Cotreatment with Histone Deacetylase Inhibitor LAQ824 Enhances Apo-2L/Tumor Necrosis Factor-Related Apoptosis Inducing Ligand-Induced Death Inducing Signaling Complex Activity and Apoptosis of Human Acute Leukemia Cells

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ABSTRACT

Present studies demonstrate that treatment with the histone deacetylase inhibitor LAQ824, a cinnamic acid hydroxamate, increased the acetylation of histones H3 and H4, as well as induced p21WAF1 in the human T-cell acute leukemia Jurkat, B lymphoblast SKW 6.4, and acute myelogenous leukemia HL-60 cells. This was associated with increased accumulation of the cells in the G0 phase of the cell cycle, as well as accompanied by the processing and activity of caspase-9 and -3, and apoptosis. Exposure to LAQ824 increased the mRNA and protein expression of the death receptors DR5 and/or DR4, but reduced the mRNA and protein levels of cellular FLICE-inhibitory protein (c-FLIP). As compared with treatment with Apo-2L/Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or LAQ824 alone, pretreatment with LAQ824 increased the assembly of Fas-associated death domain and caspase-8, but not of c-FLIP, into the Apo-2L/TRAIl-induced death-inducing signaling complex. This increased the processing of caspase-8 and Bcl-2 interacting domain (BID), augmented cytosolic accumulation of the prodeath molecules cytochrome-c, Smac and Omi, as well as led to increased activity of caspase-3 and apoptosis. Treatment with LAQ824 also down-regulated the levels of Bcl-2, Bel-xL, XIAP, and survivin. Partial inhibition of apoptosis due to LAQ824 or Apo-2L/TRAIL exerted by Bcl-2 overexpression was reversed by cotreatment with LAQ824 and Apo-2L/TRAIL. Significantly, cotreatment with LAQ824 increased Apo-2L/TRAIL-induced apoptosis of primary acute myelogenous leukemia blast samples isolated from 10 patients with acute myelogenous leukemia. Taken together, these findings indicate that LAQ824 may have promising activity in augmenting Apo-2L/TRAIL-induced death-inducing signaling complex and apoptosis of human acute leukemia cells.

INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as Apo-2L, is a member of the tumor necrosis factor family of cytokines that can bind and induce oligomerization of its agonistic cell-membrane death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5; Ref. 1). In preclinical studies Apo-2L/TRAIL has been shown to exert cytotoxic effects against human leukemia but not normal host cells (2–4). Upon binding and cross-linking by Apo-2L/TRAIL, or by agonistic antibodies, the death receptors DR4 and DR5 can trigger the activity of caspase-8 and apoptosis through the assembly of a cell-membrane associated multiprotein death-inducing signaling complex (DISC; Refs. 5, 6). Several reports have explored strategies to manipulate the levels and assembly of DISC components to ultimately increase caspase-8 activity (1, 5, 7). This, in turn, would either directly or through the recruitment of the mitochondria-based death machinery induce the processing and activity of the downstream effector caspases and apoptosis (1, 5, 8–10). Therefore, depending on the cell-type, active caspase-8 either directly cleaves and increases the activity of caspase-3 (as in type I cells) or processes the proapoptotic Bcl-2 family member Bcl-2 interacting domain (BID) into a truncated BID (tBID), which promotes the release of Smac, Omi/HtrA2, and cytochrome (cyt) c form the mitochondria (as in type II cells; Refs. 8, 10–13). Whereas the cytosolic accumulation of cyt c activates caspase-3 through Apaf-1 and caspase-9-mediated complex called the apoposome, Smac and Omi enhance the activation of the effector caspases-3 and -7 by binding and inactivating IAP family members such as XIAP and cIAP-1 and 2 (9–13). On the basis of these considerations, Apo-2L/TRAIL-induced apoptosis of leukemia cells can be augmented by increasing Apo-2L/TRAIL-induced DISC activity and/or manipulating the downstream determinants of apoptotic signaling that leads to enhanced activity of the effector caspases-3 and -7 (1, 5, 7).

Histone deacetylases and histone deacetylases (HDACs) catalyze the acetylation and deacetylation of lysine residues in the core nucleosomal histone tails, respectively, which regulates the affinity of the nonhistone protein transcriptional complexes with DNA (14, 15). Recently, HDACs have been shown to be involved in leukemogenesis (15, 16). A variety of fusion oncogenes found in leukemia, including promyelocytic leukemia (PML)/retinoic acid receptor α, promyelocytic leukemia zinc finger (PLZF)/retinoic acid receptor α, acutemyelogenous leukemia (AML)-1/ET0, recruit and complex with HDACs, and aberrantly suppress the expression of genes required for cell differentiation and growth control, which leads to the malignant transformation of hematopoietic progenitor cells (16–18). Treatment with HDAC inhibitors (HDIs) causes hyperacetylation of the N-terminal lysine residues in the nucleosomal histones and restores the expression of genes involved in cell cycle arrest differentiation and apoptosis (18, 19). Exposure to HDIs, especially hydroxamic acid analogs, suberoylanilide hydroxamic acid and cinnamic acid hydroxamate, LAQ824, have been demonstrated to induce p21WAF1 (referred as to p21) and p27KIP1 (referred to as p27), which are associated with cell cycle arrest and apoptosis of human leukemia cells (20–23). Whereas treatment with suberoylanilide hydroxamic acid has been shown to induce both caspase-dependent and caspase-independent apoptosis, LAQ824 induces only caspase-dependent apoptosis of leukemia and multiple myeloma cells (21, 22, 24–26). In the cultured T-cell leukemia CEM-CRFR and multiple myeloma MM.1S cells, caspase-independent apoptosis by suberoylanilide hydroxamic acid was demonstrated to involve the cleavage of BID and production of reactive oxygen species (24, 25).

