitors in combination with mTOR inhibitors in patients with NSCLC.

Materials and Methods

Cell culture and chemical. H460 lung cancer cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C and humidified 5% CO_2. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (BioWhittaker). ABT-737 was provided by Abbott Laboratories (Abbott Park) and rapamycin was purchased from Novartis Pharmaceutical (East Hanover).

Clonogenic assay. H460 cells were treated with DMSO, ABT-737 (500 nmol/L, 2 h), rapamycin (100 nmol/L, 2 h), or combined ABT-737 with rapamycin. Cells were irradiated with 0 to 6 Gy as described previously (14).

Immunoblotting. Cells (0.3×10^6) were treated with different combinations of radiation dose (0, 5, or 20 Gy) and drug as described above. They were collected and washed with ice-cold PBS twice before the addition of lysis buffer. Equal amounts of protein were loaded into each well and the blots were incubated overnight with caspase-3 (Cell Signaling), LC3 (Medical & Biological Laboratories), and actin antibodies for 1 h at 4°C. Membranes were then incubated with goat anti-rabbit IgG secondary (1:5,000; Santa Cruz Biotechnology) for 45 min at room temperature. Immunoblots were developed using the chemiluminescence detection system (Perkin-Elmer) according to the manufacturer's protocol and autoradiography.

Apoptosis assay. H460 cells (5×10^5) were plated into 10 mm dishes for each data point. After overnight incubation at 37°C, cells were treated with ABT-737 (500 nmol/L, 2 h) and irradiated with 5 or 20 Gy. After 24 h, cells were treated with 1 mL Accutase (Innovative Cell Technologies) for 4 min (keeping all floating cells) and counted for each sample. Cells were centrifuged and resuspended in 1× Binding Buffer at a concentration of 1×10^6/mL and 100 μL of the solution (5×10^5 cells) were transferred in a 5 mL FACS tube and combined with 1 μL Annexin V/FITC and 1 μL propidium iodide. After incubation for 30 min at room temperature in the dark, 300 μL of 1× Binding Buffer were added to each tube. The rate of apoptosis was measured using the Annexin V/FITC apoptosis detection kit I (Pharmingen) with flow cytometry.

Trypan blue assay. H460 cells (3×10^4) were plated into 24 wells for each data point and incubated and radiated as described above. After 24 h, 5 μL trypan blue solution (Invitrogen) and 4 μL PBS (Invitrogen) were combined and added to 10 μL resuspended cells. Following gentle mixing and incubation for 2 min at room temperature, the total number of cells and the number of stained cells were calculated using a hemocytometer under a microscope to determine the percentage of dead (stained) cells.

Autophagy assay. H460 cells (2×10^5) were seeded in tissue culture in a 6-well plate overnight and then were transfected with 2 μg green fluorescent protein (GFP)-LC3 expression plasmid (a gift from Dr. Norboru Mizushima, Tokyo Medical and Dental University) using Lipofectamine reagent (Invitrogen/Life Technologies). After 12 h, cells were treated with DMSO, ABT-737, rapamycin, or both and received 5 Gy radiation as described above. Cells were then incubated for 48 h at 37°C, after which GFP-LC3 fluorescence was observed under a confocal fluorescence microscope. Characteristic punctate GFP-LC3 signaling was considered a cell undergoing autophagy. The percent of punctate GFP cells per total GFP-transfected cells was calculated and experiments were conducted in triplicate.

Tumor volume assessment. H460 cells were used in a xenograft model in female athymic nude mice (*nu/nu*, 5-6 weeks old; Harlan-Sprague-Dawley). A suspension of 1×10^6 cells in 100 μL volume was injected subcutaneously into the right flank of mice using a 1 cc syringe with 27.5-gauge needle. Tumors were grown for 6 to 8 days until average tumor volume reached 0.23 cm^3. Treatment groups consisted of DMSO, ABT-737, rapamycin, combined ABT-737 with rapamycin, DMSO plus radiation, ABT-737 plus radiation, rapamycin plus radiation, and combined ABT-737 with rapamycin plus radiation. Each treatment group contained 5 mice. DMSO and ABT-737 were administered at doses of 20 mg/kg intraperitoneally and rapamycin at 2 mg/kg orally for 7 consecutive days. Mice in radiation groups were irradiated 1 h after ABT-737 and rapamycin treatment with 2 Gy daily over 5 consecutive days. Tumors on the flanks of the mice were irradiated using a X-ray irradiator (Therapax, Agfa NDT). The nontumor parts of the mice were

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shielded by lead blocks. Tumors were measured two or three times weekly in three perpendicular dimensions using a Vernier caliper. Tumor volumes were calculated using the modified ellipse volume formula [volume = (height × width × depth) / 2]. Growth delay was calculated as the number of days required to reach a tumor volume of 1.75 cm^3 for treatment groups relative to the control.

Histologic sections, von Willebrand factor, Ki-67, active caspase-3, and p62 staining. Mice were implanted with H460 lung cancer cells and treated as described above in the tumor volume studies. After 7 days of daily treatments, tumors from each mouse were resected and paraffin-fixed. Slides from each treatment group were then stained for von Willebrand factor (vWF) using anti-vWF polyclonal antibody (Chemicon). Blood vessels were quantified by randomly selecting ×400 fields and counting the number of blood vessels per field. This was done in triplicate and the average of the three counts was calculated. Ki-67, active caspase-3, and p62 staining were done in the Vanderbilt University Pathology Core Laboratory using standard protocols. The number of positive cells per ×400 fields were scored and graphed by averaging three repeated assessments.

Endothelial cell morphogenesis assay: tubule formation. HUVECs were used to examine tubule formation. HUVECs grown to ~70% confluency were treated with DMSO, ABT-737 (500 nmol/L, 2 h), rapamycin (100 nmol/L, 2 h), or combined ABT-737 with rapamycin with or without 5 Gy radiation. Cells were then trypsinized, counted, and seeded at 48,000 per well on 24-well plates coated with 300 μL Matrigel (BD Biosciences). These cells were periodically observed by microscope as they differentiated into capillary-like tubule structures. One day later, cells were stained with H&E and photographs were taken via microscope. The average number of tubules was calculated from examination of three separate microscopic fields (×100) and representative photographs were taken.

