Rapamycin Stimulates Apoptosis of Childhood Acute Lymphoblastic Leukemia Cells

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ABSTRACT

The PI3k/Akt pathway has been implicated in childhood acute lymphoblastic leukemia (ALL). Because rapamycin suppresses the oncogenic processes sustained by PI3k/Akt, we investigated whether rapamycin affects blast survival. We found that rapamycin induces apoptosis of blasts in 56% of the bone marrow samples analyzed. Using the PI3k inhibitor wortmannin, we show that the PI3k/Akt pathway is involved in blast survival. Moreover, rapamycin increased doxorubicin-induced apoptosis even in non-responder samples. Anthracyclines activate NF-κB, and disruption of this signaling pathway increases the efficacy of apoptogenic stimuli. Rapamycin inhibited doxorubicin-induced NF-κB in ALL samples. Using an siRNA approach, we demonstrate that FKBP51, a large immunophilin inhibited by rapamycin, is essential for drug-induced NF-κB-activation in human leukemia. Furthermore, rapamycin did not increase doxorubicin-induced apoptosis when NF-κB was over-expressed. In conclusion, rapamycin targets two pathways that are crucial for cell survival and chemoresistance of malignant lymphoblasts, i.e. PI3k/Akt through mTOR and NF-κB through FKBP51, suggesting that the drug could be beneficial in the treatment of childhood ALL.
INTRODUCTION

In recent decades, conventional chemotherapy has produced a dramatic improvement in survival of childhood acute lymphoblastic leukemia (cALL) (1), however, refractory or relapsed disease remains a problem. Anti-cancer drug research is aimed at developing new compounds directed against inappropriately activated cell signaling pathways that stimulate the uncontrolled growth of neoplastic cells (2). A deregulated cytokine circuitry has been proposed in the malignant transformation of lymphoid precursors in primary B- and T-lineage ALL (3). Other reports suggest that the insulin-like growth factor system plays a pathogenetic role in cALL (4, 5). Phosphorylation of cytokine (6, 7) or growth factor receptors (4, 5) generate docking sites for signaling molecules thereby activating the phosphatidylinositol 3 kinase (PI3k)/Akt-protein kinase B survival pathway (4–7), which promotes blast growth (4, 5).

The carbocyclic lactone-lactum antibiotic rapamycin, which has been approved by the Food and Drug Administration for the prevention of allograft rejection (8), exerts an anticancer effect by decreasing cell proliferation and increasing apoptosis (9, 10). Therefore, rapamycin is expected to suppress cytokine responses (11) and tumor cell survival (12). The mammalian target of rapamycin (mTOR) is a serine-threonine kinase that regulates protein translation, cell cycle progression and cellular proliferation (13–16). In response to growth factors, hormones, mitogens and amino acids (15–17), mTOR is activated through phosphorylation by Akt (18) and in turn activates two key translational regulators: the initiation factor 4E binding protein (4E-BPI) (16), and p70kDa S6 ribosomal protein kinase (p70S6 k) (14). The pathways governed by mTOR and p70S6k are involved in the survival of malignant lymphoblasts (19), which explains the anti-leukemic activity of rapamycin detected in human cell lines and in a murine model (19). Taken together, these findings prompted us to investigate whether rapamycin induces apoptosis of ALL blasts.
We recently demonstrated that rapamycin sensitized a poorly responsive human melanoma cell line to doxorubicin-induced apoptosis (20). Anthracycline compounds activate NF-κB/Rel nuclear translocation (21) and disruption of this signaling pathway increases the efficacy of apoptogenic stimuli (22). We showed that the immunophilin FKBP51 is required for IκBα degradation after stimulation with doxorubicin (20). FKBP51, which is specifically inhibited by rapamycin binding (23), exerts peptidyl-prolyl-isomerase activity that catalyzes the isomerization of peptidyl-prolyl-imide bonds in subunit α of the IKK kinase complex, and is required for IKK function (24). Given the foregoing, we investigated whether rapamycin inhibits anthracycline-induced NF-κB/Rel activity in blasts from cALL, and increases sensitivity to chemotherapy.

