Rapamycin and curcumin induce apoptosis in primary resting B chronic lymphocytic leukemia cells

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Abstract
B chronic lymphocytic leukemia (B-CLL) cells exist in patients as slowly accumulating resting as well as proliferating B cells. In this study, we examined whether Rapamycin and Curcumin, two naturally occurring compounds shown to have apoptotic effects, could selectively induce apoptosis in resting B-CLL cells. Mononuclear cells isolated from patients with B-CLL were treated with these agents and analysed by AnnexinV/propidium iodide binding, caspase activity, and changes in bcl-2/Bax ratio. Rapamycin and curcumin significantly induced apoptosis in resting B-CLL cells obtained from patients with CLL. Furthermore, rapamycin and curcumin increased caspase 9, 3 and 7 activity, decreased anti-apoptotic bcl-2 levels, and increased the pro-apoptotic protein Bax. These data suggest rapamycin and curcumin may be an effective treatment for B-CLL and are of high clinical significance considering the growing population of patients and lack of efficient treatment for this malignant disease.

Keywords: B chronic lymphocytic leukemia (B-CLL), rapamycin, sirolimus, curcumin, turmeric, caspase

Introduction
B cell chronic lymphocytic leukemia (B-CLL) is a progressive form of leukemia characterised by an increased number of circulatory lymphocytes. Representing 25% of all cases, B-CLL is the most common type of leukemia, and mainly occurs in people over 60 years of age. B-CLL is largely incurable, with treatment emphasis on maintaining an acceptable state of health and inducing remission when possible. Patients in the initial stages of the disease are generally not treated and only undergo regular check-ups to assess disease progression. For patients with advanced or progressive stages of the disease, treatment is aimed at either controlling the symptoms or prolonging survival [1].

B-CLL has long been considered a disease in which B-CLL cells accumulate due to a presumed defect in their apoptotic mechanism. Previous morphologic observations suggested that B-CLL cells are resting cells with rarely detected cell cycle activity in the blood. Recent data, however, suggest that B-CLL cells are born and proliferate at different rates that vary from patient to patient, and propose a link between cell proliferation and clinical outcome [2]. Several agents are currently under study as alternative or additive treatments in chemo-therapies for different malignancies, including B-CLL [3]. In this study, we examined two such agents:

(I) Rapamycin, also known as Sirolimus, is an inhibitor of the TORC1 complex of mTOR. TORC1 itself is a downstream target for AKT, and this signaling system is activated in numerous tumors. Activation of TORC1 induces gene
transcription that leads to cell growth. Under physiological conditions, this signaling is crucial for cell survival and development. Tumoral processes however, utilise this signaling cascade toward uncontrollable proliferation. Rapamycin was shown to suppress cell proliferation and initiate apoptotic events in many tumors, however the effect of Rapamycin on B-CLL cells has not yet been fully elucidated.

(II) Curcumin, a natural component of the rhizome of Curcuma longa (turmeric), has emerged as one of the most powerful chemopreventive and anticancer agents [4,5]. Recent studies, predominantly using actively dividing cell lines, have suggested that this compound could be used as a chemopreventative or therapeutic agent for epithelial tumors. Moreover, Curcumin has been reported to inhibit the NIK/IKK complex, downregulate NFkB, and inhibit the IKB kinase, thus suppressing proliferation and inducing apoptosis [6]. Such an activity would also be expected to induce apoptosis in B-cell malignancies. Indeed, a single study recently suggested that Curcumin might augment the efficacy of established or experimental therapies for B-CLL [3]. However, the apoptotic effects of curcumin in B-CLL have not been fully investigated.

Because all current therapies have failed to prevent relapse and eventual death of patients with B-CLL, new agents are constantly being sought to help prolong the lifespan of patients with B-CLL. We hypothesised that rapamycin and curcumin could induce apoptosis in resting B-CLL cells. Here, we show that rapamycin and curcumin induce apoptosis in resting B-CLL cells via a mechanism including caspase activation and bcl-2 downregulation.

