Antitumor effect of the histone deacetylase inhibitor LAQ824 in combination with 13-cis-retinoic acid in human malignant melanoma

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Abstract

Resistance to chemotherapy is a major hurdle in the treatment of malignant melanoma. Histone deacetylase (HDAC) inhibitors have been shown to have antitumor activity in different tumor types, including melanoma, and to reverse epigenetic repression of tumor suppressor genes, such as retinoic acid receptor β (RARβ). In this study, we tested the antitumor effect of the HDAC inhibitor LAQ824 in combination with 13-cis-retinoic acid (CRA) on two human melanoma cell lines both in vitro and in vivo. Treatment of LAQ824 showed a dose-dependent inhibitory effect on A2058 and HMV-I cell lines in a clonogenic assay. These cell lines were relatively resistant to CRA. On treatment with combination of LAQ824 and CRA, a greater inhibitory effect (up to 98%) was achieved compared with single agents. Lack of RARβ/2 gene expression was associated with histone acetylation and gene methylation at the promoter level. Treatment with LAQ824 restored retinoid sensitivity by reverting RARβ/2 epigenetic silencing. The biological effect of LAQ824 was associated with p21 induction in both cell lines but G2 cell cycle arrest in A2058 and apoptosis in HMV-I cell line. The induction of apoptosis by LAQ824 was associated with increased reactive oxygen species and induction of SM22 gene expression in HMV-I but not in A2058 cell line. Administration of the free radical scavenger l/N-acetylcysteine blocked LAQ824 + CRA-mediated apoptosis in HMV-I cells, suggesting a primary role for reactive oxygen species generation in LAQ824 + CRA–associated lethality. Combination treatment showed 61% and 82% growth inhibition in A2058 and HMV-I tumors, respectively. Greater induction of in vivo apoptosis was observed in the HMV-I but not in the A2058 tumors treated with combination therapy compared with single agents. These results suggest that the HDAC inhibitor LAQ824 has a greater antitumor activity in combination with CRA in melanoma tumors but the degree of induced apoptosis may vary. Combination of HDAC inhibitors and retinoids represents a novel therapeutic approach for malignant melanoma that warrants clinical testing. [Mol Cancer Ther 2007;6(1):70–81]

Introduction

Malignant melanoma is becoming increasingly prevalent worldwide and its incidence is growing more rapidly than other type of cancer (1). Its resistance to standard chemotherapeutic agents limits effective systemic therapy for metastatic disease. The alkylating agent dacarbazine, the only Food and Drug Administration–approved drug for the treatment of malignant melanoma as a single agent, has been reported to induce response rates of 7% to 13% in recent phase III clinical trials with 15% to 28% of patients having stable disease (2, 3). High-dose interleukin-2 has showed antitumor activity in melanoma with a response rate of 16%. Of particular interest is the fact that 6% of patients achieved a complete response and over half of those remained disease-free at >2 years (4). However, toxicity with high-dose interleukin-2 is considerable and has limited the use of this approach to highly selected patients (5). Combination chemotherapy with interleukin-2 and/or IFN-α has also failed to provide significant clinical benefit in large phase III randomized trials (6). Thus, novel therapeutic approaches are needed.

Retinoids, a group of natural and synthetic vitamin A analogues, are essential for growth and cell differentiation of epithelial tissue and may affect cancer development (7, 8). Retinoids exert their effects mainly via nuclear receptors, the retinoic acid (RA) receptors (RAR) and the retinoid X receptors, both of which are members of the nuclear receptor superfamily (9, 10). The human RARβ gene is expressed as three isoforms: β1, β2, and β4 (11). The biologically active RARβ2 isoform is under the regulation of the P2 promoter containing a high-affinity RA-responsive element, which is associated with the transcriptional activation of RARβ2 by RA in a variety of cells (12).

The clinical activity of retinoids has been limited in patients with advanced cancer, including melanoma (13).
Several melanoma cell lines are resistant to retinoids (14). Retinoid resistance in epithelial tumors has been associated with epigenetic loss of RAR/2 expression due to DNA methylation affecting the RAR/2 promoter of one or more RAR/ alleles (15, 16). Our group and others have shown that chromatin remodeling agents, such as histone deacetylase (HDAC) inhibitors, reverse epigenetic repression of RAR/2 in epithelial tumors, including prostate, renal, and breast (17–19).

Histone proteins organize DNA into nucleosomes, which are regular repeating structures of chromatin. The acetylation status of histones alters chromatin structure, which in turn is involved in gene expression. Two classes of enzyme can affect the acetylation of histones, histone acetyltransferases and HDAC. Several inhibitors have been characterized that inhibit tumor growth in vitro and in vivo and are currently under clinical testing (20, 21). LAQ824 is a HDAC inhibitor in early clinical development. It is a structurally novel hydroxamic acid derivative that inhibits HDAC at concentrations below 0.15 μmol/L. LAQ824 has also been shown to have in vivo antitumor activity against human leukemia (22), lung, colon (23), prostate (24), and multiple myeloma xenografts (25).

