Rapamycin Sensitizes Glucocorticoid Resistant Acute Lymphoblastic Leukemia CEM-C1 Cells to Dexamethasone Induced Apoptosis through both mTOR Suppression and Up-Regulation and Activation of Glucocorticoid Receptor*

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Abstract

Objective To explore the role of glucocorticoid (GC) receptor (GR) in rapamycin’s reversion of GC resistance in human GC-resistant T-acute lymphoblastic leukemia (ALL) CEM-C1 cells.

Methods CEM-C1 cells were cultured in vitro and treated with rapamycin at different concentrations with or without 1 μmol/L dexamethasone (Dex). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test was performed to assess cell proliferation. The cell cycle and cell apoptosis were analyzed by flow cytometry. The expression of GRα mRNA was determined by real-time quantitative RT-PCR. The expression of GR, p-70S6K, Mcl-1, and Bim proteins was detected by Western blot.

Results When incubated with rapamycin at different concentrations, CEM-C1 cells showed significant growth inhibition in a time- and concentration-dependent manner. The growth inhibition was synergistically increased when CEM-C1 cells were treated with rapamycin plus 1 μmol/L Dex. CEM-C1 cells treated with rapamycin alone showed no apparent apoptosis, and were arrested at G0/G1 phase. After the treatment with Dex plus rapamycin, CEM-C1 cells demonstrated apparent apoptosis and increased the cell cycle arrested at G0/G1 phase. Rapamycin combined with Dex up-regulated GRα, phosphorylated GR(p-GR), and pro-apoptotic protein Bim-EL in CEM-C1 cells, but inhibited the expression of p-p70S6K, a downstream target protein of mTOR (mammalian target of rapamycin).

Conclusion After the treatment with rapamycin plus Dex, Dex resistant CEM-C1 cells induce growth inhibition and apoptosis. The underlying mechanism may involve inhibition of the mTOR signaling pathway and also be associated with up-regulation of GR expression and activation of GC-GR signaling pathway.

Key words: Acute lymphoblastic leukemia; mTOR; Glucocorticoid resistance; Rapamycin; Glucocorticoid receptor

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INTRODUCTION

Glucocorticoids (GCs) specifically induce apoptosis in malignant lymphoblasts and are thus pivotal in treatment of lymphoid malignancies. Prednisone response in vivo is a strong and independent prognostic factor in childhood ALL[1-3]. This effect is mediated by GR, a ligand-activated transcription factor as part of the nuclear receptor superfamily. This subfamily initially resides in the cytoplasm, then gets translocated to the nucleus after ligand binding, dimerizes and finally binds to GC response elements, thereby modulating target gene expression. However, GC resistance is a major obstacle accounting for most treatment failures in childhood ALL, and the exact molecular mechanism behind GC resistance remains poorly understood. Therefore, it is of fundamental importance to develop new drugs with GC resistance revision ability or synergic effects with GC to break through the bottleneck in ALL treatment. Recent researches revealed that GC resistance in two key models of ALL occurred at a GR level due to deficiency in up-regulating GR and its downstream targets caused by mutations in the GR gene or regulatory defects or by their combined action, or perhaps more importantly, due to deficiency in GR auto-induction (i.e., up-regulation after the cells were treated with GC)[4]. This suggests that if a drug increases or synergizes with GC to strengthen auto-induction of GR, it might be helpful in overcoming GC resistance of ALL. Rapamycin, a specific mTOR inhibitor, might be a candidate.

The mTOR signaling pathway that can be constitutively activated in many types of cancers including lymphoid malignancies has attracted broad scientific and clinical interest, particularly in light of the ongoing clinical trials with mTOR inhibitors[5-10]. The mTOR inhibitors have been extensively studied in clinical trials of cancer treatment, which have shown promising results mainly in certain lymphomas with sole GC in chemotherapeutic regimens. These findings indicate that the activated mTOR signaling pathway might play an important role in inducing GC resistance in lymphoid tumors. Rapamycin, a macrolide antibiotic, specifically inhibits mTOR activity and induces cell cycle arrest at the G1 phase, but fails to induce apoptosis of tumor cells by itself[11-12]. It has been reported that combination of Dex and rapamycin can significantly improve apoptosis in a variety of cell lines, including GC resistant T-ALL cell line (CEM-C1), B-ALL cell line (697), and Burkitt’s lymphoma cell lines (Raji, NAMALWA, and Ramos)[13]. However, the precise mechanism remains to be clarified.

