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Key points:

- Bafilomycin A1 at low concentration specifically inhibits and kills in vitro, ex vivo and in vivo pediatric B-cell acute lymphoblastic leukemia cells while sparing normal cells
- Bafilomycin A1 at low concentration inhibits cytoprotective autophagy of pediatric B-cell acute lymphoblastic leukemia cells
- Bafilomycin A1 at low concentration activates caspase-independent but AIF-dependent apoptosis of pediatric B-cell acute lymphoblastic leukemia cells
Abstract

B-cell acute lymphoblastic leukemia is the most common type of pediatric leukemia. Despite plausible remission rate, current treatment regimens for pediatric B-cell acute lymphoblastic leukemia are often associated with adverse effects and central nervous system relapse, necessitating more effective and safer agents. Bafilomycin A1 is an inhibitor of vacuolar H⁺-ATPase that is frequently used at high concentration to block late-phase autophagy. Here, we show that bafilomycin A1 at low concentration (1 nM) effectively and specifically inhibited and killed pediatric B-cell acute lymphoblastic leukemia cells. It targeted both early and late stages of the autophagy pathway by activating the mammalian target of rapamycin signaling and by disassociating the Beclin 1-Vps34 complex, as well as by inhibiting the formation of autolysosomes, all of which attenuated functional autophagy. Bafilomycin A1 also targeted mitochondria and induced caspase-independent apoptosis by inducing the translocation of apoptosis-inducing factor from mitochondria to the nucleus. Moreover, bafilomycin A1 induced the binding of Beclin 1 to Bcl-2, which further inhibited autophagy and promoted apoptotic cell death. In primary cells from pediatric B-cell acute lymphoblastic leukemia patients and a xenograft model, bafilomycin A1 specifically targeted leukemia cells while sparing normal cells. An in vivo mouse toxicity assay confirmed that bafilomycin A1 is safe. Our data thus suggest that bafilomycin A1 is a promising drug candidate for the treatment of pediatric B-cell acute lymphoblastic leukemia patients.

Key Words: Pediatric B-cell acute lymphoblastic leukemia, bafilomycin A1, autophagy, apoptosis

Introduction

Most pediatric acute lymphoblastic leukemia (ALL) cases are of B-cell origin. One common B-cell acute lymphoblastic leukemia (B-ALL) subtype, first reported by Volgler et al in 1978 (1), has leukemia blasts with a specific chromosomal translocation, t(1;19)(q23;p13), resulting in the fusion of two transcription factors, E2A and PBX19 (2,3). This translocation thus causes oncogenesis through altered regulation of gene expression. Patients possessing the oncogenic E2A-PBX1 translocation had a dismal prognosis two decades ago (4,5). This adverse prognostic significance has largely been negated by the more aggressive multiagent therapies that are currently the standard of care. The treatment protocol for various B-ALL includes an intense chemotherapy regimen that yields cure rates from 15% in 1990 (6) to 80% in recent years (7,8). However, about 20% of children in remission suffer a relapse (8,9).
Currently, major approaches include new formulations of existing chemotherapeutic agents, new antimetabolites and nucleoside analogs, monoclonal antibodies directed against leukemia-associated antigens, and molecularly targeted drugs, such as tyrosine kinase inhibitors (TKIs) and FMS-related tyrosine kinase 3 inhibitors (10-13). Although the overall outcome is comparable with that of children lacking this translocation t(1;19)(q23;p13), current treatment regimens for pediatric B-ALL are often associated with adverse effects and a higher risk of central nervous system (CNS) relapse, necessitating more effective and safer agents.

Bafilomycin A1, a macrolide antibiotic isolated from the *Streptomyces* species, is an inhibitor of vacuolar H⁺ ATPase (V-ATPase). It binds to V0 sector subunit c of the V-ATPase complex and inhibits H⁺ translocation, causing an accumulation of H⁺ in the cytoplasm (14,15). Bafilomycin inhibits cell growth (16) and induces apoptosis (17,18) and differentiation (19). These anticancer effects of bafilomycin A1 are considered to be attributable to the intracellular acidosis caused by V-ATPase inhibition. Bafilomycin A1 was also found to induce growth inhibition of cancer cells under hypoxic conditions by expressing hypoxia-inducible factor-1α (20). More frequently, bafilomycin A1 has been used in the study of autophagy as an inhibitor of fusion between autophagosomes and lysosomes and as an inhibitor of lysosomal degradation (21,22). The above anticancer effects and the late-phase autophagy inhibition require a high concentration (0.1–1 μM) of bafilomycin A1 and are often associated with adverse effects because acidosis and hypoxia also occur in normal cells in physiological condition.

Apoptosis and autophagy are highly conserved and tightly regulated processes. Apart from their physiological role in the maintenance of cellular homeostasis, apoptosis and autophagy serve as main targets of tumor therapeutics (23-29). Whereas apoptosis is implicated in the removal of damaged or unwanted cells, autophagy is a cellular catabolic pathway that is involved in lysosomal degradation and recycling of proteins and organelles, and is therefore considered as an important survival mechanism for both normal cells and cancer cells in response to metabolic stress or chemotherapy. In hematologic malignancies, autophagy can either act as a chemoresistance mechanism or have tumor suppressive functions, depending on the context. In addition, autophagy is involved in other important aspects of blood cancers as it promotes immune competence and anticancer immunity, and may even help enhance patient tolerance to standard treatments (30).

Here, we present data demonstrating that low concentration bafilomycin A1 effectively inhibits and kills pediatric B-ALL cells. By using *in vitro*, *ex vivo* and *in vivo* models, we provide compelling evidence that bafilomycin A1 attenuates cytoprotective autophagy,
induces apoptosis, and delays the onset of leukemia in a xenograft mouse model and inhibits and kills leukemic primary cells. An in vivo toxicity assay confirmed that bafilomycin A1 is safe. These data validate bafilomycin A1 as a candidate novel therapeutic drug for pediatric B-ALL.

Methods

Major reagent. Bafilomycin A1 from Sigma-Aldrich (St. Louis, MO, USA) was used at a concentration of 1 nM unless indicated with different doses.

Cell lines. Leukemia cell lines RS4;11, NB4, HL-60, K562 and BV173 were purchased from the ATCC (Manassas, VA, USA). Leukemia cell lines 697 and Nalm-6 were from DSMZ, Braunschweig, Germany. The leukemia cells were grown in RPMI 1640 medium (Hyclone, USA) with 10% fetal bovine serum (Gibco, USA) in 37°C, 5% CO₂ incubator. Experimental cultures were initiated by reculturing exponentially growing cells at a density of 0.2 × 10⁶ cells/ml and sampled at the indicated times for different analyses as described. Viability of leukemia cells collected from the medium was determined by counting total and trypan blue cells under a microscope.

