

RAPAMYCIN-INDUCED AUTOPHAGY PLAYS A PRO-SURVIVAL ROLE BY ENHANCING UP-REGULATION OF INTRACELLULAR FERRITIN EXPRESSION IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Background: Elevated mammalian target of rapamycin (mTOR) signaling has been reported to correlate with poor prognosis in acute lymphoblastic leukemia (ALL) patients. Rapamycin, an mTOR kinase inhibitor, and also a potent autophagy inducer, could not only effectively reverse glucocorticoid resistance, but also promote autophagy in the ALL cells. Autophagy has been suggested to play a paradoxical role in cancer treatment. The aim of this study was to address the role of the rapamycin-induced autophagy in the leukemia treatment. **Materials and Methods:** Cell proliferation was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in ALL cell lines of CEM-C1 and CEM-C7. Western Blot analysis was performed to test protein expressions. **Results:** Inhibition of mTOR by rapamycin could reverse glucocorticoid resistance in CEM-C1 cells, and also induce autophagy in these cells by up-regulation of LC3-II and Beclin-1 expressions. This autophagy played a pro-survival role since its inhibition by 6-amino-3-methylpurine or chloroquine could enhance rapamycin-induced cell death. Rapamycin increased the expression of intracellular ferritin, and this effect could be totally blocked by 6-amino-3-methylpurine and chloroquine, suggesting that the protective role of autophagy might be mediated through up-regulation of ferritin, the major iron-binding stress protein. Cyclopirox olamine, an iron chelator, could enhance rapamycin's anti-leukemia effect by down-regulation of intracellular ferritin expression. **Conclusions:** All these findings would suggest that rapamycin-induced autophagy plays a pro-survival role in leukemia cells and this effect might be mediated by up-regulation of intracellular ferritin expression. We hypothesize that the combination of mTOR pathway inhibitors and autophagy inhibition is rational and would induce strong anti-leukemia effects in ALL.

Key Words: rapamycin, autophagy, cyclopirox olamine, ferritin, acute lymphoblastic leukemia.

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Acute lymphoblastic leukemia (ALL), the most common childhood cancer, is caused by multiple genetic and epigenetic lesions that result in aberrant arrest of normal lymphoid maturation, deregulated cellular proliferation, and evasion of programmed cell death [1–3]. Pediatric ALL is so far one of the great success stories of modern cancer therapy. With precise risk-based stratification and optimized risk-directed treatment and supportive care, together with increased reliance on both national and international collaborative studies, childhood ALL now has an overall long-term survival rate of approximately 80%, with certain subsets experiencing a greater than 98% cure rate [4, 5]. Unfortunately, 10–15% of children with ALL still have recurrent disease, esp. in high-risk subtypes, such as Ph+ B-ALL and Ph-like B-ALL, and relapsed ALL remains a leading cause of pediatric cancer mortality [6, 7]. Adults with ALL fare even more poorly with over 50%

relapse rates and only 20 to 40% overall survival, which is likely due to the higher incidence of Ph+ B-ALL in adults (30–40%) than in children (1–5%) [8, 9].

Aberrant activation of the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway has been linked to oncogenesis and therapy resistance in various malignancies including leukemia. It has been reported in up to 88% of T-cell acute lymphoblastic leukemia (T-ALL) [10, 11], and in most of the Ph+ and Ph-like B-ALL [12–15]. Physiologically the mTOR signaling pathway serves as a master regulator of cell metabolism, growth, proliferation, and survival. Many human tumors occur because of dysregulation of mTOR signaling. Currently, mTOR is an interesting therapeutic target for treating multiple cancers, both the mTOR inhibitors themselves or in combination with inhibitors of other pathways [16]. We have previously demonstrated that rapamycin (Rap), an mTOR inhibitor, can effectively reverse glucocorticoid (GC) resistance in T-ALL cell lines and Ba/F3 cells transformed by NPM-ALK [17, 18]. Accumulating evidence indicates that mTOR inhibitors are most effective against cancer cells when used in combination with other agents; for example, Rap can sensitize B-ALL cells to various chemotherapeutic agents [19, 20]. Simultaneous inhibition of PI3K and mTOR exerts profound anti-leukemic activity against a broad spectrum of B-precursor ALL and T-ALL [21, 22]. Combined inhibition of mTOR pathway with tyrosine kinase inhibitors, such as dasatinib