Finally, to assess the role of FKBP51 in NF-κB/Rel activation in lymphoid cells, we down-modulated FKBP51 levels in the Jurkat leukemic cell line using a short interfering (si)RNA approach, and investigated whether IκBα degradation and NF-κB/Rel nuclear translocation occurred in the absence of the IKKα cofactor.
MATERIALS AND METHODS

Cells and culture conditions

Bone marrow samples were obtained between April 2002 and October 2004 from 15 children affected by B-ALL and from 10 children affected by T-ALL (Table 1). Twenty-two samples were collected at diagnosis, 2 at relapse (patients 18 and 22) and 1 from a patient who partially responded to therapy (patient 8). It is declared that informed consent was provided by the parents’ patients according to the Declaration of Helsinki, for these studies conducted in the Departments of:

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Mononuclear cells from the 25 bone marrow samples were separated through Ficoll-Hypaque gradient (ICN Biomedicals, Aurora, Ohio, USA). The percentage of blasts in mononuclear cell specimens was >90%, except for refractory ALL in which it was 59.1%. Cells (1x10^6 cells/ml) were cultured in 10% FCS (Biochrom KG, Berlin, Germany) RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with antibiotic and glutamine (Cambrex Bio Science) at 37°C in a 5% CO₂ humidified atmosphere, with and without rapamycin (Rapamune, Wyeth Ayerst Laboratories. Marietta, PA, USA).

Cell transfection, plasmids and (si)RNA

Jurkat cells (2x10^7) in the logarithmic growth phase were resuspended in 400 µl of serum-free RPMI 1640 and transfected with 20 µg of plasmid DNA by electroporation at 250V
and 960 mF using the Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA). The cells were transferred to culture flasks and incubated in complete medium supplemented with 1 mg/ml G418 (Roche, Basel Switzerland) to obtain stable lines. The cDNA coding for p65 (RelA), cloned in a pCMV4 vector carrying resistance for G418, was kindly donated by Dr. Shao-Cong Sun (Pennsylvania State University College of Medicine).

For siRNA transfection, cells at a concentration of 5x10^5/ml were incubated for 24 h in six-well plates in medium without antibiotics. This was followed by transfection of the oligonucleotide 5’-ACCUAAUGUGACGUUAUAdTdT-3’ corresponding to the sense strand of the target sequence 5’-AAACCUCUAUGUGACGUUAUA-3’ of human FKBP51 (Dharmacon Research Inc.) or of a scrambled duplex as control (Dharmacon Research Inc.). The siRNA or the scrambled oligo was transfected at the final concentration of 50 nM using Metafectene (Biontex, Munich, Germany) according to the manufacturer’s recommendations. Two days later, 5 μM doxorubicin was added to the culture medium and cells were harvested 5 h later and processed for western blot analysis or electrophoretic mobility shift assay (EMSA).

Analysis of apoptosis

We used the lipophylic cation 5,5’,6,6’ tetrachloro-1,1’,3,3’-tetraethylbenzimidazol-carbocyanine iodide (JC-1) and flow cytometry to analyze the mitochondria membrane potential. In this procedure, the color of the dye changes from orange to green as the membrane potential decreases (25). Briefly, 5x10^5 cells were incubated for 10 min at 37°C, with 10 μg/ml JC-1 (Molecular Probes, Leiden, The Netherlands), washed and analyzed by flow cytometry. Phosphatidylserine externalization was investigated by annexinV staining in double or single fluorescence. Next, 5x10^5 cells were incubated with annexin V-FITC (Pharmigen/Becton Dickinson, San Diego, CA) or PE (Alexis, San Diego, CA) conjugated in 100 μl of binding buffer containing 10 mM Hepes/NaOH pH
7.5, 140 mM NaCl, 2.5 mM CaCl₂ for 15 min at room temperature in the dark. Subsequently, 400 µl of the same buffer was added to each sample and the cells were analyzed in the Becton Dickinson FACScan flow-cytometer. The mouse monoclonal, anti-CD7-FITC, -CD3-PE, -CD20-PE (Pharmigen/Becton Dickinson, San Diego, CA), added in double fluorescence tests. Caspase 3 activity was detected with the Caspase-3 Fluorometric Assay Kit (Perbio Science, Erembodegem, Belgium) according to the manufacturer's instructions. The cells (2x10⁶) were lysed in a buffer containing 10 mM Tris (pH 7.5), 130 mM NaCl, 1% Triton-X-100, 10 mM NaPi, 10 mM NaPPi, and 50 µg of protein was analyzed.

