

Rapamycin induces apoptosis of JN-DSRCT-1 cells by increasing the Bax:Bcl-xL ratio through concurrent mechanisms dependent and independent of its mTOR inhibitory activity

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Rapamycin, a complex macrolide and potent fungicide, immunosuppressant and anticancer agent, is a highly specific inhibitor of mammalian target of rapamycin (mTOR). Rapamycin has been shown to induce G1-phase cell cycle arrest in diverse tumor cell types, and its derivatives RAD001 and CCI-779 are currently in phase I and phase II clinical trials, respectively, as anticancer agents. In this study, we show that rapamycin induced the apoptotic death of JN-DSRCT-1 cells, the only available *in vitro* model for Desmoplastic Small Round Cell Tumors (DSRCT), while having only minor effects on their cell cycle. Rapamycin induced apoptosis by increasing the Bax:Bcl-xL ratio as a consequence of the concomitant downregulation of Bcl-xL and upregulation of Bax, both at the post-transcriptional level. Rapamycin also downregulated the levels of EWS/WT1, the fusion protein characteristic of DSRCT. Transient transfection studies using kinase-dead and rapamycin-resistant forms of mTOR demonstrated that only the downregulation of Bcl-xL was caused by the mTOR inhibitory action of rapamycin, which prevented cap-dependent translation initiation, whereas Bax upregulation was induced by rapamycin through a mechanism independent of its mTOR inhibitory activity. Moreover, rapamycin treatment downregulated the mRNA and protein levels of the 26S p44.5 proteasome subunit, suggesting the involvement of the proteasome complex in the mechanisms of rapamycin-induced apoptosis. Treatment of JN-DSRCT-1 cells with MG-132, a proteasome specific inhibitor, also resulted in the induction of apoptosis through a similar increase in the Bax:Bcl-xL ratio specifically caused by inhibiting Bax degradation and turnover. These results suggested that rapamycin induces apoptosis by preventing the degradation of the Bax protein by the proteasome, and that this process is independent of mTOR inhibition. Furthermore, these results strongly support the introduction of the use of rapamycin as a cytotoxic agent for the treatment of DSRCT.

Oncogene (2005) 24, 3348–3357. doi:10.1038/sj.onc.1208471
Published online 14 March 2005

Keywords: apoptosis; DSRCT; EWS/WT1; proteasome; rapamycin

Introduction

Desmoplastic Small Round Cell Tumors (DSRCT) are distinctive primitive tumors that preferentially affect young males and mostly appear in intra-abdominal sites (Biegel *et al.*, 1993). These unusual tumors carry a t(11;22)(p13;q12) recurrent chromosomal translocation (Sawyer *et al.*, 1992; Shen *et al.*, 1992) that involves the Ewing's sarcoma (*EWS*) gene and the Wilms' tumor suppressor (*WT1*) gene (Rauscher *et al.*, 1994; Gerald *et al.*, 1995). The resulting chimeric gene encodes a fusion protein that acts as an aberrant transcription factor (Ladanyi and Gerald, 1994). The gene encoding *EWS*, a member of the TET family of RNA-binding proteins (Bertolotti *et al.*, 1996), was cloned from the Ewing's sarcoma chromosomal breakpoint, where translocations produce gene fusions between *EWS* and other transcription factor-encoding genes (Delattre *et al.*, 1992). The N-terminus of *EWS* is known to be a potent transactivator domain (May *et al.*, 1993), while its C-terminal domain, which is lost in the chimeric gene product, is involved in RNA recognition (Ohno *et al.*, 1994). *WT1* is a transcription factor with four C-terminal zinc-fingers mediating sequence-specific DNA binding, and a N-terminal domain implicated in transcriptional activation. Alternative splicing leads to the generation of transcripts that encode different *WT1* isoforms (Rauscher *et al.*, 1990). One of these isoforms comes from the insertion of three amino acids (lysine, threonine, and serine (KTS)) between zinc-fingers 3 and 4. The isoform lacking this insertion, *WT1* (–KTS), binds to a specific DNA sequence and leads to the transactivation of several genes, whereas the isoform generated by this insertion, *WT1* (+KTS), does not perform these functions (reviewed in Lee and Haber, 2001). This same

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Received 1 November 2004; revised 16 December 2004; accepted 20 December 2004; published online 14 March 2005

alternative splicing event is functional in DSRCT and results in two EWS/WT1 (\pm KTS) isoforms with different DNA-binding properties (Rauscher *et al.*, 1994; Gerald *et al.*, 1995) and oncogenic activity (Kim *et al.*, 1998b).

Rapamycin and its analogues represent a novel family of anticancer agents. These compounds inhibit the function of the mammalian target of rapamycin (mTOR), a serine/threonine kinase, and potentially suppress tumor cell growth primarily by arresting cells in G1 phase, or by inducing apoptosis of cells in culture and in xenograft tumor models (Huang and Houghton, 2001). Their mode of action is based on competition with a structural analog, FK506, for binding to an M_r 12000 cytosolic protein designated FKBP-12 (Bierer *et al.*, 1990). The FKBP-rapamycin complex binds and inhibits the function of mTOR (Sabers *et al.*, 1995), thereby blocking the stimulation of ribosomal p70s6k (Chung *et al.*, 1992) and preventing the phosphorylation of 4E-BP1. Dephosphorylated 4E-BP1 then inhibits cap-dependent translation initiation by binding to the mRNA cap-binding protein eucaryotic initiation factor 4E (eIF4E) (Lin *et al.*, 1994).

To date, the JN-DRSCT-1 cell line is the only available *in vitro* model of DSRCT. This cell line exhibits the unique morphologic and genetic characteristics of DSRCT and, by RT-PCR, has been demonstrated to express a chimeric transcript in which exon 10 of the *EWS* gene is fused to exon 8 of the *WT1* gene, which generates a WT1 (-KTS) isoform (Nishio *et al.*, 2002). Therefore, the JN-DRSCT-1 cell line provides a useful *in vitro* model to understand the molecular pathology of DSRCT and to study potential new therapies.

Recently, we demonstrated that rapamycin blocks cell proliferation by promoting both cell cycle arrest at the G1 phase and the downregulation of the EWS/FLI-1 proteins in Ewing's sarcoma cells carrying various types of EWS/FLI-1 translocations (Mateo-Lozano *et al.*, 2003). Moreover, rapamycin also induced the downregulation of the EWS protein in the same cell lines (Mateo-Lozano *et al.*, 2003). It appeared as if the invariable presence of EWS in all EWS/FLI-1 fusion types was what made these proteins susceptible to downregulation by rapamycin. We reasoned that, because DSRCT express the EWS/WT1 fusion protein, they may also be susceptible to the action of rapamycin. Consequently, we studied the effects of rapamycin on the expression levels of the EWS/WT1 protein, proliferation and apoptosis in JN-DRSCT-1 cells. Our results indicate that rapamycin indeed downregulates EWS/WT1 and induces the apoptotic death of JN-DRSCT-1 cells by simultaneously inducing the downregulation of Bcl-xL through a mechanism dependent on its mTOR inhibitory activity, and the upregulation of Bax by inhibiting the proteasome complex, a process independent of mTOR inhibition. Finally, our results also indicate that, being a highly effective inducer of apoptosis, rapamycin could represent a very useful tool for the treatment of DSRCT.

