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Rapamycin increases the cellular concentration of the BCL-2 protein and exerts an anti-apoptotic effect

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Abstract

The immunosuppressant rapamycin, an immunophilin-binding antibiotic, has been studied in follicular B-cell lymphoma lines that express the highest level of the BCL-2 protein. The growth rate of human follicular B-cell lymphoma lines was slowed more efficiently than that of other human B-cell lines or non-B-cell lines. This effect was dependent on the arrest of cells in the G_1 phase; the number of apoptotic cells was not increased. Rapamycin inhibited apoptosis or caspase activation induced by cytotoxic drugs, whereas caspase activation by doxorubicin was not inhibited. The increase in the cellular concentration of BCL-2 protein was related to its concentration in the steady state and was unrelated to the amount of *bcl-2* mRNA. The increase of BCL-2 level in the cells rather than its level in the steady state may be important for drug resistance. The biochemical target of rapamycin, the mTOR kinase, may be a candidate sensitising agent for chemotherapy. This effect of rapamycin shows that G_1 arrest and protection from apoptosis are combined events susceptible to regulation by pharmacological means. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Apoptosis; BCL-2 protein; Rapamycin; G_1 arrest; B-cell lymphoma

1. Introduction

The BCL-2 protein plays a central role in cell survival and in the prevention of apoptosis induced by various agents in different cell types [1]. Many studies have provided evidence that the *bcl-2* proto-oncogene, first identified as a site involved in a t(14;18) chromosomal translocation in human follicular B-cell lymphomas [2], is involved in human malignancies, including breast cancer, lung cancer and hormone-resistant prostate cancer [3]. Moreover, BCL-2 protein has been shown to decrease cell sensitivity to chemo- and radiotherapy [4].

BCL-2 is highly expressed in neuroblastoid cells [5] and in the B-cell lineages [6] that are most sensitive to the immunosuppressant rapamycin [7]. The mechanisms by which rapamycin can inhibit the B cells and exert the anti B-cell lymphoma activity are unknown. In mammalian cells, rapamycin inhibits with high specificity the enzyme known as FRAP/RAFT/mTOR [8,9], a serine-threonine kinase homologue of TOR1 and TOR2, originally identified in yeast cells [10]. In yeast, rapamycin simulates starvation, by inhibiting protein synthesis and arresting cell growth [11]. In mammals, rapamycin mimics the withdrawal of growth factors, characterised by arrest of cells in the G_1 phase [12] and inhibition of protein synthesis [13].

Cells are arrested in the G_1 phase probably by an increase in the cellular level of the anti-mitotic protein $p27^{kip1}$ [14] and because inhibition of the initiator factor eIF4E1 prevents the synthesis of proteins [15]. Growth factor withdrawal and rapamycin treatment both slow down fundamental pathways by establishing the metabolic conditions for cells to survive in an adverse environment. Starvation might be related to the 'non-growing'-G_0 fraction in primary tumours. The relevance of the non-growing fraction to the failure of chemotherapy in human cancer is well established [16].

In preliminary studies, growth arrest was preferentially induced by rapamycin in cell lineages expressing the highest levels of BCL-2 protein, which are

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potentially endowed with a survival advantage. Our studies were designed to elucidate whether rapamycin plays a role in the expression of BCL-2 or whether the high level of BCL-2 might facilitate the anti-lymphoma activity of rapamycin.

We have compared rapamycin treatment of human follicular B-cell lymphoma lines, characterised by a high level of BCL-2 protein in the steady state [6,17], with that of lymphoma cell lines expressing a lower level. Rapamycin's anti-lymphoma activity correlated with the amount of BCL-2 expressed by the cells and rapamycin increased the cellular concentration of both $p27^{kip1}$ and BCL-2; this increase arrested cells in the G_1 phase and activated an anti-apoptotic programme.

