

ORIGINAL ARTICLE

Cytotoxic activity of the novel Akt inhibitor, MK-2206, in T-cell acute lymphoblastic leukemia

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T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive neoplastic disorder arising from T-cell progenitors. T-ALL accounts for 15% of newly diagnosed ALL cases in children and 25% in adults. Although the prognosis of T-ALL has improved, due to the use of polychemotherapy schemes, the outcome of relapsed/chemoresistant T-ALL cases is still poor. A signaling pathway that is frequently upregulated in T-ALL, is the phosphatidylinositol 3-kinase/Akt/mTOR network. To explore whether Akt could represent a target for therapeutic intervention in T-ALL, we evaluated the effects of the novel allosteric Akt inhibitor, MK-2206, on a panel of human T-ALL cell lines and primary cells from T-ALL patients. MK-2206 decreased T-ALL cell line viability by blocking leukemic cells in the G₀/G₁ phase of the cell cycle and inducing apoptosis. MK-2206 also induced autophagy, as demonstrated by an increase in the 14-kDa form of LC3A/B. Western blotting analysis documented a concentration-dependent dephosphorylation of Akt and its downstream targets, GSK-3 α / β and FOXO3A, in response to MK-2206. MK-2206 was cytotoxic to primary T-ALL cells and induced apoptosis in a T-ALL patient cell subset (CD34⁺/CD4⁻/CD7⁻), which is enriched in leukemia-initiating cells. Taken together, our findings indicate that Akt inhibition may represent a potential therapeutic strategy in T-ALL.

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Keywords: T-cell acute lymphoblastic leukemia; Akt; autophagy; targeted therapy; chemotherapy

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy characterized by the accumulation of undifferentiated thymocytes that have acquired multiple genomic aberrations affecting critical transcriptional and signaling networks.^{1–5} Survival rates at 5 years for children and adolescents with T-ALL are 70–75%, whereas for adults the rates are 35–40%.^{6,7} Therefore, there is a need for novel and less toxic treatment strategies targeting aberrantly activated signaling pathways that increase proliferation, survival and drug resistance of T-ALL cells.

The phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway has been shown to have key roles in the proliferation, survival and drug resistance of cancer cells.⁸ In particular, Akt activation and/or overexpression are often associated with resistance to chemotherapy or radiotherapy.^{9,10} Consistently, dominant-negative mutants of Akt enhanced the cytotoxicity of chemotherapeutic agents.¹¹ Thus, small-molecule inhibitors of Akt have a great potential for novel forms of cancer treatment.^{12,13} PI3K/Akt activation is detected in about 85% of T-ALL patients and portends a poorer prognosis.^{14,15} Moreover, when a constitutively active, myristoylated allele of Akt was introduced into murine hematopoietic cells, mice developed a T-cell lymphoma with high frequency (65%).¹⁶ Of note, the evolution of T-cell lymphoma to T-ALL was dependent, among other molecular alterations, also on Akt hyperactivation.¹⁷ Hence, there is a strong rationale for developing novel, molecularly targeted therapies against Akt in T-ALL.

However, the design of ATP-competitive inhibitors selective for Akt has proven challenging, although a few of them have been synthesized and tested in preclinical models of human cancers.¹⁸ MK-2206 is a novel, orally active, allosteric Akt inhibitor, which is under development for the treatment of solid tumors. MK-2206 is a potent and selective drug for Akt, and its efficacy has been proven in preclinical models of human cancers.^{19–21} Remarkably, MK-2206 displayed modest inhibitory effects on glucose transport in GLUT4-expressing adipocytes and GLUT1-rich human erythrocytes.²¹ This finding is clinically relevant, as hyperglycemia is one of the most feared side effects of Akt inhibition *in vivo*. MK-2206 has now entered phase I/II clinical trials for treating solid tumors and acute myelogenous leukemia (<http://clinicaltrials.gov/ct2/results?term=MK2206>).

Here, we documented that MK-2206 was cytotoxic against T-ALL cell lines and patient primary cells, but barely affected the proliferation of peripheral blood CD4⁺ T lymphocytes from healthy donors and the clonogenic potential of CD34⁺ cells from cord blood. Treatment of T-ALL cells with MK-2206 caused cell cycle arrest in G₀/G₁ phase of the cell cycle, apoptosis and autophagy. MK-2206 synergized with doxorubicin in drug-resistant CEM cells. Remarkably, MK-2206 targeted a T-ALL cell subset that might be enriched in leukemia-initiating cells (LICs). Taken together, our findings suggest that targeting Akt with MK-2206, alone or in combination with chemotherapeutic drugs, may be an interesting option for treating T-ALL cases that display aberrant upregulation of PI3K/Akt signaling.

