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ORIGINAL ARTICLE

Targeting the PI3K/Akt/mTOR signaling pathway in B-precursor acute lymphoblastic leukemia and its therapeutic potential

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B-precursor acute lymphoblastic leukemia (B-pre ALL) is a malignant disorder characterized by the abnormal proliferation of B-cell progenitors. The prognosis of B-pre ALL has improved in pediatric patients, but the outcome is much less successful in adults. Constitutive activation of the phosphatidylinositol 3-kinase (PI3K), Akt and the mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR) network is a feature of B-pre ALL, where it strongly influences cell growth and survival. RAD001, a selective mTORC1 inhibitor, has been shown to be cytotoxic against many types of cancer including hematological malignancies. To investigate whether mTORC1 could represent a target in the therapy of B-pre ALL, we treated cell lines and adult patient primary cells with RAD001. We documented that RAD001 decreased cell viability, induced cell cycle arrest in G_0/G_1 phase and caused apoptosis in B-pre ALL cell lines. Autophagy was also induced, which was important for the RAD001 cytotoxic effect, as downregulation of Beclin-1 reduced drug cytotoxicity. RAD001 strongly synergized with the novel allosteric Akt inhibitor MK-2206 in both cell lines and patient samples. Similar results were obtained with the combination CCI-779 plus GSK 690693. These findings point out that mTORC1 inhibitors, either as a single agent or in combination with Akt inhibitors, could represent a potential therapeutic innovative strategy in B-pre ALL.

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Keywords: B-pre acute lymphoblastic leukemia; RAD001; mTOR; targeted therapy; Akt

INTRODUCTION

B-precursor acute lymphoblastic leukemia (B-pre ALL) is characterized by malignant proliferation and accumulation of early lymphoid precursor cells in the bone marrow, blood and lymphoid organs,[1] due to acquired mutations in early B-cells.[2,3] B-pre ALL is predominantly a childhood disease but can occur at any age. Although the prognosis of B-pre ALL has improved, the outcome of relapsed and chemoresistant B-pre ALL is still poor, especially in adults, with a 35–40% survival at 5 years, whereas in children, it is about 80%.[1] Therefore, major efforts are being made to develop rationally targeted therapies against altered signaling cascades that sustain leukemia cell proliferation, survival and drug resistance.

The phosphatidylinositol 3-kinase (PI3K), Akt and the mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR) signaling pathway is frequently observed to be deregulated, thus leading to the pathogenesis of a variety of leukemias[4,5] including acute myeloid leukemia (AML),[6] T-cell ALL (T-ALL)[7] and B-pre ALL.[8]

mTOR is a serine/threonine kinase, downstream of Akt, that controls cell proliferation and survival. mTOR is the catalytic subunit of two distinct multi-protein complexes, referred to as mTORC1 and mTORC2.[6] mTORC1 phosphorylates the S6 ribosomal protein kinase (p70S6K) and the initiation factor 4E-binding protein 1 (4EBP1), which are important in translational control,[9,10] whereas mTORC2 phosphorylates Akt on Ser 473 residue.[11] Constitutive activation of PI3K/Akt/mTOR signaling negatively influences the response to therapeutic treatments and correlates with enhanced drug resistance and poor prognosis in various types of cancer.[12,13] Rapamycin, the first disclosed mTORC1 inhibitor, has been shown to exert significant *in vitro* anti-leukemic activity in ALL[14,15] and in B-pre ALL.[16] RAD001 (Everolimus; 40-O-(2-hydroxyethyl)-rapamycin) is an orally bioavailable ester derivative of rapamycin. RAD001 binds the intracellular protein FK-506-binding protein-12, forming a complex that inhibits the activity of mTORC1, thus affecting cell cycle progression, survival, angiogenesis and glycolysis.[17] RAD001 has been shown to be active against many subsets of leukemia, such as AML and acute promyelocytic leukemia,[18] non-Hodgkin's lymphoma[19] and other hematological malignancies, being under evaluation in several phase I/II clinical trials.[20] However, B-pre ALL was not included in these trials.

Here, the potential therapeutic efficacy of RAD001 was examined in B-pre ALL cell lines and adult patient primary cells. In particular, the drug induced G_0/G_1 phase cell cycle arrest, modulated the PI3K/Akt/mTOR pathway and caused apoptosis and autophagy in a dose-dependent manner. Moreover, dual treatment combining RAD001 with an allosteric Akt inhibitor, MK-2206, displayed a dramatic synergistic effect against leukemic cells. Similar results were obtained with the combination consisting of CCI-779 (Temsirolimus, another mTORC1 inhibitor[21]) and GSK 690693, an ATP-competitive Akt inhibitor.[22]

These findings indicate that mTOR inhibition, alone or in combination with additional drugs targeted to other components

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of the PI3K/Akt/mTOR signal transduction pathway, could be an attractive strategy to develop innovative therapeutic protocols for the treatment of B-pre ALL leukemia patients.

MATERIALS AND METHODS

Materials

Cell Viability Kit or MTT was from Roche Applied Science (Mannheim, Germany). Annexin V/7-AAD was from Merck-Millipore (Darmstadt, Germany). For western blotting, primary and secondary antibodies were provided by Cell Signaling Technology (Danvers, MA, USA). Signals were detected with the ECL Plus reagent by Perkin Elmer (Boston, MA, USA). RAD001, CCI-779, MK-2206 and GSK 690693 were purchased from Selleck Chemicals (Houston, TX, USA). SignalSilence control small interfering RNA (siRNA) and Beclin-1 siRNA II were obtained from Cell Signaling Technology.

Cell culture

The B-pre ALL cell lines SEM, REH, RS4;11 and NALM6 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). SEM, REH and NALM6 were grown in RPMI 1640 medium, whereas RS4;11 cells were grown in Alpha-MEM medium, both supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin.[23] The cells were grown at a density of 0.5 to 0.8 $\times 10^6$ cells/ml. Primary samples from adult B-pre ALL patients ($CD10^{+/-}$, $CD19^+$, HLA DR^+ and cytoplasmic IgM^+) were obtained with informed consent according to institutional guidelines. B-pre ALL patient lymphoblasts were cultured in triplicate in flat-bottomed 96-well plates at 37 °C with 5% CO_2 at a density of 2×10^6 cells/ml, using RPMI medium supplemented with 20% fetal bovine serum and 2 mM l-glutamine.

