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Tubacin suppresses proliferation and induces apoptosis of acute lymphoblastic leukemia cells

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Abstract

Over the past decade, histone deacetylase inhibitors have increasingly been used to treat various malignancies. Tubacin (*tubulin acetylation inducer*) is a small molecule that inhibits histone deacetylase 6 (HDAC6) and induces acetylation of α -tubulin. We observed a higher antiproliferative effect of tubacin in acute lymphoblastic leukemia (ALL) cells than in normal hematopoietic cells. Treatment with tubacin led to the induction of apoptotic pathways in both pre-

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B and T cell ALL cells at a 50% inhibitory concentration (IC₅₀) of low micromolar concentrations. Acetylation of α -tubulin increases within the first 30 min following treatment of ALL cells with tubacin. We also observed an accumulation of polyubiquitinated proteins and poly(ADP-ribose) polymerase (PARP) cleavage. Furthermore, the signaling pathways activated by tubacin appear to be distinct from those observed in multiple myeloma. In this article, we demonstrate that tubacin enhances the effects of chemotherapy to treat primary ALL cells *in vitro* and *in vivo*. These results suggest that targeting HDAC6 alone or in combination with chemotherapy could provide a novel approach to treat ALL.

Keywords

Tubacin; HDAC6; ALL; aggresome pathway

Introduction

Acute lymphoblastic leukemia (ALL) is characterized by the overproduction of immature lymphoid cells of the B and T cell lineage that replace normal hematopoietic stem and progenitor cells in the bone marrow [1]. ALL is the most common form of childhood cancer, accounting for 74% of leukemia cases among children [2]. The primary treatment for patients with ALL is chemotherapy, which results in both acute toxicities and late effects. Approximately 25–30% of patients relapse, with a relatively low salvage rate [3]. Therefore, the investigation of novel approaches to treat ALL is critical. In this study, we tested both pre-B and T cell ALL cells with an inhibitor of histone deacetylase 6 (HDAC6) both *in vitro* and *in vivo* [4]. HDAC6 is a class II histone deacetylase cytosolic protein with two catalytic domains [5,6]. The C-terminal domain is known to deacetylate α -tubulin at the Lys 40 ϵ -amino group, and the distal C-terminus possesses a conserved zinc finger domain that can directly interact with ubiquitin and deacetylate α -tubulin at the Lys 40 ϵ -amino group near the N-terminus [5–7]. Tubulin is the main component of microtubules, and it forms a heterodimer comprising α and β subunits [8]. HDAC6 recognizes and binds to ubiquitinated proteins and forms a complex with dynein, a motor protein that transports misfolded and ubiquitinated proteins to the aggresome with the help of microtubules [4,9]. The aggresome is an alternative pathway for the degradation of damaged proteins [4,10]. Tubacin is an HDAC-selective inhibitor that was previously identified in a multidimensional chemical genetic screen of a library comprising over 7000 diverse, HDAC-biased small molecules [11,12]. Tubacin is a competitive inhibitor and inhibits HDAC6 by chelating a Zn²⁺ cation in the catalytic pocket [4,10]. Tubacin synergistically increases the apoptotic effect of bortezomib, a proteasome inhibitor, by disrupting the molecular complexes of HDAC6 with dynein [9]. This complex inhibition led to the accumulation of misfolded proteins and to a synergistic increase in apoptosis with the proteasome inhibitor, bortezomib, in multiple myeloma cells [9]. Specific inhibitors of HDAC6 have not been previously studied with ALL cells. We hypothesized that the potential inhibition of the aggresome pathway in ALL cells would lead to the accumulation of ubiquitinated, misfolded proteins and, subsequently, apoptosis.

We also investigated other potential tubacin-mediated effects in ALL cells. Previous reports demonstrated that accumulation of α -tubulin acetylation led to inhibition of the Na^+, K^+ -ATPase pump, ion imbalance, and activation of caspases involved in apoptosis [13–16]. Na^+, K^+ -ATPase, an integral membrane protein found in the cell of all higher eukaryotes, uses the hydrolysis of adenosine triphosphate (ATP) to drive the transfer of sodium ions (Na^+) and potassium ions (K^+) across the cell membrane. Three Na^+ ions are pumped out of the cell and two K^+ ions are pumped into the cell [17,18]. This exchange of ions allows the cell to maintain ion homeostasis to help keep the required cell volume for the cell to function [8,19]. Na^+, K^+ -ATPase activity can be regulated by cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA), cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG), and protein kinase C through phosphorylation, serum, hormones [19], L-glutamate, and also by its association with acetylated tubulin [8,14,19–21]. Association of tubulin with the α subunit of integral Na^+, K^+ -ATPase [20] requires acetylation of α -tubulin at Lys 40 [22]. The increase in acetylated tubulin was reported to inhibit the Na^+, K^+ -ATPase activity [22]. Therefore, we examined whether tubacin-mediated increased acetylation of tubulin might inhibit the Na^+, K^+ -ATPase activity in ALL cells.