In this study, we analyzed the expression of RAR/2 in two human melanoma cell lines and tested the hypothesis that the HDAC inhibitor LAQ824 may restore retinoid sensitivity by reverting RAR/2 epigenetic silencing and have a greater antitumor effect in combination with retinoids.

Materials and Methods

Cell Lines and Reagents

The human melanoma cell line, A2058, was purchased from American Type Culture Collection (Manassas, VA), and HMV-I was kindly provided by the Department of Aging and Cancer, Tohoku University of Sendai (Sendai, Japan). A2058 and HMV-I cells were cultured in DMEM and DMEM/F12 medium (Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and 1% penicillin/streptomycin (Life Technologies), respectively, and kept in an incubator at 37°C and atmosphere containing 5% CO2. For the in vitro experiments, tumor cells were treated with 13-cis-RA (CRA; Sigma), all-trans-retinoic acid (ATRA; Sigma), LAQ824 (Novartis Pharmaceuticals, East Hanover, NJ), or vehicle (DMSO) for 72 h, the medium was replaced with medium supplemented with 10% FBS for 7 days. Cells were then washed once with PBS and stained with crystal violet (0.2% c.v. + 10% ethanol in water). Excess crystal violet solution was decanted, and the cells were destained with distilled water. Images of colony formation were captured using Kodak Image Station 440CF (Kodak, Rochester, NY), and colony counts were determined by Quantity One quantitation software (version 4.3; Bio-Rad, Hercules, CA). Percentage of growth inhibition was normalized to untreated control using the following formula: % growth inhibition (GI) = 1 – (Cexp / Cctl), where Cexp equals total colony number of experimental group and Cctl equals total colony number of untreated control. Results were reproducible in repeated experiments. Results are expressed as mean colonies number ± SE. The experiments were repeated twice with similar results.

RNA Isolation and Reverse Transcription–PCR

Tumor cell lines were treated for 24 h with 1.0 μmol/L CRA, 50 and 100 nmol/L LAQ824, or vehicle (DMSO) in DMEM/F12 medium for HMV and DMEM for A2058 supplemented with 10% FBS. Total RNA was extracted from tumor cells by Trizol (Life Technologies), and the first strand was synthesized with oligo(dT) as primer using 1 μg total RNA according to the manufacturer’s instructions. PCR was done with primers for RAR/2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; control) using cDNA synthesized from reverse transcription-PCR (RT-PCR). Primer sequences for RAR/2 covered exons 3 and 4, 5′-GACTGTATGGATGTTCGTTCAG-3′ (forward) and 5′-ATTGTCTCGCCAGACAGAACA-3′ (reverse). Primers for GAPDH are 5′-CCACCATTGGAATTTCCATGGCA-3′ (forward) and 5′-TCTAGACGGCGTACGTCACC-3′ (reverse). PCR products measured 256 and 574 bp, respectively. Results were reproducible in repeated experiments. Samples were processed in a Perkin-Elmer (Wellesley, MA) 9600 GeneAmp thermocycling system under the following conditions: 2 min denaturation step at 94°C, followed by 35 amplification cycles (30 s at 94°C for denaturation, 30 s at 60°C for primer annealing, and 45 s at 94°C for primer extension), and final extension at 72°C for 10 min.

Chromatin Immunoprecipitation Assay

The histone acetylation status of RAR/2 promoter was examined using the chromatin immunoprecipitation assay as described previously (19). An antibody specific for acetylated histone 3 (H3) was used to immunoprecipitate formaldehyde cross-linked, sonicated chromatin from cells treated with LAQ824 or the combination. Semiquantitative PCR analysis of DNA bound to immunocomplexes was done to detect a 192-bp fragment of the RAR/2 core promoter region (−165 to +27), which includes RAreponsive elements and TATA sequences (18).
Quantitative Real-time PCR Analysis