Cell viability analysis by MTT assay

MTT (3-(4,5-dimethylthythiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed as previously described.[24]

Western blot analysis

For protein extraction, 2×10^6 cells were washed in PBS and lysed with RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% SDS and 1% NP40), including protease and phosphatase inhibitors obtained from Roche Applied Science. Samples were incubated for 30 min at 4 °C, and cell extracts were sonicated and centrifuged at 13 000 *g* for 10 min at 4 °C. Protein concentration was assayed using the BCA Protein Assay (Euroclone, Milan, Italy). Samples were loaded on a polyacrylamide gel for electrophoresis separation and transferred to a nitrocellulose transfer membrane. Membranes were blocked in 5% non-fat dry-milk and incubated overnight at 4 °C with the primary antibodies. A mouse anti-β-actin antibody from Sigma-Aldrich (St Louis, MO, USA) was used as a loading control. Signals were detected with the ECL Plus reagent and a ImageQuant LAS4000 detection system (GE Healthcare Europe GmbH, Freiburg, Germany).[25]

Cell cycle analysis

Cell cycle analysis was performed using propidium iodide (PI)/RNase A staining according to standard techniques, as described elsewhere.[26] Samples were analyzed on a EPICS XL flow cytometer Beckman Coulter (Miami, FL, USA) with the appropriate software (System II, Beckman Coulter). At least 15 000 events/sample were acquired.

PI/Annexin V assay

Briefly, apoptosis analysis was performed by staining with Annexin V/7-AAD, using a Muse Cell Analyzer (Merck-Millipore). For primary cell analysis, samples were incubated with Annexin V-fluorescein isothiocyanate (FITC) and PI.[27] Samples were analyzed on a FC500 flow cytometer by Beckman Coulter.

siRNA downregulation of beclin-1

This was accomplished using 100 nM of either control (scrambled) or Beclin-1-specific siRNA, essentially as described elsewhere.[28]

Combined drug effect analysis

To characterize the effect of either RAD001/MK-2206 or CCI-779/GSK 690693 administered in combination and their potential synergy, data were evaluated from quantitative analysis of dose-effect relationship, as described previously. For each combination experiment, a combination index (CI) number was calculated using the Biosoft CalcuSyn software (Biosoft, Cambridge, UK). This method of analysis generally defines CI values from 0.9 to 1.1 as additive, from 0.3 to 0.9 as synergistic and <0.3 as strongly synergistic, whereas values >1.1 are considered as antagonistic.[29]

Statistical evaluation

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

RESULTS

The PI3K/Akt/mTOR pathway activation status and RAD001 effects in B-pre ALL cell lines

By western blotting, the PI3K/Akt/mTOR pathway activation status in B-pre ALL cell lines was first evaluated. Ser 473 p-Akt and Ser 2481 p-mTOR (indicative of mTORC2 activity)[30] were maximally phosphorylated in SEM cells and, to a lower extent, in RS4;11 cells, whereas REH cells displayed very low levels of Ser 473 p-Akt phosphorylation. Also Ser 2448 p-mTOR levels (a readout for mTORC1 activity)[30] were higher in SEM cells than in either RS4;11 or REH cells (Figure 1a). Phosphatase and tensin homolog PTEN, a negative regulator of the PI3K/Akt/mTOR pathway,[31] was much more abundantly expressed in REH cells.

We next examined the RAD001 cytotoxic effects on B-pre ALL cell lines. Cell lines were treated with increasing concentrations of RAD001 for 48 h and then analyzed by MTT assays. Cell viability decreased in a dose-dependent manner. Under these conditions, the RAD001 IC_{50} for cell lines ranged between 6.3 and 10.4 μM. It is noteworthy that SEM cells were the most sensitive to RAD001 when compared with the other cell lines (Figure 1b). The cytotoxic effects of RAD001 on SEM, REH and NALM6 cells were also studied by flow cytometry analysis of Annexin V-stained samples. Overall, the results were similar to those obtained with MTT assays, displaying a concentration-dependent increase of apoptotic cells (Figure 1c).

RAD001 modulates PI3K/Akt/mTOR signaling in B-pre ALL cell lines

We studied the effects of RAD001 on the phosphorylation levels of critical components of the PI3K/Akt/mTOR cascade. REH, SEM and RS4;11 cells were treated with increasing concentrations of RAD001 for 4 h and then analyzed by western blotting (Figure 2a). RAD001 not only decreased the phosphorylation levels of mTOR mainly on the Ser 2448 residue (a readout for mTORC1 activity) but also on the Ser 2481 phosphorylation site (a marker for mTORC2 activity). The total amount of mTOR was unchanged. mTORC1 inhibition had functional effects on two well-known mTORC1 substrates, p70S6K and 4EBP1. p70S6K was completely dephosphorylated already at 2 μM concentration of RAD001, whereas 4EBP1 was dephosphorylated starting at a higher concentration. Total levels of these two proteins were instead unaffected by RAD001.

RAD001 also decreased the levels of Ser 473 p-Akt, p-GSK3-α/β on Ser 21/9 and p-Forkhead box O3A (FoxO3A) on Ser 318/321 in SEM cells. Interestingly, FoxO3A transcription factor was fully dephosphorylated already at 2 μM RAD001 only in SEM cell line (Figure 2b).

