ABT-263 and rapamycin act cooperatively to kill lymphoma cells *in vitro* and *in vivo*

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Abstract

ABT-263 is a potent, orally bioavailable inhibitor of the antiapoptotic Bcl-2 family members Bcl-2, Bcl-x_L, and Bcl-w, which is currently in phase I clinical trials. Previous work has shown that this compound has low nanomolar cell-killing activity in a variety of lymphoma and leukemia cell lines, many of which overexpress Bcl-2 through a variety of mechanisms. Rapamycin is a macrolide antibiotic that inhibits the mammalian target of rapamycin complex, leading to cell cycle arrest and inhibition of protein translation. Rapamycin (and its analogues) has shown activity in a variety of tumor cell lines primarily through induction of cell cycle arrest. Activity has also been shown clinically in mantle cell lymphoma and advanced renal cell carcinoma. Here, we show that treatment of the follicular lymphoma lines DoHH-2 and SuDHL-4 with 100 nmol/L rapamycin induces substantial G_0-G_1 arrest. Addition of as little as 39 nmol/L ABT-263 to the rapamycin regimen induced a 3-fold increase in sub-G_0 cells. Combination of these agents also led to a significant increase in Annexin V staining over ABT-263 alone. In xenograft models of these tumors, rapamycin induced a largely cytostatic response in the DoHH-2 and SuDHL-4 models. Coadministration with ABT-263 induced significant tumor regression, with DoHH-2 and SuDHL-4 tumors showing 100% overall response rates. Apoptosis in these tumors was significantly enhanced by combination therapy as measured by staining with an antibody specific for cleaved caspase-3. These data suggest that combination of ABT-263 and rapamycin or its analogues represents a promising therapeutic strategy for the treatment of lymphoma. [Mol Cancer Ther 2008;7(10):3265–74]

Introduction

Non-Hodgkin's lymphoma consists of a family of B-cell-derived malignancies, with an estimated 63,190 newly diagnosed cases and 18,660 deaths in the United States in 2007 (1). Overexpression of Bcl-2 is frequently associated with non-Hodgkin's lymphoma, most notably in follicular lymphoma, where 80% to 90% of the these tumors harbor a t(14;18) translocation, placing the antiapoptotic Bcl-2 protein under the control of the IgH enhancer region (2). In the absence of this translocation, Bcl-2 is still frequently up-regulated in non-Hodgkin's lymphoma through other means and is an independent indicator of poor prognosis associated with decreased overall survival and increased rate of relapse (3).

ABT-263 is a small-molecule mimetic of the BH3 domain of the proapoptotic BAD protein (4). It binds with high affinity (K_i < 1 nmol/L) to the antiapoptotic family members Bcl-2, Bcl-x_L, and Bcl-w but has much lower affinity for Mcl-1 or A1 (5). ABT-263 disrupts interactions between these antiapoptotic proteins and their proapoptotic counterparts, causing rapid induction of cytochrome *c* release from mitochondria and apoptotic cell death (6). ABT-263 has shown a range of activities against a large panel of leukemia and lymphoma cell lines, with the EC_{50}s below 100 nmol/L in the most sensitive cell lines. Furthermore, ABT-263 has also been shown to synergize *in vivo* with multiple cytotoxic agents (6).

Rapamycin is a macrolide antibiotic currently marketed as an immunosuppressant (7, 8). Rapamycin binds to FKBP-12 and this complex inhibits the mammalian target of rapamycin (mTOR). Inhibition of this pathway prevents the phosphorylation of several proteins involved in translation of RNA and cell cycle progression. Currently, there are several rapamycin derivatives being tested in clinical trials for a variety of malignancies (reviewed in ref. 9). The rapamycin analogue temsirolimus has shown activity in clinical trials for mantle cell lymphoma (38% response rate with one CR; ref. 10) and advanced renal cell carcinoma [increased clinical benefit 2-fold over standard of care (IFN-α); ref. 11], ultimately leading to its approval for late-stage renal cell carcinoma in May 2007.

Several reports have linked mTOR inhibition with effects on Bcl-2 family member proteins, although some of these data have been conflicting. Damage to microtubules following nocodazole treatment led to a mTOR-dependent increase in phosphorylation of Bcl-2 that was required for effective cell killing with this agent (12). In Karpas 299 anaplastic large cell lymphoma cells, rapamycin treatment

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led to a dose-dependent decrease of Bcl-2 and Mcl-1 (13). Mcl-1 levels were also found to be decreased following rapamycin treatment in CEM-c1 acute lymphoblastic leukemia cells, although no effect was found on Bcl-2 levels (14). Bax levels increased and Bcl-x_L decreased in JN-DSRCT-1 cells following rapamycin treatment (15). Conversely, in SuDHL-4 follicular lymphoma cells, Bcl-2 levels were increased following rapamycin treatment (16).

This report describes our findings both *in vitro* and *in vivo* with a combination of ABT-263 and rapamycin in the diffuse large B-cell lymphoma (DLBCL) lines DoHH-2 and SuDHL-4 and the mantle cell lymphoma line Granta 519. In cell culture, rapamycin treatment induced significant G_0-G_1 arrest in these lines within 48 h. ABT-263, at doses as low as 20 nmol/L, was able to drive cells into apoptosis as measured by sub-G_0 accumulation and Annexin V staining. Addition of rapamycin substantially increased the apoptotic fraction of these cell lines at an equivalent dose of ABT-263. *In vivo*, significant cooperativity was observed in the efficacy of these agents across multiple xenograft lymphoma models. Whereas single-agent treatment was predominantly cytostatic or progressive, combination therapy led to a significant increase in tumor regression and overall response rates (ORR). These data suggest that the combination of ABT-263 and mTOR inhibitors such as rapamycin may provide a promising therapeutic regimen for B-cell lymphoma.

Materials and Methods

Cell Culture

DoHH-2 and SuDHL-4 DLBCL and Granta 519 mantle cell lymphoma cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen. Cells were cultured in RPMI 1640 (Invitrogen) supplemented with 1% sodium pyruvate (Invitrogen), 4.5 g/L glucose (Sigma), and 10% fetal bovine serum (Hyclone). Cells were incubated at 37°C in 5% CO_2 and 95% relative humidity.

Reagents

ABT-263 (*N*-(4-{4-[2-(4-chlorophenyl)-5,5-dimethyl-cyclohex-1-enylmethyl]-piperazin-1-yl}-benzoyl)-4-((*R*)-3-morpholin-4-yl-1-phenylsulfanylmethyl-propylamino)-3-trifluoromethanesulfonyl-benzenesulfonamide) was synthesized at Abbott Laboratories. Rapamycin used for *in vitro* experiments was purchased from Sigma. Rapamycin used for *in vivo* experiments was produced at Abbott Laboratories. Phosal 50 PG was purchased from American Lecithin. Solutol HS15 was purchased from BASF.

