L-asparaginase has been used for more than three decades in acute lymphoblastic leukemia (ALL) patients and remains an essential drug in the treatment of ALL. Poor response to L-asparaginase is associated with increased risk of therapeutic failure in ALL. However, both the metabolic perturbation and molecular context of L-asparaginase-treated ALL cells has not been fully elucidated. Here we identify that treatment with L-asparaginase results in metabolic shutdown via the reduction of both glycolysis and oxidative phosphorylation, accompanied by mitochondrial damage and activation of autophagy. The autophagy is involved in reducing reactive oxygen species (ROS) level by eliminating injured mitochondria. Inhibition of autophagy enhances L-asparaginase-induced cytotoxicity and overcomes the acquired resistance to L-asparaginase in ALL cells. The ROS-p53-positive feedback loop is an essential mechanism of this synergistic cytotoxicity. Thus, our findings provide the rationale for the future development of combined treatment of L-asparaginase and anti-autophagy drug in ALL patients.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer.1 Although treatment outcomes have been remarkably improved by the development of effective therapies and well-designed protocols, ~20% of pediatric patients develop resistance to therapy and eventually relapse, often leading to death.1,2 L-asparaginase (L-asp), one of the most important drugs used for childhood ALL therapy, is an enzyme that catalyzes the hydrolysis of asparagine (Asn) or glutamine (Gln) to aspartic acid or glutamic acid, respectively.3 Poor response to L-asp is associated with increased risk of relapse and therapeutic failure.4,5 It has been proposed that the sensitivity of ALL to L-asp is due to low or absent expression of asparagine synthetase.6 It was shown that inactivation of this enzyme confers resistance to L-asp in human lymphoblasts.7 However, genome-wide expression profiling of ALL patient samples showed conflicting results,8–11 and basal asparagine synthetase expression was shown to have no clinical significance in ALL patients.12 Thus, despite long-standing experience with L-asp therapy, both the metabolic perturbation and molecular context of L-asp-treated ALL cells remains to be fully elucidated.

One of the major cellular responses to amino-acid depletion is the induction of autophagy. Autophagy is a degradation process of proteins and organelles, which can provide metabolic intermediates such as amino acids, and can also reduce reactive oxygen species (ROS)-mediated oxidative stress by eliminating damaged mitochondria.13 Some anticancer drugs can induce cytotoxic autophagy,14 and several clinical trials using combined treatment of existing anticancer drugs and the lysosomal inhibitor chloroquine (CQ) are currently ongoing.15 Treatment with L-asp can also induce cytoprotective autophagy in human cancers.16–18 However, the biological significance of L-asp-induced autophagy or the effect of autophagy inhibition in L-asp-treated cells remains largely unknown. In this study, we sought to reveal how L-asp affects cellular processes in ALL cells, and to elucidate the implication of L-asp-induced autophagy in hopes of obtaining insight into alternative strategies for ALL therapy.

RESULTS

L-asp treatment induces metabolic shutdown and mitochondrial injury in ALL cells

We first confirmed that intracellular Asn and Gln were immediately depleted in REH cells during L-asp treatment (Figure 1a). To understand the physiological effect of L-asp treatment, we next performed the gene expression array of L-asp-treated REH cells, accompanied by gene ontology (GO) analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID)19 and gene set enrichment analysis.20 The expression levels of genes associated with several cellular metabolic pathways, including glycolysis, tricarboxylic acid cycle and oxidative phosphorylation, were significantly lower in L-asp-treated REH cells than in untreated cells (Figures 1b and c and Supplementary Table S2). Decreased expression levels of these metabolism-related genes were also confirmed in two ALL cell lines, REH and 697, by qRT–PCR (Figure 1d and Supplementary Figure S1). These findings were consistent with the decrease of intracellular ATP level (Figure 1a) and the result from the energy metabolism analysis using the XF24 extracellular flux analyzer; basal levels of both the oxygen consumption rate (OCR) for oxidative phosphorylation in the mitochondria and the extracellular acidification rate
ECAR for glycolysis were remarkably lower in L-asp-treated cells than in untreated cells (Figure 1e), suggesting that L-asp treatment effectively induces metabolic shutdown in ALL cells. In a mitochondrial stress test, treatment with oligomycin, an Fo-F1 ATPase inhibitor of Complex V, clearly reduced mitochondrial respiration in L-asp-treated and untreated cells. However, spare respiratory capacity (defined as the quantitative difference between maximal OCR after addition of mitochondrial oxidative phosphorylation uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone and the initial basal OCR) in L-asp-treated cells was significantly lower than in untreated cells (Figure 1f). These data suggested that L-asp treatment induces metabolic shutdown accompanied by reduction of both glycolysis and oxidative phosphorylation, and mitochondrial function is heavily impaired in L-asp-treated cells.

