Induction of apoptosis by HBI-8000 in adult T-cell leukemia/lymphoma is associated with activation of Bim and NLRP3

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Adult T-cell leukemia/lymphoma (ATL) is an aggressive T-cell malignancy caused by human T-cell lymphotropic virus 1. Treatment options for acute ATL patients include chemotherapy, stem cell transplantation, and recently the anti-chemokine (C-C motif) receptor 4 antibody, although most patients still have a poor prognosis and there is a clear need for additional options. HBI-8000 is a novel oral histone deacetylase inhibitor with proven efficacy for treatment of T-cell lymphomas that recently received approval in China. In the present study, we evaluated the effects of HBI-8000 on ATL-derived cell lines and primary cells obtained from Japanese ATL patients. In most cases HBI-8000 induced apoptosis in both primary ATL cells and cell lines. In addition, findings obtained with DNA microarray suggested Bim activation and, interestingly, the contribution of the NLR family, pyrin domain containing 3 (NLRP3) inflammasome pathway in HBI-8000-induced ATL cell death. Further investigations using siRNAs confirmed that Bim contributes to HBI-8000-induced apoptosis. Our results provide a rationale for a clinical investigation of the efficacy of HBI-8000 in patients with ATL. Although the role of NLRP3 inflammasome activation in ATL cell death remains to be verified, HBI-8000 may be part of a novel therapeutic strategy for cancer based on the NLRP3 pathway.

Adult T-cell leukemia/lymphoma (ATL) is an aggressive malignancy of mature T-lymphocytes that result from infection with human T-cell lymphotropic virus 1 (HTLV-1). The disease can be divided into four stages: indolent, chronic, lymphoma, and acute, with no accepted curative therapy presently available. (1–3) Outside of clinical trials, the predominant treatment option for acute, lymphoma, and unfavorable chronic type is sequential chemotherapy, whereas additional options for aggressive disease include azidothymidine and interferon-α and allogeneic hematopoietic stem cell transplantation. (2,3) Development of additional therapies has been slow, with the anti-chemokine (C-C motif) receptor 4 (CCR4) antibody mogamulizumab the only novel agent introduced in recent years. Although approximately 90% of ATL patients with aggressive disease express CCR4, (4,5) not all patients eligible to receive mogamulizumab respond to treatment; those who do often show relapse or become refractory soon thereafter. (6) Thus, the prognosis for most affected patients remains poor and there is a clear unmet need for novel therapeutic options.

Histone deacetylases (HDACs) are epigenetic modulators that regulate key cellular processes, including proliferation and survival, by modulating gene expression and protein activity. Histone deacetylase inhibitors (HDACi) induce re-expression of genes repressed during oncogenesis, including those involved in growth arrest, DNA damage repair, and apoptosis. As a consequence, HDACi have been shown to be potent inducers of growth inhibition and apoptosis in a variety of transformed cells, including those related to lymphoid malignancies. (7–8) At present, four different HDACi have been approved for treatment of T-cell lymphoma, including romidepsin and belinostat for peripheral T-cell lymphoma (PTCL), vorinostat and romidepsin for cutaneous T-cell lymphoma, and more recently the orally administered HBI-8000 (chidamide) for PTCL was approved in China. Even though there are similarities between PTCL, cutaneous T-cell lymphoma, and ATL, and while previously published data indicate that HDACi cause apoptosis of ATL cells and HTLV-1-transformed cell lines, (9,10) none of the approved HDACi have been studied in regard to clinical efficacy for ATL.

HBI-8000 is an orally bioavailable new chemical entity from the benzamidine class of HDACi rationally designed to specifically block the catalytic pocket of class I HDACs. (11,12) HBI-8000 has shown direct antitumor activity and is also known to enhance immune cell-mediated tumor cytotoxicity by both natural killer cells and tumor-specific CTLs. (12–17) In addition, its
efficacy as monotherapy for T-cell lymphomas has been proven.\(^{(18)}\)

In the present study, we evaluated the effects of HBI-8000 in ATL-derived cell lines and fresh primary ATL cells obtained from ATL patients. Our results indicate that HBI-8000 inhibits cell viability in both primary ATL cells and ATL-derived cell lines. The results provide evidence to support further investigation of the potential utility of HBI-8000 for ATL patients.