In this study, we showed that tubacin increases the acetylation of α -tubulin, which in turn leads to the accumulation of ubiquitinated misfolded proteins, and apoptosis associated with inhibition of the aggresome pathway. Furthermore, this increase in acetylation of α -tubulin also leads to the inhibition of Na^+, K^+ -ATPase, shown by the decrease of intracellular K^+ , calcium release, and caspase activation that can further contribute to apoptosis. We also found that tubacin enhances the effects of chemotherapy *in vitro* and *in vivo*. Therefore, our data suggest that targeting HDAC6 is a potential approach to treat ALL cells, either alone or in combination with chemotherapy.

Materials and methods

Cell lines and primary cells

Jurkat, Loucy, and REH T-ALL cell lines were obtained from the American Type Culture Collection (ATCC), and Nalm-6 pre-B ALL cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Deutsche Sammlung von Mikroorganismen und Zellkulturen). ALL cell lines were cultured in RPMI medium 1640 containing L-glutamine (Invitrogen), 10% fetal bovine serum (FBS; Omega Scientific), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Invitrogen). Normal T lymphocytes were obtained from the University of California, Los Angeles (UCLA) Core Facility.

LAX7R pre-B ALL cells from a patient with normal karyotype at relapse were passaged in NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice (Jackson Laboratories) and were provided by the University of Southern California (USC) Norris Comprehensive Cancer Center in compliance with Institutional Review Board (IRB) regulations (approval from the IRB of the USC Health Sciences Campus). For normal human bone marrow and ALL patient samples from UCLA, consents were obtained prior to obtaining bone marrow according to the Helsinki protocol.

Reagents

Tubacin and its inactive analog niltubacin were synthesized in-house. Ouabain octahydrate was obtained from Sigma, and MG-132 and suberoylanilide hydroxamic acid (SAHA) from Calbiochem.

Growth inhibition assay

The inhibitory effect of tubacin on ALL cells and normal T lymphocytes was assessed by colorimetric assay, 3-(4,5-dimethylthiazol-e-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma). Only active mitochondria dehydrogenase enzymes can perform cleavage of a tetrazolium ring, and therefore the amount of purple formazan generated by cells with a drug was compared with the amount of formazan produced from non-treated cells. Absorbance readings at a wavelength of 550 nm were taken on a spectrophotometer (Titertek Multiskan MCC Type 341). Cells from 72 h cultures were incubated with 10 μ L of 5 mg/mL MTT for 2 h, followed by 100 μ L isopropanol that contained 0.1 N HCl. Normal T lymphocytes were incubated with MTT for 30–37 min. All experiments were performed in triplicate.

Immunoblotting

Cells cultured with dimethylsulfoxide (DMSO) or tubacin were harvested at 1000 rpm, for 5 min, at room temperature, washed with phosphate buffered saline (PBS), and lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCL, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 [NP-40], 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]), containing protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail 2 (Sigma). Protein quantification was performed by bicinchoninic acid (BCA) protein assay procedure (Thermo Scientific). The boiled SDS–Laemmli method was used for all Western blot analyses. Whole cell lysates were subjected to SDS/ polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane (StemCell Technologies, Inc.), and immunoblotted with specific antibodies. Anti-acetylated, α -tubulin (Santa Cruz Biotechnology), goat anti-mouse immunoglobulin G–horseradish peroxidase (IgG-HRP; Santa Cruz Biotechnology), monoclonal anti- β -tubulin (Sigma), anti-poly(ADP-ribose) polymerase (PARP) antibody (Cell Signaling Technology), monoclonal anti-ubiquitin (Sigma), goat anti-rabbit IgG (H+L)-HRP conjugated (Biorad), a PhosphoPlus[®] C-Jun(Ser63) II and C-Jun(Ser73) antibody kit, and a PhosphoPlus[®] SAPK/JNK(Thr183/Tyr185) antibody kit (Cell Signaling Technology) were used.

Methylcellulose colony assays

Normal hematopoietic stem cells (2×10^5) in a volume of 0.3 mL were suspended in 3 mL of methylcellulose containing interleukin-3 (IL-3), IL-6, and stem cell factor (Methocult 3534; StemCell Technologies, Inc, Vancouver, BC). The mixture was plated in 1.1 mL duplicate cultures with varying concentrations of tubacin and was counted after 14 days.

