Chemical genetic strategy identifies histone deacetylase 1 (HDAC1) and HDAC2 as therapeutic targets in sickle cell disease

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Chemical genetic strategy identifies histone deacetylase 1 (HDAC1) and HDAC2 as therapeutic targets in sickle cell disease

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The worldwide burden of sickle cell disease is enormous, with over 200,000 infants born with the disease each year in Africa alone. Induction of fetal hemoglobin is a validated strategy to improve symptoms and complications of this disease. The development of targeted therapies has been limited by the absence of discrete druggable targets. We developed a unique bead-based strategy for the identification of inducers of fetal hemoglobin transcripts in primary human erythroid cells. A small-molecule screen of bioactive compounds identified remarkable class-associated activity among histone deacetlyase (HDAC) inhibitors. Using a chemical genetic strategy combining focused libraries of biased chemical probes and histone deacetylase (HDAC) inhibitors, we have identified HDAC1 and HDAC2 as molecular targets mediating fetal hemoglobin induction. Our findings suggest the potential of isoform-selective inhibitors of HDAC1 and HDAC2 for the treatment of sickle cell disease.

Results

Development of a Miniaturized Assay for Globin Switching in Human Hematopoietic Precursor Cells. An obstacle limiting the development of novel inducers of fetal hemoglobin has been the need for an effective, miniaturized assay capable of supporting high-throughput screening and lead optimization. We therefore developed a physiologically-relevant, high-throughput system that detects the endogenous expression of seven globin mRNA transcripts in primary human erythroid cells using a fluorescent microsphere detection system (Fig. 1 A and B) (17). In contrast to reporter-essay systems expressed in cell lines, this assay detects expression of the globin genes within their native chromatin structure in the therapeutically relevant cell type.

The assay was validated by measuring expression of the globin genes during erythroid differentiation and in response to known experimental inducers of fetal hemoglobin. We observed the expected coordinate induction of globin gene expression during erythroid differentiation, with higher levels of γ-globin in umbilical cord blood cells compared with adult bone marrow (Fig. 1C). Four well-studied activators of fetal hemoglobin (hydroxyurea, valproic acid, sodium butyrate, and 5-azacytidine), increased the γ/β-globin ratio in a dose-dependent fashion (Fig. 1D) (3, 18–20). These experiments demonstrate that our assay detects relevant changes in globin gene expression.

With an assay in hand, we next performed a small-molecule screen (see Table S1 for details) with particular emphasis on bioactive and FDA-approved compounds that could be most rapidly translated for clinical investigation. Using the γ/β-globin ratio as the primary screening measurement, previously reported inducers of fetal hemoglobin (including hemin, zileuton, rapamycin, chromomycin, mithramycin, and cisplatin) increased the γ/β ratio modestly or only at doses that substantially decreased cell viability (Fig. S1) (8, 20). The corticosteroid triamcinolone also scored highly in the assay (Fig. S1G), consistent with the known effect of corticosteroids in inducing γ-globin (21). Be-
First, we studied an expanded collection of commercial, investigational, and novel HDAC inhibitors. Notably, within this collection were 600 compounds curated from a 7,200-member diversity-oriented synthesis library of 1,3-dioxanes biased for putative HDAC inhibition by one of three metal chelating features: an ω-aminoanilide, carboxylic acid, or hydroxamic acid (26). Molecules were selected for study based on a prior demonstration of histone deacetylase inhibitory activity in a cell-based assay (26). Small molecules demonstrating the most robust γ/β-globin induction were resynthesized or purchased, as required. Several compounds from this collection were selected for further, detailed study because of potent effects on globin gene induction at concentrations permissive to cell growth and viability (Fig. S2C).

To quantify more accurately the magnitude of gene regulation, we examined the effects of compounds on globin gene expression by quantitative RT-PCR. We found that HDAC inhibitors, including NK57, increase the γ/β-globin ratio by up to 10-fold (Fig. 2D). HPLC and flow cytometric analysis of primary human erythroid cells using a fetal hemoglobin-specific antibody demonstrated an increase in both fetal hemoglobin and the percentage of F cells, a population of erythroid cells with particularly elevated levels of fetal hemoglobin, in response to HDAC inhibition (Fig. 2B and C and Fig. S3). In addition, the ε/γ-globin ratio increased by more than 300-fold with scriptaid, and by 20- to 40-fold with other compounds (Fig. 2D), although the absolute level of ε-globin expression remained low (Fig. S4). Given the regulation of ε-globin, we hypothesized that HDAC inhibition might also induce expression of ζ-globin, the embryonic form of α-globin. We found that ζ-globin was indeed induced by HDAC inhibitors by as much as 5- to 10-fold (Fig. 2E). These experiments demonstrate that HDAC inhibition reverses the ontogeny of globin gene expression at the adult, fetal, and embryonic globin genes, at both the α- and β-globin loci, suggesting a central and reversible role of globin locus histone deacetylation in the developmental silencing of globin genes.

