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Identification of Two New Synthetic Histone Deacetylase Inhibitors That Modulate Globin Gene Expression in Erythroid Cells from Healthy Donors and Patients with Thalassemia[S]

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ABSTRACT

We have identified two new histone deacetylase (HDAC) inhibitors (9 and 24) capable of inducing the expression of γ-globin and/or β-globin promoter-driven reporter genes in a synthetic model of Hb switch. Both compounds also increased, with different mechanisms, the γ/(γ+β) ratio expressed in vitro by normal human erythroblasts. Compound 9 increased the levels of γ-globin mRNA and the γ/(γ+β) ratio (both by 2-fold). Compound 24 increased by 3-fold the level of γ-globin and decreased by 2-fold that of β-globin mRNA, increasing the γ/(γ+β) ratio by 6-fold, and raising (by 50%) the cell HbF content. Both compounds raised the acetylation state of histone H4 in primary cells, an indication that their activity was mediated through HDAC inhibition. Compounds 9 and 24 were also tested as γ/(γ+β) mRNA inducers in erythroblasts obtained from patients with β^0 thalassemia. Progenitor cells from patients with β^0 thalassemia generated in vitro morphologically normal proerythroblasts that, unlike normal cells, failed to mature in the presence of EPO and expressed low β-globin levels but 10 times higher-than-normal levels of the α hemoglobin-stabilizing protein (AHSP) mRNA. Both compounds ameliorated the impaired in vitro maturation in β^0 thalassemic erythroblasts, decreasing AHSP expression to normal levels. In the case of two patients (of five analyzed), the improved erythroblast maturation was associated with detectable increases in the γ/(γ+β) mRNA ratio. The low toxicity exerted by compounds 9 and 24 in all of the assays investigated suggests that these new HDAC inhibitors should be considered for personalized therapy of selected patients with β^0 thalassemia.

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In mammals, the development of erythroid cells is characterized by sequential ontogenetic hemoglobin (Hb) switches. In man, the first embryonic-to-fetal Hb (HbF) switch occurs after 2 months of gestation and is followed by a switch from HbF to adult Hb, which begins at mid-gestation and is completed 6 months after birth. HbF expression is retained by a minority (1–3%) of normal adult red cells (Stamatoyannopoulos and Grosveld, 2001).

β Thalassemia and sickle-cell anemia are inherited genetic disorders, both arising from mutations in one of the genes, β-globin, that encode adult Hb. Sickle-cell anemia is due to a missense mutation leading to glutamate-valine substitution at position 6 of the β-globin chain. β Thalassemia may be traced to numerous genetic mutations resulting either in loss

ABBREVIATIONS: Hb, hemoglobin; HbF, fetal hemoglobin; EPO, erythropoietin; AHSP, α hemoglobin-stabilizing protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACI, histone deacetylase inhibitor; SAHA, suberoylanilide hydroxamic acid; MS-275, pyridin-3-ylmethyl *N*-[[4-[(2-aminophenyl)carbamoyl]phenyl]methyl]carbamate; APHA, aroyl pyrrolyl hydroxyamide; UBHA, uracil-based hydroxyamide; μLCR, micro locus control region; R, *Renilla reniformi*; F, firefly; DMSO, dimethyl sulfoxide; AFU, arbitrary fluorescence units; HBSS, Hanks' balanced salt solution; PCR, polymerase chain reaction; IP, immunoprecipitation; HEMA, human erythroblast massive amplification.

(more precisely defined β^0-) or reduced β-globin expression (Olivieri, 1999). Patients with β thalassemia or sickle-cell anemia are healthy until birth, when their red cells contain HbF. Furthermore, patients with β thalassemia or sickle-cell disease that coinherit genetic mutations allowing the retention of HbF production postnatally (hereditary persistence of fetal hemoglobin syndrome), have no or mild clinical phenotype (Olivieri, 1999). These observations, and additional clinical studies, have allowed us to calculate that pharmacological re-activation of γ-globin production up to ~10 to 20% of β-globin levels in adult red cells would be sufficient to ameliorate the symptoms of sickle-cell disease and β thalassemia (Noguchi et al., 1988).

