

Recombinant human arginase induced caspase-dependent apoptosis and autophagy in non-Hodgkin's lymphoma cells

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Arginase, an arginine-degrading enzyme, has gained increased attention recently as a new experimental therapeutics for a variety of malignant solid cancers. In this study, we found that recombinant human arginase (rhArg) could induce remarkable growth inhibition, cell cycle arrest, and caspase-dependent apoptosis in Raji and Daudi non-Hodgkin's lymphoma (NHL) cells through arginine deprivation. Interestingly, rhArg-treatment resulted in the appearance of autophagosomes and upregulation of microtubule-associated protein light chain 3 II, indicating that rhArg induced autophagy in lymphoma cells. Further study suggested that mammalian target of rapamycin/S6k signaling pathway may be involved in rhArg-induced autophagy in NHL cells. Moreover, blocking autophagy using pharmacological inhibitors (3-methyladenine and chloroquine) or genetic approaches (small interfering RNA targeting autophagy-related gene 5 and Beclin-1) enhanced the cell killing effect of rhArg. These results demonstrated that rhArg has a potent anti-lymphoma activity, which could be improved by in combination with autophagic inhibitors, suggesting that rhArg, either alone or in combination with autophagic inhibitors, could be a potential novel therapeutics for the treatment of NHL.

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There is a growing body of literature demonstrating that arginine deprivation could act as a targeted approach for several tumors.^{1–4} Arginine is a semi-essential amino acid for adult mammals, which has an important role in cell division, ammonia metabolism, and immune function.^{5–7} It is an essential amino acid for a large number of carcinoma cells, because these cells could not synthesize arginine resulting from argininosuccinate synthetase (ASS) or ornithine transcarbamylase (OTC) deficiency.^{2,8,9} Arginine-degrading enzymes, including arginase and arginine deiminase (ADI), could degrade arginine and lead to cell death of ASS- or OTC-deficient cells. Arginine was required for the proliferation of Burkitt lymphoma cells,¹⁰ and recent study showed that most lymphoma cell lines were lack of ASS and sensitive to ADI, an arginine-degrading enzyme, which was obtained from *Mycoplasma* spp.¹¹ Increasingly evidences suggested therapeutic potential benefits of arginase for malignant cancers including melanoma, pancreatic carcinoma, acute lymphoblastic T-cell leukemia, prostate cancer, and hepatocellular carcinomas.^{4,12–15}

It is well documented that amino-acid depletion could induce autophagy.^{11,16,17} Autophagy is a major intracellular

degradation system that engulfs cytoplasmic materials into autophagosomes, then degrades and recycles in lysosome.¹⁸ The mounting evidences indicated that autophagy is implicated in vital biological processes, such as differentiation, aging, cell death, innate, and adaptive immunity, especially in tumor occurrence and development.^{19–21} Although autophagy has a role as a double-edged sword in disease and health, most evidences indicated that autophagy serves a cytoprotective function particularly in cancer treatment.^{19,22,23}

In this study, we found that recombinant human arginase (rhArg) induced remarkable growth inhibition of Raji and Daudi non-Hodgkin's lymphoma (NHL) cells partly via caspase-dependent apoptosis, showing its potent anti-lymphoma effect *in vitro*. Meanwhile, rhArg induced the formation of abundant autophagosomes, the conversion of light chain 3 I (LC3-I) to LC3-II and the inhibition of mammalian target of rapamycin (mTOR)/S6K pathway in lymphoma cells. Furthermore, blocking rhArg-induced autophagy using pharmacological inhibitors or genetic approaches enhanced cytotoxic effect of rhArg, indicating that autophagy played a cytoprotective function in rhArg-based therapy. These results

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Abbreviations: rhArg, recombinant human arginase; NHL, non-Hodgkin's lymphoma; 3-MA, 3-methyladenine; CQ, chloroquine; ASS, argininosuccinate synthetase; OTC, ornithine transcarbamylase; mTOR, mammalian target of rapamycin; LC3, microtubule-associated 1 light chain 3; siRNA, small interfering RNA; Atg5, autophagy-related gene 5; PARP, poly (ADP-ribose) polymerase; Z-VAD-fmk, benzoyloxycarbonyl Val-Ala-Asp (O-methyl)-fluoro-methylketone; PI, propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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suggested that rhArg have a potent anti-lymphoma effect, which can be improved by in combination with autophagic inhibitors. Our study represented a novel approach for NHL treatment.

Results

rhArg inhibited the growth of lymphoma cells *in vitro*. All *in vitro* rhArg-treatment assays were performed in RPMI-1640 cell culture medium, so we first determined whether the rhArg depleted L-arginine (L-Arg) in the medium. As was shown in Figure 1a, 1 IU/ml of rhArg sharply decreased the concentration of L-Arg in the medium for indicated times. The effect of rhArg on cell growth was determined after cells were incubated with rhArg for 1 to 3 days. We found that 0.02–2 IU/ml of rhArg significantly inhibited cell growth of both lymphoma cells in a dose-dependent manner (Figure 1b). Moreover, incubation of Raji and Daudi cells in L-Arg-deficient RPMI-1640 medium for 24 or 48 h resulted in significant inhibition of cell growth (Figure 1c, Supplementary Figure 1). Furthermore, exogenous L-Arg could partially reverse the growth inhibition of rhArg on Raji and Daudi cells (Figure 1d). To determine whether cell cycle arrest was involved in rhArg-induced inhibition of lymphoma cell growth, cell cycle distribution was analyzed in this study. The percentage of lymphoma cells in G1 phase significantly increased after rhArg treatment for 1 to 3 days compared with the percentage of lymphoma cells in G1 phase in untreated groups (Supplementary Figure 2), indicating that rhArg induced G1 phase arrest of lymphoma cells.

The expression of ASS or OTC is related to cells' sensitivity to arginase treatment. ASS- and/or OTC-deficient tumor cells could be killed through arginine deprivation by arginase.⁸ Western blot analysis showed that Raji and Daudi cells were ASS deficient (Figure 1e), whereas human lung cancer cell line A549 was served as ASS-positive control² and human melanoma cell line A375 as ASS-negative control.⁴ The gene expression of OTC in these cell lines was also determined by quantitative RT-PCR. The result indicated that A549 cells expressed OTC gene and A375 cells did not express OTC as reported previously,^{2,4} whereas Raji and Daudi cells did not express OTC (Figure 1f). The sensitivities to rhArg of these cells were also examined, and rhArg could inhibit the proliferation of Raji, Daudi, and A375 cells but could not influence the proliferation of A549 cells (Supplementary Figure 3a). Moreover, after silencing ASS by small interfering RNA (siRNA), rhArg could inhibit the growth of A549 cells (Supplementary Figures 3b and c), suggesting that ASS played an important role in rhArg-induced cell death.