Statistical analysis. Analysis of study results focused on testing the differences of the mean tumor volume among treatment groups and different time points. The data analysis was completed using the restricted/residual maximum likelihood–based mixed-effect model to adjust the intracorrelation effect for the mice that had multiple measurements. The model reported in the article was selected based on the Schwarz's Bayesian criterion. All tests of significance were two-sided, and differences were considered statistically significant when $P < 0.05$. A statistical package was used for all analyses.

Results

ABT-737 increases radiation-induced apoptosis. To determine whether ABT-737 enhances radiation-induced apoptosis in

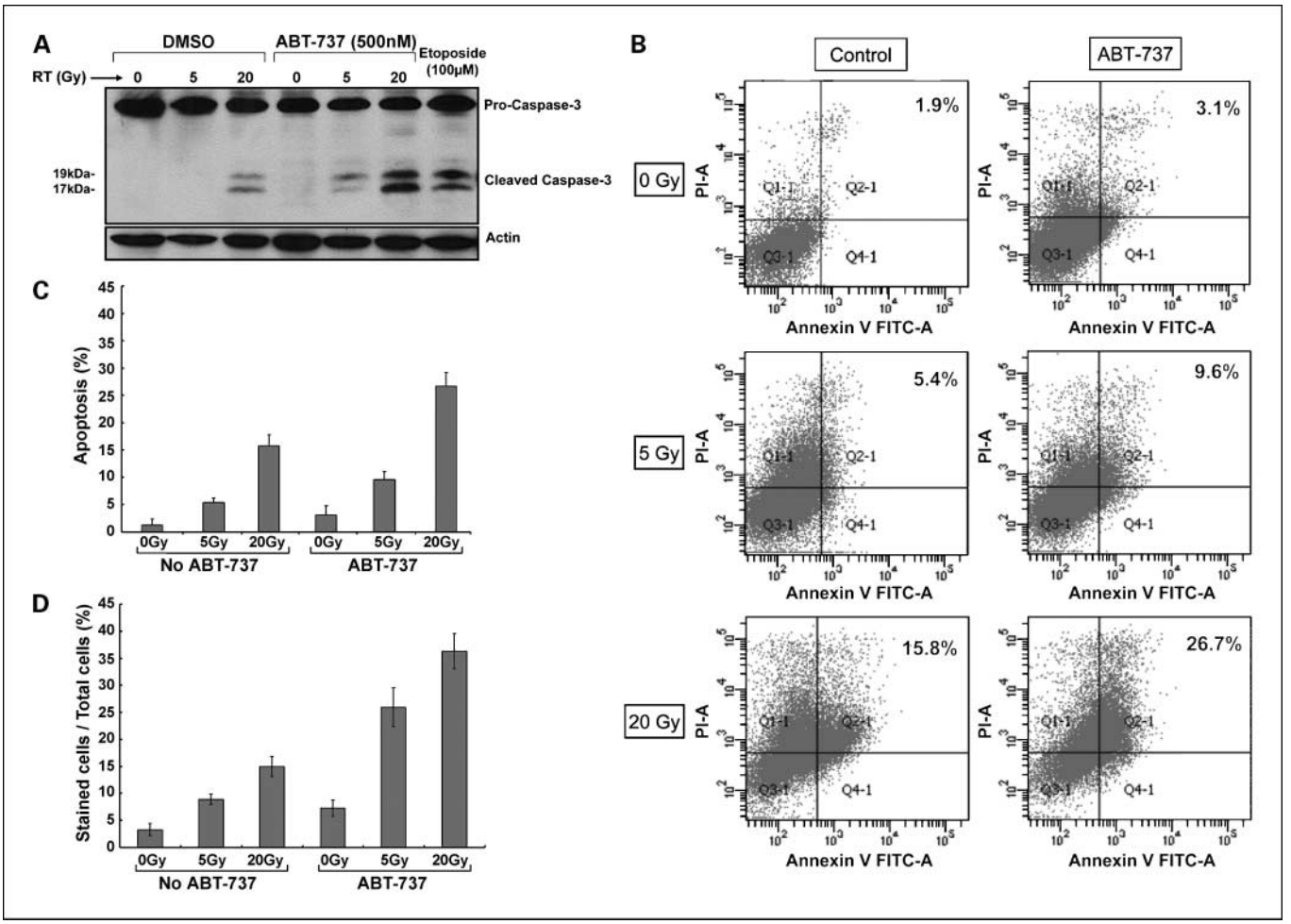

Fig. 1. ABT-737 increases radiation-induced apoptosis in H460 cells. *A,* H460 cells were pretreated with 100 nmol/L ABT-737 for 2 h followed by radiation (0, 5, or 20 Gy). Western blot analysis for active caspase-3 was done after 24 h. Actin immunoblot was used for normalization. *B,* H460 cells were treated with ABT-737 (500 nmol/L, 2 h) and immediately irradiated with 5 or 20 Gy. After 24 h, cells were stained with Annexin V and propidium iodide and analyzed by flow cytometry. This experiment was done in triplicate and representative diagrams of Annexin V assays are shown. *C,* quantitative measurement of Annexin V flow cytometry analyses showing positive apoptotic cells in response to radiation/ABT-737. *Columns,* mean; *bars,* SD. *D,* H460 cells were treated with ABT-737 (500 nmol/L, 2 h) and immediately irradiated with 5 or 20 Gy. Twenty-four hours later, they were stained with trypan blue. The total number of cells and the number of stained cells were determined using a hemocytometer under a microscope. This was repeated thrice. *Columns,* mean; *bars,* SD.