Methods

Chemicals

Rapamycin (Rapamune, Sirolimus) was purchased from Wyeth-Ayrest (Philadelphia, PA). Curcumin was purchased from Sigma-Aldrich (St Louis, MO). Curcumin stock solution was kept as 27 mM in DMSO (−20°C). Annexin-V/propidium iodide (PI) apoptosis detection kit was purchased from MBL (Naka-ku Nagoya, Japan). Annexin-V/propidium iodide (PI) apoptosis detection kit was purchased from MBL (Naka-ku Nagoya, Japan). Antibodies against bcl-2, Bax, β-actin and α-tubulin were purchased from Santa Cruz (Santa Cruz, CA). Antibodies against CD-5, CD-19, and CD-38 were purchased from IQ products (Groningen, Netherlands).

Patients

Peripheral blood (PB) was drawn from 12 patients with flow cytometry-verified B-CLL from the Hematology Institute at Kaplan Medical Center, Rehovot, Israel. The patients were in different RAI stages (0–IV) according to the RAI staging system, and with an average of 84% CD19+CD5+ or CD38+ as verified by flow cytometry (Table I). In-vitro random checks for purity assessment of CD19+ cells were performed, and no less than 98% was observed.

The study was approved by the local ethical committee and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Participants gave informed consent.

Leukemic cell isolation and culture

PB from patients with B-CLL was drawn into Vacuette EDTA tubes (Greiner Bio-One, Kremsmunster, Austria). Peripheral blood mononuclear cells (PBMCs) were isolated using gradient
centrifugation in Ficoll-Paque™ plus solution as described by the manufacturer (Amersham Biosciences, Uppsala, Sweden). In all experiments, isolated PBMCs were incubated overnight in RPMI-1640 medium (Biological Industries, Kibbutz Beit Haemek, Israel) containing 2 mM glutamine and 10% heat-inactivated fetal calf serum (Biological Industries) at 37°C in a humidified atmosphere containing 5% CO₂. After 12 h, the cells were treated as indicated.

**Annexin-V/Propidium iodide apoptosis detection assay**

PBMCs were washed in cold Phosphate buffer saline (PBS) and resuspended in 100 μL of Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). Five micro litres of Annexin-V was added 5 min before 2 μL of PI, and the solution was then incubated for 15 min at room temperature. Another 300 μL of Annexin binding buffer was added and cell fluorescence was analysed by FACStar plus (Becton Dickinson, San Jose, CA) flow cytometer using Cell Quest software. In the results, apoptotic cells count as the sum of early plus late apoptosis cells as examined by the array.

**Cell cycle distribution studies**

PBMCs were rinsed with PBS (Ca²⁺ and Mg²⁺-free) and suspended in the dark for 50 min at 4°C in 0.5 mL buffer containing 50 μg/mL PI, 0.1% sodium citrate, 0.1% Triton-X and 1 mg/mL RNase. DNA content was measured using a FACStar plus flow cytometer using Cell Quest software.

**Forward scatter/Side scatter analysis**

PBMCs were washed in cold PBS, resuspended in PBS, and then analysed by FACStar plus flow cytometer using Cell Quest software. Apoptotic cell death was examined by the morphological attributes: FSC (forward scatter)/SSC (side scatter) index of apoptotic-induced changes in cell size and granularity, respectively.

**Detection of active caspase levels**

Levels of active caspases 3, 7 and 9 were performed using Fluorescein Caspase Activity Kit (FLICA) (Alexis Biochemicals, San Diego, CA). This kit detects levels of active caspase in living cells utilising unique (carboxyfluorescein) chemistry; the fluorochrome caspase inhibitor binds covalently to the active site of the caspase enzyme. In brief, FLICA solution (30 x) was added to 300 μL (1 x 10⁶/mL) cell suspension and incubated for 1 h at 37°C. The samples were washed with wash buffer and the suspended cells were analysed by flow cytometry (FL-1 channel) using an argon ion laser at 488 nm.