Taken together, these data suggested that rhArg could deprive L-Arg and potentially inhibit cell growth of lymphoma cells *in vitro*.

rhArg induced apoptosis in lymphoma cells. As a critical pathway of cell death, apoptosis of lymphoma cells was then examined after rhArg treatment via Annexin V-FITC and JC-1 stain. Apoptotic cells significantly increased when cells were exposed to rhArg for 48 or 72 h (Figures 2a and b). Moreover, we confirmed this effect of rhArg using a mitochondrial membrane potentials detection kit (JC-1). The result showed that aggregated JC-1 within normal mitochondria was

transformed to the monomeric form after rhArg treatment for 48 h (Figures 2c and d), indicating the occurrence of apoptosis.

Collectively, these results demonstrated that rhArg induced apoptosis in lymphoma cells in a time-dependent manner.

rhArg-induced apoptosis was partially caspase dependent in lymphoma cells.

As an early marker for classic apoptosis, caspase cleavage was detected by western blot analysis. The results showed that caspase-3 was cleaved into its activated 17 and 19 kDa fragments after treatment with 1 IU/ml of rhArg for 24, 48, and 72 h in Raji and Daudi cells (Figure 3a). To further confirm the caspase-dependent cell death, a pan-caspase inhibitor benzyloxycarbonyl Val-Ala-Asp (O-methyl)-fluoro-methylketone (z-VAD-fmk) and genetic approach (siRNA against caspase-3) were employed. Treatment with 20 μ M of z-VAD-fmk notably decreased the cleavage of caspase-3 (Figure 3b). When rhArg was combined with z-VAD-fmk, the level of poly (ADP-ribose) polymerase (PARP) cleavage and the percentage of apoptosis decreased significantly (Figures 3b and d) and rhArg-induced cell viability decrease was partly rescued (Figure 3c). Caspase-3 siRNA blocked the expression of caspase-3 (Figure 3e), and significantly decreased the percentage of rhArg-induced apoptosis in Raji and Daudi cells (Figure 3f).

Collectively, these results demonstrated that rhArg-induced apoptosis was partially caspase dependent in lymphoma cells.

rhArg induced autophagy in lymphoma cells.

Nutrient deprivation including arginine deprivation is an inducing mode of autophagy,²⁴ which is a cellular adaptive process when cells were under stress such as lack of nutrients.¹⁸ In this study, we evaluated whether rhArg induced autophagy in lymphoma cells. An abundance of autophagosomes, the golden standard of autophagy, were observed through transmission electron microscopy in cells treated with rhArg for 24 h (Figure 4a). After staining of Raji and Daudi with Cyto-ID Green dye, we detected an abundance of punctuate fluorescent dots in the cells treated with rhArg for 24 h, which further confirmed rhArg-induced autophagy in these cells (Figure 4b). Moreover, western blot analysis showed the appearance of LC3-II in the cells treated with 0.1 IU/ml of rhArg, and an obvious conversion of endogenous LC3-I to LC3-II in a dose-dependent experiment (Figure 4c).

Taken together, these results strongly suggested that autophagy was induced in Raji and Daudi lymphoma cells after rhArg treatment.

Autophagy played a critical role in rhArg-induced lymphoma cell death.

Given that rhArg could induce autophagy in lymphoma cells, we explored whether rhArg-induced autophagy could affect the inhibitive efficacy of rhArg on lymphoma cells. On the one hand, we employed 3-methyladenine (3-MA) and chloroquine (CQ), a class-III PI3K-specific inhibitor and a lysosome inhibitor, respectively, to pharmacologically inhibit rhArg-induced autophagy. We found that treatment of Raji and Daudi cells with either 3-MA or CQ markedly enhanced the growth inhibition of lymphoma