RAD001 induces cell cycle arrest, apoptosis and autophagy

To assess the effects of RAD001 on cell cycle, we performed flow cytometric analysis of PI-stained samples in REH and SEM cells

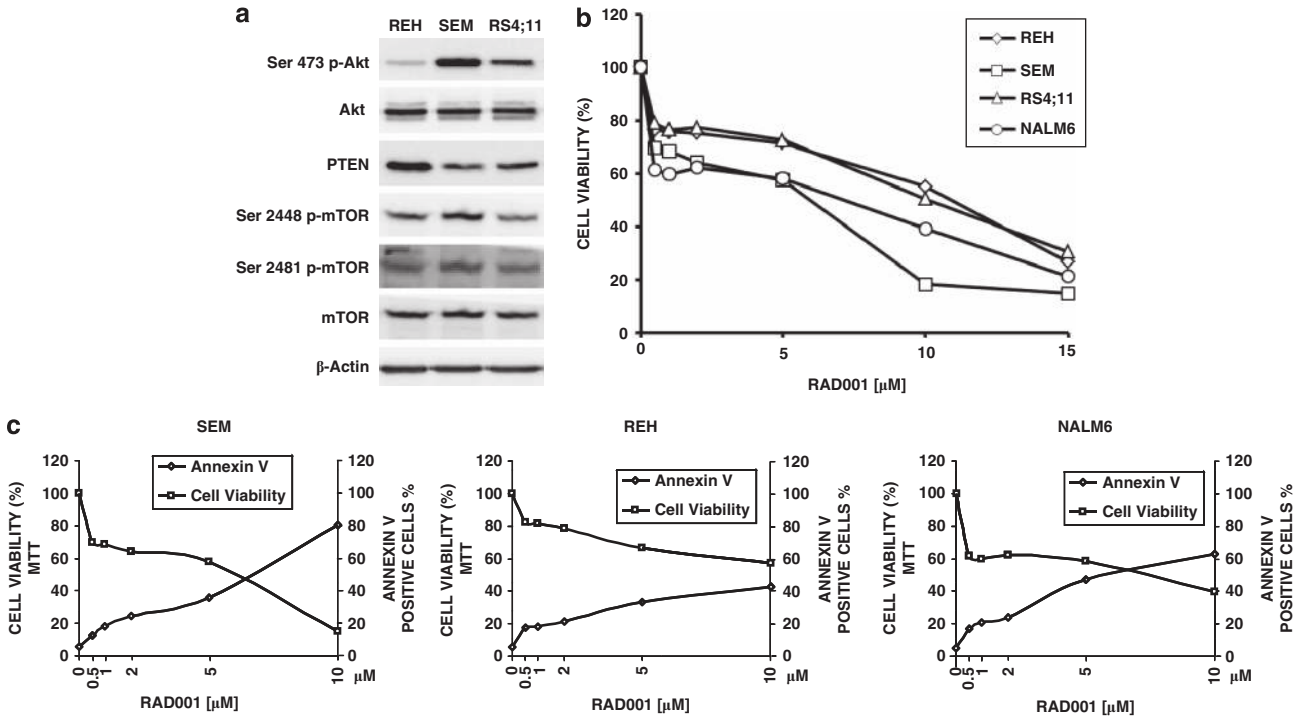

Figure 1. PI3K/Akt/mTOR signaling activation and RAD001 sensitivity of B-pre ALL cell lines. (**a**) Western blot analysis for phosphorylation levels of critical components of the PI3K/Akt/mTOR signaling network. Protein (50 μg) was blotted to each lane. An antibody to β-actin documented equal lane loading. (**b**) Cell viability of B-pre ALL cell lines treated with increasing concentrations of RAD001 for 48 h. (**c**) Assessment of cell viability and apoptosis induction by RAD001 in SEM, REH and NALM6 cells. In **b** and **c**, one representative of three different experiments that yielded similar results is shown. Viability was assessed by MTT assays, whereas apoptosis was assessed by Annexin V/7-AAD staining.

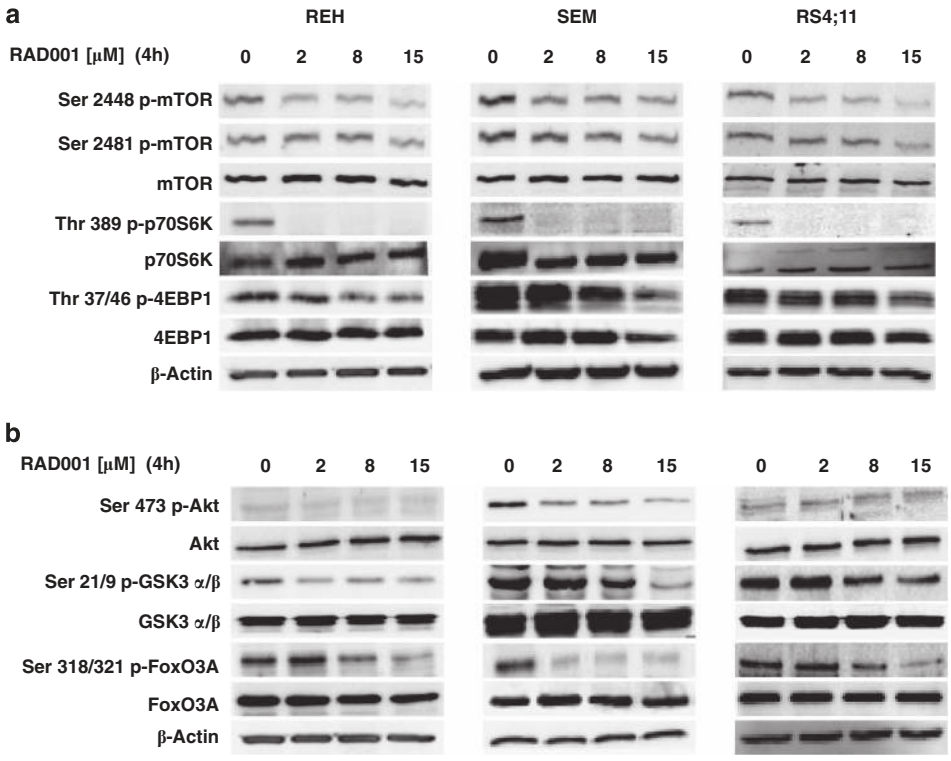

Figure 2. Effects of RAD001 on the phosphorylation levels of key components of the PI3K/Akt/mTOR pathway after 4 h of treatment with increasing concentrations of the drug. (**a**) Western blot analysis for mTOR and its downstream targets, p70S6K and 4EBP1. (**b**) Western blot analysis for Akt, GSK3-α/β and FoxO3A. In **a** and **b**, 50 μg of protein was blotted to each lane. β-actin served as a loading control.

cultured for 24 h in the presence of increasing concentrations of RAD001. RAD001 increased dose dependently the percentage of cells in the G_0/G_1 phase of the cell cycle with a concomitant decrease of cells in S and G_2/M phases (Figure 3a).