L-asp-induced autophagy is involved in reducing ROS level by eliminating injured mitochondria

To investigate whether L-asp treatment induced autophagy in ALL cells, we next evaluated autophagy flux in ALL cells by detection of LC3B, an autophagosome marker.

**Figure 1.** Induction of metabolic shutdown by L-asparaginase treatment. (a) Intracellular analysis of asparagine, glutamine and ATP. REH cells were treated with 1 U/ml of L-asp for the indicated time. Data are represented as relative ratio to control at each incubation time. (b) Expression array analysis in L-asp-treated REH cells. REH cells were treated with 1 U/ml of L-asp for 48 h. GO terms associated with metabolism from DAVID analysis. All candidate GO terms are ranked by P-value, and are listed in Supplementary Table S2. Bars indicate the counts of genes included in the respective gene set for each GO term. Line indicates P-values in log10. (c) Gene set enrichment analysis (GSEA) of microarray expression data comparing untreated and L-asp–treated REH cells. NES, normal enrichment score. (d) Quantitative RT–PCR analysis for glycolysis, tricarboxylic acid (TCA) cycle- and OXPHOS-related genes in L-asp-treated REH cells (1 U/ml of L-asp for 48 h). Expression of β-actin was used as an internal control. Expression levels relative to those in the untreated cells are indicated on the vertical axis. P-values were calculated using two-sided Student’s t-test (*P < 0.05, **P < 0.01). (e) Absolute OCR and ECAR values of untreated and L-asp–treated REH cells (1 U/ml of L-asp for 48 h). (f) Mitochondria stress test in untreated and L-asp–treated cells. OCR levels were calculated by normalization to cell number.
L-asp-treated REH cells, and these increases were clearly enhanced by addition of CQ (Figure 2c). These findings suggest that L-asp treatment can induce autophagy in ALL cells. Furthermore, intracellular amino-acid profiles revealed that both Asn and Gln levels rapidly decreased after L-asp treatment; however, the rate of decrease of these amino acids in the L-asp-treated cells did not significantly differ between cells treated with or without CQ (Supplementary Figure S2B), suggesting that autophagy might not contribute to supply a detectable amount of these amino acids. In contrast, the feature of mitochondrial injury such as the decreased mitochondrial membrane potential ($\Psi_m$) and the increase in both intracellular and mitochondrial ROS levels were remarkably enhanced autophagy-inhibited REH cells during L-asp treatment (Figures 2d and e and Supplementary Figures S2C–E). In addition, L-asp treatment significantly induced a decrease in the amount of mitochondrial DNA and mitochondrial mass (Supplementary Figures S2F and G). Taken together, these data suggested that L-asp-induced autophagy functions predominantly in mitochondrial quality control rather than in recycling intracellular amino acids. Autophagy inhibition enhances the cytotoxicity of L-asparaginase treatment. We then examined the effect of autophagy inhibition on L-asp-induced cytotoxicity in ALL cell lines. When autophagic degradation was pharmacologically inhibited by treatment with CQ simultaneously with L-asp treatment, the number of dead cells remarkably increased compared to L-asp alone (Figure 3a). Apoptotic cells, indicated by increased levels of cleaved caspase-3 and cleaved PARP, were clearly detected with combined treatment of L-asp and CQ (Figure 3b). For cell cycle analysis, the treatment with L-asp alone induced cell cycle arrest at the G1 phase, consistent with a previous report, and the combined treatment with CQ and L-asp significantly increased the sub-G1 population (Supplementary Figure S3A). Concurrent treatment with the caspase inhibitor zVAD-fmk inhibited the induction of cell death by combined treatment of L-asp and CQ (Supplementary Figure S3B). Treatment of ALL cells with L-asp showed significantly synergistic antileukemic effects in combination with autophagy inhibition using CQ (combination index at the IC50 = 0.515 in REH cells and 0.686 in 697 cells; Supplementary Figure S3C). We next investigated the effect of prolonged treatment exposure with L-asp. Almost all cells

