Tubacin Kills Epstein-Barr Virus (EBV)-Burkitt Lymphoma Cells by Inducing Reactive Oxygen Species and EBV Lymphoblastoid Cells by Inducing Apoptosis*

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Tubacin is a small molecule inhibitor of histone deacetylase 6 and blocks aggresome activity. We found that Epstein-Barr virus (EBV)-positive Burkitt lymphoma (BL) cells were generally killed by lower doses of tubacin than EBV-transformed lymphoblastoid cells (LCLs) or EBV-negative BL cells. Tubacin induced apoptosis of LCLs, which was inhibited by pretreatment with a pancaspase inhibitor but not by butylated hydroxyanisole, which inhibits reactive oxygen species. In contrast, tubacin killed EBV-positive BL cells in a caspase-3-independent pathway that involved reactive oxygen species and was blocked by butylated hydroxyanisole. Previously, we showed that bortezomib, a proteasome inhibitor, induces apoptosis of EBV LCLs and that LCLs are killed by lower doses of bortezomib than EBV-positive BL cells. Here we found that the combination of bortezomib and tubacin acted in synergy to kill EBV-positive BL cells and LCLs. Tubacin or the combination of bortezomib and tubacin did not induce EBV lytic replication. These findings suggest that the combination of a proteasome inhibitor and an HDAC6 inhibitor may represent a useful strategy for the treatment of certain EBV-associated B cell lymphomas.

 Epstein-Barr virus (EBV) is associated with several human lymphoid malignancies, including Hodgkin disease, Burkitt lymphoma (BL), T cell lymphomas, and post-transplant lymphoproliferative disease (1, 2). Tissues from patients with EBV post-transplant lymphoproliferative disease typically have a type 3 latency pattern in which each of the EBV latency-associated proteins, including EBV nuclear antigens (EBNA-1, -2, -3A, -3B, and -3C) and latent membrane proteins (LMP1 and LMP2) are expressed. A type 3 latency pattern is also seen in lymphoblastoid cell lines (LCLs), derived from primary B cells transformed with EBV in vitro. Tissues from patients with EBV-positive BL usually have a type 1 latency pattern with expression of EBNA-1 but not the other latency-associated proteins. When grown in cell culture, BL cell lines can have a type 1 or a type 3 pattern of latency.

The treatment of EBV-associated lymphoid malignancies often requires cytotoxic chemotherapy, which is not always successful. Inhibition of proteasomes and aggresomes represents new therapeutic targets for malignancies (3–5). Degradation of proteins is required for vital cell functions and is carried out both in proteasomes and aggresomes. Misfolded or unfolded proteins are polyubiquitinated by a complex of proteins and subsequently degraded by proteasomes. However, if ubiquitinated proteins escape degradation by proteasomes and aggregate, they accumulate into aggresomes (6). Aggresome formation can be abrogated by disrupting the microtubule cytoskeleton or by overexpression of the p50 subunit of dynactin (7). HDAC6 (histone deacetylase 6) is a microtubule-associated deacetylase that can induce microtubule disassembly and promote chemotactic cell motility (8–10). HDAC6 contains a dynein motor binding domain, two catalytic domains with histone deacetylase activity, and a carboxyl-terminal domain that binds polyubiquitinated misfolded proteins (11). The carboxyl catalytic domain of HDAC6 possesses α-tubulin deacetylase activity (12). HDAC6 is required for transport of misfolded proteins for aggresome formation and to prevent apoptosis in response to misfolded protein stress (11). HDAC6 inhibitors disrupt aggresomes (5). Tubacin inhibits the carboxyl catalytic domain of HDAC6, increases the level of acetylated α-tubulin, and blocks aggresome activity (4, 12, 13).