Quantitative real-time PCR for specific genes was done to confirm the differences in RARβ gene expression identified by RT-PCR (19). Single-strand cDNA was synthesized from melanoma cell total RNA (1 μg) by reverse transcription using oligo(dT) as the primer. According to the manufacturer’s protocol, quantitative real-time PCR was done using an ABI Prism 7700 Sequence Detector System (PE-Applied Biosystems, Foster City, CA) with a 2 × SYBR Green PCR Master Mix (PE- Applied Biosystems), reverse transcribed cDNA, and gene-specific primers. To quantify the amount of target mRNA in the samples, a standard curve of RARβ2 primers was prepared for each run using the plasmid containing GAPDH, as internal control. This enabled standardization of the initial mRNA content of cells relative to the amount of GAPDH. The sequences of the specific primers were as follows: RARβ2 primers are the same as in RT-PCR (256 bp), and the sense sequence was 5'-TGAACGGGAACCTC- CACCTGG-3' and the antisense sequence was 5'-TCCAC- CACCCCTTGGCTGTA-3' for GAPDH and the sense sequence was 5'-TGCCGTGATTCTGACCA-3' and the antisense sequence was 5'-CTGCCAAAGCTGCCCAAGG-3' for SM22. The relative expression of target gene was determined by the difference of the threshold cycle (Ct) between target gene and GAPDH (relative expression = 2ΔCt, where ΔCt = Ct target gene - Ct target GAPDH) and the sense sequence was 5'-CTGCCAAAGCTGCCCAAGG-3' for SM22. The relative expression of target gene was determined by the difference of the threshold cycle (Ct) between target gene and GAPDH (relative expression = 2ΔCt, where ΔCt = Ct target gene - Ct target GAPDH).

DNA Extraction and Methylation-Specific PCR

Genomic DNA was isolated from cell lines following the instruction of the DNeasy Tissue Kit (Qiagen, Valencia, CA). Approximately 1 μg DNA was modified by bisulfite treatment and subjected to methylation-specific PCR. The first methylation-specific PCR primers were designed from +80 to +284 bp (upstream 5'-TATGGYAGTTGGAGGAA- TTGGGA-3' and downstream 5'-AATATCATTTACCA- TTTTCCAAACTTA-3'). The next methylation-specific PCR primer sequences that specifically recognized methylated RARβ2 sequence (+105 to +254) were 5'-TGGCTGAACCCG- GAGCGATT-3' (upstream) and 5'-CGACCAAATCCAACC- GAAACGA-3' (downstream) and the unmethylated RARβ2 sequences (+100 to +261) were 5'-TTGGGATGTGA- GAATGTGAGTGATTT-3' (upstream) and 5'-CCTACT- CAACCAATCCCAACAAAAACAA-3' (downstream).

Flow Cytometric Analysis of Cell Cycle and Apoptosis

Cell cycle analysis was done using the Cellular DNA Flow Cytometric Analysis Kit (Roche Diagnostics, Indianapolis, IN). Cells treated with agents (1 μmol/L LAQ824, 10 μmol/L CRA, and 1 μmol/L ATRA) from 24 to 72 h were harvested by trypsin, washed, and fixed in 70% ethanol on ice followed by incubation with RNase. The cells were then stained with propidium iodide (PI) and subjected to a flow cytometry analysis of cell cycle. Aliquots of 10⁶ cells were washed thrice in PBS and resuspended in buffer. A total of 5 mL Annexin V-FITC and/or 5 mL PI was added followed by incubation at room temperature in the dark for 15 min using Annexin V-FITC Apoptosis Detection kit I (BD Biosciences). Cells were immediately analyzed by FACSCalibur (Beckton Dickinson Immunocytometry Systems, San Jose, CA) and CellQuest software (Beckton Dickinson). PI+ and Annexin V+ cells were considered apoptotic, and PI− and Annexin V− cells were considered dead cells. The experiments were repeated twice with similar results.

Tumor Growth In vivo

The animal protocol was approved by the Institutional Care and Use Committee at the Johns Hopkins Medical Institutions and was in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Male 4- to 6-week-old athymic nude mice (National Cancer Institute) were kept in a temperature-controlled room on a 12/12 h light/dark schedule with food and water ad libitum. Animals were injected s.c. in the flank region with 1 × 10⁶ tumor cells (A2058 and HMV-I) resuspended in Hank’s solution and mixed with Matrigel (1:1; Collaborative Biomedical Products, Bedford, MA) in a final volume of 0.2 mL. As the tumor volume reached a measurable size (50-100 mm³), 20 animals for each tumor were randomly placed in four groups (five animals per group): control, CRA, LAQ824, and combination. Animals in the control group were treated with a daily administration (5 days/ wk) of vehicle (polyethylenglycol) by gavage. LAQ824 (10 mg/kg/d) and CRA (20 mg/kg/d) were given by either i.p. injection or by gavage, respectively. Tumor volume was measured with a caliper twice weekly and reported as mean mm³ ± SE. The animals were treated for ~2 weeks and then euthanized by carbon dioxide inhalation. The experiments were repeated twice with similar results.