Apoptosis and autophagy, two central mechanisms for programmed cell death,[32] have important roles in the killing of malignant cells. To elucidate whether RAD001 cytotoxic effects were related to apoptosis and/or autophagy, we analyzed REH, SEM and RS4;11 cell lysates by western blotting. The cells were treated for 4 h with increasing concentrations of RAD001. Procaspase-9 and poly-(ADP-ribose)polymerase (PARP) were cleaved in a dose-dependent manner in all the three cell lines, but the apoptotic effect of RAD001 was more evident in SEM cells (Figure 3b).

Autophagy can be a form of programmed cell death, but is also involved in protective mechanisms against apoptosis.[33] Microtubule-associated protein 1 light chain 3 (LC3A/B) is a structural component in the formation of autophagosomes and is widely used as an autophagic marker, as its lipidated form (LC3A/B-II) monitors the occurrence of autophagy.[34] To evaluate whether or not the treatment with RAD001 could lead to autophagy, we detected the expression of LC3A/B-I (non lipidated) and LC3A/B-II (lipidated) by western blotting. The expression levels of LC3A/B-II gradually increased in the three cell lines in a dose-dependent manner, but were more evident in SEM cells (Figure 3c).

Another protein critically involved in autophagy is Beclin-1, which is responsible for autophagosome formation, and its deletion has been reported to increase the incidence of spontaneous tumorigenesis, abnormal proliferation of mammary epithelial cells and germinal center B lymphocytes.[33]

Therefore, we downregulated Beclin-1 expression by transfecting SEM cells with Beclin-1 siRNA (Figure 4a) and measured cell viability by MTT assay after cell exposure to increasing concentrations of RAD001. Analysis of the data showed that cells with silenced Beclin-1 were more resistant to RAD001 than the control (untreated) cells and the cells treated with scrambled siRNA (Figure 4c). Similar results were obtained with REH cells (Figures 4b–d).

These results indicated that autophagy is a critical determinant of the cytotoxic effects induced in B-pre ALL cells by RAD001.

Dual targeting of mTOR and Akt results in synergistic inhibition of proliferation in B-pre ALL cell lines

For therapeutic targeting of the PI3K/Akt/mTOR pathway, the combined inhibition at different points of the cascade often leads to more effective results than the use of a drug that acts on a single or dual targets. However, most of the studies in this field have been performed in solid tumor models.[35–38]

Therefore, we decided to evaluate whether the simultaneous administration of RAD001 and MK-2206 could lead to synergistic cytotoxic effects in B-pre ALL cell lines. MK-2206 is a novel, orally active, allosteric pan-Akt inhibitor, whose effectiveness has been proven in preclinical models of human cancers and is undergoing phase I/II clinical trials.[39,40] More than one strategy was used to determine whether the two inhibitors could synergize. In the first condition, cells were treated with the two drugs simultaneously for 48 h. In the second condition, the first drug was administered during the entire experiment (48 h), whereas the second drug was added only for the last 24 h of the treatment. Then, MTT assays were performed. As shown in Figure 5a, in SEM cells, all the conditions were tested, and the strongest synergy was observed when RAD001 was added first and MK-2206 was added second. In REH, RS4;11 and NALM6 cells, RAD001 was combined with

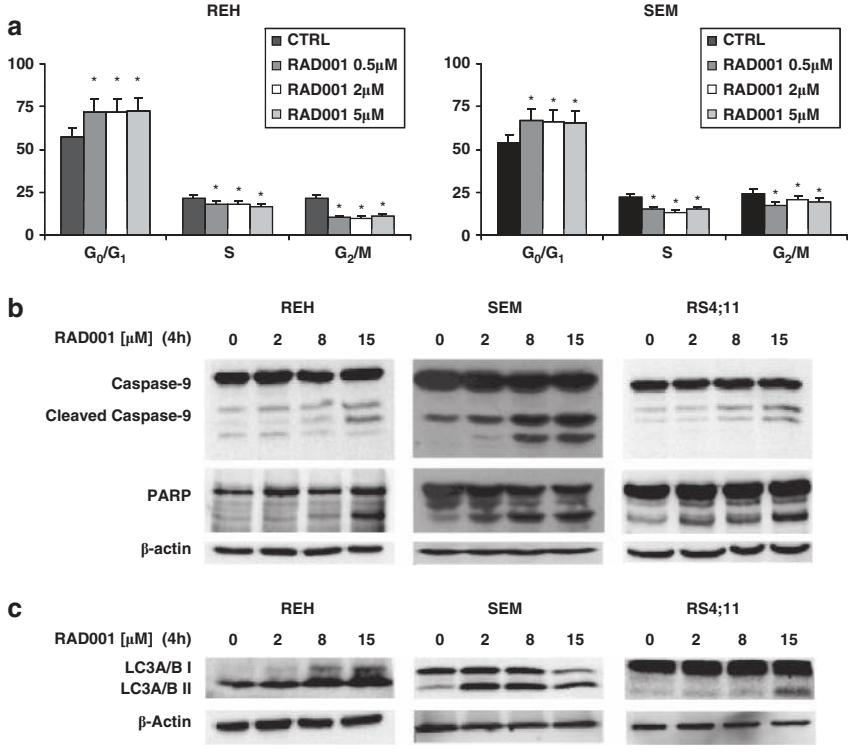

Figure 3. RAD001-induced cell cycle arrest, apoptosis and autophagy in B-pre ALL cell lines. (**a**) Cell cycle was analyzed by flow cytometry after PI staining. The results are the mean ± s.d. of three different experiments. Asterisks (*) indicate significant differences ($P < 0.05$) in comparison with control. (**b**) Western blot analysis for caspase-9 and PARP cleavage in B-pre ALL cell lines. (**c**) Western blot analysis demonstrating increased expression of the fast-migrating (lipidated) form of LC3A/B in cell lines treated with RAD001. In **b** and **c** an antibody to β-actin documented equal lane loading.