Recent reports have demonstrated that cotreatment with the histone deacetylase inhibitor sodium butyrate or trichostatin A enhances Apo-2L/TRAIL-induced apoptosis of colon carcinoma cells (27, 28). However, in these studies, the effects of the HDI on Apo-2L/TRAIL-induced DISC or the molecular determinants of the downstream signaling of apoptosis were not examined. In the present studies, we determined the effects of the HDI LAQ824 on molecular determinants of Apo-2L/TRAIL-induced DISC activity and signaling for apoptosis. 

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2580
in cultured and primary acute leukemia cells. Our findings demonstrate that treatment with LAQ824 induces DR4 and DR5 but represses cellular FLICE-inhibitory protein (c-FLIP) levels, which is associated with increased Apo-2L/TRAIL-induced DISC activity. LAQ824 treatment also increased the mitochondrial release and cytosolic accumulation of prodeath molecules cyt c, Omi, and Smac, resulting in increased activity of caspase-9 and -3, and apoptosis of human acute leukemia cells.

MATERIALS AND METHODS

Reagents. LAQ824 was kindly provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ; Refs. 23, 26). The recombinant human trimeric form of Apo-2L/TRAIL was produced in Escherichia coli and was a gift from Genentech, Inc. (South San Francisco, CA; Ref. 29). Anti-BID and anti-Smac/DIABLO antibodies were kindly provided by Dr. Xiaodong Wang of the University of Texas, Southwestern School of Medicine (Dallas, TX; Ref. 29). Monoclonal anti-XIAP antibody was purchased from Boehringer Mannheim (Indianapolis, IN). Polyclonal anti-polyl-ADP-ribose) polymerase (PARP) and monoclonal anti-cIAP-1, caspase-9, and caspase-3 antibodies were purchased from PharMingen Inc. (San Diego, CA). Polyclonal anti-caspase-8 antibody was purchased from Upstate Biotechnology (Lake Placid, NY), whereas monoclonal antisurivivin was purchased from α Diagnostic (San Antonio, TX). DR4 antibody was purchased from Alexis Corp. (San Diego, CA). Polyclonal anti-DR5 was obtained from Cayman Chemicals Co. (Ann Arbor, MI). The antibodies for the immunoblots analyses to detect the levels of p21 and p27 were obtained, as described previously (23). Monoclonal anti-toxochrome oxidase-2 antibody was purchased from Molecular Probe (Eugene, OR). z-VAD-FMK and LLLN were purchased from Calbiochem (San Diego, CA).

Cells. Jurkat T-cell leukemia and SKW6.4 B lymphoblast cells were obtained from American Tissue Culture Collection (Manassas, VA). HL-60/Bcl-2 cells with ectopic overexpression of Bcl-2 and the control HL-60/Neo cells were created and maintained in culture as described previously (30). Primary leukemia blasts from 10 patients with AML in relapse were harvested, as described previously (23, 31), from the peripheral blood or bone marrow after informed consent, as a part of a protocol study sanctioned by the local Institutional Review Board. The purity of leukemia blasts in the samples before culture in LAQ824 and/or Apo-2L/TRAIL was at least 80% or more, as determined by morphological evaluation after Wright staining (31).

Flow Cytometry Analysis of Cell Cycle Status. The flow cytometric evaluation of the cell cycle status was performed according to a method described previously (32). The percentage of cells in the G1, S, and G2-M phases were calculated using Multicycle software (Phoenix Flow Systems, San Diego, CA).

Apoptosis Assessment by Annexin-V Staining. After drug treatments, cells were resuspended in 100 μl of the staining solution containing annexin-V fluorescein and propidium iodide in a HEPES buffer (Annexin-V-FLUOS Staining kit; Boehringer-Mannheim, Indianapolis, IN). After incubation at room temperature for 15 min, annexin V-positive cells were estimated by flow cytometry, as described previously (23).

Morphological Assessment of Apoptosis. After drug treatment, 50 × 103 cells were washed and resuspended in PBS (pH 7.3). Cytospin preparations of the cell suspensions were fixed and stained with Wright stain. Cell morphology was determined by light microscopy. In all, five different fields were randomly selected for counting of at least 500 cells. The percentage of apoptotic cells was calculated for each experiment, as described previously (23, 29).

Preparation of S-100 and Western Analysis of Cytosolic Cyt c, Smac, and Omi. Untreated and drug-treated cells were harvested by centrifugation at 1,000 × g for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended with 5 volumes of buffer [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride], containing 250 mM sucrose. The cells were homogenized with a 22-gauge needle, and the homogenates were centrifuged at 10,000 × g for 10 min at 4°C. The supernatants were additionally centrifuged at 100,000 × g for 30 min. The resulting supernatants (S-100) were collected, and the protein concentrations were determined by using the BCA protein assay reagent from Pierce Biotechnology Inc. (Rockford, IL). A total of 75 μg of the S-100 fraction was used for Western blot analysis of cyt c, Smac, and Omi (33).