Antibodies

Rabbit anti-cleaved caspase-3 (Asp^{175}), mouse anti-S6 ribosomal protein, rabbit anti-phospho-Ser^{235}/Ser^{236} S6 ribosomal protein, and mouse anti-cyclin D1 were purchased from Cell Signaling. Mouse anti-Ki-67 was purchased from Dako. Rabbit anti-CD31 was purchased from Bethyl Laboratories. Goat anti-actin was purchased from Santa Cruz Biotechnology. Goat anti-rabbit IgG, goat anti-mouse IgG, and rabbit anti-goat IgG horseradish peroxidase were purchased from Pierce Biotechnology.

Combination Analysis

Cells were plated at 2.5×10^5 per well in 96-well plates (1.5×10^5 per well for Granta 519). Cells were treated for 48 to 96 h with doses of ABT-263 ranging from 10 μmol/L to 9.8 nmol/L in 1:2 dilution increments and rapamycin ranging from 100 to 0.01 nmol/L in 1:10 dilution increments. Viable cells were counted using the Viacount assay (Guava Technologies) and analyzed using a Guava EasyCyte Plus system using the CytosoftPCA96 software (Guava Technologies). Combination index was measured using Calcusyn software (Biosoft) using the methods of Chou and Talalay (17). Cells from the double vehicle-treated wells were used to generate the standard growth curve.

Cell Cycle Analysis

Cells were plated at 1×10^5/mL in six-well plates. Cells were incubated with 100, 0.5, or 0 nmol/L rapamycin for 24 to 48 h. Analysis of cell cycle was done using the Guava Cell Cycle Reagent kit (Guava Technologies). Cells were fixed and permeabilized with ice-cold 70% ethanol according to the manufacturer's protocol. DNA content was measured using ExpressPro software and gating cells on area and width within PM1 (583/26) to exclude clumped cells. Analysis was done using a Guava EasyCyte Plus system and analyzed using the CytosoftPCA96 software (Guava Technologies).

For drug combination analysis, cells were incubated from 48 to 96 h with doses of ABT-263 ranging from 2.5 μmol/L to 9.8 nmol/L in the presence of rapamycin (100 nmol/L) or vehicle (DMSO). Analysis of cell cycle was done as described above. Doses of ABT-263 that showed the largest potentiation were chosen for further analysis as described below.

Apoptosis Analysis

Cells were plated at 1×10^5/mL in 12-well plates and incubated for 48 h with ABT-263 (39 nmol/L for DoHH-2 and 156 nmol/L for SuDHL-4 and Granta 519) with or without rapamycin at 100 nmol/L. Apoptosis was measured using the Guava Nexin kit (Guava Technologies) according to the manufacturer's protocol. Fluorescence emission for Annexin V staining and 7-AAD was measured on an EasyCyte Plus system and analyzed using the CytosoftPCA96 software (Guava Technologies). Samples were run in triplicate, with duplicate analyses of each ($n = 6$).

Western Blot Analysis

Lymphoma cells were treated with ABT-263 with or without rapamycin for 48 h as described for apoptosis analysis. Cells were harvested and lysed in Cell Lysis Buffer (Invitrogen) containing 1× Complete Protease Inhibitor Cocktail (Roche Diagnostics). Protein (30 μg) was loaded into each well of a 4% to 12% Bis-Tris NuPage gradient gel (Invitrogen) and transferred to 0.20 μm supported nitrocellulose membrane (Bio-Rad) using semi-dry transfer.

Membranes were blocked using 1% polyvinylpyrrolidone in PBS-0.1% Tween 20 as described previously (18). Primary antibodies at a concentration of 1 μg/mL in