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MATERIALS AND METHODS

Materials

MK-2206 was from Selleck Chemicals (Houston, TX, USA). The kits for magnetic labeling separation of CD4⁺ or CD34⁺ cells were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Primary antibodies for western blotting analysis were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-CD34-phycoerythrin, anti-CD4-PC5, anti-CD7-PC7 and anti-Ser 473 p-Akt-AlexaFluor488 were from Beckman Coulter (Miami, FL, USA). Interleukin (IL) -2, -4, -7, -9 and -15 were from Peprotech (Rocky Hill, NJ, USA).

Cell culture and primary samples

The T-ALL cell lines, MOLT-4, CEM-S (drug-sensitive) and CEM-R (CEM VBL100, drug-resistant cells overexpressing 170-kDa P-glycoprotein) were grown in RPMI 1640, supplemented with 10% fetal bovine serum (thereafter complete medium). Patient samples, peripheral blood CD4⁺ T lymphocytes from healthy donors and cord blood CD34⁺ cells were obtained with informed consent according to institutional guidelines and isolated using Ficoll-Paque for patient lymphoblasts or magnetic labeling for CD4⁺ T lymphocytes and CD34⁺ cells. T-ALL patient lymphoblasts (2×10^6 cells/ml) were cultured in triplicate in flat-bottomed 96-well plates at 37 °C with 5% CO₂. Cultures were carried out for 72 h in complete medium supplemented with 10 ng/ml IL-7. CD4⁺ T lymphocytes (10^5 /well) were cultured in complete medium and stimulated for 48 h with a mixture of 10 µg/ml phytohemagglutinin-M and 50 ng/ml human recombinant IL-2 to induce proliferation. Then, 1 µCi [³H]-thymidine was added, and 16 h later [³H]-thymidine incorporation was measured, using a liquid scintillation counter.

Colony assays

The formation of erythroid burst-forming units, colony-forming unit (CFU)-granulo-macrophage and granulo-erythroid-megakaryocytic-monocytic CFUs from cord blood CD34⁺ hematopoietic cells, was assessed as reported elsewhere,²² while CFU-leukemia assays from primary T-ALL cells were performed by seeding bone marrow CD34⁺ cells in 35-mm petri dishes at 37 °C, 5% CO₂ in Iscove's modified Dulbecco's medium supplemented with 1.0% methylcellulose, 20% fetal bovine serum, 1% bovine serum albumin and various cytokines, including IL-2 (20 ng/ml), IL-4 (10 ng/ml), IL-7 (10 ng/ml), IL-9 (10 ng/ml) and IL-15 (20 ng/ml). Fourteen days after initiation of culture, colonies of 50 cells or more were scored using an inverted microscope.

Cell viability analysis

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed to assess the sensitivity of cells to drugs, as previously described.²³

Cell cycle analysis

Flow cytometric analysis was performed using a propidium iodide/RNase A staining according to standard procedures, as described elsewhere.²⁴ Samples were analyzed on an EPICS XL flow cytometer (Beckman Coulter) with the appropriate software (System II, Beckman Coulter). At least 15 000 events/sample were acquired.

Western blot analysis

This was performed according to standard techniques, as described elsewhere.²⁵

siRNA downregulation of Bcl-XL

This was performed as previously described,²⁶ using Bcl-XL si-Genome duplexes D-003458-01-0010 and D-003458-04-0010 from Dharmacon (Chicago, IL, USA). Scrambled siCONTROL Nontargeting siRNA no. 1 D-001210-01-20 from Dharmacon was used as negative, nonsilencing control.

Combined drug effect analysis

The combination effect and a potential synergy were evaluated from quantitative analysis of dose-effect relationship, as described previously.²⁷ For each combination experiment, a combination index number was calculated using the Biosoft CalcuSyn software (Biosoft, Cambridge, UK).

This method of analysis generally defines combination index values of 0.9–1.1 as additive, 0.3–0.9 as synergistic and <0.3 as strongly synergistic, whereas values >1.1 are considered antagonistic.

Flow cytometric analysis of putative T-ALL LIC

This was performed essentially as previously reported.²⁸ To detect apoptotic cells samples incubated with Annexin V-fluorescein isothiocyanate. In some cases, cells were permeabilized and stained with an AlexaFluor 488-conjugated antibody to Ser 473 p-Akt. Samples were analyzed on a Navios flow cytometer (Beckman Coulter) equipped with Kaluza software (Beckman Coulter).

Statistical evaluation

The data are presented as mean values from three separate experiments \pm s.d. Data were statistically analyzed by a Dunnett test after one-way analysis of variance at a level of significance of $P < 0.05$ vs control samples.