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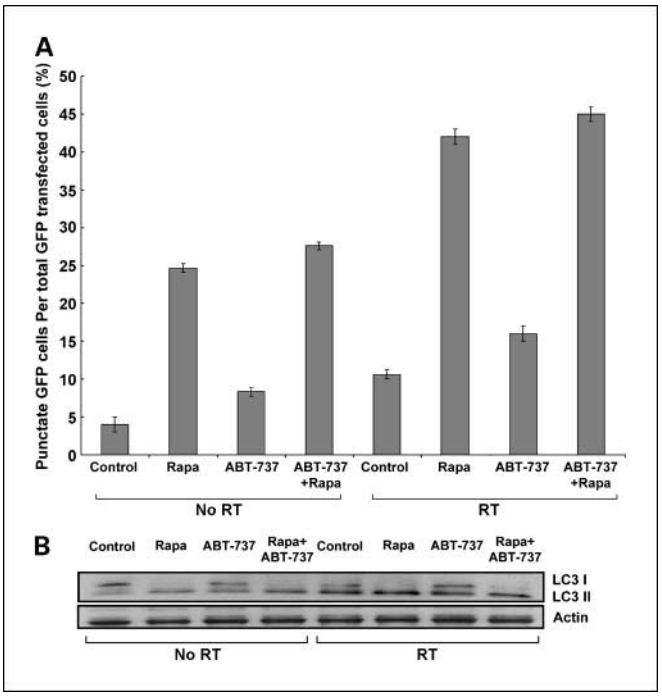

Fig. 2. Induction of autophagy by treatment with rapamycin in H460 cells. *A,* GFP-LC3 transfected cells were treated with 500 nmol/L ABT-737, 100 nmol/L rapamycin, or both for 2 h with or without radiation (5 Gy). Cells were then examined by fluorescence microscopy after 48 h. The percentage of cells with characteristic punctate GFP-LC3 fluorescence was calculated relative to all GFP-positive cells. This was repeated thrice. *Columns,* mean; *bars,* SD. *B,* LC-I and LC-II expression was determined by Western blot using lysates from H460 cells treated with 500 nmol/L ABT-737, 100 nmol/L rapamycin, or both for 2 h with or without radiation (5 Gy). Actin was probed to show equal loading.

H460 cells, cleavage of caspase-3 was examined by Western blotting (Fig. 1A). H460 cells were treated with DMSO or 500 nmol/L ABT-737 for 2 h before receiving radiation (0, 5, or 20 Gy). In addition, etoposide was used as a positive control. As shown in Fig. 1A, cleaved caspase-3 was only detected at 20 Gy, indicating radioresistance in this lung cancer cell line. In H460 cells treated with ABT-737, cleaved caspase-3 is detected at 5 Gy, with significant increase at 20 Gy. These data suggest that ABT-737 promotes apoptosis in H460 radioresistant cells. Annexin V flow cytometric analysis was used to confirm the above findings. As shown in Fig. 1B and C, 5.4% and 15.8% of Annexin V–positive cells were detected in H460 cells that received 5 and 20 Gy, respectively. Treatment with ABT-737 resulted in a further increase in apoptosis (9.6% at 5 Gy and 26.7% at 20 Gy) compared with radiation alone. Finally, trypan blue staining showed that ABT-737 increased cell death amount from 8.9% to 25.9% at 5 Gy and from 15% to 36% at 20 Gy compared with radiation alone ($P < 0.001$; Fig. 1D). ABT-737 alone yielded 7.3% positive dead cells compared with the control group. These results suggest a synergistic effect of ABT-737 in combination with ionizing radiation in H460 cells.

Rapamycin increases radiation-induced autophagy. Published studies have indicated that Bcl-2 inhibition may up-regulate autophagy (19). To determine whether combination of Bcl-2 and mTOR inhibitor enhance radiation-induced autophagy, H460 cells were transfected with 2 μg GFP-LC3 plasmid and treated with control, ABT-737 (500 nmol/L, 2 h), rapamycin (100 nmol/L, 2 h), or both followed by 5 Gy radiation. As shown in Fig. 2A, <10% of cells receiving radiation alone were found to be undergoing autophagy ($P = 0.017$) as opposed to 42% of irradiated cells treated with rapamycin ($P = 0.0002$). ABT-737 increased autophagy from 4% to 8.3% ($P = 0.02$) without radiation and from 10.7% to 16% ($P = 0.02$) with radiation. When added to rapamycin treatment, however, ABT-737 induced only autophagy from 24.6% to 27.6% ($P = 0.003$) without radiation and from 42% to 45% ($P = 0.0004$) with radiation. These results were also confirmed by assessing the level of processed LC3 protein, in which the cells treated with rapamycin showed increased levels of LC3-II proteins following irradiation in comparison with WT cells (Fig. 2B).

Combination treatment of ABT-737 and rapamycin increases radiosensitivity in H460 cells. To determine if radiosensitization is maximized by simultaneous inhibition of Bcl-2 and mTOR pathways, we performed clonogenic assays in H460 cells treated with DMSO, ABT-737 (500 nmol/L, 2 h), rapamycin (100 nmol/L, 2 h), or both and radiation (0-6 Gy). For all treatment groups, we calculated dose enhancement ratios, the ratio of radiation doses required to give an equivalent antitumor effect with radiosensitizer or without. Thus, in our study, a higher dose enhancement ratio will imply that lower doses of radiation are required to achieve a similar cytotoxicity when radiation is combined to ABT-737 and/or rapamycin compared with radiation alone. As shown in Fig. 3, the dose enhancement ratio in H460 cancer cells treated with rapamycin or ABT-737 compared with control was 1.65 ($P = 0.006$) and 2.16 ($P = 0.009$), respectively. The combination treatment group of both rapamycin and ABT-737 yielded a dose enhancement ratio of 2.47 ($P = 0002$) compared with the DMSO group. These results