Bortezomib is an inhibitor of the 26 S proteasome (3). Previously, we showed that bortezomib induces apoptosis of EBV-transformed B cells and prolongs survival of mice inoculated with EBV-transformed B cells (14). In contrast, EBV-negative Burkitt lymphoma cells were much less sensitive to killing by bortezomib. Since bortezomib has been shown to interact synergistically with tubacin to induce apoptosis in multiple myeloma cells (4), we studied the effect of tubacin on EBV-transformed B cells and Burkitt lymphoma cells both in the absence and presence of bortezomib. We show that tubacin kills LCLs by apoptosis and induction of caspase-3, whereas...
Tubacin kills EBV-positive BL cells by induction of reactive oxygen species. Bortezomib and tubacin acted in synergy to kill EBV-positive BL cells and LCLs. These findings suggest that the combination of tubacin and bortezomib may have potential as a model for the treatment of certain EBV-associated lymphomas.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Three EBV-transformed LCLs (LCLa, LCLb, and LCLc), EBV-positive BL cell lines (Akata (15), Mutu 1 (16), and Kem 1), EBV BL cells infected with EBV B95-8 (BL30-B95-8), EBV-negative BL cell lines (BJAB (17), BL30 (18), and Ramos (American Type Culture Collection, Manassas, VA)), EBV-negative primary effusion lymphoma (BCBL-1) cells, and T (Jurkat) cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. BJAB cells stably transfected with vector (Blgpt-3) or LMP1 (BJLMP-6 and BILMP-15) were a gift from Fred Wang (Brigham and Women’s Hospital, Harvard Medical School) and were grown in medium containing mycophenolic acid, xanthine, and hypoxanthine, as described previously (19). Bortezomib was purchased from Millennium Pharmaceutical Inc. (Cambridge, MA). The pancaspase inhibitor Q-VD-OPH was purchased from R&D Systems (Minneapolis, MN) and prepared in DMSO (20, 21). The HDAC6-specific inhibitor tubacin was prepared in DMSO and obtained from the Broad Institute of Harvard and MIT (12). Trichostatin A, butylated hydroxyanisole (BHA), propyl gallate, and ethoxyquin were purchased from Sigma.

Cell Viability Assay—2 x 10^5 cells/ml were cultured in 12-well plates, and cell viability was assayed by trypan blue exclusion using a Vi-Cell cell viability analyzer (Beckman Coulter, Fullerton, CA). Cells were pretreated with the pancaspase inhibitor Q-VD-OPH (50 μM) 1 h prior to the addition of tubacin.

Annexin V Analysis of Apoptosis—Apoptosis was measured using an annexin V-PE/7-AAD apoptosis assay kit (BD Pharmingen Biosciences, San Diego, CA) following the manufacturer’s instructions. Briefly, LCLs (2 x 10^6) were treated with various concentrations of tubacin for 24 and 48 h, and cells were harvested, washed in ice-cold phosphate-buffered saline, resuspended in binding buffer, and incubated with annexin V-PE for 15 min at room temperature. Cells were analyzed by flow...
cytometry, and viable cells were defined as negative for annexin V-PE and 7-AAD staining, whereas apoptotic cells were defined as positive for annexin V-PE and negative for 7-AAD staining.

Immunoblots—Cells were lysed in extraction buffer (50 mm Tris-HCl, pH 7.5, 150 mm NaCl, 1% Triton X-100, 5 mm EDTA with protease inhibitors). Equal amounts of protein were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and incubated with antibody. Antibody to caspase-3, cleaved caspase-3, and polyadenosine diphosphate-ribose polymerase (PARP), acetylated lysine (Cell Signaling Technology, Beverly, MA), β-actin and acetylated α-tubulin (Sigma), Bcl-2 (Oncogene, Cambridge, MA), Bax (Trevigene, Gaithersburg, MD), Bcl-XL and α-tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), EBV BZLF-1, LMP1 (DAKO, Carpintera, CA), and EBV early antigen D (Capricorn, Portland, ME) were used for immunoblots.

Measurement of Reactive Oxygen Species (ROS)—Cells were treated with tubacin or DMSO and stained with the Image-iT LIVE Green reactive oxygen species detection kit (Invitrogen) according to the manufacturer’s instructions. Cells were analyzed by flow cytometry to measure production of ROS.