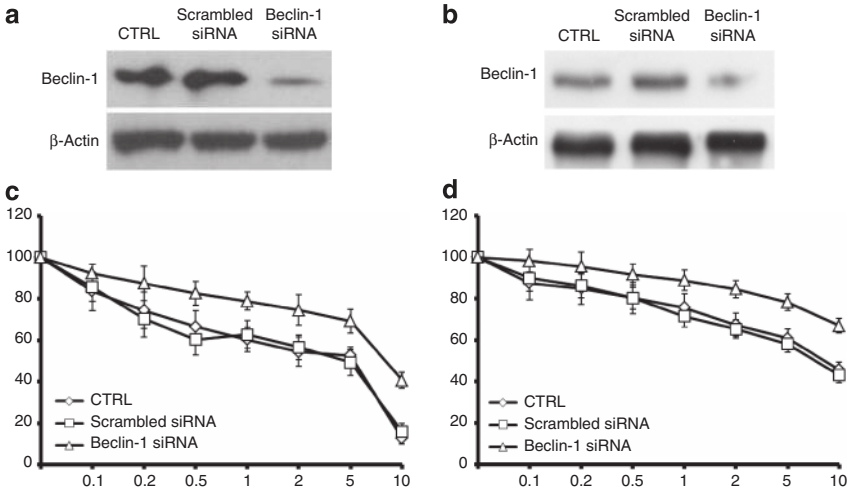

Figure 4. Beclin-1 silencing resulted in decreased RAD001 cytotoxicity in SEM and REH cells. (**a**, **b**) Western blot analysis for Beclin-1 in SEM and REH cells treated for 48 h with either scrambled (control) siRNA or Beclin-1-specific siRNA. β-actin served as the loading control. (**c**, **d**) SEM and REH cell viability, analyzed by MTT assays, after silencing of Beclin-1. Transfected cells, that is, cells with decreased levels of Beclin-1, displayed a lower sensitivity to RAD001. The results are the mean ± s.d. of three different experiments. CTRL, untreated cells.

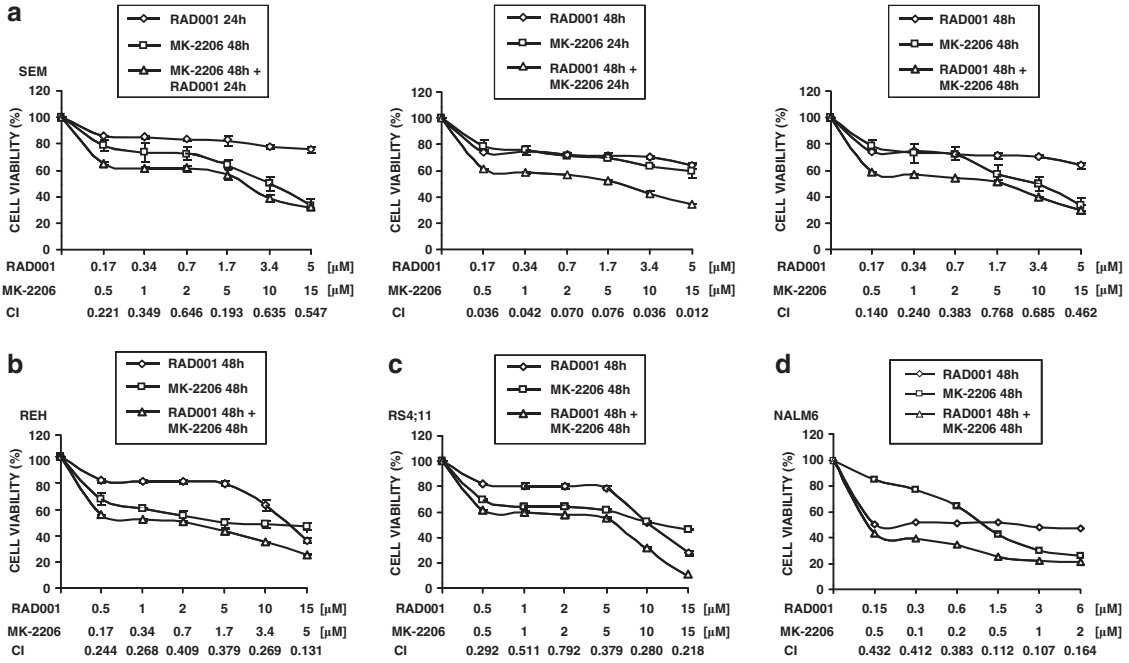

Figure 5. RAD001 and MK-2006 display a synergistic cytotoxic effect in B-pre ALL cell lines. (**a**) SEM cells were treated for 48 h with either RAD001 or MK-2206 alone, or in combination or in sequential exposure. (**b**) REH, (**c**) RS4;11 and (**d**) NALM6 cells were treated with RAD001 and MK-2206 either alone or in combination for 48 h. In **a**–**d**, one representative of three different experiments that yielded similar results is shown.

MK-2206 for 48 h. A synergistic inhibition of cell viability was observed for all cell lines (Figures 5b–d).

We next studied the effects of the RAD001/MK-2206 combination on the PI3K/Akt/mTOR pathway in SEM cells by western blotting analysis of the phosphorylation levels of 4EBP1 and GSK3-α/β. The cells were treated with 0.34 μM RAD001 for 48 h and with 1 μM MK-2206 for the last 24 h. The two proteins were much more efficiently dephosphorylated by the drug combination than that by either drug employed alone (Figure 6a).

Moreover, RAD001 and MK-2206, when administered together, increased apoptosis through extrinsic and intrinsic pathways, as documented by the increment of cleaved caspase-8, -9 and PARP (Figure 6b). Furthermore, increased levels of lipidated LC3A/B suggested that the drugs acted together for potentiating autophagy induction (Figure 6b). The effects of the RAD001 and MK-2206 combination were also analyzed in NALM6 cells. Also, in this cell line, GSK3-α/β and 4EBP1 were much more efficiently dephosphorylated by the drug combination than by either drug employed alone. Moreover, upregulated lipidation of LC3A/B indicated that the drugs synergized in potentiating autophagy induction (Figure 6c).

The increase in the percentage of G_0/G_1 phase cells and the concomitant decrease in both S and G_2/M phases were more evident when RAD001 and MK-2206 were used in combination in SEM cells (Figure 6d).

