Rapid Alteration of MicroRNA Levels by Histone Deacetylase Inhibition

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Abstract

Improved understanding of the molecular mechanisms by which small-molecule inhibitors of histone deacetylases (HDAC) induce programs, such as cellular differentiation and apoptosis, would undoubtedly assist their clinical development as anticancer agents. As modulators of gene transcript levels, HDAC inhibitors (HDACi) typically affect only 5% to 10% of actively transcribed genes with approximately as many mRNA transcripts being up-regulated as down-regulated. Using microRNA (miRNA) array analysis, we report rapid alteration of miRNA levels in response to the potent hydroxamic acid HDACi LAQ824 in the breast cancer cell line SKBr3. Within 5 hours of exposure to a proapoptotic dose of LAQ824, significant changes were measured in 40% of the >60 different miRNA species expressed in SKBr3 cells with 22 miRNA species down-regulated and 5 miRNAs up-regulated. To explore a potential functional link between HDACi induced mRNA up-regulation and miRNA down-regulation, antisense experiments were done against miR-27a and miR-27b, both abundantly expressed and down-regulated in SKBr3 cells by LAQ824. Correlating a set of genes previously determined by cDNA microarray analysis to be rapidly up-regulated by LAQ824 in SKBr3 with a database of potential 3' untranslated region miRNA binding elements, two genes containing putative miR-27 anchor elements were identified as transcriptionally up-regulated following miR-27 antisense transfection: ZBTB10/RINZF, a Sp1 repressor, and RYBP/DEDAF, an apoptotic facilitator. These findings emphasize the importance of post-transcriptional mRNA regulation by HDACi in addition to their established effects on promoter-driven gene expression. (Cancer Res 2006; 66(3): 1277-81)

Introduction

The small-molecule inhibitors of class I and II histone deacetylases (HDAC) have lately received much attention due to their promising clinical application as antimtumor agents (1–3). By altering the acetylation status of an array of substrates, including histones, transcription factors, and chaperone proteins, HDAC inhibitors (HDACi) reprogram the cellular machinery to induce such processes as cell cycle arrest, differentiation, and apoptosis (1–3). This redirecting of cellular processes largely results from alteration of the transcriptional profile where genes such as p21, which promotes cell cycle arrest, are up-regulated, whereas genes, such as HER2/ERBB2, which promotes a mitogenic response, are down-regulated (3). However, there is still little mechanistic understanding of why only 5% to 10% of transcribed genes are perturbed by an effective HDACi dose with an approximately equal number of transcripts being either elevated or suppressed. Thus, to explore additional avenues of HDACi influence, changes to the microRNA (miRNA) expression profile of the breast cancer cell line SKBr3 following treatment with the potent hydroxamic acid HDACi, LAQ824, were examined. As small noncoding RNAs, miRNAs are processed from an initial stem-loop structure of ~70 nucleotides by several dsRNA-specific endonucleases and ultimately delivered as mature 20- to 25-nucleotide species to RNA-induced silencing complex where they engage in either translational arrest or degradation of targeted transcripts through imperfect base pairing with the 3' untranslated regions (UTR) of the targeted transcripts (4, 5). Their role in malignancies was first realized when molecular characterization of the 13q14 deletion in chronic lymphocytic leukemia identified mir-15a and mir-16-1 genes as the target of this deletion (6). Recently, miRNA profiling has revealed its utility in characterizing various cancers, whereas other studies have underscored that both amplification and deletion of various miRNA loci may play pathogenic roles in malignant progression (7–9). The studies presented here show that a potent HDACi, LAQ824, can rapidly alter cellular miRNA levels in SKBr3 cells. To validate that LAQ842 treatment may modulate cellular miRNA levels through miRNA targeting, an antisense strategy against two LAQ824-down-regulated miRNAs (mir-27a and mir-27b) produced increases in the level of two transcripts also up-regulated by LAQ824.

Materials and Methods

Microarray construction. Printing, postprocessing, and analysis of the microarrays were done as described. At the time of printing, the arrays contained all of the annotated human miRNA genes (~200) listed in the miRNA Registry as of November 2004. Oligonucleotide probes identical to the sense orientation of the mature human miRNA sequences were synthesized by Operon Biotechnologies (Huntsville, AL) and duplicate spotted in 3× SSC on Gold Seal microslides (Becton Dickinson, Bedford, MA) coated with poly-l-lysine using a linear servo arrayer at the University of California at San Francisco Core Facility for Genomics and Proteomics. The probes on these microarrays consisted of tandem repeats or dimers of the mature miRNA sequences printed in duplicate.

