Oncogene (2001) 20, 6172–6180

www.nature.com/onc

Damaged microtubules can inactivate BCL-2 by means of the mTOR kinase

Angela Calastretti[1], Anna Bevilacqua[1], Cristina Ceriani[1], Simona Viganò[1], Paola Zancai[2], Sergio Capaccioli[3] and Angelo Nicolin*[,1]

[1]*Department of Pharmacology, University of Milan, Milan, Italy;* [2]*Division of Experimental Oncology, Centro di Riferimento Oncologico, Aviano, Italy;* [3]*Institute of General Pathology, University of Florence, Florence, Italy*

Rapamycin, a specific inhibitor of the serine/threonine mTOR kinase, markedly inhibited both cell growth and apoptosis in human B-cell lines. Besides arresting cells in G_1 by increasing $p27^{kip1}$, rapamycin tripled the cellular level of the BCL-2 protein. The activity was dose-dependent and specific for the $p27^{kip1}$ and BCL-2 proteins. Rapamycin did not affect *bcl-2* mRNA although it increased cellular BCL-2 concentration by inhibiting phosphorylation, a mechanism initiating the decay process. To add new insight, we combined rapamycin treatment with treatment by taxol, which, by damaging microtubules, can phosphorylate BCL-2 and activate apoptosis. It was found that the mTOR kinase was activated in cells treated with taxol or with nocodazole although it was inhibited in cells pre-treated with rapamycin. BCL-2 phosphorylation, apoptosis and hyperdiploidy were also inhibited by rapamycin. In contrast, taxol-induced microtubule stabilization or metaphase synchronization were not inhibited by rapamycin. Taken together, these findings indicate that mTOR belongs to the enzymatic cascade that, starting from damaged microtubules, phosphorylates BCL-2. By regulating apoptosis, in addition to the control of a multitude of growth-related pathways, mTOR plays a nodal role in signaling G_1 and G_2-M events. *Oncogene* (2001) **20,** 6172–6180.

Keywords: apoptosis; BCL-2; mTOR; microtubules; p27; rapamycin

Introduction

Besides human follicular B-cell lymphomas, in which the *bcl-2* gene was originally found (Tsujimoto *et al*., 1984; Bakhshi *et al*., 1985; Cleary *et al*., 1986), *bcl-2* over-expression has been observed in a variety of human tumors, thus providing the first indication of BCL-2 involvement in human cancer (Silvestrini *et al*., 1994; Korsmeyer *et al*., 1990). Many other studies in experimental systems (McDonnell *et al*., 1996) such as *bcl-2* transgenic (Strasser *et al*., 1990; Adams and Cory, 1991) or knock-out mice (Nakayama *et al*., 1993; Korsmeyer, 1998; Veis *et al*., 1993) have directly related BCL-2 antiapoptotic activity to neoplastic transformation and/or progression. BCL-2 protein has been shown to lower cell sensitivity to chemotherapy and radiotherapy (Reed *et al*., 1996; Kroemer, 1997). On the contrary, reduced *bcl-2* expression has been associated to degenerative disorders including Parkinson's disease or Alzheimer's disease (Saille *et al*., 1999).

Many attempts have been made to develop biological or pharmacological means of modulating *bcl-2* expression (Konig *et al*., 1997; Itano *et al*., 1996; Siegel *et al*., 1998). Antisense compounds have shown biological activity in switching off *bcl-2* expression in cell (Capaccioli *et al*., 1996; Morelli *et al*., 1996, 1997), and are currently under clinical evaluation for anti-lymphoma activity (Webb *et al*., 1997) and for chemosensitization (Jansen *et al*., 2000). Both microtubule-stabilizing agents such as taxol and microtubule-damaging compounds such as nocodazole have been shown to mediate phosphorylation of BCL-2 protein at serine residues in the loop region (Srivastava *et al*., 1999; Blagosklonny *et al*., 1996); phosphorylation inactivates the antiapoptotic effect. Microtubule damage induced by pharmacological means may trigger apoptosis in cancer cells through a mechanism involving BCL-2 phosphorylation (Haldar *et al*., 1996). Moreover, arrest of cells in G_2-M phase consequent on damage to the mitotic apparatus may be responsible for triggering a physiological mechanism of cell demise. The biochemical pathways that link microtubule damage with the BCL-2 phosphorylation-inactivation leading the cells to activate apoptotic programs, are not yet well known (Yamamoto *et al*., 1999; Li *et al*., 1998).

Previously, in lymphoid cells, we observed that the cell cycle arrest in G_1 phase by rapamycin was regulated by the amount of the BCL-2 protein expressed at the steady state. These observations prompted to study whether rapamycin might regulate the BCL-2 pathway and apoptosis in cells treated with taxol, a pro-apoptotic compound that damages microtubules and down-regulates BCL-2.

*Correspondence: A Nicolin, Department of Pharmacology, University of Milan, Via Vanvitelli 32, Milan 20129, Italy;
E-mail: angelo.nicolin@unimi.it
Received 20 December 2000; revised 14 June 2001; accepted 26 June 2001

In mammalian cells, the immunophilin-binding immunosuppressant rapamycin was shown to inhibit mammalian target of rapamycin (mTOR/RAFT-1/FRAP), a serine/threonine kinase whose function is not entirely understood (Sehgal, 1998). In yeast, rapamycin-mediated inhibition of TOR1 and TOR2, two kinases homologous to human mTOR, induces a starvation-like response (Schmidt *et al*., 1998) and can hinder the cell cycle-dependent organization of the cytoskeleton (Bickle *et al*., 1998). In human, mTOR is mainly implicated in the signaling of cell growth and proliferation from growth factors and amino acids abundance (Hara *et al*., 1998; Nave *et al*., 1999). More recently, mTOR has been shown to control an unusually abundant and diverse set of readouts, eliciting to the spatial and temporal control of balanced cell growth (Schmelzle and Hall, 2000; Choi *et al*., 2000).

In this study human follicular B-cell lymphoma lines endowed with high levels of BCL-2 at steady state were exposed to rapamycin. We found that rapamycin was able to increase cellular levels of BCL-2 protein in addition to $p27^{kip1}$ protein. Prior treatment of cells with rapamycin inhibited taxol-induced apoptosis and DNA endoreduplication (Stewart *et al*., 1999), while having no effect on microtubule stabilization and on arrest in the G_2-M phase. Prediction was made that the mTOR kinase was activated by antimicrotubules agents and might be included in the kinase cascade whereby the apoptosis triggered by microtubule damage is brought about.