DNA degradation assay

Nalm-6 cells were cultured with tubacin at varying concentrations for 24 h, harvested at $250 \times g$ for 5 min, washed with PBS, fixed with chilled 70% ethanol, and kept at -20°C . On the day of DNA staining the cells were centrifuged at $250 \times g$ for 5 min, and the pellet was

treated with DNA staining buffer (3.4 mM sodium citrate [Sigma], 0.03% Triton-X 100 [Sigma-Aldrich], 100 $\mu\text{g/mL}$ propidium iodide [Calbiochem], 20 $\mu\text{g/mL}$ Ribonuclease A [Sigma]) and gently vortexed. The cells were incubated for 15 min and the cell cycle profile was determined using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Na⁺,K⁺-ATPase K⁺ transport and enzymatic activity assay

Jurkat cells (3×10^5 cells/mL) were first incubated for 6 h with DMSO or tubacin and then the Na⁺,K⁺-ATPase K⁺ transport was measured using a FluxOR Thallium Detection Kit (Invitrogen). Cells are initially incubated with FluxOR dye; K⁺ channels are permeable to thallium (Tl⁺), and as the K⁺ goes down its concentration gradient the fluorescence increases. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a plate reader (DTX880; Beckman Coulter).

The enzymatic activity of the Na⁺,K⁺-ATPase pump was determined by direct *ex vivo* enzymatic assay (adapted from [23,24]) and a Malachite Green Phosphate Assay Kit (BioAssay Systems). Jurkat cells were harvested by centrifugation at 500 $\times g$, for 5 min, at 4°C. The pellet was washed with 1 mL 280 mM HEPES buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], centrifuged as before, resuspended with 280 mM HEPES buffer (5.0×10^7 cells/mL), and stored at -80°C. The cells were lysed by the freeze-thaw method in 280 mM HEPES buffer containing 0.1 mM SDS (2.5×10^7 cells/mL) for 10 min, at room temperature. The cell lysates were then incubated with ATPase assay buffer (127 mM NaCl, 20 mM KCl, 5 mM MgCl₂, 3 mM Na₂-ATP, 30 mM histidine, pH 7.4, 1 mM ethylene glycol tetraacetic acid [EGTA]) and treated with tubacin, ouabain, or DMSO for 15 min at 37°C, followed by a 30 min incubation with Malachite Green Reagent also at 37°C. The absorbance was measured at 620 nm on a spectrophotometer (Titertek Multiskan MCC Type 341).

Xenograft system of primary leukemia samples and *in vivo* drug treatment

Primary cells from the peripheral blood or bone marrow of patients with pre-B ALL were injected into sub-lethally irradiated (250 cGy) immunodeficient NOD/SCID mice and serially passaged as described previously [25,26]. For *in vivo* drug treatment, primary ALL cells were intravenously injected into sub-lethally irradiated NOD/SCID mice (5×10^4 cells/mouse). Leukemia progression was monitored by non-invasive bioluminescent imaging as described previously [26]. Recipient mice were treated for 4 weeks intraperitoneally with saline ($n = 3$), 50 mg/kg/day tubacin only ($n = 3$), vincristine, dexamethasone, and L-asparaginase (VDL) ($n = 7$), or VDL + tubacin ($n = 7$). Weights were monitored daily. Mantel-Cox survival analysis was performed.

Results

Tubacin inhibits proliferation of pre-B and T ALL cells

We studied the effects of tubacin and its inactive analog niltubacin on the proliferation of T-ALL cells, Jurkat and Loucy, and pre-B ALL Nalm-6 cells and REH cells, using a MTT viability assay (Figure 1). Cells were treated for 72 h with varying concentrations of tubacin ranging from 0.5 to 2.5 μM or controls (DMSO or no treatment). Our results showed that

tubacin inhibited the growth of ALL cells in a dose-dependent manner, with 50% inhibitory concentrations (IC₅₀) ranging from 1.2 to 2 μ M. HDAC6 was also overexpressed in ALL cell lines and an ALL patient sample compared to normal proliferating lymphocytes (Supplementary Figure 1).

To study the effects of tubacin on the proliferation of normal cells, we treated human lymphocytes stimulated with IL-2 and human bone marrow progenitor cells with tubacin for 72 h [Figure 2(A)]. A subset of primary cells from patients with ALL was also found to be sensitive to tubacin *in vitro* (Supplementary Figure 2). We observed a therapeutic window of approximately 10-fold between ALL cells and normal lymphocytes and bone marrow progenitors [Figure 2(A)]. We further tested an inactive analog of tubacin, niltubacin, for 72 h and demonstrated that treatment with niltubacin had no effect on cell growth of T-ALL, pre-B ALL, or normal lymphocytes [Figure 2(B)]. These results suggest that ALL cells have a greater sensitivity to tubacin compared to normal cells.