Western Blot Analysis

To evaluate the effect of LAQ824 and CRA in vivo, HMV-I xenographs from mice treated with LAQ824 and CRA were homogenized in M-PER mammalian protein extraction reagent (Pierce, Rockford, IL) with protease inhibitor cocktail (Roche Diagnostics). Proteins (10 μg/lane) from the homogenized tissues were applied to 4% to 15% Tris-Glycine SDS-PAGE gels, which were then transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in TBST, incubated with the primary antibodies for acetyl H3 or α-tubulin, washed in TBST, and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000). After washing, enhanced chemiluminescence was used with an X-ray film. The expressions of acetyl H3, α-tubulin, and β-actin were determined by comparing the density of the bands to β-actin (as an internal control) by using Quantity One software (Bio-Rad) and are shown in Figure 5. The bands were almost identical in all groups.
were terminated by incubation in 2× SSC for 15 min. Tissues were washed in PBS and counterstained with a 300 nmol/L solution of 4′,6-diamidino-2-phenylindole in PBS for 20 min. After a final round of washes, slides were mounted with Prolong (Molecular Probes) and cover slides. Images were captured with a Nikon E800 microscope (Nikon, Tokyo, Japan), an Interline 5 mHz 3CCD camera, and Metamorph software (Universal Imaging Corp., Downingtown, PA). Percentages of TUNEL-positive cells were counted with a minimum of 200 cells counted (range, 200-300).

Immunohistochemistry

Immunohistochemistry for Ki67 was done using DAKO Envision + System Peroxidase (Carpinteria, CA) according to the manufacturer’s protocol with minor modification (primary antibody was incubated for 1 h at room temperature). Horseradish peroxidase enzyme–labeled polymer conjugated with secondary antibody incubation was carried out for 30 min at room temperature. Ki-67–positive cells were counted as the number of positively stained cells per total cells counted in each field, with a minimum of 200 cells counted (range, 200-300) in four randomly chosen fields.

Detection of Intracellular Reactive Oxygen Species

5-Chloromethyl-2,7′-dichlorodihydrofluorescein diacetate was used to measure intracellular peroxide levels. This agent diffuses into cells and is trapped by de-esterification. Subsequent reaction with peroxides generates intensely fluorescent 5-chloromethyl-2,7′-dichlorofluorescein. After treatment with CRA and/or LAQ for 24 h, cells were incubated in PBS containing 10 μmol/L 5-chloromethyl-2,7′-dichlorodihydrofluorescein diacetate at 37°C for 30 min in the dark, washed with PBS, and analyzed on the Fluorescence channel of a Beckman Coulter (Fullerton, CA) Multimode Detector. H2O2 and/or NAC were added for 5-chloromethyl-2,7′-dichlorodihydrofluorescein diacetate in some experiments. The experiments were repeated twice with similar results.

Figure 1. Effect of combination of LAQ248 and CRA on A2058 and HMV-I melanoma cell growth in vitro. A, A2058 and HMV-I melanoma cells were treated with increasing dose of LAQ248 (0.05-1 μmol/L), fixed concentration of CRA (10 μmol/L), or combination, and colony numbers were assessed. Columns, mean percentage of controls; bars, SE. *, P < 0.05 versus untreated controls; **, P < 0.05 versus single agents. B, colony formation assay was done in HMV-I tumor cells treated for 72 h with increasing doses of LAQ248 (50, 100, and 200 μmol/L) in combination with increasing doses of CRA (0.5, 1.0, and 2.5 μmol/L). Left, results are expressed as means of percentage of inhibition; right, representative colony formation (left, shaded area): Untreated control (top left), 0.5 μmol/L CRA (top right), 50 nmol/L LAQ248 (bottom left), and combination (bottom right).
Statistical Analysis
Differences between means of unpaired samples were evaluated by Student’s *t* test using the Sigmastat program; *P* < 0.05 was considered of statistical significance.

Results
LAQ824 Restores Retinoid Sensitivity in RARβ2-Negative Melanoma Cell Lines *In vitro*
To determine the antiproliferative effect of CRA and LAQ824, we used a clonogenic assay. A2058 and HMV-I cells were exposed to increasing doses of LAQ824 (0.1-1.0 μmol/L) and CRA (0.5-10 μmol/L) for 72 h (Fig. 1A and B). CRA treatment did not induce a significant inhibition of cell proliferation in either cell lines. Treatment with LAQ824 induced a dose-dependent inhibition as single agent; however, the combination of LAQ824 with CRA had a greater inhibitory effect (up to 99% growth inhibition).