Result

Rapamycin induces the selective accumulation of BCL-2 and $p27^{kip1}$ proteins

In a program to study the molecular mechanisms regulating *bcl-2* expression, we found that rapamycin inhibited the cell growth of follicular lymphoma cell lines very efficiently. In three human follicular t(14; 18)-positive cell lines, cells were arrested in the G_1 phase of the cell cycle at doses not effective in t(14; 18)-negative cell lines (Table 1). The activity of rapamycin was dose-dependent and at 1 ng/mL the percentage of G_1 fraction was 36% higher than in the untreated control. Moreover, rapamycin did not increase the hypodiploid cell fraction and in preliminary studies apoptosis induced by pharmacological means was reduced.

These findings and those of others (Kitamura *et al*., 1997; Migita *et al*., 1997) prompted us to measure the cellular levels of BCL-2 protein and of the relevant mRNA. These last were not modified upon exposure to rapamycin (not shown), whilst those of the BCL-2 protein increased up to threefold upon the same treatment. As expected, the cellular level of the $p27^{kip1}$ protein underwent the same fate (Figure 1a). In the same assays, the concentrations of the apoptosis-related protein BCL-X_L or BAX and of the cell-cycle regulators p21, CDK 2 and Cyclin E were not augmented. The increases of BCL-2 and $p27^{kip1}$ proteins were dependent on the rapamycin concentration. The levels reached a peak after 24 h and were still high after 48 h (Figure 1b).

These findings indicated that a post-transcriptional mechanism could underlie the rapamycin-mediated BCL-2 increase as it does with $p27^{kip1}$ (Vlach *et al*., 1997).

Rapamycin inhibits taxol-induced BCL-2 phosphorylation, DNA endoreduplication and apoptosis

The rapamycin-mediated inactivation of the kinase cascade starting from mTOR might account for the rapamycin-mediated inhibition of BCL-2 phosphorylation. By contrast, taxol treatment can phosphorylate BCL-2 causing functional inactivation. Cells were incubated 24 h with rapamycin and then treated with taxol for further 24 h. As shown in Figure 2, rapamycin prevented taxol-induced BCL-2 phosphorylation in a dose-dependent fashion. In these studies taxol has been used within the clinical relevant doses

Table 1 Growth rate and cell cycle analysis of lymphoma cells treated with rapamycin

Cell treatments		*Growth rate*		*Cell cycle*			
Cell line	*Rapamycin* (ng/mL)	*Cell number* $\times 10^3 \pm$ *s.e.m.*	*MTT assay OD* $\times$ *s.e.m.*	*Hypo*	G_1	S	G_2/M
$DOHH_2$	–	424 ± 33	1250 ± 78	8	38	32	22
	3	132** ± 27	483** ± 50	9	59**	17	15
	1	196** ± 21	539** ± 42	9	49**	25	17
	0.3	217** ± 29	667** ± 61	8	44*	27	21
SU-DHL 4	–	640 ± 50	1416 ± 69	7	36	36	21
	3	169** ± 21	450** ± 36	7	52**	22	19
	1	268** ± 33	610** ± 51	7	48**	24	21
	0.3	380* ± 37	825** ± 59	5	40*	35	20
K-422	–	563 ± 49	1322 ± 75	10	31	33	26
	3	258** ± 15	611** ± 63	10	48**	17	25
	1	373* ± 36	803** ± 86	10	45**	20	25
	0.3	437* ± 41	989* ± 91	8	37*	32	23

Rapamycin was given as a single dose at the indicated concentrations on day 0. On day 3, cultures were arrested and cells were counted under the microscope and analysed by the MTT assay. Assays were performed in triplicate and data are representative of 4–6 experiments. Cell cycle was studied in cells incubated for 24 h in the presence of 3, 1 or 0.3 ng/mL of rapamycin, respectively. The percentage distribution over the cell cycle was measured by flow cytometry. $^{**}P \leqslant 0.005$, $^{*}P \leqslant 0.05$

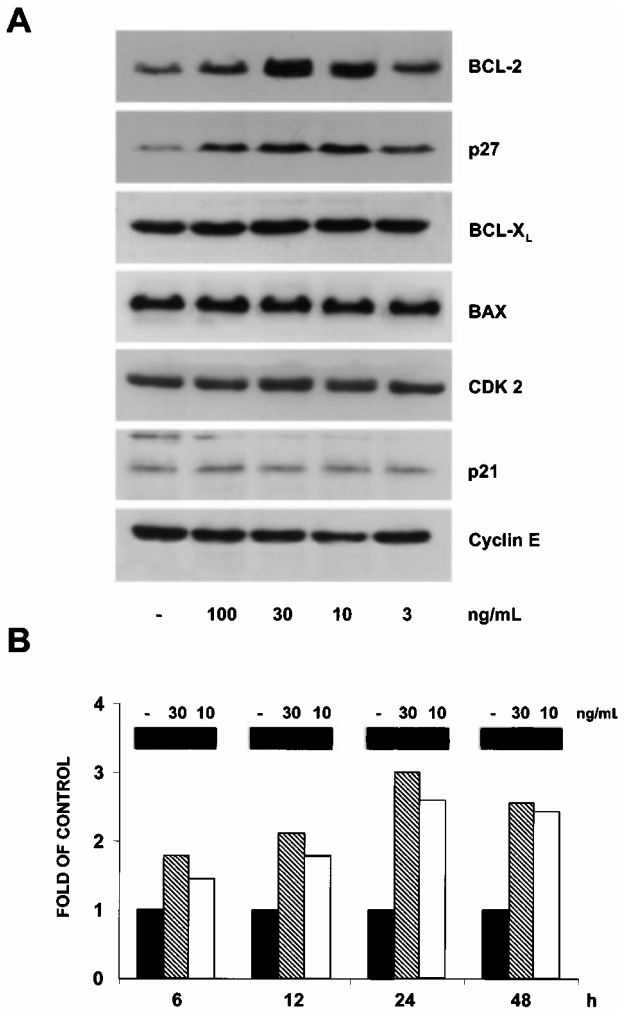

Figure 1 Increased expression of BCL-2 and p27^{kip1} proteins in lymphoma line treated with rapamycin. (**a**) $DOHH_2$ cells were treated for 24 h with rapamycin at the indicated doses. Cell extracts, 25 μg of proteins or 12 μg for BCL-2 determination, were separated by SDS–PAGE, Western blotted and analysed for the expression of the relevant proteins as indicated. All blots were probed for β-actin and assays were repeated three times. (**b**) $DOHH_2$ cells, treated with 30 or 10 ng/mL rapamycin, were analysed for BCL-2 protein expression by Western blot for the indicated periods

(5–200 nM) (Blagosklonny and Fojo, 1999) or at higher doses to understand the magnitude of inhibition of phosphorylation by a low dose of rapamycin.