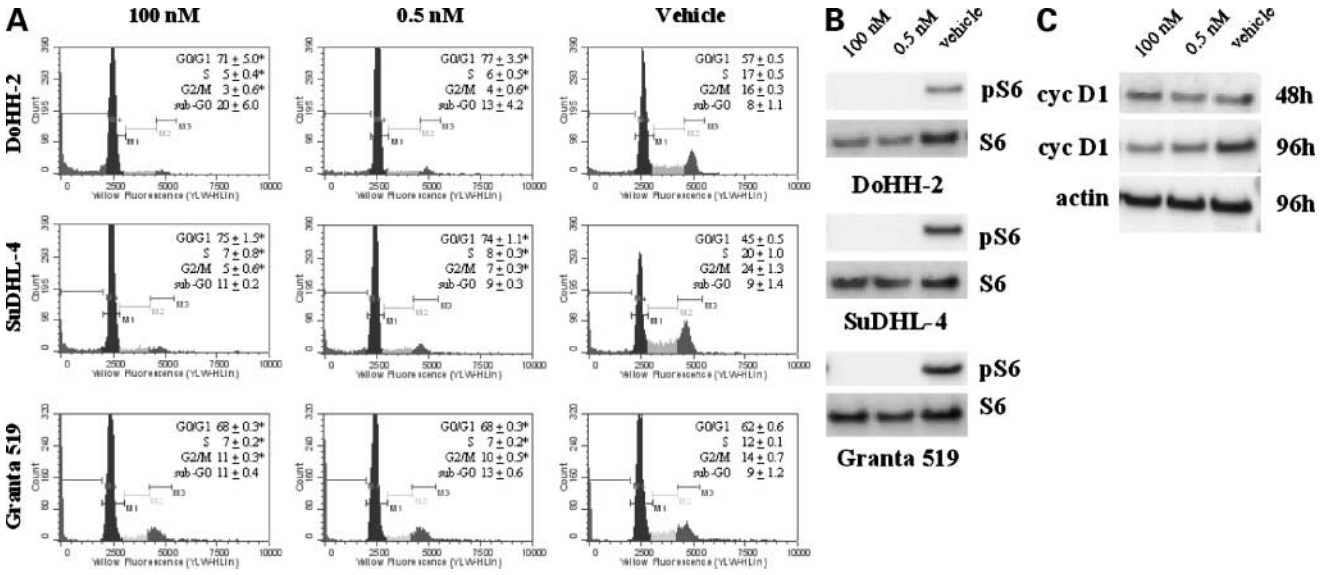

Figure 1. Analysis of cell cycle effects in DLBCL and mantle cell lymphoma cell lines following treatment with rapamycin. DoHH-2, SuDHL-4, and Granta 519 cells were treated for 48 h with rapamycin at 100, 0.5, and 0 nmol/L. Cells were analyzed for DNA content and S6 phosphorylation. **A,** analysis of cell cycle arrest in DoHH-2, SuDHL-4, and Granta 519 cells. DNA content was measured using the Guava Cell Cycle kit as described in Materials and Methods. *Inset,* relative percentage of cells in each stage of the cell cycle within the respective histogram. DoHH-2 and SuDHL-4 cells showed substantial G_0-G_1 arrest with no dose dependency, whereas Granta 519 was modestly but significantly arrested. *, $P < 0.05$ versus vehicle-treated cells. **B,** biomarker analysis for rapamycin activity in lymphoma cells. Phosphorylation of S6 ribosomal protein was determined by Western blot following 48 h exposure to rapamycin at 100, 0.5, or 0 nmol/L. Total S6 protein level was measured as a normalization control. In all lines, rapamycin eliminated S6 phosphorylation, showing mechanistic activity. **C,** Western analysis for cyclin D1 levels in Granta 519. At 48 h, cyclin D1 levels were unchanged with rapamycin treatment, which may explain the inability of rapamycin to substantially arrest these cells at this time point. Following 96 h of exposure, cyclin D1 levels were reduced approximately 2-fold by rapamycin with no dose dependency, but no increase in G_0-G_1 arrest over 48 h was observed (Fig. 2A).

PBS-0.1% Tween 20 were exposed to the membrane overnight followed by secondary antibodies at a dilution of 1:20,000 in PBS-0.1% Tween 20 for 1 h. Labeled proteins were visualized using SuperSignal West Pico (Pierce Biotechnology). Detection of chemiluminescence was done in a Fluorochem 8900 Imaging System and processed using AlphaEaseFC imaging software (AlphaInnotech).

In vivo Xenografts

All animal studies were conducted in accordance with the guidelines established by the internal Institutional Animal Care and Use Committee. C.B.-17 *scid* mice (DoHH-2 and Granta 519) or C.B.-17 *scid*-bg mice (SuDHL-4; Charles River Laboratories) were inoculated with 1×10^6 (DoHH-2), 3×10^6 (SuDHL-4), or 5×10^6 (Granta 519) cells s.c. in the right flank. Inoculation volume was 0.2 mL consisting of a 50:50 mixture of cells in growth medium and Matrigel (BD Biosciences). Tumor volume was estimated by two to three weekly measurements of the length and width of the tumor by electronic calipers and applying the following equation: $V = L \times W^2 / 2$. Tumors were allowed to reach approximately 250 mm^3, and mice were size-matched (day 0) into treatment and control groups. All animals were ear-tagged and monitored individually throughout the experiment. ABT-263 was administered orally at a dose of 100 mg/kg/d in a vehicle consisting of Phosal 50 PG/polyethylene glycol 400/ethanol (60:30:10) once daily for 21 days. Rapamycin was administered i.p. at a dose of 20 mg/kg/d in a vehicle consisting of ethanol/Solutol HS15/5% dextrose in water (4:10:86) once daily for 17 (DoHH-2 and SuDHL-4) or 21 (Granta 519) days. ABT-263 was administered approximately 1 h before rapamycin injection. Tumor growth inhibition was calculated based on the difference between the mean tumor volumes for the treated group relative to the appropriate vehicle control at the end of the dosing period. Complete response (CR) was defined as regression of an established tumor below the level of physical detection. Partial response (PR) was defined as regression of an established tumor to below 50% of the relative starting volume, excluding complete responders. ORR is the sum of complete and partial responders. The percent increase in lifespan (%ILS) of monotherapy and combination therapy was calculated as described previously (19). All xenograft trials were composed of 9 or 10 mice per group.

Immunohistochemistry

Tumor samples were harvested at each time point in triplicate and fixed in either Streck's tissue fixative (Streck Laboratories) or 10% neutral buffered formalin (Richard Allen Scientific) for 24 h. Samples were then processed, embedded, and mounted at a thickness of 5 μm. Antibody dilutions were as follows: rabbit anti-CD31 (1:25), rabbit anti-cleaved caspase-3 (1:100), and mouse anti-Ki-67 (1:50). Quantification of staining for cleaved caspase-3 and Ki-67 was done using AxioVision software (Zeiss). Quantification was done on three non-necrotic fields from each tumor at ×100 magnification ($n = 9$).