Statistical Analysis—The Chou-Talalay method was used to determine if drug combinations were synergistic or additive (22). The combination index (CI) was determined based on the formula, CI = (D1/(Dx)1 + (D2/(Dx)2, where (D1 and (D2 are the concentrations of drug 1 and drug 2 that have effect x when used together, and (Dx)1 and (Dx)2 are the concentrations of drug 1 and drug 2 that have the same effect x when used alone. A CI of <1 is indicative of a synergistic effect, CI of 1 is indicative of an additive effect, and CI of >1 is indicative of an antagonistic effect.

RESULTS

EBV-positive BL Cells Tend to Be More Sensitive to Tubacin-induced Cell Death than Lymphoblastoid Cells—Various EBV-positive and -negative B cells were treated with different concentrations of tubacin for 48 h. EBV-positive BL cells with a type 1 latency pattern (Akata, Mutu 1, and Kem 1) were killed by tubacin with ≥40% loss of cell viability at ≤10 μM (Fig. 1A). In contrast, EBV-transformed primary B cells (LCLA and LCLc) were less sensitive to tubacin-induced cell death with <30% loss in viability at 10 μM tubacin. EBV-negative B cells (BL30, Ramos, and BCBL) showed little loss in viability even at 10 μM tubacin. EBV-negative B cells stably expressing EBV LMP1 (BJLMP-6 and BJLMP-15) or EBV-negative B cells infected with EBV B95-8 that stably express LMP1, EBNA-1, and EBNA-2 (BL30-B95-8) showed no increased susceptibility to tubacin when compared with their parental cell lines that do not express EBV proteins (supplemental Fig. 1). Akata cells that lost their EBV genomes showed no reduction in susceptibility to tubacin compared to the parental EBV-positive Akata cells (supplemental Fig. 1). Thus, the original status of the tumor cell (before EBV genes are added or are lost from the cell) tends to determine its susceptibility to tubacin.

Tubacin reduced the number of viable LCLA and LCLb cells in a dose-dependent manner with ~40% cell loss at 50 μM tubacin (Fig. 1B). Treatment of LCLA and EBV-positive BL cells with tubacin over longer periods of time resulted in increasing cell death (Fig. 1C). Although tubacin is specific for inhibiting HDAC6, trichostatin A inhibits HDAC1 to -11 (23). In contrast to tubacin, trichostatin A showed little difference in killing LCLs or EBV-positive BL cells (Fig. 1D). To test the toxicity of tubacin, human peripheral blood mononuclear cells were treated with 5 or 10 μM tubacin for 48 h. No significant cell death was observed (data not shown), consistent with a previous report (4).

Tubacin Induces Apoptosis and Caspase-3 Cleavage in LCLs but Little or No Apoptosis of EBV-positive BL Cells—EBV-positive BL cells and LCLs were treated with various concentrations of tubacin for 24 and 48 h, and apoptosis was analyzed by flow cytometry after annexin V staining. LCLA cells treated with tubacin showed increasing numbers of apoptotic cells (annexin V-positive, 7-AAD-negative) in a dose-dependent manner compared to untreated cells (Fig. 1). Similar results were observed in LCLb cells (data not shown). In contrast, two EBV-positive BL cells, Akata and Kem 1, showed few apoptotic cells with tubacin treatment but a large number of necrotic cells (annexin V-positive, 7-AAD-positive). Although the number of

FIGURE 2. Tubacin induces apoptosis of LCLs but not EBV-positive BL cells. LCLA, Akata, or Kem 1 cells were treated with various concentrations of tubacin. Apoptosis was evaluated at 24 or 48 h after treatment by annexin V-7AAD staining using flow cytometry. The percentage of annexin V^-7AAD^- cells is indicated in the lower right corner of each panel.
apoptotic cells was elevated in Kem 1 cells treated with 20 μM of tubacin at 48 h, the proportion of apoptotic to necrotic cells was low. EBV-negative BJAB cells showed a similar response to tubacin as the EBV-positive BL cells (data not shown).