Materials and Methods

Sample preparation and cell cultures. Eleven samples were taken from 10 patients diagnosed with aggressive type ATL, based on Shimoyama’s diagnostic criteria (Table S1).\(^{(1)}\) The diagnosis of ATL was confirmed by monoclonal integration of HTLV-1 proviral DNA (Southern blotting; data not shown) in all cases.\(^{(19)}\) Peripheral blood mononuclear cells were obtained from ATL patients and healthy adult volunteers by density gradient centrifugation using a Lympho-prep (Axis Shield, Oslo, Norway). CD4+ lymphocytes from healthy donors were purified from PBMCs by the magnetic bead method (CD4+ T Cell Isolation Kit, Miltenyi Biotec, Auburn, CA, USA). The PBMCs from ATL patients contained more than 85% leukemic cells and were maintained in medium supplemented with 10 ng/mL interleukin-2 (IL2). Among 10 patients with ATL, ATL cases 5, 7, 9, and 10 had cells of sufficient quantity to carry out Western blotting or protein expression array following approval by the Ethics Committee of Nagasaki University Hospital (No. 13102878; Nagasaki, Japan), all patient samples were obtained after receiving informed consent.

Cell lines and cell cultures. The ATL-derived lines KOB, KK1, ST1, SO4, LMY1, and LMY2 were established from patients with aggressive type ATL and kept in our laboratory.\(^{(20,21)}\) LMWTS is a newly established cell line from PBMCs from a patient with acute type ATL. The primary ATL origin of the cells was confirmed by concordance of the results of Southern blotting (data not shown). Human T-cell leukemia cell line Jurkat and human monocytic leukemia cell line THP-1 were obtained from ATCC (Manassas, VA, USA). ST1, Jurkat, and THP-1 cells were maintained in medium supplemented with 10% FBS in an IL2-independent manner. Other cell lines were maintained in medium supplemented with 10 ng/mL IL2. In a previous study, we clarified that KOB, LMY1, LMY2, and ST1 cells have wild-type p53.\(^{(22)}\)

Chemicals and assays of cell viability, apoptosis, and cell cycle. HBI-8000 (HYUA Bioscience International, San Diego, CA, USA) was used in this study. The MTS cell viability assay was carried out using a Cell Titer 96 Aqueous Cell Proliferation Assay kit (Promega, Madison, WI, USA). Cells were prepared for cell cycle evaluation using a Cell Cycle Test kit (BD Biosciences, San Jose, CA, USA). Apoptosis induction was determined by annexin V/prodipid iodide (PI) (Bender Medsystems, Vienna, Austria), mitochondrial outer membrane permeability using a Mitochondrial Membrane Potential (MMP) Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), and caspase-3 activation with a fluorometric assay (MBL, Woburn, MA, USA). A cell cycle test, annexin V/PI assay, MMP assay, and caspase-3 assay were carried out by FlowCytometry (FCM) using a FACS Diva software (BD Biosciences).

DNA microarray analysis. Total RNA was extracted using a PureLink RNA micro kit (Life Technologies, Carlsbad, CA, USA). RNA integrity was assessed using a 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). Double-stranded cDNA and biotinylated cRNA were synthesized using a BioArray RNA labeling kit (Enzo, Farmingdale, NY, USA). Labeled cRNA was then fragmented and hybridized to SurePrint G3 Human Gene Expression 8x60K version 2.0 (Agilent). The arrays were scanned using The SureScan Microarray Scanner G2600D (Agilent) and analyzed with Agilent Feature Extraction Software Version 11.5.1.1(Agilent).

Mutation analysis of p53. Reverse transcription–PCR was carried out to amplify the sequence targeting the ORF of p53 (GenBank Accession number NM_000546) as described previously.\(^{(22)}\)

Quantitative real-time RT-PCR. The mRNA levels for Bim, nucleotide-binding domain and leucine-rich repeat containing gene (NLR) family, pyrin domain containing 3 (NLRP3), p21\(^{waf1/cip1}\), and porphobilinogen deaminase were measured using a LightCycler-480 PCR System (Roche Diagnostics, Basel, Switzerland) as described previously.\(^{(23)}\) Primers and probes used in this study were summarized in Table S2. To normalize the results for variability in concentration and integrity of RNA and cDNA, the PBGD gene was used as an internal control for each sample.