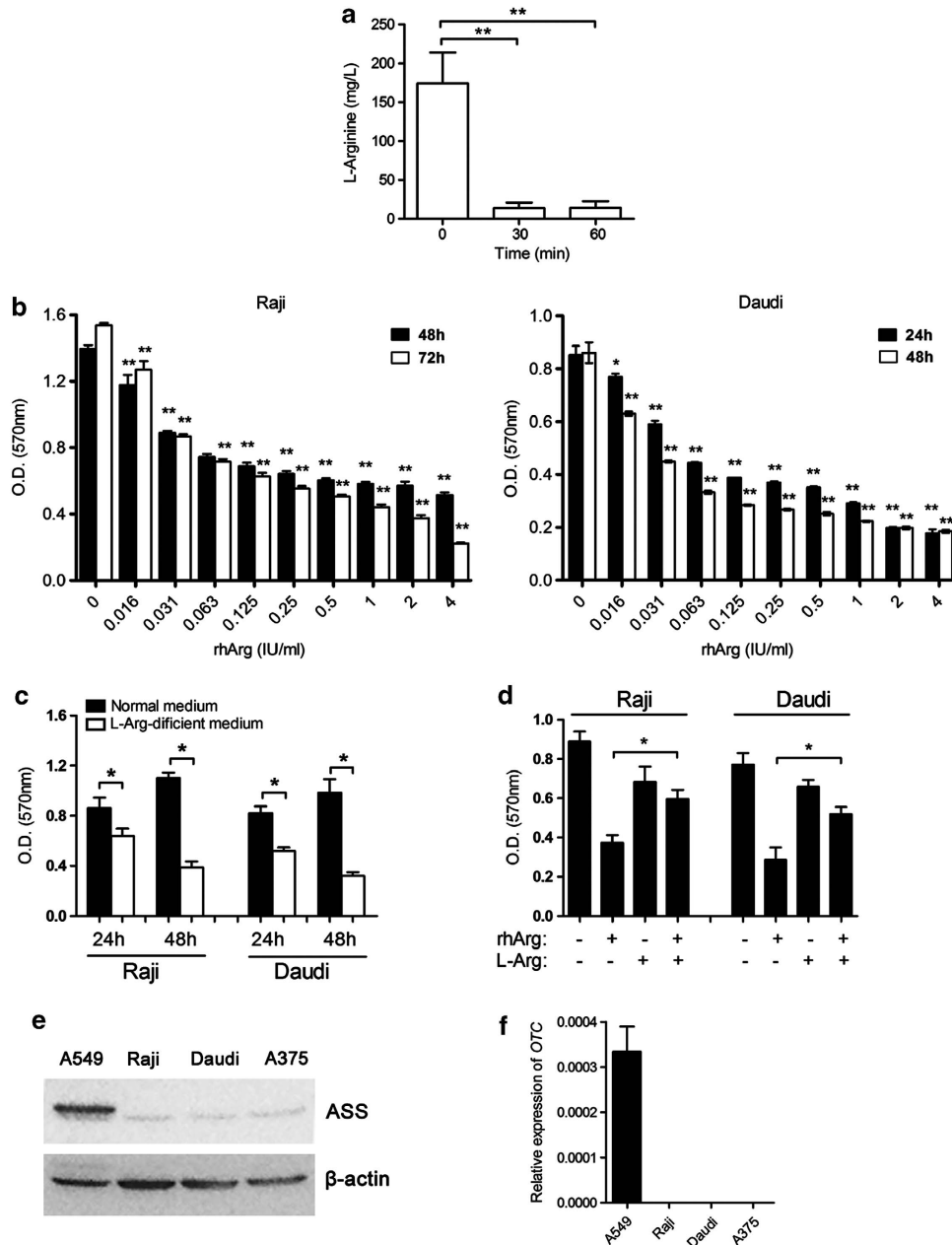


Figure 1 rhArg depleted L-Arg and potentially decreased cell viability of NHL cells *in vitro*. (a) RPMI-1640 cell culture medium was co-cultured with 1 IU/ml of rhArg for 30 or 60 min at 37 °C in cell incubator. The concentration of L-Arg was detected by HPLC. (b) Raji and Daudi cells (1×10^4 cells/well) were incubated for 24, 48, or 72 h in the presence or absence of different concentrations of rhArg. *: *versus* control, $P < 0.05$; **: *versus* control, $P < 0.01$ (c) Raji and Daudi cells were cultured in normal RPMI-1640 medium or L-Arg-deficient medium for 24 or 48 h. (d) Raji and Daudi cells were supplied with L-Arg (10 mM) for every 12 h and treated with or without 0.5 IU/ml of rhArg for 48 h. (e) A549, Raji, Daudi, and A375 cells were examined for ASS protein expression by western blot analysis. A549 and A375 cells were served as ASS-positive and ASS-negative control, respectively. (f) Gene expression of OTC was detected by using quantitative RT-PCR. Relative mRNA expression was estimated by normalization with GAPDH expression. Error bars showed standard deviation from triplicates. A typical result from three independent experiments was presented. * $P < 0.05$, ** $P < 0.01$

cells induced by rhArg, respectively (Figure 5a). Western blot analysis showed that rhArg-induced autophagy was successfully inhibited by 3-MA and CQ (Figure 5b). CQ could inhibit the fusion of autophagosome and lysosome, which led to the aggregation of autophagosomes and subsequent increase of LC3-II. To further understand the biological role of autophagy in rhArg-induced cell death, we examined the changes in rhArg-induced apoptosis.

The results demonstrated that rhArg in combination with 3-MA or CQ induced more cleavage of PARP (Figure 5b) and a higher percentage of apoptotic cells (Figure 5c) when compared with rhArg-treated alone, whereas neither 3-MA nor CQ alone treatment showed inhibitory effects on lymphoma cells. On the other hand, we used siRNA-targeting autophagy-related gene 5 (Atg5) and Beclin-1 to block rhArg-induced autophagy, as they are required for initiating

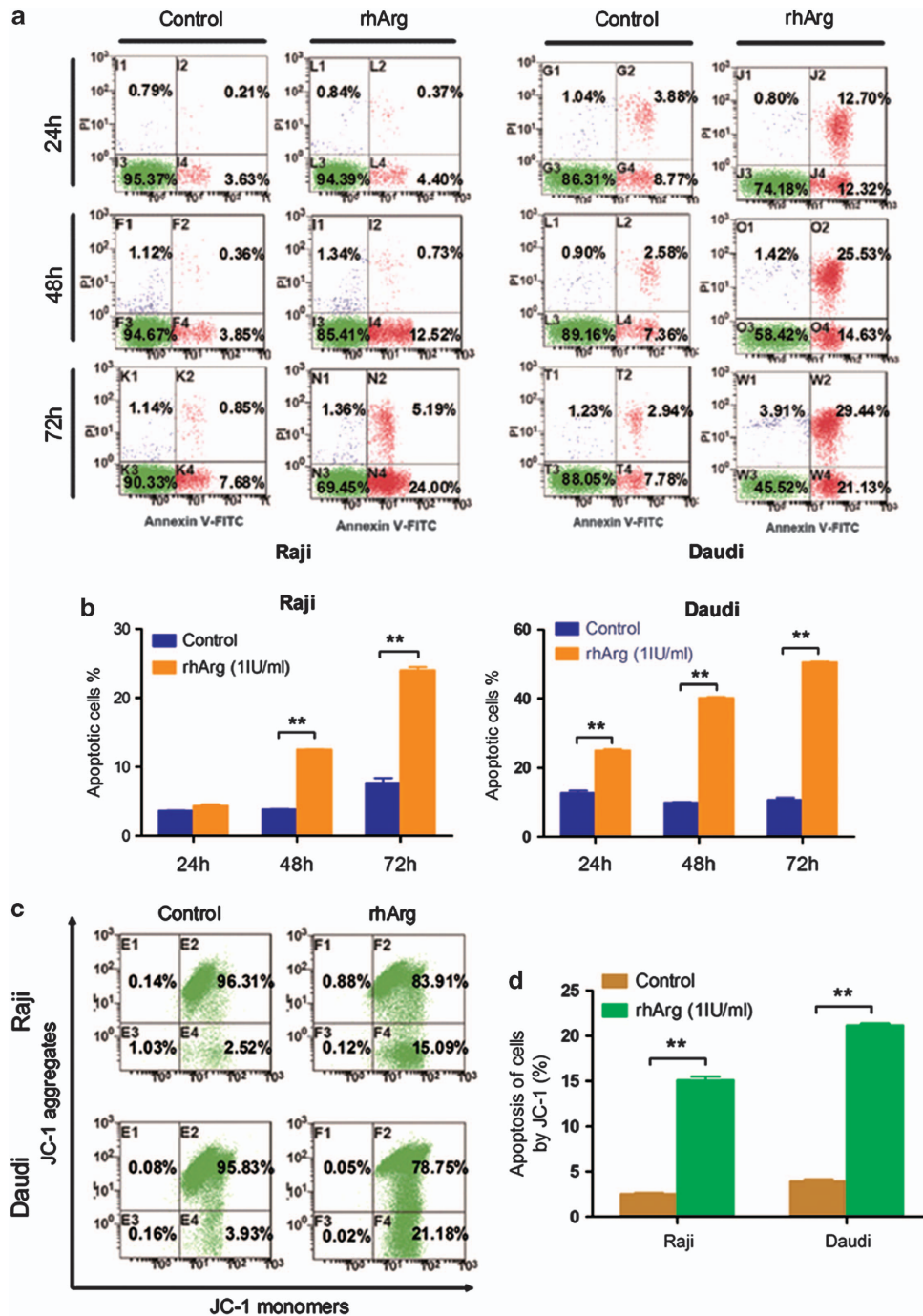


Figure 2 rhArg induced apoptosis in NHL cells. (a) Raji and Daudi cells were treated with 1 IU/ml of rhArg for 24, 48, and 72 h, and stained with Annexin V/PI and analyzed by flow cytometry. (b) The percentage of Raji and Daudi cells positive for Annexin V staining were presented in bar charts. (c) Raji and Daudi cells were incubated with 1 IU/ml of rhArg for 48 h, and then stained with JC-1 probe. $\Delta\Psi_m$ changes were detected by flow cytometry. (d) The percentage of Raji and Daudi cells characterized by JC-1 monomers was presented in bar charts. In flow cytometry scatter plots, data in each quadrant were expressed as mean of triplicate determinations. Error bars showed standard deviation from triplicates. A typical result from three independent experiments was presented. ** $P < 0.01$