Tubacin induces apoptosis in ALL cells

To understand the effects of tubacin on ALL cells, we tested whether tubacin induced apoptosis. Jurkat and Nalm-6 cells were treated for 24 h and Western blot analyses were performed to examine PARP cleavage. A band at 85 kDa was observed, suggesting that apoptosis occurs [Figure 3(A)]. We further confirmed tubacin-mediated DNA degradation by staining the cells with propidium iodide, which showed increased DNA degradation after 24 h of tubacin treatment [Figure 3(B), top right panel]. In contrast, tubacin did not induce apoptosis of normal T lymphocytes [Figure 3(B), bottom right panel]. These results suggest that tubacin-mediated apoptosis is specific to ALL cells.

Tubacin increases acetylation of α -tubulin

Previously, tubacin was demonstrated to increase acetylation of α -tubulin through inhibition of HDAC6 [4,9]. To determine whether tubacin increased α -tubulin acetylation in ALL cells, Jurkat, Loucy, and Nalm-6 were treated with tubacin for 1 and 3 h. Western blot analyses were performed with treated lysate using acetylated α -tubulin antisera. We observed an increase in acetylated α -tubulin with all three ALL cell lines at both time points [Figure 3(C)].

Accumulation of polyubiquitinated proteins in cells treated with tubacin

Tubacin can enhance the effects of bortezomib, resulting in increased ubiquitination of proteins and apoptosis in myeloma cells [9]. To determine whether tubacin increased levels of polyubiquitinated proteins in ALL cells, we performed Western blot analysis using anti-ubiquitin antisera. Our results demonstrated that tubacin alone resulted in the accumulation of polyubiquitinated proteins by 12 h after treatment of Loucy cells and 24 h after treatment of Jurkat cells [Figure 3(D)]. These data suggest that single drug treatment of ALL cells with tubacin is sufficient to induce accumulation of polyubiquitinated proteins.

Tubacin induces apoptosis through JNK/SAP kinase-independent pathways

Previous reports suggest that inhibition of the proteasome by MG-132 generates an accumulation of polyubiquitinated proteins that are toxic to cells, leading to activation of the

SAPK/JNK signaling pathway that leads to apoptosis [9,27–29]. Activated JNK phosphorylates c-Jun on Ser63 and Ser73 [30]. Enhanced α -tubulin acetylation was induced by treatment of Jurkat cells with tubacin or SAHA [Figure 4(A)], a potent inhibitor of HDACs. PARP cleavage was also observed in the cells that were treated with tubacin, SAHA, or MG-132 [Figure 4(A)]. As expected, an increase in ubiquitinated proteins was generated by MG-132, SAHA, or tubacin; however, only MG-132 induced activation of the SAPK/JNK, as seen by the presence of the double bands produced by phospho-SAPK/JNK [Figure 4(A)]. SAPK/JNK and c-Jun were expressed at equivalent levels in all samples. Tubacin alone did not induce phosphorylation of SAPK/JNK as indicated by the absence of phospho-Jun at serine 63 or 73, although phosphorylation was observed in cells treated with SAHA or MG-132 [Figure 4(A)]. To demonstrate whether tubacin could also induce apoptosis in human T-ALL Jurkat cells, we examined PARP cleavage from cells harvested at the same time [Figure 4(B)]. Previous reports showed that inhibition of HDAC6 resulted in hyperacetylation of heat shock protein 90 (Hsp90) in acute myeloid leukemia (AML) cells [31]. Our results demonstrated no increase in Hsp90 acetylation in Jurkat or Nalm-6 cells treated with tubacin (data not shown). These results suggest that unlike myeloma or AML cells, ALL cells treated with tubacin undergo apoptosis through caspase activation but through an SAPK/JNK- and Hsp90-independent pathway.

Acetylation of α -tubulin was previously reported to inhibit the Na^+, K^+ -ATPase pump [14–16]. In order to test whether the tubacin-mediated increase in acetylation inhibited Na^+, K^+ -ATPase in ALL, Jurkat cells were treated with tubacin or ouabain, a Na^+, K^+ -ATPase inhibitor, and the transport of K^+ by the Na^+, K^+ -ATPase pump was measured using the FluxOR Thallium Detection Kit. Tubacin and ouabain similarly inhibited intracellular K^+ transport by Na^+, K^+ -ATPase [Figure 5(A)]. The Na^+, K^+ -ATPase activity was also tested by direct *ex vivo* enzymatic assay, where the absorption value is directly proportional to the concentration of inorganic phosphate (P_i). Our results demonstrated that ouabain directly inhibits the Na^+, K^+ -ATPase activity but tubacin-mediated inhibition of Na^+, K^+ -ATPase is non-enzymatic [Figure 5(B)], since acetylation of α -tubulin is required [32]. Within 30 min following tubacin treatment, acetylated tubulin increased [Figure 5(C)]. This correlated with PARP cleavage 14 h after tubacin treatment. Finally, we observed increased calcium levels in Jurkat and Nalm-6 cells treated with tubacin for 12 h [Figure 5(D)]. Therefore, treatment of ALL cells with tubacin is associated with significant changes in ion homeostasis, including K^+ and Ca^{2+} ; however, this is most likely not due to direct inhibition of the Na^+, K^+ -ATPase pump.