PathScan stress and apoptosis signaling antibody array analysis. The PathScan Stress and Apoptosis Signaling Antibody Array Kit (Cell Signaling Technology, Beverly, MA, USA) allows for simultaneous detection of 19 different signaling molecules. Whole-cell lysates were prepared and incubated on the slides overnight, followed by a biotinylated detection antibody cocktail. Streptavidin-conjugated HRP and LumGLO Reagent, containing in the kit, were then used to visualize by chemiluminescence. Slide images were captured with an image analyzer LAS3000 (Fujifilm, Tokyo, Japan) and spot signals were quantified (Multigauge version 3.0; Fujifilm).

Western blotting and antibodies. Western blot analysis was performed as previously described.\(^{(24)}\) Analyses were undertaken using antibodies to p53 (DO-1), acetylated histone-H3 and -H4 (Merck, Darmstadt, Germany), caspase-1, cleaved caspase-1, Bim, BAX, Bcl-2, Bcl-xL, p21, IKKB, IkxB, B kinase (IKK)x, IKKbeta, IKKgamma, and NLRP3 (Cell Signaling Technology), and β-actin (Sigma, St. Louis, MO, USA).

Transfection and siRNA experiments. Transfection was performed with a Neon Transfection System MPK5000S (Invitrogen, Carlsbad, CA, USA). The transfection programs for KOB and LMY1 (No. 24) were run in such a manner that cell viability and transfection efficiency would be compatible (data not shown). Twelve hours after transfection, cells were treated with or without HBI-8000 and processed for experiments. Two different siRNAs were prepared against each target as follows: Bim, Silencer Select Validated siRNA s195011 (#1) and Silencer Select siRNA s195012 (#2); NLRP3, Silencer Select siRNA s41555 (#1), s41556 (#2). As a control siRNA, Silencer negative control #1 (Applied Biosystems, Foster City, CA) was used.

Statistical analysis. Student’s \(t\)-test with Welch’s correction was used to calculate statistical significance. **P-values < 0.01 and *P-values < 0.05 were regarded as significant.

Results

HBI-8000 inhibits cell viability in ATL-derived cell lines. To investigate the activity of HBI-8000 in ATL cells, two model systems were used, both based on cells derived from ATL patients. First, the activity of HBI-8000 was evaluated using seven ATL cell lines and the non-ATL T-cell line Jurkat,
which is known as a good responder against HDACi.\(^8\) All cases and two unfavorable chronic type cases, responded to HBI-8000 treatment (Fig. 1a). ST1 was relatively unresponsive and showed only modest inhibition. The IC\(_{50}\) values of ATL cells showing a response ranged from 0.7 to 1.7 µM (mean, 1.16 µM). These results indicated that sensitivities of ATL cell lines to HBI-8000 are not inferior compared to Jurkat cells. HTLV-1 infection, IL2-dependence, or p53 status did not have an effect on the response to HBI-8000.

**Apoptosis induction by HBI-8000 in ATL cells associated with annexin-V binding, loss of mitochondrial membrane potential, and caspase-3 activation.** Apoptosis induction was measured using four commonly used assays: annexin-V binding (Fig. 1b), accumulation of cells in the sub-G\(_1\) cell cycle analysis peak (Fig. 1c), the loss of MMP (Fig. 1d), and activation of caspase-3 (Fig. 1e). The data obtained from the four different methods used to detect cell death were consistent, and revealed that a significant level of apoptosis was induced in the KOB, LM1, LM2Y1, LM2T5, and Jurkat lines, whereas the KK1, SO4, and ST1 lines showed only modest apoptosis. Consistent with previous findings,\(^2\) HBI-8000 also induced G\(_1\) cell cycle arrest in KK1, SO4, and ST1 cells (Fig. 1f).

**HBI-8000 induces apoptosis in primary ATL cells.** The effect of HBI-8000 was also assessed using cells freshly obtained from ATL patients (Table S1). The effect of HBI-8000 was evaluated with various concentrations of HBI-8000 for 72 h, after which cell viability was evaluated using an MTS assay. For the first screening step, we treated seven ATL samples with 1.25–100 µM HBI-8000 (Table S1). For more detailed observations, we used 20 µM as the maximum dose and carried out MTS assays with six ATL samples, which included four new ATL samples, as well as samples 1 and 7 from the screening experiment (Fig. 2a, Table S1). Primary ATL cells, including two relapsed cases and two unfavorable chronic type cases, responded to HBI-8000 (Fig. 2b). For more detailed observations, we used MTS assay with ATL samples (Fig. 2b). The ATL samples tested showed decreased cell viability after 72 h of treatment with various concentrations of HBI-8000. Interestingly, normal CD4 lymphocytes are harmed to some extent (Fig. S1) and the histones were acetylated (Fig. 3b) but their tumour suppressor genes were not activated by HBI-8000 (Fig. 3d).