Combination of LAQ824 and CRA Induces RARβ2 Reexpression in RARβ2-Negative Melanoma Cell Lines
Based on previous evidence of RARβ2 induction by HDAC inhibitors in other tumor cell lines, we hypothesized that the greater inhibitory effect of the combination of LAQ824 and CRA was due to RARβ2 induction and consequent restoration of retinoid sensitivity. To assess the status of RARβ2 expression and associated retinoid resistance in the human melanoma cell lines, RT-PCR assay was done. A2058 and HMV-I cell lines were treated with increasing doses of LAQ824 in the presence or absence of CRA (1 μmol/L) for 24 h. RT-PCR analysis revealed reinduction of RARβ2 by LAQ824 in the presence of retinoid (Fig. 2A). There was no induction of RARβ2 in CRA-only–treated cells. Quantitative PCR analysis of RARβ2 gene expression was done and revealed a synergistic induction of RARβ2 by combination treatment with LAQ824 and CRA in both A2058 and HMV-I cell lines (Fig. 2B). The next step was to determine whether reexpression of RARβ2 was due to the direct effect of LAQ824 on histone acetylation by chromatin immunoprecipitation assay analysis. The results showed a high constitutive H3 deacetylation at the RARβ2 promoter in both melanoma cell lines, and treatment with CRA alone did not increase histone acetylation status. However, LAQ824 induced a significant increase of histone acetylation associated with the RARβ2 promoter after 24 h of treatment (Fig. 2C). To determine whether lack of RARβ2

**Figure 2.** Effect of LAQ824 and CRA on RARβ2 gene expression in melanoma cell lines. A, reexpression of RARβ2 in HMV-I and A2058 cell lines treated for 24 h with indicated concentrations of CRA and LAQ824 as single agents or in combination. RT-PCR was used to assess gene expression of RARβ2 and GAPDH (internal control) as described in Materials and Methods. B, RARβ2 relative expression by quantitative real-time PCR in A2058 and HMV-I cell lines treated with increased concentrations of LAQ824 and CRA (10 μmol/L). C, RARβ2 promoter acetylation was assessed by chromatin immunoprecipitation assay. RARβ2-negative cell lines were treated with LAQ824 (1 μmol/L), CRA (10 μmol/L), or combination (Comb). Chromatin acetylation associated with RARβ2 promoter in A2058 and HMV-I cell lines at baseline and following treatments was determined by using anti–acetyl H3 antibody. Bottom, DNA input as internal control. D, methylation-specific PCR analysis of RARβ2 in A2058 and HMV-I cell lines was done. DNA from MDA-MB-231 (RARβ2 methylation) and RCC 1.11 (RARβ2 unmethylation) cell lines were used as controls. U, unmethylated; M, methylated.
expression was due to aberrant methylation at the promoter level, the RARβ2 promoter was analyzed by methylation-specific PCR. The results showed that A2058 cell line presented both unmethylated and methylated bands, whereas HMV-I cell line showed only a methylated band (Fig. 2D).

**p21 Status and Modulation by LAQ824 and CRA in A2058 and HMV-I Human Melanoma Cell Lines**

p21 represents a critical checkpoint in cell cycle regulation and its gene expression modulation is considered a hallmark of HDAC inhibition. Thus, we analyzed the status of p21 in the human melanoma cell lines following treatment with LAQ824 and CRA. p21 gene and protein expression was induced by LAQ824 and LAQ824 + CRA in both cell lines as assessed by RT-PCR (Fig. 3A) and Western blot analysis (Fig. 3B), respectively. p14 gene expression was present in HMV-I but not in A2058, whereas p16 and p27 gene expression was present in both cell lines by RT-PCR (data not shown). No modulation of these genes by LAQ824 was observed (data not shown).

**LAQ824 Induces G2 Arrest in A2058 and Apoptosis in HMV-I Cell Line**

The melanoma cell lines were cultured with 0.1 to 1.0 μmol/L LAQ824 and/or 10 μmol/L CRA and assayed for cell cycle analysis at different time points by the PI staining method. Combination treatment with 1.0 μmol/L LAQ824 and CRA for 24 h induced A2058 cell line in G2-M phase (32% in CRA + LAQ824 versus 24.2% in medium only; Fig. 4A). The induction of G2-M phase was also dose dependent following 72 h of treatment (Fig. 4B). HMV-I cells were induced in sub-G0 phase (33.5% in 1 μmol/L CRA + LAQ824 versus 3.5% in medium only; Fig. 4A). The induction of sub-G0 phase was also dose dependent following 72 h of treatment (Fig. 4B). Administration of 0.1 to 1.0 μmol/L LAQ824 and/or 10 μmol/L CRA for 24 h induced apoptosis in both melanoma cell lines by Annexin V and PI staining (Fig. 4C). Treatment with 0.5 μmol/L CRA + LAQ824 induced greater apoptosis in HMV-I cells compared with A2058 cells (51.2% and 9.5%, respectively; Fig. 4C). Dead cell fraction (Annexin V+ and PI+ cells) increased in a dose-dependent manner following 72 h of treatment (Fig. 4D). These results were consistent with the cell cycle analysis data.