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Abbreviations used: 3-MA – 6-amino-3-methylpurine; ALL – acute lymphoblastic leukemia; CPX – cyclopirox olamine; CQ – chloroquine; Dex – dexamethasone; DMSO – dimethyl sulfoxide; GC – glucocorticoid; HRP – horseradish peroxidase; mTOR – mammalian target of rapamycin; PI3K – phosphatidylinositol 3-kinase; Rap – rapamycin; T-ALL – T-cell acute lymphoblastic leukemia.

in ABL-rearranged Ph⁺ and Ph-like B-ALL had promising anti-leukemia efficacy in preclinical models [14, 23, 24].

mTOR is also the key molecule in regulation of autophagy, which as an evolutionarily conserved catabolic process has been implicated in the regulation of various aspects of biological process, including cell survival/death, proliferation, differentiation, and senescence. In recent years, a large body of evidence has indicated that dysregulation of autophagy links to carcinogenesis [25–27]. It is well known that the PI3K-AMPK-mTOR signaling pathways play an important role in determining the levels of autophagy in cancers [28]. Autophagy has been suggested to play a paradoxical role in cancer treatment. In some types of tumor, autophagy shows a pro-survival role against anticancer treatment; however, others go through autophagic cell death [29]. Nevertheless, increasing preclinical data suggest that autophagy is active in leukemia as a means of promoting cell survival in response to chemotherapy, and coupling autophagy inhibition strategies with current cytotoxic chemotherapy could provide new therapeutic opportunities for patients with leukemia [30].

In the present study, we demonstrated that inhibition of the mTOR pathway by Rap induced autophagy while reversing GC resistance in CEM-C1 cells. The up-regulated autophagy played a pro-survival role since its inhibition would augment leukemia cell death. Interestingly, we also showed that the increased autophagy activity enhanced intracellular ferritin expression. Since our previous work demonstrated that ciclopirox olamine (CPX), an iron chelator, could reverse GC resistance by down-regulation of intracellular ferritin expression in T-ALL cell lines, we assume that Rap-induced autophagy might protect the leukemia cells from apoptotic cell death by up-regulation of the intracellular ferritin expression, so blockage of the ferritin expression would enhance Rap's anti-leukemia effect. Indeed, we proved here that inhibition of the intracellular ferritin expression by CPX could enhance Rap's anti-leukemia effect. We hypothesize that the combination of mTOR pathway inhibitors and autophagy inhibition is rational and would induce strong anti-leukemia effects in ALL.

MATERIALS AND METHODS

Cell lines and culture. Both cell lines of GC-resistant CEM-C1 and GC-sensitive CEM-C7 were kindly provided by Dr. E. Brad Thompson (University of Texas Medical Branch). The cells were cultured in RPMI 1640 Medium (Gibco, Carlsbad, CA, USA) supplemented with 10–20% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), 2 mM L-glutamine and antibiotics (Gibco) at 37 °C in a humidified 5% CO₂ in-air atmosphere.

Reagents and antibodies. Rap (Calbiochem, La Jolla, CA, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and used at the concentration of 10 nM. Dexamethasone (Dex, Sigma, St. Louis, MO, USA) was dissolved in ethanol and used at the concentration of 1 μM. CPX (Sigma, St. Louis, MO, USA) was dissolved in ethanol to make the concentration of the stock solution of 10⁵ μM. The final concentrations of DMSO and ethanol in the medium were 0.05% and 0.1%, respectively,

at which cell proliferation/growth or viability was not obviously altered. 6-Amino-3-methylpurine (3-MA) was dissolved in phosphate-buffered saline to make the stock solution of 200 mM. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and propidium iodide were purchased from Sigma. Annexin V-PI Kit was purchased from Keygen (Nanjing, China). Antibodies to phospho-4E-BP1 and phospho-p70S6K were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to ferritin and Beclin-1 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody to LC3 was purchased from Sigma. Secondary antibodies of horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody and HRP-conjugated sheep anti-mouse antibody were all obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). P62 antibody was a gift from Dr. Zhixin Zhang of Sichuan University. Anti-GAPDH antibody was obtained from Kangchen Bio-Tech (Shanghai, China).