**DNA microarray and antibody array analysis.** To address the vital mechanisms of HBI-8000-induced ATL cell death, we used DNA microarray and protein expression array analyses. For the DNA microarray analysis, we used KOB and LM1, which showed apoptotic changes with HBI-8000 treatment, and compared gene expression profiles between treated and untreated cells. Genes with known functions related to apoptosis, cell cycle, cell death, or cell growth are arranged in order of change in the expression (log\(_2\) ratio) in either direction (Table 1). Among commonly observed changes, the upregulation of Chemokine (C-C Motif) Ligands (CCL2 and CCL4) Interleukin (IL) 9, IL13, and as well as molecules related to cell-growth signaling such as the JAK/Signal transducer and activator of transcription pathway (IL9, IL13, and signal transducer and activator of transcription 4), and inflammasome-related molecules (IL1β, tumor necrosis factor, γ-interferon, and NLRP3) were notable. In contrast, apoptosis facilitating molecules such as FAS (CD95) and breast cancer 1, early onset were downregulated. These results seem to be in contradiction to the observed HBI-8000-induced ATL cell death. A possible explanation is that small numbers of apoptosis facilitators are activated by HBI-8000 that can overcome anti-apoptotic or cell-growth signals. Considering that KOB and LM1 showed acetylation of histones following HDAC inhibition can increase the gene expression of various tumor suppressors, such as p53 and p21. We confirmed that HBI-8000 was effective for increasing the acetylation of histones H3/H4 in ATL cell lines and primary ATL cells (Fig. 3a,b), and then sought to determine the effect of HBI-8000 on protein expressions of p53 and p21 by Western blot analysis. Previous results suggested that p53 status does not determine the response to HBI-8000; however, cells with wild-type p53 (KOB, LM1) showed increased expression of p53 and p21. KK1 cells with mutated p53 showed accumulated p53 proteins and were not changed by HBI-8000 treatment, but they nevertheless showed increased p21 expression (Fig. 3c). In primary ATL cells, ATL9 and 10 have wild-type p53 (Table S1) and showed activation of p53. Reminiscent of the cell line KK1, ATL7 may possess mutant p53 in that it too showed accumulated p53 proteins. Surprisingly, none of the primary ATL samples showed increased p21 expression following HBI-8000 treatment (Fig. 3d), yet they do not require p21 activation for HBI-8000-induced apoptosis.\(^2\) Only three cases (ATL1, 5, and 8), but we further investigated the expression of p21 mRNA after HBI-8000 treatment. These cases have mutant p53, however, ATL1 showed upregulation of p21 mRNA. Thus, some ATL cells may have the ability to upregulate p21 mRNA or protein by HBI-8000 but did not correlate with sensitivities to HBI-8000. Our results also suggested that ATL cells with wild-type p53 may be able to reactivates p3 with the addition of HBI-8000. Interestingly, normal CD4 lymphocytes are harmed to some extent (Fig. S1) and the histones were acetylated (Fig. 3b) but their tumour suppressor genes were not activated by HBI-8000 (Fig. 3d).