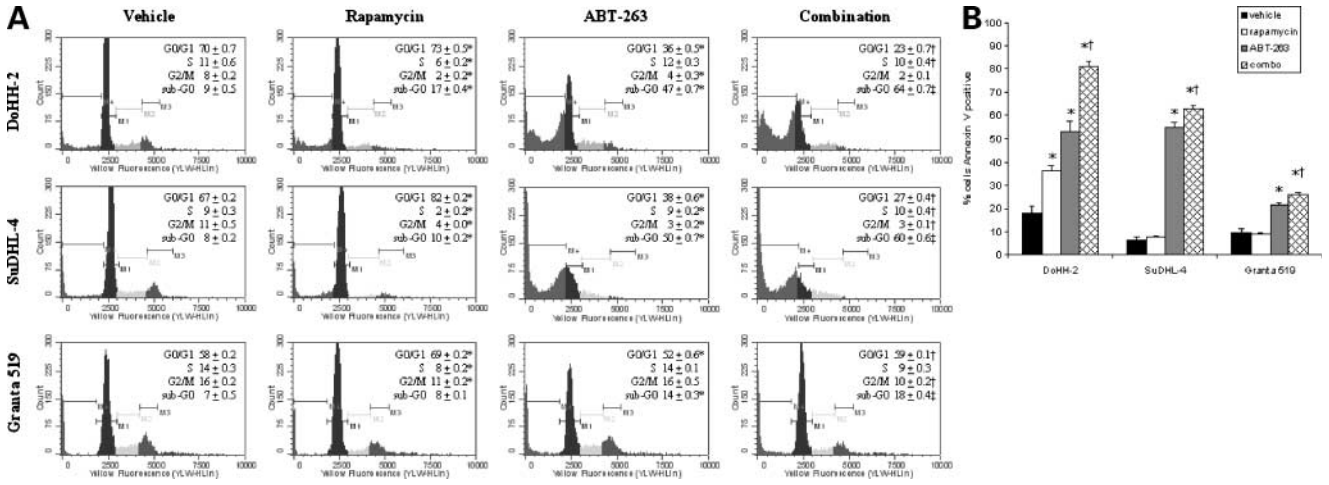

Figure 2. **A,** cell cycle analysis of lymphoma cells following 96 h exposure to ABT-263 and/or rapamycin. Cells were treated for 96 h with rapamycin at 100 nmol/L with or without ABT-263 at 39 nmol/L for DoHH-2 or 156 nmol/L for SuDHL-4 and Granta 519. DNA content was measured using the Guava Cell Cycle kit as described in Materials and Methods. *Inset,* relative percentage of cells in each stage of the cell cycle within the respective histogram. All lines showed significant G_0-G_1 arrest at 96 h with 100 nmol/L rapamycin. ABT-263 monotherapy induced a significant increase in sub-G_0 cells over control in all cases, and combination with rapamycin enhanced this population. The combination activity was stronger in DLBCL cell lines (47% versus 64% and 50% versus 60% for DoHH-2 and SuDHL-4, respectively) than in the Granta 519 mantle cell lymphoma line (14% versus 18%). *, $P < 0.05$ versus vehicle; †, $P < 0.05$ versus rapamycin monotherapy; ‡, $P < 0.05$ versus ABT-263 monotherapy. **B,** analysis of apoptosis in DoHH-2, SuDHL-4, and Granta 519 cells treated with ABT-263 with or without rapamycin. Cells were treated for 48 h with vehicle (*black*), rapamycin at 100 nmol/L (*white*), ABT-263 as described above (*gray*), or both in combination (*hatched*). Apoptosis was measured by Annexin V binding using the Guava Nexin kit as described in Materials and Methods. In all lines, ABT-263 monotherapy significantly increased apoptotic cells above control (299%, 828%, and 222%, respectively). Additionally, DoHH-2 cells showed a significant increase in apoptotic cells with rapamycin alone (204%). Combination of ABT-263 and rapamycin significantly increased the percentage of apoptotic cells over control (457%, 947%, and 264%, respectively) or ABT-263 monotherapy (154%, 114%, and 121%, respectively). *, $P < 0.05$ versus vehicle; †, $P < 0.05$ versus ABT-263 alone. $n = 6$ per treatment group.

Statistical Analysis

Significance between groups in the flow cytometry assays and immunohistochemistry quantitation were done using a Student's *t* test. Significance within *in vivo* experiments for tumor growth inhibition and tumor growth delay were done by Wilcoxon rank-sum analysis and Kaplan-Meier log-rank analysis, respectively. Significance for ORR was done by Fisher's exact test.

Results

Rapamycin Induces Cell Cycle Arrest in Lymphoma Cell Lines

Rapamycin is a macrolide antibiotic that inhibits the mTOR protein with exquisite specificity (7, 8, 20). Rapamycin, at concentrations of 100 and 0.5 nmol/L, was administered to all three lymphoma lines for 48 h. The percentage of DoHH-2 and SuDHL-4 cells in G_0-G_1 significantly increased from 57% and 45% in vehicle-treated to 71% and 75% after treatment with 100 nmol/L rapamycin, respectively. Similar effects were observed with 0.5 nmol/L rapamycin (Fig. 1A). Rapamycin induced a modest but significant increase in the sub-G_0 population in all cell lines following 96 h treatment, most prominently in DoHH-2 (Fig. 2A). Arrest was significant but modest in the Granta 519 line following 48 h rapamycin treatment (62% versus 68%). mTOR inhibition at 48 h was verified in all lines by the loss of phosphorylation of S6 ribosomal protein, a downstream target of mTOR, as detected by Western analysis (Fig. 1B). The hallmark of mantle cell lymphoma is overexpression of cyclin D1 due to translocation placing the cyclin D1 gene under the control of the IgH enhancer (21). Blotting for cyclin D1 in Granta 519 revealed no difference in expression level following 48 h treatment (Fig. 1C). However, after 96 h exposure to rapamycin, cyclin D1 levels dropped approximately 50% in Granta 519 (Fig. 1C). This may explain the attenuated G_0-G_1 arrest seen in this cell line. In contrast, neither DoHH-2 nor SuDHL-4 expressed detectable levels of cyclin D1 (Supplementary Fig. S1).[1]

ABT-263 Kills Lymphoma Cells as a Single Agent

ABT-263 is an orally bioavailable, potent ($K_i < 1$ nmol/L) inhibitor of antiapoptotic Bcl-2 family members Bcl-2, Bcl-x_L, and Bcl-w. ABT-263 shows significant single-agent activity against a subset of tumor types *in vitro* and *in vivo*, including small cell lung cancer and leukemia/lymphoma (6, 22). Cell killing with ABT-263 is Bax/Bak dependent and is associated with release of cytochrome *c* from mitochondria and cleavage of caspase-3. The IC_{50} values for DoHH-2, SuDHL-4, and Granta 519 in 10% fetal bovine serum for ABT-263 are 16, 25, and 299 nmol/L, respectively (Fig. 3). ABT-263 induced an accumulation of cells in the sub-G_0 compartment as early as 48 h in a dose-dependent

[1] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

fashion. Prolonging exposure to 96 h led to enhancement of cell killing, with as little as 39 nmol/L ABT-263 inducing a significant increase in sub-G_0 population, while having no effect on the cell cycle (Fig. 2A).

Combination of ABT-263 and Rapamycin Increases Cell Killing in Lymphoma Cell Lines

Isobolographic analysis was done on lymphoma cells using doses of ABT-263 ranging from 10 μmol/L to 9.8 nmol/L and rapamycin ranging from 100 to 0.01 nmol/L. Cells were treated for 48 h and counted using the Viacount assay on a Guava EasyCyte system. DoHH-2 cells showed a synergistic response to ABT-263 and rapamycin, with CI values at the ED_{75} ranging from 0.53 at a ratio of 3.9:1 to 0.64 at a ratio of 39:1. SuDHL-4 and Granta 519 showed an additive response (Fig. 3), which is in good agreement with responses seen in the cell cycle and Annexin V assays (Fig. 2). Continuing treatment for 96 h did not enhance the CI in DLBCL (data not shown).