**Western blot analysis**

Protein samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Tris-glycine running buffer. Gels were then electroblotted using semi-dry transfer apparatus (Bio-Rad) in transfer buffer containing 0.025 mol/L Tris base, 0.15 mol/L glycine, and 10% (v/v) methanol for 1.5 h at 15 V onto a nitrocellulose membrane (Bio-Rad). The membranes were then incubated in blocking buffer (5% non-fat milk in 20 mmol/L Tris-HCL, pH 7.5, 137 mmol/L NaCl, 0.2% Tween-20) for 1 h at 23°C. The membrane was incubated overnight at 4°C with a primary antibody. After washing three times (5 min per wash) with Tris-buffered saline-T (20 mmol/L Tris-HCL, pH 7.5, 137 mmol/L NaCl, 0.2% Tween-20), the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody. After washing five times (5 min per wash) with Tris-buffered saline-T, the membrane was incubated with chemoluminescent substrate, enhanced chemiluminescence (Pierce Endogen, Rockford, IL) for 5 min, and chemoluminescent signals were visualised by exposing the membrane to X-ray film (Kodak X-ray film; InterScience, Mississauga, ON, Canada). Densitometry was performed using National Institute of Health (NIH) ImageJ software and represents percent of control.

**Statistical analysis**

The results were analysed using a paired one-tailed independent Student’s t-test, ANOVA repeated measures, or Pearson correlation, as indicated for every experiment. Results represent mean ± SEM. Statistical significance was defined as p ≤ 0.05.

**Results**

**Rapamycin induces apoptosis in resting B cell chronic lymphocytic leukemia cells**

The ability of Rapamycin to induce apoptosis of primary resting cultured B-CLL cells was tested using the Annexin-V/PI assay. Control cells in all experiments were in G1 phase of the cell cycle, as shown in Figure 1(A**). As early as Day 1, rapamycin (5 ng/mL and 50 ng/mL (5.47 and 54.7 nM) elevated apoptosis levels in B-CLL cells, however this increase was not significant (data not shown). Rapamycin (5 and 50 ng/mL), significantly
elevated apoptosis above control levels by Day 3 (32.16 ± 5.7% and 36.91 ± 6.2%, (n = 9), respectively; control 22.75 ± 4.4, n = 9; p < 0.001) [Figure 1(A)]. However, compared to the PBMCs of healthy donors, there was no significant change in apoptosis induced by Rapamycin after 3 days of treatment [Figure 1(D)]. Rapamycin (5 and 50 ng/mL), significantly elevated apoptosis levels on Day 7 (40.33 ± 8.1% and 62.55 ± 10.9%, (n = 8), respectively; control 20.47 ± 4.9, (n = 8); p < 0.001) [Figure 1(A)]. Further, while PBMCs of healthy donors did not progress in apoptosis after 7 days, there was a significant progression in the PBMCs of patients with CLL [Figures 1(A) and 1(D)].

The apoptotic effect conferred by Rapamycin on B-CLL cells was correlated with morphology changes in both cell granularity (side-scattering; SSC) and size (forward-scattering; FSC) as measured by FACS analysis. Consistent with classic plots of apoptosis in cultured primary lymphocytes, the
cells shift toward lower FSC and higher SSC parameters [7] [Figures 1(BI) (control cells) vs. 1(BII) (Rapamycin-treated cells)]. Apoptotic cells (exhibiting low FSC/high SSC) shown in Figure 1(B) were also stained with Annexin-V/PI. This method was significantly correlated to the Annexin-V/PI method on Day 3 (Pearson correlation $p < 0.001; r = 0.928; N = 8$), and Day 7 (Pearson correlation $p < 0.0001; r = 0.993; n = 7$), and was also correlated to caspase-9 levels (Pearson correlation $p < 0.05; r = 0.984; n = 4$) and caspases 3 and 7 levels (Pearson correlation $p < 0.001; 0.999; n = 4$). After 3 and 7 days of treatment, Rapamycin (5 and 50 ng/mL) significantly elevated apoptosis above basal levels (control day $3 = 21.61 \pm 3.8\% (n = 9)$; control Day 7 $- 21.19 \pm 4\% (n = 8)$; rapamycin Day 3 $- 31.08 \pm 5\%$ and 39.2 $\pm 6.3\%$, respectively, $p < 0.001$; Day 7 $- 40.64 \pm 8.4\%$ and 64.84 $\pm 11.4\%$, respectively, $p < 0.001$) [Figure 1(B)].