Based on the facts that rapamycin induces cell growth inhibition and cell cycle arrest through suppression of mTOR, and that GC resistance might be associated with inability of GR auto-induction, we speculate that rapamycin, in addition to inhibiting the mTOR signal pathway, might also synergize with GC to overcome GC resistance of ALL cells through GR auto-induction.

The present study has demonstrated that rapamycin could sensitize GC-resistant T-ALL CEM-C1-15 cells to Dex-induced apoptosis through up-regulation and activation of GR and inhibition of mTOR.

MATERIALS AND METHODS

Cell Lines

The GC-resistant T-ALL cell line CEM-C1-15 (CEM-C1) and its sister cell line, GC-sensitive CEM-C7-14 (CEM-C7) were kindly provided by E. B Thompson (University of Texas Medical Branch, Galveston, TX). Cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St Louis, MO, USA) and antibiotics (penicillin 100 U/mL and streptomycin 50 µg/mL) at 37 °C in a humidified, 5% CO2 incubator.

Chemicals and Antibodies

Rapamycin and Dex were purchased from Sigma (St. Louis, MO, USA), dissolved in ethanol and used as 100 ng/mL (rapamycin) or 1 µmol/L (Dex). The final concentration of ethanol in the medium was 0.05%, at which cell proliferation/growth or viability was not obviously altered. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma. Annexin V-PI Kit was purchased from Jingmei (Shenzhen, China). The antibodies used for Western blot were as follows: GR, phospho-p70S6K (Thr421/Ser424), and Bim from Santa Cruz Biotech (Santa Cruz, CA, USA), phosphor-GR (Ser211) from Cell Signaling Technology (Beverly, MA, USA), Mcl-1 and p70S6K from ABZzoom Biotech (Changsha, China), and anti-GAPDH from Kangchen Bio-Tech (Shanghai, China).
Cell Culture

Logarithmically growing cells were harvested and seeded into 96- or 6-well sterile plastic culture plates (Corning Inc., Acton, MA, USA), to which 100 ng/mL rapamycin (Rap group), 1 μmol/L Dex (Dex group), 100 ng/mL rapamycin plus 1 μmol/L Dex (Rap+Dex group) and 0.05% ethanol (Control group) were added respectively. At the end of the incubation, cells were collected and pelleted through centrifugation at 400 g at room temperature for 5 min, and prepared for the analysis as described below.

Proliferation Assay

200 μL cell suspensions were seeded in 96-well plates (7500/mL) and incubated for 24, 48, and 72 h. Then MTT was added to each well (with a final concentration of 0.5 mg/mL) and the cells were incubated for additional 4 h and centrifuged at 1000 rpm for 10 min. The supernatant was gently removed from the cell pellet and 100 μL of DMSO (Sigma, USA) was added to each well. The plate was shaken gently for 10 min, and the absorbance at 570 nm was measured with an ELISA reader. Three separate experiments were conducted and each was performed in triplicate. The ratio of growth inhibition was calculated based on the formula: Inhibition rate ( \% ) = (1- absorbance of the experimental group/absorbance of the control group) × 100%.

Cell Morphology

Both CEM-C1 and CEM-C7 cells were harvested after treatment and washed with phosphate-buffered saline (PBS) twice. Cells were centrifuged onto slides by cytopsin, stained with Wright’s-Giemsa dye and observed under microscopy.

Cell Cycle Analysis

For each analysis, 10^6 cells were harvested 24 and 48 h after treatment and fixed overnight in 70% ethanol at 4 °C. Cells were then washed by cold PBS and stained with 5 μg/mL PI in the presence of DNase free RNase (Sigma). After 30 min at room temperature, the cells were analyzed via flow cytometry (Beckman Coulter, Inc., Miami, FL, USA).

Apoptosis Assay

Both CEM-C1 and CEM-C7 cells were harvested after treatment, washed with cold PBS twice and re-suspended in 500 μL of binding buffer containing 5 μL of Annexin V-FITC stock solution and 10 μL of PI for determination of phosphatidylserine exposure on the outer plasma membrane. After incubation for 10 min at room temperature in the dark, 1×10^4 cells were collected and the apoptosis was detected through flow cytometry (FACS Aria, BD).