autophagy. Raji and Daudi cells were transiently transfected with Atg5 or Beclin-1 siRNA followed by co-incubation with rhArg for indicated time, then cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, or cells were lysed and subjected to western blot analysis. The protein level of Atg5 and Beclin-1

was downregulated by siRNA as measured by western blot analysis (Figures 5d and e, right). Compared with cells treated with rhArg alone, cells treated with rhArg and siRNA transfection showed more cleavage of PARP (Figures 5d and e, right) and lower cell viability (Figures 5d and e, left).

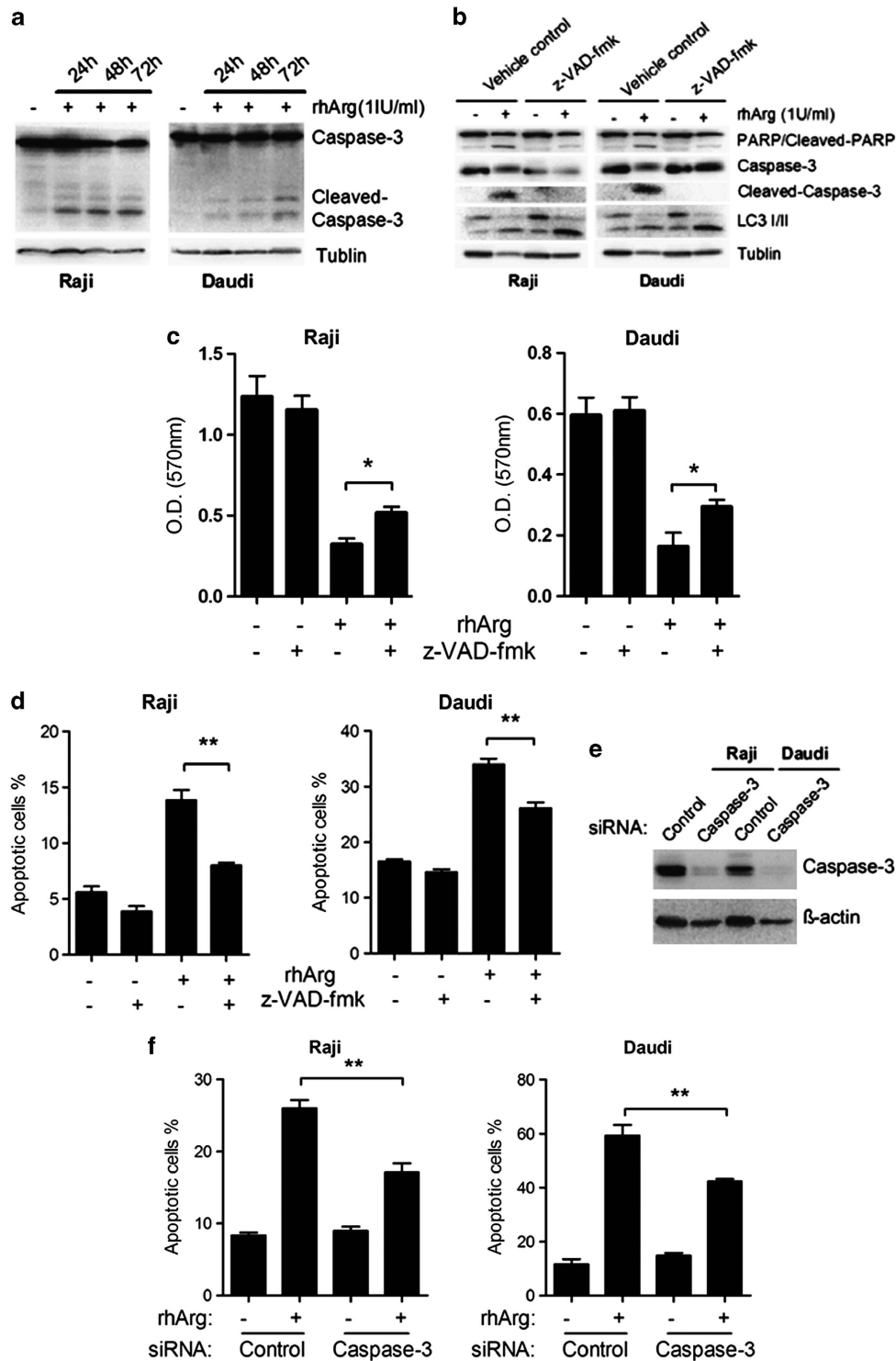


Figure 3 rhArg-induced apoptosis was caspase-dependent in NHL cells. (a) Raji and Daudi cells were incubated with 1 IU/ml of rhArg for 24, 48, and 72 h and cell lysates were subjected to Western blotting using anti-caspase-3 and tubulin antibodies. (b–d) Raji and Daudi cells were incubated with 1 IU/ml of rhArg or in combination with 20 μ M z-VAD-fmk for 48 h. (b) The LC3 conversion, PARP, and caspase-3 protein levels were detected by western blot analysis. (c) Cell viability was measured by MTT assay. (d) Cells were stained with Annexin V/PI, and analyzed by flow cytometry. The percentage of Raji and Daudi cells positive for Annexin V staining were presented in bar charts. (e and f) Raji and Daudi cells transfected siRNA against caspase-3 followed by treatment of 1 IU/ml of rhArg for 48 h. (e) The low expression of caspase-3 of Raji and Daudi cells resulted in siRNA was detected by western blot. (f) The percentage of Raji and Daudi cells positive for Annexin V staining were presented in bar charts. The percentages of positive cells were calculated from triplicates of one representative experiment. Error bars showed standard deviation from triplicates of one typical experiment. Similar results were obtained from three independent experiments. * $P < 0.05$, ** $P < 0.01$