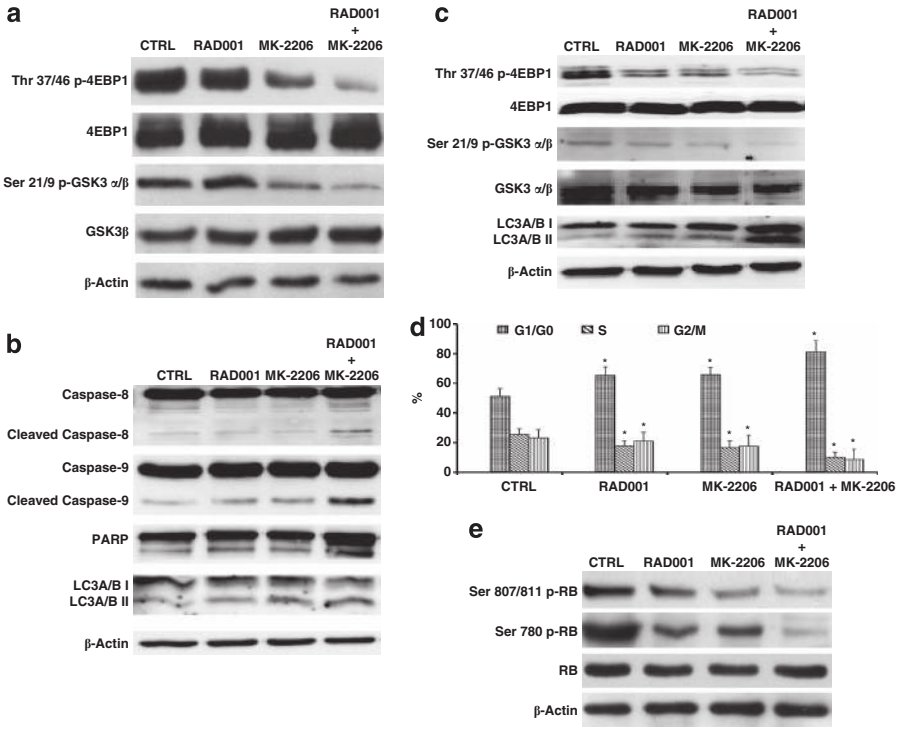

Figure 6. Effects of the RAD001/MK-2206 drug combination on phosphorylation of critical components of the PI3K/Akt/mTOR pathway, apoptosis, autophagy and cell cycle in SEM (**a**, **b** and **d**, **e**) and NALM6 (**c**) cells. (**a**) Western blot analysis on the phosphorylation status of 4EBP1 and GSK3-α/β. (**b**) Western blot analysis for caspase-8, -9 and PARP cleavage, and LC3A/B expression. (**c**) Western blot analysis on the phosphorylation status of 4EBP1 and GSK3-α/β, and LC3A/B expression. (**d**) Flow cytometric analysis of the cell cycle performed using PI-stained samples. The results are the mean ± s.d. of three different experiments. Asterisks (*) indicate significant differences ($P < 0.05$) in comparison with control. (**e**) Western blot analysis for the levels of total Rb and Ser 807/811 or Ser 780 p-Rb. In **a**, **b** and **d**, **e**), 0.34 μM RAD001 for 48 h and MK-2206 at 1 μM for the last 24 h were used. In panel **c**, 0.6 μM RAD001 and MK-2206 at 0.2 μM for 48 h were used. CTRL, untreated cells.

The tumor suppressor protein retinoblastoma (Rb) in the phosphorylated state (p-Rb) is active and exert its role by promoting cell cycle progression from G_1 to S phase.[41] Western blotting analysis documented that in SEM cells, dual treatment with RAD001 and MK-2206 maximally reduced the levels of phosphorylated Rb protein on Ser 807/811 and Ser 780 residues (Figure 6e). This effect was consistent with the arrest of B-pre ALL cells in G_0/G_1 phase of the cell cycle.

To confirm these findings, we employed a different mTORC1/Akt inhibitor combination, consisting of CCI-779 and GSK 690693. SEM and NALM6 cells were treated with the two drugs simultaneously for 48 h. Then, MTT assays were performed. As shown in Figures 7a and b, the two drugs synergized to inhibit cell viability. We next studied the effects of the drug combination by western blotting analysis of the phosphorylation levels of 4EBP1 and GSK3-α/β. The two proteins were much more efficiently dephosphorylated by the drug combination than that by either drug employed alone (Figures 7c and d). As different inhibitors yielded similar results, it was concluded that there were no off-target drug effects implicated in the described findings.

B-pre ALL lymphoblasts are sensitive to combined mTOR/Akt inhibition

To establish the efficacy of the inhibitors as potential therapeutic agents for B-pre ALL, we analyzed four B-pre ALL adult patient samples isolated from peripheral blood/bone marrow. Available patient characteristics are presented in Supplementary Table T1.

Samples were treated with either RAD001 or MK-2206 alone or with the combination of 0.34 μM RAD001 and 1 μM MK-2206 for 72 h. In the two patients shown in Figure 8a, RAD001 induced a downregulation Ser 473 p-Akt, Ser 2448 p-mTOR and Thr 389 p-p70S6K levels. In the same two patients, we tested the synergistic effects of RAD001 and MK-2206 on 4EBP1 and GSK3-α/β. The decrease in the phosphorylation levels of the two proteins was much higher when the two drugs were used in combination than as single agents. Autophagic induction in the primary samples was also more effective when the two drugs were combined together as indicated by the levels of lipidated LC3A/B (Figure 8b).

Dual targeting of mTOR and Akt also increased apoptosis in patient primary cells. Cells were stained by PI/Annexin V-FITC and analyzed by flow cytometry. After 48 h of treatment, when the two drugs were administered together, the apoptotic cells raised to 50%, whereas RAD001 and MK-2206 alone induced apoptosis in a percentage of 14% or 25%, respectively (Figure 8c).

In Figure 8d are shown the results of MTT assays of four representative patients analyzed for cell viability after *in vitro* treatment with either drugs used as single agents or combined together. In all the patients studied, the drug combination induced a stronger decrease in cell viability. Overall, these findings demonstrated that the combination consisting of RAD001 and MK-2206 reduced the growth of B-pre ALL primary cells. Moreover, also the combination of CCI-779 and GSK 690693 caused a stronger decrease in cell viability than that by either drug employed alone (Supplementary Figure S1).