To ascertain a possible mechanism by which HDAC inhibitors may increase expression of the globin locus, we examined their effect on the α/βCH, in which the regulatory sequences from the β-globin locus control region (LCR) are brought in proximity to promoters of individual globin genes (8). We employed chromosome conformation capture (3C) to monitor the chromatin structure of this locus in response to compound treatment with two high-scoring HDAC inhibitors, SAHA and NK57 (27). Both compounds induced a conformational change characterized by increased contact between the LCR and the γ- and ε-globin promoters, similar to the chromatin state observed in erythroid cells from umbilical cord blood (Fig. 2F and Fig. S5). Importantly, the compounds did not alter LCR interactions with other regions of the β-globin locus. These results are unique in providing direct evidence for chemical modulation of the structure of the β-globin locus.

**HDAC Inhibitors Induce Fetal Hemoglobin and Modulate Chromatin Structure.** First, we studied an expanded collection of commercial, investigational, and novel HDAC inhibitors. Notably, within this collection were 600 compounds curated from a 7,200-member diversity-oriented synthesis library of 1,3-dioxanes biased for putative HDAC inhibition by one of three metal chelating features: an ω-aminoanilide, carboxylic acid, or hydroxamic acid (26). Molecules were selected for study based on a prior demonstration of histone deacetylase inhibitory activity in a cell-based assay (26). Small molecules demonstrating the most robust γ/β-globin induction were resynthesized or purchased, as required. Several compounds from this collection were selected for further, detailed study because of potent effects on globin gene induction at concentrations permissive to cell growth and viability (Fig. S2C).

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**HDAC1 and HDAC2 Repress γ-Globin Gene Expression.** The experiments described above all point to small-molecule HDAC inhibitors as a potential route to fetal hemoglobin induction for sickle cell disease. However, all clinically-used HDAC inhibitors are perceived to be relatively nonspecific, acting to inhibit multiple class I and II deacetylases (28). It is then conceivable that combinatorial inhibition of HDACs is required for fetal hemoglobin induction. Alternatively, the inhibition of individual HDACs might account for the effect. To resolve this uncertainty, we first correlated the fetal hemoglobin inducing effects of 14 structurally diverse HDAC inhibitors (Fig. S6) with their biochemical HDAC inhibitory activity, using in vitro assays for each of nine deacetylases (HDAC1-9). Hierarchical clustering of these data pointed to a strong correlation between γ-globin induction and biochemical inhibition of HDAC1, HDAC2, and HDAC3, but not the other HDACs (Fig. 3A). Highlighting this observation, a cluster of in-
vestigational benzamides (CI-994, MGCD0103, and MS-275) powerfully activate γ-globin expression but inhibit only HDAC1-3 without observable effects on HDAC4-9 (Fig. 3 B and C and Table S2). In contrast, the HDAC6-selective tool compound tubacin failed to elicit marked induction of γ- and ε-globin at high experimental concentrations (Fig. S2C). These experiments suggest that inhibition of HDAC1-3, either alone or in combination, is sufficient to regulate the globin locus.

To further dissect the role of HDAC1-3 in regulating globin gene expression, we turned to a genetic perturbation strategy. Primary erythroid cells were infected with lentiviruses harboring short hairpin RNAs (shRNAs) targeting individual HDAC transcripts. Knockdown of HDAC1-3 by directed shRNAs was first validated at the RNA and protein levels (Fig. S7). Multiple effective shRNAs targeting HDAC1 and HDAC2 increased the relative expression of γ- and ε-globin, but shRNAs targeting HDAC3 had no effect (Fig. 3 D and E). These experiments functionally validate HDAC1 and HDAC2 as requisite corepressors for full repression of fetal and embryonic globin expression observed in adult erythroid cells.

**Selectivity of HDAC1 or HDAC2 for Fetal Hemoglobin Induction.** Current clinical and investigational HDAC inhibitors are limited in their broad utility by class-associated side effects, such as fatigue, malaise, thrombocytopenia, and cardiotoxicity. Whereas pharmaceutical HDAC inhibitors (SAHA and LBH-589) exhibited a dose-dependent induction of bulk chromatin acetylation, selective knock-down of HDAC1 or HDAC2 failed to elicit patterns of nuclear hyper-acetylation, potentially indicating at least partial reducency of these factors in global histone acetylation (Fig. 4/4). Notably, all perturbations induced fetal hemoglobin to comparable levels.