2. Materials and methods

2.1. Cell lines

Human cell lines, follicular B-cell lymphoma lines $DOHH_2$, SU-DHL-4 and K422, carrying the t(14;18) chromosomal translocation [18,19], human t(14;18)-negative B-cell lines Burkitt lymphoma Raji and Namalwa [20], human T-cell leukaemias Jurkat and MOLT-4 [21], human erythroleukaemia K562 and promyelocytic leukaemia HL-60, all mycoplasma-free, were routinely maintained at 37 °C and 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium containing 1% L-glutamine, 1% penicillin-streptomycin and 10% heat-inactivated fetal calf serum (HyClone Laboratories, UT, USA). The nucleotide sequence of the *bcl-2*/IgH joining region of the t(14;18)-translocated cells was frequently checked.

NIH 3T3 fibroblasts transfected with plasmids inserted with the rabbit β-globin-ARE of the *bcl-2* RNA, transcriptionally driven by the serum-inducible c-*fos* promoter, were obtained as described in Ref. [22], and maintained in Dulbecco's modified Eagle's medium.

2.2. Growth rate assay and cell cycle analysis

Cell viability and growth rate were determined by the Trypan Blue dye-exclusion assay and by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based colorimetric assay [23]. Cells seeded in 24-well plates were treated with rapamycin kindly supplied by Dr Sehgal (Wyeth-Ayerst Research, NJ, USA). Doxorubicin, etoposide and paclitaxel (Sigma-Aldrich, Milan, Italy) were used in rapamycin pretreated cells.

The distribution of DNA in the cell cycle was studied by flow cytometry. Cells were washed, permeabilised and exposed for 30 min at 4 °C to 800 μl of DNA-staining solution in 0.1% Nonidet P-40 (Sigma-Aldrich, Milan, Italy) and 25 μg/ml propidium iodide (Sigma-Aldrich) [24]. The cellular DNA content was analysed by fluorescent activated cell sorter (FACS)can (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) using Cell Quest software system for histograms of cell frequency versus propidium iodide fluorescence intensity.

2.3. Northern and western blot analysis

Total RNA, extracted by the guanide isothiocyanate method, was determined by Northern blot analysis [25]. Briefly, 15–30 μg of RNA was electrophoresed on 1% denaturing agarose gel, blotted onto a nylon membrane (Roche Diagnostics, Monza, Italy), hybridised overnight with digoxigenin-labelled DNA probes in 50% dimethylformamide at 50 °C, and visualised by chemiluminescence using secondary antibodies coupled to alkaline phosphatase. The autoradiograms were quantified by densitometry. *Bcl-2* mRNA level was normalised to α-glyceraldehyde-3-phosphate dehydrogenase (*α*/*GAPDH*) RNA.

The BCL-2 protein level was determined as follows. Briefly, 4×10^6 cells, treated with rapamycin as indicated, were collected, washed twice in phosphate-buffered solution (PBS) plus 1 mM sodium orthovanadate. The cell lysates obtained with cold radio-immune precipitation assay buffer (RIPA) buffer and protease inhibitors were centrifuged, mixed with reducing buffer and heated to 99 °C for 2 min. Opportune amounts of protein were analysed by 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) blotted onto PVDF membranes (Immobilon P, Millipore, Bedford, MA, USA) in a BIO-RAD Trans-blot apparatus (Hercules, CA, USA) at 100V 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 antibody 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 β-actin with appropriate antibodies (Sigma-Aldrich, Milan, Italy).

2.4. Caspase activation assay

Three μg/3 μl of proteins, obtained from cells (1×10^6) resuspended in 100 μl of lysis buffer (10 mmol/l HEPES, pH 7.4; 0.1% CHAPS; 2 mmol/l ethylene-diammine-tetra-acetic acid (EDTA); 2 mmol/l dithiothreitol (DTT)), were incubated in 500 μl of reaction buffer (20 mmol/l HEPES, pH 7.4, 10% (v/v) glycerol, 2 mmol/l DTT). Cleavage of Ac-Asp-Glu-Val-Asp-alpha-(4-methyl-coumaryl-7-amide) (DEVD-MCA, Peptide Co, Osaka, Japan), 20 μmol/l in the dark at 37 °C for 2 h, was determined fluorimetrically and read in a Kontron spectrophotofluorometer (Perkin-Elmer, Monza, Italy) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm [26].