RESULTS

MK-2206 displays cytotoxic pro-apoptotic effects on T-ALL cell lines

To determine whether MK-2206 could affect viability of T-ALL cell lines, MOLT-4, CEM-R and CEM-S cells were incubated in the presence of increasing concentrations of MK-2206 for either 24 or 48 h. Then, the rates of cell survival were analyzed by MTT assays. The experiments documented that all three cell lines were sensitive to MK-2206. After 24 h of incubation with the drug, the IC₅₀ was 2.6 µM for MOLT-4, 4.1 µM for CEM-R and 6.9 µM for CEM-S cells (data not shown). After 48 h of treatment, the cytotoxic effect was slightly more evident, being the IC₅₀ 1.7 µM for MOLT-4, 3.3 µM for CEM-R and 5.1 µM for CEM-S cells (Figure 1a). To establish whether the decreased viability was related to apoptosis, extracts from MOLT-4 and CEM-R cells, treated for 4 h with MK-2206 concentrations ranging from 1 to 10 µM, were analyzed by western blotting, which demonstrated cleavage of procaspase-8, procaspase-9, procaspase-3 and of poly(ADP-ribose)polymerase (Figure 1c).

MK-2206 blocks cells in the G₀/G₁ phase of the cell cycle

Given the importance of the PI3K/Akt/mTOR signaling pathway in the regulation of cell proliferation,²⁹ the effects of MK-2206 on cell cycle progression were also investigated. MOLT-4 cells were treated with MK-2206 for 24 h. Flow cytometric analysis of propidium iodide-stained cells documented a concentration-dependent increase in cells in the G₀/G₁ phase of the cell cycle and a concomitant decrease in cells in both S and G₂/M phase (Figure 1b).

Overall, these findings demonstrated that MK-2206 potently reduced the growth of T-ALL cell lines and that this effect was due to apoptosis and G₀/G₁ cell cycle arrest.

MK-2206 affects PI3K/Akt/mTOR signaling in T-ALL cell lines

As MK-2206 is an allosteric Akt inhibitor, it was analyzed whether treatment with this drug resulted in downregulation of Akt phosphorylation. Upon 4 h of incubation with MK-2206, a concentration-dependent decrease in both Thr 308 and Ser 473 p-Akt levels was detected in all the cell lines analyzed (Figure 2a). Total Akt levels were unaffected by MK-2206. Akt inhibition had functional consequences on the phosphorylation levels of two well-established Akt substrates, GSK3- α/β and FoxO3A. Both of these proteins displayed dephosphorylation at amino acid residues (Ser 21/9 for GSK3- α/β and Thr-32 for FoxO3A) that are targeted by Akt. In contrast, expression of total GSK3- α/β and FoxO3A was unaffected by treatment with MK-2206.

MK-2206 affected also mTOR complex 1 (mTORC1) activity, as it dephosphorylated p70S6K on Thr 389 and 4E-BP1 on Thr 37/46. MK-2206 also diminished mTOR complex 2 (mTORC2) activity, as

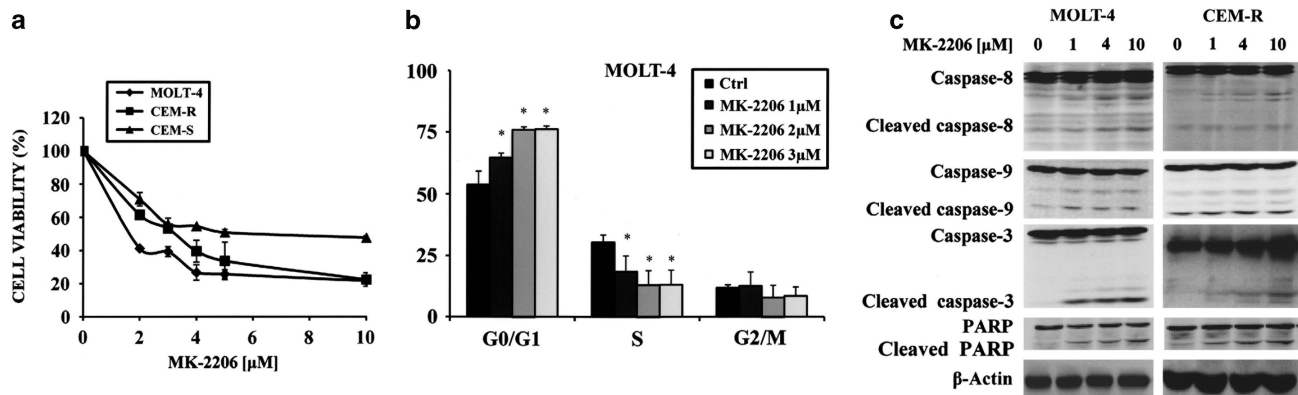


Figure 1. MK-2206 is cytotoxic to T-ALL cell lines and induces cell cycle arrest and apoptosis. **(a)** MTT assay of T-ALL cell lines treated with increased concentrations of MK-2206 for 48 h. **(b)** MOLT-4 cells were treated with increasing concentrations of MK-2206 for 24 h. Then cell cycle analysis was performed by flow cytometry. MK-2206 treatment resulted in an increase in cells in the G₀/G₁ phase and in a decrease in cells in S phase. CTRL, control (untreated) cells. Asterisks indicate significant differences compared with CTRL. In **(a)** and **(b)** results are mean of three different experiments ± s.d. **(c)** Western blot analysis documenting caspase-8, -9, -3 and poly(ADP-ribose)polymerase cleavage in MOLT-4 and CEM-R cells, treated for 4 h with MK-2206. Antibody to β-actin served as a loading control.

documented by a decrease in the levels of Ser 2481 p-mTOR, a readout for mTORC2 activity (Figure 2b).