Labeling of miRNAs and hybridization. Cell culture lysates were enriched for miRNAs using the mirVana miRNA Isolation kit following the manufacturer's protocol (Ambion, Austin, TX). Samples enriched for miRNA were labeled with the Array900 miRNA kit (Genisphere) according to the

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http://www.sanger.ac.uk/Software/Rfam/mirna/.

http://derisilab.ucsf.edu/microarray/.
manufacturer protocol. Briefly, 200 ng enriched miRNA was used in the initial tailing reaction. Four replicates of each experimental condition were done. Hybridization was done according to the product literature. The hybridization mix containing the Cy3- and Cy5-tagged miRNA was hybridized to the array under a glass coverslip at 46°C for 16 hours in a Hybex hybridization oven (SciGene, Sunnyvale, CA). After the washing procedure, the arrays were scanned with a GenePix 4000B Scanner (Axon, Foster City, CA) and raw pixel intensities were extracted with Axon software.

Bioinformatic and statistical methods. Primary array data have been submitted to Gene Expression Omnibus (accession number GSE3978). Cy3 and Cy5 median pixel intensity values were background subtracted, and Cy3/Cy5 ratios were obtained. Cy3/Cy5 ratios were log transformed (base 2) and hierarchically clustered (average linkage correlation metric) using the Cluster program from Stanford University. Database calculations were done and expression maps were generated with Significance Analysis of Microarrays (SAM) for Excel. The Cy3/Cy5 ratios were compared between the untreated and treated SKBr3 cells in SAM using a one class analysis.

Cells, drug, cDNA probes, and primers. The human breast cancer cell line SKBr3 was obtained from the American Type Culture Collection (Manassas, VA) and maintained in McCoy's medium (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum. LAQ824 was a kind gift from Novartis Pharmaceutical, Inc. (East Hanover, NJ) with 1 mol/L stock solutions kept in DMSO at −20°C protected from light. Primers to amplify the following RNA transcripts were designed against the 5′ UTR and included the following: ZBTB10 (Hs.205742) forward primer 5′-CAATATCCCACTCTACTAATCCGACT-3′ and reverse primer 5′-TGGGACATCATGGAAGAGTC-3′ (229 bp); BTG3 (Hs.181751) forward primer 5′-CTTCTGACAGTGGCAACGACG-3′ (332 bp); PTGER4 (Hs.199248) forward primer 5′-TATGTTGCACTGATCCGTAGTCAGACGCGTTA-3′ and reverse primer 5′-CTGAGTGGTTTGATGGACAGAG-3′ (297 bp); SH3BGRL3 (Hs.109051) forward primer 5′-CAATTGGCTATGTCGATCGCATCACT-3′ and reverse primer 5′-TTCTTCCCCTGCGAGATTTACTG-3′ (313 bp); DUSP5 (Hs.2128) forward primer 5′-GAAACAGCAGAACCAGAGCAG-3′ and reverse primer 5′-methyl RNA oligonucleotides used for miRNA antisense degradation (13). Thus, SKBr3 cells were transfected with antisense 2′-O-methyl RNA probes against miRNAs effectively inhibited their suppression. Previous studies have established that antisense miR treatment. As shown in Fig. 1B, drug treatment. As shown in Fig. 1C, the array of miRNAs to the HDACi LAQ824.

Results

Rapid alteration of miRNAs levels following treatment with the HDACi LAQ824. To assess the response of miRNAs to the HDACi LAQ824, the expression profile of miRNAs from the breast cancer cell line SKBr3 following 5 hours of 20 μmol/L treatment was determined by miRNA microarray analysis. By selecting the relatively early time point of 5 hours for analysis, only primary events initiated by HDACi treatment would influence results. Figure 1D displays the relative Cy3 intensity levels, averaged over the four separate miRNA microarrays used in these experiments, of the 67 different miRNAs whose expression levels were detected significantly over background. As shown in the Fig. 1D heat map, following LAQ824 treatment, the expression level of many SKBr3 miRNAs were altered with 22 miRNAs down-regulated and 5 miRNAs up-regulated. To validate these miRNA microarray results, Northern analysis for several of the more abundantly expressed miRNAs was done. As shown in Fig. 1C, Northern blots showing the up-regulation of miR-320 and the down-regulation of let7a and miR-27a following LAQ824 treatment confirmed the miRNA microarray results for these miRNAs.

To place these miRNA alterations in the context of a phenotypic response induced by LAQ824, inspection of SKBr3 cellular morphology clearly suggested progression toward apoptosis with membrane blebbing and cellular collapse culminating 20 hours of drug treatment. As shown in Fig. 1D, the appearance of caspase-3-mediated PARP1 cleavage, first evident following 5 hours of treatment, increased substantially following 20 hours of 20 μmol/L LAQ824.