**Cell lysates and Western blot analysis**

For IκBα detection, cytosolic extracts were obtained by resuspending the cells in lysing buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol, 1mM PMSF, 50 µg/ml antipain, 40µg/ml bestatin, 20 µg/ml chymostatin, 0.2% v/v Nonidet P-40) for 15 min in ice. For Akt, phospho-Akt, p65 and FKBP51 detection, whole cell lysates were prepared by homogenization in modified RIPA buffer (150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate, 5 µg/ml of aprotinin, 5 µg/ml of leupeptin). Cell debris was removed by centrifugation. Protein concentration was determined with the Bio-Rad protein assay. The cell lysate was boiled for 5 min in 1x SDS sample buffer (50 mM Tris-HCl pH 6.8, 12.5% glycerol, 1% sodium dodecylsulfate, 0.01% bromophenol blue) containing 5% beta-mercaptoethanol, run on 10% SDS polyacrylamide gel electrophoresis, transferred onto a membrane filter (Cellulosenitrate, Schleider and Schuell, Keene, NH) and incubated with the primary antibody. The anti-IκBα and –p65(RelA) were rabbit polyclonal antibody
from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Akt, anti-phospho-Akt (Thr308) and anti-phospho-Akt (Ser473) were rabbit polyclonal antibodies purchased from Cell Signaling (Beverly, MA). Anti-FKBP51 was a rabbit polyclonal antibody purchased from Abcam Ltd. (Cambridge, UK). After a second incubation with peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) or anti-mouse IgG (Santa Cruz Biotechnology), the blots were developed with the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ).

**Nuclear extracts, EMSA, and oligonucleotides**

Cell nuclear extracts were prepared by cell pellet homogenization in two volumes of 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 10% glycerol v/v. Nuclei were centrifuged at 1,000 g for 5 min, washed and resuspended in two volumes of the above-specified solution. KCl was added to reach 0.39 M KCl. Nuclei were extracted at 4°C for 1 h and centrifuged at 10,000 g for 30 min. The supernatant was clarified by centrifugation and stored at −80°C. Protein concentration was determined with the Bio-Rad protein assay. The NF-κB consensus 5′-CAACGGCAGGGGAATCTCCCTTCTCTTT-3′ oligonucleotide was end-labeled with [γ-32P] ATP (Amersham Pharmacia Biotech) using a polynucleotide kinase (Roche). End-labeled DNA fragments were incubated at room temperature for 15 min with 5 μg of nuclear protein, in the presence of 1 μg poly(dI-dC), in 20 μl of a buffer consisting of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT and 5% glycerol v/v. In competition assays, a 50X molar excess of NF-κB or NFAT cold oligonucleotide was added to the incubation mixture. Protein-DNA complexes were separated from free probe on a 6% polyacrylamide w/v gel run in 0.25X Tris borate buffer at 200 mV for 3 hrs at room temperature. The gels were dried and exposed to X-ray film (Kodak AR).
Statistical analysis

Statistical analysis of the apoptosis data was performed by means of the paired Student’s $t$ test. The chi square test was used to compare categorical data.
RESULTS