Patient samples. Primary samples from leukemia patients either cytogenetically identified for myeloid leukemia cells or sorted against CD19⁺ for B-ALL cells were obtained with informed consent according to institutional guidelines and were cultured in triplicate in flat-bottomed 24-well plates at 37°C with 5% CO₂ at a density of 2 x 10⁶ cells/ml, using IMDM medium supplemented with 20% BIT, 55 mM β-mercaptopethanol and 1% glutamine.

Animals and B-ALL xenograft model. Male and female mice were used equally in all experiments and littermates were used as controls. The 697 B-ALL cells were injected at 5x10⁶ cells/animal into 6- to 8-week-old male NOD-SCID mice or C57/BL/6J control mice. Cells were allowed to proliferate in vivo for 6 days and then the transplanted mice were injected intraperitoneally (i.p.) with PBS or bafilomycin A1 (0.1 mg/kg or 1 mg/kg). Mice were killed on days 30 after starting the treatment. Peripheral blood, bone marrow, livers and spleens were analyzed for the presence of leukemic cells by flow cytometry. Engraftment was detected by flow cytometry using antibodies recognizing E2A/PBX1 (BD, USA). Liver and spleen cells were collected for analysis. All experiments with animals are complied with the institutional protocols on animal welfares and approved by the Ethics Committee of Soochow University.
**ImageStream analysis.** B-ALL cells (5×10^6) of different treatment groups were collected with an ImageStreamX MarkII (Amnis, Seattle, USA). Samples were visualized and analyzed for the expression of marker proteins with IDEAS 5.0 software (Amnis, Seattle, USA). Cells were gated for single cells with the area and aspect ratio features or, for focused cells, with the Gradient RMS feature. These subsets were plotted for log intensities of channel with LC3. LC3 spot count was analyzed by the spot count wizard. Autophagy levels were calculated by measuring cell percentage with LC3^{high} spots cells.

Cell proliferation assay, small RNA interference, fluorescence microscopy, analysis of mitochondrial membrane potential, analysis of intracellular free Ca^{2+} concentration, measurement of intracellular pH, blood routine examination were described in the online supplementary information.

**Statistical analysis.** Results are shown as mean ±SD of at least three independent experiments. For comparison between groups, the Student’s t test was used, with a p value less than 0.05 considered significant.

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**Results**

**Bafilomycin A1 preferentially inhibits in vitro growth of pediatric B-ALL cells**

Bafilomycin A1 between 0.1 and 1 μM is often used to inhibit autophagic flux (21,22,32). While studying autophagy in leukemia cells, we unexpectedly observed an unusual sensitivity of a certain type of leukemia cells to bafilomycin A1, which prompted us to investigate the effect of bafilomycin A1 on various leukemia cell lines including 697, Nalm-6, RS4;11, NB4, HL-60, K562 and BV173 representing B-ALL (697, Nalm-6, RS4;11), acute myeloid leukemia (AML: NB4, HL-60), and chronic myeloid leukemia (CML: K562, BV173), respectively. The cells were cultured in the presence of increasing concentrations of bafilomycin A1 (0 nM, 0.5 nM, 1 nM). Cell proliferation was measured by using an MTT assay. The results showed that various concentrations of bafilomycin A1 profoundly inhibited the growth of three pediatric B-ALL cells in culture (Fig. 1A). A flow cytometric assay also revealed that bafilomycin A1 effectively inhibited cell division of the three pediatric B-ALL cell lines (Fig. 1B). In contrast, AML and CML cell lines were virtually insensitive to the action of this growth inhibitory agent (Fig. 1A,B; Fig. S 1A,B).

The 697, Nalm-6 and RS4;11 cell lines are derived from pediatric B-ALL patients with distinct oncogenic chromosome translocations. Although the remission rate of these types of
B-ALL is high, it is often associated with CNS relapse. To understand the mechanisms by which bafilomycin A1 at low concentration (1 nM) inhibits the growth of pediatric B-ALL cells, we performed flow cytometric analysis of propidium iodide (PI)-stained samples of the B-ALL cell lines cultured for 72 hours in the presence of bafilomycin A1. Bafilomycin A1 increased the percentage of B-ALL 697 and Nalm-6 cells in the G0/G1 phase of the cell cycle with a concomitant decrease in cells in S and G2/M phases (Fig. 1C, Fig. S 1C). To elucidate how bafilomycin A1 slows down the cell cycle progression, we examined the protein levels of critical components of cell cycle regulation in the B-ALL cells. The time course of bafilomycin A1 treatment revealed that p21, but not p27, was increased in the 697 cells (became obvious at 24 h treatment) and Nalm-6 cells (became obvious at 48 h treatment) (Fig. 1D, Fig. S 1D). Cyclin D3 in the 697 cells and cyclin D1, D3 in Nalm-6 cells were decreased at 72 h of bafilomycin A1 treatment (Fig. 1D, Fig. S 1D). Cyclin E2 was decreased at late stage of bafilomycin A1 treatment in both the 697 cells and Nalm-6 cells, but the irregular change in cyclin E2 level in the early stage of culture and drug treatment of the 697 cells remains an open question (Fig. 1D, Fig. S 1D). Bafilomycin A1 treatment also decreased both total Rb and phosphorylated Rb in the 697 cells and Nalm-6 cells (Fig. 1D, Fig. S 1D).

Although discrepancy exists in the dynamic changes of the cell cycle regulators in different B-ALL cell lines, the overall pattern appears to be similar that low concentration bafilomycin A1 upregulates the protein levels of certain cell cycle-dependent kinase (CDK) inhibitors, but downregulates the protein levels of certain cyclin members.

Notably, the discrepancy between the percentage of G0/G1 cells and the CFSE flow cytometry data or cell cycle profile in response to bafilomycin A1 treatment suggest that, in addition to inhibiting proliferation, bafilomycin A1 also causes cell death. Therefore, the growth arrest may also result from fewer cells in the S phase after bafilomycin A1 treatment. Nevertheless, the above data suggest that bafilomycin A1 may induce cell cycle arrest and inhibit proliferation of pediatric B-ALL cells, at least in part, by upregulating negative regulators and downregulating positive regulators of the cell cycle.

**Bafilomycin A1 at low concentration effectively inhibits autophagy of pediatric B-ALL cells via multiple targets**

To confirm that bafilomycin A1 targets acidic vesicle lysosomes in pediatric B-ALL cells, we analyzed the long-term effects of bafilomycin A1 on the pH of intracellular vesicles in B-ALL cell lines. LysoSensor staining showed that 1 nM bafilomycin A1 induced alkalization of intracellular acidic compartments in 697 cells (Fig. 2A), suggesting that
bafilomycin A1 induces early alterations in the lysosomal compartment consisting of lysosomal pH alkalinization and lysosomal membrane permeabilization. The above results suggest that 1 nM bafilomycin A1 is sufficient to compromise cytoprotective autophagy by targeting lysosomes, leading to an elevated cell mortality of the B-ALL cells. Thus, inhibiting autophagy may be one of the mechanisms by which bafilomycin A1 at low concentration functions in the B-ALL cells.