Cell treatment. Logarithmically growing cells were collected and re-cultured in 96- or 6-well sterile plastic culture plates (Corning Inc., Acton, MA, USA), to which 10 nM Rap (Rap group), 1 μM Dex (Dex group), 10 nM Rap plus 1 μM Dex (Rap+Dex group), and 0.05% DMSO plus 0.1% ethanol (Control group) were added respectively. At the end of the incubation period, cells were harvested and transferred to sterile centrifuge tubes, pelleted by centrifugation at 400 g at room temperature for 5 min, and prepared for analysis as described below.

Proliferation assay. The cells were then seeded in 96-well plates (20,000/mL) and incubated for 48 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium with the final concentration of 0.5 mg/mL was added to each of the wells for 4 h at 37 °C. After that, 100% (v/v) of a solubilization solution (10% SDS in 0.01 M HCl) was added to each of the wells, and the plates were re-incubated for 24 h at 37 °C. Spectrophotometric absorbance was measured at 570 nm (reference 690 nm) using a multi-plate reader (Multiskan Spectrum, Thermo Electron Co., Vantaa, Finland). Values were obtained by comparing these cells with their respective controls.

Western Blot analysis. After treatment, cells were washed twice in cold phosphate-buffered saline, and then collected and lysed by Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). Proteins were separated on 6 to 12% 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 mM Tris-HCl, pH 7.6, 137 mM 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 HRP-conjugated donkey anti-rabbit antibody or HRP-conjugated sheep anti-mouse antibody diluted 1:5000 in TTBS with 5% non-fat milk. Proteins were visualized by ECL plus (Amersham Biosciences, Inc., Piscataway, NJ).

Statistical analysis. All experiments were carried out in triplicate, and data were expressed as mean values ± SD. The Student's *t*-test was performed to compare

two groups. *P*-value < 0.05 was considered statistically significant.

RESULTS

Rap reversed GC resistance in acute CEM-C1 cells by inhibiting mTOR signaling pathway. Rap at the concentration of 10 μM had an inhibitory effect on both GC-sensitive CEM-C7 cells and GC-resistant CEM-C1 cells. However, it had marked synergistic inhibitory effect with GC in CEM-C1 cells (Fig. 1). Western Blot analysis indicated that Rap decreased the phosphorylation of both mTOR itself and its two downstream effectors of p70S6K and 4E-BP1, while Dex itself had no obvious inhibition on mTOR pathway, however, when Dex was used with Rap, there was a marked synergistic inhibitory effect on mTOR signaling (Fig. 2).

Rap induced autophagy played a protective role in GC-resistant cells. Rap is an inducer of autophagy. We found that Rap could induce autophagy in CEM-C1 cells as demonstrated by up-regulation of Beclin-1 and LC3-II, and also down-regulation of p62 (Fig. 3). 3-MA, an autophagy inhibitor, could counteract Rap’s effect by down-regulation of Beclin-1 and LC3-II, and also up-

regulation of p62 (Fig. 4). Interestingly, after inhibiting the autophagy using chloroquine (CQ), a marked growth-inhibitory effect on leukemia cells could be observed, even the concentration of the CQ decreased from 3 μM to 1 μM (Fig. 5). These data suggested that Rap induced autophagy played a protective role in GC-resistant cells.