DISCUSSION

Deregulation of the PI3K/Akt/mTOR pathway contributes to cancer cell survival, promotes chemotherapy resistance through

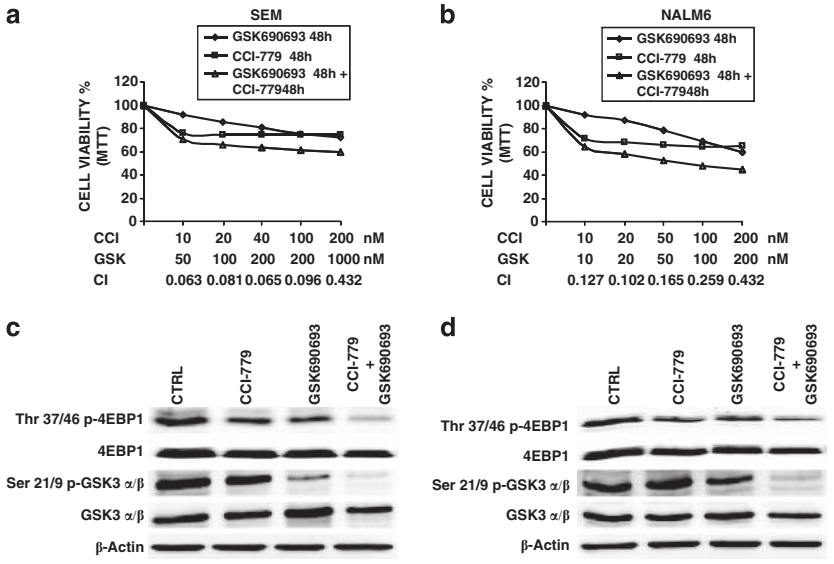

Figure 7. CCI-779 and GSK 690693 display a synergistic cytotoxic effect in B-pre ALL cell lines and affect the phosphorylation status of critical components of the PI3K/Akt/mTOR pathway. (**a**) SEM cells were treated for 48 h with either CCI-779 or GSK 690693 alone or in combination. (**b**) NALM6 cells were treated for 48 h with either CCI-779 or GSK 690693 alone or in combination. In **a** and **b**, one representative of three different MTT experiments that yielded similar results is shown. (**c**, **d**) Western blot analysis on the phosphorylation status of 4EBP1 and GSK3-α/β in SEM and NALM6 cell lines, respectively.

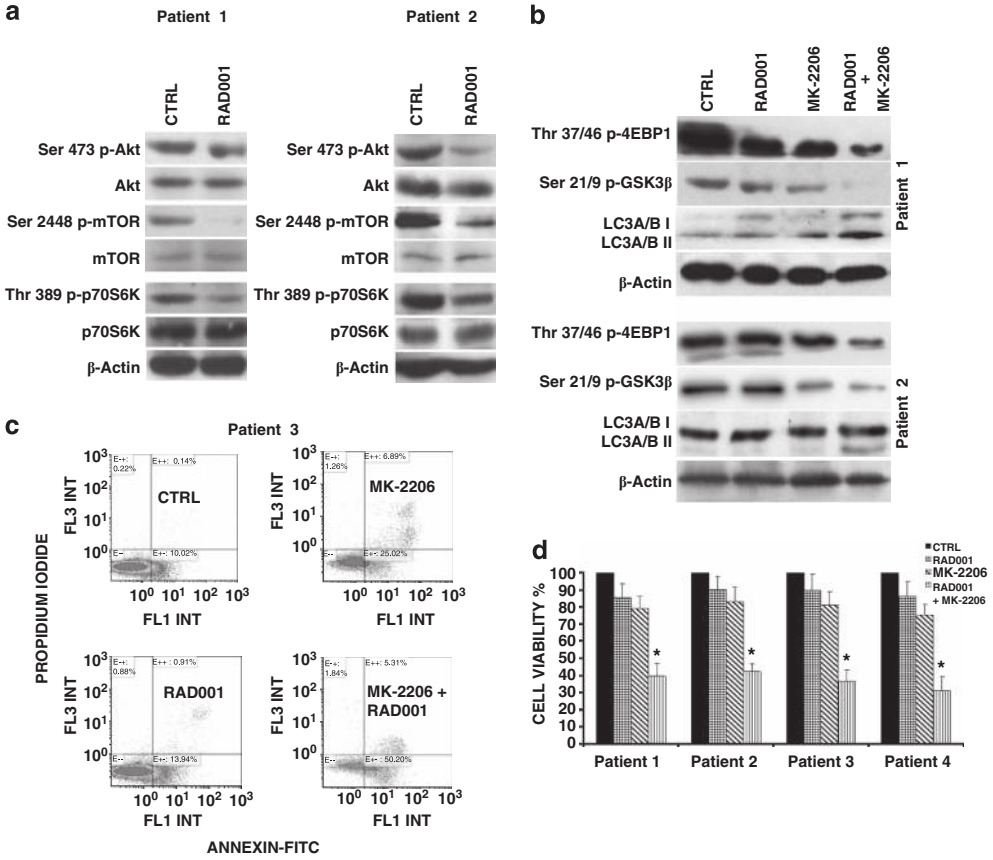

Figure 8. The RAD001/MK-2206 combination is cytotoxic to B-pre ALL lymphoblasts. (**a**) Effects of RAD001 alone on the phosphorylation status of Akt, mTOR and p70S6K in two representative patient samples. (**b**) Western blot analysis for 4EBP1, GSK3-α/β and LC3A/B in two patient samples after dual targeting with RAD001 and MK-2206. (**c**) Flow cytometric analysis of Annexin V-FITC/PI-stained patient lymphoblasts. One representative of three different experiments that yielded similar results is shown. (**d**) Cell viability analysis, as documented by MTT assays, performed on four patient samples. The results are the mean ± s.d. of three different experiments. CTRL, untreated cells. Asterisks (*) indicate significant differences ($P < 0.05$) in comparison with control. In **a**–**d**, samples were treated with 0.34 μM RAD001 and 1 μM MK-2206 for 72 h.

disruption of apoptosis and initiates cap-dependent translation of mRNAs essential for cell cycle progression, differentiation and growth.[42] Activation of this pathway is a common feature of a wide range of human cancers[43] and is an indicator of poor prognosis.[44–48] Thus, it is reasonable to assume that targeting mTOR using the orally active rapamycin derivative RAD001 can effectively contribute to treat tumors with hyperactivation of PI3K/Akt/mTOR signaling. In this study, we demonstrated the efficacy of RAD001 as a potential therapeutic inhibitor of the PI3K/Akt/mTOR pathway in B-pre ALL cells.