Effects of tubacin in combination with standard chemotherapy

The prognosis of ALL resistant to standard chemotherapy is poor. We first examined the effects of tubacin either alone or in combination with vincristine or bortezomib on the growth of pre-B ALL Nalm-6 cells. After 72 h, we observed decreased viability of cells with combined treatment compared to tubacin alone [Figures 6(A) and 6(B)]. These results indicate that tubacin can potentiate the effects of a microtubule poison such as vincristine or proteasome inhibition.

To study the effects of tubacin on pre-B ALL Nalm-6 cells *in vivo*, we systemically injected cells also expressing luciferase into NOD/SCID mice for bioluminescence imaging. After approximately 35 days, we observed significant bone marrow infiltration of CD19+ and CD38+ Nalm-6 cells by fluorescence activated cell sorting (FACS) analysis (Supplementary Figures 3 and 4). We then treated these mice, starting on day 28, with 100 mg/kg tubacin, intraperitoneally, daily. Our results demonstrated that tubacin alone was able to suppress leukemic progression (Supplementary Figure 5).

We next examined the effects of tubacin on patient-derived pre-B ALL cells *in vivo*, by injecting primary leukemia cells from a patient with relapsed ALL into NOD/SCID mice and treating them with tubacin in combination with VDL. The groups treated with saline ($n = 3$) (median survival time [MST] 40 days post-leukemia injection) or tubacin ($n = 3$) (MST 39 days) died rapidly, as did the group treated with VDL only ($n = 7$) (MST 69 days) [Figure 6(C)]. In marked contrast, the mice treated with the VDL + tubacin combination ($n = 7$) (MST 77 days) showed a significantly prolonged survival compared with the VDL-only group ($p = 0.0171$) [Figure 6(C)]. These results indicate that tubacin is active in suppressing the growth of ALL cells both *in vitro* and *in vivo*.

Discussion

In this article, we report that tubacin inhibits ALL cell proliferation and has a greater effect against ALL cells than normal T lymphocytes or bone marrow progenitor cells, yielding a 10-fold therapeutic window (Figures 1 and 2). These results suggest that HDAC6 inhibition should be considered as a possible approach for ALL therapy. To investigate its mechanism of action, we focused on the effects of tubacin on apoptosis, signaling pathways, and metabolism. Previous studies reported that tubacin inhibits HDAC6, leading to an increase of tubulin acetylation in multiple myeloma (MM) cells [9]. We found that tubacin increases acetylation of α -tubulin in ALL cells and this in turn generates an increase in polyubiquitinated proteins that is sufficient to induce apoptosis, as shown by PARP cleavage and the increase of cells in the sub-2n population. These findings indicate that tubacin also inhibits the aggresome pathway in ALL cells, similarly to MM cells [9].

An increase in α -tubulin acetylation was previously reported to inhibit the Na^+, K^+ -ATPase pump [14–16], hence, we tested whether tubacin could mediate this inhibition in ALL cells. We found that a tubacin-mediated increase in α -tubulin acetylation correlates with inhibition of the Na^+, K^+ -ATPase K^+ transport and, as expected, this inhibition was not direct. It may be possible that tubacin induces an alteration in membrane fluidity that leads to inactivation of Na^+, K^+ -ATPase. In fact, there is evidence that HDAC6 and the aggresome pathway play a role in the integrity of the cytoskeleton and migration of lymphocytes [4,33]. The reduction of intracellular K^+ concentration was able to induce the endoplasmic reticulum (ER) to release greater levels of Ca^{2+} into the cytosol and activate downstream caspases, as shown by PARP cleavage (Figure 4), contributing to apoptosis (Figure 6). In a different study, apoptotic insults led to impaired Na^+, K^+ -pump activity as a mechanism of neuronal death due to a combination of energy deficiency and production of reactive oxygen species [34]. Based on previous reports, potential tubacin-mediated mitochondria stress mechanisms that lead to apoptosis in ALL cells need to be studied. Furthermore, since our results did not

demonstrate that tubacin increased phosphorylation of JNK/SAP kinases as seen with myeloma cells, activation of alternative signaling pathways warrants examination.

Recently, it was reported that tubacin-mediated HDAC6 inhibition significantly increased the cell death effect of topoisomerase II inhibitors, etoposide and doxorubicin, and SAHA, selectively in transformed cells, LNCaP and MCF-7 [35]. We investigated whether tubacin in combination with other chemotherapy drugs could increase their effect in ALL cells. A low concentration of tubacin (0.6 μ M) increased the effect of standard chemotherapy drugs, such as vincristine or Velcade, *in vitro*. Furthermore, tubacin combined with vincristine, dexamethasone, and L-asparaginase increased the survival of NOD/ SCID mice that were previously injected with primary leukemia cells from a patient with relapsed ALL. These results make tubacin an attractive drug to be used in combination with standard chemotherapy treatment in order to improve the survival prognosis of patients with ALL.