Rap induced intracellular ferritin expression and inhibition of autophagy down-regulated the ferritin expression. Rap could also induce intracellular ferritin expression in both GC-sensitive CEM-C7 cells and GC-resistance CEM-C1 cells, and this effect could be a consequence of increased autophagy since inhibition of autophagy by 3-MA could down-regulated the ferritin expression (Fig. 6).

CPX enhanced Rap’s inhibition effect by down-regulation of intracellular ferritin expression. We previously demonstrated that CPX had an anti-leukemia effect by down-regulation of intracellular ferritin expression, and pretreatment of the T-ALL cell lines with Fe³⁺ could induce the expression of the intracellular ferritin and totally blocked the anti-leukemia effect of CPX [31]. However, Fe³⁺ had no obvious effect on the anti-leukemia effect of Dex, Rap, or the combination of Dex and Rap on the CEM-C7 cells (Table). Therefore, we deduced that CPX could counteract Rap’s effect of inducing ferritin expression and enhancing its growth-inhibitory function

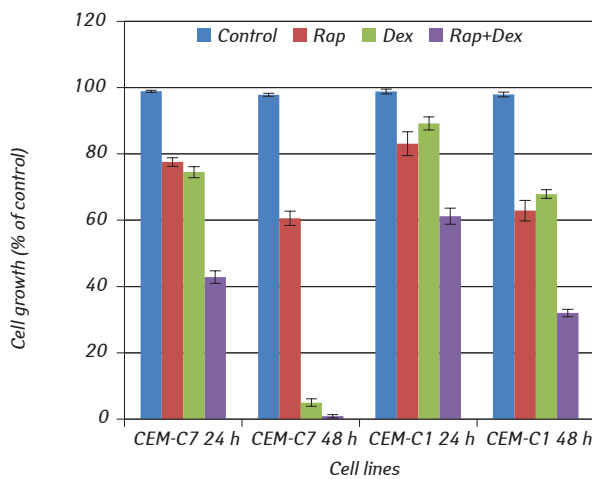


Fig. 1. Rap re-sensitizes GC-resistant CEM-C1 cells to Dex treatment. Both Dex (1 μM) and Rap (10 nM) had mild inhibitory effect on GC-resistant CEM-C1 cells, however, a strong synergistic growth inhibition was induced when they were used together, especially after 48 h of culture

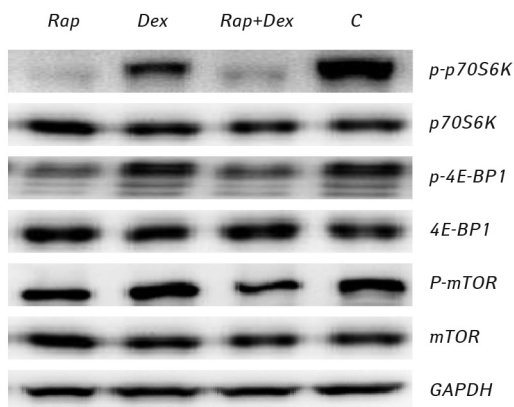


Fig. 2. Rap inhibited mTOR signaling pathway. Rap (10 nM) inhibited not only the phosphorylation of mTOR itself, but also two of the important downstream targets, pSK70 and 4E-BP1: C — control

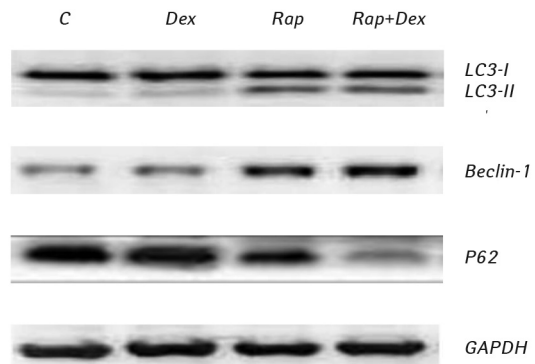


Fig. 3. Rap induced autophagy in CEM-C1 cells. Western Blot analysis showed that Rap (10 nM) increased the transition of LC3-I to LC3-II and Beclin-1 protein expression and decreased the expression of p62: C — control