**LAQ824 Restores Retinoid Sensitivity in RARβ2-Negative Cell Lines In vivo**

To determine the effect of LAQ824 and CRA on in vivo tumor growth, melanoma cells were injected s.c. in nude mice. Once the tumors were established, animals received either control vehicle, CRA (20 mg/kg/d), LAQ824 (10 mg/kg/d), or combination. Tumor weight analysis showed that treatment with LAQ824 had a significant inhibitory effect on both A2058 and HMV-I tumor growth (Fig. 5A). CRA had no significant inhibitory effect. Combination of LAQ824 and CRA had a greater inhibitory effect than LAQ824 alone. Then, we assessed the pharmacodynamic effect of this combination. Protein extracts from HMV-I tumor samples were analyzed for histone acetylation. Animals treated with either LAQ824 or combination presented increased tumor histone acetylation (Fig. 5A). We also did TUNEL immunofluorescence in tumor sections counterstained with 4',6-diamidino-2-phenylindole to quantify the percentages of in vivo apoptosis. The results confirmed that LAQ824 and the combination of LAQ824 and CRA stimulated significant increases in apoptosis in HMV-I but not in A2058 xenografts (Fig. 6A). Staining for the proliferation marker Ki-67 in the same sections showed that combination treatment produced a decrease in proliferation signal in HMV-I but not in A2058 tumors.

**Exposure to LAQ824 and CRA Induces Reactive Oxygen Species Production in HMV-I but not in A2058 Cells**

HDAC inhibitors have been reported to induce reactive oxygen species (ROS) production in transformed cells (27). HMV-I cells cultured with 1.0 μmol/L LAQ824 and 10 μmol/L CRA for 24 h revealed a greater accumulation of ROS than cells cultured with single agents (Fig. 7A). Treatment with NAC markedly diminished ROS production (Fig. 7A). Consistent with these findings, NAC significantly blocked CRA/LAQ–mediated apoptosis in HMV-I cells (Fig. 7B). A2058 cells cultured with 1.0 μmol/L LAQ824 and 10 μmol/L CRA for 24 h had no detectable increased accumulation of ROS compared with cells cultured in absence of any agents (Fig. 7A).

**LAQ824 Induces SM22 Expression in HMV-I but not A2058 Cell Line**

Based on previous evidence of the role of actin dynamics in regulating ROS generation (28), we tested the hypothesis whether the different proapoptotic effect of combination of LAQ824 and CRA in the two melanoma cell lines was attributable to modulation of specific genes linked to cytoskeleton organization. The SM22/transgelin gene has homology with the actin-bundling protein Sc1p linked to the aging process in yeasts (29). Thus, we assessed SM22 gene expression modulation in the human melanoma cell lines by RT-PCR analysis. A2058 and HMV-I cell lines were treated with 1.0 μmol/L LAQ824 in the presence or absence of 10 μmol/L CRA for 24 h. RT-PCR analysis revealed induction of SM22 by LAQ824 and/or CRA in HMV-I (Fig. 8A and B). No significant modulation was observed in A2058 cells treated with LAQ824 and CRA.
Figure 4. Effect of LAQ824 and CRA on cell cycle in melanoma cell lines. A2058 and HMV-I cells treated with 1.0 μmol/L LAQ824 and/or 10 μmol/L CRA for 24 h. Cell cycle analysis (A) and apoptosis assessment (C) by flow cytometry were done. Cell cycle analysis (B) and apoptosis assessment (D) by flow cytometry were repeated following 72 h of treatment with increasing LAQ824 doses. Points, mean percentages; bars, SE.
Discussion

In this study, we reported that the loss of RARβ2 gene expression in two human melanoma cell lines was associated with histone hypoacetylation and methylation at the promoter level and retinoid resistance. Treatment with the hydroxamic acid derivative HDAC inhibitor LAQ824 increased histone 3 acetylation at the RARβ2 promoter level and induced reexpression of RARβ2 in the presence of CRA. Restoration of RARβ2 expression in these melanoma cell lines was associated with a greater inhibitory effect of the combination of LAQ824 with CRA on tumor growth both *in vitro* and *in vivo* compared with single agents.

Figure 5. Effect of combination of LAQ24 and CRA on HMV-I and A2058 tumor growth *in vivo*. A, animals bearing established A2058 and HMV-I tumors were treated with either vehicle, CRA (20 mg/kg/d), LAQ824 (10 mg/kg/d), or combination for 2 wks. Columns, mean tumor weight; bars, SE. *, P < 0.05 versus vehicle control; **, P < 0.05 versus single agents. B, HMV-I tumor samples from mice treated with LAQ824, CRA, and combination were analyzed for protein expression of acetyl H3 and α-tubulin (loading control). Protein samples from two control and three each of the treatment groups.