To test whether 1 nM bafilomycin A1 was able to block the fusion between autophagosomes and lysosomes, we treated 697 B-ALL cells with this concentration of the drug. An autophagic flux assay, which detects whether processed LC3-II accumulates in response to autophagy inhibition at the lysosomal degradation phase, is a reliable methodology for detecting a functional or complete autophagy process. Western blotting showed that conversion of LC3-II from LC3-I increased upon bafilomycin A1 treatment, suggesting that bafilomycin A1 blocks the basal autophagic flux (Fig. 2B). Confocal imaging further showed that, in both nutrient-rich medium (Ctrl) and nutrient-depleted medium (HBSS), 1 nM bafilomycin A1 caused enhanced puncta of GFP-LC3 (Fig. 2C), reflecting accumulation of LC3 in acidic vesicular organelles due to a blockade of lysosomal degradation. The confocal data, however, did not tell us to what extent autophagic degradation of LC3-II is inhibited or whether autophagy is effectively inhibited at the stage of the fusion between autophagosomes and lysosomes or at the stage of lysosomal degradation.

Imaging flow cytometry is a useful tool that provides both fluorescence images indicating autophagic markers and numerical data revealing the accurate degree of the autophagic markers, thereby making it possible to statistically measure autophagy activation or inhibition. To this end, we analyzed the formation of GFP-LC3 puncta, a marker reflecting autophagy inhibition at its late stage, with the ImageStreamX Mark II imaging flow cytometer (Amnis, Seattle, USA). The results demonstrated that while the image data was comparable to that obtained from the confocal analysis, the statistical data indicated that GFP-LC3 puncta were increased by only about 30% by bafilomycin A1 treatment in both nutrient-rich (regular medium) and nutrient-poor (HBSS medium) conditions (Fig. 2D), suggesting that 1 nM bafilomycin A1 caused partial inhibition of autophagy at the late stage of autolysosome formation, and that there may be other mechanisms involved in inhibiting and/or killing the leukemia cells.

Interestingly, examination of the mammalian target of rapamycin (mTOR) signaling cascade revealed that, 1 nM bafilomycin A1 upregulated mTOR signaling starting at or about 6 hours of the treatment, manifested by an increase in the phosphorylation levels of mTOR and its upstream regulator Akt and downstream effectors, p70S6K and 4EBP1 in B-ALL 697
cells (Fig. 2E). These results suggest that bafilomycin A1 may largely inhibit autophagy by activating mTOR signaling in pediatric B-ALL cells.

Formation of the Beclin 1-Vps34 complex is a critical step in initiating autophagy activation, which is normally inhibited by mTOR, a gatekeeper in regulating autophagy (31). Consistent with the mTOR activation seen with bafilomycin A1 of low concentration (Fig. 2E), a co-immunoprecipitation assay disclosed that bafilomycin A1 treatment at low concentration inhibited the formation of the Beclin 1–Vps34 complex (Fig. 2F). Therefore, activation of mTOR signaling may be the main mechanism by which bafilomycin A1 inhibits autophagy. This result explains, at least in part, why bafilomycin A1 of low dose (1 nM) effectively inhibits the B-ALL cells.

Furthermore, bafilomycin A1 treatment resulted in an increased formation of the Beclin 1–Bcl-2 complex (Fig. 2F). Bcl-2 is an apoptosis inhibitor and formation of a Beclin 1–Bcl-2 complex attenuates inhibition of apoptosis by Bcl-2 (32). The association between disassembly of the Beclin 1–Vps34 complex and formation of the Beclin 1–Bcl-2 complex suggests that bafilomycin A1 treatment may simultaneously cause autophagy inhibition and apoptosis activation by possibly removing Beclin 1 from autophagy machinery and also removing Bcl-2 from anti-apoptosis regulatory machinery, both of which may deteriorate the cytoprotective autophagy and activate the apoptotic cascade.

The activation of mTOR signaling and Beclin1 association with Bcl-2 but disassociation with Vps34 by 1 nM bafilomycin A1 were also seen in Nalm-6 cell line (Fig. S 1E,F). Hence, unlike bafilomycin A1 of high concentration (0.1–1 μM), which solely targets V-ATPase and the late phase of autophagy in other cell types (33), bafilomycin A1 of low concentration may inhibit the autophagy pathway at multiple targets in pediatric B-ALL cells. It activated mTOR signaling and effectively disrupted the formation of the Beclin 1–Vps34 complex to possibly inhibit the induction of autophagy activation; it also partially inhibited late-phase autophagy and targeted the lysosomal membrane potential. Furthermore, it promoted the formation of the Beclin 1–Bcl-2 complex, which may further activate or promote apoptosis of pediatric B-ALL cells.

**Bafilomycin A1 induces caspase-independent but apoptosis-inducing factor (AIF) dependent apoptosis of pediatric B-ALL cells**

To examine whether bafilomycin A1-induced formation of Beclin 1–Bcl-2 is truly associated with activation of apoptosis in B-ALL cells, we treated B-ALL 697 cells for 72
hours with bafilomycin A1 and then labeled the cells with PI and FITC-conjugated annexin V. The results of these annexin V assays showed that bafilomycin A1 induced apoptosis in the B-ALL cells (Fig. 3A). A TUNEL assay also revealed that bafilomycin A1 induced apoptosis (Fig. 3B). However, procaspase-3 and poly-(ADP-ribose) polymerase (PARP) were not cleaved in the cells treated with bafilomycin A1 over 72 hours, whereas treatment with the positive control camptothecin (Cam) resulted in caspase cleavage (Fig. 3C). Caspase inhibition through the pan-caspase inhibitor z-VAD-fmk was unable to rescue bafilomycin A1-induced apoptotic death of the leukemia cells (Fig. 3D). These data suggest that caspase-independent apoptosis was induced by bafilomycin A1.

We evaluated the effects of bafilomycin A1 treatment on mitochondrial membrane potential. The 697 B-ALL cells were treated for 72 hours with bafilomycin A1 and changes in the mitochondrial membrane potential were analyzed by flow cytometric analysis. Depolarization of the mitochondrial membrane potential was detected (Fig. 3E). We also found that apoptosis-inducing factor (AIF), which is a noncanonical proapoptotic protein, is uniformly upregulated in leukemia cells and not in normal blood cells (Fig. 3F), prompting us to investigate the role of AIF in pediatric B-ALL cells. Immunoblotting revealed that AIF relocalized from the cytoplasm to the nucleus after 72 hours of bafilomycin A1 treatment (Fig. 3G). This bafilomycin A1-induced nuclear relocalization of AIF was further seen with confocal microscopy (Fig. 3H). In the cytoplasm, AIF is exclusively localized in the mitochondria. The caspase inhibitor z-VAD failed to block the bafilomycin A1-induced nuclear relocalization of AIF from mitochondria (Fig. 3I). To examine whether AIF plays a role in the regulation of apoptosis in the B-ALL cells treated with bafilomycin A1, we silenced AIF gene in 697 cells by RNA interference and treated the cells with bafilomycin A1. Knockdown of AIF significantly attenuated bafilomycin A1-induced apoptosis (Fig. 3J). These data indicate the involvement of AIF in the execution of the apoptotic process upon bafilomycin A1 treatment.