Numerous studies have been undertaken to identify HbF inducers for the cure of hemoglobinopathies. In primates, HbF has been induced by cell cycle-specific cytotoxic drugs [cytosine arabinoside (Papayannopoulou et al., 1984) and hydroxyurea (Letvin et al., 1984)]. Both drugs also induced HbF in phase I-II clinical trials in patients with sickle-cell disease and β thalassemia (Platt et al., 1984; Veith et al., 1985). These trials have indicated hydroxyurea as the treatment of choice for sickle-cell anemia (Bradai et al., 2003). This drug, however, is not devoid of counter-indications, is not effective in all patients with sickle-cell disease and has modest effects in the patients with β thalassemia. The search for additional and less toxic agents, therefore, continues (Atweh and Schechter, 2001).

Two enzyme superfamilies, histone acetyltransferases (HATs) and histone deacetylases (HDACs), exert antagonistic epigenetic controls on gene expression through chromatin re-modeling (Hassig and Schreiber, 1997). HATs induce histone acetylation, favoring chromatin relaxation and exposing gene regulatory regions to the transcription machinery. HDAC catalyze histone deacetylation, resulting in the formation of tightly supercoiled, transcriptionally silent, "heterochromatin" regions (Felsenfeld and Groundine, 2003). Specific histone acetylation patterns have recently been shown to play a role in the murine β-globin switch, suggesting that HDACs might participate in the silencing complex that represses γ-globin expression (Forsberg et al., 2000). Hence, the hypothesis that HDAC inhibitors (HDACIs) might represent pharmacological reactivators of HbF (Cao, 2004).

The proof of concept for the use of HDACI as pharmacologic HbF inducers was provided by the observation that sheep fetuses continuously infused with sodium butyrate, a well known albeit weak (millimolar range) HDACI, displayed delayed HbF-to-adult-Hb switch (Perrine et al., 1988). Subsequent studies showed that butyrate, its analogs phenylbutyrate and valproic acid, additional short chain fatty acids, and their hydroxyamide derivatives induce HbF synthesis in human erythroid cultures (Perrine et al., 1989), in adult baboons (Constantoulakis et al., 1989), in some patients with β thalassemia (Perrine et al., 1993; Sher et al., 1995), and in the majority of patients with sickle-cell disease (Atweh et al., 1999). The rapid metabolism, inconvenient mode of application, and weak HbF-inducing activity of these compounds, however, prompted the search for alternative HbF-inducing HDACIs. A variety of HDACIs, mostly with unrelated chemical structures [trichostatin A, trapoxin, suberoylanilide hydroxamic acid (SAHA), MS-275, apicidin, scriptaid, and analogs], have been shown to be capable of inducing HbF in vitro and/or in vivo (Cao, 2004; Cao et al., 2004). Their potential use for the cure of hemoglobinopathies remains unclear because of their modest effects as HbF inducers and their high cell toxicity.

The aim of this study was to identify new, possibly less toxic, HDACI capable of inducing HbF, using a two step screening strategy. First, HDACI were screened for their potential to increase expression of a $^A\gamma$-driven reporter in GM979 cells stably transfected with a dual luciferase reporter construct (Skarpidi et al., 2000). Second, selective compounds were evaluated for their capability to increase the $\gamma/(\gamma+\beta)$ ratio expressed in vitro by normal adult erythroblasts (Migliaccio et al., 2002). The two most potent compounds identified with this second screening were, finally, tested for their ability to restore the impaired maturation of erythroblasts obtained in vitro from patients with β^0 thalassemia.