Figure 2. Induction of autophagy by L-asparaginase treatment. (a) Western blot analysis of REH and 697 cells. Fold change of LC3B-II level (normalized to $\beta$-actin) relative to that of untreated cells is indicated in the graph in the lower panel. (b) Immunofluorescence analysis of LC3B protein. Square areas are enlarged and shown in the lower panel. Scale bars represent 10 µm. (c) Representative images of electron microscopic analysis. Arrow and arrowhead indicate an autolysosome and autophagosome, respectively. Numbers and areas of these autophagic vacuoles per cell were analyzed using ImageJ. Fifty cells were investigated per group. Scale bars represent 500 nm. Data represent as mean ± s.d. (d) Mitochondrial membrane potential assay with TMRE. Fluorescence intensity was measured using flow cytometry. (e) Measurement of intracellular and mitochondrial ROS level. Treated REH cells were stained with 10 µM of DCFDA (intracellular ROS) or 2.5 µM of MitoSox (mitochondrial ROS). REH cells were treated with 1 U/ml of L-asp for 48 h and/or 10 µM of CQ for the last 3 h (a–d) or for 48 h (d, e). Data in a, c–e represent as mean ± s.d. (n = 3); *$P$ < 0.05, ***$P$ < 0.001. $P$-values were calculated using one-way analysis of variance (ANOVA).
treated with the combination of L-asp and CQ died after 6–9 days, whereas cells treated with L-asp or CQ alone remained viable and continued to slowly proliferate during treatment (Figure 3c). This combined effect was also observed when autophagy was inhibited by small interference RNA (siRNA)-mediated knockdown of the essential autophagy genes including ATG7, ATG5, and BECN1 (Beclin1; Figure 3d and Supplementary Figure S3D). Furthermore, we examined the inhibitory effect of autophagy process at the early stage by treatment with 3MA or at the late stage by treatment with ALLN, a cathepsin inhibitor. As the result, the clear synergistic effect was shown in both combined treatment, suggesting that blocking autophagy flux can enhance L-asp-induced toxicity in ALL cells (Supplementary Figures S3E and F). Particularly, the synergistic effect by CQ treatment was not shown in the cells whose autophagy was clearly inhibited by treatment with 3MA (Supplementary Figure S3G), suggesting that CQ-mediated sensitization is dependent on inhibiting autophagy. To test whether autophagy inhibition can overcome the resistance to L-asp treatment, we generated the acquired resistant cells from 697 cells by prolonged exposure to L-asp (parental 697 cells; IC50 = 0.74 and 697-R cells, a resistant 697 cell; IC50 = 2.4; Figure 3e). Combined treatment of L-asp and CQ induced significant cell death, including in 697-R cells (Figure 3f). Importantly, LC3B-II levels were increased in 697-R cells more than in parental 697 cells by L-asp treatment, and this increase was significantly enhanced by CQ treatment, suggesting that the activity of L-asp-induced autophagy in 697-R cells was higher than that of the parental 697 cells (Figure 3g). Thus, these findings suggest that autophagy inhibition may be a useful strategy to overcome L-asp resistance. Combination treatment of L-asp and chloroquine suppresses leukemia growth in vivo. To examine the therapeutic potential of the combined treatment using an in vivo ALL xenograft model, REH cells stably expressing luciferase (REH-Luc2) were injected into the tail vein of non-obese diabetic/severe combined immunodeficient mice. Quantification of leukemia-associated bioluminescence at 7 days after transplantation demonstrated no significant differences among the four treatment groups (Figure 4a). The mice then received daily intraperitoneal injections of PBS, 6 U/g L-asp, 50 mg/kg CQ or both L-asp and CQ. Asn levels in the plasma of mice treated with L-asp were completely depleted (Figure 4b). A decrease in leukemia burden and increase in outcome improvement were observed in mice