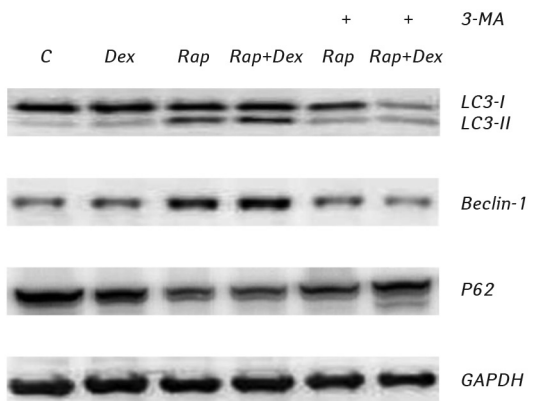


Fig. 4. 3-MA inhibited autophagy induced by Rap. Western Blot analysis indicated that 3-MA (10 mM) decreased both the expression of LC3 and Beclin, and also inhibited the transition of LC3-I to LC3-II, but increased the expression of p62: C — control

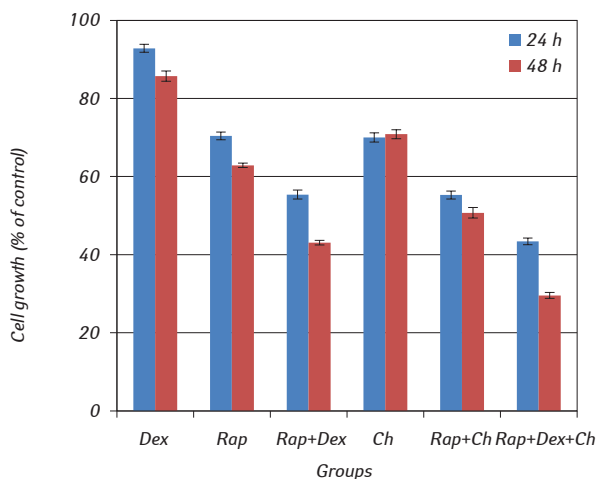


Fig. 5. Autophagy inhibitor chroloquine enhanced Rap's anti-leukemia effect. When chroloquine (1 μ M) was used together with Dex (1 μ M) and Rap (10 nM), cell growth inhibition was even stronger: Ch — chroloquine

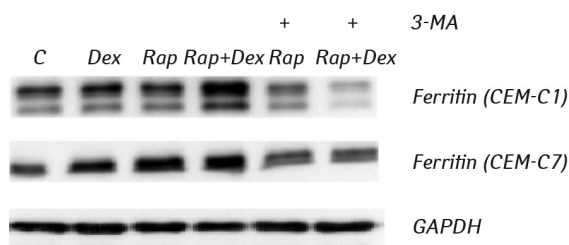


Fig. 6. Western Blot analysis showed that Rap induced the intracellular ferritin expression in both GC-sensitive CEM-C7 and resistant CEM-C1 cells and this effect could be blocked by autophagy inhibitor 3-MA (10 mM): C — control

in leukemia cells. Indeed, *in vitro* cell culture study showed that CPX blocked the up-regulation of intracellular ferritin expression in both GC-sensitive and -resistant cells (Data not shown), and CPX augmented the Rap's anti-leukemia effect (Fig. 7).

Table. The effect of iron on the survival of CEM-C7 cells ($\bar{X} \pm$ SD)

Fe ³⁺	Control	Dex	Rap	Dex + Rap
-	97.73 \pm 2.22	5.03 \pm 1.14	77.56 \pm 1.29	0.84 \pm 0.46
+	99.00 \pm 0.59 ^a	4.57 \pm 0.25 ^a	78.49 \pm 3.21 ^a	1.31 \pm 0.87 ^a

Note: ^a*p* > 0.05 compared with the corresponding group in the absence of Fe³⁺.