The biochemical mechanisms underlying taxol activity were studied in some detail in the rapamycin-pretreated cells, with more emphasis in the BCL-2 overexpressing lymphoma B-cell lines. At the end of the pharmacological treatments, genomic DNA was stained with propidium iodide and analysed by flow cytometry. Figure 3 shows that combined treatment with rapamycin and taxol caused the arrest in G_2-M phase of a higher number of cells (70% of total cells) than taxol alone (50% of cycling cells). Taxol was probably more effective than rapamycin in arresting cell cycle causing G1 arrested cells by rapamycin to accumulate into the more definitely G_2-M phase arrest.

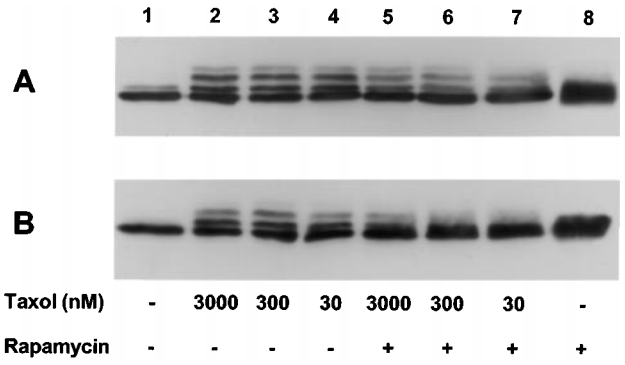

Figure 2 Inhibition of taxol-induced BCL-2 phosphorylation by rapamycin. $DOHH_2$ cell line (**a**) or K422 cell line (**b**) treated with rapamycin, 10 ng/mL at time 0, on day 1 were untreated or treated with taxol at the indicated doses for a further 24 h. Cell extracts were analysed in Western blot with an anti-BCL-2 MoAb. The hypophosphorylated form of BCL-2, not reduced by taxol, lanes 2–4 *versus* lane 1, was reduced in rapamycin pre-treated cells, lanes 5–7 *versus* lane 8

A second observation was that rapamycin totally inhibited the ability of taxol to induce mitotic slippage shown as >4N in Figures 3 and 4. Thus, taxol did not relieve the inhibition of DNA synthesis by rapamycin, which entirely blocked endoreduplication. Cytometry analysis also showed that rapamycin protected cells from taxol-induced apoptosis.

The DNA distribution in rapamycin pre-treated cells exposed to increasing doses of taxol was extensively studied in lymphoma lines including three t(14; 18) lymphoma lines. Rapamycin was confirmed to inhibit with high efficiency the biological effects of taxol as studied by flow cytometry. In particular, apoptosis was reduced significantly and hyperploidy completely, in spite of the heavy alteration produced by a high dose of taxol as shown in Figure 4.

Taxol-mediated microtubule stabilization is not impaired in cells pretreated with rapamycin

Since the main biochemical effect of taxol is to stabilize the microtubule apparatus, which activates the apoptotic program, we studied the effect of taxol on microtubule stabilization in rapamycin-pretreated cells. Figure 5a was obtained by staining microtubules with fluorescent α-tubulin antibodies and shows that the stabilization of microtubules by 2 h exposure of cells to taxol was not impaired by prior treatment with rapamycin. Figure 5b shows that the accumulation of cells treated with taxol in the prometaphase is not significantly inhibited by a pre-treatment with rapamycin.

Taxol or nocodazole activate the serine/threonine mTOR kinase

Because taxol phosphorylates BCL-2 protein and rapamycin inhibits this phosphorylation by acting on mTOR, we investigated whether taxol might induce the activation of mTOR. Taxol indeed induced the

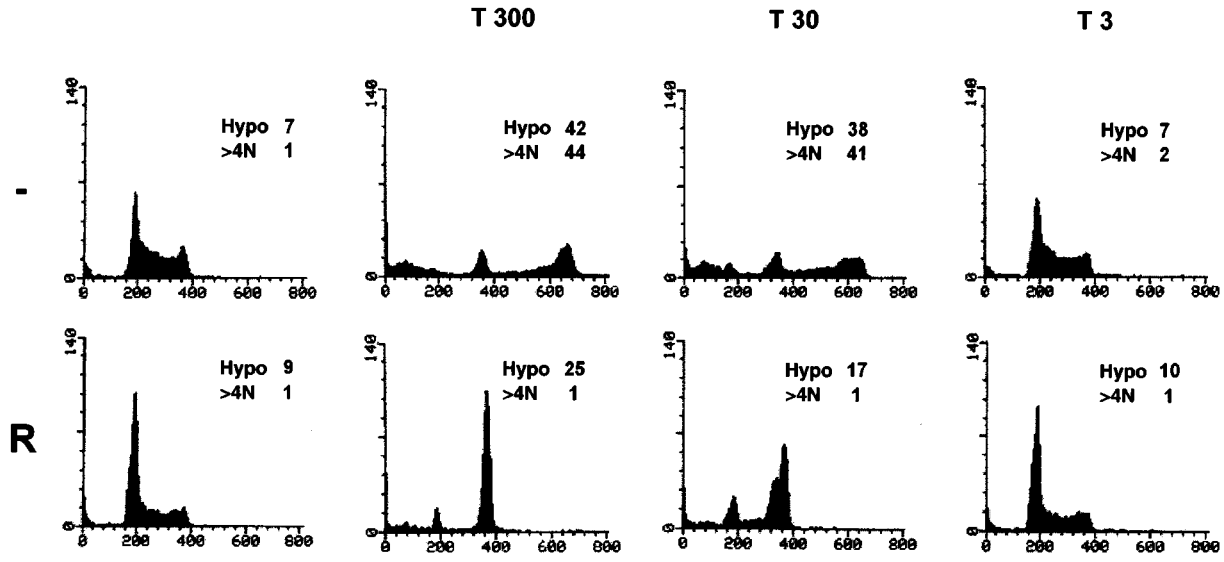

Figure 3 DNA distribution in rapamycin pre-treated cells exposed to taxol treatments. $DOHH_2$ cells, treated with rapamycin, 10 ng/mL at time 0, on day 1 were untreated or treated with taxol at 300, 30 or 3 nM for a further 24 h. Cells, stained with propidium iodide, were analysed by flow cytometry. Upper rows, no rapamycin (−); lower rows, rapamycin 10 ng/mL (R). In the top-right of the histograms are indicated the percentage of hypodiploid cells (Hypo) or hyperdiploid cells (>4N), respectively. Histograms are representative of 4–5 experiments

phosphorylating activity of mTOR (Figure 6, upper panel) without affecting its steady-state level in cells, as shown in Figure 6 (lower panel). Extracts from K422 cells, treated 24 h with different doses of taxol, were immunoprecipitated with both mTab1 and mTab2 rabbit antibodies and resuspended in the kinase buffer containing recombinant PHAS-I or recombinant BCL-2. mTOR immunoprecipitates from cells treated with 300 or 30 nM of taxol were able to phosphorylate PHAS-I, while 3 nM was almost ineffective. However, a direct activity of mTOR on BCL-2 might be excluded since recombinant BCL-2 in the kinase buffer was not phosphorylated by activated mTOR (not shown).