Rapamycin-induced B-CLL cell apoptosis was correlated with elevated levels of the active form of caspase-9. After 7 days, Rapamycin (5 ng/mL and 50 ng/mL) significantly elevated active caspase-9 levels (32.76 $\pm 11.8\%$ and 56.01 $\pm 17.1\%$, respectively) above control cells (18.21 $\pm 8.7\%$, $n = 5$, $p < 0.05$) [Figure 1(CI)]. Because caspase-9 acts as an initiator caspase that activates effector caspases, we next determined whether rapamycin also increased activation of caspases 3 and 7. Rapamycin (5 and 50 ng/mL) significantly elevated levels of caspases 3 and 7 (28.41 $\pm 10.3\%$, $n = 5$ and 54.86 $\pm 12.7\%$, $n = 7$, respectively; control 17.72 $\pm 5\%$, $n = 7$, $p < 0.05$) [Figure 1(CII)]. This indicates that rapamycin-induced apoptosis acts through the intrinsic caspase activation cascade.

To confirm the induction of the intrinsic caspase activation pathway, we next examined Bax and bcl-2 protein levels. The intrinsic caspase activation pathway is mediated through an increase in the levels of Bax protein and a concomitant decrease in bcl-2 levels. Western-blot analysis revealed that both 5 and 50 ng/mL Rapamycin increased Bax and decreased bcl-2 protein levels, resulting in a decreased bcl-2/Bax ratio in rapamycin-treated cells (untreated $= 1.17$; 5 ng/mL $= 0.83$; 50 ng/mL $= 0.22$) [Figure 1(CIII)]. These ratios were obtained by dividing the arbitrary units of bcl-2 by Bax, with no reference to actin or tubulin.

**Curcumin induces apoptosis in B cell chronic lymphocytic leukemia cells**

The ability of Curcumin to induce apoptosis of primary cultured B-CLL cells was tested using the Annexin-V/PI assay. 10 $\mu$M Curcumin was used in all experiments. Curcumin significantly elevated apoptosis levels on Days 3 and 7 above basal levels (Day 3- control $25 \pm 3.54$, $n = 12$; curcumin $54.01 \pm 10.35\%$, $n = 12$, $p < 0.002$; Day 7 - control $20.47 \pm 4.91$, $n = 8$; curcumin $45.84 \pm 12.44\%$, $n = 8$, $p < 0.02$) [Figure 2(A)]. PBMCs from healthy donors did not exhibit a notable elevation in apoptosis after 3 or 7 days [Figure 2(D)].

Similar to rapamycin-treated cells, Annexin-V/PI positive cells following curcumin administration were also correlated with lower FSC and higher SSC, as shown in Figure 2(B). Curcumin significantly elevated apoptosis levels on Days 3 and 7 (Day 3 – control $24.42 \pm 3.67$, $n = 11$; curcumin $52.16 \pm 11.13\%$, $n = 11$, $p < 0.005$; Day 7 – control $21.19 \pm 4.8$, $n = 8$; curcumin $47.73 \pm 12.8\%$, $n = 8$, $p < 0.002$).