Materials and Methods

Construction of the HDACI Library. The synthetic schemes, experimental preparation procedures, physical and chemical data, and registry number for the new compounds 2, 3, 13 to 19, and 23 to 25 are reported in the Supplemental Data. Compounds 1, 4 to 12, and 20 to 22 were synthesized as described previously (see references in Supplemental Data). SAHA (Vorinostat) was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). The HDACI belong to the chemical classes of aroyl pyrrolyl hydroxyamides (APHAs; compounds 1–6), aryloxopropenylpyrrolyl hydroxyamides (compounds 7–19), and uracil-based hydroxyamides (UBHAs; compounds 20–25) and were tested against the maize deacetylases HD2, HD1-B (class I HDAC), and HD1-A (class II HDAC). Inhibitory assays were performed according to established procedures (Brosch et al., 1996b; Kölle et al., 1998) based on the ability of a compound to inhibit the maximal amount of tritiated acetic acid, as quantified by scintillation counting, liberated from radioactively labeled chicken core histones by each purified enzyme. In brief, maize HDACs (50 μl) were first preincubated with increasing concentrations of compounds for 15 min on ice and then incubated for 30 min at 30°C with total [^{3}H]acetate-prelabeled chicken reticulocyte histones (10 μl of a 2 mg/ml solution). The reaction was stopped by adding 36 μl of 1 M HCl/0.4 M acetate and 800 μl of ethyl acetate. After centrifugation (10,000*g*, 5 min), the radioactivity present in the supernatant (600 μl) was counted in a scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA). SAHA was included in the assay as reference, and blank solvents were used as negative controls. Maize HDACs were purified as described previously (Brosch et al., 1996a; Kölle et al., 1998). IC_{50} values were calculated with Microsoft Excel software (Microsoft Corp, Redmond, WA) and expressed as mean (± S.D.) of triplicate determinations as summarized in Supplemental Table 3. All of the compounds inhibited maize HDACs, albeit with a wide range of efficiency (IC_{50} between 0.004 and 39 μM). Four of them (compounds 8, 9, 11, and 12), were selectively active on class II enzymes (selectivity ratio >10). None of them was selective for the class I enzyme. As expected on the basis of previous results (see references in Supplemental Data), the six compounds of the UBHA family expressed IC_{50} values lower than those of SAHA. In particular, compound 24 was 2- to 30-fold more potent than SAHA in inhibiting the maize HD1-B and HD1-A.

Activation of $^A\gamma$-Driven Reporter Expression. The μLCRβprRluc$^A\gamma$prFlucGM979 cell line was obtained by stably transfecting the murine erythroleukemia GM979 cell line with a dual-luciferase reporter containing a 3.1-kilobase pair μLCR cassette including the DNase I hypersensitive core of the 5′ hypersensitive sites HS1, HS2, HS3, and HS4, linked to 315 base pairs of the human β-globin promoter and 1.4-kb of the $^A\gamma$-globin promoter driving the *Renilla reniformis* (R) and the firefly (F) luciferase genes, respectively (Skarpidi et al., 2000). μLCRβprRluc$^A\gamma$prFlucGM979 cells,

indicated from now on as GM979 for brevity, were frozen within 1 month from transfection and thawed when requested for the assay. As such, they may not be representative of the cells established by Skarpidi et al. (2000). GM979 cells were maintained in RPMI 1640 medium containing 10% (v/v) fetal calf serum (EuroClone, Milan, Italy), 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, and 400 μg/ml G418 as described previously (Migliaccio et al., 2005). The effect of HDACI on the expression of the $^{A}\gamma$-driven reporter was evaluated by incubating the cells with increasing concentrations of each compound solubilized in DMSO [final concentration, 0.1% (v/v)]. Negative controls were represented by cells incubated with DMSO alone. After 4 days of incubation, cells were harvested and $^{A}\gamma$-F and β-R luciferase activities determined in triplicate using the Dual Luciferase Reporter Assay System (Promega, Madison, WI), as described by the manufacturer. Luminescence was measured with the Lumat LB9507 Luminometer (Berthold Technologies, Bad Wildbad, Germany) and expressed in arbitrary fluorescence units (AFU). Results are expressed as mean ($\pm$ S.D.) of triplicate assays and are presented both as absolute values and as activity ratios ($^{A}\gamma$-F AFU/$^{A}\gamma$-F AFU + 2β-R AFU).