Figures 7 and 8 show that mTOR activity triggered by taxol or nocodazole, respectively, was significantly reduced in the immunoprecipitated samples from cells pre-treated with rapamycin. The Western blot determination of mTOR in the immunoprecipitates ruled out the possibility that reduced activity might be dependent on the failure of antibody to immunoprecipitate the rapamycin-FKBP12-mTOR complex.

Therefore, mTOR, the molecular target for the biological effects of rapamycin, recently strengthened by studies in cells carrying mTOR resistant to rapamycin, strongly associate it to the kinase cascade that phosphorylates BCL-2 in mitotic arrested cells (Brunn *et al*., 1997).

Discussion

Very important pathways of cell life such as protein synthesis, cell proliferation and apoptosis are often controlled in a coordinated fashion (Lundberg and Weinberg, 1999; Guo and Bruce, 1999) by growth factors. Rapamycin, by inhibiting protein synthesis (Crossin and Carney, 1981; Terada *et al*., 1995) and arresting the cell-cycle in G_1 (Hashemolhosseini *et al*., 1998; Metcalfe *et al*., 1997) is able to induce a 'growth-factor withdrawal' condition (Schmidt *et al*., 1998). Whether starvation conditions induced by rapamycin can play some role in the regulation of the apoptotic programs activated by pharmacological means is not yet well known (Abraham, 1998).

Rapamycin activates an anti-apoptotic program

The biochemical events triggered by rapamycin were studied in three human follicular B-cell lymphoma lines that contain a high concentration of BCL-2 protein (Aiello *et al*., 1992; Steube *et al*., 1995) and are most sensitive to rapamycin. The lowest doses of rapamycin inhibited growth and arrested the cell lines in the G_1 phase. In these studies it was also noticed that cell cultures were protected from spontaneous apoptosis and from apoptosis induced by treatments with taxol. Inactivation of the apoptotic program prompted our studies on the expression of the *bcl-2* gene in cells exposed to rapamycin. We observed that the cellular concentration of the *bcl-2* mRNA was unchanged, whereas that of BCL-2 protein was significantly increased in a dose-dependent fashion and correlated with the increased level of $p27^{kip1}$ protein (Koff and Polyak, 1995; Alessandrini *et al*., 1997; Cordon-Cardo *et al*., 1998). The increased level of the proteins by rapamycin was specific for BCL-2 and $p27^{kip1}$, since the cell concentrations of neither the apoptosis-related proteins BCL-X_L and BAX nor the cell-cycle regulators p21, CDK-2 and cyclin-E were changed. This observation suggested that BCL-2 and

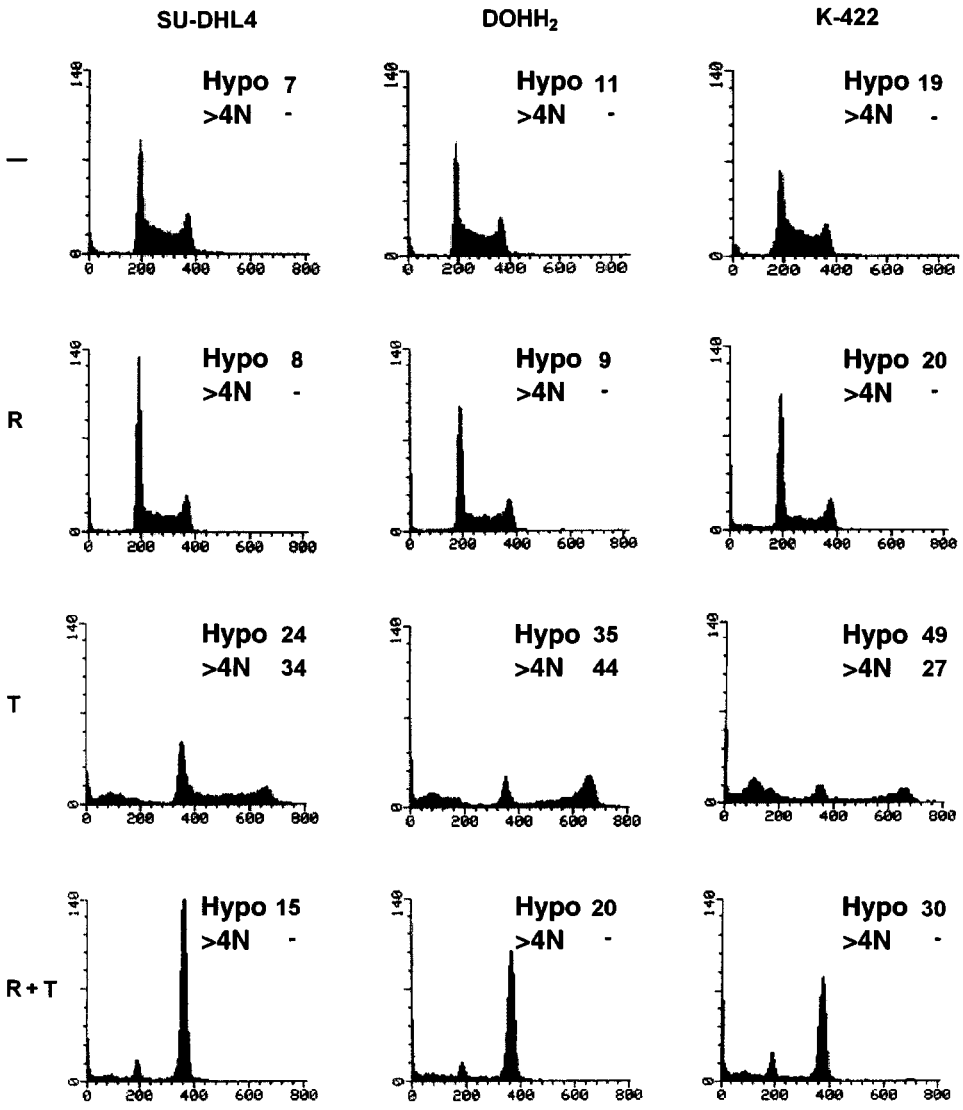

Figure 4 DNA distribution in rapamycin pre-treated t(14; 18) cells exposed to taxol at high dose. SU-DHL-4, $DOHH_2$ and K422 cell lines, were untreated (−), treated with rapamycin, 48 h at 10 ng/mL (R), treated with taxol, 24 h at 300 nM (T), or pre-treated with rapamycin, 24 h at 10 ng/mL, and with taxol for a further 24 h at 300 nM (R + T). Cells were processed as in Figure 3 and histograms are representative of three experiments

$p27^{kip1}$ could be modulated by common pathways or a coordinated mechanism. Such biochemical modifications are associated with the activation of an anti-apoptotic program and with the arrest of cells in the G_1 phase.