In summary, our findings indicate that tubacin treatment leads to an accumulation of acetylated α -tubulin, which could lead to altered membrane stiffness. Effects on membranes could reduce the activity of K^+/Na^+ -ATPase and the lack of K^+/Na^+ -ATPase activity could lead to an increase of intracellular Na^+ and osmotic stress. This osmotic stress would lead to an intracellular calcium release that could subsequently trigger apoptosis (see model, Figure 7). Taken together, the cumulative damage to cells by inhibiting HDAC6 and the aggresome leads to the apoptosis of ALL cells, thereby providing a rationale to target this pathway for therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. The Leukemia & Lymphoma Society. Facts 2010–2011 [Internet]. 2010. Available from: <http://www.lls.org/resourcecenter/freeeducationmaterials/generalcancer/facts>
2. American Cancer Society. Cancer Facts & Figures 2010. Atlanta: ACS; 2010.
3. Reichmann R. Acute lymphoblastic leukaemia: a review. *Trinity Stud Med J*. 2000; 1:26–28.
4. Aldana-Masangkay GI, Sakamoto KM. HDAC6 and its role in cancer. *J Biomed Biotech*. 2011; 2011:875824.
5. Matsuyama A, Shimazu T, Sumida Y, et al. In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *EMBO J*. 2002; 21:6820–6831. [PubMed: 12486003]
6. Valenzuela-Fernandez A, Roman-Cabrero J, Serrador JM, Sanchez-Madrid F. HDAC6: a key regulator of cytoskeleton, cell migration and cell-cell interactions. *Trends Cell Biol*. 2008; 18:291–297. [PubMed: 18472263]

7. Cabrero JR, Serrador JM, Barreiro O, et al. Lymphocyte chemotaxis is regulated by histone deacetylase 6, independently of its deacetylase activity. *Mol Biol Cell*. 2006; 17:3435–3445. [PubMed: 16738306]
8. Arce CA, Casale CH, Barra HS. Submembrane microtubule cytoskeleton: regulation of ATPases by interaction with acetylated tubulin. *FEBS J*. 2008; 275:4664–4674. [PubMed: 18754775]
9. Hideshima T, Bradner JE, Wong J, et al. Small-molecule inhibition of proteasome and aggresome function induces synergistic antitumor activity in multiple myeloma. *Proc Natl Acad Sci USA*. 2005; 102:8567–8572. [PubMed: 15937109]
10. Rodriguez-Gonzalez A, Lin T, Ikeda AK, Simms-Waldrup T, Fu C, Sakamoto KM. Role of the aggresome pathway in cancer: targeting histone deacetylase 6-dependent protein degradation. *Cancer Res*. 2008; 68:2557–2560. [PubMed: 18413721]
11. Haggarty SJ, Koeller KM, Wong JC, Grozinger CM, Schreiber SL. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. *Proc Natl Acad Sci USA*. 2003; 100:4389–4394. [PubMed: 12677000]
12. Haggarty SJ, Koeller KM, Wong JC, Butcher RA, Schreiber SL. Multidimensional chemical genetic analysis of diversity-oriented synthesis-derived deacetylase inhibitors using cell-based assays. *Chem Biol*. 2003; 10:383–396. [PubMed: 12770821]
13. Boulares AH, Yakovlev AG, Ivanova V, et al. Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. *J Biol Chem*. 1999; 274:22932–22940. [PubMed: 10438458]
14. Casale CH, Previtali G, Barra HS. Involvement of acetylated tubulin in the regulation of Na⁺,K⁺-ATPase activity in cultured astrocytes. *FEBS J*. 2003; 276:115–118.
15. James PF, Grupp IL, Grupp G, et al. Identification of a specific role for the Na,K-ATPase alpha 2 isoform as a regulator of calcium in the heart. *Mol Cell*. 1999; 3:555–563. [PubMed: 10360172]
16. Grupp I, Im WB, Lee CO, Lee SW, Pecker MS, Schwartz A. Relation of sodium pump inhibition to positive inotropy at low concentrations of ouabain in rat heart muscle. *J Physiol*. 1985; 360:149–160. [PubMed: 2580969]
17. Rakowski RF, Gadsby DC, de Weer P. Stoichiometry and voltage dependence of the sodium pump in voltage-clamped, internally dialyzed squid giant axon. *J Gen Physiol*. 1989; 93:903–941. [PubMed: 2544655]
18. Lingrel JB, Kuntzweiler T. Na⁺,K⁺-ATPase. *J Biol Chem*. 1994; 269:19659–19662. [PubMed: 8051040]
19. Koksoy AA. Na⁺,K⁺-ATPase: a review. *J Ankara Med Sch*. 2002; 24:73–82.
20. Fujiki Y, Hubbard AL, Fowler S, Lazarow PB. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J Cell Biol*. 1982; 93:97–102. [PubMed: 7068762]
21. Alonso A del C, Nunez-Fernandez M, Beltramo DM, Casale CH, Barra HS. Na⁺,K⁺-ATPase was found to be the membrane component responsible for the hydrophobic behaviour of the brain membrane tubulin. *Biochem Biophys Res Commun*. 1998; 253:924–927.
22. Santander VS, Bising CG, Purro SA, Casale CH, Arce CA, Barra HS. Tubulin must be acetylated in order to form a complex with membrane Na⁺,K⁺-ATPase and to inhibit its enzyme activity. *Mol Cell Biochem*. 2006; 291:167–174. [PubMed: 16733802]
23. Nobel CS, Aronson JK, van den Dobbela DJ, Slater AFG. Inhibition of Na⁺-K⁺-ATPase may be one mechanism contributing to potassium efflux and cell shrinkage in CD95-induced apoptosis. *Apoptosis*. 2000; 5:153–163. [PubMed: 11232243]
24. Esmann M. ATPase and phosphatase activity of Na⁺,K⁺-ATPase: molar and specific activity, protein determination. *Meth Enzymol*. 1979; 156:105–115. [PubMed: 2835596]
25. Trageser D, Iacobucci I, Nahar R, et al. Pre-B cell receptor-mediated cell cycle arrest in Philadelphia chromosome-positive acute lymphoblastic leukemia requires IKAROS function. *J Exp Med*. 2009; 206:1739–1753. [PubMed: 19620627]
26. Klemm L, Duy C, Iacobucci I, et al. The B cell mutator AID promotes B lymphoid blast crisis and drug resistance in chronic myeloid leukemia. *Cancer Cell*. 2009; 16:232–245. [PubMed: 19732723]