Results

Rapamycin induces the dose- and time-dependent death of JN-DRSCT-1 cells

To study the effects of rapamycin on DSRCT, JN-DRSCT-1 cells were exposed to increasing concentrations of rapamycin for 24 h. By using the trypan blue exclusion assay, we determined that rapamycin induced a dose-dependent increase in cell death that reached up to about 40% at 100 ng/ml (Figure 1a), a concentration previously shown to induce apoptosis in rhabdomyosarcoma cells (Hosoi *et al.*, 1999). Therefore, we used this concentration to test the effect of rapamycin in time-course experiments. Figure 1b shows that cell death induced by rapamycin reached around 70% after 72 h of continued exposure to the drug. Terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling (TUNEL) assays demonstrated that JN-DRSCT-1 cells died by apoptosis, and showed the presence of about 20% apoptotic cells already after 16 h exposure to rapamycin, whereas little apoptosis could be detected in untreated cells (Figure 2a). Rapamycin has been shown to induce cell cycle arrest in G1 phase in various tumor cell types (reviewed in Huang *et al.*, 2003). Therefore, we tested if treatment of JN-DRSCT-1 cells with rapamycin (100 ng/ml) induced any cell cycle alterations. As shown in Figure 2b, exposure to rapamycin induced only minor delays at both G1 and

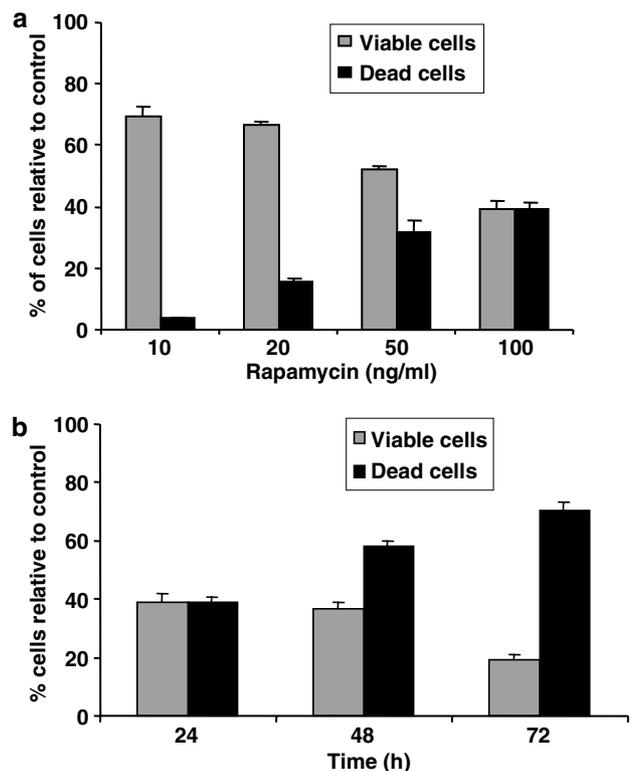


Figure 1 Rapamycin induces cell death in JN-DRSCT-1. Cells were treated with the indicated concentrations of rapamycin (a) or with 100 ng/ml of rapamycin for various times (b), and the induction of cell death was determined by the trypan blue exclusion assay

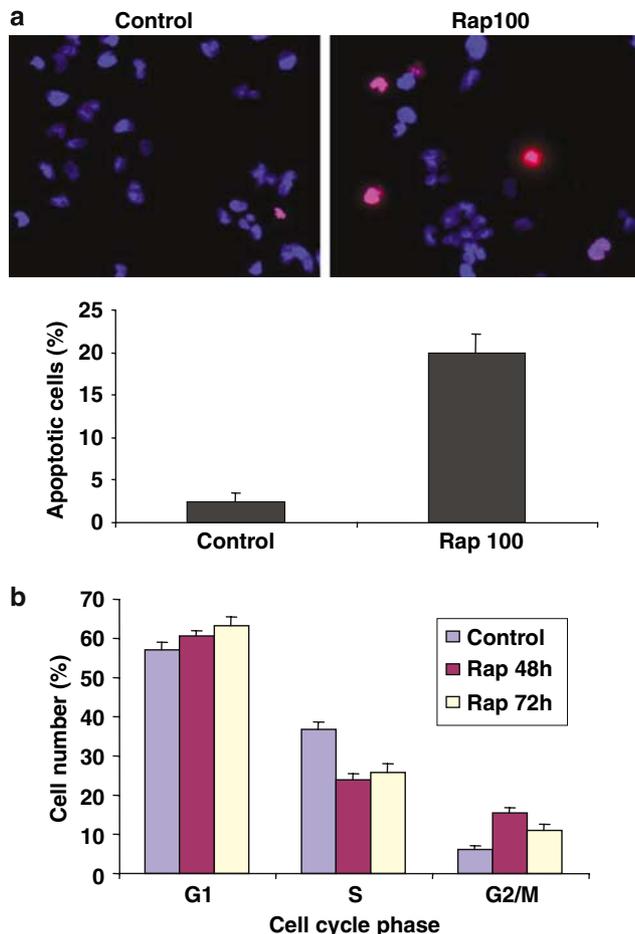


Figure 2 Rapamycin induces cell death by apoptosis and has little effect on the cell cycle. (a) JN-DSRCT-1 cells were grown in slide chambers and treated with 100 ng/ml of rapamycin (Rap 100) for 16 h. Apoptosis was measured by TUNEL assays, and quantitative data (bottom panel) were obtained by counting TUNEL-positive cells in five different fields per slide. Similar results were obtained in three independent experiments. (b) JN-DSRCT-1 cells were treated with rapamycin (Rap), at 100 ng/ml, for 48 or 72 h, then stained with propidium iodide, and the DNA content was determined by flow cytometry

G2/M phases of the cell cycle, which remained below the 10% level even after 72 h of treatment. In rapamycin-exposed cell cultures, there was a noticeable decrease in the proportion of S-phase cells (Figure 2b), with a concomitant increase in the sub-G1 cell fractions detected by flow cytometry (18.41 ± 3.5 at 48 h, and 21 ± 1.06 at 72 h).