**Fig. 1.** Apoptosis in adult T-cell leukemia/lymphoma (ATL)-derived cell lines induced by HBI-8000. (a) ATL-derived cell lines and Jurkat cells (2–5 × 10\(^5\)/mL) were treated with either the vehicle or indicated concentrations of HBI-8000 for 72 h, then cell viability was evaluated using MTS assay. All experiments were carried out in triplicate and the results are expressed as the mean ± SD. (b-d) ATL-derived cell lines and Jurkat cells (2–5 × 10\(^5\)/mL) were treated with either the vehicle or 4 µM HBI-8000 for 48 h, and collected. (b) Annexin-V/propidium iodide staining was carried out and the percentage of annexin-V-positive cells was evaluated. Experiments were carried out in triplicate and the results are expressed as the mean ± SD. (c) Cell cycle analysis. The percentage of cells in the sub-G\(_1\), G\(_1\), S, and G\(_2\)/M phase was evaluated. Increased numbers of cells in the G\(_2\)/M phase with G\(_1\) arrest were observed among KK1 and SO4 cells treated with HBI-8000. (d) Evaluation of loss of mitochondrial membrane potential. Cells were incubated with JC-1 dye and analyzed using FCM to determine the percentage with low JC-1 red fluorescence. Except for KK1 cells, most of the cell lines showed decreased mitochondrial membrane potential (ΔΨ\(_m\)). (e) Activated (cleaved) caspase-3 was evaluated using an Apopcyto caspase-3 colorimetric assay kit. Values for fold induction of signaling (HBI-8000-treated cells/non-treated cells) are indicated.
undergo apoptosis by way of the intrinsic pathway (Fig. 1d), upregulation of BCL2L11 (Bim) is consistent with the observation.

The findings from the protein expression array assays showed that cleavage of caspase-3 and -7, and poly(ADP-ribose) polymerase(26) are common phenomena in cell lines and primary ATL samples (Table S3). These results support previous findings showing apoptosis induced by HBI-8000 in ATL cells. Among other molecules, an apparent upregulation of IκBα was a common phenomenon in ATL cells except KK1.(27) Western blotting revealed that there were tendencies for increased expression of IκBα and decreased expression of IKKs in both the cell lines and primary ATL cells (Fig. S2). These results indicate that HBI-8000 contributes to ATL cell death partly through nuclear factor-κB inhibition.

Analysis of intrinsic apoptotic pathway focusing on activation of Bim. Bim is a pro-apoptotic protein that induces apoptosis by binding to and antagonizing anti-apoptotic members of the Bcl-2 family.(28) To investigate the behavior of Bim, we undertook quantitative PCR for Bim and Western blot analysis for key molecules interacting with Bim (Fig. 4). Quantitative PCR confirmed upregulation of Bim by HBI-8000 at the mRNA level. Importantly, this upregulation was commonly observed in KOB and LMY1 cells, as well as the other cells, but not in normal CD4 lymphocytes. In accordance with the PCR results, the protein expression of Bim was remarkably upregulated in KOB, LMY1, and all of the three primary ATL cells examined. Downregulation of Bcl-2 and Bcl-xL was apparent, whereas Bax showed no change in the KOB and LMY1 cell lines. Among the primary ATL cells, ATL9 and 10 showed...
Table 1. Microarray analysis of KOB and LMY1 adult T-cell leukemia/lymphoma cells

| Symbol | Gene | KOB | LMY1 |
|--------|------|-----|------|
| **Upregulated genes** | | | |
| IL1B  | Homo sapiens interleukin 1, β (IL1B), mRNA [NM_000576] | 11.0 | 2.5 |
| CCL4  | Homo sapiens chemokine (C-C motif) ligand 4 (CCL4), transcript variant 1, mRNA [NM_002984] | 7.8 | 3.7 |
| IL9   | Homo sapiens interleukin 9 (IL9) | 7.5 | 6.0 |
| SERPINB2 | Homo sapiens serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2) | 7.3 | 4.6 |
| CCL2  | Homo sapiens chemokine (C-C motif) ligand 2 (CCL2) | 6.3 | 5.3 |
| BCL2L11 | Homo sapiens BCL2-like 11 (apoptosis facilitator) (BCL2L11) | 5.3 | 3.2 |
| CTLA4 | Homo sapiens cytotoxic T-lymphocyte-associated protein 4 (CTLA4) | 5.2 | 1.6 |
| IL13  | Homo sapiens interleukin 13 (IL13) | 5.1 | 4.6 |
| IL3RA | Homo sapiens interleukin 3 receptor, α (low affinity) (IL3RA) | 4.8 | 2.5 |
| GZMA  | Homo sapiens granzyme A (granyme 1, cytocytic T-lymphocyte-associated serine esterase 3) (GZMA) | 4.7 | 3.4 |
| ID1   | Homo sapiens inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (ID1) | 4.6 | 1.7 |
| NLRP3 | Homo sapiens NLR family, pyrin domain containing 3 (NLRP3) | 4.4 | 2.4 |
| IER3  | Homo sapiens immediate early response 3 (IER3) | 4.4 | 1.7 |
| BCL2A1| Homo sapiens BCL2-related protein A1 (BCL2A1) | 4.0 | 1.8 |
| IFNG  | Homo sapiens interferon, gamma (IFNG) | 3.9 | 2.8 |
| TNF   | Homo sapiens tumor necrosis factor (TNF) | 3.8 | 1.6 |
| SNAI1 | Homo sapiens snail homolog 1 (Drosophila) (SNAI1) | 3.6 | 2.5 |
| STAT4 | Homo sapiens signal transducer and activator of transcription 4 (STAT4) | 3.5 | 2.2 |
| DHRS2 | Homo sapiens dehydrogenase/reductase (SDR family) member 2 (DHRS2) | 3.0 | 2.6 |
| LGMN  | Homo sapiens legumain (LGMN) | 2.7 | 4.6 |
| IL24  | Homo sapiens interleukin 24 (IL24) | 2.5 | 6.1 |

