A Histone Deacetylase Inhibitor LBH589 Downregulates XIAP in Mesothelioma Cell Lines Which is Likely Responsible for Increased Apoptosis With TRAIL

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Purpose: Tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL) is a member of tumor necrosis factor family and it is important for ligand induced apoptosis in tumor cells. TRAIL has been shown to be synergistic with a variety of chemotherapies and targeted agents. In the study, a combination of TRAIL and a histone deacetylase inhibitor LBH589 was studied in mesothelioma cell lines.

Experimental Design: Five mesothelioma cell lines and two normal cell lines were tested for cell growth inhibition and apoptosis using high-throughput assays in the presence of LBH589, TRAIL and a combination of the two. Caspase induction was studied and levels of X-linked inhibitor of apoptosis (XIAP) were tested using Western blotting. A combination of a direct inhibitor of XIAP was also tested in combination with TRAIL.

Results: In mesothelioma cell lines, a combination of LBH589 and TRAIL markedly increased cell growth inhibition and apoptosis when compared with the effect on normal cell lines. LBH589 and TRAIL appeared to induce higher levels of caspase 3 and 7 and this appeared to be closely related to ability of LBH589 to degrade XIAP. In addition, a direct inhibitor of XIAP was also sensitized cells to TRAIL apoptosis, providing an indirect confirmation for XIAP degradation as a possible mechanism of synergy.

Conclusions: In mesothelioma cell lines, LBH589 increases the sensitivity to TRAIL. In addition, at least partly, the mechanism of this induction of TRAIL sensitivity is due to LBH589 related degradation of XIAP. These results provide initial evidence for testing this combination in clinical trials.

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Tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL) is a member of tumor necrosis factor (TNF) superfamily and exhibits antitumor activity in a variety[1,2] of tumor cells.[1,2] TRAIL signals primarily through two receptors—DR4 and DR5 that carry a cytoplasmic death domain. Upon binding to its receptors, TRAIL induces activation of caspases 8 and 10 that (in turn) lead to activation of effector caspases 3, 6, and 7 that are responsible for apoptosis. Several factors may be responsible for induction of TRAIL sensitivity to cancer cells. These include decoy receptors (DcR1 and DcR2 that lack a functional cytoplasmic death domain), c-FLIP (which is an inhibitor of activation of caspase 8 and 10), ability to induce intrinsic or mitochondrial pathway of apoptosis by cleavage of Bid or activation of XIAP (XIAP that directly binds and inactivates caspase 3 and 9). Several cancers including pancreatic cancers, neuroblastomas, breast cancer, urothelial cancers demonstrate resistance to effects of TRAIL.[3–6] In earlier reports, combinations of histone deacetylase inhibitors (HDAIs) and TRAIL were found to demonstrate synergistic activity against leukemia and prostate cancer cell lines.[7,8]

Mesothelioma is a malignancy that is rare and is caused by asbestos exposure. Treatment of mesothelioma is unsatisfactory and is primarily surgical. A minority of patients, however, demonstrate long- term disease control with surgery alone. Chemotherapy remains the mainstay of therapy for advanced and recurrent mesothelioma. Standard chemotherapy regimen includes cisplatin and pemetrexed that improve survival in these patients but are often associated with unpleasant toxicities. In addition, there are no approved therapies for patients who fail this particular treatment or cannot tolerate it. There is some clinical activity of HDAIs in mesothelioma. In a recently published report, 4 of 13 patients with mesothelioma demonstrated prolonged stable disease and two patients had partial responses with suberoylanilide hydroxamic acid which is a HDAI.[9]

In the report, we tested a combination of TRAIL and a novel deacetylase inhibitor LBH589 in a panel of mesothe-

lioma cell lines. Our results demonstrate that this putative combination is synergistic across a panel of cell lines. In addition, a marked down-regulation of XIAP was observed on treatment with LBH589 which contributed to this synergy between this inhibitor and TRAIL.

MATERIALS AND METHODS

Cells Lines

Human mesothelioma (NCI-H2452, NCI-H2052, and MSTO-211H) cancer cell lines were obtained from American Type Culture Collection (ATCC) (CRL-5946, CRL-5915, and CRL-2081, respectively). ME13 and ME 16 were gracious gifts from Dr. Pass and are unique mesothelioma cell lines obtained from patients (NYU School of Medicine, NY, NY). Normal cell lines were studied and included human dermal fibroblasts (HDFA) obtained from Sciencell Research Laboratories (2320) and normal human lung cells (Met5A) also gifted from Dr. Pass. Cell lines were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cell lines NCI-H2452, NCI-H2052, and MSTO-211H were grown in RPMI 1640 Medium (ATCC) medium containing 10% FBS (Gibco/Invitrogen Corp) and penicillin-streptomycin 1 ml/100 ml (Hyclone). Cell lines ME13 and ME16 were grown in Dulbecco Modified Eagle Medium/ATCC) medium containing 5% FBS (Gibco/Invitrogen Corp) and penicillin-streptomycin 1 ml/100 ml (Hyclone). HDFA cells were grown in fibroblast medium containing 2% FBS and 1% penicillin-streptomycin. Met5A cells were grown in Dulbecco Modified Eagle Medium.