Rapamycin induces apoptosis by altering the Bax : Bcl-xL ratio at the post-transcriptional level

To study possible mechanisms involved in the apoptosis of JN-DSRCT-1 cells, we analysed the expression levels of known apoptosis-associated proteins. Western blot analyses demonstrated that rapamycin induced a time-dependent upregulation of the Bax protein and a parallel downregulation of the Bcl-xL levels, thereby causing an increased Bax : Bcl-xL ratio (about fourfold)

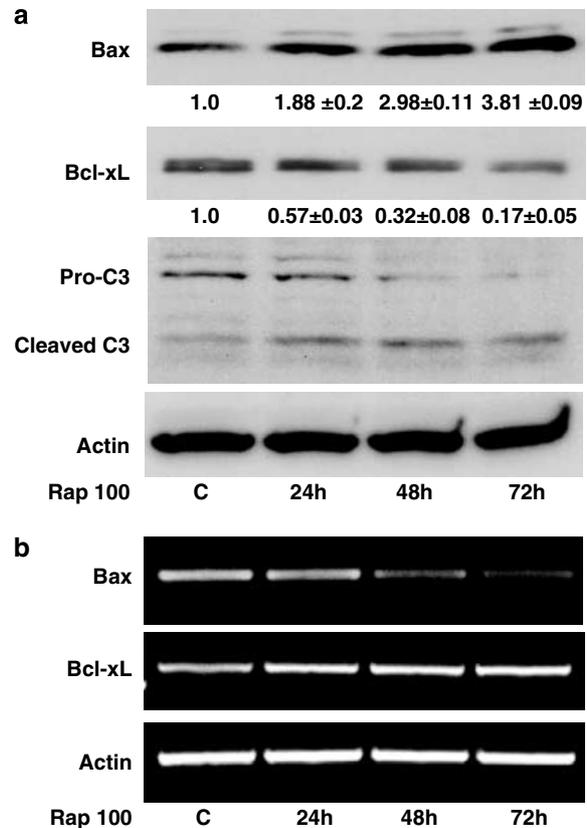


Figure 3 Rapamycin-induced apoptosis is mediated by an increase in the Bax : Bcl-xL ratio at the post-transcriptional level. JN-DSRCT-1 cells were treated with 100 ng/ml of rapamycin (Rap 100) for the times indicated. The cells were then lysed and protein levels of Bax and Bcl-xL, as well the cleavage of caspase-3 (C3) were analysed by Western blot using specific antibodies (a), and the mRNA levels of Bax and Bcl-xL were analysed by semiquantitative RT-PCR (b). Actin was used as the loading control. C, untreated controls. Quantitative data indicated below each lane for the Bax and Bcl-xL Western analyses (a) were normalized for loading and represent the mean densitometric values (\pm s.d.) from four independent experimental replicas

that resulted in the activation of caspase-3 (Figure 3a). However, semiquantitative RT-PCR assays demonstrated that the mRNA levels of *Bax* and *Bcl-xL* did not correlate with the changes observed at the protein level after rapamycin treatment (Figure 3b). In fact, interestingly, *Bax* mRNA decreased over time, whereas *Bcl-xL* mRNA increased during the same period. These data demonstrated that rapamycin induced the observed alterations of the Bax and Bcl-xL proteins at a translational or post-translational level.

Rapamycin prevents the phosphorylation of mTOR kinase targets and downregulates the expression of the EWS/WT1 protein

Previously, we demonstrated that rapamycin induced the downregulation of the EWS/FLI1 protein in Ewing's sarcoma cells by inhibiting the p70s6k kinase, a known target of the mTOR kinase (Mateo-Lozano *et al.*, 2003).

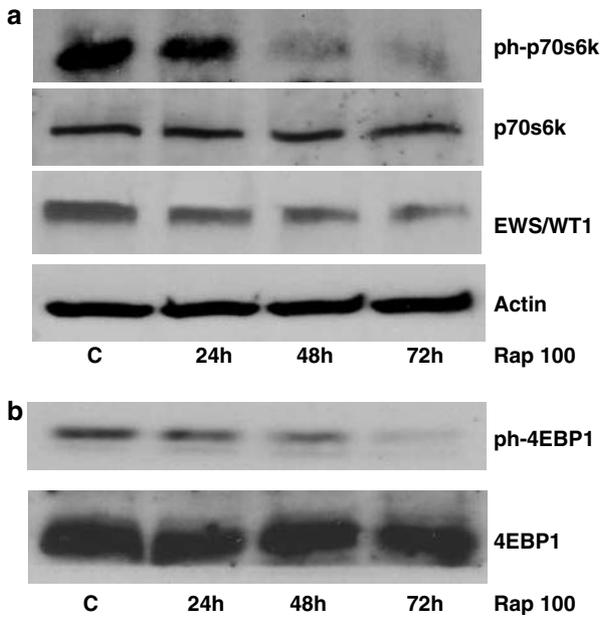


Figure 4 Rapamycin downregulates mTOR kinase targets and the EWS/WT1 protein. JN-DSRCT-1 cells were treated with 100 ng/ml rapamycin (Rap100) for 24, 48, and 72 h. The cells were then lysed and the phosphorylation status and the protein levels of p70s6k, and the protein levels of EWS/WT1 (a) as well as the phosphorylation status and the protein levels of 4E-BP1 (b) were analysed by Western blot using specific antibodies. C, untreated cells. Actin was used as a loading control

Therefore, we tested whether rapamycin would inhibit the expression of the EWS/WT1 protein in JN-DSRCT-1 cells by a similar mechanism. Results showed that, over time, rapamycin prevented the phosphorylation of p70s6k, and that this inhibition correlated with a progressive downregulation of the EWS/WT1 protein levels, which was maximal after 72 h of treatment (Figure 4a). As it has been shown that the translation initiation of the Bcl-xL protein is cap dependent (Li *et al.*, 2003), we studied the effect of rapamycin exposure on the phosphorylation state of 4E-BP1, a key mediator of this process. For this analyses, we used an anti-phospho-4E-BP1 antibody directed to the early 4E-BP1 phosphorylation sites (Thr 37 and Thr 46) because these are the mTOR-dependent, rapamycin-sensitive, relevant sites, and it has been shown that phosphorylation of these early sites is required for subsequent phosphorylations of 4E-BP1 (Gingras *et al.*, 1999). Consequently, it can be safely assumed that by preventing early phosphorylation of 4E-BP1, rapamycin also inhibits the late phosphorylation events, while the reverse would not be true. Results showed an almost complete lack of phosphorylation of 4E-BP1 that occurred as a result of exposure to rapamycin for 72 h (Figure 4b). Moreover, immunoprecipitation experiments demonstrated a greater binding between 4E-BP1 and eIF-4E proteins after 72 h of exposure to rapamycin (data not shown), an expected consequence of the dephosphorylation of 4E-BP1. Therefore, these results indicated that the downregulation of Bcl-xL was due at least in part to the inhibition of cap-dependent

translation brought about by the inhibitory action of rapamycin on mTOR and, consequently, of its targets.