| **Downregulated genes** | | | |
| IL4   | Homo sapiens interleukin 4 (IL4) | −3.7 | −1.2 |
| FAS   | Homo sapiens Fas (TNF receptor superfamily, member 6) (FAS) | −3.0 | −1.3 |
| TSC22D3 | Homo sapiens TSC22 domain family, member 3 (TSC22D3) | −2.4 | −2.1 |
| CHAC1 | Homo sapiens ChaC, cation transport regulator homolog 1 (E. coli) (CHAC1) | −2.3 | −2.1 |
| JMY   | Homo sapiens junction mediating and regulatory protein, p53 cofactor (JMY) | −2.3 | −1.4 |
| SERPINH1 | Homo sapiens serpin peptidase inhibitor, clade H (HSP47), member 1, (SERPINH1) | −2.2 | −1.2 |
| HDAC9 | Homo sapiens histone deacetylase 9 (HDAC9) | −2.1 | −1.1 |
| PPP1R15A | Homo sapiens protein phosphatase 1, regulatory subunit 15A (PPP1R15A) | −2.1 | −1.1 |

Table 1 (Continued)

| Symbol | Gene | KOB | LMY1 |
|--------|------|-----|------|
| RNF7  | Homo sapiens ring finger protein 7 (RNF7) | −2.0 | −1.9 |
| HDAC7 | Homo sapiens histone deacetylase 7 (HDAC7) | −1.9 | −1.1 |
| IRAK4 | Homo sapiens interleukin-1 receptor-associated kinase 4 (IRAK4) | −1.9 | −1.1 |
| INPP5D | Homo sapiens inositol polyphosphate-5-phosphatase, 145 kDa (INPP5D) | −1.7 | −1.9 |
| IL9R  | Homo sapiens interleukin 9 receptor (IL9R) | −1.6 | −1.7 |
| ADA   | Homo sapiens adenosine deaminase (ADA) | −1.6 | −1.5 |
| BNI3  | Homo sapiens BCL2adenovirus E1B 19 kDa interacting protein 3 (BNI3) | −1.5 | −1.5 |
| FHL2  | Homo sapiens four and a half LIM domains 2 (FHL2) | −1.3 | −2.0 |
| CENPH | Homo sapiens centromere protein H (CENPH) | −1.2 | −1.5 |
| RAD51 | Homo sapiens RAD51 homolog (S. cerevisiae) (RAD51) | −1.2 | −1.7 |
| BRCA1 | Homo sapiens breast cancer 1, early onset (BRCA1) | −1.1 | −1.9 |
| MUC1  | Homo sapiens mucin 1, cell surface associated (MUC1) | −1.1 | −1.9 |
| CCNG2 | Homo sapiens cyclin G2 (CCNG2) | −1.0 | −2.1 |

Cells (2–5 × 10⁶/mL) were treated with either vehicle or 4 µM HBI-8000 for 16 h, then DNA microarray analysis was carried out. Upregulated genes and downregulated genes in KOB cells are arranged in descending and ascending orders of fold change (log2 ratio), respectively. Among them, we selected those with known functions related to apoptosis, cell cycle, cell death, and cell proliferation.

downregulation of Bcl-xL, and ATL7 and 10 showed moderate upregulation of Bax. These results support the previously noted speculation that small numbers of apoptosis facilitators are activated by HBI-8000, which are able to overcome anti-apoptotic or cell-growth signals. Western blotting for normal CD4 lymphocytes was undertaken to compare with that of ATL samples and they did not alter expressions of Bax, Bim, Bcl-2, or Bcl-xL by HBI-8000 (Fig. 4d).