Damaged microtubules activate the mTOR kinase

Taxol can induce BCL-2 phosphorylation at position serine (Srivastava *et al*., 1999; Haldar *et al*., 1997) causing the inactivation of BCL-2 (Chadebech *et al*., 1999) and consequent loss of anti-apoptotic function. BCL-2 phosphorylation and the pro-apoptotic program triggered by taxol in the lymphoma cells were greatly inhibited by prior treatment with rapamycin in a dose-response manner. Moreover, prior treatment with rapamycin completely inhibited the taxol-induced hyperdiploidy but did not prevent taxol-induced microtubule damage.

The inhibition of BCL-2 phosphorylation despite microtubule alteration, prompted us to study the biochemical signaling activated by microtubules. Our attention has been focused on the threonine/serine mTOR kinase. The immunoprecipitation studies provided evidence that treatment with taxol or nocodazole activated mTOR which indicated an important role in the kinase cascade regulating BCL-2 phosphorylation and loss of function.

In the first part of the work we show that rapamycin is able to increase the cellular level of the BCL-2 protein and protect lymphoma cells from apoptosis. The increase of BCL-2 protein is achieved by inhibition of BCL-2 phosphorylation and might be more evident in cells containing a large amount of

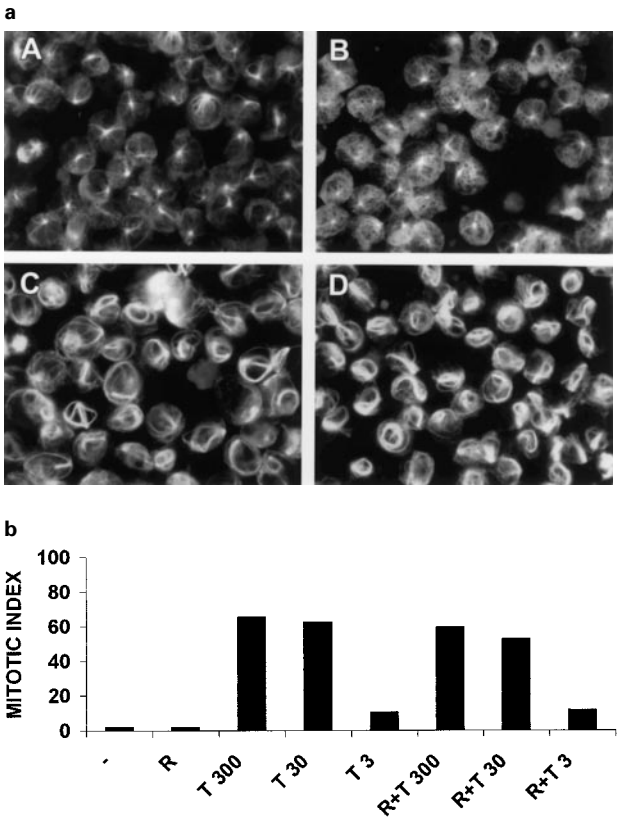

Figure 5 (**a**) Microtubule stabilization by taxol in cells previously treated with rapamycin. $DOHH_2$ cells were: untreated (**a**), treated with rapamycin, 22 h at 10 ng/mL (**b**), untreated 22 h and treated with taxol, 2 h at 200 nM (**c**), treated with rapamycin, 22 h at 10 ng/mL and with taxol 2 h at 200 nM. Cells were stained with fluoresceinated anti-tubulin monoclonal antibody and photographed at 63 × magnification. (**b**) Mitotic Index. $DOHH_2$ cells, treated with rapamycin, 10 ng/mL at time 0, on day 1 were untreated or treated with taxol at 300, 30 or 3 nM for a further 24 h, and then stained with eosine-hematoxylin. The mitotic index was determined by scoring 1500 cells on each sample. Values are expressed as the percentage of the mitotic index of control cells. Samples were run in triplicate and data represent mean values from three independent experiments

BCL-2 in the steady state. We show here that agents that damage microtubules can induce BCL-2 phosphorylation-inactivation through a pathway that involves the mTOR kinase. This pathway might be a physiological mechanism for the elimination of cancer cells with damaged mitotic apparatus (Haldar *et al*., 1997). In contrast, impaired mTOR activity, such as that mediated by rapamycin, might hinder the elimination of damaged cells inducing resistance to anticancer therapy or protecting tissues from degeneration.

In this scenario, the role of $p27^{kip1}$ remains to be elucidated. Taxol has never been directly demonstrated to inactivate $p27^{kip1}$ by phosphorylation. The concept of $p27^{kip1}$ inactivation might not be in contrast with the observation of hyperdiploidy in cells hampered to divide because of damage of mitotic apparatus. However, the possibility that taxol does not enter in the regulation of $p27^{kip1}$ already inactivated by signals from the growth factors cannot be ruled out.

In conclusion, damaged microtubules can induce an apoptotic program by activating a biochemical cascade that includes serine/threonine kinases and mTOR. The findings discussed here might indicate a key role for the mTOR kinase in relevant metabolic steps of the G_2-M phase in addition to the role of central controller of cell growth. A coordinate regulation of cell cycle and apoptosis by means of mTOR might be envisaged.

Material and methods

Cell lines and drugs

The human follicular B-cell lymphoma lines $DOHH_2$ (Kluin-Nelemans *et al*., 1991), SU-DHL-4 (Cleary *et al*., 1986) and K422 (Dyer *et al*., 1990), carrying the t(14; 18) chromosomal translocation, all mycoplasma-free, were grown in RPMI

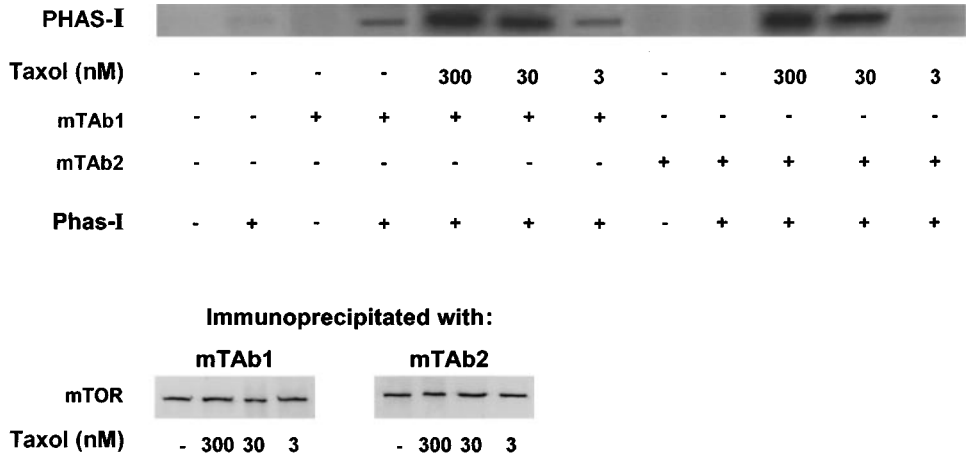

Figure 6 mTOR protein kinase activity from cells treated with taxol. K422 cells, treated with taxol, 24 h at 300, 30 or 3 nM, were lysed and extracts, quantitated by Western blottings (lower panel), were immunoprecipitated either with mTAb1 or with mTAb2 rabbit antibodies, respectively. The substrate for the kinase reactions was the human recombinant PHAS-I. The products were separated by SDS–PAGE and detected by autoradiography. Data are representative of three assays