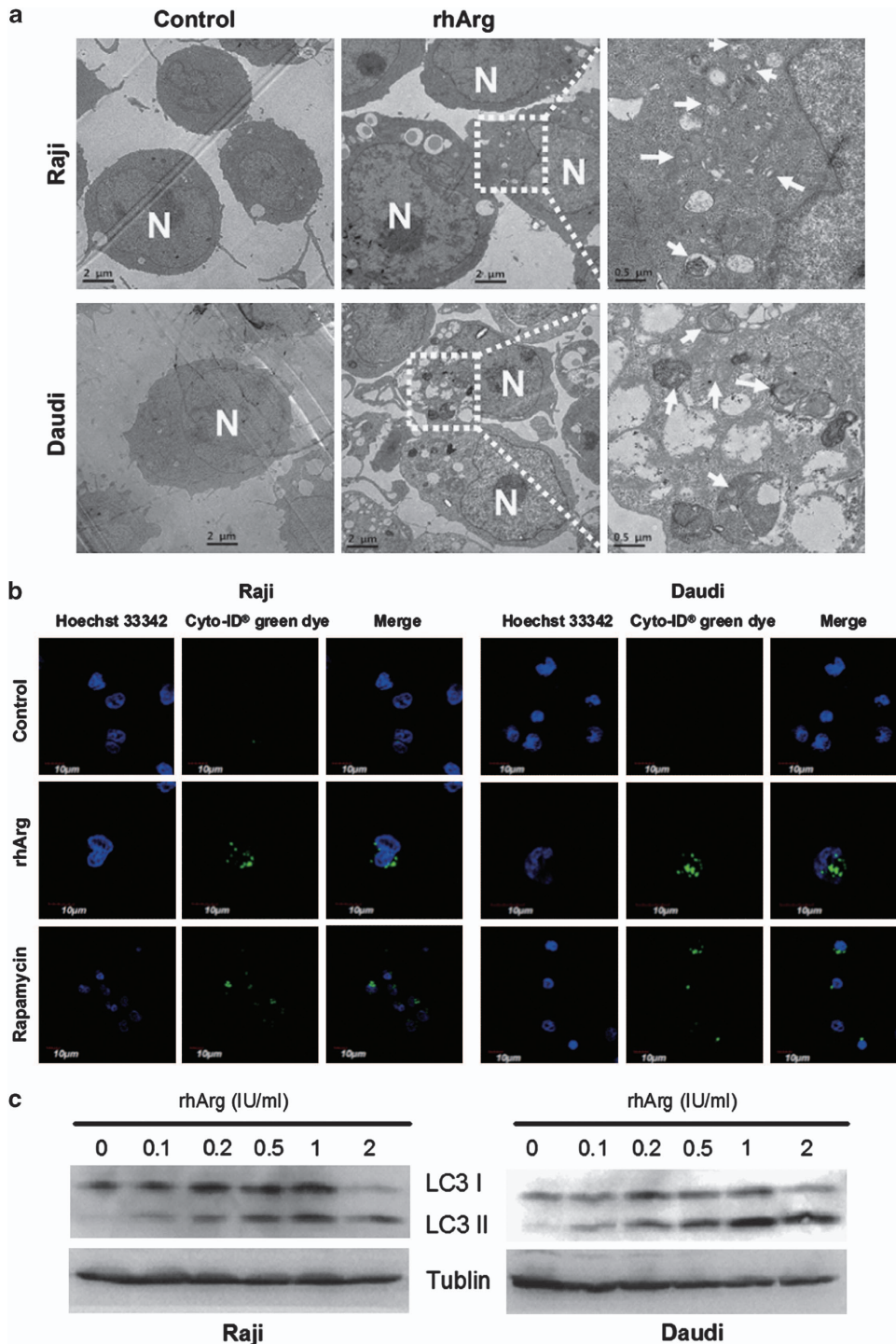


Figure 4 rhArg induced autophagy in NHL cells. (a) Raji and Daudi cells were treated with 1 IU/ml of rhArg for 24 h. Electron microphotographs were taken at $\times 5000$ (left) or $\times 20\,000$ (right) magnification. ('N': nucleus; 'white arrows': autophagosomes). (b) Raji and Daudi cells were treated with 1 IU/ml of rhArg for 24 h or with 500 nM rapamycin for 12 h. Then cells were stained with Cyto-ID Green autophagy dye and analyzed by Confocal microscopy. (c) Raji and Daudi cells were treated with 0.1, 0.2, 0.5, 1, and 2 IU/ml of rhArg for 48 h, lysed, and subjected to western blot analysis with anti-LC3 antibodies to monitor autophagy

All these data indicated that autophagy acted as a cellular protective role in rhArg-treated cells, and inhibition of autophagy might be a potential strategy to enhance the anti-lymphoma efficacy of rhArg-based therapy.

mTOR/S6K pathway was likely to be involved in rhArg-induced autophagy in lymphoma cells. As an increasingly important anti-cancer target, autophagy has a complex signaling transduction pathway. The molecular