Western Analyses of Proteins. Western analyses of DR4, DR5, Apo-2L, Fas-associated death domain (FADD), Caspase-8, c-FLIP, and cFlip, big BID, Caspase-9, Caspase-3, PARP, XIAP, cIAP-1, survivin, and β-actin were performed using specific antisera or monoclonal antibodies according to protocols reported previously (30-32). To estimate the levels of acetylated histones H3 and H4 by immunoblot analyses, histones were extracted from the cells according to a method described previously (34). Horizontal scanning densitometry was performed on Western blots by using acquisition into Adobe PhotoShop (Apple, Inc., Cupertino, CA) and analysis by the NIH Image Program (NIH, Bethesda, MD). The expression of β-actin was used as a control.

Apo-2L/TRAIL-Induced DISC Analysis. Untreated or LAQ824-treated SKW6.4 or Jurkat cells were suspended at a final concentration of 106 cells/ml in a prewarmed, complete RPMI 1640. Cells were treated with 100 ng/ml of Apo-2L/TRAIL for 2 h at 37°C, followed by washing with 1.0 ml of ice-cold PBS. Cells were lysed in 500 μl lysis buffer [25 mM Tris-HCl (pH 7.2), 150 mM NaCl, 25 mM NaF, 1 mM benzamidase, 1.0% Triton X-100, 2 μg/ml apotinin, 2 μg/ml leupeptin, 1 μg/ml pepstin-A, and 0.1 μg/ml phenethylmethylsulfonyl fluoride] for 30 min on ice (29). In the untreated controls, 100 ng/ml Apo-2L/TRAIL was added after lysis of cells to immunoprecipitate nonstimulated Apo-2L/TRAIL receptors. One-hundred μg of the lysates were incubated at 4°C for 2 h with 1 μg each of anti-Apo-2L/TRAIL receptor 1 and 2 (DR4 and DR5) antibodies, kindly provided by Immunex Corp. (Seattle, WA). The immune complexes were incubated overnight at 4°C with 20 μl of protein A-agarose beads (Roche, Indianapolis, IN). The beads were recovered by centrifugation and washed twice with the lysis buffer. The pellet was resuspended in the sample buffer, and immunoblot analyses using antibodies against caspase-8, DR5, DR4, and FADD were performed (30-32).

Transfection of Dominant-Negative FADD cDNA. Viable Jurkat cells were transfected with the cDNA of dominant-negative FADD, which encodes for an 80–208 amino acid death effector domain-containing NH2-terminus-deleted fragment (NF-D-4) cloned into the pcDNA 3.1 plasmid (In vitrogen Corp., Carlsbad, CA) or with the control vector (pcDNA 3.1 Zeo; Ref. 35), using LipofectAMINE PLUS reagent (Invitrogen Corp.), as described previously (29). The transfectants were treated with Apo-2L/TRAIL and/or LAQ824. After this, the percentage of apoptotic cells was estimated.

Chromatin Immunoprecipitation Assay. Chromatin immunoprecipitation analysis was performed by a slight modification of a method described previously (20, 23). Cells were incubated overnight at a density of 0.25 × 106 cells/ml at 37°C with 5% CO2. The next day, cells were cultured with 0, 50, 100, or 250 μM of LAQ 824 for 24 h. Formaldehyde was then added to the cells to a final concentration of 1%, and the cells were gently shaken at room temperature for 10 min. After this, the cells were pelleted and suspended in 1 ml of ice-cold PBS containing protease inhibitors (Complete; Boehringer Mannheim). Cells were again pelleted, resuspended in 0.5 μl of SDS lysis buffer [1% SDS, 1.0 mM EDTA, and 50 mM Tris-HCl (pH 8.1)], and incubated on ice for 20 min. Lysates were sonicated with 15-s bursts. Debris was removed from samples by centrifugation for 20 min at 15,000 × g at 4°C. An aliquot of the chromatin preparation (100 μl) was set aside and designated as Input Fraction. The supernatants were diluted 3-fold in the immunoprecipitation buffer [0.01% SDS, 1.0% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), and 150 mM NaCl], and 80 μl of 50% protein A Sepharose slurry containing 20 μg sonicated salmon sperm DNA and 1 mg/ml BSA in the TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA] was added and incubated by rocking for 2 h at 4°C. Beads were pelleted by centrifugation, and supernatants were placed in fresh tubes with 5 μg of the anticetified histone H3 antibody, or 5 μg of rabbit serum and incubated overnight at 4°C. Protein A Sepharose slurry (60 μl) was added, and the samples were rocked for 1 h at 4°C. Protein A complexes were centrifuged and washed three times for 5 min each with immunoprecipitation buffer and two times for 5 min each with immunoprecipitation buffer containing 500 mM NaCl. Immunecomplexes were eluted twice with 250 μl of elution buffer (1% SDS and 1 M NaHCO3) for 15 min at room temperature. Twenty μl of 5 M NaCl was added to the combined eluates, and the samples were incubated at 65°C for 24 h. EDTA, Tris-HCl (pH 6.5), and proteinase K were then added to the samples at a final concentration of 10 μg, 40 μg, and 0.04 μg/μl, respectively. The samples were incubated at 37°C for 30 min. Immunopre-
LAQ824 ENHANCES TRAIL-INDUCED APOPTOSIS