Rapamycin upregulates Bax protein expression by a mechanism independent of its mTOR inhibitory activity

To ascertain whether the apoptotic effect of rapamycin was indeed mediated by its ability to inhibit mTOR, we transiently transfected a kinase-dead, dominant-negative mutant of mTOR (mTOR-KD) into JN-DSRCT-1 cells and tested them for their response to rapamycin. Results showed that, relative to cells transfected with the vector control, the percentage of apoptotic cells observed after 48 h in mTOR-KD-transfected cultures was about 25%, a level much lower than the near 60% caused by rapamycin treatment of untransfected JN-DSRCT-1 cells for a similar period of time (data not shown). Nevertheless, expression of mTOR-KD efficiently decreased the level of phosphorylation of both p70s6k (~fourfold) and 4E-BP1 (~threefold) proteins, compared to cells transfected with vector DNA alone (Figure 5a). Moreover, this reduction in phosphorylation correlated with a marked downregulation of the EWS/WT1 protein (Figure 5b), most likely due to the decreased p70s6k phosphorylation. Interestingly, the reduction in EWS/WT1 protein seen following mTOR-KD transfection (Figure 5b) was more dramatic than that observed when cells were treated with rapamycin (Figure 4a). This differential effect was probably due to the greater inhibitory specificity of the dominant negative construct for mTOR compared to rapamycin, although it could also be an effect of the different relative inhibitor concentrations. In addition, Bcl-xL protein downregulation also correlated with the decrease in 4E-BP1 phosphorylation. However, interestingly, despite of its ability to inhibit the phosphorylation of both mTOR targets, and in contrast to the effect of rapamycin treatment, inhibition of mTOR by expression of the inactive mTOR-KD mutant did not induce simultaneously a significant upregulation of Bax. With the downregulation of Bcl-xL as the only variable, the Bax:Bcl-xL ratio increased, but such increase was of lower magnitude than that elicited by rapamycin treatment (Figure 5b), which concurrently increased Bax levels. Consequently, these data explain why expression of mTOR-KD resulted in lower levels of cell death than those induced by rapamycin treatment. These results strongly suggested that the apoptotic effect of rapamycin on JN-DSRCT-1 cells was mediated, at least in part, by a mechanism independent of its mTOR inhibitory activity, which caused the upregulation of Bax.

To further demonstrate that the induction of apoptosis by rapamycin was not exclusively due to its ability to inhibit mTOR, JN-DSRCT-1 cells were transiently transfected with a rapamycin-resistant mTOR mutant (mTOR-RR) (Sekulic *et al.*, 2000) and, 24 h after transfection, treated with rapamycin (100 ng/ml) for 48 h. Both, control cells transfected with vector alone and those transfected with mTOR-RR showed indistinguishable levels of apoptosis (data not shown). Interestingly, despite of this similar response to the drug, the levels

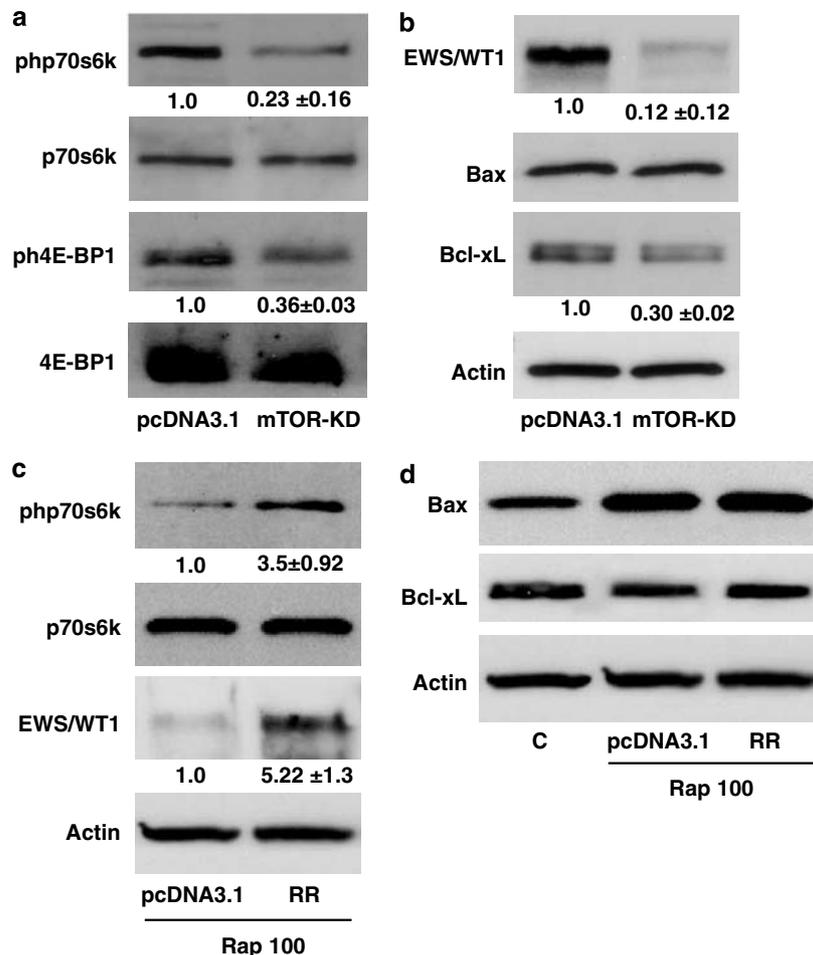


Figure 5 Expression of a kinase-dead mTOR mutant (mTOR-KD) hinders the activation of mTOR targets, whereas expression of a rapamycin-resistant mTOR mutant (mTOR-RR) does not provide protection against rapamycin-induced apoptosis. JN-DSRCT-1 cells were transiently transfected with pcDNA3.1 vector alone, a kinase-dead mutant of mTOR, or a rapamycin-resistant mTOR construct (RR). After transfection (48 h), the cells were lysed and phosphorylation status and protein levels of p70s6k, 4E-BP1 (a) and the protein levels of EWS/WT1, Bax, and Bcl-xL (b) were analysed by Western blot using specific antibodies. Total p70s6k and 4E-BP1 (a) and actin (b) were used as loading controls. On the other hand, after transfection (24 h), the cells were treated with 100 ng/ml of rapamycin (Rap100) for 48 h. At that point, the cells were lysed and the phosphorylation status and protein levels of p70s6k and the protein levels of EWS/WT1 (c), Bax, and Bcl-xL (d) were analysed by Western blot using specific antibodies. Actin was used as loading control. Quantitative data indicated below each lane for some of the Western analyses (panels a, b, and c) were normalized for loading and transfection efficiency and represent mean densitometric values (\pm s.d.) from four independent experimental replicas

of EWS/WT1 were downregulated only in cells transfected with the vector alone, but not in cells transfected with mTOR-RR. Moreover, the levels of phosphorylation p70s6k were decreased only in the cells transfected with the vector alone (Figure 5c), further demonstrating that EWS/WT1 is a target for downregulation by rapamycin. However, this downregulation did not correlate with the levels of rapamycin-induced apoptosis. Both the control cells transfected with vector alone and those transfected with mTOR-RR showed upregulation of the Bax protein, but only the cells transfected with vector alone showed downregulation of the Bcl-xL protein (Figure 5d). Taken together, these results confirmed that a mechanism other than mTOR inhibition was mediating the upregulation of Bax, an important component of the apoptotic activity of rapamycin.