To further analyze the mechanism of tubacin-induced cell death, caspase cleavage, a hallmark of apoptosis, was investigated by immunoblotting. Tubacin (50 μM for 12 h) induced cleavage of caspase-3, caspase-7, and PARP in LCLa cells (Fig. 3A). Similar results were seen in LCLb cells (data not shown). In contrast, tubacin failed to induce cleavage of caspase-3 in EBV-positive BL cells (Akata and Kem 1), although PARP and caspase-7 were cleaved in cells treated with tubacin. Although caspase-3 is important for cleavage of a number of substrates, including PARP, and functions as an “executioner” for apoptosis, caspase-7 has been shown to cleave PARP but few other substrates and by itself has little effect on apoptosis (24). PARP cleavage, in the absence of caspase-3 cleavage, was also seen in Akata and Kem 1 cells treated with 10 μM tubacin (data not shown). Tubacin induced caspase-3 cleavage in BJAB cells (data not shown).

To determine whether inhibition of caspases reduces apoptosis due to tubacin, cells were incubated with Q-VD-OPH (a pan-caspase inhibitor) for 1 h followed by treatment with tubacin (10 μM) for 48 h. Pretreatment with Q-VD-OPH inhibited tubacin-induced cell death of LCLa cells but not EBV-positive BL cells (Akata and Kem 1) (Fig. 3B). Similar results were observed in LCLb cells treated with Q-VD-OPH (data not shown). Q-VD-OPH also inhibited caspase-3 cleavage induced by tubacin in LCLa cells; the

FIGURE 3. Tubacin induces caspase-3 cleavage in LCLs but not EBV-positive BL cells. A, LCLS, Akata, or Kem 1 cells were treated with 50 μM tubacin, and lysates were blotted for caspase-3, cleaved caspase-3, caspase-7, cleaved caspase-7, PARP, and β-actin. Cells were also incubated with Q-VD-OH (Q-VD) for 1 h prior to incubation with tubacin and immunoblotted. The upper β-actin blot corresponds to the cells in the panels immunoblotted with caspase-3 antibodies; the lower β-actin blot corresponds to cells immunoblotted with caspase-7 and PARP antibodies. B, Q-VD-OPH inhibits tubacin-induced apoptosis. LCLa, Akata, or Kem 1 cells were incubated with or without Q-VD-OPH for 1 h and then incubated with tubacin (50 μM for LCLs, 10 μM for Akata or Kem 1 cells) for 48 h.
p20 peptide, but not the mature p17 peptide required for apoptosis (25), was detected in Q-VD-OH-treated LCLa cells (Fig. 3A). Q-VD-OPH inhibited PARP and caspase-7 cleavage, induced by tubacin in LCLa, Akata, and Kem 1 cells (Fig. 3A). These data indicate that tubacin-induced cell death of LCLs is mediated by caspase-3-dependent apoptosis.

Since tubacin inhibits the carboxyl catalytic domain of HDAC6, which has α-tubulin deacetylase activity, we studied the effect of tubacin on acetylation of α-tubulin in EBV-transformed B cells. Tubacin, but not bortezomib, induced acetylation of α-tubulin in LCLa, Kem 1, and Akata cells (supplemental Fig. 2A). The addition of bortezomib had no effect on acetylation of α-tubulin by tubacin.

To verify that acetylation of α-tubulin was specific, LCLs, Kem 1, and Akata cells were treated for 6 h with various concentrations of tubacin, and lysates were immunoblotted with antibody to acetylated lysine. At concentrations of tubacin that induced cell death (10 μM for Kem 1 or Akata cells or 50 μM for LCLs), tubulin acetylation was markedly increased relative to acetylation of histones H3 or H4 (supplemental Fig. 2B).