Lymphoma cells were treated with 100 nmol/L rapamycin with or without ABT-263 at concentrations ranging from 2.5 μmol/L to 9.8 nmol/L. Cotreatment of lymphoma cells with 100 nmol/L rapamycin and ABT-263 led to enhanced sub-G_0 accumulation in all three cell lines compared with ABT-263 alone (Fig. 2A). In the DoHH-2 line, addition of 39 nmol/L ABT-263 to rapamycin led to an increase in the sub-G_0 population from 47% to 64% compared with ABT-263 treatment alone. In the SuDHL-4 and Granta 519 lines, maximal effect was achieved at a slightly higher concentration of ABT-263. Addition of 156 nmol/L ABT-263 to rapamycin led to an increase in sub-G_0 population from 50% and 14% with ABT-263 alone to 60% and 18% with combination treatment, respectively. In all three cell lines, G_0-G_1 population of cells was the most significantly affected by combination treatment compared with rapamycin treatment alone (Fig. 2A). G_0-G_1 populations decreased from 73%, 82%, and 69% to 23%, 27%, and 59% for DoHH-2, SuDHL-4, and Granta 519, respectively. Treatment of cells with the less active enantiomer of ABT-263 did not increase the sub-G_0 population with or without rapamycin (data not shown). Standard growth curves showed a reasonably linear growth rate for these cells through 96 h (Supplementary Fig. S2).[1]

To confirm that the increase in sub-G_0 cells was due to apoptosis, cells were treated with ABT-263 with or without rapamycin and analyzed for Annexin V staining. Following 48 h exposure to ABT-263, Annexin V staining increased 300%, 828%, and 222% over vehicle alone for DoHH-2, SuDHL-4, and Granta 519, respectively (Fig. 2B). In agreement with cell cycle results, 100 nmol/L rapamycin induced a 204% increase in Annexin V-stained cells in DoHH-2 while showing no increase in staining in SuDHL-4 or Granta 519. Combination of both agents induced a significant increase in positive staining of 153%, 114%, and 121% versus ABT-263 alone for each cell line, respectively, in good agreement with cell cycle data (Fig. 2B).

Combination of Rapamycin and ABT-263 Leads to Tumor Regression in DLBCL *In vivo*

ABT-263 administered orally at 100 mg/kg/d once daily for 3 weeks resulted in significant inhibition of tumor growth in both DoHH-2 and SuDHL-4 models (Table 1; Fig. 4). In both models, tumor growth rate was inhibited by approximately 50% coupled with a roughly 2-fold increase in median time for tumors to reach 1 cc relative to vehicle control. ABT-263 administered orally at 100 mg/kg/d had a more modest effect in the Granta 519 model with 22% tumor growth rate inhibition and a significant increase in time for tumors to reach 1 cc (Table 1; Fig. 4).

Rapamycin administered i.p. at 20 mg/kg/d for 17 to 21 days was significantly more efficacious than ABT-263 monotherapy in all three xenograft models. Plasma levels of rapamycin at this dose reached a C_{max} of 2.5 μmol/L at approximately 2 h with a long half-life (8 h). Activity was particularly robust in both DoHH-2 and SuDHL-4 models with approximately 80% inhibition of tumor growth rate and a marked effect on tumor growth delay. However, aside from the 30% PR rate seen in DoHH-2, significant tumor regression was not observed with rapamycin monotherapy. Rapamycin was also more efficacious than

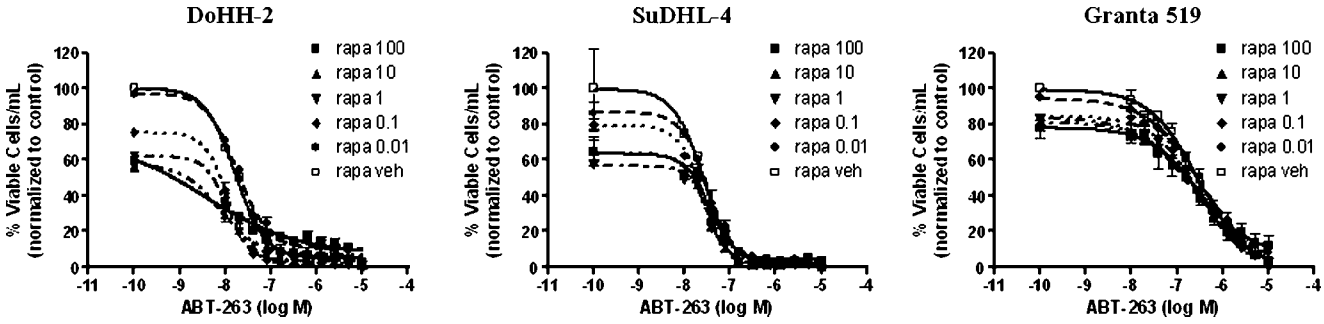

Figure 3. Combination analysis of rapamycin and ABT-263 in DoHH-2, SuDHL-4, and Granta 519 cells. Cells were plated at 2.5×10^5/mL (1.5×10^5/mL for Granta 519) and treated with ABT-263 and rapamycin as described in Materials and Methods. $n = 2$ per dose pair. DoHH-2 cells showed a synergistic response, with CI values of approximately 0.6 at 39:1 to 0.39:1 ratios of ABT-263/rapamycin. SuDHL-4 and Granta 519 showed no synergy in this assay.

Table 1. Efficacy of ABT-263 and rapamycin in lymphoma xenograft models

Cell line	Compound	Dose* (mg/kg/d)	Average ± SE tumor volume	%TGI†	%ILS‡	%PR§	%CR‖	%ORR¶
DoHH-2	ABT-263	100	1,718 ± 285	52**	56**	0	10	10
		0	3,584 ± 322					
	Rapamycin	20	625 ± 79	82**	657**	30	0	30
	ABT-263 + rapamycin	100 + 20	50 ± 16	>99**	>710**	30	70††	100††
		0 + 0	3,511 ± 364					
SuDHL-4	ABT-263	100	1,019 ± 117	52**	50**	0	0	0
	Rapamycin	20	388 ± 85	82**	257**	11	0	11
	ABT-263 + rapamycin	100 + 20	116 ± 56	95**	414**	25	75††	100††
		0 + 0	2,125 ± 269					
Granta 519	ABT-263	100	2,085 ± 199	22	25**	0	0	0
		0	2,687 ± 239					
	Rapamycin	20	1,048 ± 206	58**	88**	0	0	0
		0	2,477 ± 182					
	ABT-263 + rapamycin	100 + 20	545 ± 112	77**	175**	10	0	10
		0 + 0	2,346 ± 225					

NOTE: Tumors were size matched (day 0) to approximately 250 mm^3 before therapy. $n = 9$ or 10 per group. In all cases, ABT-263 was dosed once daily for 21 d. In DoHH-2 and SuDHL-4, rapamycin was dosed once daily for 17 d. In Granta 519, rapamycin was dosed once daily for 21 d.

*"0" or "0 + 0" is vehicle(s) for group with which it is associated.

†% Tumor growth inhibition (measured at end of dosing period).