Real-Time Quantitative RT-PCR

Total RNA was extracted from cells through TRIZOL reagent (TIANGEN, Peking, China). 1 μg of total RNA was reverse transcribed into cDNA by using Omniscript reverse transcriptase (Takara, Japan). Reverse transcriptase minus control was included in all runs to exclude DNA contamination. The amplifications were performed on a RotorGene 3000 apparatus (Corbett Instruments, Australia), a rotor-based real-time PCRycler. The amplified PCR products were detected by using PrimerScript™ RT-PCR Kit (Takara, Japan). PCR primers (F, R) and probes (P) used were shown in Table 1.

### Table 1. Primers for GRα and GAPDH

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers and Probes (5’-3’)</th>
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<tbody>
<tr>
<td>GRα</td>
<td>F: GACCTACATCAAAAGAGCTA</td>
</tr>
<tr>
<td></td>
<td>R: AACCAAGAGTTTTGTCAGTT</td>
</tr>
<tr>
<td></td>
<td>P: FAM-TGGCTGGAGTTCTCCCCCTC-TAMRA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: GAAAGTGGAGTGGGAGTC</td>
</tr>
<tr>
<td></td>
<td>R: GAGATGGTGATGGGATTTC</td>
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<tr>
<td></td>
<td>P: FAM-CAAGCTTCCGTTCTCAGCC-TAMRA</td>
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</tbody>
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Each PCR reaction was carried out in a total volume of 25 μL containing 10 μmol/L of each primer, 12.5 μL master mix, and water. Real-time PCR conditions designed to ensure linearity were 95 °C for 10 sec, followed by 40 cycles of 95 °C for 10 sec and 60 °C for 30 sec. Each sample was prepared in triplicate and the experiments were repeated on separate occasions to ensure reliability and reproducibility of the results.

Western Blotting

Cells (10^6) were washed twice in cold PBS, and then lysed by lysis buffer (Keygen Nanjing, China). Protein concentration was determined by using a BCA protein assay kit (Keygen Nanjing, China).
Samples were boiled for 5 min at 100 °C. Proteins were separated on 10% or 15% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (0.45 μm, Millipore, São Paulo, SP, Brazil). Nonspecific-binding sites were blocked with 5% non-fat dry milk dissolved in TBS (10 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl) with 0.1% Tween 20 (TTBS) for 1 h at room temperature, followed by incubation with primary antibody at 4 °C overnight. The membranes were then washed 3 times in TTBS and incubated for 1 h at room temperature with secondary horseradish peroxidase (HRP)–conjugated sheep anti-rabbit antibody (Santa Cruz) diluted 1:5000 in TTBS with 5% non-fat milk. Proteins were visualized by ECL kit (Santa Cruz). All experiments were carried out independently at least 3 times. The level of the GAPDH protein was used as a control of the amount of protein loaded into each lane.

Statistical Analysis

All assays were performed in triplicate, and data are expressed as mean values±s.d. The Student’s t-test was used to compare means of two groups and one-way ANOVA was used to compare means of multiple groups. Results were considered significant with P-value <0.05.

RESULTS

Comparison of Dex Induced Growth Inhibition, Apoptosis and Auto-induction of GR between CEM-C1 and CEM-C7 Cells

Growth Inhibition of Dex on CEM-C1 and CEM-C7 Cells To test the Dex induced growth inhibition in CEM-C1 and CEM-C7 cells, we first used 1 μmol/L Dex to treat both cells for different periods of time. The survival rates were (97.8±4.8)%, (88.4±4.3)%, (81.9±2.8)% for CEM-C1 cells and (90.2±5.4)%, (11.0±2.0)%, (2.3±1.2)% for CEM-C7 cells at 24, 48, and 72 h, respectively, indicating that CEM-C1 cells were highly resistant to Dex while CEM-C7 cells were very sensitive to Dex (Figure 1).