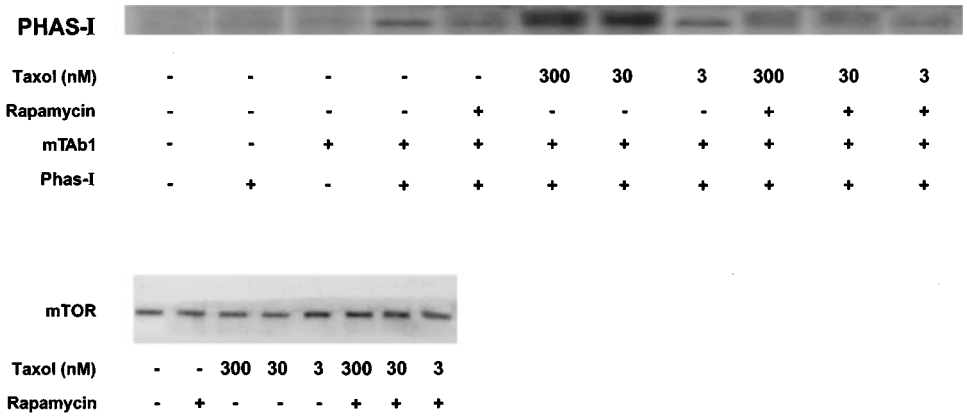

Figure 7 mTOR protein kinase activity from cells pre-treated with rapamycin and treated with taxol. K422 cells, treated with rapamycin, 10 ng/mL at time 0, on day 1 were untreated or exposed to taxol for a further 24 h at the doses indicated. Cell extracts were obtained and processed as described in Figure 6. Data are representative of three assays

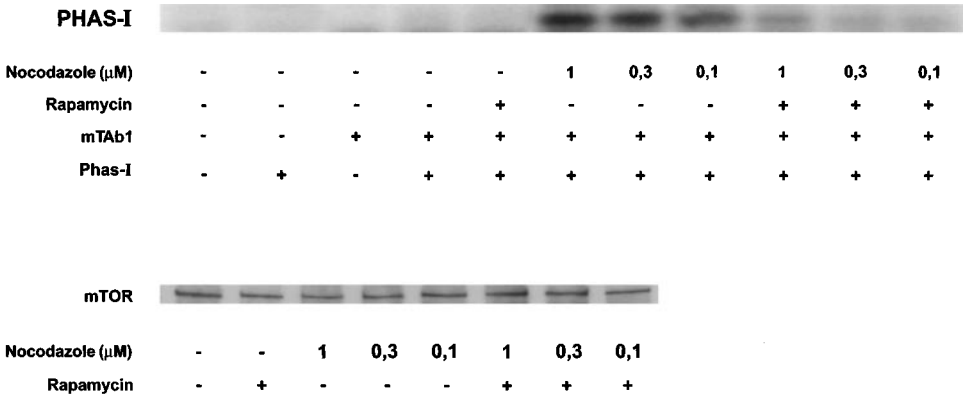

Figure 8 mTOR protein kinase activity from cells pre-treated with rapamycin and treated with nocodazole. DOHH2 cells, treated with rapamycin, 10 ng/mL at time 0, on day 1 were untreated or exposed to 1, 0.3, 0.1 μM nocodazole for a further 24 h at the doses indicated. Cell extracts were obtained and processed as described in Figure 6. Data are representative of three assays

1640 medium (Mascia Brunelli, Milan, Italy) containing 10% heat-inactivated FCS (HyClone Laboratories, UT, USA), 2 mM glutamine, 50 IU/mL penicillin and 50 μg/ml streptomycin (Sigma-Aldrich, Milan, Italy) at 37°C in a 5% CO_2 atmosphere. The nucleotide sequence of the bcl-2/IgH joining region of the t(14; 18)-positive cells were frequently checked. Rapamycin, kindly supplied by Dr Sehgal (Wyeth-Ayerst Research, NJ USA), was dissolved in absolute ethanol to generate a 2 mM solution and small aliquots of the stock solution were stored at −80°C. Taxol and Nocodazole (Sigma-Aldrich) were dissolved in DMSO to generate a 1 mM solution or 10 mM solution, respectively and small aliquots of the stock solutions were stored at −20°C.

Growth rate assay

Cultures, $25-100\times10^3$ cells/mL in 24-well plates (Greiner), split twice weekly to avoid crowding, were washed, resuspended in RPMI 1640 medium (Mascia Brunelli) containing 10% FCS and the same glutamine and antibiotic concentrations as above and exposed to rapamycin as indicated. Growth rate and viability of the cells were determined by the Trypan blue exclusion assay and by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based colorimetric assay (Mosmann, 1983).

Western blot analysis of the proteins

Samples of 4×10^6 cells, treated with rapamycin as indicated, were collected by low-speed centrifugation and washed twice in ice-cold PBS plus 1 mM sodium orthovanadate. The cell pellet was resuspended in 60 μL of lysis buffer (ice-cold RIPA buffer with freshly added protease inhibitors), vortexed for 3 s and incubated on ice for 30 min. The lysates were centrifuged at high speed for 20 min at 4°C. Five μL of the supernatant was removed for protein determination, transferred to a microfuge tube, mixed with reducing buffer and heated to 99°C for 2 min. Equal amounts of proteins, unless specified, were analysed by 12% SDS–PAGE, blotted onto PVDF membranes (Immobilon P, Millipore, Bedford, MA, USA) in a Bio-Rad Trans-blot apparatus at 100 V for 90 min. Blots were processed by an enhanced chemiluminescence (ECL Plus) detection kit as instructed by the supplier (Amersham Pharmacia Biotech, UK). The blots were probed with a mouse anti-BCL-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by a horseradish peroxidase, conjugated secondary antibody. The same blots were then

probed for $p27^{kip1}$ and BAX (DAKO, Milan, Italy), p21 (NeoMarkers, Freemont, CA, USA), BCL-X_L (Pharmingen, San Diego, CA, USA), CDK2 and Cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and for β-actin (Sigma-Aldrich) using appropriate antibodies.