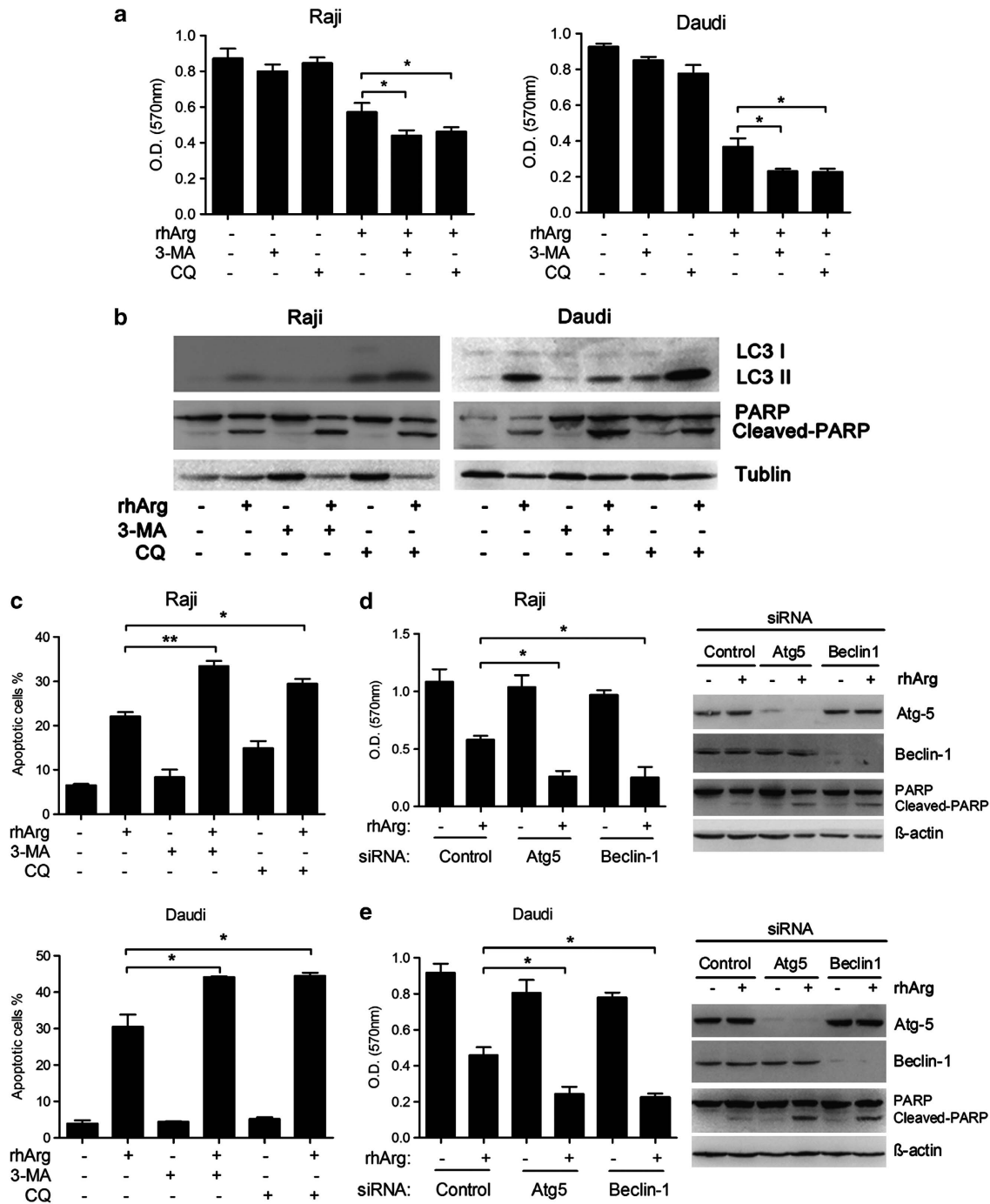


Figure 5 Autophagy played a critical role in rhArg-induced NHL cell death. (a and b) Raji and Daudi cells were treated with 1 IU/ml of rhArg, either alone or in combination with 2 mM 3-MA or 20 μ M CQ, for 24 h. (a) The cell viability was measured by MTT assay and expressed with optical density (OD) values. (b) Cell lysates were analyzed by western blot analysis. (c) Lymphoma cells were treated with 1 IU/ml of rhArg, either alone or in combination with 2 mM 3-MA or 20 μ M CQ, for 48 h (Raji) or 24 h (Daudi). Raji and Daudi cells were stained for Annexin V/PI and analyzed by flow cytometry. The percentage of Annexin V-positive cells was presented in bar charts. The percentages of positive cells were calculated from triplicates of one representative experiment. (d and e) Raji and Daudi cells were transiently transfected with Atg5 or Beclin-1 siRNA followed by treatment of 1 IU/ml of rhArg for 48 h (Raji) or 24 h (Daudi). Raji (d) and Daudi (e) cell lysates were subjected to western blot analysis to assess the protein level of Atg5, Beclin-1, and PARP and cell viability was measured by MTT assay. Error bars showed standard deviation from triplicates of one typical experiment. Similar results were obtained from three independent experiments. * $P < 0.05$, ** $P < 0.01$

mechanisms of rhArg-induced autophagy were also investigated in this study. AMPK/TSC/mTOR/S6K signal pathway is a key mechanism that is sensitive to the change of intracellular energy status.²⁵ During lack of nutrient,

activation of AMPK inhibits the phosphorylation of mTOR via TSC2. As was shown in Figure 6a, increase of the protein level of LC3-II after rhArg treatment indicated that autophagy was induced in lymphoma cells. rhArg treatment significantly

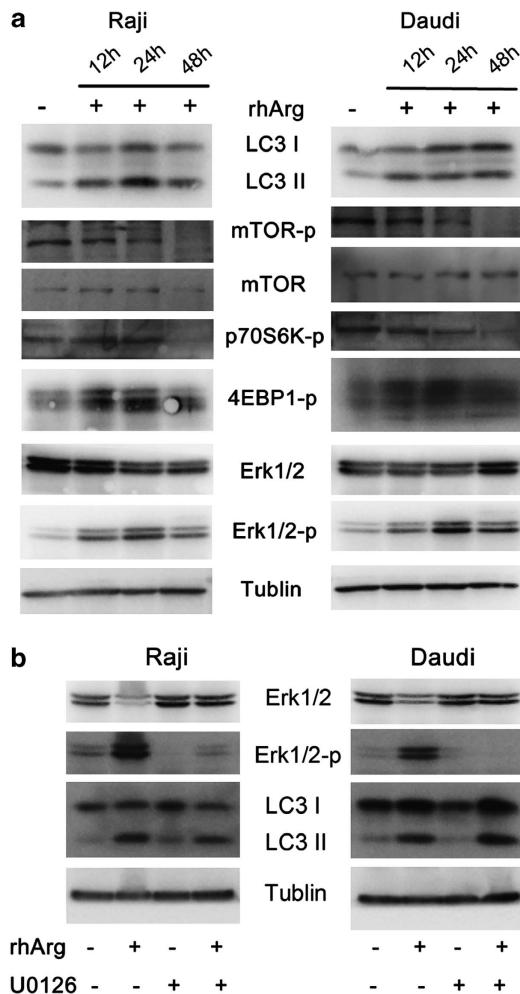


Figure 6 mTOR/S6K pathway was involved in rhArg-induced autophagy in NHL cells. (a) Raji and Daudi cells were treated with 1 IU/ml of rhArg for 12, 24, and 48 h. Cell lysates were analyzed by western blot analysis. (b) Analysis of LC3 and Erk by western blot analysis in the presence or absence of U0126 (20 μ M) after rhArg (1 IU/ml) treatment for 24 h

decreased the protein level of phosphorylated mTOR and phosphorylated p70S6K, which reflected the activity of mTOR, indicating that rhArg inhibited mTOR/S6K signaling pathway during autophagy. MEK/Erk is another important pathway involved in autophagy. rhArg activated the phosphorylation of Erk1/2 in lymphoma cells (Figure 6a). Whereas inhibiting of the MEK/Erk pathway using U0126, a MEK1/2-specific inhibitor, did not decrease the protein level of LC3-II (Figure 6b), indicating that MEK1/2 inhibitor did not affect rhArg-induced autophagy in Raji and Daudi cells. So, MEK/Erk pathway was not involved in rhArg-induced autophagy in lymphoma cells.

Collectively, these data suggested that mTOR/S6K pathway was likely to be involved in rhArg-induced autophagy in lymphoma cells.