27. Dai Y, Rahmani M, Grant S. Proteasome inhibitors potentiate leukemic cell apoptosis induced by the cyclin-dependent kinase inhibitor flavoripidol through a SAPK/JNK and NF-kappa β -dependent process. *Oncogene*. 2003; 22:7108–7122. [PubMed: 14562039]
28. Tang G, Xu Z, Golman JE. Synergistic effects of the SAPK/ JNK and the proteasome pathway on glial fibrillary acidic protein (GFAP) accumulation in Alexander disease. *J Biol Chem*. 2003; 281:38634–38643. [PubMed: 17038307]
29. Yu X, Hong S, Faustman EM. Cadmium-induced activation of stress signaling pathways, disruption of ubiquitin dependent protein degradation and apoptosis in primary rat Sertoli cell-gonocyte cocultures. *Toxicol Sci*. 2008; 104:385–396. [PubMed: 18463101]
30. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell*. 2000; 103:239–252. [PubMed: 11057897]
31. Rao R, Fiskus W, Yang Y, et al. HDAC6 inhibition enhances 17-AAG-mediated abrogation of hsp90 chaperone function human leukemia cells. *Blood*. 2008; 112:1886–1893. [PubMed: 18591380]
32. Kulikov A, Eva A, Kirck U, Boldyrev A, Scheiner-Bobis G. Ouabain activates signaling pathways associated with cell death in human neuroblastoma. *Biochim Biophys Acta*. 2007; 1768:1691–1702. [PubMed: 17524349]
33. Boyault C, Sadoul K, Pabion M, Khochbin S. HDAC6, at the crossroads between cytoskeleton and cell signaling by acetylation and ubiquitination. *Oncogene*. 2007; 26:5468–5476. [PubMed: 17694087]
34. Wang XQ, Xiao AY, Sheline C, et al. Apoptotic insults impair Na⁺,K⁺-ATPase activity as a mechanism of neuronal death mediated by concurrent ATP deficiency and oxidant stress. *J Cell Biol*. 2003; 116:2099–2110.
35. Namdar M, Perez G, Ngo L, Marks PA. Selective inhibition of histone deacetylase 6 (HDAC6) induces DNA damage and sensitizes transformed cells to anticancer agents. *Proc Natl Acad Sci USA*. 2001; 107:20003–20008. [PubMed: 21037108]

Figure 1. Tubacin inhibits cell growth in ALL cells. Chemical structures of (A) tubacin and (B) its inactive carboxylic acid analog, niltubacin. (C) Effects of tubacin treatment for 72 h on proliferation of lymphoblastic leukemia cell lines. IC₅₀ = 2 μM for Jurkat cells, IC₅₀ = 1.2 μM for Loucy cells, IC₅₀ = 1.2 μM for Nalm-6 cells, and IC₅₀ = 1.4 μM for REH cells. Proliferation measurement was performed by the MTT method. Absorbance at 540 nm correlates with living cells. The bars in the graphs show the mean values of a single experiment performed in triplicate, representative of at least three independent experiments.