‡Median % increase in time for tumors to reach 1 cc endpoint relative to vehicle.

§% Partial regression.

‖% Complete regression.

¶% Overall response.

**$P < 0.05$ versus vehicle or rapamycin monotherapy (Wilcoxon rank-sum test for % tumor growth inhibition; Kaplan-Meier log-rank analysis for % increase in lifespan).

††$P < 0.05$ versus rapamycin monotherapy (Fisher's exact test).

ABT-263 in the Granta 519 model. However, as was observed with ABT-263 monotherapy, the Granta 519 model was more resistant to rapamycin therapy than either of the DLBCL lines.

The combination of ABT-263 plus rapamycin was significantly more efficacious than either monotherapy in all three xenograft models. The effect was most pronounced in the DLBCL models where the combination

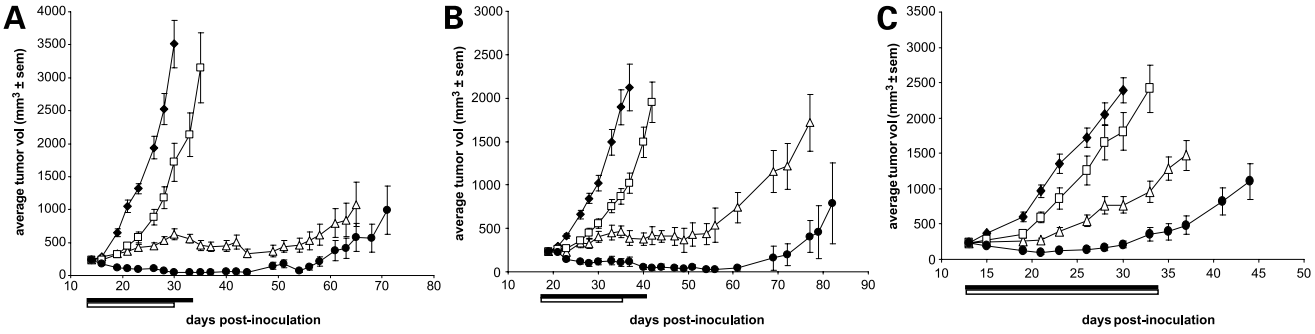

Figure 4. Xenograft sensitivity to ABT-263 with or without rapamycin. Mice were grafted with either DoHH-2 (**A**), SuDHL-4 (**B**), or Granta 519 (**C**) cells and treated following size matching at approximately 225 mm^3 (day 0). Animals were dosed with either double vehicle (♦), ABT-263 100 mg/kg/d (□), rapamycin 20 mg/kg/d (Δ), or both agents (●). Dosing schedules for ABT-263 (*filled columns*) and rapamycin (*open columns*). Representative trial for each lymphoma tumor model. *Points,* mean; *bars,* SE. All lines showed a modest but significant response to ABT-263 monotherapy (DoHH-2 and SuDHL-4 significant throughout dosing; Granta 519 significant days 15-21). Rapamycin monotherapy induced a cytostatic response in both DLBCL lines but failed to fully arrest tumor growth in Granta 519 (significance from vehicle control in all three tumors throughout dosing). Combination therapy led to significant tumor regression throughout dosing in both DLBCL lines, with a 100% ORR, and significant improvement in tumor growth inhibition versus rapamycin monotherapy through day 61 in DoHH-2 and day 77 in SuDHL-4. Granta 519 tumors responded more fully to combination of both agents compared with either agent alone; however, regression of the tumors was not seen, and the ORR was only 10%. Tumor growth inhibition was significantly better than either vehicle or rapamycin monotherapy from day 19 forward in all models.

led to significant tumor regression. In both of these models, nearly complete inhibition of tumor growth was observed. Indeed, a significant increase in complete responders and ORR versus rapamycin monotherapy was observed in both lines (Table 1). Whereas rapamycin monotherapy resulted in PR on the scale of 10% to 30% in DoHH-2 and SuDHL-4, combination with ABT-263 induced greater than 70% CR in both models. A significant increase in inhibition of tumor growth rate and enhancement of tumor growth delay was also noted in the Granta 519 model relative to monotherapy. However, there was only a modest effect on tumor regression, and no significant change in response rates was observed (Table 1).

Immunohistochemical Analysis of Drug-Treated Tumors

Pharmacodynamic analysis was done on xenograft tumors following a single dose of ABT-263, rapamycin, or both agents in combination, looking specifically at cell cycle inhibition and induction of apoptosis. DoHH-2 tumors were size matched at 500 mm^3 and dosed with a single dose of either ABT-263 (100 mg/kg), rapamycin (20 mg/kg), or both in combination. Tumors were harvested at 8, 48, and 96 h post-dose and analyzed by immunohistochemistry. Staining of Ki-67 was used to measure proliferative index, and apoptosis induction was monitored by levels of cleaved caspase-3.

ABT-263 monotherapy induced a significant increase in cleaved caspase-3 levels of 229% in DoHH-2 at 8 h post-dose (Fig. 5B and C). Elevated cleaved caspase-3 levels were maintained throughout the 96 h time course (data not shown). However, ABT-263 monotherapy showed no effect on cell cycle, with Ki-67 levels remaining unchanged at all time points following ABT-263 monotherapy (Fig. 5A and C). In contrast, rapamycin monotherapy had no significant effect on induction of cleaved caspase-3 levels