Apoptosis in CEM-C1 and CEM-C7 Cells Induced by Dex Both CEM-C1 and CEM-C7 cells were treated with 1 μmol/L Dex for 48 h. Then, the morphological changes of cells were observed under a light microscope and the apoptosis was detected with flow cytometry. As showed in Figure 2, Dex could induce remarkable apoptosis in CEM-C7 cells, whereas no apoptosis was virtually observed in CEM-C1 cells. These results further demonstrated that CEM-C1 cells were highly resistant to Dex.

Up-Regulation of GR Induced by Dex in CEM-C1 and CEM-C7 Cells To evaluate the role of GR in Dex induced apoptosis of CEM cells, we used quantitative real time PCR and Western blot to check the expression levels of GRα after treatment with Dex. As shown in Figure 3, the basic expression levels of GRα mRNA and protein in CEM-C7 cells were both higher than those in CEM-C1 cells. After the treatment with 1 μmol/L Dex for 18 and 24 h, GR, especially activated GR (phosphorylated GR, p-GR), was significantly up-regulated in CEM-C7 cells at 18 h, while slight increase in GR at 18 h and p-GR at 24 h was observed in CEM-C1 cells, indicating the presence of a defect in GR auto-induction, especially the p-GR induced by Dex in GC-resistant CEM-C1 cells compared with GC-sensitive CEM-C7 cells.

Combination of Rapamycin and Dex Synergistically Reversing GC Resistance in CEM-C1 Cells

Rapamycin and Dex Synergistically Inhibiting Growth of CEM-C1 Cells When incubated with rapamycin at different concentrations (1000 ng/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, and 0.1 ng/mL, respectively), CEM-C1 cells showed significant growth inhibition in a time- and concentration-dependent manner. Combination of rapamycin with Dex enhanced the growth inhibitory effect compared with application of rapamycin or Dex alone, with P<0.05 (Figure 4).
Figure 2. Morphological changes and apoptosis of CEM-C1 and CEM-C7 cells treated with Dex (1 µmol/L) for 24 and 48 h. Then, the apoptosis was detected with Annex V-FITC/PI double staining and flow cytometry. Remarkable apoptosis was observed in CEM-C7 cells.

Figure 3. The expression levels of GR mRNA and proteins of GR and p-GR in CEM-C1 and CEM-C7 cells induced by 1 µmol/L Dex. The basic expression level of GR in CEM-C7 cells was higher than that in CEM-C1 cells. After the incubation with Dex, GR was slightly increased and p-GR was remarkably up-regulated in CEM-C7 cells at 18 h, while only slight increase in GR at 18 h and p-GR at 24 h was observed in CEM-C1 cells.
Figure 4. CEM-C1 cells were incubated with rapamycin plus Dex (1 µmol/L) at different concentrations for 72 h. Rapamycin and Dex synergistically inhibited the growth of CEM-C1 cells in a dose-and time-dependent manner. *: P<0.05 vs Rap group or Dex group.

Rapamycin Sensitizing CEM-C1 Cells to Dex-Induced Apoptosis  When CEM-C1 cells were incubated with 100 ng/mL rapamycin (Rap group) or 1 µmol/L Dex (Dex group) alone, no obvious changes in cell morphology were observed. After the treatment with 1 µmol/L Dex combined with 100 ng/mL rapamycin (Rap+Dex group), CEM-C1 cells exhibited obvious apoptosis at 48 h (Figure 5).

The apoptosis of CEM-C1 cells were further confirmed through Annexin V-FITC/PI double staining. When treated with rapamycin or Dex alone, no obvious apoptosis in CEM-C1 cells was observed. However, remarkable apoptosis in CEM-C1 cells was detected after cells were treated with Dex plus rapamycin (Rap+Dex group) for 48 h, P<0.05 (Figure 5).