These data establish regulation of the globin locus by HDAC1 and HDAC2, irrespective of global hyperacetylation, a pharmacodynamic feature associated with cytotoxicity of HDAC inhibitors. To extend these specificity studies and further explore the transcriptional networks regulated by HDAC1 and HDAC2, we performed global gene-expression profiling. Using three distinct HDAC1 shRNAs and three HDAC2 shRNAs to minimize the off-target effects of individual shRNAs, we identified both distinct and overlapping effects of HDAC1 and HDAC2 knockdown. We defined three gene sets: genes responsive to HDAC1 shRNAs only, genes responsive by HDAC2 shRNAs only, and genes modulated by either HDAC1 or HDAC2 shRNAs (Fig. 4B). Using gene-set enrichment analysis, we confirmed that NK57, an HDAC inhibitor with high potency (IC$_{50}$ 30 nM) for HDAC1-3 (Table S2), powerfully activated all three gene sets (Fig. 4B). The HDAC1- and HDAC2-responsive genes comprise a minority of...
the gene expression consequences of NK57 or SAHA treatment (Fig. 4C), indicating that selective inhibition of HDAC1 or HDAC2 would be expected to activate fetal hemoglobin with a markedly reduced effect on global gene expression.

**Induction of Fetal Hemoglobin in Human Subjects with LBH-589.** Despite the compelling activity of HDAC inhibitors in cell-culture systems, it is possible that HDAC inhibitors could fail to induce fetal hemoglobin expression when tested in vivo, because of pharmacologic considerations. To address this issue, we first profiled two well-established, pharmacologic inducers of \( \gamma \)-globin for inhibitory potency against the panel of biochemically-active human histone deacetylases (HDAC1-9), using the optimized HDAC assay platform. Although markedly less potent than current pharmaceutical agents, sodium butyrate and valproate demonstrate a clear preference for class I enzymes (HDAC1, HDAC2, HDAC3, and HDAC8) and dose-dependent inhibition of HDAC1 and HDAC2 well within pharmacologically-achievable concentrations (Fig. 5A and B).

The characterization of these agents as HDAC inhibitors was discerned decades after the phenotypic identification of these compounds as clinically active substances. Modern HDAC inhibitors have emerged through biomarker-directed drug development using biochemical assays to drive the potency of clinical candidates. Exemplifying these principles is LBH-589 (panobinostat; Novartis Pharmaceuticals) (29). In comparative biochemical studies, we observe greater than 1,000-fold increase in potency for HDAC1 (IC\(_{50}\) 0.5 nM) and HDAC2 (IC\(_{50}\) 2 nM) compared with either sodium butyrate or valproate (Fig. 5B and Table S2).

We explored the effect of HDAC1 and HDAC2 inhibition on fetal hemoglobin production in three adult patients with Hodgkin lymphoma treated with LBH-589 in the context of a prospective clinical trial. Serial hemoglobin electrophoresis before and after treatment indicated that fetal hemoglobin increased by approximately 2-fold in all patients examined over 3 mo of therapy (Fig. 5C). Notably, this magnitude of fetal hemoglobin induction has previously been associated with amelioration of symptoms and complications of sickle cell disease (5).

**Discussion**

Taken together, our results demonstrate that HDAC1 and HDAC2 are highly attractive therapeutic targets for sickle cell disease, establishing a compelling rationale for the further chemical optimization and preclinical development of isoform-selective...
small molecule HDAC1 and HDAC2 inhibitors. Small molecules that inhibit these HDAC isoforms, and shRNAs that decrease expression of their genes, powerfully induce the expression of γ- and ε-globin. Moreover, we found that selective knockdown of an individual HDAC is capable of inducing fetal hemoglobin without alterations in global histone acetylation, with less extensive effects on global gene expression, and without inhibiting the cell cycle.

Recent human genetic studies support a role for HDAC1 and HDAC2 as regulators of the globin locus. A genome-wide association study searching for regulators of fetal hemoglobin identified BCL11A as a candidate (12, 13, 15), and a follow-up study of BCL11A observed direct interaction with HDAC1 and HDAC2 by coimmunoprecipitation (14). Genetic ablation of BCL11A increases the expression of γ-globin in vitro and in vivo (14, 16). The convergence of our chemical genetic approach with human genetics studies heightens the candidacy of HDAC1 and HDAC2 for future drug development efforts.