We also observed that bafilomycin A1 downregulated cytochrome c (Fig. 3K). The downregulation of cytochrome c in response to bafilomycin A1 was confirmed by confocal microscopy (Fig. 3L). The caspase inhibitor z-VAD did not reverse the bafilomycin A1-induced depletion of cytochrome c in mitochondria but this depletion of cytochrome c was reversed by the ubiquitination inhibitor MG132, suggesting that bafilomycin A1 induces degradation of cytochrome c in mitochondria and that this degradation is sensitive to ubiquitination inhibition (Fig. 3M). Normally, release of cytochrome c from mitochondria to the cytoplasm is a critical signal for the activation of apoptosis (34), but we did not detect release of cytochrome c to the cytoplasm, even when proteasomal degradation was blocked.
with MG132 (Fig. 3M). Similar results on activating non-classical apoptosis by 1 nM bafilomycin A1 was also found in Nalm-6 cells (Fig. S 2A). Therefore, our above data suggest that bafilomycin A1 targets the mitochondrial membrane to trigger apoptosis via the AIF pathway in a unique manner in pediatric B-ALL cells.

**Bafilomycin A1 extends the survival and improves the pathology of xenograft mice by targeting leukemia cells**

The *in vivo* effect of bafilomycin A1 on solid tumor growth has previously been evaluated in xenografted pancreatic tumors. Bafilomycin A1 (1 mg/kg) did not inhibit tumor growth until tumors reached 300 mm$^3$(15). Another study suggests that bafilomycin A1 (1 mg/kg) can inhibit the growth of large tumors subjected to hypoxia more effectively than small tumors (20). Bafilomycin A1 is thus considered a useful therapeutic agent for large solid tumors.

Leukemia, however, is a nonsolid tumor, and circulating leukemia cells are not confronted with hypoxia. To study the effect of bafilomycin A1 *in vivo*, we examined the ability of the compound to suppress and kill B-ALL cells in the mouse model of pediatric B-ALL. We transplanted 697 B-ALL cells into NOD-SCID mice and allowed the leukemic cells to proliferate for 6 days while the animals manifested signs of advanced disease, including enlarged lymph nodes, hepatosplenomegaly, and elevated levels of white blood cells. Then, 0.1 mg/kg or 1 mg/kg bafilomycin A1 was administered daily for 3 days. Bafilomycin A1 extended survival in the bafilomycin A1-treated B-ALL mice with advanced disease compared with control mice, as shown in a Kaplan-Meier curve (Fig. 4A). The average time from diagnosis to death was 30.9 days for the vehicle-treated disease control mice (range 20-39 days; n=20) vs. 37.2 days for 0.1 mg/kg bafilomycin-treated mice (range 30-46 day; n=15, p<0.001). More significantly, 1 mg/kg bafilomycin A1 increased survival with a median survival of 42.5 days (range 30-48 days; n=15, p<0.001). (Fig. 4A). Animal weights, monitored once a week over four weeks, displayed no difference between the treated and control groups (Fig. S 2B). Peripheral blood counts were conducted at sacrifice on post-transplantation day 30. In the disease model group, white blood cells and lymphocytes remarkably increased, whereas these two types of cells in the bafilomycin A1-treated group were significantly normalized within 30 days of initiating therapy (Fig. 4B). However, by the time of death, the mean counts of white blood cells and lymphocytes of the bafilomycin A1-treated mice were above normal (the control group). The red blood cell, hemoglobin, and particularly platelet counts in the B-ALL model group reduced significantly, whereas the red
blood cell, hemoglobin, and platelet counts in the bafilomycin A1-treated group were significantly normalized (Fig. 4B). These data suggest that bafilomycin A1 was well tolerated in these mice. Bafilomycin A1 treatment reduced tumor burden without causing significant anemia or thrombocytopenia.

In the disease model group, the mice had seriously hepatosplenomegaly compared with the control group. However, the size and weight of livers and spleens in the bafilomycin A1-treated group were significantly normalized compared with the disease model group (Fig. 4C). The livers from the disease model group mice were significantly infiltrated. In contrast, considerably fewer ALL cells showed infiltration in the livers of bafilomycin A1-treated mice compared with the model group mice (Fig. 4D). In contrast, there was no infiltration in the spleens from the mice of the model group (Fig. S 2C).

In mice sacrificed on day 30, flow cytometric analysis of bone marrow cells and peripheral blood cells from the four groups of mice, using human E2A/PBX1 antibodies, showed that bafilomycin A1 treatment significantly reduced the number of E2A/PBX1-positive leukemia cells in the bone marrow (Fig. 4E) and eliminated virtually all circulating E2A/PBX1-positive leukemia cells compared with the model group and normal mouse control (Fig. 4F).

These data suggest that bafilomycin A1 dramatically inhibits pediatric B-ALL cell engraftment by targeting leukemia cells in vivo while sparing normal cells.

**Bafilomycin A1 inhibits the primary cells from B-ALL patients**

Because bafilomycin A1 exhibited a clear cytotoxic effect on in vitro pediatric B-ALL cells and in the pediatric B-ALL xenograft mouse model, we next investigated whether such cytotoxicity could also be observed ex vivo in bone marrow-derived leukemic cells directly isolated from patients with different kinds of leukemia. Bone marrow cells were either cytogenetically identified for myeloid leukemia cells or stained and sorted with antibodies against CD19 for B-ALL cells. The resultant cells were set up in suspension culture to evaluate proliferative ability in the presence of bafilomycin A1 (1 nM or 10 nM). In line with the results from the in vitro system and animal model, where profound inhibition of the growth of the B-ALL cells was shown (Figs. 1,4), significant growth inhibition on B-ALL primary cells was found after 72 hours of treatment with 1 nM bafilomycin A1. Specifically, low concentration bafilomycin A1 induced a clear cytotoxicity in B-ALL primary cells (n=12) compared with untreated controls. Conversely, bafilomycin A1 had no toxic effect on bone marrow cells isolated from AML patients (n=3), CML patients (n=3), CLL patients (n=3) and
healthy subjects (n=3) (Fig. 5A).

To determine if the drug really kills the human primary leukemic cells, we analyzed the primary cells from patient samples (sorted against CD19+) with Annexin V-FITC/PI. The flow cytometric result shows that bafilomycin A1 of 1 nM caused apoptotic death in the pediatric B-ALL primary cells at 72 h treatment, confirming that low concentration bafilomycin A1 truly kills pediatric B-ALL cells (Fig. 5B).