RESULTS

LAQ824 Treatment Induces p21 and p27, and Causes Cell Cycle G1 Phase Accumulation and Apoptosis of Jurkat and SKW 6.4 Cells. We had reported previously that treatment with LAQ824 (5–250 nm) inhibits the in vitro HDAC activity of the HeLa cell nuclear extracts in a dose-dependent manner. In the present studies, we first determined the effect of LAQ824 on histone acetylation, p21 and p27 levels, as well as on growth arrest and apoptosis of human acute leukemia Jurkat and SKW 6.4 cells. Treatment of Jurkat cells with 20 or 50 nm or SKW 6.4 cells with 50 or 200 nm of LAQ824 for 24 h increased the acetylation of histone H3 (Fig. 1) and histone H4 (data not shown). LAQ824-mediated histone hyperacetylation was associated with a dose-dependent increase in the levels of p21 in SKW 6.4 but not Jurkat cells (Fig. 1). In contrast, although exposure to 50 nm LAQ824 increased the intracellular levels of p27 in both SKW 6.4 and Jurkat cells, treatment with 200 nm of LAQ284 attenuated the p27 levels in both cell types (Fig. 1). These results are consistent with the previous reports that LAQ824 induces the hyperacetylation of nucleosomal histones associated with the p21 but not the p27 gene promoter, thereby augmenting the transcription of p21 but increasing p27 levels by an alternative nontranscriptional mechanism (23). Down-regulation of p27 levels observed after exposure to higher concentrations of LAQ824 may be due to degradation of p27. The effect of LAQ824 on the cell cycle status of SKW 6.4 and Jurkat cells is shown in Fig. 2, A and B, respectively. The results show that exposure to LAQ824 for 24 h markedly increased the percentage of cells in the G1 phase and caused a decline in the percentage of cells in the S phase of the cell cycle. Similar effects were observed in the AML HL-60 cells (data not shown). Importantly, exposure to 10–200 nm of LAQ824 for 24 h induced apoptosis in a dose-dependent manner, more in Jurkat (Fig. 2D) than in SKW 6.4 cells (Fig. 2C). Although not shown, treatment with 100 or 200 nm LAQ824 induced apoptosis in >90% of Jurkat cells. These findings with respect to LAQ824 induced apoptosis were also confirmed by the morphological assessment of apoptosis (data not shown).

LAQ824 Induces DR4, DR5, and Apo-2L/TRAIL Levels But Attenuates the Levels of FLIP, Bcl-2, and IAP Family of Proteins. On the basis of its ability to induce apoptosis, we determined the effect of LAQ824 on the intracellular levels of the molecular determinants of the extrinsic and intrinsic pathway of apoptosis in SKW 6.4 and Jurkat cells. Fig. 3A shows that exposure to LAQ824 for 24 h induced Apo-2L/TRAIL, DR4, and DR5 levels. However, LAQ824 treatment did not affect the expression of the decoy receptors for Apo-2L/TRAIL, i.e., DcR1 and DcR2 (data not shown). In contrast, treatment with LAQ824 attenuated the levels of c-FLIPL in both SKW 6.4 and Jurkat cells (Fig. 3A). The attenuation of c-FLIPL occurred to a lesser extent in SKW 6.4 and only slightly in Jurkat cells. This discrepancy in the attenuation of c-FLIPL versus c-FLIPs in the two cell types could be due to different kinetics of down-regulation. Regardless, this was associated with the processing of caspase-9 and -3, suggesting that treatment with LAQ824 not only induces the intrinsic pathway but also primes the cells to the extrinsic pathway induced by Apo-2L/TRAIL. In addition, treatment with LAQ824 also attenuated the levels of Bcl-xL, Bcl-2, XIAP, c-IAP, and survivin (Fig. 3B), which may collectively further lower the threshold to apoptosis.

The reaction was performed at 95 °C for 5 min, and was followed by 30 cycles of denaturing at 95°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. The PCR products were separated on 2% agarose/ethidium bromide gel. The size of the amplified product was 253 bp. The ratio between the immunoprecipitated DNA and Input DNA was calculated for each treatment and primer set. The amplified product was 253 bp. The ratio between the immunoprecipitated DNA and Input DNA was calculated for each treatment and primer set. The fold increases after treatment with LAQ824 was calculated from the indicated ratio (20, 23).

RNase Protection Assay. A RiboQuant MultiProbe RNase Protection Assay System was used according to the manufacturer’s instructions (BD/PharMingen), and as described previously (33). A probe set, hAPO-3d [FADD-like IL-1β converting enzyme (FLICE)], FAS, DR5, DR4, and TRAIL was used for T7 RNA-polymerase directed synthesis of [α-32P]UTP-labeled antisense RNA probes. The probe set contains the DNA templates, including glyceraldehyde-3-phosphate dehydrogenase used as internal control. The probes (1 x 10⁶ cpm/reaction) were hybridized with 20 µg of RNA isolated from the SKW 6.4 and Jurkat leukemia cells, after treatment with 100 nm LAQ824 at different time points using the RNeasy Mini kit (Qiagen, Valencia, CA). After overnight hybridization, samples were digested with RNase to remove single-stranded (nonhybridized) RNA. The remaining probes were resolved on 5% denaturing polyacrylamide gel and analyzed by autoradiography.