3. Results

3.1. Growth rate of rapamycin-treated cells

We focused on the biological activity of rapamycin in human lymphoma cell lines SU-DHL-4, K422 and $DOHH_2$, all carrying the t(14;18) chromosome translocation, in t(14;18)-negative B-cell lines Raji or Namalwa, in T-cell lines Jurkat or MOLT-4, in human erythroleukaemia K562 or in promyelocytic leukaemia HL-60. Table 1 shows that 72 h of exposure to rapamycin reduced the number of viable cells in a dose-response manner. The t(14;18)-positive cell lines were the most sensitive.

Fig. 1 shows cell-growth assays in 4-week cultures. Cells were treated with rapamycin at 3 ng/ml and counted by microscopy. Viable cells were split at the initial concentration twice a week and re-exposed to the original concentration of rapamycin. The total cell number was calculated at the end of the culture × the

Table 1
Viability in lymphoid cell lines treated with rapamycin

Cell line	Rapamycin (ng/mL)	Viable cells (10^3/ml±S.E.M.) (%)	(%)	BCL-2 level
$DOHH_2$	–	788±48	–	+++ [6]
	1	643±30	(18)	
	3	476±31**	(40)	
	10	466±36**	(41)	
	30	300±35**	(62)	
SU-DHL-4	–	1066±52	–	+++ [17]
	1	943±55	(13)	
	3	843±42*	(21)	
	10	728±55*	(32)	
	30	603±40**	(44)	
K422	–	936±35	–	+++ [6]
	1	726±38*	(22)	
	3	588±46**	(37)	
	10	520±40**	(44)	
	30	498±32**	(47)	
Raji	–	1253±88	–	++ [27]
	1	1183±77	(7)	
	3	1091±68	(13)	
	10	957±58*	(24)	
	30	891±62*	(29)	
Namalwa	–	1150±65	–	++ [6]
	1	1010±71	(12)	
	3	990±41	(14)	
	10	903±55*	(21)	
	30	810±42*	(30)	
Jurkat	–	1150±50	–	+ [6]
	1	1060±70	(8)	
	3	985±65	(14)	
	10	921±70*	(20)	
	30	871±60*	(24)	
MOLT-4	–	1300±56	–	+ [6]
	1	1260±63	(3)	
	3	1103±74	(15)	
	10	981±80*	(25)	
	30	931±71*	(28)	
K562	–	1250±73	–	– [6]
	1	1155±65	(8)	
	3	1086±61	(13)	
	10	980±50*	(22)	
	30	895±52*	(28)	
HL-60	–	1075±68	–	+ [6,28]
	1	1045±68	(3)	
	3	915±50*	(15)	
	10	845±61*	(21)	
	30	785±55*	(27)	

Cells were treated with rapamycin for 72 h at the indicated doses. Viability of cells were assessed by the trypan blue assay. Experiments were performed in triplicate. Data are means±standard error of the mean (S.E.M.) of 3–5 experiments. $^{**}P \leqslant 0.001$, $^{*}P \leqslant 0.01$ versus no rapamycin by Student's *t*-test.