Rapamycin induced apoptosis of cALL blasts

We evaluated apoptosis in cultured mononuclear cells from 25 bone marrow samples obtained from cALL patients (15 B-cALL and 10 T-cALL) and exposed to rapamycin. We found that rapamycin significantly \( p=0.004 \) increased basal cell death in 14 (7 B-cALL and 7 T-cALL) of the 25 leukemia samples (56%) (Table 1). Thus, 46.6% of B-cALL and 70.0% of T-cALL samples responded to the drug (responder group). Interestingly, the response of blasts to rapamycin in vitro correlated with prednisone response in vivo \( p=0.01 \). Indeed, 11 of the 14 patients who responded to prednisone in vivo responded to rapamycin in vitro. Similarly, 8 of the 11 poor responders to prednisone in vivo, including the 2 hyperdiploid patients, did not respond to rapamycin in vitro. It is noteworthy that the patient with the t(4;11) rearrangement (patient 18) was among the 3 poor responders to glucocorticoids who responded to rapamycin. Rapamycin caused mitochondrial depolarization after 6 h of incubation (Fig. 1, panel A). After a further 19 h, the proportion of annexin V+ cells was increased more than 80% with an enhancement of caspase 3 catalytic activity of more than 100%. Figure 1B shows dose/response curves relating to five donors. At 20 ng/ml, rapamycin increased basal cell death by at least 25%. The apoptotic effect increased as the rapamycin dose increased. The active doses of rapamycin were within the range considered to be therapeutically achievable (26). Indeed, although the maximum serum level should not exceed 15 ng/ml, it must be taken into account that approximately 95% of rapamycin is vehicled by red blood cell because of its high lipophilicity (26). Rapamycin did not appear to affect the survival of normal peripheral T- and B-lymphocyte (Fig. 1B). The mean values of apoptosis in the rapamycin-responsive samples are shown in panel C, each experiment was in triplicate.
Normal bone marrow cells were less sensitive than cALL blasts to rapamycin-induced apoptosis

We next evaluated if rapamycin is cytotoxic for the normal hematopoietic counterpart of cALL cells. To this aim, we used annexin V in double fluorescence and flow cytometry to examine the effect of rapamycin in diverse bone marrow cell subpopulations from a refractory-cALL (patient 8). Figure 2 shows the flow cytometry diagrams of rapamycin-induced apoptosis of normal mononuclear cells (CD3+ or CD7−) compared to that of CD3−/CD7+ blasts. At a rapamycin concentration between 20-100 ng/ml, the extent of apoptosis of normal bone marrow mononuclear cells was <15%. These results suggested that rapamycin counteracted a cell signaling pathway that is deregulated in cALL blasts.

Akt was activated in rapamycin-sensitive samples

To determine if the phosphatidylinositol pathway is involved in blast survival, we investigated the effect of the PI3k inhibitor wortmannin (27) on cALL cell death. Our data show that wortmannin induced levels of apoptosis comparable to those induced by rapamycin in 11 of the 15 samples from the responder group, whereas none of the samples from non-responder patients underwent apoptosis when stimulated with wortmannin. Figure 3A shows the flow cytometry diagrams of annexin V binding to cALL blasts from two samples, cultured for 24 h with rapamycin (100 ng/ml) or wortmannin (1 µM). Analysis of phospho-Akt by western blotting assay revealed high expression of both Ser473- and Thr301-phospho-Akt in patient 1 and of Thr301-phospho-Akt in patient 2 (Fig. 3B). These findings suggest that the PI3k/Akt pathway is involved in the survival of malignant lymphoblasts.

Rapamycin enhanced doxorubicin-induced apoptosis
Anthracycline compounds are widely used to treat leukemias. We investigated if rapamycin could enhance doxorubin-induced cALL blast apoptosis. To this end, we cultured primary leukemic cells from 15 bone marrows (8 samples responding and 7 non responding to rapamycin) for 24 h with 500 nM doxorubicin, in the presence and not of rapamycin (100 ng/ml), and then evaluated apoptosis by measuring annexin V staining in flow cytometry. In responder samples (Fig. 4, panel A), both rapamycin and doxorubicin significantly increased cell death over basal levels (p=0.000 and 0.005, respectively). The addition of rapamycin to doxorubicin-exposed cells increased apoptosis versus cells exposed to doxorubicin alone, in both responder (Fig. 4A) (p =0.001) and non responder samples (Fig. 4B) (p=0.001). Therefore, rapamycin exerts a pro-apoptotic effect even when it is unable to directly activate cell death. As is shown in Fig. 4C, the cooperative effect of rapamycin plus doxorubicin was detected at doses ≥20 ng/ml. Figure 4D shows a sample in which rapamycin or doxorubicin alone did not induce cell death, whereas the two drugs together enhanced basal apoptosis by more than 90%.