Figure 3. Effect of autophagy inhibition on L-asparaginase-induced cytotoxicity in ALL cells. (a, b) Apoptotic analysis in ALL cell lines REH and 697 cells with or without CQ treatment by flow cytometry (a) and western blotting (b). The proportion of dead cells was measured by flow cytometry using Annexin-V staining. Cells were treated with the indicated concentrations of L-asp and/or CQ for 48 h. (c) Cell survival assay during prolonged treatment. According to the clinical method for administering L-asp, which is repeated every 3 days in patients, ALL cells were cultured with PBS (control) or repeated (0, 72, 144 h) administration of L-asp and/or CQ. Viable cells were counted using trypan blue staining every 24 h. ALL cells were treated with repeated administration of L-asp and/or CQ. (d) Sensitivity to L-asp treatment in REH cells transfected with ATG7-siRNA. Cells transfected with control-siRNA (si-control) or ATG7-siRNA (si-ATG7) were treated with the indicated concentrations of L-asp for 48 h. Viable cells were counted by flow cytometry using Annexin-V staining. (e) Cell survival assay of parental cells and L-asp-resistant cells generated from 697 cells (697-R). (f) Apoptotic analysis of 697-R cells. (g) Western blot analysis of parental cells and 697-R cells. Data in a and d–f are represented as mean ± s.d. (n = 3; *P < 0.05, ***P < 0.001). P-values were calculated using two-sided Student’s t-test (a, f), and one-way ANOVA (c).
administered L-asp and CQ combined treatment, compared with mice treated with L-asp or CQ alone (Figures 4c and d and Supplementary Figure S4A). This therapeutic effect by combined treatment with L-asp and CQ was also observed in other xenograft models using 697-Luc2 cells (Supplementary Figures S4B and C).

As hepatomegaly is caused by infiltration of leukemia cells, the liver weight of mice with leukemia is used as the indicator of objective response to antileukemic agents on some occasions. Mice treated with L-asp or CQ alone were found to have significantly decreased body weight and increased liver weight compared with mice administered L-asp and CQ combined treatment (Figures 4e and f), which indicate a remarkable antileukemic response. In addition, we could not find any signs of the treatment-related complication including hemorrhage or infarction in the killed mice. Importantly, LC3B-positive puncta were observed to be accumulated in ALL cells that remained within the bone marrow, peripheral blood and central nervous system of the mice receiving combined treatment, indicating the therapeutically sufficient autophagy inhibition (Supplementary Figure S5). These findings strongly suggested that combined treatment with L-asp and CQ may be therapeutically useful for ALL.

The ROS-p53-positive feedback loop is an essential mechanism of the combined treatment of L-asp and CQ

We attempt to determine the molecular mechanism underlying the synergistic effect of L-asp-induced cytotoxicity and autophagy inhibition. Although the apoptosis pathway via the ATF4-CHOP axis is known to be involved in L-asp-induced cytotoxicity, this pathway was not significantly activated in cells treated with the combination of L-asp and CQ, compared with the cells treated with L-asp alone (Supplementary Figure S6), suggesting that other mechanisms may contribute to the induction of cell death by...
combined treatment with L-asp and CQ. We then focused on the production of ROS as a possible mechanism because ROS levels were remarkably increased during combined treatment (Figure 2e). ROS scavenger N-acetyl-L-cysteine rescued the induction of cell death by combined treatment with L-asp and CQ (Figure 5a and Supplementary Figure S7A), indicating that excessive ROS accumulation is critical for the induction of cytotoxicity by the combination.

Severe ROS accumulation leads to irreparable DNA damage, which may induce the ROS–DNA damage-p53-positive feedback loop. ROS–DNA damage-p53-positive feedback loop. The protein expression levels of p53 and PUMA were remarkably increased, and was accompanied by DNA damage indicated by an increased level of γH2AX, in cells treated with the combination of L-asp and CQ compared with cells treated with L-asp alone in both REH and 697 cells with TP53 wild type (Figure 5b and Supplementary Figure S7B).