**Analysis of activation of NLRP3 inflammasome signals.** The inflammasome pathway is responsible for activation of inflammatory processes and induces cell pyroptosis, a process of programmed cell death distinct from apoptosis. (29) NLRP3 is an inflammasome-forming nucleotide-binding oligomerization domain-like receptor that activates procaspase-1, leading to maturation of IL1β. Like other caspsases, procaspase-1 is activated in a proteolytic manner (cleaved caspase-1). (30) As our microarray analysis results strongly suggested the activation of NLRP3 inflammasome-related molecules, we carried out further quantitative PCR assays for NLRP3, and Western blotting for NLRP3 and caspase-1 (Fig. 4b, e, f). In accordance with the results of microarray analysis, quantitative PCR confirmed upregulation of NLRP3 by HBI-8000 at the mRNA level in the ATL cell lines and primary ATL cells examined. Meanwhile, the level of mRNA and protein in normal CD4 lymphocytes did not upregulate NLRP3 and caspase-1 in response to HBI-8000. Western blot analysis showed that KOB cells did

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not express NLRP3 or procaspase-1α, but rather showed decreased expression of procaspase-1 isoforms. In contrast, LMY1 showed apparent upregulation of NLRP3 and procaspase-1α, while the expression of caspase-1 isoforms was decreased. Among the primary ATL cells, ATL7 and 10 showed decreased expression of procaspase-1α and increased expression of cleaved caspase-1, and ATL9 showed decreased expression of procaspase-1 isoforms and increased expression of cleaved caspase-1. Therefore, depending on the cell types, there was a tendency for NLRP3 and caspase-1 to be activated by HBI-8000.

Bim plays critical role in initiating HBI-8000-induced apoptosis.

Cell viability assays revealed that targeting Bim (siRNA#1 and #2) significantly repressed HBI-8000-induced cell death in KOB and LMWT5 cells (Fig. 5a). Similar inhibitory effects by si-NLRP3 were observed in annexin-V/PI assay findings (Fig. 5b). Significant inhibition of apoptosis was observed in LMWT5 cells by use of siRNAs against NLRP3 and a similar tendency was seen in KOB cells, although it was not statistically significant. Western blot analysis revealed that si-NLRP3 suppressed the activation of NLRP3 but not that of Bim in LMWT5 cells (Fig. 5c). Together, these results suggest that activation of NLRP3 is important for HBI-8000-induced cell death of LMWT5 cells.

Possible contribution of NLRP3 inflammasome in HBI-8000-induced cell death.

We also carried out siRNA experiments targeting NLRP3. Cell viability assays revealed that siRNAs against NLRP3 significantly repressed HBI-8000-induced cell death in KOB and LMWT5 cells (Fig. 5a). Similar inhibitory effects by si-NLRP3 were observed in annexin-V/PI assay findings (Fig. 5b). Significant inhibition of apoptosis was observed in LMWT5 cells by use of siRNAs against NLRP3 and a similar tendency was seen in KOB cells, although it was not statistically significant. Western blot analysis revealed that si-NLRP3 suppressed the activation of NLRP3 but not that of Bim in LMWT5 cells (Fig. 5c). Together, these results suggest that activation of NLRP3 is important for HBI-8000-induced cell death of LMWT5 cells.
Fig. 5. Knockdown experiments using siRNA of Bim and NLRP3 in HBI-8000-induced cell death. At 12 h after transfection, cells (1–2 × 10⁵/mL) were incubated for 24 h with either the vehicle or 1–2 μM HBI-8000. (a) Cell viability relative to untreated cells in each experiment was evaluated using MTS assay. (b) The percentage of annexin-V-positive cells was evaluated by FCM. Results in 5 (a, b) are expressed as the mean ± SD for three independent experiments and were analyzed using Student’s t-test with Welch’s correction (*p < 0.05, **p < 0.01) as compared with HBI-8000-treated si-Control cells. (c) After transfection using si-Control, siRNA#1, or #2, cells (1–2 × 10⁵/mL) were incubated for 24 h with either vehicle or 1–2 μM HBI-8000. Cells were harvested and Western blot analysis was carried out. Representative results using siRNA#1 are shown.
Discussion