Rapamycin inhibited doxorubicin-induced NF-κB/Rel activation in cALL cells

To identify the mechanism by which rapamycin enhanced doxorubicin-induced apoptosis in cALL blasts, we investigated if rapamycin was able to counteract the induction of NF-κB/Rel transcription factors (20) in cALL blasts. In fact, anthracycline compounds activate transcription factors that play an important role in chemoresistance (21). As shown in Fig. 5A, rapamycin, but not wortmannin, inhibited the translocation of NF-κB/Rel complexes in blast nuclei. Furthermore, rapamycin but not wortmannin enhanced doxorubicin-induced apoptosis (Fig. 5B). These results suggest that rapamycin can sensitize cALL blasts to anthracycline drugs by inhibiting activation of NF-κB/Rel transcription factors through a mechanism independent of PI3k/Akt inhibition.
The enhancement of apoptosis by rapamycin was antagonized by p65(RelA) hyperexpression

To determine if rapamycin-induced enhancement of apoptosis was related to NF-κB down-modulation, we investigated whether rapamycin increased apoptosis when NF-κB was over-expressed. In these experiments we used the Jurkat leukemic cell line in which we transfected PCMV4 vector carrying the p65 subunit of NF-κB and resistance to genetycin, thus obtaining stable transfectants (Fig. 6, panel A). As shown in Fig. 6B, doxorubicin activated NF-κB in Jurkat cells, whereas rapamycin antagonized this effect. Moreover, as expected, p65 transfectants constitutively expressed NF-κB in nuclei, but control cells did not. We subsequently incubated p65- and void vector-stable transfectants both with and without doxorubicin for 24 h, and analyzed apoptosis by propidium iodide incorporation. Rapamycin did not enhance apoptosis in cells hyper-expressing p65. In fact, it increased apoptosis in control cells by 59.4% (p=0.032) and by only 3.9% in RelA hyper-expressing Jurkat cells (Fig. 6, panel C). We therefore conclude that down-modulation of NF-κB/Rel transcription factors is a mechanism by which rapamycin enhances apoptosis and that rapamycin can cooperate with NF-κB-inducing drugs.

The rapamycin-binding protein FKBP51 controls NF-κB activation in leukemia

The immunophilin FKBP51 is required for IKK-α functioning (23). Rapamycin specifically binds to FKBP51 and inhibits its peptidyl-prolyl-isomerase activity (22). FKBP51 was first cloned in lymphocytes in which it was abundant (22). To assess the role of FKBP51 in the NF-κB activation pathway in human leukemia, we down-modulated immunophilin levels in Jurkat cells using the (si)RNA technique. As shown in Fig. 7A, the expression levels of FKB51 were remarkably decreased in Jurkat cells transfected with FKBP51 siRNA than in cells incubated with control medium or transfected with a
scrambled oligonucleotide. We then investigated the ability of doxorubicin to induce IκBα degradation and NF-κB nuclear translocation when FKBP51 was down-modulated. Figure 7B shows that IκBα levels decreased in control- or scrambled-oligo-transfected cells cultured with doxorubicin, but not when FKBP51 was down-modulated. Accordingly, NF-κB complexes were not detected by EMSA in nuclear extracts from FKBP51 siRNA-transfected cells (Fig 7C). These findings suggest that FKBP51 controls NF-κB activation in human leukemia.
DISCUSSION

The mammalian target of rapamycin mediates PI3k/Akt-driven cell proliferation and survival (12, 15–18). In line with this finding, rapamycin has been reported to exert its major anti-cancer effect in neoplasias that lack the PI3k antagonist, PTEN (12, 28) (phosphatase and tensin homolog). mTOR controls the synthesis of proteins essential for cell cycle progression and cellular proliferation (14–17) by phosphorylating two key translational regulators: the initiation factor 4E binding protein (4E-BPI) (16), and the 70-kDa S6 ribosomal protein kinase (p70S6k) (14). Several lines of evidence support the view that abnormal survival signals from the phosphatidylinositol cascade lead to neoplastic transformation of lymphoid precursors (5–7, 19). The finding that rapamycin exerts antiproliferative and apoptotic effects on B-precursor leukemia, \textit{in vitro} and \textit{in vivo}, by mechanisms involving the inhibition of mTOR and p70S6k (19), provided the rationale for new therapeutic strategies against acute lymphoblastic leukemia. In agreement with these findings, we show that rapamycin induces apoptosis of blasts in 56% of bone marrow samples from cALL patients. Moreover, we found that apoptosis can be induced, in responder samples, also by inhibiting phosphatidylinositol 3-kinase using the specific inhibitor wortmannin (27). These findings, together with the detection of constitutive activation of Akt in two different cALL bone marrow samples, support previous evidence (5–7, 19) that the phosphatidylinositol pathway is involved in blast survival.