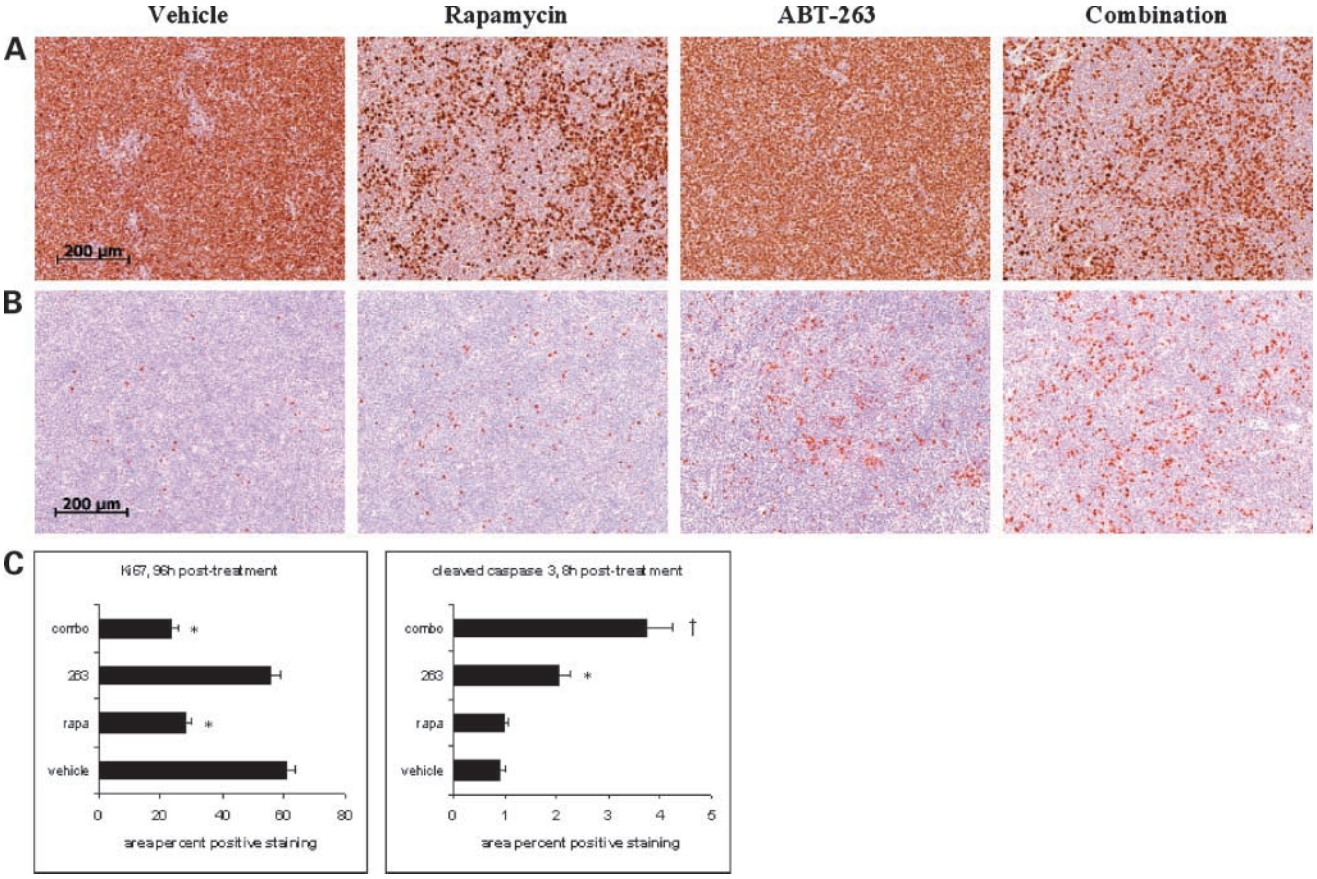

Figure 5. Immunohistochemical analysis of DoHH-2 xenograft tumors following treatment with rapamycin, ABT-263, or both. **A** and **B,** mice bearing DoHH-2 xenografts approximately 500 mm^3 in size were injected with either vehicle, rapamycin (20 mg/kg/d), ABT-263 (100 mg/kg/d), or both in combination. Tumors were harvested 8, 48, and 96 h post-injection. $n = 3$ tumors per treatment group. Representative of nonnecrotic areas of all samples. Results for DoHH-2 tumors. Inhibition of proliferation and induction of caspase were also seen with SuDHL-4 and Granta 519 with ABT-737. **A,** Ki-67 expression after 96 h of treatment. Rapamycin significantly inhibited proliferation of DoHH-2 tumors in the presence or absence of ABT-263. **B,** cleaved caspase-3 staining 8 h after injection. Significant induction of apoptosis was seen with ABT-263 monotherapy, and this was enhanced by the addition of rapamycin. **C,** quantitation of Ki-67 and cleaved caspase-3 staining. Three nonnecrotic areas from each tumor were selected and analyzed at a magnification of ×100 ($n = 9$). *, $P < 0.05$ versus vehicle; †, $P < 0.05$ versus ABT-263 monotherapy.

at any time point (Fig. 5B and C). A significant decrease in Ki-67 expression was observed although not at earlier time points (data not shown). At 96 h post-dose, Ki-67 levels were decreased 53% in rapamycin treated tumors compared with vehicle control (Fig. 5A and C). Consistent with previous reports (23–25), a significant decrease in CD31 staining was observed in tumors treated with rapamycin compared with those treated with either ABT-263 alone or vehicle, indicative of decreased blood vessel density (data not shown).

Combination of ABT-263 and rapamycin showed significant potentiation of caspase-3 cleavage at 8 h post-dose, with cleaved caspase staining increasing 183% above ABT-263 monotherapy and 410% over vehicle control (Fig. 5B and C). Proliferation of co-treated tumors as measured by Ki-67 expression was decreased 61% compared with vehicle control at 96 h post-dose; however, this response was not significantly different from rapamycin monotherapy (Fig. 5C).

Discussion

The mTOR protein is a master regulator protein that monitors nutrient levels available to cells and regulates growth and metabolism accordingly (23). Constitutive activation of the mTOR pathway has been shown in a variety of tumors (7–9). Inhibition of mTOR, by agents such as rapamycin and its analogues, leads to a G_0-G_1 cell cycle arrest through inhibition of cyclin D1 translation (26) and stabilization of the cyclin-dependent kinase 2 inhibitor $p27^{Kip1}$ (27, 28). Cell cycle arrest and apoptosis are intricately linked through a variety of mechanisms, as reviewed by Maddika et al. (29), and mTOR has been shown to regulate several members of the Bcl-2 family (12–16). Thus, we hypothesized that the combination of a cell cycle arresting agent and an inducer of apoptosis could potentially act in a synergistic fashion.

Here, we describe the activity of the combination of ABT-263 and rapamycin in several models of B-cell lymphoma. ABT-263 monotherapy led to a dose-dependent increase in cell killing, with DoHH-2 cells being the most sensitive (Figs. 2 and 3). Induction of apoptosis was achieved most potently in DoHH-2, where 39 nmol/L ABT-263 induced 50% of cells to bind Annexin V after 48 h, and less potently in SuDHL-4 and Granta 519, where 156 nmol/L ABT-263 induced 50% and 20% positive staining, respectively (Fig. 2B). Treatment of DLBCL lines with rapamycin alone induced substantial G_0-G_1 arrest following 48 h of exposure (Fig. 1A). Granta 519 cells were also arrested with rapamycin but to a lesser extent (Fig. 1A). Western blotting for phospho-S6 ribosomal protein showed that in all three lines complete mTOR inhibition was achieved at 48 h (Fig. 1B). However, in Granta 519 cells, cyclin D1 levels remained elevated, and these cells failed to fully arrest (Figs. 1 and 2). Neither DLBCL line showed detectable cyclin D1 expression (Supplementary Fig. S1), which suggests that this factor may provide some resistance to the effects of ABT-263.