**Tubacin Induces Production of ROS in EBV-positive B Cells**—Besides apoptosis, other pathways can induce cell death, including production of ROS. Cell lines were labeled with carboxy-2',7'-dichlorofluorescein diacetate and treated with tubacin, and ROS was measured using production of carboxyl dichlorodihydrofluorescein using flow cytometry. ROS was induced in Akata, Kem 1, or LCLa treated with tubacin (Fig. 4A). Similar results were seen with LCLb (data not shown). BHA inhibits cell death induced by ROS. BHA (40 μM) prevented tubacin-induced death of Akata or Kem 1 cells but did not inhibit tubacin-induced death of LCLs (Fig. 4B). Other ROS inhibitors (propyl gallate and ethoxyquin) showed similar results. These results suggest that tubacin induces death of EBV-positive BL cells, but not LCLs, by production of ROS. BHA did not inhibit tubacin-induced cell death of BJAB cells (data not shown). Pretreatment
of Akata cells with BHA followed by tubacin resulted in reduced ROS compared with cells not treated with BHA (Fig. 4C). Similar results were seen in Kem 1 cells.

Tubacin Does Not Induce Lytic Gene Expression in EBV-positive BL Cells or LCLs—To determine if tubacin induces expression of EBV lytic proteins, we examined the expression of the EBV immediate early protein BZLF1 and early antigen D in tubacin-treated cells. No difference was noted in the level of EBV BZLF1 or early antigen D in Akata, Kem 1, or LCLb cells treated with the two drugs for 6 h when compared with untreated cells (supplemental Fig. 3A). Similar results were seen when cells were treated for 24 h.

To determine if tubacin induces apoptosis of LCLs by regulating the expression of pro- or antiapoptosis proteins, we examined the expression of several of these proteins in LCLs. There was no detectable change in the level of Bcl-2, Bcl-XL, or Bax (supplemental Fig. 3B) in LCLs or in BL (Kem 1 or Akata) cells treated with tubacin. Similarly, there was no change in the level of these proteins in LCLs or BL cells treated with bortezomib or tubacin combined with bortezomib. Since some HDAC inhibitors increase the expression of EBV LMP1 (26), we tested whether tubacin might also increase expression of the protein. Treatment of LCLs or BL cells with tubacin, bortezomib, or tubacin and bortezomib did not change the expression of EBV LMP1.

The Combination of Bortezomib and Tubacin Act in Synergy to Induce Killing of EBV-positive BL Cells—Previously, we found that bortezomib induces apoptosis of LCLs; however, the drug was much less effective for EBV-positive BL cells (14). Therefore, we investigated whether the combination of bortezomib and tubacin could enhance the killing of LCLs and EBV-positive BL cells compared with tubacin alone.

LCLa cells treated with 5–20 μM tubacin alone for 48 h showed a maximum loss in cellular viability of 18% compared with no drug, and incubation with 5–20 nM bortezomib alone resulted in a maximum loss in viability of 49% compared with untreated cells. However, when the cells were cultured with these concentrations of both agents together, the loss in viability was 74% (Fig. 5A). An isobologram analysis (Fig. 5B) showed synergy with each combination of tubacin and bortezomib, and this was confirmed by combination indices of less than 1.0 for each of the four combinations of drugs (Fig. 5C).

Akata cells treated with 5–20 μM tubacin alone showed loss of cellular viability of up to 52% compared with control cells, and incubation with 5–20 nM bortezomib alone resulted in a maximum loss of viability of 43% compared with untreated cells; however, when the cells were cultured with both drugs the maximum loss of viability was 73% (Fig. 5D). An isobologram showed synergy of the two drugs for killing Akata cells (Fig. 5E) and the combination indices were all ≤0.4, indicative of synergy (Fig. 5F).

Treatment of LCLa with 10 μM tubacin alone did not result in significant loss of viability (Fig. 1A) or cleavage of caspase-3 (Fig. 5G). However, 10 μM tubacin enhanced caspase-3 cleavage of LCLa cells by bortezomib, compared with bortezomib alone. Similar results were seen with LCLb cells (data not shown). Although 2.5 μM tubacin did not result in cleavage of caspase-3 in Akata or Kem 1 cells, 2.5 μM tubacin enhanced caspase-3 cleavage of these cells by bortezomib compared with bortezomib alone.