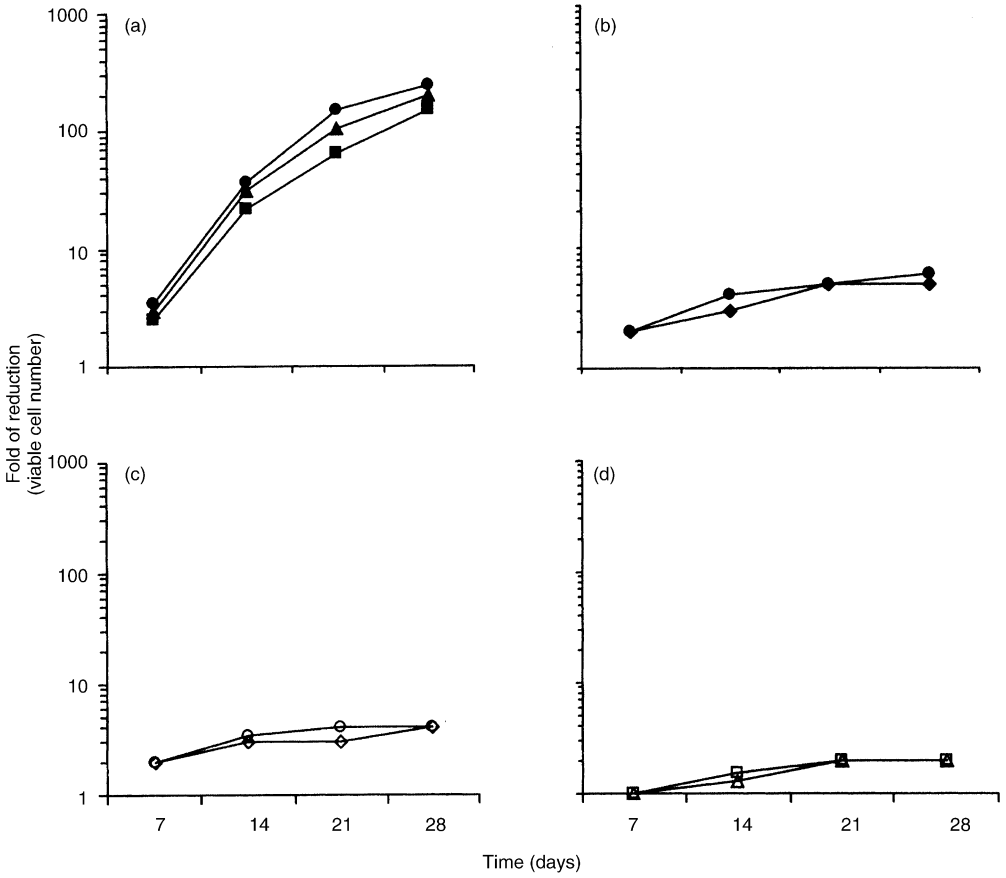

Fig. 1. Growth inhibition of lymphoid cell lines exposed to 3 ng/ml rapamycin for 4 weeks. (a) SU-DHL-4 (■), $DOHH_2$ (▲) and K422 (■); (b) Raji (●) and Namalwa (◆); (c) MOLT-4 (○) and Jurkat (◇); (d) K562 (□) and HL-60 (△).

number of splits × the fold dilutions at each splitting and the reduction was calculated compared with untreated controls. The dose-dependent response and the preferential action on defined cell types were confirmed.

These studies show that growth inhibition was not associated with an increased percentage of dead cells (see also Fig. 2 and Table 2), but was apparently related to the steady-state level of BCL-2 in the cells [6,17,27,28].

3.2. Cell-cycle analysis and caspase activation

The potent inhibition of growth and the lack of cytotoxic effects as observed under the microscope prompted us to study the DNA distribution in the cell cycle, as assessed by flow-cytometry. Fig. 2 shows that very low doses of rapamycin were able to arrest follicular cells in the G_1 phase. The cell line SU-DHL-4 (a), representative of the t(14;18) cells, increased the G_1 fraction by 23 or 43% with rapamycin 0.3 or 1 ng/ml, respectively, (from 40 to 49 or to 57%), whereas the G_1 fraction increased only by 30% at 1 ng/ml (from 50 to 63%) in Raji cells (b) and not at all in Jurkat cells (c) (49 to 50%). In agreement with the microscopic observations, the percentage of hypodiploid apoptotic cells or the percentage of cells stained by Annexin V (data not shown) did not increase even at doses as high as 100 ng/ml of rapamycin.