**In vivo toxicity evaluation reveals bafilomycin A1 to be a potent yet safe compound**

Treatment-related toxicities are a major issue in the approach to patients with hematologic cancers as they significantly affect patient quality of life. To determine the single-dose maximum tolerated dose (MTD), bafilomycin A1 was injected into C57BL/6 mice by daily administration through i.p. injection for 3 days at doses ranging from 0.1 to 25 mg/kg. After injection, general appearance and body weight were monitored for 3 weeks. At 25 mg/kg, the mice experienced weight loss of up to 30% within 12 days of injection, but at 10 mg/kg, the mice did not undergo weight loss during the same period of treatment (Fig. 6A). Peripheral complete blood counts (Fig. 6B) and the organ coefficients (Fig. 6C) of mice treated with different doses of bafilomycin A1 were not significantly different. At 25 mg/kg, the liver had a little enlargement, but at 10 mg/kg, the liver maintained a normal size (Fig. 6C). Analysis of serum for AST/ALT (Fig. 6D) and histological examination of the liver (Fig. 6E) did not reveal signs of tissue damage or liver toxicity in the 10 mg/kg bafilomycin A1-injected mice compared with controls. At 25 mg/kg, however, the AST/ALT was increased and the liver showed elevated transaminases, indicative of liver damage. Thus, doses of bafilomycin A1 up to 10 mg/kg were well tolerated with no detectable toxicity. However, 25 mg/kg was toxic. Accordingly, the MTD of bafilomycin A1 in mice was determined to be 10 mg/kg.

**Discussion**

Bafilomycin A1 is a specific inhibitor of the vacuolar type H⁺-ATPase in cells and suppresses the acidosis of organelles containing this enzyme (14,15,33). It has frequently been used as an inhibitor at high doses (0.1–1 \( \mu \)M) to block the fusion between autophagosomes and lysosomes, or inhibiting lysosomal activity, a step critical to late-stage autophagy (21,22). Our present study indicated that bafilomycin A1 at low concentration (1 nM) effectively and specifically inactivates autophagy and activates apoptosis at multiple targets in pediatric...
Manipulating autophagy pharmacologically has been reported to enhance the activity of anticancer agents. We previously found that autophagy is essential for vitamin D3-induced differentiation of myeloid leukemia cells (32). Other researchers showed that, in addition to apoptosis, arsenic trioxide induces autophagy in the human T-cell lymphocytic leukemia cell line Molt4 (35-36). Indeed, the downregulation of the PML/RARa oncoprotein is mainly affected by autophagy, and its degradation in cells treated with arsenic trioxide or all-trans retinoic acid depends upon and is related to increased autophagic activity. Autophagy also induces the differentiation of NB4 acute promyelocytic cells, suggesting that this degradation pathway potentiates therapy-induced differentiation of acute promyelocytic cells (37). RAD001 induced G0/G1 phase cell cycle arrest, modulated the PI3K/Akt/mTOR pathway, and caused apoptosis and autophagy in a dose-dependent manner. Moreover, dual treatment of RAD001 with an Akt inhibitor or mTOR inhibitor displayed a dramatic synergistic effect against leukemic cells (38). Unlike the mechanism we revealed in this study, the above antileukemic effects were largely attributable to the activation of autophagy, which either non-specifically overdigests intracellular components leading to cellular demise or specifically digests oncogenic fusion proteins to promote terminal differentiation of leukemia cells.

One of the current strategies in the treatment of leukemia is to identify new autophagy inhibitors that could have better pharmacological properties, better tolerance, and improved activity. Imatinib and its improved compounds dasatinib or nilotinib are tyrosine kinase inhibitors (TKIs) that are currently used in CML treatment (39). These compounds stimulate autophagy in CML cells, and the activated autophagy causes increased chemoresistance, possibly through inhibition of the PI3K-AKT-mTORC1 axis (40). BCR-ABL signaling in leukemia cells leads to activation of the PI3K/AKT pathway and mTOR. In line with this, it has been demonstrated that BCR-ABL–expressing cells have low basal levels of autophagy but are highly dependent on this process (41). Inhibition of BCR-ABL by TKIs has now been shown to not only induce apoptosis, but also autophagy (42). Therefore, inhibition of autophagy may improve the antileukemic effect of TKIs. Pharmacological inhibition of autophagy often occurs in the late stage of autophagy. For instance, clarithromycin, at a very high dose, increases TKI-induced cell death in CML cells (43,44). Although the mechanism by which this drug inhibits autophagy remains unclear, autophagy inhibitors, combined with TKIs, could be used to improve clinical outcomes in CML and myeloma.

Targeting apoptosis has been the overwhelming focus of methods aimed at killing leukemia cells. Mer tyrosine kinase is aberrantly expressed in 30% of pediatric B-ALL patients,
including most patients with an E2A-PBX1 translocation. Mer inhibition induced apoptotic cell death and chemosensitization and prolonged survival in a xenograft model (45). SYK is a master regulator of anti-apoptotic signaling pathways in B-lineage leukemia cells. Inhibitors designed to target SYK may overcome the resistance of malignant B-lineage lymphoid cells to apoptosis and thereby provide the foundation for more effective multimodal treatment regimens for poor prognosis B-precursor ALL. C61, a liposomal nanoparticle formulation of a SYK substrate-binding site inhibitor, has been developed as a nanomedicine candidate against poor prognosis and relapsed B-cell ALL by inducing apoptosis in radiation-resistant primary leukemic cells (46,47).

In the present study, bafilomycin A1 at low concentration not only inhibited late-stage autophagy, specifically fusion between autophagosomes and lysosomes, but also inhibited autophagy at its early signaling by activating mTOR and attenuating the formation of the Beclin 1-Vps34 complex, a critical event needed for autophagy induction (Fig. 2). Inhibition of autophagy can cause apoptosis (48). Thus, identifying the mechanisms in the regulation of the crosstalk between apoptosis activation and autophagy inhibition is an essential step for the development of optimal chemotherapeutic approaches for leukemia treatment. In addition to inhibiting autophagy, we found that bafilomycin A1 activated caspase-independent but AIF-dependent apoptosis of the B-ALL cells (Fig. 3). Notably, bafilomycin A1 also promoted the formation of the Beclin 1–Bcl-2 complex (Fig. 2), which in turn may compete for Beclin 1 away from the autophagy machinery and also remove Bcl-2 from the anti-apoptotic machinery, ultimately leading to inhibited autophagy and activated apoptosis. We previously reported that Beclin 1 bridges autophagy and apoptosis (49). A link between proteins from the autophagy apparatus and apoptotic cascades has further been illustrated by Rubinstein and colleagues, identifying Atg12 as a positive mediator of mitochondrial apoptosis by showing that this protein binds and inactivates the anti-apoptotic Bcl-2 family members Bcl-2 and Mcl-1 (50).