Combination of ABT-263 and rapamycin led to a significant enhancement of ABT-263-induced apoptosis and cell killing (Fig. 2). Interestingly, enhanced cell killing required an extended period, because there was no significant increase in sub-G_0 population versus ABT-263 alone following 48 h of treatment (data not shown) but required longer exposure times (Fig. 2A). One possible explanation is that rapamycin-induced cell cycle effects were required for effective killing, because enhancement was not seen until after DLBCL cells were substantially arrested, and the combination efficacy was significantly blunted in the Granta 519 cells, which never fully arrested (Fig. 2). Conceivably, arrested cells could be solely dependent on Bcl-2 or Bcl-x_L for survival during arrest or as they emerge from arrest. This is supported by the fact that cells in the G_0-G_1 phase of the cell cycle disappeared at a greater rate than other phases of the cell cycle as the sub-G_0 population increased (Fig. 2A). Experiments are ongoing to address this question.

A significant increase in efficacy *in vivo* was also observed with the combination of ABT-263 and rapamycin. ABT-263 administered at 100 mg/kg/d decreased tumor growth rate by approximately 50% in both DLBCL lines while having a more modest effect in Granta 519 (Fig. 4). Rapamycin administered at 20 mg/kg/d generated a more robust response in all tumor lines, with approximately 80% inhibition in growth rate in DLBCL and 60% in Granta 519. However, both agents failed to generate significant tumor regression as a monotherapy (Table 1).

Combination of both agents led to significant and robust tumor responses, particularly in DLBCL lines, where 100% ORR (CR + PR) were achieved with greater than 95% inhibition in tumor growth rate. Additionally, rapamycin induced 10% to 30% PR with no CR when used alone, whereas the combination with ABT-263 induced greater than 70% CR (Table 1). The combination in Granta 519 tumors, while still showing enhancement of response, did not produce significant tumor regression, although tumor growth inhibition was increased to 77%. All three tumor models showed a significant tumor growth delay in response to combination therapy (Table 1).

Inhibition of mTOR is currently being investigated clinically in a variety of tumor types including lymphoma (9). Overexpression of Bcl-2 has been shown to be a resistance factor to rapamycin treatment. *Eμ-myc/PTEN*$^{+/-}$ cells engineered to overexpress Bcl-2 were shown to be selectively enhanced in a mixed population with untransfected cells treated *in vivo* with rapamycin (30). In ovarian carcinoma, SK-OV-3 cells that express Bcl-2 were more resistant to rapamycin therapy than the Bcl-2-negative line IGROV-1 (31). Although previous reports have shown modulation of Bcl-2 family members following rapamycin treatment (12–16), we did not detect significant changes in protein levels of several Bcl-2 family members following rapamycin treatment in the DLBCL lines (Supplementary Fig. S3A). Modulation of Akt phosphorylation by mTOR inhibitors has also been reported (32, 33). However,

changes in Akt phosphorylation at Ser^{473} were not observed following rapamycin treatment through 48 h (Supplementary Fig. S3B). Further experiments are ongoing to determine what factors in the intrinsic apoptotic pathway are influencing sensitivity to ABT-263 in the presence of rapamycin.

Granta 519 tumors were more resistant to both agents used either alone or in combination. The hallmark signature of mantle cell lymphoma is aberrant cyclin D1 expression, generated by translocation of the IgH enhancer region upstream of the cyclin D1 gene (21). Mantle cell lymphomas have been examined as targets for rapamycin or its analogues, because they have been shown to block translation of the cyclin D1 message via mTOR inhibition (10). In our hands, cyclin D1 levels were not eliminated rapidly following rapamycin treatment of Granta 519 cells, and complete cell cycle arrest could not be achieved *in vitro* through 96 h (Figs. 1 and 2).

Peponi et al. found that inhibition of mTOR using the phosphatidylinositol 3-kinase inhibitor LY-294002 at 20 μg/mL in several mantle cell lymphoma lines eliminated cyclin D1 protein expression within 48 h and induced a 28% increase in Annexin V-positive cells and a 34% decrease in cell viability as measured by ATP production and bromodeoxyuridine incorporation. This activity was associated with the loss of Mcl-1 protein expression (34). However, LY-294002 is a fairly nonspecific inhibitor (20), and it is possible that the activity described in this report could be unrelated to mTOR. Consistent with off-target activity, significantly less apoptosis was achieved when rapamycin was substituted for LY-294002 (34).

As described in previous reports (23–25), rapamycin not only decreased proliferation in lymphoma xenografts but also significantly decreased tumor vasculature as measured by CD31 staining (data not shown). This anti-angiogenic response could contribute to the combination activity seen in these models, although to what extent it is difficult to determine. Anoxic cell death is known to involve the intrinsic apoptotic pathway, including modulation of several members of the Bcl-2 family, particularly elimination of Mcl-1 (reviewed in ref. 35). Although a rapamycin-mediated effect on angiogenesis contributing to the observed results *in vivo* cannot be ruled out, it is not clear why some models (DoHH-2) responded more robustly than others (Granta 519).

These results suggest that modulators of apoptotic cascades such as ABT-263 may have particular utility in combination with agents that induce cell cycle arrest. Conceivably, cells that have been arrested at a cell cycle checkpoint may be particularly reliant on the Bcl-2 pathway for survival as they attempt to emerge from arrest. Indeed, del Gaizo Moore et al. showed that chronic lymphocytic leukemia cells were particularly sensitive to the related Bcl-2 inhibitor ABT-737 (mean cellular EC_{50}, 5 nmol/L). This is of particular interest in that primary chronic lymphocytic leukemia cells are completely arrested in G_0-G_1, similar to rapamycin therapy (36). Furthermore, Granta 519 tumors were more resistant to the combination of rapamycin and ABT-263 and were less susceptible to cell cycle arrest induced by rapamycin *in vitro*. Additional experiments are required to more fully examine the mechanism of the observed combination synergy.

In conclusion, these data show that the combination of the orally bioavailable anti-apoptotic Bcl-2 family inhibitor ABT-263 and rapamycin may provide an effective treatment strategy for B-cell lymphoma and suggest that the rapamycin-induced cell cycle arrest is the critical factor in potentiation. Future efforts are under way to explore in more detail the connection between cell cycle arrest and potentiation of apoptosis by agents such as ABT-263.

Disclosure of Potential Conflicts of Interest

All authors are employees of Abbott Laboratories.

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