Figure 6. Effect of combination of LAQ24 and CRA on HMV-I and A2058 apoptosis and cell proliferation *in vivo*. A, representative TUNEL-stained and anti-Ki-67–stained sections obtained from A2058 and HMV-I tumor-bearing animals treated with LAQ24, CRA, or combination. Green, TUNEL staining; blue, 4′,6-diamidino-2-phenylindole (total cell nuclei); brown, anti-Ki-67 staining. B, quantitative analysis of *in vivo* tumor cell apoptosis and proliferation. Columns, mean percentage of TUNEL-positive cells and proliferation marker Ki-67–positive cells of four independent fields per tumor slide; bars, SE. *, P < 0.05 versus control; **, P < 0.05 versus single agents.
Retinoids have been found to be ineffective against melanoma in clinical trials, and several human melanoma cell lines have shown resistance (30, 31). As reported in other tumor types, we hypothesized that the epigenetic silencing of \( RAR_b^2 \) gene expression may be in part responsible for retinoid resistance in melanoma. Recent studies have documented the presence of a hypermethylated \( RAR_b^2 \) promoter in tumor and blood samples from melanoma patients (32, 33). In a recent report, the combination of the HDAC inhibitor sodium butyrate and RA synergistically activated transcription of a RA-dependent reporter gene in a RA-sensitive murine melanoma cell line but not in a human RA-resistant cell line (34). The authors concluded that HDAC inhibitors may enhance the activity of RA in RA-responsive melanoma cells. Our results suggest for the first time that a specific HDAC inhibitor may restore sensitivity in human RA-resistant melanoma cell lines both in vitro and in vivo. The restored sensitivity to

![Figure 7](image1)

**Figure 7.** Effect of combination of LAQ824 and CRA on ROS generation. A, A2058 and HMV-I cells were incubated with 1.0 \( \mu \text{mol/L} \) LAQ824 and/or 10 \( \mu \text{mol/L} \) CRA for 24 h ± the free radical scavenger NAC (24 mmol/L). Following the incubation, cells were labeled with an oxidative-sensitive dye (dichlorodihydrofluorescein diacetate) and detected on the Fluorescence channel of a Beckman Coulter Multimode Detector. As controls, cells were treated with 10 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \) for 50 min and analyzed in parallel. *, \( P < 0.05 \) versus control; **, \( P < 0.05 \) versus single agents. B, the effect of increasing concentrations of LAQ824 in the presence of CRA on sub-\( G_0 \) fraction in the presence of the scavenger NAC was assessed. HMV-I cells were treated for 24 h with 0.01 to 1.0 \( \mu \text{mol/L} \) LAQ and CRA (10 \( \mu \text{mol/L} \)) in the absence or presence of 24 mmol/L NAC. *, \( P < 0.05 \) versus NAC.

**Figure 8.** Effect of LAQ824 on \( SM22 \) gene expression in melanoma cell lines. \( SM22 \) and \( GAPDH \) (internal control) gene expression in A2058 and HMV-I melanoma cells treated with LAQ824 (1 \( \mu \text{mol/L} \)), CRA (10 \( \mu \text{mol/L} \)), or combination was analyzed by RT-PCR (A) and quantitative real-time PCR (B) as described in Materials and Methods.

![Figure 8](image2)
retinoid was associated with reexpression of epigenetically repressed RARβ2. We are currently investigating whether RARβ2 reexpression is associated with restoration of RA-dependent gene activation.

HDAC inhibitors have been reported to have antitumor activity in melanoma preclinical models (35–38). Most of the cultured melanoma cells undergo apoptosis following treatment with the HDAC inhibitors, such as trichostatin A, FK-228, and valproic acid via a mitochonrdial and caspase-dependent pathway. These agents have been shown to induce growth arrest in several tumor cell types by affecting different phases of the cell cycle (39). Most of the HDAC inhibitors cause growth arrest in G1. Treatment of untransformed human fibroblasts with HDAC inhibitors have been reported to induce G2 checkpoint, which caused cell cycle arrest with little or no cytotoxicity (40). The growth arrest has been reported to be mediated by p53-independent induction of p21WAF1/CIP1, loss of activity of cycle-dependent kinases, and transcriptional inactivation of CTP synthesis in S phase (41–43). G2 arrest has been detected in some tumor cell lines and, in general, requires higher dose of HDAC inhibitors than G1 arrest (41, 43). Cells with an intact G2 checkpoint are growth arrested by HDAC inhibitors, whereas cells with a defective G2 checkpoint undergo apoptosis within hours from the mitotic exit (44). Recent studies have shown that Gadd45 expression induced by the HDAC inhibitor trichostatin A causes cell cycle arrest at the G2-M transition phase (45). The molecular events responsible for HDAC inhibitor–induced G2 checkpoint remain unclear (39).