Rapamycin and Dex Synergistically Inducing G1 Arrest in CEM-C1 Cells  When CEM-C1 cells were treated with Dex alone, there was no obvious change in cell cycle and the percentage of cells at G1 phase was 47.8% after application of Dex for 48 h. However, rapamycin alone induced slight G1 arrest of CEM-C1 cells with the percentage in G1-phase being 49.7% after its application for 48 h. The G1 arrest of cell cycle was significantly increased when CEM-C1 cells were treated with both drugs simultaneously. The percentage in G1-phase was 63.8% after application of both Dex and rapamycin for 48 h (Figure 6).
Figure 5. Rapamycin sensitizes CEM-C1 cells to GC treatment by enhancing apoptosis. Morphological changes of CEM-C1 cells induced by rapamycin (100 ng/mL) and/or Dex (1 µmol/L) at 48 h were observed under a light microscope. The apoptosis was detected through Annexin V-FITC/PI staining at 24 and 48 h, respectively. A higher apoptosis rate (Q2+Q4) in cells treated with rapamycin plus Dex was observed. Rap, rapamycin; Dex, dexamethasone; Rap+Dex, rapamycin+ dexamethasone.
**Rapamycin and Dex Synergistically Inducing Up-Regulation of Pro-apoptotic Protein Bim-EL in CEM-C1 Cells**

We then checked the effect of rapamycin and Dex on the expression of pro-apoptotic protein Bim-EL and anti-apoptotic protein Mcl-1, both of which were intimately associated with GC resistance in ALL cells. No up-regulation in Bim-EL expression was observed in CEM-C1 cells after treatment with Dex alone, but there was up-regulation in Bim-EL expression after treatment with rapamycin. A synergistic effect was observed when CEM-C1 cells were treated with Dex plus rapamycin. The expression of Mcl-1 in CEM-C1 cells was inhibited by rapamycin and no obvious synergistic effect was observed when CEM-C1 cells were co-treated with Dex and rapamycin (Figure 7). These results suggested that rapamycin sensitized CEM-C1 cells to Dex induced apoptosis by synergistically enhancing the expression of pro-apoptotic protein Bim-EL.

**Figure 7.** The expression of pro-apoptotic protein Bim-EL and anti-apoptotic protein Mcl-1 in CEM-C1 cells treated with rapamycin and Dex alone or with both for 24 h. Rap, rapamycin; D, dexamethasone; Rap+Dex, rapamycin+ dexamethasone.

**Rapamycin and Dex Synergistically Inhibiting mTOR Signaling Pathway in CEM-C1 Cells**

To evaluate the effect of rapamycin and Dex alone or in their combination on mTOR signaling pathway, we checked the phosphorylation of p-70S6K, a key downstream effector molecule of mTOR. The results showed that rapamycin or Dex alone had a slight inhibitory effect on mTOR, but when they were used together, the mTOR activation was almost completely inhibited, suggesting a strong synergic inhibitory effect of rapamycin and Dex on mTOR signaling (Figure 8). These results indicated that inhibition of the mTOR signaling pathway might potentiate the cytotoxic effect of Dex on CEM-C1 cells.

**Figure 8.** A strong synergic inhibition of mTOR signaling was demonstrated by de-phosphorylation of p70S6K in CEM-C1 cells co-treated with rapamycin and Dex for 24 h. Rap, rapamycin; Dex, dexamethasone; Rap+Dex, rapamycin+ dexamethasone.

**Rapamycin Sensitizing CEM-C1 Cells to Dex-Induced Apoptosis through Up-Regulation of GR Expression**

To test whether the rapamycin sensitizing CEM-C1 cells to Dex was achieved through up-regulation of GRα expression, real-time quantitative RT-PCR and Western blot analysis were performed to detect the expression change of GR. The results reveal that the expression levels of GRα mRNA and protein in CEM-C1 cells were slightly increased after treatment with rapamycin, but significantly increased levels of GRα mRNA and protein were observed in CEM-C1 cells when they were treated with Dex plus rapamycin. Moreover, the combination of Dex with rapamycin also remarkably induced up-regulation of activated GR (p-GR) (Figure 9), suggesting that, in addition to suppression of mTOR, rapamycin’s sensitization of GC-resistant CEM-C1 cells to Dex-induced apoptosis might also be realized through GC-GR signaling pathway.
DISCUSSION

The GC sensitivity in childhood ALL is a major determining factor of its clinical outcome. GC resistance is a well-documented feature for predicting relapse\(^{[14-16]}\). Despite clinical importance of the drug, the exact mechanism involved in GC sensitivity and the development of GC resistance remain largely unclear\(^{[17-18]}\). Studies of in vitro models have demonstrated that regulatory and/or structural defect in GR might be the major reason for GC resistance in ALL cells\(^{[19-22]}\). However, it is not always the case in ALL patients\(^{[23]}\). Recently, increasing evidence indicated that rapamycin could be used as a potential GC sensitizer through inhibition of mTOR\(^{[13,24-25]}\). In this study, we further demonstrated that rapamycin sensitizing GC resistant T-ALL CEM-C1 cells to Dex was realized through not only inhibition of mTOR but also up-regulation of the GR.