We also found that rapamycin increased doxorubicin-induced cell death, even in non-responder samples, whereas the PI3k inhibitor wortmannin did not act in concert with doxorubicin. These findings suggest that rapamycin may also exert a pro-apoptotic activity by mechanisms independent of PI3k/Akt/mTOR inhibition. We demonstrate that the immunophilin FKBP51 controls drug-induced NF-κB activation in human leukemia,
which explains the pro-apoptotic effect of rapamycin. Immunophilins are the first target of
the drug (22, 29) and, in fact, the binding of rapamycin to FKBP12 is crucial for mTOR
inhibition (29). Immunophilins are abundant cytosolic proteins endowed with inherent
peptidyl-prolyl cis-trans isomerase activity that is inhibited by drug ligand binding (22,
29). Given the biological relevance of this class of proteins (24,34-36) it is not surprising
that rapamycin induces effects independent of PI3k/Akt/mTOR inhibition.

In conclusion, our findings suggest that rapamycin might be effective in the
treatment of cALL, despite the biological heterogeneity of the disease. Finally, our study,
in agreement with other reports showing that immunophilins are involved in a host of
diseases that do not necessarily involve the same signaling pathway (37, 38), opens the
way to the development of a range of new drugs specifically targeting immunophilins (38).
REFERENCES


FIGURE LEGENDS

Figure 1. Rapamycin induces apoptosis of primary malignant lymphoblasts.
(A) Analysis of mitochondrial membrane potential, phosphatidyl-serine externalization and caspase 3 catalytic activity of cALL blasts cultured with rapamycin (50 ng/ml). After 6 h of incubation mitochondrial potential was analyzed by calculating the amount of JC-1 monomers by flow cytometry. Annexin V binding and caspase-3 activity were measured by flow cytometry and fluorometric assay, respectively, after 24 h of incubation. (B) Dose/response curve to rapamycin. Malignant lymphocytes from 5 different samples and peripheral B or T lymphocytes, from a normal donor, were incubated with rapamycin at different concentrations. After 24 h, the cells were harvested and apoptosis was evaluated by annexin V staining and flow cytometry. For B and T lymphocyte analysis, the whole PBMC was acquired in flow cytometry, after which B or T cells were gated on the basis of CD3/SSc or CD20/SSc parameters, and the percentage of annexin V^+ cells was calculated. (C) Mean values of rapamycin-induced apoptosis in responder samples. Apoptosis was evaluated by annexin V staining of ALL blasts, from the indicated patients, after 24 h of incubation with or without rapamycin (100 ng/ml).

Figure 2. Normal bone marrow mononuclear cells display low sensitivity to cell death stimuli compared to blasts.
Flow cytometry evaluation of apoptosis of leukemic- (CD3^+/CD7^+) or normal- (CD3^+/CD7^+, CD3^-/CD7^+) mononuclear cell populations (pt.8), incubated for 24 h with 25 ng/ml rapamycin. The cells were gated on the basis of FL1 (CD7-FITC)/SSc or FL2(CD3-PE)/SSc parameters and the percentage of annexin positive cells was measured.
Figure 3. The PI3k/Akt pathway plays a role in ALL blast apoptosis

(A) Flow cytometry analysis of apoptosis, by annexin V-FITC staining, of ALL blasts incubated for 24 h with and without rapamycin (100 ng/ml) or wortmannin (1 µM). (B) Western blotting assay of phospho-Akt, at Ser 473 or Thr 308, in cell lysates from the same samples.

Figure 4. Rapamycin enhances doxorubicin-induced apoptosis of ALL cells.