These observations stimulated studies on the effects of a prior treatment with rapamycin in lymphoid cells, either high and low BCL-2 expressors, exposed to apoptotic compounds such as etoposide, paclitaxel or doxorubicin. Table 2 shows that SU-DHL-4 cells pretreated with rapamycin were highly protected from apoptotic death, as assessed by caspase activation or flow cytometry. Table 2 shows that caspases activated by pro-apoptotic compounds etoposide and paclitaxel were substantially inhibited, in a dose–response manner, by pretreatment with rapamycin, whereas rapamycin was less efficient in protecting from apoptosis and almost ineffective in inhibiting caspase activation induced by doxorubicin.

Studies of the DNA distribution in cell samples treated as above showed that rapamycin significantly reduced the fraction of hypodiploid cells produced by the anticancer drugs. Caspase inhibition and the anti-apoptotic activity of rapamycin chiefly occurred in cells expressing high amounts of BCL-2. However, since the high level of BCL-2 under basal conditions did not protect these cells from drug-induced apoptosis, we

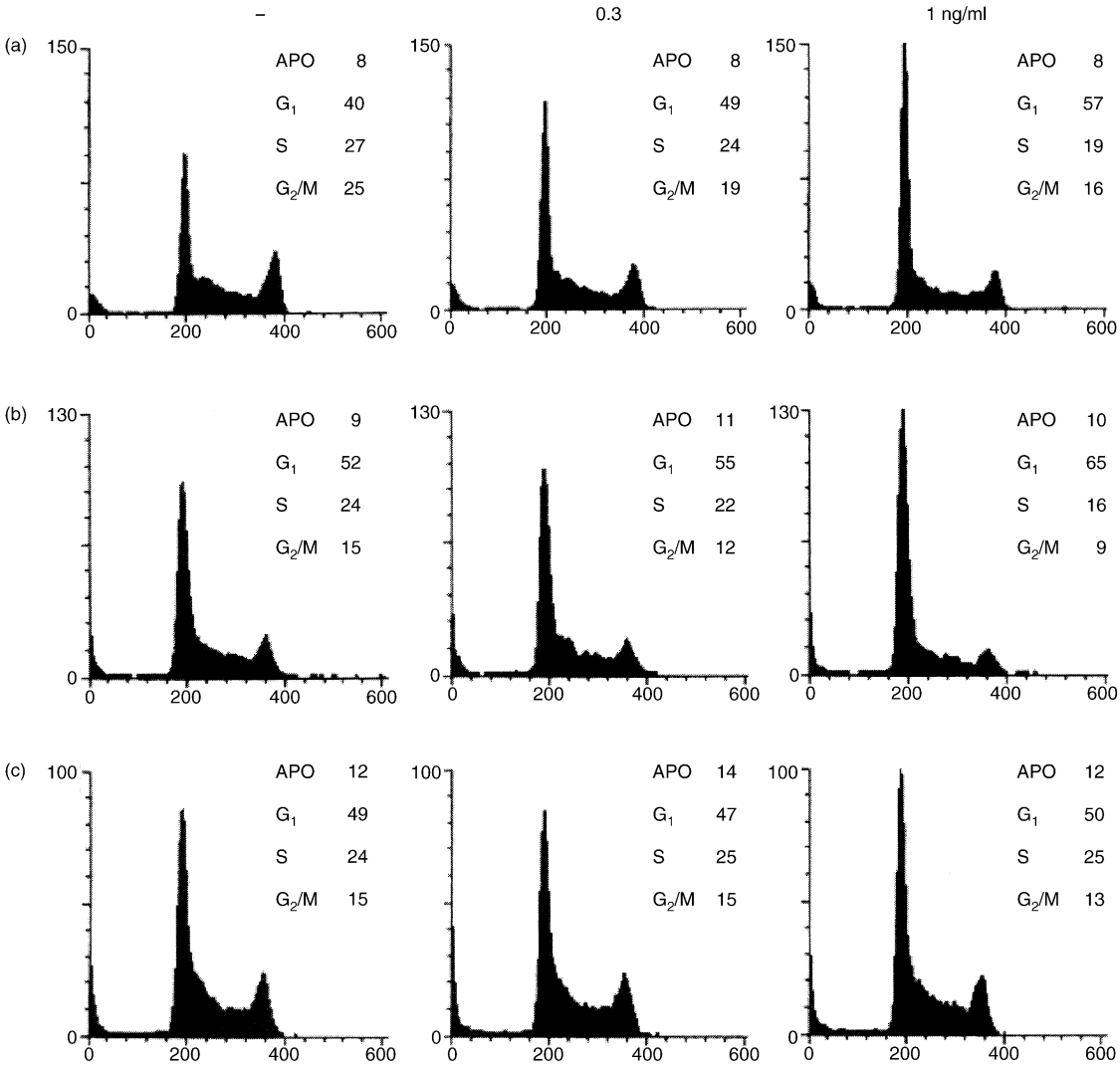

Fig. 2. Cell-cycle analysis of lymphoid cells treated with rapamycin. SU-DHL-4 (a), Raji (b) or Jurkat (c) were treated for 24 h with rapamycin at 0.3 or 1 ng/ml. Hypodiploid cells (APO).

studied the expression of the *bcl-2* gene in cells treated with rapamycin.