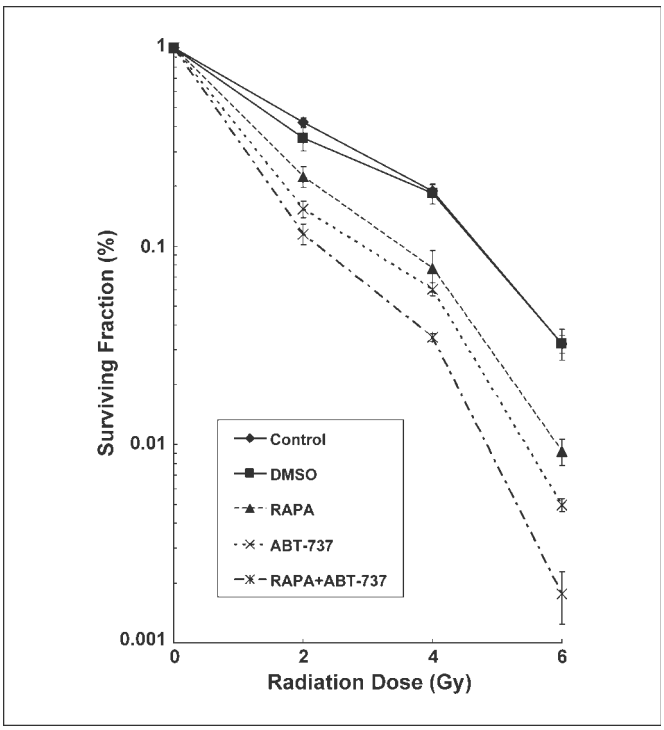

Fig. 3. Combined ABT-737 and rapamycin treatment sensitizes H460 cells to radiation. Clonogenic assay revealing radiosensitization of H460 cells treated with ABT-737 (500 nmol/L, 2 h), rapamycin (100 nmol/L, 2 h), or combination of both and were irradiated with the indicated doses of radiation. After 8 d, surviving colonies were stained and the scored colonies were graphed. Mean ± SD of three separate repeated experiments.

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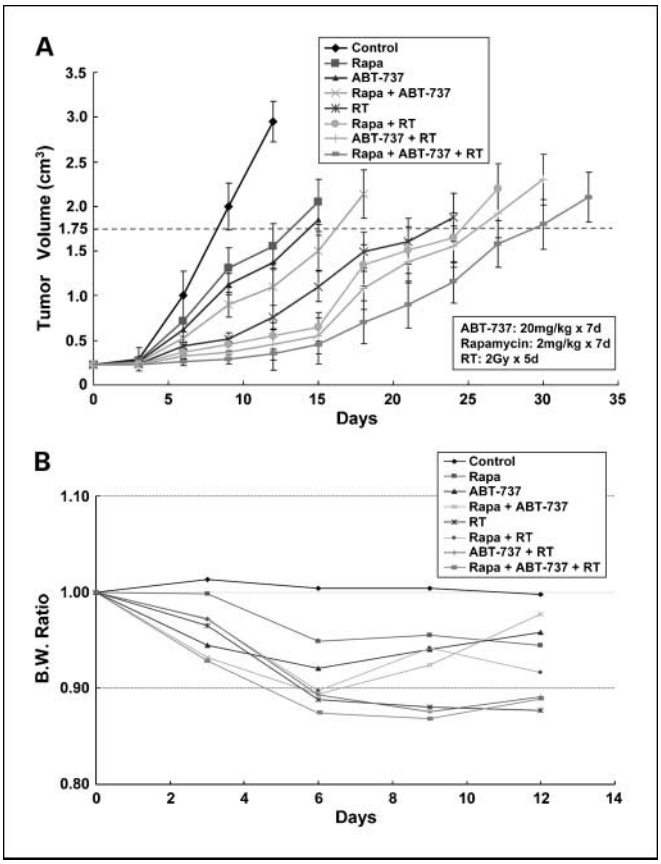

Fig. 4. Extended tumor growth delay with combined ABT-737/rapamycin and radiotherapy at well-tolerated doses in xenografted H460 model. H460 lung cancer cells were injected subcutaneously in athymic female mice and grown for 8 to 10 d until tumor volume averaged 0.23 cm^3. Mice were treated daily for 7 d with vehicle control (DMSO), ABT-737 (20 mg/kg/d), rapamycin (2 mg/kg/d), or both in combination and then were treated 1 h after drug treatment with 2 Gy radiation daily over 5 consecutive days (detailed in Materials and Methods). *A,* tumors were measured regularly and volume was plotted versus time. Growth delay was calculated as the number of days required to reach a tumor volume of 1.75 cm^3 for treatment groups relative to control tumors. *B,* body weights were measured every 5 d and body weight ratio was calculated relative to baseline measurement.

suggest that either mTOR inhibition by rapamycin or Bcl-2 inhibition by ABT-737 increases radiation sensitivity and that dual inhibition of these pathways maximizes radiosensitivity in H460 lung cancer cells.

Combination treatment of ABT-737, rapamycin, and radiation results in extended tumor growth delay in lung xenograft model. Having established the *in vitro* effects of combined Bcl-2 and mTOR inhibition on lung cancer radiosensitivity, mouse heterotopic xenograft models were used to confirm the biological effects of ABT-737, rapamycin, and radiation *in vivo*. The treatment groups consisted of DMSO (20 mg/kg intraperitoneally), ABT-737 (20 mg/kg intraperitoneally), rapamycin (2 mg/kg orally), or combination of ABT-737 and rapamycin consecutively for 7 days with or without 10 Gy radiation (2 Gy/d for 5 days). Growth delay was calculated as the number of days required to reach a tumor volume of 1.75 cm^3 for treatment groups relative to control tumors. As shown in Fig. 4A, a significant tumor growth delay was seen with combination therapy of ABT-737, rapamycin, and radiation compared with irradiation alone (22 versus 15 days; $P = 0.002$), whereas ABT-737 or rapamycin alone did not significantly affect the tumor growth compared with control. Similarly, combination therapy of ABT-737/radiation and rapamycin/radiation resulted in a significant tumor growth delay, 3 days ($P = 0.01$) and 2 days ($P = 0.001$), respectively, compared with irradiation alone. In addition, mouse body weights monitoring suggested that all treatments were relatively well tolerated (Fig. 4B). Taken together, these results suggest that the combination therapy of ABT-737 and rapamycin increase lung cancer response to radiotherapy *in vivo*.