The inhibition of ROS production by treatment with N-acetyl-L-cysteine led to the reduction of both DNA damage and p53 expression (Figure 5c and Supplementary Figure S7C). TP53 knockdown resulted in the reduction of ROS and DNA damage in cells treated with the combination of L-asp and CQ (Figures 5d and e and Supplementary Figures S7D and E). TP53 is known to have multiple roles in the regulation of autophagy. In western blot analysis, knockdown of TP53 did not exert a sever influence on L-asp-induced autophagy (Supplementary Figures S7F). Importantly, when TP53 expression was inhibited by differentiating two knockdown systems using short hairpin RNA (shRNA) or siRNA, the synergistic cytotoxic effect of L-asp and CQ combined treatment was abrogated (Figure 5f and Supplementary Figures S7G and H).

Activated p53 transcriptionally upregulated the pro-oxidant genes, including PUMA, TP53I3, SCO2, and downregulated the anti-oxidant gene HK2 (Supplementary Figure S7I). In addition, in overall survival analysis of mice transplanted with TP53-knockdown or control ALL cells treated with L-asp and CQ combination, poor outcome was observed in mice transplanted with TP53-knockdown ALL cells (sh-p53), similar to those receiving no treatment (Figure 5g). We finally examined the functional role of p53 on the synergistic effect of L-asp and CQ combination treatment in seven primary ALL samples, including six cases with...
Role of autophagy in L-asparaginase-treated ALL cells
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![Figure 6. Schematic diagram. L-asparaginase treatment indues mitochondrial injury. Autophagy contributes to the prevention of oxidative DNA damage accumulation in L-asparaginase-treated cells. Autophagy inhibition with L-asp treatment triggers the ROS–DNA damage-p53 feedback loop, which leads to marked apoptosis in ALL cells.](image)

Wild-type TP53 and one case with mutant TP53 (R248Q). These samples were derived from five newly diagnosed patients and two relapsed patients. Clinical features of these patients are shown in Supplementary Table S3. As expected, the synergistic effect of L-asp and CQ was observed in ALL cells derived from the six wild-type TP53 samples, but not in ALL cells from the mutant TP53 sample (Figure S5h). In addition, adenovirus-mediated expression of exogenous TP53 into TP53-mutated ALL cells from No.7 patient or TP53-mutated ALL cell lines, Jurkat and CCRF-CEM, induced the synergistic effect by combined treatment with L-asp and CQ (Figure S5i and Supplementary Figure S7J). These findings strongly suggest that p53 function is essential for this synergistic effect.

DISCUSSION

In the present study, we clarified the physiological effect of L-asp and the biological significance of L-asp-induced autophagy, and additionally demonstrated the therapeutic effectiveness of autophagy inhibition by CQ in combination with L-asp treatment in ALL cells. The treatment with L-asp results in metabolic shutdown via the reduction of both glycolysis and oxidative phosphorylation, accompanied by mitochondrial injury and ROS production. Importantly, we demonstrated that the inhibition of autophagy using CQ enhances L-asp-induced cytotoxicity and overcomes the acquired resistance to L-asp in ALL cells via ROS-p53-positive feedback loop as an essential mechanism of this synergistic cytotoxicity Figure 6.

Several studies have suggested that autophagy may act as a cytoprotective mechanism in tumor cells and that therapy-induced cell death can be enhanced upon autophagy inhibition.18,28–30 It has been reported that cytoprotective autophagy was induced by treatment with L-asp and autophagy inhibition enhanced L-asp-induced cytotoxicity in K562 cells, a chronic myeloblastic leukemia cell line. However, the functional role of L-asp-induced autophagy has not been clarified. While it has been believed that L-asp-induced autophagy contributes to the supply of amino acids including Asn and Gln,16–18,31 our data in the current study suggest that L-asp-induced autophagy predominantly have a function as elimination of damaged mitochondrial rather than supply of intracellular amino acids by recycling. In treatment of patients with ALL, L-asp is used in combination with vincristine and prednisone.32 Prednisone is reported to induce autophagy, which is required for cell death.32–34 Thus, the modulation of autophagy by CQ needs to consider autophagic effect on combined drugs other than L-asp in the clinical setting of ALL.