Figure 2.

(A) Sensitivity of normal lymphocytes and bone marrow hematopoietic stem cells to tubacin treatments. MTT assay of normal T lymphocytes (2×10^6 cells/mL) treated with DMSO or increasing concentrations of tubacin for 0 and 72 h. IC₅₀ = 16 μ M for normal lymphocytes and IC₅₀ = 20 μ M for colony formation in bone marrow hematopoietic stem cells. (B) Comparison of the effect in proliferation by tubacin and niltubacin treatments. Proliferation measurement was performed by the MTT method. Absorbance at 540 nm correlates with living cells. The bars in the graphs show the mean values of a single experiment performed in triplicate, representative of at least three independent experiments.

Figure 3.

Tubacin treatment induces apoptosis specifically in leukemia cells. (A) Western blot of PARP protein cleavage after treating Jurkat and Nalm-6 with 2.5 μ M of tubacin for 24 h. (B) Comparison of cell cycle profile by flow cytometry, corresponding to Jurkat and Nalm-6 cells treated with 2.4 μ M tubacin for 24 h. Cells were stained with propidium iodide, showing the 2n and 4n DNA picks. Apoptosis is indicated by a black arrow as a sub-2n population. The histograms show a single experiment, representative of at least three independent experiments with similar findings. Induction of acetylated tubulin and polyubiquitinated protein accumulation by tubacin treatments in leukemia cell lines. (C) Western blot of acetylated α -tubulin protein levels after treating Jurkat, Loucy, and Nalm-6 cells with 2.5 μ M of tubacin for the indicated times. (D) Western blot of polyubiquitinated protein levels after treating Jurkat and Loucy cells with 2.5 μ M of tubacin for the indicated times. These experiments were repeated three times.

Figure 4.

Polyubiquitinated protein accumulation by tubacin treatments in leukemia cells induces apoptosis through a SAPK/JNK-independent signaling pathway. (A) Western blots of acetylated α -tubulin, β -tubulin, polyubiquitinated protein, PARP cleavage, phospho-SAPK/JNK, total SAPK/JNK, 63Ser phospho-Jun, 73Ser phospho-Jun, and c-Jun protein levels after treating Jurkat with the indicated compounds and concentrations for 14 h. (B) Western blot of PARP protein cleavage after treating Jurkat cells with 2.5 μ M of tubacin for the indicated times.

Figure 5.

Accumulation of acetylated α -tubulin in Jurkat cells by tubacin correlates in time with an inhibition of cellular K^+/Na^+ transportation, an increase of cytosolic concentration of calcium and apoptosis. (A) Effect of increasing concentrations of tubacin in K^+/Na^+ transportation in whole cells. (B) Effect of increasing concentrations of tubacin assayed directly on K^+/Na^+ -ATPase activity. (C) Densitometry corresponding to a Western blot of acetylated α -tubulin protein levels after treating Jurkat and Loucy with increasing concentrations of tubacin for 30 min. (D) Western blot of PARP protein cleavage after treating Jurkat with increasing concentrations of tubacin for 14 h. (E) Levels of cytoplasmic calcium in tubacin-treated Jurkat and Nalm-6 cells.

Figure 6.

Tubacin enhances the effect of chemotherapy and small molecules *in vitro* and *in vivo*. Tubacin increases apoptosis of ALL cells with vincristine and bortezomib (Velcade). Nalm-6 human pre-B ALL cells were treated with either (A) vincristine (V) or (B) Velcade; tubacin alone; or both together at the indicated concentrations for 72 h. We observed a significant decrease in viable cells with both together compared to each drug alone. MTT absorbance of 0.2 is maximal inhibition within the linear range. Experiments were performed in triplicate. (C) Primary ALL cells were intravenously injected into sub-lethally irradiated NOD/SCID mice (5×10^4 cells/mouse). Recipient mice were treated for 4 weeks intraperitoneally with saline ($n = 3$), 50 mg/kg/day tubacin only ($n = 3$), vincristine, dexamethasone, and L-asparaginase (VDL) ($n = 7$), or VDL + tubacin ($n = 7$). Log-rank (Mantel–Cox) test for survival analysis was performed ($p = 0.0171$).

Figure 7. 'Domino effect' triggered by tubacin treatment. Tubacin treatment leads to an accumulation of acetylated α -tubulin. Effects on membranes potentially reduce the activity of K⁺/Na⁺-ATPase and the lack of K⁺/Na⁺-ATPase activity would lead to an increment of intracellular Na⁺ and osmotic stress. This osmotic stress would lead to an intracellular calcium release that could subsequently trigger apoptosis.