In summary, bafilomycin A1 exhibits highly sensitive toxicity for pediatric B-ALL cells while sparing normal hematopoietic cells treated with the same doses. Unlike other drugs, bafilomycin A1 inhibits and kills pediatric B-ALL cells at multiple targets. It not only targets early autophagy signaling, but also targets H⁺-ATPase of lysosomes and blocks late-phase autophagy activity. Furthermore, it targets mitochondria to activate the AIF-dependent apoptotic cell death pathway and promotes Beclin 1 binding to Bcl-2, further decreasing cytoprotective autophagy and activating apoptotic cell death. Our data thus propose that bafilomycin A1 may be developed to treat pediatric B-ALL. Identification of its direct binding target(s) in the unique autophagy and apoptosis pathways in pediatric B-ALL cells
will be a major focus of our future study into bafilomycin A1.

**Authorship:** NY and JW designed the experiments and wrote the paper. NY, LS, SZ, WL, YC, FX, YF, ZW, HZ, XL, ZW, JC, JW, YZ, XM, WZ, SH and SC conducted the experiments.

**Disclosure of Conflict of Interest:** The authors declare no conflicts of interest.

Supplementary information is available at Haematologica's website.

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A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct Funct* 1998; 23(1):33-42.


**Figure legends**

**Figure 1. Bafilomycin-A1 at low concentration preferentially inhibits the growth of pediatric B-ALL cells.**

(A) Effect of bafilomycin A1 on the growth of various leukemia cell lines measured by MTT assay. Bafilomycin A1 of low dose preferentially inhibits B-ALL cells over 96 hours of bafilomycin A1 treatment with concentration indicated. (B) Effect of bafilomycin A1 on the proliferation measured by CFSE assay. Cell division of various leukemia cell lines was measured by labeling with CFSE. The cells were cultured for 72 hours with or without bafilomycin A1. (C) Effect of bafilomycin A1 on the cell cycle of B-ALL cells. The B-ALL cells were treated bafilomycin A1, and then the cell cycle was analyzed by flow cytometry after PI staining. Bafilomycin A1 increased the percentage of cells in the G0/G1 phase and decreased the percentage of cells in the S and G2/M phase of the cell cycle. The representative histogram data and the percentage of cells in each stage of the cell cycle after 72h of treatment are shown. (D) Effect of bafilomycin A1 on cell cycle regulators measured by western blotting analysis.

**Figure 2. Bafilomycin A1 at low concentration inhibits the cytoprotective autophagy of pediatric B- ALL cells**

(A) Bafilomycin A1 induced the elevation of \([\text{Ca}^{2+}]_i\). The pre-B ALL 697 cells were loaded with 5 mM Fluo-4 AM dye for a total of 30 min before analysis by flow cytometry (left). The variation of intra-vesicular pH and the status of lysosomal permeability were measured by flow cytometric analysis of bafilomycin A1-treated B-ALL cells stained with LysoSensor.
Green DND-189 and Lysotracker or acridine orange as described in Methods (right). (B) Western blotting for the conversion of LC3-II from LC3-I for the 697 cells treated with or without 1 nM bafilomycin A1. (C) Confocal microscopic analysis of autophagy activity. A GFP-LC3 stable expressing transfectant 693 cell line was generated. The cells were treated with or without bafilomycin A1 for 72 hours. The nucleus was stained with Hoechst 33258. (D) Image Flow cytometric analysis of autophagy activity. The GFP-LC3 697 cells were treated as in (C) and the green GFP-LC3 puncta was measured by Amnis ImageStreamX Mark ll and the data were analyzed with Amnis IDEAS 4.0 software. Original magnification×600 (left) and the quantitative data (right). (E) Western blotting analysis for mTOR and its upstream regulator Akt and downstream targets, p70S6K and 4EBP1. The B-ALL cells were treated with or without bafilomycin A1 for a time course specified. The numbers below the blots indicate relative intensity of phosphorylation-specific bands for mTOR, AKT, p70S6K and 4E-BP1 relative to total mTOR, AKT, p70S6K or 4E-BP1. (F) Co-immunoprecipitation of Vps34 or Bcl-2 with Beclin1 as a bait after the B-ALL cells were treated with or without bafilomycin A1 under regular or starvation (HBSS) medium condition.

Figure 3. Bafilomycin A1 induces caspase-independent but AIF-dependent apoptosis of pediatric B-ALL cells.

The 697 cells were cultured with or without bafilomycin A1 of 1 nM for 72 hours. (A) Left, a representative flow cytometric plot for Annexin V-FITC/PI stained 697 cells. Right, statistical results on apoptotic level of the 697 cells. Apoptosis was defined as the percentage of Annexin V–positive cells. The results are the mean ± SD of three different experiments. Asterisks indicate significant differences (p<0.05) in comparison with control. (B) Left, a representative flow cytometric analysis of TUNEL. Right, the statistical result of bafilomycin A1-induced apoptosis of the 697 cells. (C) Western blotting analysis for caspase-3 and PARP cleavage in the 697 cells, and camptothecin (CAM) is a positive control. (D) Effects of pan-caspase inhibitor z-VAD on apoptosis of the 697 cells induced by bafilomycin A1. (E) A representative flow cytometric analysis of the mitochondrial membrane potential by using JC-1 staining on 697 cells at 72h after treatment with bafilomycin A1. (F) Western blotting analysis of total cellular AIF in various leukemia cells indicated. (G) Western blotting analysis of the AIF in the cytoplasm and in the nucleus of the 697 cells treated with or without bafilomycin A1. (H) Confocal microscopic observation of bafilomycin A1-induced AIF subcellular localization. The nucleus was stained with Hoechst 33258 (blue) and AIF by antibody (red). AIF was observed a relocalization from cytoplasmic compartments to the nucleus upon bafilomycin A1 treatment. (I) Western blotting analysis of the AIF localization in response to bafilomycin A1 and/or z-VAD treatment. (J) AIF silencing by siRNAs in the
697 cells. Left, Quantitative real time PCR of AIF mRNA expression (top) and Western blotting analysis of AIF expression (bottom); right, apoptosis was analyzed after the 697 cells transfected with AIF siRNA and cultured with or without bafilomycin A1 for 72 hours. (K) Western blotting analysis of the total cytochrome c in response to bafilomycin A1 over 72 h treatment. (L) Confocal microscopic observation of cytochrome C subcellular localization. The mitochondria were stained by MitoTracker (green) and cytochrome c by antibody (red). Co-localization is shown in merged images. (M) The bafilomycin A1-induced decrease of cytochrome c in the mitochondria was reversed by the MG132 treatment.