3.3. Bcl-2 *mRNA determinations in rapamycin-treated cells*

We initially asked whether rapamycin might regulate the cellular concentration of *bcl-2* mRNA by a mechanism requiring an Adenine-uridine Reach Element (ARE) in the 3′ untranslated region regulating the rate of decay [29]. In the SU-DHL-4 cell line, 24 h exposure to 30 or 100 ng/ml of rapamycin did not affect the cellular level of *bcl-2* mRNA as measured by northern blotting (Fig. 3). These findings were confirmed in NIH 3T3 fibroblasts stably transfected with a rabbit *β-globin* gene fused at the 3′ end to human *bcl-2* ARE. Decay of the rabbit *β-globin* transcript, which accelerates

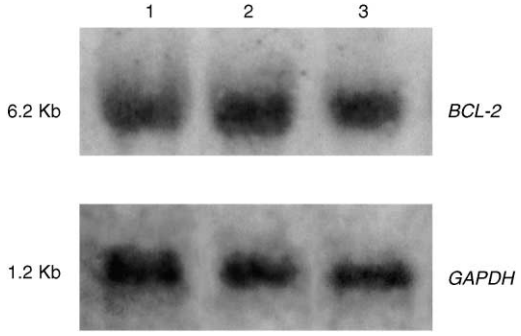

Fig. 3. Expression of *bcl-2* mRNA in DHL-4 cells treated with rapamycin. Untreated cells (lane 1) and cells treated for 24 h with rapamycin, 30 ng/ml (lane 2) and 100 ng/ml (lane 3) were analysed by Northern blot. The lower panel shows rehybridisation with a digoxigenin-labelled glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe.

in apoptotic cells [22], was not altered by 30 ng/ml of rapamycin (Fig. 4).

In sharp contrast, the amount of the BCL-2 protein, evaluated by western blotting in the lymphoid cells, was significantly increased in the rapamycin-treated samples. Fig. 5 shows that 10–30 ng/ml of rapamycin increased the SU-DHL-4 cellular level of BCL-2 protein 3–4 times over that of the untreated controls, while the β-actin level was unchanged.