Rapamycin upregulates Bax protein expression by inhibiting the proteasome complex

Several mTOR-independent activities have been reported recently for rapamycin (Zhou *et al.*, 2003; Hleb *et al.*, 2004; Schoffstall *et al.*, 2005). Among them, rapamycin has been shown to induce mRNA downregulation of three subunits of the 26S proteasome (p55, p44.5, and p27), suggesting that rapamycin inhibits the proteasome at the transcriptional level (Grolleau *et al.*, 2002). As proteasome inhibition could prevent normal Bax degradation and turnover and, consequently, result in Bax upregulation after rapamycin exposure, we analysed the mRNA levels of the proteasome subunit 26S p44.5 in JN-DSRCT-1 cells after treatment with 100 ng/ml of rapamycin for 24 h. As shown in Figure 6a, rapamycin induced about twofold downregulation of

the 26S p44.5 mRNA. Moreover, this level of down-regulation was maintained when rapamycin treatment was extended up to 72 h (Figure 6b). Most importantly, the observed changes in 26S p44.5 mRNA were closely paralleled by the effects of increasing rapamycin concentrations (Figure 6a) or treatment times (Figure 6b) on the protein levels of the 26S p44.5 subunit. These results supported the notion that the upregulation of the Bax protein induced by rapamycin exposure may occur as a consequence of the inhibition of the proteasome complex by the drug. In order to test this possibility, we treated JN-DSRCT-1 cells with MG-132, a specific inhibitor of the proteasome complex. Interestingly, results revealed that JN-DSRCT-1 cells

are highly sensitivity to MG-132. Figure 7a shows that the levels of cell death attained by exposing the cells for 24 h to a low dose (0.1 μ M) of MG-132 were very similar (about 60%) to those obtained with rapamycin after 48 h, but the levels of cell death reached almost 100% when cells were treated with higher inhibitor concentrations (10–20 μ M). Western blot analyses demonstrated that MG-132 also induced an increase in the expression levels of the Bax protein (Figure 7b), although no changes in Bax mRNA expression were detected (data not shown). Moreover, combinations of TUNEL assays and detection of cleaved caspase-3 by immunofluorescence demonstrated that the type of cell death induced by MG-132 (0.5 μ M) was apoptotic and caspase-3 dependent (Figure 7c). These results confirmed that proteasome inhibition is the likely mechanism by which rapamycin upregulates Bax levels during the induction of apoptosis of JN-DSRCT-1 cells.

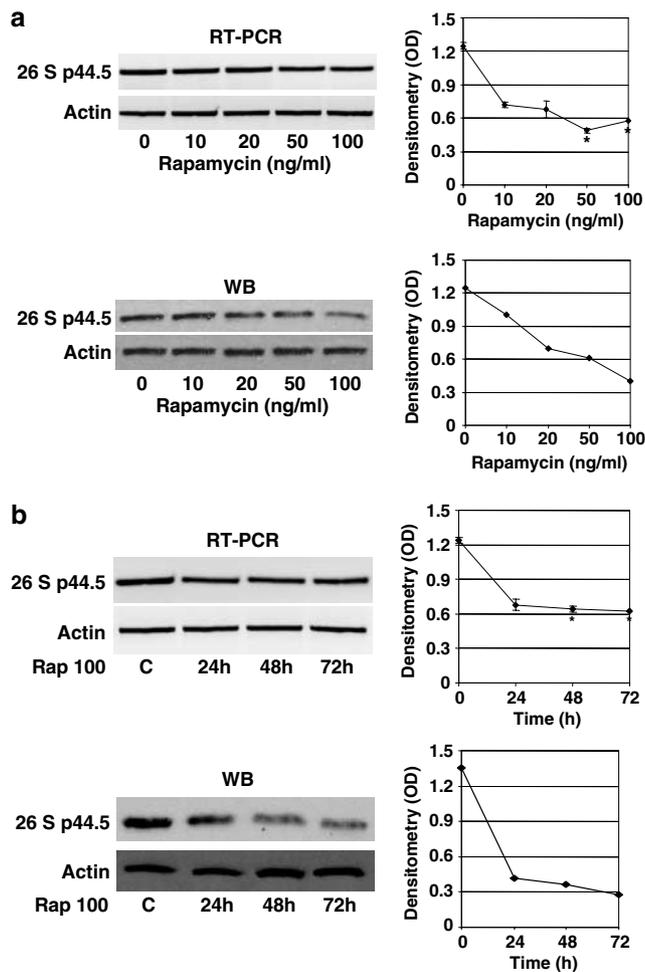


Figure 6 Rapamycin induces downregulation of the proteasome subunit 26S p44.5 mRNA and protein. Exposure of JN-DSRCT-1 cells to the indicated concentrations of rapamycin for 24 h resulted in a dose-dependent downregulation of the 26S p44.5 mRNA levels, as detected by semiquantitative RT-PCR, and of the 26S p44.5 protein, as detected by Western blot (WB) analysis (a). Time-course experiments with 100 ng/ml of rapamycin also showed the time-dependent downregulation of the 26S p44.5 levels of mRNA and protein (b). Panels at right of each gel image show the densitometric evaluation of results from RT-PCR or WB analyses. For the RT-PCR data, densitometric values are expressed as mean \pm s.d., and an ANOVA test was used to assess the significance of differences between groups. * $P < 0.05$ was regarded as significant