Combination treatment of ABT-737, rapamycin, and radiation reduces tumor proliferation index and induces both apoptosis and autophagy in irradiated H460 xenografts. To further characterize the effects of ABT-737 and rapamycin shown in the tumor growth delay model, we examined fixed H460 tumor sections in all treatment groups for proliferation (Ki-67 staining; Fig. 5C), apoptosis (active caspase-3 staining; Fig. 5A), and autophagy (P62 staining; Fig. 5B). The treatment groups were identical to those used for the tumor growth delay study. As shown in Fig. 5C, Ki-67 staining revealed a significant decrease in cell proliferation in the radiation combined to ABT-737 (22.3 ± 2 versus 39 ± 1; $P = 0.01$) or rapamycin (28 ± 2 versus 39 ± 1; $P = 0.002$) groups compared with radiation alone, respectively. The greatest reduction in Ki-67 proliferation index results from the combination of ABT-737, rapamycin, and radiation (10 ± 1 versus 39 ± 1; $P = 0.001$) compared with radiation alone. Apoptosis levels in fixed H460 tumor sections were assessed using active caspase-3 staining. As shown in Fig. 5A, radiation plus ABT-737 increased apoptotic cells compared with radiation alone (16.3 ± 1 versus 8 ± 1; $P = 0.001$), whereas the addition of rapamycin to radiation had no increase in apoptosis compared with radiation alone. When rapamycin was combined with radiation and ABT-737, there was only a minor increase in apoptosis compared with radiation plus ABT-737 alone. These results suggest that ABT-737 increases radiation-induced apoptosis and further reduces tumor cell proliferation after irradiation.

To further explore the mechanisms of cell death resulting from the tri-combination treatment *in vivo*, we also examined fixed H460 tumor sections in all treatment groups for autophagy (P62 staining). P62 interacts and binds to LC3 and is removed in lysosomes by autophagy, which controls its turnover. Representative histologic photographs of P62 staining on lung tumor sections are shown in Fig. 5B. As shown in Fig. 5B, rapamycin combined with radiation reduced P62 protein staining by 6-fold compared with radiation alone (59 ± 1.5 versus 10 ± 1.5; $P < 0.001$), whereas the ABT-737 plus radiation group exhibited an insignificant increase in autophagic level. There was no significant change in p62 staining with the addition of ABT-737 to rapamycin with radiation treatment, suggesting that mTOR inhibition is primarily responsible for autophagic cell death *in vivo*.

Combination treatment of ABT-737, rapamycin, and radiation reduces vascular density in irradiated H460 xenografts and sensitizes HUVECs to radiation. To determine the effects of Bcl-2/mTOR inhibition on tumor vasculature, vascular density study was done using vWF staining in each lung cancer xenograft treatment groups. The number of vessels per microscopic field was then quantified for each treatment group. As shown in Fig. 6A, combination therapy with ABT-737 and rapamycin with radiation resulted in a 3-fold reduction relative to radiation therapy alone (4 ± 1.5 versus 12.3 ± 0.5; $P = 0.01$). To further

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investigate the effects of Bcl-2/mTOR inhibition on blood vessel formation, an endothelial cell morphogenesis assay was done to examine the ability of treated HUVECs to produce capillary-like tubular structures. A representative image and the mean number of tubules from three separate (×400) fields are shown in Fig. 6C and D. Treatment with rapamycin (2.6 ± 0.6 versus 5.3 ± 0.6; $P = 0.05$) or ABT-737 (3 ± 1 versus 5.3 ± 0.6; $P = 0.07$) combined with radiation reduced tubule formation compared with radiation alone, respectively. The greatest reduction in tubule formation was seen following treatment with combination of rapamycin, ABT-737, and radiation ($P = 0.001$). These results suggest that both ABT-737 and rapamycin have antiangiogenic effects in addition to their radiosensitization effect.

Discussion

In the present report, we showed the effects of ABT-737, a Bcl-2 inhibitor, and rapamycin, a mTOR inhibitor, which resulted in the effective radiosensitization of lung cancer cells *in vitro* and