Discussion

Recently, targeting cellular metabolism especially arginine metabolism as an approach of cancer therapy is undergoing a

renaissance. ADI, an arginine-depleting enzyme, has shown strong anti-tumor effect in many types of malignancies. Unfortunately, a series of problems, such as its bacterial origin, potential ammonia toxicity due to its catalysis, and resistance, have limited its application.^{26–29} In contrast, because of its human origin, rhArg has less antigenicity compared with ADI, and its catalytic product is ornithine instead of citrulline and ammonia. In arginine-degrading enzyme-treated cells, ornithine cannot be recycled to arginine, but citrulline can be readily recycled to reutilize by cells.³⁰ Moreover, many ASS-positive cell lines, which were found to be resistant to ADI treatment, were sensitive to rhArg because of the absence of OTC expression in these cells.² Thus, rhArg might be a better option for anti-tumor therapy than ADI.

In this study, we reported that rhArg could significantly induce cell death of lymphoma cells. rhArg could deplete arginine in our system and exhibit the anti-lymphoma effect, which could be reversed by exogenous L-Arg, confirming that rhArg killed lymphoma cells through arginine depleting. Raji and Daudi cells did not express ASS and OTC, so these cells' growth relied on exogenous arginine and were sensitive to rhArg treatment. Furthermore, our results suggested that rhArg activated caspase-3 and led to partially caspase-dependent apoptosis, which might mainly contribute to rhArg-induced cell death. In addition to inducing apoptosis, rhArg could also induce cell cycle arrest in NHL cells, and this effect of arginase has been demonstrated in other cell types.^{3,4}

Previous investigators have demonstrated that amino-acid starvation could induce autophagy.^{16,31} In this study, we showed that autophagy was induced in lymphoma cells after rhArg treatment, protecting cells from rhArg-induced inhibition of cell growth and apoptosis. Several experimental procedures were undertaken to validate the appearance of autophagy in rhArg treatment process. First, we observed the appearance of characteristic autophagosomes through transmission electron microscopy, which is the gold standard for detecting autophagy. Second, we detected an abundance of punctuate fluorescent dots in rhArg-treated cells using Cyto-ID green autophagy dye. Last, as a widely used marker of autophagy, the conversion of LC3-I to LC3-II was examined by Western blotting. The protein level of LC3-II was notably increased after rhArg treatment.

Downregulation of intracellular energy status through arginine deprivation changed the ratio of AMP/ATP, which activated AMPK. AMPK signals the nutrients deficiency to the mTOR complex via TSC2. Activation of AMPK could inhibit mTOR/S6K and subsequently induce autophagy in cancer cells.²⁵ In rhArg-treated lymphoma cells, the appearance of the autophagosomic form of LC3-II correlated with the inhibition of mTOR and p70S6K phosphorylation, and the activation of 4EBP1 phosphorylation, indicating that rhArg inhibited the mTOR/S6K pathway. MEK/Erk, an important regulator of autophagy under a variety of stimuli, was activated in ADI-induced autophagy in prostate cancer cells.¹⁷ In our study, the activation of Erk1/2 phosphorylation was also observed; however, the increase of the protein level of LC3-II in rhArg-treated cells was not affected after inhibition of Erk1/2 phosphorylation with a specific MEK1/2 inhibitor U0126,

suggesting that MEK/Erk pathway was not involved in rhArg-induced autophagy in Raji and Daudi cells.

Recently, there are an increasing number of studies showing that autophagy played both a pro-survival role and a cell death mechanism in chemotherapy, immunotherapy, and radiotherapy.^{32–34} ‘What is the biological function of rhArg-induced autophagy’ is a worthy question to be explored. We used two pharmacological autophagy inhibitors (a PI3K inhibitor 3-MA and an autophagolysosome inhibitor CQ) and siRNA (targeting Atg5 and Beclin-1) to block rhArg-induced autophagy. We found that rhArg-induced cell death was significantly enhanced by combination with autophagy inhibitors 3-MA or CQ or Atg5 and Beclin-1 siRNA, indicating that rhArg-induced autophagy might have a cytoprotective mechanism in lymphoma cells. To further understand the protective role of rhArg-induced autophagy, we tested the relation between rhArg-induced autophagy and apoptosis. Our study showed that inhibition of autophagy could enhance rhArg-induced apoptosis, which referred to the increase of the percentage of Annexin V-positive cells and PARP cleavage. Whereas CQ and 3-MA or Atg5 and Beclin-1 siRNA themselves had little effect on lymphoma cells. These data confirmed that rhArg-induced autophagy acted as a cellular protective mechanism in B lymphoma cells and inhibiting autophagy could enhance efficacy of rhArg on NHL cells. Moreover, combination of rhArg with CQ for NHL treatment has certain feasibility. CQ has been used worldwide for treatment of malaria and autoimmune diseases for more than half a century and is a FDA-approved autophagy inhibitor for clinical use.³⁵ Recently, there were many ongoing clinical trials evaluating enhanced efficacy of anti-cancer drugs via inhibiting autophagy using CQ.³⁶

In conclusion, our study demonstrated that rhArg had potential cytotoxicity on lymphoma cells *in vitro*. We found that rhArg inhibited cell growth via inducing cell cycle arrest in G1 phase and apoptosis of lymphoma cells, which partly depended on caspase activation. Furthermore, our data showed that combination of rhArg and autophagy inhibitors or siRNA could significantly accelerate cell death of lymphoma cells, indicating a protective mechanism autophagy played in rhArg-induced lymphoma cell death. These results provided a new approach to improve the efficacy of rhArg for lymphoma therapy. Finally, our results suggested that mTOR/S6K pathway was likely to be involved in rhArg-induced autophagy in lymphoma cells. This study might contribute to explore novel approaches for the treatment of NHL.

Materials and Methods

Cell lines and culture. Two human NHL cell lines, Raji and Daudi, human lung cancer cell line A549, and human melanoma cell line A375 were obtained from Cell Bank of Chinese Academy of Sciences, Shanghai Branch (Shanghai, China). L-Arg-deficient RPMI-1640, RPMI-1640, and DMEM medium were purchased from Invitrogen (San Diego, CA, USA). All cancer cells were maintained in RPMI-1640 or DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin in a humidified incubator with 5% CO₂ and 95% air at 37 °C.

Preparation of rhArg. pET30a(+)/ARGC plasmid, contained a pET30(+) backbone and the human arginase gene (containing non-coding sequence), was used to transform competent BL21 (DE3) *E. coli* cells on LB plates containing kanamycin. Single colonies were picked and transferred into LB media. The cells were fermented at 37 °C at 250 r.p.m. IPTG was added to induce rhArg

expression. rhArg was purified using nickel column.³⁷ The specific activity of rhArg was approximately 200 IU/ml and the purity of the recombinant protein is above 95%. One international unit of rhArg is defined as the amount of enzyme that produces 1 µM urea per minute at 30 °C, pH 8.5.