MK-2206 induces autophagy

It has been reported that MK-2206 induces autophagy in human glioma cells and this protected tumor cells against apoptosis.²⁰ Therefore, we investigated whether MK-2206-induced autophagy also in T-ALL cell lines. MK-2206 increased the amount of cleaved (14-kDa form) LC3A/B, a well-established autophagy marker (Figure 3a). Interestingly, the increased cleavage was detected by western blot in MOLT-4 and CEM-S, but not in CEM-R cells.

Beclin-1, an essential initiator of autophagy, interacts with BH3 domain proteins such as Bcl-2, Bcl-XL and Mcl-1 and these interactions could inhibit beclin-1-mediated induction of autophagy.³⁰ Therefore, the expression levels of Bcl-2, Bcl-XL and Mcl-1 were investigated by western blot in CEM-R and CEM-S cells. Although we did not detect major differences between Bcl-2 and Mcl-1, Bcl-XL was expressed to a much higher extent in CEM-R when compared with CEM-S cells (Supplementary Figure S1). Bcl-XL levels were then downregulated by siRNA in CEM-R cells and the cleavage of LC3A/B was investigated by western blot. In Figure 3b, we document that specific siRNA (but not scrambled siRNA) to Bcl-XL lowered the levels of Bcl-XL in CEM-R cells. Moreover, when cells treated with BCL-XL-specific siRNA were treated with MK-2206, the cleavage of LC3A/B was much more evident than in cells treated with scrambled siRNA. Overall, these findings demonstrated that the levels of Bcl-XL are important for determining the induction of MK-2006-dependent autophagy in CEM-R cells.

We then inhibited autophagy using either bafilomycin A1 or chloroquine and measured cell viability by MTT assays. Both bafilomycin A1 and chloroquine, when used alone, displayed only limited cytotoxic effects against CEM-S cells. However, when they were combined with MK-2206, it was possible to detect an increased cytotoxicity in CEM-S cells (Figure 3c). Overall, these findings suggested that autophagy could protect T-ALL cells by the cytotoxic effects of an Akt inhibitor.

MK-2206 synergizes with doxorubicin

We examined whether MK-2206 could synergize with the anthracycline antibiotic, doxorubicin. Doxorubicin is frequently included in established protocols to treat T-ALL patients.³¹ Two different strategies were explored to determine whether synergy

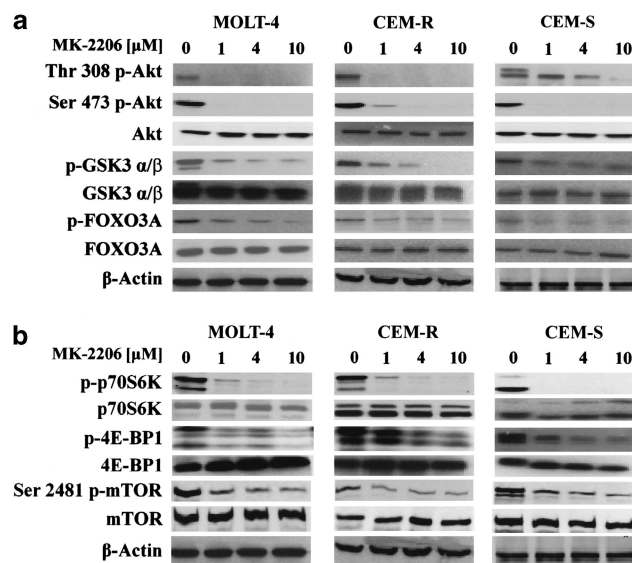


Figure 2. Effects of MK-2206 on the phosphorylation status of critical components of the PI3K/Akt/mTOR signaling pathway. **(a)** Western blot analysis for Akt, GSK3 α/β and FoxO3A. **(b)** Western blot analysis for mTOR and its downstream targets, p70S6K and 4E-BP1. In **(a)** and **(b)**, MK-2206 treatment was for 4 h, and β-actin served as a loading control.

occurred. In the first protocol, T-ALL cell lines were incubated with both MK-2206 and doxorubicin in combination for 48 h. In the second protocol, one drug was added before the other, the first drug was administered for 48 h (throughout the entire experiment) while the second drug was added only for the last 24 h of treatment. In all the experiments, the drugs were used at a fixed ratio (doxorubicin: MK-2206, 1:30 for MOLT-4 cells and 1:1 for CEM-R cells). MTT assays were then performed. In MOLT-4 cells, synergistic effects were observed when MK-2206 was added with doxorubicin together at low concentrations (10–30 nM of doxorubicin) after 48 h of treatment. Also in MOLT-4 cells, both of the sequential treatments resulted in synergistic results, that is, synergy was observed when MK-2206 was added first and doxorubicin was added second, or when doxorubicin was added first and MK-2206 was added second (Figure 4a).