We demonstrated in an ALL xenograft model that autophagy inhibition using CQ with L-asp treatment is therapeutically effective. Notably, the effect of L-asp and CQ combined treatment could be observed in ALL cells that remained within the bone marrow and central nervous system, suggesting the potency of autophagy inhibition with CQ in combination with L-asp treatment in intractable ALLs. In addition to clinical trials utilizing the inhibition of autophagy by CQ for treatment of solid tumors in adults, CQ is clinically used as an FDA-approved drug for treatment of pediatric patients with several diseases, such as malaria and interstitial lung disease.35,36 However, CQ may cause severe side effects, including irreversible retinal toxicity.36 Moreover, CQ is not a specific autophagy inhibitor, but also modulates various additional signal transduction pathways.37 Therefore, development of therapeutic agents that can specifically inhibit the autophagy pathway is required for the clinical use.

We showed that functional p53 is needed for the synergistically cytotoxic effect of L-asp and CQ combined treatment in ALL cells. While autophagy was shown to be required for the development of Ras-driven pancreatic tumors in a previous study, autophagy inhibition by CQ promoted tumorigenesis in developing tumors lacking p53.38 Another study reported that p53 has an important role in the combined effect of temozolomide and CQ in glioblastoma.39 Thus, these previous reports together with the present results suggest that functional p53 has an essential role in autophagy inhibition-mediated cytotoxicity. Because mutations of the TP53 gene are observed in ~6–8% of pediatric ALL patients,40 the majority of pediatric patients may benefit from the combined effect of autophagy inhibition and L-asp treatment.

In summary, we reported molecular evidence supporting the development of a novel therapeutic strategy of combined L-asp and autophagy inhibition for ALL. It will be important for ALL patients to evaluate the autophagy flux before, after or during L-asp treatment. Further validation of this strategy, together with determination of p53 status, in a large cohort of patients is warranted to effectively evaluate its impact on the treatment of ALL.

MATERIALS AND METHODS

Cell culture and reagents

TP53-intact ALL cell lines, REH and 697, or TP53-mutated ALL cell lines, CCRF-CEM and Jurkat, were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. Culture medium was changed 24 h before treatment for each experiment. REH (CRL-8246), CCRF-CEM (CRL-8436) and Jurkat (TIB-152) are commercially available from the American Type Culture Collection, and 697 is available from DSMZ (Germany, catalog code ACC 42). LEUNASE was used for L-asp treatment, purchased from Kyowa Hakko Kirin Co. (Tokyo, Japan). Chloroquine diphosphate, Bafilomycin A1 and N-acetyl-L-cysteine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Patients

Bone marrow with more than 90% blast content was obtained from six patients (five patients with newly diagnosed ALL and one patient with...
Cell viability assay and apoptosis assay

ALL cells were plated at 1 × 10^5 cells in six-well plates and treated with the appropriate reagents. Viable and dead cells were counted using trypan blue assay by the TC20 Automated Cell Counter (BioRad Laboratories, Richmond, CA, USA). Apoptosis was assessed by flow cytometry using Annexin-V/propidium iodine staining (MEBCYTO-Apoptosis Kit, MBL Co., Ltd., Nagoya, Japan). All experiments were performed in triplicate.

Cell cycle analysis

Cells were washed in PBS and fixed in 70% cold ethanol overnight at −20 °C. Fixed cells were washed in PBS, incubated in PBS containing RNase (250 μg/ml) for 30 min at 37 °C and then stained with propidium iodide (Thermo Scientific). Fluorescence intensities were measured by flow cytometry and cell population analysis was performed using the FlowJo software (Tree-star Inc., Ashland, OR, USA).

Generation of L-asp-resistant cells

L-asp-resistant cells were established by sequential incubation of parental cells with increasing concentrations of L-asp from 0.01 to 1.0 μM for 6 months in resistant cells from 697 cells (697-R).

Measurement of mitochondrial DNA copy number and mitochondrial mass

The relative ratio of mitochondrial DNA to nuclear genomic DNA was measured using the Human Mitochondrial DNA Monitoring Primer Set Ratio kit (Takara Bio, Shiga, Japan). For measurement of mitochondrial mass, cells were incubated with 100 nm of MitoTracker green (Thermo Scientific) for 30 min, and fluorescence intensities were measured by flow cytometry.