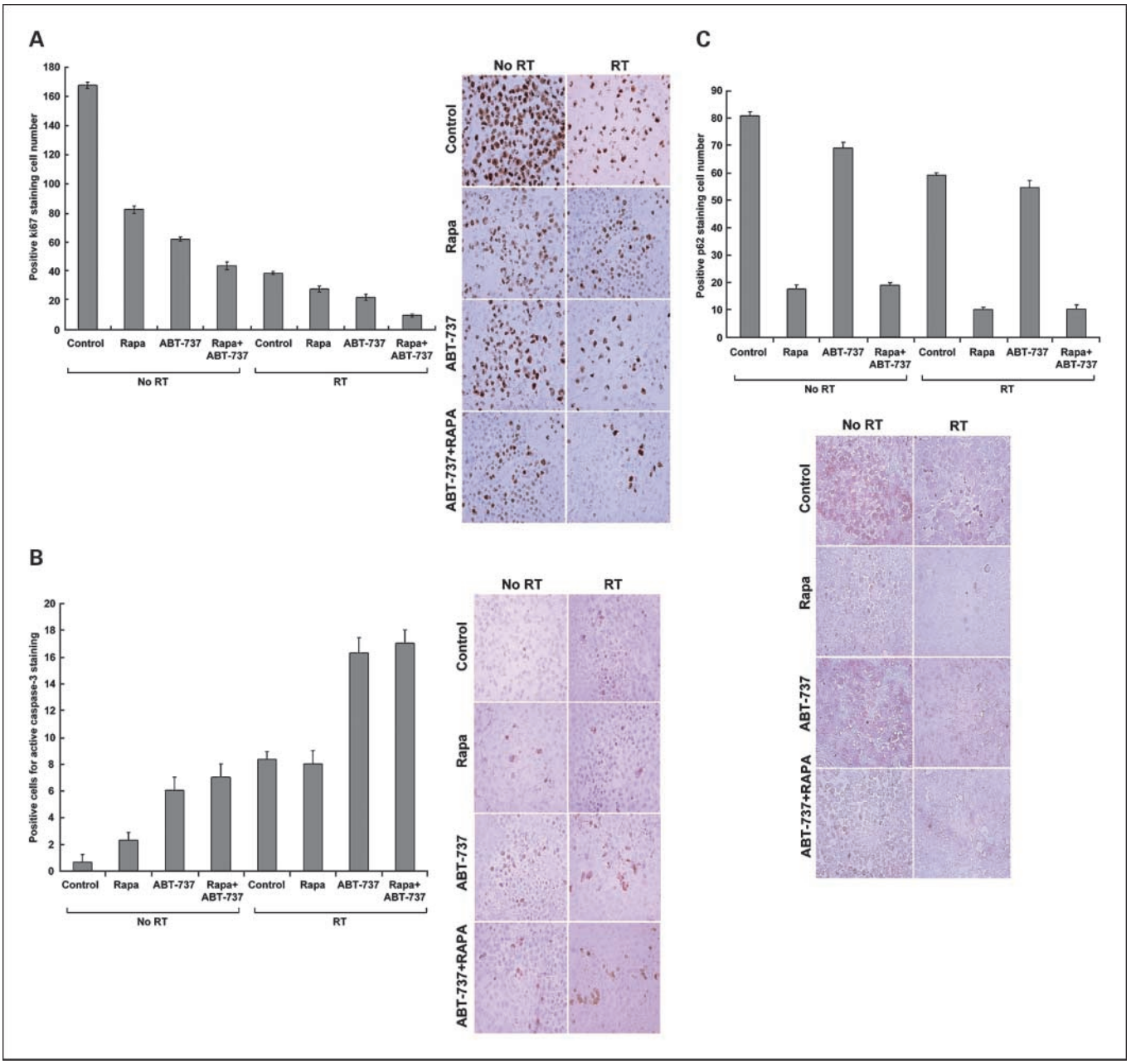

Fig. 5. Combined ABT-737 and rapamycin therapy reduces cell proliferation and increases apoptosis and autophagy *in vivo*, respectively. Histologic sections were obtained from the tumors of the mice in each treatment group from the tumor volume study. The number of positive cells per ×400 field was scored and graphed by averaging three repeated experiments. Representative histologic photographs and quantitative graphs are shown for each type of staining. *A,* average Ki-67 proliferative index of each treatment group was determined by the percent of Ki-67–positive cells per microscopic field. This was repeated thrice. *Columns,* mean; *bars,* SD. *B,* active caspase-3 staining was done on lung tumor sections, and apoptotic index was calculated by counting positive active caspase-3–stained cells per microscopic field. This was repeated thrice. *Column,* mean; *bars,* SD. *C,* P62 antibody staining was also done, and autophagy index was similarly calculated by counting positive P62-stained cells per microscopic field. This was repeated thrice. *Columns,* mean; *bars,* SD.

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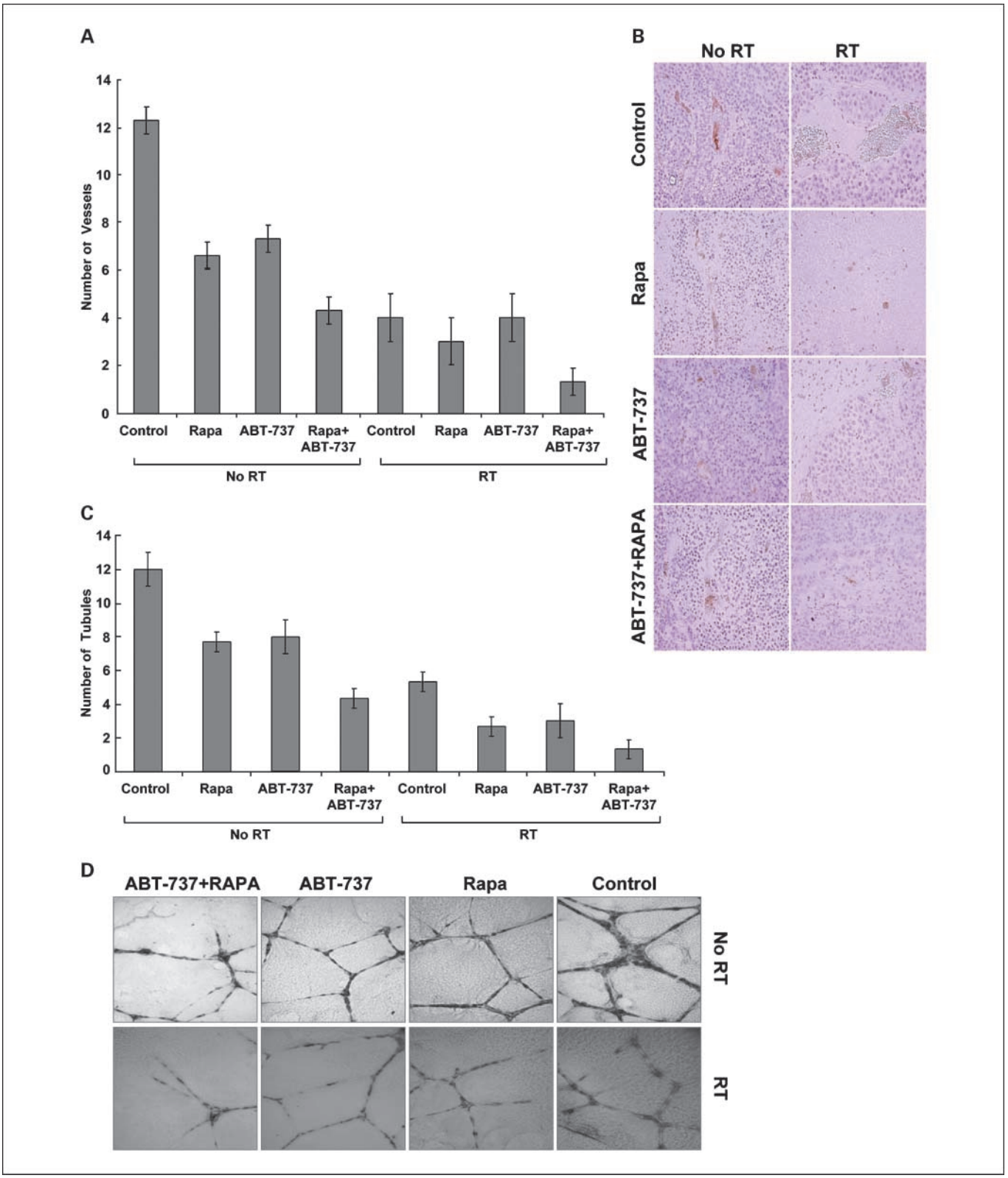