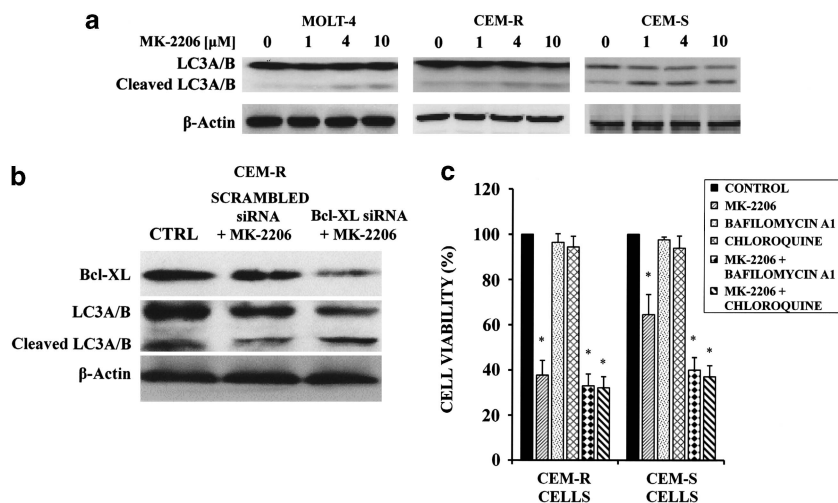


Figure 3. MK-2206 induces autophagy and siRNA downregulation increases MK-2206-dependent cleavage of LC3A/B. (a) Western Blot analysis for LC3A/B in MOLT-4, CEM-R and CEM-S cells. Cells were treated with MK-2206 for 4 h. (b) The levels of Bcl-XL were downregulated by a 48-h-long treatment with siRNA, then cells were incubated for 4 h with 4 μ M MK-2206 and western blot analysis was performed. In (a) and (b), β -actin served as a loading control. (c) MTT assays documenting the effects of bafilomycin A1 (4 μ M) or chloroquine (25 μ M) on viability of CEM-S cells treated for 24 h with MK-2206 (4 μ M). Results are mean of three different experiments \pm s.d. Asterisks indicate significant differences compared with CTRL (untreated cells).

In contrast, different results were observed with the drug-resistant CEM-R cell line. Namely, if the two drugs were administered together for 48 h, synergism was observed at concentrations of doxorubicin ranging from 2.5 to 7 μ M. In the sequential exposure experiments, synergism was dependent upon which drug was added first. When MK-2206 was added after doxorubicin treatment, synergism was detected at all the concentrations tested. In contrast, when the reverse sequence was performed, where cells were exposed to MK-2206 for 48 h followed by post-treatment with doxorubicin for 24 h, antagonism was frequently observed (Figure 4b). Thus, in the drug-resistant CEM-R cells, addition of doxorubicin for the entire period was required to detect synergy with the Akt inhibitor MK-2206.

T-ALL lymphoblasts are sensitive to MK-2206

To better assess the efficacy of the inhibitors as potential therapeutic agents in T-ALL, we examined six T-ALL pediatric patient samples isolated from bone marrow, for their sensitivity to MK-2206. All of the patients displayed enhanced phosphorylation of Ser 473 p-Akt (data not shown). T-ALL lymphoblast samples, cultured in the presence of IL-7, which functions as a powerful proliferative stimulus for these cells,^{32,33} were treated with increasing concentrations of MK-2206, and cell survival was analyzed by MTT assays. A marked reduction in cell viability at 72 h was detected. Under these conditions, the MK-2206 IC₅₀ for patient samples ranged between 0.75 and 1.35 μ M (Figure 5a). In contrast, CD4⁺ T lymphocytes isolated from the peripheral blood of healthy donors and stimulated with phytohemagglutinin-M plus IL-2 were much less sensitive to MK-2206 concentrations up to 10 μ M, as far as their proliferation (assayed by [³H]-thymidine incorporation) was concerned (Figure 5b). Overall, these findings demonstrated that MK-2206 reduced the growth of T-ALL primary cells. Moreover, they also suggested that the drug could have a favorable therapeutic index, as it affected proliferation of normal CD4⁺ T lymphocytes to a much lower extent.

MK-2206 inhibits the clonogenic growth of T-ALL progenitors without affecting normal hematopoiesis

Clonogenic cultures of primary T-ALL were generated from CD34⁺ cells treated with 0.5–5.0 μ M MK-2206. The levels of CFU-leukemia formation from three different T-ALL samples were

markedly reduced with a mean decrease from 63 to 21%, after treatment with 0.5–5.0 μ M MK-2206 (Figure 5c). In contrast, MK-2206 did not affect in a statistically significant manner the clonogenic growth and differentiation of cord blood CD34⁺ hematopoietic progenitors, even at the maximal concentration of 5.0 μ M. Indeed, the number of the erythroid burst-forming units, or granulo-monocytic (CFU-granulo-macrophage), or granulocyte, erythroid, macrophage and megakaryocyte (CFU-granulo-erythroid-megakaryocytic-monocytic), colonies was not significantly decreased by the drug (Figure 5c).