Western blot analysis for the phosphorylated form of BCL-2 protein

Samples of 8×10^6 cells, treated with rapamycin and taxol as indicated, were collected and resuspended in 320 μL of Laemmli buffer (Laemmli, 1970), boiled for 2 min, protease inhibitors were added and the mixture was sonicated for 20 s. Supernatants containing protein extracts were collected and a 1 : 10 volume of BMG (glycerol:β-mercaptoethanol: bromophenol-blue, 1%/2 : 5 : 5) was added; they were then boiled for 3 min and stored at -20°C. Protein concentration was determined using the Micro BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Aliquots of 130 μg were size-separated by SDS–PAGE (12%, 1.5 mm thick gel −0.8% Bis-acrylamide/30% acrylamide) at 80 V overnight and blotted onto a PVDF membranes (Immobilon P) in a BIO-RAD Trans-Blot semi-dry apparatus at 15 V for 60 min. Immunodetection was carried out using an anti-BCL-2 (DAKO, Milan, Italy) monoclonal antibody (1 : 100), and blots were successively developed with peroxidase anti-mouse antibody (1 : 2000), using the enhanced chemiluminescence kit (Pratesi *et al*., 2000).

Analysis of cellular DNA content by flow cytometry

Cells treated as indicated were collected and washed in PBS for 10 min at 1500 r.p.m., then permeabilized with 200 μL of PBS containing 0.1% Saponin and 0.5 mg/mL RNAse (type IIIA, Sigma-Aldrich) for 30–40 min at 37°C. Eight hundred μL of DNA-staining solution, 0.1% saponin and propidium iodide (25 μg/mL in PBS), were added for 30 min at 4°C (Nicoletti *et al*., 1991). The cellular DNA content was analysed by flow cytometry with a FACscan (Becton Dickinson, CA, USA). Histograms of cell frequency *vs* propidium iodide fluorescence intensity, which is proportional to DNA content, were analysed using the Lysis II software system (Becton Dickinson).

Microtubule staining and Mitotic Index

The distribution of α-tubulin was analysed by the indirect immunofluorescence technique (Schiff and Horwitz, 1980). The cells, treated as indicated, were fixed and permeabilized for 5 min with methanol at -20°C, washed with PBS 1X and incubated with monoclonal anti-α-tubulin conjugated with FITC (1 : 75 in PBS + 0.05% Triton X) (Sigma-Aldrich), in a humid atmosphere for 90 min at 37°C and in the dark. After two washes with PBS and with water, respectively, the preparations were mounted with 70% glycerol, 10% PBS and 1 mg/mL p-phenylenediamine (Sigma-Aldrich). The cover-slips were viewed with a Zeiss Axioskop (Germany) microscope equipped with epifluorescent optics. Pictures were taken with an oil-immersion objective (63 ×) on Ektachrome 400 Kodak film.

$DOHH_2$ cells, treated with rapamycin, 10 ng/mL at time 0, on day 1 were untreated or treated with taxol at 300, 30 or 3 nM for a further 24 h and then collected, washed twice in PBS for 10 min at 1500 r.p.m. and stained with eosine-hematoxylin. The mitotic index was determined by scoring at least 1500 cells on each sample.

mTOR activity

mTOR was immunopurified as described in Brunn *et al*. (1997) with minor modifications. Briefly, K422 cells, treated with taxol, were washed in cold PBS and lysed at 4°C in a solution containing 50 mM Tris/HCL, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 0.1 mM PMSF, 5 μg/mL leupeptin, 1 μg/mL aprotinin, 0.2% Triton X-100, 0.3% Nonidet P-40. Lysates, clarified by centrifugation at 13 000 r.p.m. for 25 min at 4°C, were immunoprecipitated by the rabbit antibodies mTAb1 or mTAb2 (kindly provided by Dr JC Lawrence) (Brunn *et al*., 1997). The immune complexes were suspended in 50 μL of kinase buffer containing 10 mM $MgCl_2$, 20 mM Tris/HCl pH 7.4, 1 mM DTT, 30 μM ATP, 1 μg [His6]-PHAS-I (Stratagene, CA, USA), 5 μCi of [γ-^{32}P]ATP and analysed by SDS–PAGE electrophoresis. Radiolabeled PHAS-I was detected by autoradiography. Incorporation of ^{32}P into PHAS-I was quantified with an Ambis Imaging system.

Acknowledgments

We thank Dr JC Lawrence for the Tab1 and Tab2 antibodies, Dr F Navone for the assistance in microtubule staining, Dr P Castagnoli and Dr A Costa for assistance in the flow cytometry and Prof R Silvestrini for assistance in the mitotic index. Finally, we thank Drs D Delia, JC Lawrence and A Quattrone for invaluable suggestions. This work was supported by grants from AIRC, Milan; CNR, Project ACRO; MURST and Istituto Superiore di Sanità, Rome.

References

Abraham RT. (1998). *Curr. Opin. Immunol.,* **10,** 330–336.
Adams JM and Cory S. (1991). *Science,* **254,** 1161–1167.
Aiello A, Delia D, Borrello MG, Biassoni D, Giardini R, Fontanella E, Pezzella F, Pulford K, Pierotti M and Della Porta G. (1992). *Cytometry,* **13,** 502–509.
Alessandrini A, Chiaur DS and Pagano M. (1997). *Leukemia,* **11,** 342–345.
Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL and Korsmeyer SJ. (1985). *Cell,* **41,** 899–906.
Bickle M, Delley PA, Schmidt A and Hall MN. (1998). *EMBO J.,* **17,** 2235–2245.
Blagosklonny MV and Fojo T. (1999). *Int. J. Can.,* **83,** 151–156.
Blagosklonny MV, Schulte T, Nguyen P, Trepel J and Neckers LM. (1996). *Cancer Res.,* **56,** 1851–1854.
Brunn GJ, Fadden P, Haystead TAJ and Lawrence Jr JC. (1997). *J. Biol. Chem.,* **272,** 32547–32550.
Brunn GJ, Hudson CC, Sekulic A, Williams JM, Hosoi H, Houghton PJ, Lawrence Jr JC and Abraham RT. (1997). *Science,* **277,** 99–101.
Capaccioli S, Quattrone A, Schiavone N, Calastretti A, Copreni E, Bevilacqua A, Canti G, Gong L, Morelli S and Nicolin A. (1996). *Oncogene,* **13,** 105–115.
Chadebech P, Brichese L, Baldin V, Vidal S and Valette A. (1999). *Biochem. Biophys. Res. Comm.,* **262,** 823–827.