Curcumin-induced B-CLL apoptosis was also correlated with elevated levels of the active form of caspase-9. After 7 days of treatment, curcumin significantly elevated active caspase-9 (30.95 $\pm 12.74\%$), in contrast to control cells (18.21 $\pm 8.7\%$, $n = 5$, $p < 0.04$) [Figure 2(CII)]. Levels of activated caspases 3 and 7 were also significantly elevated following curcumin administration [control 18.6 $\pm 5.15\%$, curcumin $37.7 \pm 12.25\%$, $n = 7$, $p < 0.05$; Figure 2(CII)], indicating that curcumin induces apoptosis in B-CLL cells through the intrinsic caspase activation cascade.

Curcumin treatment induced a mild increase in Bax protein levels and decrease in bcl-2 levels, resulting in a lowered bcl-2/Bax ratio (control 0.93, curcumin 0.73) These changes, however, were less notable than those achieved with Rapamycin [Figure 2(CIII)].

**Discussion**

In the present study, we examined the effect of Rapamycin and Curcumin on non-stimulated B-CLL cells extracted from patients. For this purpose, we used blood from 12 patients at different stages of RAI (0–IV). The current paradigm on B-CLL cells suggests the existence of two subsets of cells: (I) a non-dividing circulating subset of cells; and (II) bone marrow cells that possess an active cell cycle [8]. Consistent with other reports, the majority of B-CLL cells extracted from patients were arrested in the G1 phase of their cell cycle. Decker et al. found that Rapamycin (50 ng/mL) induced apoptosis of B-CLL cells stimulated with IL-2 and CpG oligodeoxynucleotides (cells with an active cell cycle), but not of non-stimulated B-CLL cells (non-dividing cells) [8]. No study has yet shown an apoptotic effect of Rapamycin on resting B-CLL cells. In contrast to the findings of Decker et al., we did observe a significant apoptotic effect of Rapamycin on
non-stimulated B-CLL cells. We found that 3-day incubation with 5 ng/mL Rapamycin was the minimum requirement to induce apoptosis of resting B-CLL cells, but 7 days of treatment were needed to produce significant apoptosis levels when compared to normal PBMCs. Previous studies failed to observe this effect, possibly because they did not use the same conditions. In CLL cells, after 3 days of Rapamycin treatment, the elevation of apoptosis was not higher than in the PBMCs of healthy donors. However, in CLL cells, there was a significant progression in the apoptosis levels during the time, while in the PBMCs derived from healthy donors, almost no progression could be seen. Thus, after 7 days of treatment, there was a significant difference between the PBMCs of healthy donors and patients with CLL. Seven days of treatment should be the ideal time for Rapamycin usage.

Since normal PBMCs contain mostly T-lymphocytes, and PBMCs derived from patients with CLL contain mostly B-lymphocytes, we cannot directly compare these two cell populations. Thus, normal PBMCs obtained from healthy donors were used as toxicity control, in order to rule out toxicity of curcumin and rapamycin in non-malignant cells. Our data indicate that Rapamycin induces activation of caspases 9, 3 and 7, which implies an intrinsic apoptotic mechanism [9]. To further emphasise the apoptosis involved in this treatment, we attempted to determine whether Rapamycin altered the expression of the anti-apoptotic protein bcl-2 and the pro-apoptotic protein Bax [10]. We found that...
Rapamycin decreased the bcl-2 levels and increased Bax levels, as shown in Figure 1(C). Consequently, the bcl-2/Bax ratio was reduced dose dependently in treated cells. These results show transition from a clearly anti-apoptotic to a pro-apoptotic signal (1.17 in control cells, 0.83 in 5 ng/mL Rapamycin, and 0.22 in 50 ng/mL Rapamycin), as shown in Figure 1(C). Interestingly, in addition to Bax increase, we show that Rapamycin induced a decrease in tubulin expression. Since Rapamycin is capable of inducing apoptosis of both resting and proliferative B-CLL cells, it is plausible that it might affect the majority of the tumoral B-CLL cell population.