Reverse Transcription-PCR Assay for c-FLIP mRNA Levels. Total RNA was isolated from cells using a TRizol LS reagent (Invitrogen). Reverse transcription-PCR analysis was performed, as described previously (23, 33). The RNA (1.0 µg) was reverse-transcribed into cDNA by using SuperScript II RT (Invitrogen) according to the manufacturer’s protocol. For the c-FLIP PCR, the primer sequences were as follows, forward primer: 5'-GCC CGA GCA CGG AGA GTA CGA TA ACG CG-3' and reverse primer: 5'-AGG GAG GGC GAG CTG TGA GAC-3'. For β-actin, the forward primer was 5'-CTA CAA TGA GCC GCT GGT TT G-3' and the reverse primer was AAG GAA GGC TGG AAG AGT GC. The PCR reaction contained 0.2 mM concentration of dATP, dCTP, dGTP, and dTTP, 200 nM of each DR5 promoter primer, 1.5 mM of MgCl₂, and 10 x PCR buffer containing Tris-HCL (pH 8.0), 500 mM KCl, and 1 unit of Tag polymerase (Invitrogen). The reactions were performed at 95°C for 5 min, and followed by 30 cycles of denaturing at 95°C for 45 s, annealing at 52°C for 45 s, and the extension at 72°C for 1 min. The PCR products were separated on a 2% agarose/ethidium bromide gel. The size of the amplified products was 395 bp for the c-FLIP and 527 bp for β-actin product, respectively.

Statistical Analyses. Data were expressed as mean ± SE. Comparisons used Student’s t test or ANOVA, as appropriate. Ps of <0.05 were assigned significance.
LAQ824 ENHANCES TRAIL-INDUCED APOPTOSIS

Fig. 2. LAQ824 induces accumulation in the G1 phase of the cell cycle and apoptosis. SKW 6.4 (A, C) and Jurkat (B, D) cells were treated with the indicated concentration of LAQ824 for 24 h. After this, the cell cycle status and the percentage of apoptotic cells were determined by flow cytometry. Values represent mean of three experiments; bars, SEM.

Due to Apo-2L/TRAIL. Fig. 3C shows that in Jurkat cells, these effects were evident after exposure intervals to LAQ824 of \( \leq 16 \) h. Similar observations were made when SKW 6.4 cells were exposed to 200 nM LAQ824 (data not shown). Previous reports have suggested that during apoptosis, several of the determinants of apoptosis belonging to the Bcl-2 and IAP family may be processed by caspases and/or degraded by the proteasome (36–39). However, cotreatment with z-VAD-fmk, which inhibited the processing of caspase-3 and PARP, or cotreatment with the proteasomal inhibitor ALLnL, did not reverse the attenuating effect of LAQ824 on XIAP, Bcl-2, Bcl-xL, c-FLIP\(_L\), and c-FLIP\(_S\) in Jurkat cells (data not shown). Next, we determined, whether LAQ824 treatment increases the cell surface expression of DR5, DR4, and Apo-2L/TRAIL. Fig. 4 demonstrates that treatment of Jurkat cells with LAQ824 induced the cell-membrane expression of DR5, as determined by flow cytometry. However, LAQ824 treatment only slightly increased DR4 levels, and Apo-2L/TRAIL expression was not increased in Jurkat or SKW 6.4 cells (data not shown).

LAQ824 Increases the mRNA Levels of DR4 and DR5 But Depletes the mRNA of c-FLIP\(_L\). Next, we investigated the effect of LAQ824 on the mRNA levels of c-FLIP\(_L\), DR5, DR4, and Apo-2L/TRAIL in Jurkat and SKW 6.4 cells, using a multiprobe RNase protection assay. Fig. 5A demonstrates that treatment with LAQ824 increased the mRNA expression of DR5 (2.4-fold) in both cell types and FAS expression in SKW 6.4 cells (1.5-fold). There was no apparent increase in FAS expression in Jurkat cells. DR4 levels also increased by 2.2-fold only in SKW 6.4 cells. Exposure to LAQ824 only minimally affected the mRNA levels of Apo-2L/TRAIL and caspase-8 (FLICE). We next determined whether the promoter of DR5 is associated with acetylated histones, which would explain why LAQ824, by inducing histone acetylation, would up-regulate DR5 mRNA levels. The results of the chromatin immunoprecipitation analyses performed on the lysates of the untreated or LAQ824-treated Jurkat cells showed that treatment with 100 and 200 nM LAQ824 for 8 h increased the level of the DR5 promoter associated with acetylated histones H3 and H4 by 3.3- and 5.7-fold, respectively (mean of three experiments; Fig. 5, B and C). Similar results were also obtained in LAQ824-treated SKW 6.4 cells (data not shown). As has been reported previously (20, 23), LAQ824 also increased the association of p21\(^{WAF1}\) promoter DNA with acetylated histones in Jurkat and SKW 6.4 cells (data not shown). In contrast to the increase in the DR5 and DR4 mRNA levels, exposure to LAQ824 for 8 h inhibited the mRNA level of c-FLIP\(_L\) by 75%, as determined by a reverse transcription-PCR assay (Fig. 6A). This was reversed by cotreatment with LAQ824 and cycloheximide (Fig. 6B). These results indicate that LAQ824-mediated repression of the c-FLIP\(_L\) message required new protein synthesis. These results also support the interpretation that LAQ824 augments the levels and activity of a transcriptional repressor for c-FLIP\(_L\), an outcome that is neutralized by cotreatment with cycloheximide.