Fig. 6. ABT-737 and rapamycin reduce vascular density in irradiated H460 tumors *in vivo* and sensitize vascular endothelial cells to radiation *in vitro*. *A,* histologic sections were obtained from the tumors of the mice in each treatment group from the *in vivo* tumor volume study and stained for blood vessels using an antibody for vWF. Blood vessel density of each treatment group was determined by counting the number of blood vessels per ×400 microscopic field. This was done in triplicate and the average of the three counts was calculated. *Columns,* average; *bars,* SD. *B,* representative histologic photographs following vWF staining. *C,* HUVECs were treated with DMSO, ABT-737 (500 μmol/L, 2 h), rapamycin (100 nmol/L, 2 h), or combination therapy and then irradiated with either 0 or 3 Gy. Six hours later, cells were trypsinized and replated on 24-well plates coated with Matrigel. After 24 h, cells were fixed and stained with H&E. The slides were examined by microscopy (×100), and stained tubules were then counted in three separate, randomly selected fields. *Columns,* mean; *bars,* SD. *D,* representative histologic photographs of H&E-stained HUVECs showing tubule formation.

Published OnlineFirst September 22, 2009; DOI: 10.1158/1078-0432.CCR-09-0589

in a lung cancer xenograft model. This study also suggests that the combination treatment of ABT-737 and rapamycin increases the effects of radiation on vasculature, which may partially explain the extended tumor growth delay. Interestingly, we found that both apoptosis and autophagy can simultaneously be induced and further enhance radiosensitivity of lung cancer.

It has been shown that ABT-737, a BH3 mimetic, binds to antiapoptotic Bcl-2 proteins and disrupts the sequestering and neutralizing of proapoptotic proteins. ABT-737 and its oral analogue, ABT-263, have been shown to promote apoptosis and cause *in vitro* regression of several hematologic malignancies and a variety of solid tumors, including small cell lung cancer (20). The drug has shown efficacy on administration as both a monotherapy and in combination with cytotoxic therapies (20–22). Unfortunately, data were not consistent across all cell lines. In a study of a panel of NSCLC cell lines, however, ABT-737 (0-50 μmol/L, 48 h) showed mixed results in several resistant cell lines, with apoptosis levels remaining at ≤30% (18). Consistently, our study similarly suggested that ABT-737 (500 nmol/L, 2 h) was sufficient to further promote apoptosis in irradiated H460 cells but remained relatively low (Fig. 1). Even the use of higher dose of radiation (20 Gy) failed to result in cell death of >35% of cells (Fig. 1C). Although some of the results are not synergistic, the clonogenic assays, however, clearly showed synergistic results with the tri-therapy compared with any other combinations (Fig. 3). In addition, the trypan blue assay, which detects the total amount of cell death, also showed synergistic effects for the combination ABT-737/rapamycin/radiation over radiation alone (Fig. 1D). We believe that this is probably due to the fact that some cells will undergo other types of cell death, such as necrosis, in addition to apoptosis or autophagy, which is also suggested by Fig. 1B. Together, the results confirmed that, like many NSCLC lines, H460 is relatively radioresistant and ABT-737 alone remains limited for an adequate induction of cell death at reasonable doses of radiation.

As we know, defects in apoptosis are not limited to Bcl-2 or Bcl-xL proteins, as they occur at several cellular levels, which could potentially cause resistance to anticancer agents (23). Therefore, Bcl-2 inhibition by ABT-737 alone may not be effective enough in the induction of apoptosis on its own. Indeed, previous studies have suggested that, to effectively induce apoptosis, multiple points in the apoptotic pathway may need targeting, such as Mcl-1 inhibition or Bak induction (18). One can suggest that successful sensitization may require individually tailored molecular therapies targeting all the potential defects in the apoptotic pathway. As a result, the use of alternate mechanisms of cell death such as autophagy becomes an attractive strategy to overcome defects in apoptosis.

Consistent with previous findings (13, 14, 24, 25), we found that rapamycin alone significantly sensitizes H460 cells to radiation (Fig. 3) and extends tumor growth delay in irradiated lung xenografts (Fig. 4A), suggesting that it is possible to take advantage of autophagy to enhance radiation therapy in lung cancer. However, the enhancement of radiation by rapamycin may be limited by relative resistance to autophagy in cancer cells (13). Among the mechanisms known to restrict autophagy, a relevant example is the association of NSCLC with mutations in LKB1 tumor suppressor, which negatively regulates mTOR signaling and is involved in the stimulation of autophagy (26). In addition, there is suggestion of several resistance mechanisms to mTOR inhibitors that could potentially limit the clinical efficacy of these agents (27). Therefore, there is a rationale for combination therapy with mTOR inhibitors to induce autophagy and Bcl-2 inhibitors to induce apoptosis.