DISCUSSION

The PI3K/AKT/mTOR pathway is frequently abnormally activated in ALL, especially in T-ALL, and in Ph+ and Ph-like B-ALL, and also in relapsed ALL patients [10–15, 32–34]. Interestingly, GC resistance is frequently seen in those cases above, which contributes to one of the leading causes of treatment failures in pediatric ALL. Our group and others have demonstrated that mTOR inhibitors could re-sensitize both *in vitro* cell lines and *in vivo* xenograft animal models to GC [17–20]. Promising results have been reported in ALL patients treated with conventional chemotherapeutics and Rap [35]. All these data suggest that mTOR inhibitor, as a target therapy agent, could be successfully used in ALL.

Autophagy is considered to be a pro-survival mechanism in cancer therapy [17]. mTOR pathway is a master regulator of autophagy, and Rap is a potent autophagy inducer. In this study, we observed that Rap suppressed

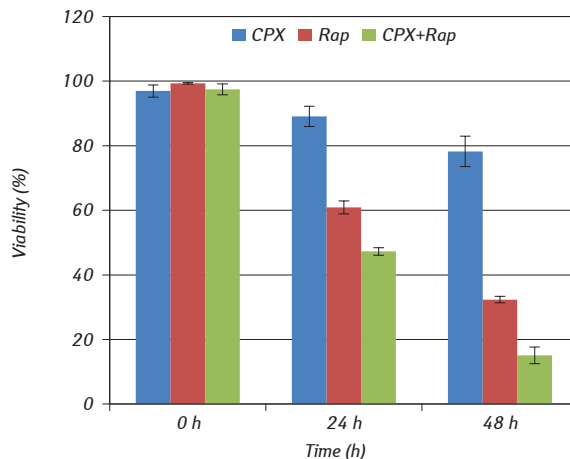


Fig. 7. Increased expression of intracellular ferritin induced by autophagy protected leukemia cells from oxidative stress. 1 μ M CPX had no obvious growth inhibitory effect, but when it was used with Rap, a strong synergistic growth inhibition was induced in CEM-C1 cells

the phosphorylation of 4E-BP1 and pSK6 and induced autophagy. Therefore, the big concern is whether this autophagy activity is pro-survival or pro-apoptosis. Recent studies indicated that mTOR inhibition induced resistance to methotrexate and 6-mercaptopurine in Ph+ and Ph-like B-ALL [15]. Our study indicated that Rap induced autophagy played a protective role, since inhibition of the autophagy by 3-MA or CQ could enhance the Rap-induced cell death. Thus, autophagy inhibition should be taken into account when using mTOR inhibitor in ALL treatment.

In the setting of chemotherapy, radiation therapy and immunotherapy, autophagy serves as a major means of resistance to cytotoxic stress [25–27, 29]. Ferritin is a ubiquitous intracellular protein that stores iron, which is an essential element for mammalian cell growth and proliferation. The concentration of ferritin can be induced in response to stresses such as anoxia and inflammation, and high ferritin expression can enhance cell growth and improve resistance to oxidative stress in tumor cells [36]. We found that increased autophagy induced by Rap enhanced intracellular ferritin expression, since inhibition of autophagy by 3-MA block the ferritin up-regulation and augmented cell death. We demonstrated that inhibition of ferritin expression by CPX could also facilitate Rap's anti-leukemia effect. These data would suggest that the induced autophagic activity by mTOR inhibition enhanced the recycling of intracellular ferritin, which protects the leukemia cells from oxidative stress by chemotherapeutic drugs.

CONCLUSIONS

In conclusion, in this study we demonstrated that Rap induced autophagy while sensitizing T-ALL CEM-C1 cells to GC. The autophagy might enhance the recycling of intracellular ferritin and thus protect the leukemia cells from oxidative stress from the chemotherapeutic drugs. Therefore, combination of mTOR pathway inhibitors and autophagy inhibition is rational and would induce strong anti-leukemia effects in ALL, and iron chelator, CPX, might be an ideal agent in combination with mTOR inhibitors in ALL treatment.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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