LAQ824 Enhances Apo-2L/TRAIL-Induced DISC Assembly, and Activity and Apoptosis. We next determined the effects of LAQ824 on Apo-2L/TRAIL-induced DISC and apoptosis, because agents that lower c-FLIP levels, and increase DR5 and DR4 levels have been shown previously to enhance Apo-2L/TRAIL-induced DISC activity, and apoptosis of leukemia and epithelial cancer cells (1, 5, 7). Fig. 7, A and B, demonstrates that cotreatment with LAQ824 and Apo-2L/TRAIL induced significantly more apoptosis of Jurkat and SKW 6.4 cells, as compared with treatment with either agent alone (\( P \leq 0.05 \)). Concomitantly, combined treatment with LAQ824 (20 nM) and Apo-2L/TRAIL (10 ng/ml) for 24 h, versus treatment with LAQ824 or Apo-2L/TRAIL alone, induced greater processing of caspase-8 and BID, as well as increased processing and PARP cleavage activity of caspase-3 (Fig. 7C). This involved an increase in the mitochondrial injury, because cotreatment with LAQ824 and Apo-2L/TRAIL, versus LAQ824 or Apo-2L/TRAIL alone, also caused more accumulation of the prodeath molecules cyt c, Smac, and Omi into the cytosome (Fig. 7D). To determine the effect of treatment with LAQ824 on Apo-2L/TRAIL-induced DISC, we compared the recruitment of caspase-8, FADD, and c-FLIP\(_L\) into the immunoprecipitates of DR5 and DR4 after treatment with Apo-2L/TRAIL (100 nM for 2 h) versus...
treatment with LAQ824 (100 nM for 24 h) followed by Apo-2L/TRAIL. As shown in Fig. 7E, pretreatment with LAQ824 induced more recruitment of FADD and caspase-8 but not c-FLIP_L into the immunoprecipitates of DR4 and DR5, resulting in greater processing of caspase-8 but less of c-FLIP_L. We were unable to detect any recruitment of c-FLIP_S to Apo-2L/TRAIL-induced DISC in the cells pretreated with LAQ824 (data not shown). To determine whether the increased assembly and activity of DISC due to up-regulation of DR4 and DR5, and down-regulation of c-FLIP_L and c-FLIP_S contributed to enhancement of Apo-2L/TRAIL-induced apoptosis, we determined the effect of transient transfection of the DED-depleted cDNA of DN-FADD on apoptosis of Jurkat cells induced by Apo-2L/TRAIL or cotreatment with LAQ824 and Apo-2L/TRAIL. As compared with Jurkat cells transfected with the control vector alone (Jurkat-Zeo cells), apoptosis induced by treatment with Apo-2L/TRAIL or by cotreatment with LAQ824 and Apo-2L/TRAIL was significantly inhibited in Jurkat cells transfected with DN-FADD (P < 0.05; Fig. 7F). Importantly, the sensitizing effect of LAQ824 on Apo-2L/TRAIL-induced apoptosis was reduced in Jurkat-DN FADD versus Jurkat-Zeo cells (Fig. 7F). These findings suggest that LAQ824-induced modulations of the components and activity of Apo-2L/TRAIL-induced DISC contribute toward the overall potentiating effect of LAQ824 on Apo-2L/TRAIL-induced apoptosis.

Combined Treatment with LAQ824 and Apo-2L/TRAIL Overcomes the Inhibition of Apoptosis by Bcl-2 Overexpression. The effects of LAQ824 and/or Apo-2L/TRAIL were compared in HL-60/Bcl-2 cells that possess ectopic overexpression of Bcl-2 (5-fold) versus the control HL-60/Neo cells (29, 30). As shown in Fig. 8A, differences in the baseline expression of p21, DR5, DR4, FLIP_L, and FLIP_S were also noted in the untreated HL-60/Neo versus HL-60/Bcl-2 cells. These differences may be due to the clonal selection of HL-60 cells stably transfected with Bcl-2 carried out by the limiting dilution technique and the transfectants maintained for several months under the selection pressure. Fig. 8A demonstrates that LAQ824-mediated increase in p21, p27, DR4, and DR5 levels, as well as decline in FLIP_L and FLIP_S levels were approximately similar, as compared with the untreated in HL-60/Bcl-2 versus HL-60/Neo cells. As has been reported previously, Apo-2L/TRAIL-induced apoptosis was inhibited in HL-60/Bcl-2 versus HL-60/Neo cells (Fig. 8B). Although after treatment with 50 nM LAQ824, the PARP-cleavage activity of caspase-3 and processing of caspase-8 was also inhibited in HL-60/Bcl-2 cells, exposure to higher level of LAQ824 (100 nM) resulted in similar processing of PARP and caspase-8 in HL-60/Bcl-2 and HL-60/Neo cells (Fig. 8A). Additionally, cotreatment with 50 ng/ml of Apo-2L/TRAIL and LAQ824 (50 or 100 nM) induced more apoptosis than either agent alone in HL-60/Bcl-2 cells (Fig. 8B). This was consistently above 50% in HL-60/Bcl-2 cells, and was associated with more processing of caspase-8 and BID, with generation of higher levels of tBID (Fig. 8C). It was also associated with increased PARP cleavage activity of caspase-3 and down-regulation of XIAP (Fig. 8C). These findings suggest that Bcl-2 overexpression inhibits apoptosis due to Apo-2L/TRAIL and due to lower levels of LAQ824, but this inhibition can be overcome by exposure to higher levels of LAQ824, or at least in part overcome by cotreatment with Apo-2L/TRAIL and LAQ824.