Mean values of apoptosis of ALL blasts, from 8 responder (patients 1, 2, 3, 6, 11, 14, 18, 19) (A) and 7 non responder samples (patients 4, 5, 7, 9, 12, 15, 20), (B) incubated for 24 h with and without rapamycin (100 ng/ml) or doxorubicin (0.5 µM). (C) Dose/response effect of rapamycin on doxorubicin-induced apoptosis. ALL cells (patient 7) were cultured with and without 0.5 µM doxorubicin and with or without rapamycin at the indicated concentrations; 24 h later, apoptosis was measured by annexin V staining. (D) Flow cytometry diagrams of apoptosis of blasts, from patient 20, cultured with doxorubicin at the indicated concentrations, with and without rapamycin (100 ng/ml).

Figure 5. Rapamycin inhibits doxorubicin-induced NF-κB activation in ALL blasts.

(A) EMSA analysis of nuclear extracts from ALL cells (patient 5) cultured for 5 h with 0.5 µM doxorubicin, with and without rapamycin (100 ng/ml) or wortmannin (1 µM). A competition assay performed with the same –κB cold oligo or an unrelated oligo (see Materials and Methods) indicated the specificity of the NF-κB band. (B) Flow cytometry diagrams of apoptosis of ALL blasts, from the same patient, cultured for 24
h with 0.5 μM doxorubicin, with and without rapamycin (100 ng/ml) or wortmannin (1 μM).

Figure 6. Rapamycin does not enhance doxorubicin-induced apoptosis in RelA-hyperexpressing transfectants.

(A) Western blotting analysis of p65 (RelA) expression levels in cell lysates obtained from wild type Jurkat cells and void vector- or RelA-stably transfectants. (B) EMSA analysis of nuclear extracts obtained from Jurkat wild type cells cultured for 5 h with and without 5 μM doxorubicin and with or without rapamycin (100 ng/ml), and RelA- or void vector- stably transfectants. (C) Analysis of apoptosis of RelA- or void vector-stable transfectants cultured with and without 5 μM doxorubicin and with and without rapamycin (100 ng/ml). After 24 h of incubation, cells were harvested and cell death was analyzed by propidium iodide incorporation in flow cytometry. Results are from four different experiments, each of which was in triplicate.

Figure 7. FKBP51 controls drug-induced NF-κB activation in human leukemia.