Our results show that rapamycin, a strong inhibitor of mTOR with minimal cytotoxic effect by itself, significantly sensitized Dex resistant CEM-C1 cells to Dex-induced apoptosis as manifested by increased inhibition of cell growth, cell cycle arrest at G1 and apoptosis. Previous studies have demonstrated that Bcl-2 family members are critical regulators of the intrinsic apoptotic pathway\(^{[27]}\). Among them, Bim and Mcl-1 represent a pair of pivotal factors playing pro- and anti-apoptotic roles in GC-induced apoptosis of ALL cells. Mcl-1 selectively inhibits Bim and is essential for both early lymphoid development and later maintenance of mature lymphocytes\(^{[26]}\). Stam et al.\(^{[27]}\) confirmed that Mcl-1 expression appeared to be significantly higher in MLL-rearranged infant ALL patients with a poor response to prednisone in vivo than in prednisone good responders and high levels of Mcl-1 expression were also found to be associated with GC resistance in vitro\(^{[13,28]}\). Our previous studies have also shown that up-regulation of pro-apoptotic protein Bim induced by Dex was one of the most important mechanisms of apoptosis in GC-sensitive ALL cells, and lack of or insufficient up-regulation of Bim after Dex treatment might be the fundamental reason for GC-resistance\(^{[29-30]}\). In this study, we demonstrated that the combination of rapamycin and Dex had a synergistic effect in Bim-EL induction, whereas there was no obvious synergistic inhibition in Mcl-1 expression, suggesting that rapamycin sensitized GC-induced apoptosis in CEM-C1 cells mainly through modulation of Bim expression.

The general biological effects of Dex are practically mediated by GR. In the present study, we found that the basic expression levels of GR\(\alpha\) mRNA and protein in CEM-C7 cells were both higher than those in CEM-C1 cells. Our results also showed that Dex could significantly inhibit proliferation and induce cell apoptosis in GC-sensitive CEM-C7 cells, which was achieved by significant up-regulation of GR and especially p-GR. However, in GC-resistant CEM-C1 cells, auto-induction of p-GR was markedly deficient, which might be one of the most important molecular mechanisms underlying GC resistance in CEM-C1 cells. It was also interesting to find that the combination of rapamycin and Dex could reverse GC resistance in CEM-C1 cells by not only inhibiting...
mTOR but also increasing auto-induction of GR, especially phosphorylated GR. To the best of our knowledge, there has been no report on whether rapamycin’s reversion of GC resistance is associated with GR or not\cite{31-32}. Our studies indicate that a cross talk between mTOR and GC-GR signaling pathways might exist, as the combination of rapamycin and Dex is able to both inhibit the mTOR signaling pathway as confirmed by down-regulation of p-p70S6K, and up-regulate the expression of GR mRNA and total GR protein, especially phosphorylated GR (activated GR). Further studies need to be performed to confirm such interaction.

In conclusion, we show in this study that the combination of rapamycin and Dex induces growth arrest and apoptosis in GC resistant T-ALL CEM-C1 cells via both suppression of mTOR and auto-induction of GR, especially auto-induction of activated GR (p-GR). Therefore, addition of rapamycin to current ALL protocols might be an attractive new therapeutic approach for GC resistant T-ALL patients.

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**AUTHORS’ CONTRIBUTIONS**

GUO Xia performed experiments, analyzed data and drafted the paper; ZHOU Chen Yan prepared rapamycin and assisted in conducting the experiments; LI Qiang designed the study, analyzed data, revised the paper and gave the final approval of the paper; GAO Ju and ZHU Yi Ping assisted in data analyses; GU Ling assisted in performing the experiments; and MA Zhi Gui assisted in the study design, data analysis and paper revision.

**DECLARATION OF INTEREST**

The authors declare no conflict of interest.

**REFERENCES**