Fig. 4. Treatment with LAQ824 increases DR5 expression on the cell membrane. Jurkat cells were exposed to 20, 30, or 50 nM of LAQ824 for 24 h. After this, the cell membrane expression of DR5 was determined in untreated or LAQ824-treated cells by staining with anti-DR5 antibody followed by flow cytometry. The histograms in A–E are representative of three experiments and derived from cells treated as follows: A, isotype control; B, untreated control cells; C, LAQ824, 20 nM; D, LAQ824, 30 nM; and E, LAQ824, 50 nM. Values in each panel represent the percentage of cells showing positive staining.
LAQ824 ENHANCES TRAIL-INDUCED APOPTOSIS

DISCUSSION

Consistent with the previous reports focused on the antileukemia activity of the other HDIs (16, 21–23), the present studies demonstrate that treatment with LAQ824 also induces p21 and p27, inhibits cell cycle progression, and triggers caspase-dependent intrinsic (mitochondria initiated) pathway of apoptosis of acute myeloid and lymphoid leukemia cells. In addition, the present findings show for the first time that treatment with LAQ824 can modulate the expression of the key determinants of Apo-2L/TRAIL-induced apoptosis. LAQ824 treatment results in increased Apo-2L/TRAIL-induced DISC assembly and activity in acute leukemia cells, which involves autoactivation of caspase-8 by proteolysis. Additionally, by down-modulating the expressions of the key antiapoptotic Bcl-2 and IAP family members, LAQ824 treatment additionally facilitates both the mitochondria-initiated and common pathway of apoptosis, downstream of Apo-2L/TRAIL-induced processing and activation of caspase-8 followed by BID.

A number of preclinical studies using a variety of tumor models have shown that pretreatment with chemotherapeutic agents, including DNA damaging drugs, e.g., topoisomerase inhibitors, antimetabolites, and antimicrotubule agents, increase the expression of DR4 and DR5, and enhance Apo-2L/TRAIL-induced DISC activity and apoptosis (7, 40–43). Consistent with these findings, the studies presented herein also demonstrate that LAQ824-mediated induction of DR5 and DR4 levels is associated with increased Apo-2L/TRAIL-induced DISC assembly and activity. However, we were unable to detect any effect of LAQ824 on the expression of DcR1 and DcR2, suggesting a lack of their role in LAQ824-mediated enhancement of Apo-2L/TRAIL-induced DISC assembly and activity in acute leukemia cells. This is consistent with the prior reports that failed to show any correlation between DcR expression and Apo-2L/TRAIL sensitivity (41, 44). Although LAQ824 treatment increased the intracellular levels of Apo-2L/TRAIL, its surface expression was not increased, discounting its role in LAQ824-induced apoptosis of acute leukemia cells. In a previous report, treatment of neuroblastoma cells with a hybrid polar HDI M-carboxy-cinnamic acidbishydroxamide was shown to induce the expression of Fas and Fas ligand within 12 h (36).

Cotreatment with LAQ824 Overcomes Resistance to Apo-2L/TRAIL-Induced Apoptosis of Leukemia Blasts from Patients with AML in Relapse. We next determined the sensitivity of fresh AML cells procured from patients with relapsed AML to Apo-2L/TRAIL and/or LAQ824-induced apoptosis. Table 1 shows that all 10 samples of AML blasts were resistant to apoptosis induced by Apo-2L/TRAIL (100 ng/ml). In contrast, exposure to LAQ824 (100 nm) induced apoptosis of the primary AML cells to a variable extent. However, in every sample evaluated, cotreatment with LAQ824 and Apo-2L/TRAIL induced more apoptosis than treatment with either agent alone. In some samples, e.g., samples 2 and 5, the apoptotic effect is additive. These data are similar to those derived from HL-60/Bcl-2 cells, in that the resistance of primary AML cells to Apo-2L/TRAIL-induced apoptosis could be overcome by cotreatment with LAQ824 plus Apo-2L/TRAIL. On the basis of the availability of adequate sample of primary AML cells, we also determined the effect of LAQ824 on the determinants of Apo-2L/TRAIL-induced DISC. As shown in Fig. 9A, in a representative sample of primary AML blasts, and similar to the cultured acute leukemia cells, treatment with 100 or 250 nm LAQ824 for 24 h induced the acetylation of histones H3 and H4 (data not shown). LAQ824 treatment also increased DR4 and DR5 levels, as well as down-regulated the levels of FLIPL and c-FLIPs (Fig. 9A). Corresponding to the increase in the intracellular levels of DR5 determined by Western analysis, treatment of the primary AML sample with 100 and 250 nm LAQ824 also increased the DR5 expression on the cell membrane, as determined by flow cytometry, from a baseline of 17.5% to 33.2% and 62.4% of cells, respectively (Fig. 9B).

Dashed lines indicate time intervals (hours), mRNA expression levels of the indicated genes were indicated for each treatment. The ratio between input DNA and precipitated DNA was calculated for each treatment. The lanes represent culture conditions with or without LAQ824, 50 nM cycloheximide, and/or LAQ824-induced apoptosis. Table 1 shows that all 10 samples of AML in Relapse.
However, neither the mechanism underlying this effect nor its impact on Fas ligand-induced apoptosis was determined (36). In contrast, present studies demonstrate that treatment with LAQ824 increases the mRNA levels of DR4 and DR5. With respect to DR5, this was most likely due to LAQ824-induced increased association of DR5 promoter with acetylated histones, which may facilitate the binding of a trans-activator to the DR5 promoter, as is the case with the p21 promoter (20). LAQ824 may also induce the acetylation of lysine residues on the trans-activator, thereby increasing its DNA binding. This possibility is supported by previous reports that the acetylation of specific lysine residues may affect the DNA binding and/or activity of p53, GATA-1, and E2F1 (16). There is also the possibility that the stability of DR4 and DR5 mRNA is increased by treatment with LAQ824, which adds to the increase in the mRNA levels of DR4 and DR5.