Immunofluorescence analysis

Cells were fixed in cold methanol for 5 min. After blocking with PBS containing 1% bovine serum albumin and 0.01% Triton X-100 for 1 h at 4 °C, the cells were incubated with anti-LC3B (Sigma-Aldrich Co.) and/or human CD45-FITC antibodies (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) overnight at 4 °C. Bound antibodies were visualized using Alexa Fluor 488 anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA). The cells were mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were obtained by confocal fluorescence microscopy (Nikon, Tokyo, Japan).

Electron microscopy

The cells were fixed with 2.5% glutaraldehyde in 0.1 M PBS overnight. They were washed with 0.1 M PBS and post-fixed with 1% O2O2 buffered in 0.1 M PBS for 2 h. Then, the cells were dehydrated in a graded series of ethanol solutions and embedded in Epon 812. Ultrathin (90 nm) sections were collected on copper grids, double-stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (H-7100, Hitachi, Tokyo, Japan).

Gene expression array analysis

Gene expression profiling of ALL cells was performed as previously reported.42 GO analysis was performed using DAVID (https://david.ncifcrf.gov/home.jsp) and gene set enrichment analysis (http://www.broadinstitute.org/gsea/index.jsp). The microarray data from this publication have been submitted to the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and assigned the identifier GSE94289.

Real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (PCR) was carried out using TaqMan polymerase with SYBR Green fluorescence (KAPA SYBR FAST qPCR Master Mix: NIPPON Genetics, Tokyo, Japan) on an ABI PRISM 7300 Sequence Detector (Applied Biosystems, Concord, ON, Canada). Real-time RT-qPCR analysis was performed using specific primers (Supplementary Table S1).
Western blotting
Western blotting analysis was performed as previously reported. Antibodies for LC3B (L7543), β-actin (A5441) and asparagine synthetase (A6485) were purchased from Sigma-Aldrich; ATF4 (L0911) was from Santa Cruz Biotechnology (Dallas, TX, USA); p53 (OP43L) was from Calbiochem (San Diego, CA, USA); cleaved PARP (#9541), cleaved CASP3 (#9661), CASP3 (#9662), CHOP (#2895), BECN1 (#4122) and ATG5 (#12994) were from Cell Signaling (Danvers, MA, USA); γH2AX (ab11174) was from Abcam.

Transduction of shRNA or siRNA
shRNA oligonucleotides for TP53 (target sequence: 5′-GACTCCAGTG GTAATCTAC-3′) were annealed and inserted into the pGreenPuro vector (System Biosciences, Palo Alto, CA, USA). Lentivirus was prepared using HEK293T cells and the pPACK Packaging Kit (System Biosciences). Cells were infected with 5 multiplicity of infection (PFU/cell) of lentivirus with either an empty vector (as a control) or p53-shRNA vector using TransDux (System Biosciences).

The siRNA for TP53 (M-003329-03-0005), BECN1 (M-010552-01-0005), ATG5 (M-004374-04-0005), ATG7 (M-020112-01-0005) and non-targeting negative control (D-001206-14-05) were obtained from Thermo Scientific Dharmacon. Cells were transfected with 10 nM of each siRNA using the HVJ Envelope Vector Kit (GENOMEONE-Neo, Ishihara Sangyo), according to the manufacturer's instructions.

Recombinant Adenovirus infection
The TP53 adenovirus was prepared and cells were infected as previously described. As a control, an Ad-LacZ adenovirus encoding the β-galactosidase gene was constructed from the cosmID pAxCaLacZ (Takara Bio).

Mutation analysis in TP53 by direct sequencing
Mutations within coding exons in the TP53 gene were analyzed by direct DNA sequencing. DNA fragments were amplified by PCR using primer pairs described previously (http://www-ps3.iarc.fr) and then PCR products were sequenced by primer for each exon.

Statistics
The experiments performed in ALL cell lines were performed independently in triplicate. All P-values were two-tailed and considered significant at < 0.05. Statistical analyses were performed using the statistical software EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan). We analyzed drug synergism using the Chou-Talalay median-effect method and used CalcSYN software (Biosoft, Cambridge, UK) to calculate the combination index and perform isobologram analysis of drug interactions.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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