DISCUSSION

We have found that tubacin usually kills EBV-positive BL cells at lower concentrations of drug than LCLs or EBV-negative BL cells. Tubacin inhibits HDAC6 (12, 13), which belongs to the class IIb family of HDACs. HDAC inhibitors result in covalent modification of histones, with chromatin remodeling and increased expression of multiple genes.

HDAC inhibitors have been studied for their effects on EBV-positive B cells. Depsipeptide induced apoptosis of LCLs in vitro and improved survival of mice with severe combined immunodeficiency inoculated with LCLs but had little activity
in killing EBV-positive BL cells (27). Valproic acid, an inhibitor of class I and IIa HDACs, had a modest effect in killing LCLs \textit{in vitro} or in mice with severe combined immunodeficiency but enhanced the ability of cytotoxic drugs to kill LCLs \textit{in vitro} and in the mice (28). Trichostatin A inhibits class I, II, and IV HDACs and induces apoptosis of EBV-positive and EBV-negative BL Akata cells (29). Arginine butyrate, which inhibits class I and IIa HDACs, induces lytic EBV gene expression to potentiate the toxic effect of ganciclovir on LCLs (30). Some HDAC inhibitors (e.g. butyrate and trichostatin A) up-regulate LMP1 (26); however, tubacin did not increase LMP1 (supplemental Fig. 3B). Thus, tubacin is more selective than the other inhibitors that have previously been used to kill EBV-infected cells.

Tubacin-induced apoptosis and caspase-3 cleavage in LCLs but had little effect on caspase-3 cleavage in EBV-positive BL cells. Tubacin-associated cell death in LCLs was inhibited by a pancaspase inhibitor, whereas death of EBV-positive BL cells was not affected by the inhibitor. LCLs express each of the EBV latency proteins, while EBV-positive BL cells express only EBNA-1. The EBV latency proteins apparently are not required for tubacin-induced apoptosis of B cells, since tubacin induces apoptosis and caspase cleavage of multiple myeloma B cells (4).

ROS were induced in LCLs and EBV-positive BL cells treated with tubacin. Accumulation of ROS can cause cell damage and death (31). HDAC inhibitors can result in accumulation of ROS in some tumor cells, and free radical scavengers can reduce cell death (23). HDAC inhibitors can induce ROS-mediated cell death, which is caspase-independent and not blocked by caspase inhibitors (32). Activation of c-Myc can induce DNA damage, induction of ROS, and loss of cell viability without induction of apoptosis; antioxidants can reduce ROS and increase survival (33). We found that the EBV-positive Burkitt lymphoma cell lines, Akata and K and 1 cells showed high levels of c-Myc, relative to LCLa and LCLb (data not shown), consistent with production of ROS and improved survival of the Burkitt cell lines after treatment with ROS inhibitors.

BHA protected EBV-positive BL cells, but not LCLs, from tubacin-induced cell death. In contrast, tubacin-treated LCLs, but not BL cells, underwent apoptosis with caspase-3 cleavage and were protected from cell death by Q-VD-OPH. These results indicate that tubacin kills LCLs by caspase-induced apoptosis, whereas the ROS pathway is important for tubacin-induced cell death of BL cells.

We found that the combination of tubacin and bortezomib acted in synergy to enhance apoptosis of EBV-positive Akata cells and LCLs. Bortezomib interacted synergistically with the HDAC inhibitor suberoylanilide hydroxamic acid or sodium butyrate to induce apoptosis of leukemic or multiple myeloma cells (34, 35). Bortezomib induced aggresome formation in pancreatic tumor cells or multiple myeloma cells that were disrupted with induction of apoptosis after treatment with the HDAC inhibitor trichostatin A or suberoylanilide hydroxamic acid (5). Although bortezomib also acted in synergy with tubacin to induce apoptosis of multiple myeloma cells (4), our study is the first to demonstrate this in EBV-positive B cells.

Administration of bortezomib to mice with severe combined immunodeficiency inoculated with LCLs results in significantly prolonged survival in these mice; however, some animals

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