Tumor cell death has been reported to be induced by HDAC inhibitors via generation of ROS (46). ROS production leads to activation of caspase cascade and degradation of critical proteins, such as p21CIP1/WAF1, p27Kip1, Bcl-2, and pRb. LAQ824 at low concentrations has been shown to trigger cell cycle arrest in G1 phase, relatively delayed generation of ROS, and cellular maturation in leukemia cells, whereas at higher concentrations induced apoptosis associated with early ROS generation, G2-M arrest, and generation of ceramide (47). In an acute T-cell leukemic cell line, suberylanilide hydroxamic acid has also been shown to induce cell death pathway acting via cleavage of Bid and production of ROS (48). HDAC inhibitors have been reported to activate both the death-receptor and the intrinsic apoptosis pathway (46). Specific either genetic or epigenetic defects affecting regulation of the cell cycle and apoptosis may be involved. In our study, equivalent doses of LAQ824 and CRA induced A2058 cells to undergo primarily G2-M arrest, whereas HMV-I cells underwent apoptosis via generation of ROS. We observed a minimal induction of apoptosis in A2058 cell line with combination of LAQ824 and CRA (up to 19%). No differences in caspase-3 activity on treatments were observed between A2058 and HMV-I cell lines (data not shown). The combination treatment induced also p21 gene and protein expression in both cell lines. The mechanism responsible for the difference in sensitivity to combination-induced lethality between the two cell lines remains to be elucidated. Preclinical results suggest that generation of ROS is necessary but not sufficient for HDAC inhibitor–induced lethality in transformed cells (46, 47). The increased induction of ROS induced by LAQ824 and CRA in HMV-I cells compared with A2058 translated into higher degree of apoptosis both in vitro and in vivo and in some extent greater antitumor activity in vivo. Identification of the molecular mechanisms responsible for this susceptibility to ROS induction and consequent lethality will be critical for the optimal clinical development of HDAC inhibitors.

Induction of the cell cycle inhibitors p16 and p21 and high levels of ROS have been linked to programmed cell death pathways and replicative senescence (49, 50). Replicative senescence plays an important role in maintaining tissue integrity and is impaired during tumorigenesis (51). Recent reports have suggested that changes in the dynamics of the actin cytoskeleton are associated with senescence, ROS release from mitochondria, and subsequent cell death (52). Increased actin turnover, which can be induced by gene mutation or by deleting the gene for the actin-bundling protein Scp1, leads to decreased production of ROS and to increased cell viability (53). Homology between Scp1 and mammalian SM22/transgelin, which itself has been isolated in senescence screens and once deleted can increase life span, suggests a conserved mechanism linking aging to actin stability (54, 55). HDAC inhibitors have been shown recently to induce premature senescence in normal human fibroblast (55). Reduced HDAC1 expression level in senescent cells has been reported to mediate the transition to senescent phenotype. Interestingly, overexpression of HDACs has been reported to decrease, whereas trichostatin A treatment stimulates SM22 promoter activity in smooth muscle cells (56). Chromatin immunoprecipitation assay showed that trichostatin A treatment induces chromatin hyperacetylation in the SM22 gene. Our results showed that SM22 gene expression was induced by LAQ824 + CRA treatment in HMV-I cell line, which underwent apoptosis via ROS production but was not modulated in the A2058 cell line, which did not present increased ROS generation. The free radical scavenger NAC blocked LAQ824 + CRA–mediated ROS generation and apoptosis, suggesting a primary role for oxidative injury in LAQ824 + CRA lethality. Our data also suggest that SM22 induction by LAQ824 + CRA may be associated with the difference in biological effects between HMV-I and A2058 cell lines. Additional experiments are needed to determine the possible role of SM22 gene and protein expression and actin dynamics in HDAC inhibitor–induced lethality.

Several HDAC inhibitors are currently in clinical trials both in solid and hematologic malignancies (57). Some studies have already provided important information on the pharmacodynamics of these novel agents (58, 59). Preliminary reports have also shown promising clinical activity in different tumor types, including melanoma (60). Based on preclinical studies, several rational strategies are being developed with combinations of HDAC inhibitors and either molecular targeted or standard therapies.
In summary, this study reports for the first time that retinoid sensitivity can be restored in retinoid-resistant melanoma by a targeted therapy with RARβ2 agonists and chromatin remodeling drugs. This therapeutic approach induced promoter epigenetic changes at expression RARβ2 promoter. Inducible RARβ2 expression may represent a rational predictor for tumor response in patients undergoing 'differentiation' therapy with the combination of a HDAC inhibitor and a retinoid. The clinical success of HDAC inhibitors will require rational combination strategies with other compounds that may enhance the cell cycle blocking and proapoptotic activity of this novel class of agents. A Cancer Therapy Evaluation Program-National Cancer Institute–sponsored phase I clinical study of the HDAC inhibitor MS-275 in combination with CRA in metastatic progressive cancer is currently accruing patients at our institution.

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References


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