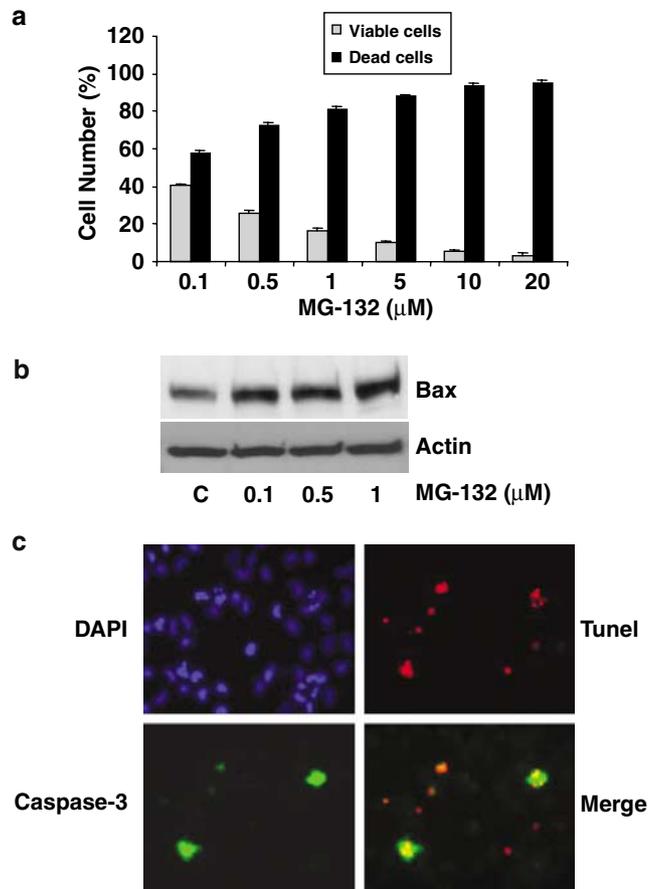


Figure 7 Proteasome inhibitor MG-132 induces apoptosis in JN-DSRCT-1 cells. Treatments of JN-DSRCT-1 cells with the indicated MG-132 concentrations were carried out for 24 h and the induction of cell death was evaluated by the trypan blue exclusion assay (a). JN-DSRCT-1 cells were treated with MG-132 (0.1, 0.5, 1 μ M), the cells were lysed and levels of the Bax protein were analysed by Western blot (b), using actin as the loading control. JN-DSRCT-1 cells were grown in slide chambers, treated with MG-132 (0.1 μ M) for 12 h. Then, apoptosis was measured by TUNEL assays (red), cleaved caspase-3 (green) was visualized by immunofluorescence using a specific antibody, and nuclear morphology was examined by staining with DAPI (c)

Discussion

DSRCT are characterized by an extremely poor prognosis due to their very limited response to conventional chemotherapy regimens. Consequently, there is a desperate need for new therapies that may improve the outcome for patients afflicted by this disease. In the majority of cases, rapamycin has been shown to exert its antitumor activity by inducing G1-phase cell cycle arrest of the various cancer cell types examined (reviewed in Huang and Houghton, 2001). A limited number of studies have reported that, besides cell cycle arrest, rapamycin induced apoptotic cell death (Hosoi *et al.*, 1999; Frost *et al.*, 2004; Majumder *et al.*, 2004). In our experimental system, results demonstrate that apoptosis induction is the primary mechanism of action of rapamycin on JN-DSRCT-1 cells, whereas it only has minor effects on the cell cycle.

Members of the Bcl-2 family are key regulators of mitochondrial cell death. This family includes some pro- (Bax, Bak, Bad) and anti- (Bcl-2, Bcl-xL) apoptotic members (reviewed in Cory *et al.*, 2003). Enhancement of the relative ratio between the protein levels of Bax and Bcl-2 or Bcl-xL has been shown to be associated with the efficient induction of apoptosis in several cancer cell lines (Mohiuddin *et al.*, 2001; Lebedeva *et al.*, 2002; Aranha *et al.*, 2003; Liu *et al.*, 2004). JN-DSRCT-1 cells express normal levels of Bax, high levels of Bcl-xL and nearly undetectable levels of Bcl-2 (data not shown). Given this pattern of protein expression, we studied the effects of rapamycin on Bax and Bcl-xL, and showed an enhancement in the Bax:Bcl-xL ratio and a concomitant activation of caspase-3. These changes were demonstrated to be at the post-transcriptional level because neither upregulation of *Bax* mRNA nor downregulation of *Bcl-xL* mRNA were observed.

As in the Ewing's sarcoma family of tumors (ESFT), a chromosomal translocation involving the *EWS* gene has been described for DSRCT (Sawyer *et al.*, 1992). Given that we previously demonstrated that rapamycin induced the downregulation of the EWS/FLI1 proteins (Mateo-Lozano *et al.*, 2003), we hypothesized that the EWS/WT1 protein may also be a target for rapamycin-induced downregulation and that this downregulation might also negatively impact cells expressing the EWS/WT1 fusion protein. Indeed, our results demonstrated that rapamycin inhibited mTOR targets, p70s6k and 4E-BP1, in a time-dependent manner and that, in parallel to p70s6k inhibition, EWS/WT1 protein levels were downregulated. However, EWS/WT1 downregulation did not correlate with rapamycin-induced apoptosis, suggesting that the known targets of this aberrant transcription factor are most likely not involved in the apoptotic process induced by rapamycin. In agreement with its distinct DNA-binding specificity relative to WT1 (Kim *et al.*, 1998a), microarray analysis has shown that several apoptosis-related genes are regulated by EWS/WT1 (-KTS) (Ito *et al.*, 2003). Interestingly, the normal WT-1 protein is also expressed in DSRCT (Ladanyi and Gerald, 1994), and its activity is believed to be overcome by the stronger activity of EWS/WT1 (Scharnhorst

et al., 2001). As the WT1 protein levels were not affected by the treatment with rapamycin (data not shown), it seems possible that WT1 activities are recovered by downregulation of EWS/WT1 and that any transcriptional consequence resulting from this downregulation is counteracted by the restored WT1 normal function.

Translation of the Bcl-xL protein has been demonstrated to be cap dependent (Polunovsky *et al.*, 1996; Tan *et al.*, 2000; Li *et al.*, 2003). Cap-dependent translation initiation is a process regulated by mTOR through phosphorylation of its known target, 4E-BP1. Once phosphorylated, 4E-BP1 releases eIF-4E, which then binds to eIG-4E forming a complex that initiates translation. Inhibition of mTOR kinase by rapamycin prevents the phosphorylation of 4E-BP1 and its subsequent binding to eIF-4E, thereby inhibiting translation initiation (reviewed in Bjornsti and Houghton, 2004). Our results indicated that, indeed, Bcl-xL protein downregulation correlated with the reduced levels of phosphorylated 4E-BP1 induced by rapamycin, thereby driving the balance towards the Bax protein in the Bax:Bcl-xL ratio. These results suggested that the mechanism by which rapamycin induced apoptosis of JN-DSRCT-1 cells was mediated, at least in part, by its ability to inhibit the mTOR-dependent pathway.