Human Subjects. Buffy coats from the peripheral blood of at least 15 to 20 different healthy donors were obtained from the Italian Red Cross Blood Bank (Rome, Italy). Blood from five patients with β^0 thalassemia was collected before routine transfusion at the Center for Studies on Thalassemia, University of Cagliari, Italy. All the patients were homozygous for the nonsense β^0 39 mutation (Trecartin et al., 1981). This mutation reduces β-globin mRNA expression through a nonsense-mediated mRNA decay mechanism (Zhang et al., 1998) and should have no consequence on the conformation of the β-globin locus. Human blood was collected according to guidelines established by the local ethical committee for human subject studies.

Cell Processing. Mononuclear blood cells were separated by centrifugation at 400*g* for 30 min over Ficoll-Hypaque (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Light-density cells were collected, washed with Hanks' balanced salt solution (HBSS) supplemented with 1% (w/v) bovine serum albumin and either cultured directly or cryopreserved in 10% dimethyl sulfoxide (Sigma).

Activation of the Endogenous γ-Globin Gene in Primary Human Erythroblasts. Human proerythroblasts were obtained by culturing light-density blood cells (10^6 cells/ml) in Iscove's modified Dulbecco's medium (Mascia Brunelli, Milan, Italy) containing fetal bovine serum [20% (v/v); Hyclone, Logan, UT], stem cell factor (10 ng/ml; Amgen, Thousand Oaks, CA), erythropoietin (EPO; 1 U/ml) (Epoetina alfa, Dompè Biotec, Milan, Italy), interleukin-3 (1 ng/ml) (Bouty, Milan, Italy), dexamethasone (10^{-6} M) (Sigma), and estradiol (10^{-6} M) (Sigma), as described previously (Migliaccio et al., 2002). The homogeneous population of proerythroblasts generated after 8 to 12 days in these cultures mature in 4 days up to the stage of orthochromatic erythroblasts once transferred in cultures supplemented with fresh medium and EPO (1 U/ml) alone (Calbiochem, Darmstadt, Germany). The effect of HDACI on the expression of the endogenous globin genes was determined by adding each of them at increasing concentrations at the beginning of the maturation culture. Cells were then harvested 4 days later for further analyses.

Phenotypic Analysis. Cell morphology was analyzed according to standard criteria on cytocentrifuged (Shandon, Astmoor, England) smears stained with May-Grünwald-Giemsa (Sigma).

RNA Isolation and Quantitation of Globin Gene Expression by Real-Time PCR. Total RNA was isolated from 10^6 cells using TRIzol (Invitrogen, Carlsbad, CA). Total RNA (1 μg) was reverse-transcribed with 250 ng of random primers, 1 μl of dNTP (10 mM), and 1 μl of RNase OUT (recombinant RNase inhibitor, 40 U/μl) (Invitrogen), as described by the manufacturer. Quantitative real-time PCR was carried out in a Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), using the TaqMan Master Mix containing AmpliTaq Gold DNA polymerase with 5′-3′ nuclease activity, which hydrolyzes a dual fluorescently labeled, target-specific oligonucleotide (TaqMan probe). The sequence of the amplification primers and of the probes used for γ- and β-globin were described previously (Di Baldassarre et al., 2007). Primers and probes for α-globin were represented by: forward, 5′-CTCTTCTG-GTCCCCACAGACT-3′; reverse, 5′-GGCCTTGACGTTGGTCTTG-3′; probe, 5′- 5-carboxyfluorescein-ACCATGGTGCTGTCTCCTGCCG-5-carboxytetramethylrhodamine-3′ (Applied Biosystems, Warrington, Cheshire, UK). α Hemoglobin stabilizing protein (AHSP) mRNA was evaluated with an assay on demand (TaqManGene expression assays; Applied Biosystems). For multiplex PCR in real-time relative quantization, target and endogenous reference control were amplified in the same tube with the TaqMan hGAPDH, which contained the selected primer/probe set (20× solution; Applied Biosystems) according to the manufacturer's instructions. Each determination was performed in triplicate. The level of a specific mRNA (X) was expressed in arbitrary units, using hGPDH as calibrator, according to the following algorithm: $\Delta C_t = [C_tX - C_tGPDH]$, where C_t is the X threshold cycle, and presented as $2^{-\Delta C_t}$. $\gamma/(\gamma+\beta)$ and α/non-α expression ratios were calculated as $2^{-\Delta C_t}\gamma/2^{-\Delta C_t}\gamma+2^{-\Delta C_t}\beta$ and $2^{-\Delta C_t}\alpha/2^{-\Delta C_t}\gamma+2^{-\Delta C_t}\beta$, respectively.