Reagents. Cyto-ID Autophagy Detection Kit was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). 3-MA, CQ, L-Arg, and 5-Aza-dC were purchased from Sigma (St. Louis, MO, USA). The MEK1/2 inhibitor U0126, and antibodies to LC3, Atg5, Beclin-1, β-actin, Phospho-mTOR (Ser2448), mTOR, Phospho-p44/42 MAPK(Erk1/2)(Thr202/Tyr204), p44/42 MAPK(Erk1/2), and Caspase-3 were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibodies to p70 S6 Kinase Phospho(pS371) and PARP-1 Phospho(p116/p85) were obtained from Epitomics (Burlingame, CA, USA). The mouse antibody to Arginino Succinate Synthetase (ASS) and Annexin V-FITC Apoptosis Detection Kit were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The secondary antibodies horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit immunoglobulin G (IgG) were obtained from MR Biotech (Shanghai, China). z-VAD-fmk and JC-1 probe were provided by Beyotime Institute of Biotechnology (Haimen, China). All other reagents were purchased from Sigma (St. Louis, MO, USA).

Cell viability assay. Cell viability was measured using the MTT assay, as previously described.³⁸ Briefly, about 1 × 10⁴ cells per well were seeded in 96-well plates and then exposed to rhArg or autophagic inhibitors at indicated concentrations. The cells were incubated with MTT solution (0.5 mg/ml) for 4 h at 37 °C. Then, 100 µl of 20% SDS in dimethyl formamide/H₂O (1:1, v/v; pH 4.7) was added to each well to lyse cells and then dissolve formazan for measurement. The optical density was measured at an absorbance wavelength of 570 nm.

Apoptosis assay. Apoptosis was measured using the Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's guides. Analysis was performed using a FACSCalibur flow cytometer (Becton-Dickinson, Fullerton, CA, USA). In the present study, apoptotic cells contained Annexin V⁺PI⁻ and Annexin V⁺PI⁺ cells. As a symbol of early apoptosis, the mitochondrial membrane potential (ΔΨ_m) changes were monitored after staining with a JC-1 probe as described elsewhere.³⁹ ΔΨ_m depolarization in Raji and Daudi cells was characterized by transformation of JC-1 dye aggregates (red-orange fluorescence at 590 nm) to JC-1 monomers (green fluorescence at 529 nm), which indicated that rhArg induced early apoptosis. Lymphoma cells cultured with or without rhArg were labeled with JC-1 probe as manufacturer's instruction and analyzed by flow cytometry.

Cell cycle analysis. After incubation with rhArg for 24, 48, and 72 h, Raji or Daudi cells were fixed in 70% ethanol at -20 °C for over night, washed twice with cold PBS, and stained with PI for 30 min at 37 °C in darkness. After washing twice again with cold PBS, stained cells were analyzed using flow cytometry. To analyze the DNA content, 10 000 cells per sample were evaluated using FACSCalibur flow cytometer (Becton-Dickinson).

Quantitative RT-PCR. Total RNA was extracted from Raji, Daudi, and A549 cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The RNA was reverse-transcribed into cDNA using Fermentas K1622 RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was subjected to quantitative RT-PCR (iQ5 Multicolor Real-Time PCR Detection System, Bio-Rad, Richmond, CA, USA) using the FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics, Indianapolis, IN, USA). Relative mRNA expression was estimated by normalization with GAPDH expression. The following primers were used: OTC-S: 5'-TTTTCAAGGGCATAGAATCGTC-3' (from 17 to 38 nt); OTC-AS: 5'-CTTTTCCCATAAACCAACTCA-3' (from 1248 to 1269 nt).² Gene copy numbers were expressed with the comparative CT method for relative gene expression quantification against GAPDH.

Detection of L-Arg level in cell culture medium. The concentration of L-Arg in the cell culture medium was detected using HPLC analysis as previously described.⁴⁰ In brief, RPMI-1640 medium samples were subjected to precolumn derivatization with O-Phthaldialdehyde (containing mercaptoethanol) for 2 min at ambient temperature followed by HPLC analysis.

siRNA transfection. siRNA against Atg5 (sense sequence: 5'-GUGAGUA UGGUUGAAUA-3'; antisense sequence: 5'-CACUCUAUACCAAACUUAU-3'),

against Beclin-1 (sense sequence: 5'-CAGUUUGGCACAAUCAAU-3'; antisense sequence: 5'-GUCAAACCGUUGUAGUUAU-3'), against caspase-3 (sense sequence: 5'-AGUGAAGCAAUCAGAAAC-3'; antisense sequence: 5'-UCACUUCGUUUAGUCUUUG-3'), against ASS (sense sequence: 5'-GCUAUGACGUCAUUGCCUA-3'; antisense sequence: 5'-CGAUACUGCAGUAACGGAU-3'), and a negative control siRNA were purchased from Guangzhou RiboBio Co., Ltd (Guangzhou, China). For siRNA transfection, Raji and Daudi cells (5×10^5 cells/ml) or A549 cells (1×10^5 cells/ml) transfected siRNA using X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics) according to the manufacturer's instructions, and cultured for 48 h for further treatments or western blot assays.

Western blot analysis. For western blot analysis, cells were harvested and washed with cold phosphate-buffered saline (PBS) and kept on ice for at least 20 min in Cell Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China).⁴¹ The lysates were centrifuged and the supernatants were collected. Equal amounts of protein were run on SDS-PAGE gels and subsequently transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat milk for 1 h at room temperature, then incubated overnight at 4 °C with primary antibodies. Membranes were incubated with peroxidase-conjugated secondary antibodies for 2 h at room temperature, and then the signals were developed using an enhanced chemiluminescent detection kit (Pierce, Rockford, IL, USA).

Confocal immunofluorescence. Raji and Daudi cells were treated with 1 IU/ml of rhArg for 24 h. Cells were stained with Cyto-ID autophagy detection dye according to the manufacturer's instructions.⁴² Briefly, after staining with Cyto-ID Green dye and Hoechst 33342 for 30 min, lymphoma cells were washed and re-suspended with $1 \times$ assay buffer. A drop of the cell suspension were applied onto a glass microscope slide and overlaid with a coverslip and immediately analyzed by confocal microscopy. As a positive control, cells were treated with 500 nM of rapamycin for 12 h and disposed with same procedures.

Transmission electron microscopy. Raji and Daudi cells were incubated with 1 IU/ml of rhArg for 24 h, then cells were harvested and processed as described.⁴³ Samples were analyzed with a JEM 1230 transmission electron microscope (JEOL Ltd., Tokyo, Japan). Micrographs were taken at $\times 5000$ or $\times 20\,000$ magnification.

Statistics analysis. Statistics analysis was carried out with GraphPad Prism 5. The results were expressed as means \pm S.D. or means \pm S.E.M. Student's t-test (two-tailed) or one-way ANOVA with *post-hoc* Bonferroni test was used for statistical analysis. *P*-value < 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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