Transfection of a catalytically inactive mTOR-KD mutant has been shown to inhibit mTOR kinase activity in K562 erythroleukemia cells (Brunn *et al.*, 1997). Transfection of this same mutant into JN-DSRCT-1 cells decreased the phosphorylation of the p70s6k and 4E-BP1 proteins, at the same time that induced the downregulation of the EWS/WT1 and Bcl-xL proteins. However, inhibition of mTOR by transfection of the mTOR-KD mutant did not result in Bax upregulation or high levels of apoptosis. These results strongly suggested that the Bax upregulation component of the apoptotic process induced by rapamycin was not dependent on the ability of the drug to inhibit mTOR. To demonstrate that the increase in Bax protein was indeed independent of the inhibition of mTOR by rapamycin, we treated JN-DSRCT-1 cells with the drug after transfecting them with a rapamycin-resistant mTOR construct (mTOR-RR). This construct was used previously to demonstrate mTOR-dependent activities in other systems (Brunn *et al.*, 1997; Iiboshi *et al.*, 1998; Hosoi *et al.*, 1999; Sekulic *et al.*, 2000; Mateo-Lozano *et al.*, 2003). In our case, the mTOR-RR construct did not protect JN-DSRCT-1 cells from rapamycin-induced apoptosis. Furthermore, rapamycin was able to induce the upregulation of Bax in cells transfected with mTOR-RR construct as efficiently as in cells transfected with vector alone. The levels of apoptosis in mTOR-RR-transfected cells were slightly lower than the level reached in cells transfected with the vector alone. This may be explained by the fact that the cap-dependent translation initiation was not inhibited by rapamycin in mTOR-RR-transfected cells and the levels of Bcl-xL were higher than in vector-transfected cells. So, although there was an increase in the Bax:Bcl-xL ratio, it was of lower magnitude than that observed in untransfected, rapamycin-treated cells, as was also the activation of

caspase-3 (data not shown). This could account for the lower levels of rapamycin-induced apoptosis in mTOR-RR-transfected cells. These results confirmed that the Bax upregulation component of the apoptotic activity of rapamycin is mediated by a mechanism independent of its mTOR inhibitory activity.

Rapamycin has been reported to repress proteasome activity (Wang *et al.*, 1997) and, more recently, it was shown to downregulate the levels of mRNA of different proteasome subunits (Grolleau *et al.*, 2002) through a process independent of mTOR. Moreover, rapamycin itself has been shown to directly bind ubiquitin-1 (Davis and Soldin, 2000). Our results confirmed that rapamycin induces the transcriptional downregulation of the proteasome subunit 26S *p44.5*, suggesting that proteasome inhibition may play a role in its apoptotic effect on JN-DRSCT-1 cells. In this regard, the inhibitory action of rapamycin on the proteasome complex, and the subsequent inhibition of the normal degradation and turnover of Bax, which would allow its accumulation in the cells, seemed the most likely explanation for the upregulation of Bax after rapamycin treatment. To test this notion, we treated JN-DRSCT-1 cells with a proteasome inhibitor (MG-132). Results demonstrated that MG-132 induced levels of apoptosis similar to those induced by rapamycin, and increased the expression of the Bax protein, in agreement with previous reports (Chang *et al.*, 1998). However, in contrast to rapamycin-induced apoptosis, MG-132 induced these effects at low doses and within 24 h. These results are consistent with the facts that proteasome inhibitors induce apoptosis, have *in vivo* antitumor efficacy, and sensitize diverse malignant cells and tumors to the proapoptotic effects of conventional chemotherapeutic drugs and radiation (reviewed in Voorhees *et al.*, 2003). Therefore, the effects mediated by MG-132 at higher concentrations may involve apoptotic pathways different from those activated during the apoptotic process induced by rapamycin. Although mTOR has been reported to be involved in the transcriptional regulation of certain genes (Mayerhofer *et al.*, 2002; Erbay *et al.*, 2003), to date there are no data implicating the mTOR pathway in the transcriptional regulation of any of the proteasome subunits.

In summary, our results demonstrated that rapamycin induces apoptosis in JN-DRSCT-1 cells through a process mediated by the concurrent upregulation of Bax and downregulation Bcl-xL. Our results also demonstrated that the downregulation of the Bcl-xL protein is caused by the mTOR-dependent inhibition of its cap-dependent translation initiation, and strongly supported that the upregulation of the Bax protein may be promoted by inhibition of the proteasome complex caused by rapamycin in an mTOR-independent fashion. By working simultaneously, these processes increased the Bax:Bcl-xL ratio, thereby resulting in caspase-3 activation and triggering apoptosis.

Rapamycin, a complex macrolide antibiotic with potent fungicide action frequently used as an immunosuppressant, has been shown recently to have anticancer effects (Geoerger *et al.*, 2001; Guba *et al.*, 2002; Huang and Houghton, 2002; Frost *et al.*, 2004) and its

derivatives RAD001 and CCI-779 are currently in phase I and phase II clinical trials, respectively, as anticancer agents. The primary mechanism by which rapamycin and its analogues potentially suppress tumor cell growth is by arresting cells at the G1 phase of the cell cycle. However, in the vast majority of the cases, rapamycin and its analogues do not induce apoptosis of the tumor cells and, consequently, their potential application as single therapeutic agents is limited. In contrast, the fact that rapamycin is a potent apoptosis inducer in JN-DRSCT-1 cells, strongly suggests that this compound will most likely show high level of efficacy against DRSCT. Our results strongly suggest the inclusion of rapamycin in therapeutic protocols for the treatment of patients diagnosed with DSRCT, a tumor type highly refractory to other more conventional therapies.

Materials and methods

Culture conditions, antibodies, and general reagents

JN-DRSCT-1 cells were a gift from Dr Jun Nishio (Fukuoka University, Japan). Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (Biofluids, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA) and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) at 37°C, in an atmosphere of 5% CO₂ and 95% air. Polyclonal antibodies against Bax, Bcl-xL, caspase-3, p70s6k, phospho-p70s6k, eIF-4E, 4E-BP1, and phospho(Thr37/46)-4E-BP1 were purchased from Cell Signaling Technology (Beverly, MA, USA). The WT-1 antibody used to detect the EWS/WT1 fusion was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The proteasome 26S *p44.5* subunit (PSMD11) was detected with a polyclonal antibody from Orbigen (San Diego, CA, USA). Oligonucleotide primers were from Bio-Synthesis (Lewisville, TX, USA). Rapamycin and MG-132 were purchased from Sigma (St Louis, MO, USA). Trypan blue was obtained from Gibco.