MK-2206 affects PI3K/Akt signaling in T-ALL patient samples

T-ALL lymphoblast samples, cultured as described above, were treated with 1 μ M MK-2206 for 48 h and then analyzed by western blot. In all the patients analyzed ($n=5$), the drug induced a downregulation of Akt phosphorylation (Figure 5d).

MK-2206 dephosphorylates Akt and induces apoptosis in the CD34⁺/CD7⁻/CD4⁻ subset of patient lymphoblasts

Finally, we investigated whether MK-2206 could dephosphorylate Akt and induce apoptosis in a T-ALL patient lymphoblast subpopulation (CD34⁺/CD7⁻/CD4⁻), which is enriched in putative LIC,³⁴ using quadruple staining and flow cytometric analysis. After electronic gating on the CD7⁻/CD4⁻ lymphoblast subset, cells were analyzed for CD34⁺ expression and positivity to either Ser 473 p-Akt or Annexin V-fluorescein isothiocyanate staining. After 48 h of treatment, the drug markedly dephosphorylated Akt at Ser 473 (Figure 6a) and induced apoptosis in the CD34⁺/CD7⁻/CD4⁻ subpopulation, as documented by Annexin-V fluorescein isothiocyanate staining (Figure 6b).

DISCUSSION

The PI3K/Akt/mTOR signaling cascade is a pivotal pathway that is deregulated in a wide variety of human cancers and strongly contributes to both tumorigenesis and therapy resistance. Considering the crucial role had by aberrantly activated Akt in the pathogenesis of T-ALL,^{16,17} we studied the efficacy of MK-2206, a novel allosteric Akt inhibitor,¹⁹ as a potential therapeutic agent. MK-2206 decreased the viability of T-ALL cell lines, in a concentration-dependent manner. All these cell lines are

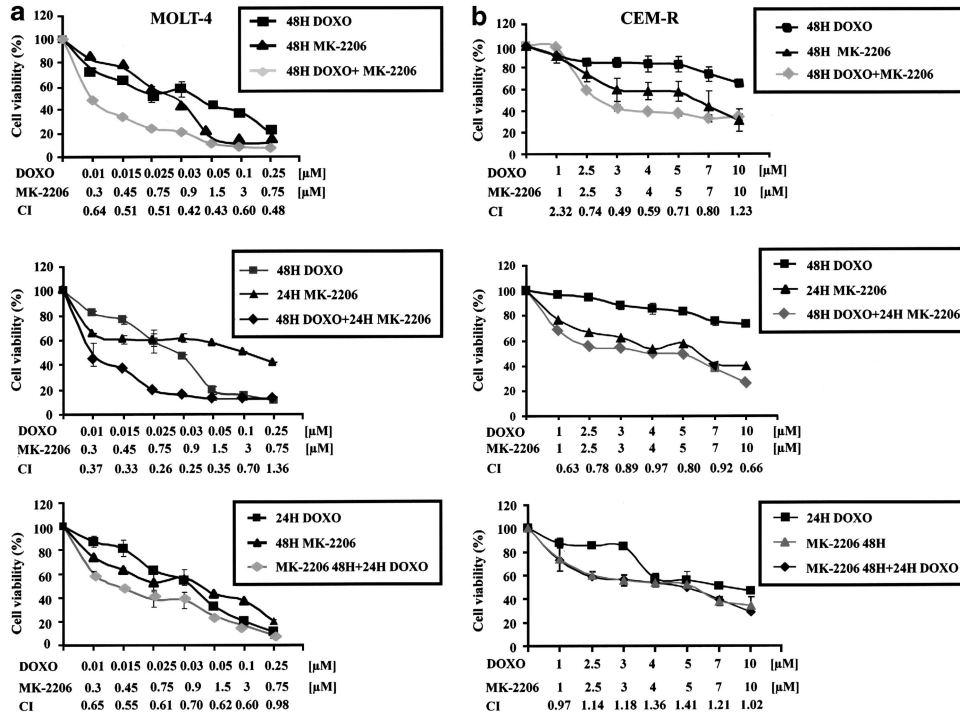


Figure 4. Synergistic effects of the MK-2206 plus doxorubicin combination in MOLT-4 and CEM-R cells. (a) MOLT-4 cells were treated for 48 h with MK-2206 or doxorubicin, either alone, in combination or in sequential exposure (relative concentration ratio, MK-2206:doxorubicin, 30:1). (b) CEM-R cells were treated for 48 h with MK-2206 and doxorubicin, alone, in combination or in sequential exposure (relative concentration ratio, MK-2206:doxorubicin, 1:1). Viability was then analyzed by MTT assays. Results are mean of three different experiments \pm s.d. Combination index (CI) value for each data point was calculated with the appropriate software for dose effect analysis.