Identification of miRNA transcripts up-regulated by antisense miR-27 treatment. Recent experiments have clearly documented the ability of a miRNA to promote the degradation of miRNAs bearing partial complementary sites to the miRNA in their 3′ UTR (5, 11, 12). To establish a functional link between LAQ824-induced changes to miRNA and mRNA levels, we sought to detect the up-regulation of selected miRNA transcripts resulting from miRNA suppression. Previous studies have established that antisense 2′-O-methyl RNA probes against miRNAs effectively inhibited their activity and blocked small interfering RNA-mediated miRNA degradation (13). Thus, SKBr3 cells were transfected with antisense 2′-O-methyl RNA probes against miRNAs 27a and 27b by PCR using the reverse transcription-PCR (RT-PCR) purified product bands with the respective reverse primer. Autoradiograph band intensities were quantified using a GS-710 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA).

Western analyses of poly(ADP-ribose) polymerase cleavage. The Western blot was prepared, probed, and detected as described previously (10). The antibody to the caspase cleaved p85 fragment of poly(ADP-ribose) polymerase (PARP) was obtained from Promega, Inc. (Madison, WI).

To select mRNA transcripts most likely targeted by both miR-27a and miR-27b, a database of potential miRNA 3′ UTR anchor elements (15) was searched against a list of genes rapidly up-regulated by LAQ824 that was generated from an earlier cDNA array analysis of LAQ824-treated SKBr3 cells (16). From the top 200 up-regulated LAQ824 genes, 14 with putative miR-27 anchor elements were identified. For initial analysis, 6 of these genes with established biological properties were selected and PCR primers directed against their 3′ UTR prepared (see Materials and Methods).

Transfections of SKBr3 cells for 7 hours were done using 2′-O-methyl RNA oligonucleotides complementary to miRNAs 27a and 27b with a scrambled miR-27a 2′-O-methyl RNA oligonucleotide serving as a negative control. The 7-hour transfection time was chosen to be long enough for sufficient cellular uptake of the oligonucleotides yet short enough to simulate the 5-hour LAQ824 treatment period.

Aliquots of the RNA harvested following the transfection period were reversed transcribed and subjected to PCR analysis with inclusion of primers to either GAPDH or GUS as internal normalization controls with successive cycles examined to assure linearity. Of the six putative miR-27 responsive genes examined, two genes, ZBTB10/RINZF (17) and RYBP/DEDAF (18, 19), consistently displayed increased transcript levels in antisense miR-27a and miR-27b transfected cells relative to control transfected cells with results for miR-27a shown in Fig. 2. Also shown in Fig. 2 are RT-PCR results for p21, a gene known to be up-regulated by HDACi but displaying no differential response between antisense and control oligonucleotides.

**Figure 1.** A, profile of miRNA expression in LAQ824-treated SKBr3 cells. The relative Cy3 intensity of the 67 miRNAs from control SKBr3 cells that achieved at least a 10-fold signal above background. B, quantitation of the significant miRNA changes between LAQ824 (LAQ)-treated cells (Cy5) and untreated control cells (Cy3) according to SAM analysis. Results for the four array slides used in the experiment with colors indicative of relative signal intensities (red, significantly higher in treated cells; green, significantly lower in treated cells in comparison with untreated cells). Right, relative change in expression averaged over the four arrays. C, Northern blots validating array results for miR-320, which is up-regulated, and let7a and miR-27a, which are both down-regulated. Blots were normalized with a probe for arginine tRNA. In addition to the mature miRNAs, the Northern blots also detected the pre-miRNAs as indicated. D, Western blot of nuclear extract from control (C) and 5- and 20-hour LAQ842 (20 μM)–treated SKBr3 cells probed with an antibody to the p85 PARP fragment.

**Figure 2.** RT-PCR showing that RYBP and ZBTB10 expression is up-regulated in 2′-O-methyl RNA antisense miR-27a-transfected SKBr3 cells. A, RT-PCR results for RYBP relative to GUS for SKBr3 cells transfected with antisense 27a oligonucleotides or control (C1) oligonucleotide (left) and SKBr3 treated for 5 hours with 20 μM LAQ824 (+) or vehicle control (--; right). The ratio of RYBP to GUS in the 27a antisense lane and LAQ824 (+) lane were normalized by the value obtained from the control lane and LAQ824 (--) lane, respectively. B, RT-PCR results for ZBTB10 as in (A). C, RT-PCR results for BTG1 as in (A). D, RT-PCR results for p21 using GAPDH as the internal normalizing control as in (A).
and BTG1, another candidate miR-27 responsive gene that, although up-regulated by LAQ824, showed no significant response to antisense miR-27 treatment.