Drugs

TRAIL was obtained commercially (EMD Biosciences). LBH589 (LBH) and LBW-242 (LBW) were provided by Novartis. LBH and LBW stock was prepared in DMSO and dilutions from stock were conducted in media. Z-V-A-D (OMe)-FMK (ZVAD) was purchased through R&D Systems (FMK001). ZVAD stock was prepared in DMSO according to manufacturer's instruction at a 20 mM concentration and dilutions from stock were conducted in FBS supplemented media. MG-132 was obtained from Sigma (C2211) and was dissolved in DMSO to prepare stock solutions.

Cell Growth Inhibition

Cell Growth Inhibition was assessed using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) which uses the MTS reagent. Metabolically active cells contain dehydrogenase enzymes which can convert the MTS into aqueous soluble formazan. The quantity of formazan product is measured by the amount of absorbance and is directly proportional to the number of living cells. Approximately 20 hours before experiments, 3500 cells per well with 200 μl of the appropriate media were plated onto a 96 well tissue culture plate. Cells were allowed to adhere overnight in a 37°C, humidified, 5% CO_2 incubator. Cells were centrifuged at 500 $\times$ g for 5 minutes and media was replaced with appropriate volumes so that various concentrations of chemotherapeutic agents (single and in combination) could be added. After various exposure times to TRAIL, LBH, or LBH–TRAIL combinations (sequential and/or concurrent), the plates were retrieved from the incubator and an MTS assay applied according to protocol. The cells were allowed to incubate with the assay for 3 hours after which the absorbance was measured at 490 and 630 nm using the Biotek μQuant plate reader and from which the Delta optical density was calculated.

Cell Death Detection Assay for Apoptosis

Induced cell death was evaluated using the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) or CDD (Roche Applied Science) which quantifies histone complexed DNA fragments released from the cytoplasm after apoptosis induction. 3500 cells per well with 200 μl of the appropriate media were plated onto a 96 well tissue culture plate. Cells were allowed to adhere overnight in a 37°C, humidified, 5% CO_2 incubator. Cells were centrifuged at 500 $\times$ g for 5 minutes and media was replaced. Cells were treated as a single agent and concurrent combination of the compounds LBH and TRAIL. Cells were incubated for 6 and 24 hours before beginning the CDD protocol and conducted according to manufacturer's instructions. For LBH589 and MG-132 experiments, cells were treated normally with LBH589. MG-132 was added for last 6 hours before conducting the CDD assay.

Caspase 3, 7, 8, and 9 Activity Assays

Caspase-Glo 3, 7, 8, and 9 were purchased from Promega and the assay application was followed according to protocol. For all caspase assays, approximately 20 hours before experiments, 3500 cells/well were seeded in 96-well plates (Greiner Bio-One for luminescence assays). The plates were retrieved from the incubator after overnight incubation, spun down at 500 x g for 5 minutes, media removed and replaced with appropriate volumes so that various concentrations of chemotherapeutic agents could be added. Cells were treated as a single agent and concurrent combination of the compounds LBH and TRAIL. The plates were read at exposure time ranging from 1 to 48 hours. Luminescence was recorded with Bio-Tek FL$\times$800 Microplate Fluorescence Reader and results were recorded as relative light units with blank subtracted.

Immunoblotting

Histone Acetylation

For histone acetylation immunoblotting, cells were treated with 100 nM LBH for 48 hours, trypsin-treated, counted by means of Trypan Blue exclusion then washed with 1X Phosphate Buffered Saline (PBS) 3 times. Cell pellets were resuspended in Triton Extraction Buffer (PBS containing 0.5% Triton X 100, 2 mM phenylmethanesulfonyl fluoride, 0.02% NaN_3) at a cell density of 1×10^7 cells per ml. Cells were lysed on ice for 10 minutes with gentle mixing then centrifuged at 4800 $\times$ g for 10 minutes at 4°C. The supernatant was discarded, and the cell pellet was washed with Triton Extraction Buffer a second time. The pellet was resuspended in 0.2 N HCl at a cell density of 4 $\times$ 107 cells per ml and the histones were acid extracted overnight at 4°C. The supernatant containing the histones were collected at