Choi JH, Adames NR, Chan T, Zeng C, Cooper JA and Zheng XFS. (2000). *Curr. Biol.,* **10,** 861–864.
Cleary ML, Smith SD and Sklar J. (1986). *Cell,* **47,** 19–28.
Cordon-Cardo C, Koff A, Drobnjak M, Capodieci P, Osman I, Millard SS, Gaudin PB, Fazzari M, Zhang ZF, Massague J and Scher HI. (1998). *J. Natl. Cancer Inst.,* **90,** 1284–1291.
Crossin KL and Carney DH. (1981). *Cell,* **27,** 341–350.
Dyer MJ, Fischer P, Nacheva E, Labastide W and Karpas A. (1990). *Blood,* **75,** 709–714.
Guo M and Bruce AH. (1999). *Curr. Opin. Cell Biol.,* **11,** 745–752.
Haldar S, Basu A and Croce CM. (1997). *Cancer Res.,* **57,** 229–233.
Haldar S, Chintapalli J and Croce CM. (1996). *Cancer Res.,* **56,** 1253–1255.
Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C and Avruch J. (1998). *J. Biol. Chem.,* **273,** 14484–14494.
Hashemolhosseini S, Nagamine Y, Morley SJ, Desrivieres S, Mercep L and Ferrari S. (1998). *J. Biol. Chem.,* **273,** 14424–14429.
Itano Y, Ito A, Uehara T and Nomura Y. (1996). *J. Neurochem.,* **67,** 131–137.
Kitamura Y, Kosaka T, Shimohama S, Nomura Y and Taniguchi T. (1997). *Jpn. J. Pharm.,* **75,** 195–198.
Kluin-Nelemans HC, Limpens J, Meerabux J, Beverstock GC, Jansen JH, De Jong D and Kluin PM. (1991). *Leukemia,* **5,** 221–224.
Koff A and Polyak K. (1995). *Prog. Cell Cycle Res.,* **1,** 141–147.
Konig A, Schwartz GK, Mohammad RM, Al-Katib A and Gabrilove JL. (1997). *Blood,* **90,** 4307–4312.
Korsmeyer SJ. (1998). *Trends Genet.,* **11,** 101–105.
Korsmeyer SJ, McDonnell TJ, Nunez G, Hockenbery D and Young R. (1990). *Curr. Top. Microbiol. Immunol.,* **166,** 203–207.
Kroemer G. (1997). *Nat. Med.,* **3,** 614–620.
Jansen B, Wacheck V, Heere-Ress E, Schlagbauer-Wadl H, Hoeller C, Lucas T, Hollenstein U, Wolff K and Pehamberger H. (2000). *Lancet,* **356,** 1728–1733.
Laemmli UK. (1970). *Nature,* **227,** 680–685.
Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC and Altieri DC. (1998). *Nature,* **396,** 580–584.
Lundberg AS and Weinberg RA. (1999). *Eur. J. Cancer,* **35,** 1886–1894.
McDonnell TJ, Beham A, Sarkiss M, Andersen MM and Lo P. (1996). *Experientia,* **52,** 1008–1017.
Metcalfe SM, Canman CE, Milner J, Morris RE, Goldman S and Kastan MB. (1997). *Oncogene,* **15,** 1635–1642.
Migita K, Eguchi K, Ichinose Y, Kawabe Y, Tsukada T, Aoyagi T and Nagataki S. (1997). *Clin. Exper. Immunol.,* **108,** 199–203.
Morelli S, Alama A, Quattrone A, Gong L, Copreni E, Canti G and Nicolin A. (1996). *Anti Cancer Drug Des.,* **11,** 1–14.
Morelli S, Delia D, Capaccioli S, Quattrone A, Schiavone N, Bevilacqua A, Tomasini S and Nicolin A. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 8150–8155.
Mosmann T. (1983). *J. Immunol. Meth.,* **65,** 55–63.
Nakayama K, Nakayama K, Negishi I, Kuida K, Shinkai Y, Louie MC, Fields LE, Lucas PJ, Stewart V, Alt FW and Loh DY. (1993). *Science,* **261,** 1584–1588.
Nave BT, Ouwens M, Withers DJ, Alessi DR and Shepherd PR. (1999). *Biochem. J.,* **344,** 427–431.
Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C. (1991). *J. Immunol. Met.,* **139,** 271–279.
Pratesi G, Polizzi D, Perego P, Dal Bo L and Zunino F. (2000). *Biochem. Pharmacol.,* **60,** 77–82.
Reed JC, Miyashita T, Takayama S, Wang HG, Sato T, Krajewski S, Aime-Sempe C, Bodrug S, Kitada S and Hanada M. (1996). *J. Cell. Biochem.,* **60,** 23–32.
Saille C, Marin P, Martinou JC, Nicole A, London J and Ceballos-Picot I. (1999). *Neurosci.,* **92,** 1445–1463.
Schiff PB and Horwitz SB. (1980). *Proc. Natl. Acad. Sci. USA,* **77,** 1561–1565.
Schmelzle T and Hall MN. (2000). *Cell,* **103,** 253–262.
Schmidt A, Beck T, Koller A, Kunz J and Hall MN. (1998). *EMBO J.,* **17,** 6924–6931.
Sehgal SN. (1998). *Clin. Biochem.,* **31,** 335–340.
Siegel DS, Zhang X, Feinman R, Teitz T, Zelenetz A, Richon VM, Rifkind RA, Marks PA and Michaeli J. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 162–166.
Silvestrini R, Veneroni S, Daidone MG, Benini E, Boracchi P, Mezzetti M, Di Fronzo G, Rilke F and Veronesi U. (1994). *J. Natl. Cancer Inst.,* **86,** 499–504.
Srivastava RK, Mi QS, Hardwick JM and Longo DL. (1999). *Proc. Natl. Acad. Sci. USA,* **96,** 3775–3780.
Steube KG, Jadau A, Teepe D and Drexler HG. (1995). *Leukemia,* **9,** 1841–1846.
Stewart ZA, Leach SD and Pietenpol JA. (1999). *Mol. Cell. Biol.,* **19,** 205–215.
Strasser A, Harris AW, Bath ML and Cory S. (1990). *Nature,* **348,** 331–333.
Terada N, Takase K, Papst P, Nairn AC and Gelfand EW. (1995). *J. Immunol.,* **155,** 3418–3426.
Tsujimoto Y, Finger LR, Yunis JJ, Nowell PC and Croce CM. (1984). *Science,* **226,** 1097–1099.
Veis DJ, Sorenson CM, Shutter JR and Korsmeyer SJ. (1993). *Cell,* **75,** 229–240.
Vlach J, Hennecke S and Amati B. (1997). *EMBO J.,* **16,** 5334–5344.
Webb A, Cunningham D, Cotter F, Clarke PA, Di Stefano F, Ross P, Corbo M and Dziewanowska Z. (1997). *Lancet,* **349,** 1137–1141.
Yamamoto K, Ichijo H and Korsmeyer SJ. (1999). *Mol. Cell. Biol.,* **19,** 8469–8478.