**Figure 4. Bafilomycin A1 specifically inhibits pediatric B-ALL cell engraftment in NOD/SCID mice.**

NOD/SCID mice, injected with 5×10⁶ B-ALL 697 ALL cells, were treated with vehicle or bafilomycin A1 (0.1 mg/kg or 1 mg/kg) starting day 6 after the injection of the B-ALL cells. n=15/group. C, untreated normal mouse control; M, model mice engrafted with the 697 cells, treated with vehicle; T1, treatment1, mice engrafted with the 697 cells and treated with bafilomycin A1 (0.1 mg/kg); T2, treatment2, mice engrafted with the 697 cells and treated with bafilomycin A1 (1 mg/kg). (A) Effect of bafilomycin A1 treatment on body weight. (B) Analysis at day 30 for the indicated blood cells, as measured by complete blood count. WBC, white blood cell; LYM, lymphocytes; LYM%, the percentage of lymphocytes; RBC, red blood cells; HGB, hemoglobin; PLT, platelets. (C) Left, representative photographs of livers and spleens recovered from mice engrafted with the 697 cells after the indicated days of treatment with bafilomycin A1 or vehicle; right, the statistical result on liver coefficient (the ratio of the weight of liver to total body weight) and spleen coefficient (the ratio of the weight of spleen to total body weight). (D) Representative hematoxylin and eosin–stained sections from liver of the mice engrafted with the 697 B-ALL cells 30 days after the completion of treatment with bafilomycin A1 or vehicle. Regions of leukemic infiltration are indicated by arrows. (E) Flow cytometric analysis detecting the human ALL cells (CD19/GFP) in the BM of mice engrafted with the 697 B-ALL cells. Left, a representative histogram results; right, the statistical percentage of pediatric B-ALL cells in the BM of the mouse disease model. (F) Flow cytometric analysis detecting the B-ALL cells (CD19-APC/GFP-FITC) in the peripheral blood of mice engrafted with 697 B-ALL cells. Left, a representative histogram results; right, the statistical percentage of pediatric B-ALL cells in the peripheral blood of the mouse disease model.

**Figure 5. Bafilomycin A1 preferentially inhibits pediatric B-ALL primary cells.**
Bone marrow cells from pediatric leukemia patients were either cytogenetically identified for myeloid leukemia cells or sorted against CD19 for B-ALL cells. The resultant cells were treated with 1 nM bafilomycin A1 for 72 h, followed by cell counting. (A) Cell viability of patient BM cells (sorted against CD19+ cells) treated with 1 nM bafilomycin A1 for 72 h. (B) Flow cytometric analysis of Annexin V-FITC/PI stained patient BM cells (sorted against CD19+ cells) treated with 1 nM bafilomycin A1. Apoptosis was defined as the percentage of Annexin V–positive cells. The results are the mean ± s.d. of three independent experiments. Asterisks indicate significant differences (p<0.05) in comparison with control.

**Figure 6. In vivo toxicity evaluation of bafilomycin A1 in mice.**

C57BL6J mice (n = 6 per group) were treated with bafilomycin A1, i.p. at doses between 0.1 and 25 mg/kg. (A) Weight changes of the mice in response to received doses of bafilomycin A1 as indicated. (B) Effect of bafilomycin A1 on the peripheral blood count of mice. WBC, white blood cell; LYM, lymphocytes; LYM%, the percentage of lymphocytes; RBC, red blood cells; HGB, hemoglobin; PLT, platelets. (C) Effect of bafilomycin A1 on organs weight after treatment with different doses of bafilomycin A1. (D) The aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio after treatment with different doses of bafilomycin A1 to indicate the extent of liver injury. (E) H&E staining of liver injuries after treatment with different doses of bafilomycin A1.
Bafilomycin A1 targets both autophagy and apoptosis pathways in pediatric B-cell acute lymphoblastic leukemia

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Supplementary Methods

**Cell proliferation assay.** Cells were collected and resuspended in serum-free 1640 medium at a density of 4×10^6 cells/mL and mixed gently immediately after adding the dye [5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Sigma-Aldrich)] into the suspension to a final working concentration of 3 μM, then incubated the mixture in the dark for 15 minutes at 37°C, after which 5 fold volume of cold 1640 medium containing 10% FBS was added to terminate reactions. The resultant cells were washed twice with culture medium. Finally, cell suspension (stained with CFSE) was dispensed into 24 well plate and added different drugs into corresponding wells. Cells were harvested at appropriate time, washed twice and resuspended with PBS, followed by analysis on a BD Calibur flow cytometer. Cell group not treated with CFSE served as a negative control.

**Cell cycle analysis.** To analyze cell-cycle status, cells were pelleted by centrifugation for 5 minutes at 200 g, washed in PBS, and resuspended in 70% ice-cold ethanol. Cells were fixed overnight and then stained with propidium iodide. The pretreated cells were analyzed using a BD Calibur Flow Cytometer.

**Western blotting, co-immunoprecipitation and antibodies.** Primary and secondary antibodies were provided by Cell Signaling Technology (Danvers, MA, USA). Signals were detected with the ECL Plus reagent by Perkin Elmer (Boston, MA, USA). Cell was collected and resuspended in lysis buffer containing protease inhibitor cocktail and PhosSTOP phosphatase...
inhibitor cocktail (Roche, USA). The resuspended cell pellet was vortexed for 20 seconds and then incubated on ice for 30 min and centrifuged at 13,000 g for 20 min at 4°C. The supernatants were collected and the protein concentration was determined using the BCA protein assay kit (Thermo, USA) and heated at 95 °C for 5 min. Equal amounts of protein were subjected to SDS-PAGE. Samples were loaded on a polyacrylamide gel for electrophoresis separation and transferred to a polyvinylidenedifluoride membrane (Millipore, USA). Membranes were blocked in 5% non-fat dry-milk and incubated overnight at 4°C with the primary antibodies. The primary antibodies were revealed using an appropriate horseradish peroxidase (HRP)–conjugated secondary antibody and detected by an enhanced chemiluminescence kit (Pierce, USA). For Co-immunoprecipitation, 50 μg of antibody against each bait protein was immobilized in a coupling gel, and each 1 mg of cell lysates prepared from different treatments was incubated with the antibody-immobilized coupling gel using Co-Immunoprecipitation kit (Pierce, USA) following the manufacturer’s protocol.

**Autophagic flux assay.** To induce autophagy, cells were incubated with nutrient-depleted medium (HBSS) for 12 hours. To study autophagic flux, we constructed a 697 stable transfectant leukemia cell line expressing a reporter GFP-LC3, and fluorescence images were taken using Olympus Confocal Microscope and ImageStreamX Mark II (Amnis, Seattle, USA) and analyzed with IDEAS 4.0 software by measuring total number of green dots.

**Apoptosis assay.** Cells were seeded into 24-well plates at a density of 1×10^5 cells/well. After 72 h incubation, cells were assessed by Annexin V staining using Annexin V-FITC apoptosis detection kit (BD Bioscience, USA) followed the manufacturer’s instructions and analyzed by flow cytometry using a BD Calibur Flow Cytometer and BD Cell Quest program software (Becton Dickinson, USA).