(A) Western blotting analysis of FKBP51 expression levels in cell lysates obtained from transfected or not transfected Jurkat cells, with FKBP51 siRNA or the scrambled oligo as control. (B) Western blotting analysis of IκBα expression levels in cells transfected with FKBP51 siRNA and cultured with or without doxorubicin (5μM) for 5 hrs. (C) EMSA analysis of nuclear extracts from Jurkat cells transfected with FKBP51 siRNA and cultured with or without doxorubicin (5 μM) for 5 h. A competition assay, with the same –κB cold oligo or an unrelated oligo (see Materials and Methods), indicated the specificity of the NF-κB band.
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<td>7.4</td>
<td>100</td>
<td>T</td>
<td>314.950</td>
<td>CD3cy, CD4, CD8, CD10, CD7, CD5, TdT</td>
<td>–</td>
<td>Poor</td>
<td>Yes</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>12.3</td>
<td>90</td>
<td>T</td>
<td>334.900</td>
<td>CD2, CD3, CD4, CD8, CD7</td>
<td>Partial del. chrom.4</td>
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<td>Yes</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>4.0</td>
<td>100</td>
<td>B</td>
<td>45.500</td>
<td>CD19, CD34, CD10</td>
<td>–</td>
<td>Poor</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>14.8</td>
<td>100</td>
<td>B</td>
<td>20.240</td>
<td>CD10, CD19, CD20, DR, CD34cy, TdT</td>
<td>–</td>
<td>Good</td>
<td>Yes</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>1.8</td>
<td>100</td>
<td>B</td>
<td>17.810</td>
<td>CD10, CD19, DR, TdT</td>
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<td>Good</td>
<td>Yes</td>
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<tr>
<td>15</td>
<td>F</td>
<td>3.7</td>
<td>100</td>
<td>B</td>
<td>108.700</td>
<td>CD34, CD19, CD10, DR</td>
<td>Hyperdiploid</td>
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<td>No</td>
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<tr>
<td>16</td>
<td>F</td>
<td>2.7</td>
<td>100</td>
<td>B</td>
<td>196.000</td>
<td>CD10, CD19, CD20, DR, CD34</td>
<td>Hyperdiploid</td>
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<tr>
<td>17</td>
<td>M</td>
<td>2.0</td>
<td>98</td>
<td>T</td>
<td>92.500</td>
<td>CD5, CD2, CD7, CD3cy, CD10, CD4, CD8</td>
<td>–</td>
<td>Poor</td>
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<tr>
<td>18</td>
<td>F</td>
<td>1.9</td>
<td>98</td>
<td>B</td>
<td>124.560</td>
<td>CD34, CD19, DR</td>
<td>t(4;11)</td>
<td>Poor</td>
<td>Yes</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>5.7</td>
<td>100</td>
<td>T</td>
<td>16.800</td>
<td>CD2, CD5, CD7, TdT, CD10cy</td>
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<td>Good</td>
<td>Yes</td>
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<tr>
<td>20</td>
<td>F</td>
<td>3.5</td>
<td>98</td>
<td>B</td>
<td>78.400</td>
<td>TdT, CD10, CD19, CD20, DR</td>
<td>t(12;21)</td>
<td>Good</td>
<td>No</td>
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<td>21</td>
<td>M</td>
<td>8.11</td>
<td>98</td>
<td>T</td>
<td>21.990</td>
<td>CD3cy, CD7, CD5, TdT, CD34, TCR</td>
<td>–</td>
<td>Good</td>
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<td>22</td>
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<td>10.7</td>
<td>60</td>
<td>B</td>
<td>3.630</td>
<td>CD10, CD19, CD22, CD34, TdT</td>
<td>NT</td>
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<td>Yes</td>
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<tr>
<td>23</td>
<td>F</td>
<td>9.10</td>
<td>99</td>
<td>B</td>
<td>34.190</td>
<td>CD19, CD34, TdT</td>
<td>–</td>
<td>Poor</td>
<td>No</td>
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<td>24</td>
<td>M</td>
<td>11.1</td>
<td>99</td>
<td>T</td>
<td>310.000</td>
<td>CD7, CD2, CD5, CD8, cyCD3, TdT</td>
<td>–</td>
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<td>25</td>
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<td>9.3</td>
<td>90</td>
<td>B</td>
<td>6.970</td>
<td>CD10, CD19, CD33 CD34, TdT</td>
<td>–</td>
<td>Good</td>
<td>Yes</td>
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*G.R.= in vivo response to glucocorticoids after a 7 day prednisone prophase
°R.R.= in vitro response to rapamycin
Figure 1

A

B

C

Avellino et al. Fig.1
Avellino et al. Fig. 2

Blasts
\(CD3^- / CD7^+\)
(59.10 %)

Normal
T lymphocytes
\(CD3^+ / CD7^+\)
(0.89 %)

Normal mononuclear cells
\(CD3^- / CD7^-\)
(39.03 %)

Rapamycin

\(\quad - \quad +\)

\[
\begin{array}{cc}
25.51 \% & 48.35 \% \\
6.93 \% & 10.91 \% \\
10.50 \% & 8.99 \%
\end{array}
\]
Avellino et al. Fig. 3
Avellino et al. Fig. 4
A

Doxorubicin - - + + + + +
Rapamycin - + - + + - -
Wortmannin - - + - + - -

NF-κB

Competition

B

Doxorubicin

Counts

28.9 %

Counts

30.5 %

Counts

31.4 %

Counts

Annexin V - FITC

57.5 %

84.1 %

57.6 %

None Rapamycin Wortmannin

Avellino et al. Fig. 5
Avellino et al. Fig. 6
Avellino et al. Fig. 7
Rapamycin Stimulates Apoptosis of Childhood Acute Lymphoblastic Leukemia Cells

Raffaella Avellino, Simona Romano, Rosanna Parasole, Rita Bisogni, Annalisa Lamberti, Vincenzo Poggi, Salvatore Venuta and Maria F Romano