In addition, there is some evidence of crosstalk between those two pathways. Recent studies have shown that Bcl-2 interacts with autophagy, via Beclin-1, a haploinsufficient tumor suppressor that is essential for autophagy (28, 29). It has been shown that Beclin-1 mediates its interactions with Bcl-2 and Bcl-xL through a BH3 domain. This property allows the competitive inhibition of Beclin-1/Bcl-2 interaction by ABT-737, which results in stimulation of autophagy in HeLa cells (29). Thus, Bcl-2 acts as an antiautophagy protein in addition to its antiapoptotic function, suggesting a role for Bcl-2 in maintaining low apoptosis and autophagy levels for cell survival. In our study, the application of ABT-737 also resulted in an increase in autophagy, particularly in combination with radiation (Fig. 2). In contrast, the concurrent use of ABT-737 and rapamycin yielded a lower increase in autophagy only compared with rapamycin alone. Similar findings were also observed after p62 staining *in vivo* (Fig. 5B). These data suggest that ABT-737 does not influence rapamycin-induced autophagy in our lung cancer models, although it may disrupt the interaction between Bcl-2 and Beclin-1 proteins.

Alternatively, mTOR has been correlated to apoptosis, which can be promoted by rapamycin and its analogues dependent on the cell type (30). Although it has been shown that rapamycin-induced apoptosis is minimal on its own, it has potential to enhance the effects of DNA damaging agents, such as cisplatin (31). Interestingly, it has been suggested that expression of Bcl-2 was associated with "resistance" to rapamycin and analogues (32, 33) and that sensitivity to rapamycin was restored by Bcl-2 antisense (32). Another study showed that rapamycin (100 nmol/L) in conjunction with ABT-263 (39 or 156 nmol/L) resulted in increased apoptosis in lymphoma cell lines (34). Consistently, similar studies in neuroblastoma, lymphoid, and hepatocellular carcinoma showed that inhibition of mTOR induces or sensitizes cells to apoptosis (35–38). In this study, we only observed a small effect of rapamycin when administered alone for induction of apoptosis *in vivo*, whereas combination with ABT-737, radiation, or both did not significantly promote apoptosis (Fig. 5A). Differences in findings may be due to the intrinsic nature of the hematologic cell lines as opposed to the solid NSCLC xenograft tumor or to the differences in concentration of the Bcl-2 inhibitor (39 or 156 versus 500 nmol/L). Taken together, our results suggest that a potential clinical benefit from the combination of ABT-737 and rapamycin will probably be secondary to the individual targeting of each pathway rather than a crosstalk between them.

In contrast, we previously illustrated a crosstalk at a different level between inhibition of apoptosis and up-regulation of autophagy (14). More precisely, H460 radiosensitivity was increased when rapamycin was administered in presence of Z-DEVD, a caspase-3 inhibitor (14). Thus, it appears that autophagy may serve as a backup death mechanism when apoptosis is unavailable. In the present study, instead of channeling radiation-induced cell death through autophagy only, we wanted to take advantage of the two death pathways simultaneously to maximize cell death. By doing this, we found that the targeting of both pathways is better than the induction of one pathway alone, but one drug did not significantly induce a synergistic effect on the alternate pathway. This also illustrates

Published OnlineFirst September 22, 2009; DOI: 10.1158/1078-0432.CCR-09-0589

the complex role of autophagy and suggests that more studies are needed to further determine the mechanisms of autophagy.

To investigate autophagy *in vivo*, we stained histologic sections for p62 antibody (Fig. 5B). *In vivo* detection of autophagy has long been a challenge and p62 detection may offer an important solution. p62, or sequestosome 1 (SQSTM1), is a common component of protein aggregates, responsible for linkage of polyubiquitinated proteins to autophagic machinery (39, 40). Both p62- and LC3-positive bodies are degraded in autolysosomes and inhibition of autophagy leads to an increase in p62 protein levels (41). Detection of p62 *in vivo* has been proposed previously as a way to monitor autophagic flux, although, to our knowledge, previous use of this protocol has not been published (42). Currently, there is no other means of detecting autophagy *in vivo* and we believe that obtained data are a good representation of autophagy levels in examined histologic sections. Indeed, *in vivo* results suggested that rapamycin and not ABT-737 resulted in autophagy induction both with and without radiation (Fig. 5B). This is consistent with our *in vitro* experiments, which showed similarly that ABT-737 does not result in a significant increase in autophagosome formation as opposed to rapamycin (Fig. 2) and suggests that the p62 *in vivo* staining may be used in future investigations.

Because tumor neovascularization is a poor prognostic factor in NSCLC patients (43), we also investigated the effects of Bcl-2 and mTOR inhibition on vascular density and angiogenesis. Ionizing radiation is known to exhibit contrasting effects on vascularization, resulting in an increase in proangiogenic factors such as the vascular endothelial growth factor as well as antivascular effects (44–46). We report here that the triple-combination ABT-737/rapamycin/radiation reduced the vessels density (vWF staining) compared with radiation alone ($P = 0.01$; Fig. 6A). We confirmed the vascular effects observed *in vivo* by determining the ability of treated endothelial cells to produce capillary-like tubular structures *in vitro* (Fig. 6B). Although the bi-combination was not previously explored, antiangiogenic effects of Bcl-2 (47) and mTOR (48) inhibitors were individually reported. Additionally, it has been shown that rapamycin exerts antiangiogenic effects possibly by reducing vascular endothelial growth factor production and also by blockage of vascular endothelial growth factor–induced endothelial cell signaling (49). We also recently reported that rapamycin and analogues decreased clonogenic survival of HUVECs and sensitized them to apoptosis (50). Further studies are needed to fully elucidate the complex mechanisms by which ABT-737 and rapamycin mediate their antivascular effects.

In conclusion, this preclinical study supports the therapeutic potential for the combination treatment of ABT-737, a Bcl-2 inhibitor, and rapamycin, a mTOR inhibitor, to sensitize lung cancer to radiotherapy. The rational therapeutic targeting of Bcl-2 and mTOR pathway simultaneously is a promising strategy to overcome possible resistance in NSCLC to standard radiotherapy. Clinical trials are warranted to determine if this novel strategy might benefit patients with NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Published OnlineFirst September 22, 2009; DOI: 10.1158/1078-0432.CCR-09-0589

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Combined Bcl-2/Mammalian Target of Rapamycin Inhibition Leads to Enhanced Radiosensitization via Induction of Apoptosis and Autophagy in Non–Small Cell Lung Tumor Xenograft Model

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Clin Cancer Res Published OnlineFirst September 22, 2009.