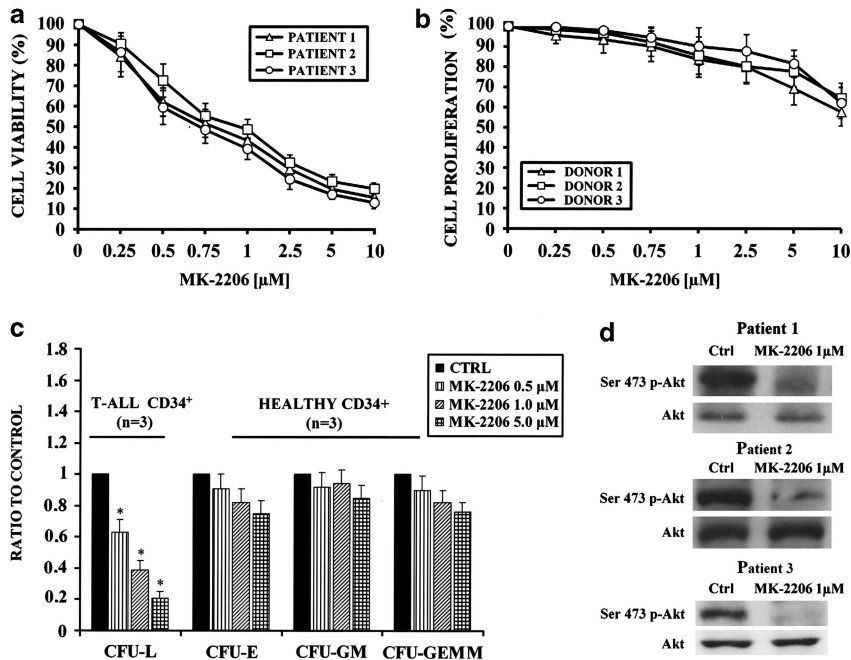


Figure 5. MK-2206 is cytotoxic to primary T-ALL cells. (a) Three representative T-ALL patient lymphoblast samples, cultured in the presence of IL-7, were treated with increasing concentrations of MK-2206 for 72 h. Cell viability was then analyzed by MTT assays. (b) CD4⁺ T lymphocytes, isolated from the peripheral blood of healthy donors, stimulated with phytohemagglutinin-M plus IL-2 for 72 h, were assayed for their proliferation by [³H]-thymidine incorporation in the presence of MK-2206. (c) Clonogenic activity of CD34⁺ cells from three representative T-ALL patients or from cord blood CD34⁺ cells obtained from three healthy donors. Cells were cultured for 14 days, then colony formation was assessed under an inverted microscope. In (a–c), results are mean of three different experiments \pm s.d. Asterisks indicate significant differences compared with CTRL (untreated samples). (d) Western blot analysis on T-ALL lymphoblast samples cultured as in a. In all three patients, the phosphorylation status of Ser 473 p-Akt was tested before and after MK-2206 (1 μ M for 48 h) treatment *in vitro*.

phosphatase and tensin homologue deleted on chromosome 10 negative and display a nonfunctional p53 pathway.³⁵ The efficacy of MK-2206 in decreasing the viability of T-ALL cell lines was due to both cell cycle arrest and caspase-dependent apoptosis.

MK-2206 dephosphorylated Akt on both Thr 308 and Ser 473, its downstream targets, GSK3 α/β and FoxO3A and two mTORC1 downstream targets, that is, p70S6K and 4EB-P1. Downregulation of Ser 2448 p-mTOR levels were indicative of an inhibition of mTORC1 (data not shown), whereas decreased phosphorylation of Ser 473 p-Akt indicated an indirect targeting of mTORC2 by MK-2206.

The mechanisms that control mTORC2 activity have only begun to be revealed. mTORC2 activation requires PI3K and the TSC1/TSC2 complex.³⁶ As Akt is upstream of TSC1/TSC2, MK-2206, by inhibiting Akt, could also downregulate mTORC2 activity.

In addition to apoptosis, MK-2206 caused autophagy in T-ALL cell lines. Autophagy induction by MK-2206 could be related to mTORC1 inhibition, as mTORC1 inhibits autophagy through phosphorylation of two autophagy-promoting factors, unc-51-like kinase 1 and autophagy-related gene 13.³⁷ However, the induction of autophagy, as indicated by cleavage of LC3A/B, was much stronger in MOLT-4 and CEM-S cells than in drug-resistant CEM-R cells. As downregulation of Bcl-XL by siRNA increased LC3A/B cleavage in CEM-R cells, we could infer that the differential induction of autophagy in CEM-R vs CEM-S cells was related to the fact that CEM-R cells express Bcl-XL to a much higher extent than CEM-S cells. This observation is consistent with findings obtained in other cell types.³⁸

Autophagy is a lysosomal degradation pathway that is essential for survival, differentiation development and homeostasis. Tumor cells can exploit autophagy as a survival mechanism to tolerate metabolic stress and to contrast apoptosis.³⁹ Interestingly, both bafilomycin A1 and chloroquine, two

inhibitors of autophagy, sensitized CEM-S cells to MK-2206, by increasing apoptosis. This finding demonstrated that autophagy is a prosurvival mechanism that protected CEM-S cells from MK-2206-induced apoptosis. Therefore, the use of autophagy inhibitors in combination with MK-2206 should be considered in future clinical trials.