Rapamycin and its two analogues, Temsirolimus (Torisel) and Everolimus (RAD-001), exhibited antitumor activity in in-vitro and in-vivo models and are currently under clinical trials for prostate and bladder cancer. Phase II and III trials have already established the clinical efficacy of Temsirolimus in renal cancer [11]. Temsirolimus, when administered weekly in doses of 25 mg (i.v.) to patients with advanced renal cell carcinoma and poor prognostic features, exhibited related adverse events that were primarily metabolic and easily controlled medically. These effects did not negatively impact patient quality of life [12]. A phase II pilot trial of Everolimius administered orally in doses of 5 mg/day in patients with advanced B-CLL, showed some degree of activity. However, the trial was stopped because of toxicity concerns [13]. Decker et al. suggest focusing on different treatment schedules, adequate anti-infectious prophylaxis, or combinations of cytotoxic drugs, for further investigations.

Curcumin was recently suggested to augment the efficacy of established or experimental therapies for B-CLL [3]. Our results with Curcumin treatment of B-CLL cells are consistent with those of Everett et al. [3]. In this regard, we further explored the mechanism behind its apoptotic effect and found it to be mediated through the intrinsic caspase cascade, which involves the activation of caspases 9, 3 and 7, as well as a slight decrease in the bcl-2/Bax ratio in Curcumin-treated cells [9]. In addition, as can be seen in the results presented in Figure 2, while treating B-CLL cells with Curcumin, some patients' blood samples reacted mildly to the treatment and showed small changes in the levels of apoptosis, while other patients strongly responded to the treatment. In an attempt to classify these subpopulations of patients, no correlation was found between them and any of the following parameters: RAI stage, age, gender, the absolute lymphocyte count, or the CD19+CD5+ percentage of the patients’ blood samples. It is very important to investigate, distinguish, and classify these two subpopulations to find a common denominator to improve decisions concerning the efficacy of clinical treatments in patients with B-CLL. Consistent with the knowledge that turmeric has been used for centuries in diet and in traditional medicine [14], and with another report that Curcumin does not induce apoptosis in normal human PBMCs [15], we found that Curcumin almost did not induce apoptosis in PBMCs of healthy donors after 3 or 7 days.

The chances for a single agent to effectively eliminate tumoral cells in vivo while allowing full recovery of the patients are slim. It is, therefore, a necessity to treat these cells with multiple drugs simultaneously to reduce the chances of drug resistance. However, no significant results were shown while B-CLL cell were treated with combination of Rapamycin and Curcumin (data not shown). Combination of each treatment with well known cytotoxic chemotherapies may prove beneficial for these patients. Curcumin has already been found to augment the efficacy of established or experimental therapies for B-CLL [3]. However, Gotze et al. reported on a single patient with relapsed CLL, who was treated with Everolimus within a phase II clinical trial that was discontinued after 32 weeks due to progression. After the treatment was stopped, fludarabine-based chemotherapy was started. The patient subsequently developed a rapidly fatal Epstein-Barr-virus-associated lymphoproliferative disorder, clonally unrelated to the CLL. Gotze et al. suggest caution when using new immunosuppressive drugs for treatment of CLL, especially in the context of additional cytotoxic therapy [16].

Several mechanisms have been proposed to account for the ability of Rapamycin and Curcumin to induce apoptosis in malignant cell lines in general and in B-CLL cells in particular. Rapamycin is known as an mTor inhibitor, while activated mTOR is phosphorylated on Ser2448 [17]. In preliminary results, we found decreased levels of pSer2448 mTor in Curcumin-treated cells (data not shown). These results imply inhibition of the mTOR pathway, but further investigation is required.

Despite new treatments, combinations of chemotherapies, and the introduction of monoclonal antibodies, there is a high percentage of relapse in treated patients with B-CLL. Thus, a constant search for alternative treatments to replace or to be added to previous non-responsive treatments must be conducted. In this study, we suggest that Rapamycin and Curcumin could be effective as apoptosis inducers for resting B-CLL cells.

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