These data provide evidence that rapamycin increases the cellular concentration of BCL-2 protein by acting at the post-transcriptional level. The BCL-2 level was increased in a dose-dependent manner, most evident in the follicular B cells, much less in the t(14;18)-negative B cell lymphoma lines and not at all in the T lymphoid cells (data not shown). The inhibition of the growth rate, the arrest in G_1 phase, the anti-apoptotic activity and the degree of increase in BCL-2 by rapamycin seem to correlate with the basal level of BCL-2 in the cells.

Table 2
Prevention of caspase activation and hypodiploidy by Rapamycin

Drug treatment	DEVD-MCA Hydrolytic activity[a]		% Hypodiploid cells		
	Rapamycin		Rapamycin		
	–	+	–	+	%[b]
–	69±5	54±6	12	10	–
E45	412±32	147±15*	49	39	27
E15	321±28	86±8*	40	34	21
E5	142±15	49±5*	31	22	47
–	77±7	56±6	10	10	–
T100	286±31	196±21*	46	36	28
T30	226±21	150±15*	41	30	35
T10	179±15	123±14*	34	23	46
–	94±7	85±7	15	13	–
D180	231±18	200±19	48	41	21
D60	172±19	154±16	43	35	29
D20	164±18	144±16	31	25	38

SU-DHL-4 cells, treated with rapamycin 10 ng/ml on day 0, and with etoposide 45 μM (E45), 15 μM (E15) and 5 μM (E5) or with paclitaxel 100 nM (T100), 30 nM (T30) and 10 nM (T10) or with doxorubicin 180 nM (D180), 60 nM (D60) and 20 nM (D20) on day 1, were processed on day 2 as indicated in Materials and methods. Data represent three experiments in triplicate. *$P \leqslant 0.01$ by Student's *t*-test.

[a] Fluorescence Arbitrary Units±standard error of the mean (S.E.M.).

[b] % protection from hypodiploidy.

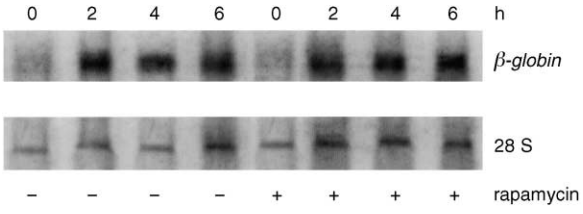

Fig. 4. Expression of rabbit *β-globin* mRNA in cells treated with rapamycin. At the indicated times, total RNA was extracted from starved cells treated for 24 h with rapamycin 30 ng/mL and fetal calf serum and (FCS) and was hybridised with digoxigenin-labelled *β-globin* probe.

4. Discussion

A number of compounds have been studied in recent years in the hope of reducing the BCL-2 level in tumour cells with the aim of obtaining a direct anti-tumour activity or a potentiation of chemo and radiotherapy [30,31]. These efforts, currently under clinical evaluation, have been pursued with chemicals acting at the protein level [32] or with antisense oligonucleotides acting at the transcription level [19,33,34]. Because of the relevance of BCL-2 in cancer therapy, we have studied the anti-B-cell activity of rapamycin in lymphoid cell