We have also documented that MK-2206 synergized with doxorubicin. Analysis of the results demonstrated that in MOLT-4 the synergism of MK-2206 with doxorubicin was relevant at low concentrations (10–30 nM of doxorubicin) at 48 h of treatment. The sequential exposure to MK-2206 after doxorubicin treatment for a total of 48 h resulted in relevant synergism at concentrations ranging from 10 to 100 nM doxorubicin, with an even stronger synergism using the reverse sequence. In CEM-R cell line, if the two drugs were administered together for 48 h, the synergism was evident at intermediate concentrations. The sequential exposure to MK-2206 after doxorubicin treatment for a total of 48 h resulted in relevant synergism (Figure 4b). We are presently investigating the reasons that could explain these findings. Nevertheless, it is remarkable that in MOLT-4 cells a synergism was detected at concentrations that were well below the MK-2206 IC₅₀ for this cell line. This finding could have a clinical relevance for T-ALL patients, as a combination of MK-2206 and doxorubicin increased the cytotoxic activity of MK-2206 and allowed for the use of a much lower concentration of the inhibitor. This could considerably attenuate the possible MK-2206 toxic side effects. However, MK-2206 is generally well tolerated at doses that result in plasma concentrations portending activity in preclinical models.⁴⁰ MK-2206 demonstrated marked anti-leukemic effects *in vitro* against T-ALL patient lymphoblasts. The cytotoxic effects were specific to leukemic cells, as the drug only slightly affected the proliferation of CD4⁺ T lymphocytes from healthy donors. Moreover, MK-2206 spared normal hematopoiesis *in vitro*, while it affected the clonogenic activity of T-ALL CD34⁺ cells. Taken together, these findings suggest that MK-2206 could result in a favorable therapeutic window also *in vivo*.

The difficulty in eradicating tumors might result from the conventional treatments targeting the bulk of the tumor cells, but not LICs.⁴¹ Therefore, strategies aimed to eliminating these cells could have significant clinical implications. Of note, MK-2206 dephosphorylated Akt and induced apoptosis in a T-ALL lymphoblast subpopulation (CD34⁺/CD7⁻/CD4⁻), which has been reported to be enriched in LIC in pediatric patients.³⁴

In conclusion, our preclinical findings strongly suggest that MK-2206, either alone or combined with traditional chemotherapeutic drugs, could be a valuable compound for treating those T-ALL patients displaying activation of PI3K/Akt signaling and who are still facing a poor prognosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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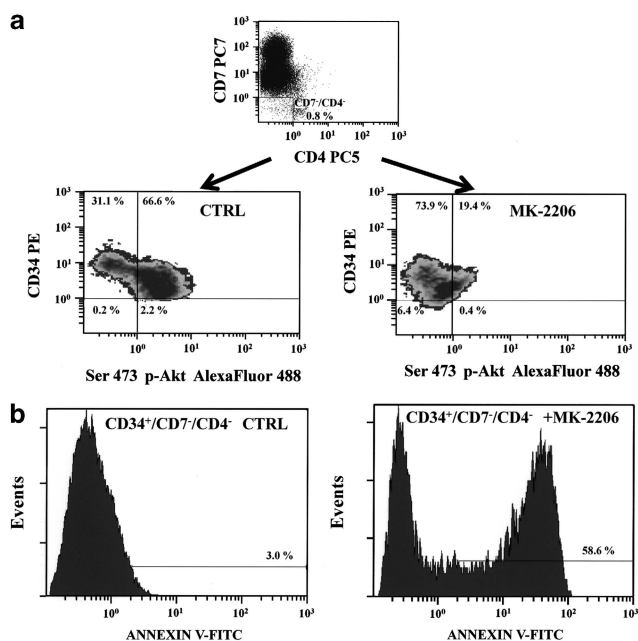


Figure 6. MK-2206 causes Akt dephosphorylation and apoptosis in the CD34⁺/CD7⁻/CD4⁻ subset of patient lymphoblasts. (a) Primary T-ALL cells were incubated with MK-2206 (1 μ M for 48 h). After electronic gating on the CD7⁻/CD4⁻ lymphoblast subset, cells were analyzed for CD34 expression and positivity to Ser 473 p-Akt. (b) The CD34⁺/CD7⁻/CD4⁻ subset was stained with Annexin-fluorescein isothiocyanate (FITC) to analyze apoptosis induction by MK-2206 (1 μ M for 48 h). In (a, b), one representative of four different patients is shown. CTRL, untreated cells.

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Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)