Accumulated evidence supports the notion that HDACi have therapeutic value for ATL. However, none of the approved HDACi therapies have been studied in regard to clinical efficacy for ATL. The China Food and Drug Administration recently granted approval for use of HBI-8000, the world's first orally available HDACi for treatment of relapsed or refractory PTCL. Consistent with its activity for PTCL, HBI-8000 induced cell cycle arrest and apoptosis in ATL-derived cell lines and, perhaps more importantly, induced apoptosis of treatment-naïve or relapsed patient-derived ATL cells. A phase I study of HBI-8000 in non-Hodgkin's lymphoma including ATL patients is underway in Japan, with phase II studies for ATL/PTCL in the planning stage.

The pro-apoptotic molecule Bim has recently attracted increasing attention as a target for tumor therapy, with ima tinib, gefitinib, bortezomib, and the Bim protein itself spotlighted as current and future Bim-targeting therapeutic agents. Bolden et al. investigated the ability of HDACi to affect the growth and survival of tumor cells whilst leaving normal cells relatively unharmed, and found that Bim is a key molecule in vorinostat-induced apoptosis of cancer cells. It has been shown that HTLV-1 Tax has a pro-survival role in infected T cells by enhancing expression of the Bcl-2 family of anti-apoptotic proteins. Very recently, several reports pointed out the importance of the role of Bim in HTLV-1 infected cells. Mühleisen et al. reported that HTLV-1 Tax downregulates Bim expression by enhancing hypoxia-inducible factor-1α protein expression, while siRNA knockdown of the hypoxia-inducible factor-1α increased the expression level of Bim, and death receptor induced apoptosis in HTLV-1-infected leukemic T-cell lines. Furthermore, Tanaka-Nakanishi et al. found that an HTLV-1 bZIP factor impairs transcription of Bim by disrupting FoxO3a function and acts to promote viability of HTLV-1-infected T cells by blocking their apoptosis. In addition, our previous studies indicated that death receptor-mediated apoptotic signals in ATL cells are strongly inhibited at the mitochondrial level through the intrinsic apoptotic pathway. In the present study, we found that HBI-8000 is a novel Bim activator and, as such, may be a promising agent for treatment of ATL.

Inflammasomes are protein complexes assembled following recognition of infection or cell damage signals, and play important roles in apoptotic and pyroptotic cell death. The NLRP3 proteins show a variety of functions during innate immune response, among which NLRP3 is the most well known. It has been shown that deregulation of NLRP3 is associated with cancer pathogenesis, and viral infections may activate the NLRP3 inflammasome. Previous reports have shown the role of the NLRP3 inflammasome in antiviral immunity towards influenza virus in vivo, and a very recent study indicated that the NLRP3 inflammasome pathway caused quiescent CD4 T-cell death in HIV-infected hosts by caspase-1-mediated pyroptosis. Therefore, it is not surprising that HTLV-1-infected CD4 T cells harbor an NLRP3-induced cell death pathway. However, there is no useful method for detection of pyroptosis, such as the annexin-V/PI assay for apoptosis, and distinguishing apoptotic cells from pyroptosis is difficult.

Our purpose in the present study did not include clarifying the mechanism of pyroptosis. Nevertheless, our microarray analysis results strongly suggest activation of NLRP3 inflammasome signaling. We focused on NLRP3 and caspase-1, the initiator and executioner, respectively, of this pathway, and noted a clear tendency for some types of ATL cells to activate NLRP3 and caspase-1 following treatment with HBI-8000. Furthermore, siRNA experiments revealed that activation of NLRP3 partly contributed to HBI-8000-induced ATL cell death.

The importance of our results regarding NLRP3 activation may be explained from two aspects. First, there is no known previous report showing that antitumor drugs such as HDACi are responsible for inflammasome-mediated cell death. Second, our results suggest that the antiviral response by NLRP3 inflammasome is reactivated by HBI-8000 in ATL cells. This indicates the possibility that HBI-8000 may be effective as an antiviral agent for patients who are HTLV-1 carriers as well as an antitumor agent for patients with ATL.

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Disclosure Statement

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