**Small RNA Interference.** Expression of AIF in leukemia cell lines was transiently silenced using small interfering RNA (siRNA) of AIF. The sense and antisense strands of AIF-1 siRNA (5-AUGCAGAACUCCAAGCAGTT-3 and 5- CGUGCUUGGAGUUCUGCAUTT-3), AIF-2 (5-GAUCUCUCCCCGAAUACCUCTT -3 and 5- GAGGUAUUCGGGGAGGAUCTT-3), the negative control siRNA (5-UUCUCCGAACGUGUCAGCUTT-3 and 5-ACGUGACACGUUCGGAGAATT-3) were synthesized by GenePharma company (Shanghai, China). Briefly, cells were transfected with 100 nM siRNA for 48 hours using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After siRNA transfection, AIF mRNA levels were detected by Q-PCR and AIF protein expression levels were detected by western blotting.

**Fluorescence microscopy.** The B-ALL cells were pelleted by centrifugation for 5 minutes at
200 g, washed in PBS, fixed in 4% paraformaldehyde at RT for 10 min. After rinse three times in PBS for 5 min each time, the cells were blocked with 5% goat serum in PBS (with 0.3% Triton X-100) for 60 minutes. The cells were incubated first with the primary antibody (diluted in PBS supplemented with 0.3% Triton X-100 and 1% bovine serum albumin, overnight in a humid chamber at 4°C) and then with FITC-conjugated secondary antibodies (diluted 1:1000, for 1 h at 37°C in a humid chamber). Excess of unbound antibody was removed at each step by three washes with PBS. The nuclear material was stained with 20 μg/ml Hoechst 33342 (Invitrogen, USA) at room temperature for 10 min. The cells were washed twice with PBS. The images were obtained using an Olympus confocal microscope.

**Analysis of mitochondrial membrane potential.** The status of the mitochondrial membrane potential (ΔΨ) was evaluated by using the JC-1 probe (Molecular Probes, USA). Cells were stained with 10 μmol/L JC-1 for 15 min at 37°C and then immediately transferred on ice and analyzed by flow cytometry.

**Analysis of intracellular free Ca²⁺ concentration.** Cells were cultured in six-well plates and pretreated with bafilomycin A1 (1 nM) for 72 h, Fluo-4/AM was used as an intracellular free Ca²⁺ fluorescent probe to analyze [Ca²⁺]i. Briefly, the harvested cells were incubated with Fluo-4/AM (5 mM final concentration) for 30 min at 37°C in the dark, washed with PBS, and analyzed on a BD Calibur flow cytometer. Intracellular [Ca²⁺]i levels were represented by fluorescent intensity. Fluorescent intensity was recorded by excitation at 494 nm and emission at 516 nm. The data were analyzed by Cell Quest program (Becton Dickinson, USA), and the mean fluorescence intensity was obtained by histogram statistics.

**Measurement of intracellular pH.** After the treatment with 1 nM bafilomycin A1 for 72 h, B-ALL cells were washed and incubated with 1 μM LysoSensor Green DND-189 (Life Technologies, USA) for 30 min. Cells were then harvested, pelleted, and resuspended in PBS. Cells were kept on ice and protected from light during immediate transport to the BD Calibur flow cytometer for analysis.

**Blood routine examination.** 20 μl mouse peripheral blood was added into 500 μl CPK-303A solution (37°C), and blood routine examination was performed using Sysmex KX-21N (Sysmex, USA).

**Supplementary Figures with Legends**
Figure A: HL60 and BV173 cells treated with Baf-A1 concentrations 0.5nM and 1nM over time (0h, 24h, 48h, 72h).

Figure B: Flow cytometry histograms for cell cycle distribution under Baf-A1 treatment.

Figure C: Histograms showing cell cycle distribution under Baf-A1 treatment.

Figure D: Western blots for cyclins, P21, P-Rb, and GAPDH under Baf-A1 treatment at 24h, 46h, and 72h.

Figure E: Protein expression levels of mTOR, Akt, and their phosphorylated forms at different time points (0h, 6h, 12h, 24h) under Baf-A1 treatment.

Figure F: Immunoprecipitation (IP) analysis of Beclin1 with input and IP Beclin1 under Baf-A1 treatment with or without starvation (Starv.).
Figure S1. Bafilomycin A1 preferentially inhibits pediatric B-ALL cells.

(A) Low concentration bafilomycin A1 treatment did not inhibit cell growth of AML HL-60 and CML BV173 cells as determined at 0, 24, 48 and 72 h of the treatment by MTT assay. (B) Low concentration bafilomycin A1 did not inhibit cell division of HL60 and BV173 cells as determined at 72 h of the treatment by flow cytometry with CFSE labeling. (C) Cell cycle analysis on B-ALL Nalm-6 cells. The Nalm-6 cells were treated with bafilomycin A1, and the cell cycle was analyzed by flow cytometry after PI staining. Bafilomycin A1 increased percentage of the cells in G0/G1 phase and decreased percentage of the cells in the S and G2/M phase of the cell cycle. The percentage of cells in each stage of the cell cycle after 72h of treatment as indicated is shown. Data are from three independent experiments. *: p ≤ 0.05, **: p ≤ 0.01. (D) Cell cycle regulators modulated by bafilomycin A1 in Nalm-6 cells. Nalm-6 cells were cultured with 1 nM Baf-A1, and cell cycle regulatory proteins were measured at 24, 48, and 72 h of treatment by western blotting. (E) Bafilomycin A1 activated mTOR signaling in Nalm-6 cells. The Nalm-6 cells were cultured with 1 nM Baf-A1 for the time course indicated. Akt, p-Akt, mTOR, p-mTOR and its downstream targets, p70S6K and 4EBP1, were measured by western blotting. The numbers below the blots indicate the relative intensity of phosphorylation-specific bands for mTOR, AKT, p70S6K and 4E-BP1 relative to total mTOR, AKT, p70S6K and 4E-BP1. (F) Co-immunoprecipitation of vps34 or Bcl-2 with Beclin1 as a bait of the Nalm-6 cells after either HBSS starvation or bafilomycin A1 treatment.
Figure S2. Bafilomycin A1 preferentially inhibits pediatric B-ALL cells.

(A) Bafilomycin A1 induced a caspase-independent apoptosis of Nalm-6 cells. The Nalm-6 cells were cultured with 1 nM bafilomycin A1 for 72 hours. Left, Flow cytometric analysis of Annexin V-FITC/PI stained Nalm-6 cells. Right, statistic data on bafilomycin A1-induced apoptosis level in Nalm-6 cells. Apoptosis was defined as the percentage of Annexin V–positive cells. Data are from three independent experiments. *: p≤0.05, **: p≤0.01. (B) Animal weights, monitored once a week over four weeks, displayed no difference between bafilomycin A1-treated groups and the control. (C) Hematoxylin and eosin–stained sections from spleen of the mice engrafted with B-ALL 697 cells, examined on day 30 after the completion of treatment with vehicle or bafilomycin A1.