The molecular basis of sickle cell disease is well understood, yet drug development has been limited by the absence of specific therapeutic targets. Using a strategy which integrates forward chemical genetics, enzymology, and systematic reverse genetics by RNA interference, we have identified a corepressor function for HDAC1 and HDAC2 in the regulation of the γ- and ε-globin loci. We demonstrate functionally that high potency inhibitors of these targets up-regulate fetal hemoglobin in vitro and in human subjects. The development of small molecules specific to HDAC1 or HDAC2 has the potential to generate an increasingly effective, well-tolerated therapy for sickle cell disease. More immediately, HDAC inhibitors that are currently in clinical trials for other diseases represent promising candidates for immediate clinical development, alone or in combination with hydroxyurea.

Methods

Culture of Primary CD34+ Cells. Cryopreserved human bone marrow CD34+ cells were obtained from Cambrex (Poeitics, Cambrex). Umbilical cord blood was harvested at Brigham and Women’s Hospital under an institutional review board-approved protocol. Erythroid differentiation was induced in vitro in two steps. For the first 7 d, cells were cultured in serum-free expansion medium (Stem Cell Technologies) supplemented with 100 U/mL penicillin/streptomycin, 2 mM glutamine, 100 ng/mL stem cell factor, 10 ng/mL interleukin-3, 40 µg/mL lipids, and 0.5 µM/L erythropoietin (Epo). After 7 d, cells were cultured in the same medium supplemented with 3 µg/mL Epo.

RNA capture, reverse transcription, and LMA were performed as described previously (17, 31). See SI Methods for details.

Viability. As an assessment of cytotoxicity, samples from screens were analyzed with the CellTiter-Glo bioluminescent assay (Promega).

Western Blots. Western blots were performed as described previously. Antibodies against HDAC1, HDAC2, HDAC3, and β-actin were purchased from Santa Cruz Biotechnology.

F-Cell Quantitation. For F-cell assays, 2 to 4 × 10^5 cells were harvested and fixed with 0.05% glutaraldehyde for 10 min at room temperature. Cells were washed and labeled with an anti-HbF monoclonal antibody (Caltag Laboratories). For each sample, 50,000 events were detected using a FACScan flow cytometer (Becton Dickinson).
Chromatographic Separation of Hemoglobin Chains. Erythroid cell hemolysates were prepared as described previously (32). Chromatographic separation of the globin chains was performed on an Agilent 1100 Capillary LC system at 45 °C with a Jupiter 300 A C4 reversed-phase column 5 μ, 250 × 0.5 mm (Phenomenex), using Buffer A: 0.108% TFA, and Buffer B: 80% acetonitrile/0.07% TFA at 9 μL/min over 140 min. Cell lysates were acidified with 0.1% TFA before reverse-phase chromatography.

The 3C was performed as described (33). A control PCR template was generated by EcoRI digestion and random ligation of a series of BAC clones covering the j-globin region, as well as a region on chromosome 16 (as described in Dostie et al. (34)). See SI Methods for details.

Biochemical Profiling of HDACs. The inhibition of HDAC proteins (HDAC1-9) was determined using a homogenous, kinetic assay, as reported previously (30). Calculation of IC50 is determined by logistic regression (GraphPad Prism Software).

Gene-Expression Profiling. RNA was purified from mononuclear cells using TRizol (Invitrogen). Linear amplification of 20 ng of total RNA was performed using the Ovation Biotin RNA Amplification and Labeling Nucleus (Nugen). Labeled RNA was hybridized to Affymetrix HG_U133A oligonucleotide microarrays. Raw expression values were normalized using robust multivariate averaging. Markers of HDAC1 or HDAC2 knockdown were identified using the signal-to-noise metric, selecting probe sets with P ≤ 0.1 and greater than 1.5 mean fold-change. The dataset is available at GEO, accession GSE22369.

Real-Time RT-PCR. RNA was reverse transcribed using SuperScript II (Invitrogen) using oligo(dT) primers. TaqMan primers and probes for real time PCR were obtained from Applied Biosystems. Each quantitative RT-PCR was performed in triplicate using a Prism 7900 HT instrument (Applied Biosystems). The mean threshold cycle (Ct) for each assay was used for further calculations. The expression of γ, ε, and δ globin were normalized to β-globin (ΔΔCt). The ΔΔCt value was calculated by normalizing the ΔCt value to a vehicle-treated control sample.

Statistical Analysis. The signal-to-noise metric was used to identify marker genes in microarray experiments and to analyze chromatin configuration capture data. For gene x, the signal to noise metric, $S_x$, is calculated as: $S_x = (x_0 - μ)/σ = μ + σ$, where $x_0$ and $σ$ are the mean and SD for gene x in class 0, and $μ$ and $σ$ are the respective values for class 1.

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