In a recent report the histone deacetylase inhibitor Depsipeptide was shown to down-regulate c-FLIP, which was associated with casapse-8 and casapse-3 activation, and apoptosis of CLL cells (45). Additionally, Hernandez et al. (27) showed that treatment with sodium butyrate decreased c-FLIP levels and sensitized Apo-2L/TRAIL-resistant human colon carcinoma cells to Apo-2L/TRAIL-induced caspase-3 activity and apoptosis. Consistent with these observations, our studies also showed that LAQ824 treatment decreased the mRNA transcript and protein levels of c-FLIP. Importantly, LAQ824-mediated increase in DR4 and DR5, and the decline in c-FLIP were also observed in a primary sample of AML blasts (Fig. 9). Although the precise underlying mechanism was not established, this effect may be playing an important role in the sensitizing effect of LAQ824 on Apo-2L/TRAIL-induced DISC activity and apoptosis (Fig. 7E). This inference is strongly supported by the previous reports demonstrating that c-FLIP is a potent inhibitor of Apo-2L/TRAIL-
induced DISC activity and apoptosis (46, 47). In addition, down-modulation of c-FLIP levels has been shown to increase Apo-2L/ TRAIL-induced processing of caspase-8, and apoptosis in human leukemia and cancer cells (48, 49). Furthermore, the finding that the sensitizing effect of LAQ824 on Apo-2L/ TRAIL at the indicated doses for 24 h. After this, the percentage of apoptotic cells was determined by annexin-V staining and flow cytometry. Values represent mean of three experiments; bars, ± SE. C, HL-60/Bcl-2 cells were treated with 50 nM LAQ824 and 50 ng/ml Apo-2L/TRAIL. After this, the cell lysates were obtained and immunoblot analyses of caspase-8, BID, tBID, PARP, and XIAP were performed.

Table 1

<table>
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<th>Patients</th>
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<th>LAQ824</th>
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</table>

*TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.
In summary, due to the poor long-term clinical outcome in the adult patients with several forms of acute leukemia, novel treatment strategies are needed to overcome resistance and sensitize the leukemia blasts to the extrinsic and intrinsic pathway of apoptosis. Treatment with LAQ824 and Apo-2L/TRAIL alone has been recognized to induce apoptosis of leukemia blasts, but the intrinsic mechanisms of resistance limit the antileukemia activity of either agent when administered alone. Therefore, the preclinical findings presented in this report create a strong rationale for investigating the mechanism and activity of the combination of LAQ824 and Apo-2L/TRAIL in the in vivo setting.

REFERENCES

3. Plasillos M, Zivny J, Jelinek J, et al. TRAIL is superior to treatment with either agent alone. Therefore, the preclinical findings presented in this report create a strong rationale for investigating the mechanism and activity of the combination of LAQ824 and Apo-2L/TRAIL in the in vivo setting.

In promoting the activity of the Apaf-1-mediated “apoptosome” by increasing the cytosolic accumulation of cys c (39, 54), treatment with LAQ824 attenuated XIAP levels. This is consistent with the report by Mitani et al. (24), who showed that treatment with suberoylanilide hydroxamic acid also attenuated XIAP and cIAP2 levels in the multiple myeloma MM.1S cells (24). LAQ824 and Apo-2L/TRAIL also resulted in increased accumulation of Smac and Omi, which would exert anti-IAP effects and additionally potentiate Apo-2L/TRAIL-induced apoptosis (11–13, 51, 52). Because LAQ824 treatment alone attenuated XIAP levels (Fig. 3, B and C), not surprisingly, the combined treatment with LAQ824 and Apo-2L/TRAIL was associated with increased attenuation of XIAP. This is consistent with the recent reports that the accumulation of Omi, a serine protease, into the cytosol during Apo-2L/TRAIL-induced apoptosis is involved in the processing of XIAP (13, 55). Previous reports have indicated that increased expression of XIAP and survivin is common and associated with poor clinical outcome in acute leukemia (56, 57). Therefore, the combined treatment with LAQ824 and Apo-2L/TRAIL would be an attractive treatment strategy to test against acute leukemia, especially where resistance to Apo-2L/TRAIL-induced apoptosis in the leukemia blasts is due to overexpression of Bcl-2 and/or IAP family of proteins. The preliminary data derived from 10 primary AML samples presented in Table 1 support this view by indicating that combined treatment with LAQ824 and Apo-2L/TRAIL is superior to treatment with either agent alone.

In summary, due to the poor long-term clinical outcome in the adult patients with several forms of acute leukemia, novel treatment strategies are needed to overcome resistance and sensitize the leukemia blasts to the extrinsic and intrinsic pathway of apoptosis. Treatment with LAQ824 and Apo-2L/TRAIL alone has been recognized to induce apoptosis of leukemia blasts, but the intrinsic mechanisms of resistance limit the antileukemia activity of either agent when administered alone. Therefore, the preclinical findings presented in this report create a strong rationale for investigating the mechanism and activity of the combination of LAQ824 and Apo-2L/TRAIL in the in vivo setting.
Cancer Research


Cotreatment with Histone Deacetylase Inhibitor LAQ824 Enhances Apo-2L/Tumor Necrosis Factor-Related Apoptosis Inducing Ligand-Induced Death Inducing Signaling Complex Activity and Apoptosis of Human Acute Leukemia Cells

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