RAD001 was cytotoxic to B-pre ALL cell lines in a concentration-dependent manner, which was demonstrated by both MTT assays and flow cytometric analysis of Annexin V-stained samples. Cytotoxicity was independent from the t4;11 translocation, which occurs in SEM and RS4;11 cell lines.

In B-pre ALL cell lines, RAD001 not only downregulated mTORC1 activity (as documented by dephosphorylation of Ser 2448 p-mTOR, Thr 389 p-p70S6K and Thr 37/46 p-4EBP1) but also decreased mTORC2 activity, as demonstrated by decreased phosphorylation of Ser 473 p-Akt and Ser 2481 p-mTOR.

The downmodulation of mTORC2 by RAD001 had been reported in AML.[49] However, in AML RAD001 was effective in inhibiting mTORC2 formation only when used for long incubation times (at least 24 h), whereas in B-pre ALL cell lines, a 4 h incubation was sufficient to block mTORC2 activity. This could indicate that B-pre ALL cell lines are very sensitive to RAD001.[50]

The cytotoxic effects of RAD001 on B-pre ALL cell lines could be related to cell cycle arrest in G_0/G_1, and induction of either apoptosis or autophagy (or both). Remarkably, both apoptosis and autophagy were more evident in SEM cells, consistently with the highest sensitivity of this cell line to RAD001.

Cancer cells can gain benefit of autophagy as a survival mechanism, which gives them a selective advantage to tolerate metabolic stress and to contrast apoptosis.[51] We found that silencing Beclin-1, a protein essential for the assembly of the autophagy initiation complex that leads to autolysosomes formation,[33] resulted in a lower sensitivity of both SEM and REH cells to the cytotoxic effects of RAD001. Therefore, RAD001-induced autophagy is very important for the reduction of cell viability. Although the induction of autophagy by RAD001 in B-pre ALL cells has been previously reported,[52] this is the first time that the functional significance of autophagy was explored. We aimed in investigating the effect of combined Akt and mTOR inhibition on B-pre ALL cells using the novel allosteric, clinically available pan-Akt inhibitor MK-2206.[29] This combination has been demonstrated to be effective in hepatocellular carcinoma cell lines,[53] cholangiocarcinoma cell lines[38] and in an *in vivo* model of castration-resistant prostate cancer.[54] Moreover, we used a second combination consisting of CCI-779 and GSK 690693.

There is a strong rationale for targeting Akt in B-pre ALL cells, as a recent work has documented that Akt activation is associated in this leukemia subset with poor prognosis and resistance to a set of chemotherapeutic drugs, including prednisolone, dexamethasone, vincristine and adriamycin.[48]

We observed a synergistic cytotoxic effect of the RAD001/MK-2206 drug combination. The combination caused a dose-dependent decrease of cell viability, and this was visible even at low concentrations in all cell lines, but especially in SEM cells. After testing different strategies for combining the two drugs together, we observed that the highest synergistic effects were detectable in SEM cells when RAD001 was administered for 48 h and MK-2206 was added after 24 h. We are at present investigating the mechanisms underlying this peculiar behavior.

The combination consisting of RAD001 and MK-2206 was more effective than either treatment alone in inducing cell cycle arrest in G_0/G_1, and this correlated with a more effective dephoshorylation of Rb on both Ser 807/811 and Ser 780. These two residues are key determinants of Rb activity. Indeed, it has been documented that a Rb mutant with alanine substitutions at Ser 807/811 had enhanced growth-suppressing activity.[55] Moreover, phosphorylation of Ser 807/811 led to an inactivation of Rb tumor suppressor activity in uveal melanoma.[56] The Ser 807/811 and Ser 780 residues are targets of cyclin E/CDK2 and cyclin D/CDK4, respectively.[57] It should be reminded here that mTORC1 controls the translation of several proteins that are of critical importance for cell cycle progression, including CDK2.[50] It is important to emphasize that *Rb* gene activation is an unfavorable prognostic predictor in initial and relapsed childhood ALL.[58] Therefore, drugs that affect the Rb pathway activity may be important for development of individual-targeted therapies.

The dual mTOR/Akt inhibition was more effective than single drugs in lowering cell viability and affecting phosphorylation of critical components of the PI3K/Akt/mTOR pathway, also if we employed a different drug combination consisting of CCI-779 and GSK 690693.

These findings may have a clinical relevance for B-pre ALL patients, because the synergistic combination of the drugs led to an increase of the cytotoxic activity at lower concentrations of the inhibitors. If this would be translated into the clinic, an attenuation of toxic side effects could be expected.

RAD001 alone displayed anti-leukemic effects also against B-pre ALL patient samples, acting on Ser 473 p-Akt, Ser 2448 p-mTOR and Thr 389 p-p70S6K.

Consistently, also in B-pre ALL lymphoblasts, we observed a striking synergistic effect of the combination of RAD001/MK-2206 that resulted in increased dephosphorylation of key components of the PI3K/Akt/mTOR pathway, as well as in enhanced apoptosis and decreased cell viability. Also the CCI-779 and GSK 690693 combination affected B-pre ALL lymphoblasts viability more effectively than either drug alone.

In conclusion, we have documented that RAD001 is cytotoxic to B-pre ALL cells, and that autophagy has an important role in decreasing cell viability. Moreover, our findings suggest that rapamycin derivatives can be effectively used in combination with other inhibitors targeting key nodes of the PI3K/Akt/mTOR pathway, in order to reduce drug concentration and hence systemic side effects. Therefore, dual targeting of PI3K/Akt/mTOR signaling pathway might represent a new promising therapeutic strategy for treatment of adult B-pre ALL patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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