Transient transfections

Expression vectors encoding AUI-tagged kinase dead (mTOR-KD) and rapamycin-resistant (mTOR-RR) mTOR cDNA mutants were generously provided by Dr Robert Abraham (The Burnham Institute, La Jolla, CA, USA). These vectors and the pcDNA3.1 control plasmid (4 µg of each) were transfected using Lipofectamine (Life Technologies) as recommended by the manufacturers. Transfection efficiency, which ranged from 72 to 76% in all cases, was monitored by Western analysis using a monoclonal antibody specific for the AUI tag (Covance, Berkeley, CA, USA) and by amplification of a 262 bp ampicillin fragment from the vector sequences that was carried out using the following primers: CGATACGGGAGGGCTTAC (upper) and GCCATACCAAACGACGAG (lower).

Apoptosis and cell cycle assays

Apoptosis was evaluated by viable cell counting and TUNEL assays. Cell viability was determined by the trypan blue exclusion method: cells were suspended in 0.04% trypan blue in phosphate-buffered saline (PBS), placed on a hemocytometer, and counted under the microscope (Lohrum and Vousden, 1999). TUNEL assays were performed for the *in situ* detection of apoptotic cells using the TMR red-based In Situ Death Detection kit from Roche Diagnostics

(Indianapolis, IN, USA). Cells were cultured in two-chamber slides (Nunc, Naperville, IL, USA) to a density of 5×10^4 cells per chamber. At 16 h after rapamycin exposure, cells were washed with PBS and incubated with the TUNEL reaction mixture for 1 h, at 37°C, in a humidified atmosphere in the dark. TUNEL-positive cells were visualized with a Nikon E600 fluorescence microscope. For cell cycle analysis, cells were harvested 24, 48, and 72 h after exposure to rapamycin, washed once in PBS, fixed in citrate buffer, pH 7.6, resuspended in PBS containing 20 µg/ml of propidium iodide (Calbiochem-Novabiochem Corp., San Diego, CA, USA), and incubated for 30 min at 37°C before flow cytometric analysis on a FACScan instrument (Becton Dickinson, San Jose, CA, USA), performed at the Flow Cytometry/Cell Sorting Shared Resource of the Vincent T Lombardi Comprehensive Cancer Center.

RT-PCR assays

RNA was extracted with the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA (3 µg) was reversed transcribed using 200 U of Superscript II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in a 20 µl reaction volume, in the presence of 25 µg/ml Oligo(dT), first-strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂), 10 mM dithiothreitol, and 10 mM of each dATP, dGTP, dCTP, and dTTP. The mixture of RNA and Oligo(dT) was heated at 70°C for 10 min and cooled to 4°C; then, all other reagents were added and reverse transcription was performed at 42°C for 50 min. PCR primers for *Bax*, *Bcl-xL*, and *actin* were designed using the Oligo 6.0 software from National Biosciences (Plymouth, MN, USA), with GenBank published sequences as templates. The primer set for *EWS/WT1* has been previously published (Nishio *et al.*, 2002). A 279-bp *Bax* fragment was amplified with primers GAGGATGATTGCC GCCGTGGAC (upper) and CGGTGGTGGGGGTGAGGAGG (lower). A 715-bp fragment of *Bcl-xL* was amplified with primers GAGTGAGCAGGTGTTTTGGAC (upper) and CCACAGTCATGCCCGTCAG (lower). A 239-bp fragment of proteasome subunit *26S p44.5* was amplified with primers ACTCCATCGTGAAGCGTGAC (upper) and GACCTCCTGCCCTGTAGC (lower). The primer set CGGGACCTGACTGACTACCTC (upper) and CTTCATTGTGCTGGGTGC (lower) was used to amplify actin, as standard for semiquantitative RT-PCR determinations. Amplifications were carried out using a 2700 Perkin-Elmer thermocycler (Applied Biosystems, Foster City, CA, USA) and consisted of amplification cycles in which denaturation was performed at 94°C for 15 s; annealing was at 60°C for *Bax*, 58°C for *Bcl-xL*, and 55.3°C for *26S p44.5*; and extension at 72°C for 45 s. As a routine housekeeping reference, conditions for actin amplification were always identical to those used for the transcript under investigation. For each set of cDNA-specific primers, the number of cycles were adjusted so that the reaction end points fell within the exponential phase of product amplification (Coenen Schimke *et al.*, 1999; Wang *et al.*, 1999), thus providing us with a semiquantitative estimate of relative mRNA abundance. PCR products were resolved on 1.5% agarose gels and quantified using the Molecular Analyst Macintosh data analysis software and a Bio-Rad (Hercules, CA, USA) Image Analysis System.

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Amplification products were purified using the QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions, and sequenced using an ABI Prism 310 system (Perkin-Elmer). All experiments involving RT-PCR were carried out twice, each time in duplicate, thus allowing the analysis of four replicated RNA preparations.

Immunoprecipitation and Western blot analysis

Cells were lysed in TNES buffer (immunoprecipitation) or RIPA buffer (Western blot) containing protease inhibitors (1 mM PMSF, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) and the lysates were centrifuged at 13 000 g, at 4°C, for 30 min. Protein content in the supernatants was determined with the BCA Protein Assay System (Pierce, Rockford, IL, USA). Immunoprecipitations were performed at 4°C by incubation of lysates with 1 µg/ml specific antibodies and 50 µl of protein A agarose beads (Upstate, Lake Placid, NY, USA) for 2–3 h in TNES buffer. After washing, the immunoprecipitated proteins were analysed by SDS-PAGE. For Western blot experiments, proteins (30 µg) in cell extracts were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in PBS containing 0.2% Tween-20, membranes were incubated at 4°C overnight with the different antibodies. Blots were then incubated for 1 h at room temperature with HRP-conjugated secondary antibody (1:2000) and the peroxidase activity was analysed with the ECL chemiluminescence substrate system (Amersham Biosciences, Piscataway, NJ, USA).

Immunofluorescence

Cells grown in two-chamber slides were fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature, rinsed and permeabilized with 0.4% Triton X-100 in PBS, for 30 min. Permeabilized cells were then incubated for 30 min at room temperature with 10% donkey serum in PBS to block nonspecific binding. After thorough rinsing with PBS, cells were incubated for 1 h at 37°C with anticaspase-3 antibody followed by 30 min at 37°C with fluorescein isothiocyanate (FITC)-coupled anti-rabbit antibody. Cells were rinsed again with PBS and twice with distilled water, and mounted in Vectashield® mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA, USA), which provided nuclear fluorescent counterstaining, and visualized using a Nikon E600 fluorescence microscope. Appropriate controls were maintained by substituting the primary antibody with normal donkey and/or rabbit serum to check for nonspecific binding.

Acknowledgements

This work was supported by US Public Health Service Grant PO1-CA74175 from the National Cancer Institute, NIH. Partial support was also provided by the Microscopy and Imaging Macromolecular Analysis and the Flow Cytometry/Cell Sorting Shared Resources of the Vincent T Lombardi Comprehensive Cancer Center, funded through US Public Health Service Grant 2P30-CA51008.

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