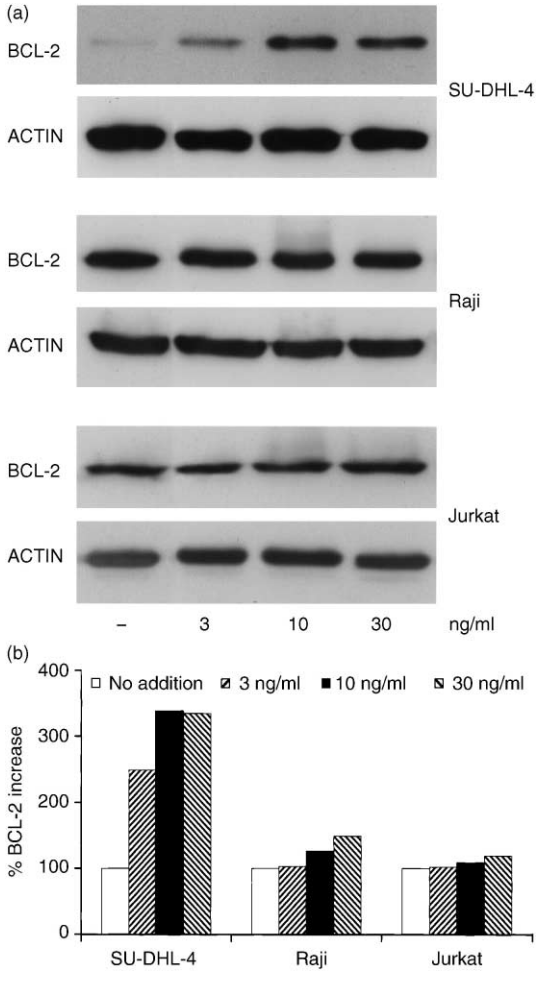

Fig. 5. (a) BCL-2 protein determination in lymphoid cells treated with rapamycin. BCL-2 and β-actin protein were evaluated by western blotting of protein samples from SU-DHL-4 cells (12 μg of proteins), Raji and Jurkat (75 μg of proteins) cells treated for 24 h with rapamycin. (b) Densitometric analysis of BCL-2 protein expression. Histograms show the percentage of BCL-2 level over untreated controls as determined by densitometric analysis of the gels.

lines expressing different amounts of BCL-2 in their steady state.

In this study, rapamycin was more effective in inhibiting the growth rate of the t(14;18)-positive human follicular B-cell lymphoma lines, which are high BCL-2 expressors, than the t(14;18)-negative lymphoid B-cell lines, lymphoid T-cell lines or myeloid cell lines, which are medium or low expressors. Cells were arrested in the G_1 phase at concentrations of rapamycin that were usually ineffective in cell lines expressing less BCL-2. The degree of activity of rapamycin depended on the basal cellular level of BCL-2 protein. The strong inhibition of the growth rate by rapamycin was not associated with an increase in cell death, nor with an increase in the hypodiploid fraction that is believed to represent the apoptotic fraction.

Further studies provided direct evidence for the anti-apoptotic activity and for the inhibition of caspase activation by rapamycin. Rapamycin was less active in doxorubicin-treated cells which suggests that doxorubicin can activate caspases and apoptosis by a biochemical mechanism poorly regulated by BCL-2. Analysis of DNA distribution in cells exposed to the same pharmacological agents confirmed the protective activity of rapamycin.

The dependence of the biological activity of rapamycin on the cellular level of BCL-2 prompted us to study the expression of *bcl-2* gene in cells treated with rapamycin. At the transcriptional level, no change in the amount of *bcl-2* RNA was observed, nor was any change observed in the rate of decay of a reporter system regulated by the ARE motif [35]. In sharp contrast, the cellular level of BCL-2 protein was increased 3–4 times over the basal level.

Although the biochemical mechanisms capable of increasing the basal level of BCL-2 protein are not yet known, modifications at the post-translational level must be involved since the higher the steady-state level of BCL-2 in the lymphoid cells. the greater the increase of BCL-2. The kinase mTOR, the biochemical target of rapamycin, might be involved in these processes (data not shown).

Our findings also indicate that apoptosis might be regulated by the increase in BCL-2 protein rather than by its basal level. The forced overexpression of BCL-2 by pharmacological means might alter an equilibrium, for instance the ratio between BCL-2 and the pro-apoptotic proteins, thus enhancing the anti-apoptotic activity.

In conclusion, rapamycin can arrest the cell cycle by augmenting the cellular level of the $p27^{kip1}$ protein [36] and can activate an anti-apoptotic programme by increasing the cellular level of the BCL-2 protein. These studies have disclosed a new pathway balancing cell proliferation and apoptosis in a coordinated manner that may be relevant for cancer chemotherapy.

Acknowledgements

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