Northern blots confirmed the up-regulation of RYBP/DEDAF and ZBTB10/RINZF transcripts by antisense treatment. As shown in Fig. 3, although RYBP/DEDAF, ZBTB10/RINZF, and BTG1 were all up-regulated by LAQ824, only RYBP/DEDAF and ZBTB10/RINZF were also up-regulated by antisense miR-27 treatment relative to control treatment when normalized against GAPDH. To directly compare the up-regulated RYBP/DEDAF and unchanged BTG1 transcript levels, the Northern blot shown in Fig. 3D was probed for both transcripts and quantified by densitometry to show that antisense miR-27 treatment.

Discussion

Mechanistic understanding of the antitumor programs initiated by HDACi through the transcriptional regulation of key cellular genes, at both transcript and protein levels, remains a challenging problem (1–3). There seems as yet no simple paradigm to explain why inhibition of protein acetylation leaves the transcriptional status of most genes unchanged, although a few undergo extensive reprogramming. The demonstration here that treatment of a breast cancer cell line SKBr3 with a proapoptotic dose of the HDACi LAQ824 rapidly changes the levels of ~40% of the cell’s expressed miRNA offers new insight into the cellular mechanisms modulated by antitumor HDACi. Furthermore, the identification of two HDACi up-regulated mRNA transcripts, RYBP/DEDAF and ZBTB10/RINZF, whose levels can also be up-regulated by antisense suppression of miRNAs 27a and 27b, which are similarly affected by LAQ824, calls greater attention to the post-transcriptional effects of HDACi in mediating their antitumor activity.

Nearly a third of all human genes are estimated to be regulated by miRNAs (4). However, because the bulk of the binding specificity of a miRNA is conferred by the first half-dozen 5′ “seed” nucleotides (4, 15), identifying mRNA targets regulated by specific miRNAs remains a major bioinformatic challenge. Additionally, it is proposed that multiple miRNAs may synergize to coordinately regulate a target transcript (15). Given the observation here that nearly 30 SKBr3 miRNAs were rapidly altered by HDACi treatment, it is not unreasonable to speculate that many HDACi responsive mRNA transcripts are being modulated post-transcriptionally by these altered miRNA levels. Although our demonstration that suppression of a single miRNA was sufficient to up-regulate the ZBTB10 and RYBP transcripts, clearly the level of up-regulation was well below that achieved by HDACi treatment (Fig. 3). In that regard, depletion of miRNAs 27a or 27b did not induce an apoptotic response in the SKBr3 cells following 8 hours of treatment, as did HDACi treatment; moreover, additional apoptotic regulators not under miR-27 control may be affected by HDACi treatment. Thus, it will be of future interest to assess what effect the down-regulation of multiple miRNAs has on prospective mRNA targets. For example, as ZBTB10/RINZF is predicted to contain a let7 target element (15), the down-regulation of both miR-27 and the LAQ824 down-regulated let7 would be anticipated to further enhance ZBTB10/RINZF levels.

The Sanger miRNA Registry currently annotates 321 human miRNAs (release 7.0), a dramatic increase from the ~200 human miRNAs listed a year ago and used as input to our miRNA array. Newer estimates that the human genome may contain ~1,000

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**Figure 3.** Northern blots showing RYBP and ZBTB10 are up-regulated in 2′-O-methyl RNA antisense miR-27a and miR-27b transfected SKBr3 cells. Designated transcript sizes, annotated in Ensemble (http://www.ensemble.org) for RYBP and BTG1 and ref. 17 for ZBTB10, were consistent with their gel migration using the 18S (1.9 kb) and 28S (5.0 kb) ribosomal bands as size markers. A, RYBP Northern results for SKBr3 cells treated for 5 hours with 20 μmol/L LAQ824 (+) compared with vehicle control (−; left) and SKBr3 cells transfected with antisense 27a, antisense 27b, or control (C1) oligonucleotides (right). The ratio of RYBP to GAPDH in the 27a and 27b antisense lanes and the RYBP to GAPDH ratio in the LAQ824 (+) lane were normalized by the value obtained from the control lane and LAQ824 (−) lane, respectively. B, results for ZBTB10 in (A). C, results for BTG1 as in (A). D, Northern blot probed for RYBP and BTG1 with the ratios of RYBP to BTG1 in the 27a and 27b antisense lanes were normalized by the value obtained from the control lane. Results for 5-hour LAQ824 (20 μmol/L)–treated (+) compared with untreated (−) SKBr3 cells (left).
miRNAs with a subset unique to humans (20) underscores the incipient state of our understanding of miRNA-mediated gene regulation. The present observation that miRNAs are rapidly and differentially regulated by a HDACi suggests that miRNAs might serve as targets for therapeutic intervention and indicates that further studies are needed to understand their response to HDACi.

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