Determination of Cell HbF Content. Cells were washed twice in HBSS and lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate. Lysates were centrifuged for 10 min at 12,000*g*, and supernatants were used for HbF determination with a specific enzyme-linked immunosorbent assay kit (Bethyl Lab Inc., Montgomery, TX), using the HbF standard provided by the kit, as calibrator. The amount of the immunoreaction was assessed by densitometry at 450 nm with a Victor^3 Multilabel Counter 1420 (PerkinElmer Life and Analytical Sciences–Wallac Oy, Turku, Finland).

Determination of H4 Acetylation by Flow Cytometry. Levels of H4 acetylation were measured as described by Ronzoni et al. (2005). In brief, 1×10^6 cells were fixed for 15 min in 1% formaldehyde in HBSS on ice and permeabilized with 200 μl of 0.1% Triton X-100 in HBSS for 10 min at room temperature. Cells were first incubated with an anti-acetyl-Histone H4 (1:50 dilution; Upstate, Charlottesville, VA) for 1 h at room temperature and then with the R-phycoerythrin–conjugated AffiniPure F(ab′)2 Fragment Donkey Anti-Rabbit IgG (H+L) (1:100 dilution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h at room temperature in the dark. Cell fluorescence was analyzed with an Epics Elite ESP (Beckman Coulter). Nonspecific fluorescence signals were gated on cells incubated with R-phycoerythrin–conjugated AffiniPure F(ab′)2 Fragment Donkey Anti-Rabbit IgG (H+L) alone.

Human Cell Lines. The human nonerythroid U937 cell line and the human breast cancer ZR75.1 cell line were cultured in RPMI 1640 medium with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, 10 mM HEPES, and 2 mM glutamine.

In Vitro HDAC Inhibition Assay against Human HDAC1 and HDAC4. The functional complexes containing human HDAC1 and HDAC4 were purified by immunoprecipitation (IP) from U937 and ZR75.1 cells, respectively. Cells were lysed in IP buffer (50 mM Tris-HCl, pH 7.0, 180 mM NaCl, 0.15% Nonidet P-40, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM $NaMO_4$, and 0.5 mM NaF) with a protease inhibitor cocktail (Sigma), for 10 min on ice and centrifuged at 14,700*g* for 30 min. Extracts (1000 μg/ml of protein) were precleared by incubating with 20 μl of A/G plus Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for up to 1 h on a rocking table at 4°C. Supernatants were then transferred into a new tube and incubated again either with an anti-HDAC1 (Abcam, Cambridge, MA) or anti-HDAC4 (Sigma) antibody (3 μg) or with an irrelevant IgG (Santa Cruz Biotechnology), as negative control, overnight at 4°C on a rocking table. The following day, 20 μl of A/G plus Agarose (Santa Cruz) was added to each IP and incubation continued for 2 h. The beads were washed twice in PBS and re-suspended in 20 μl of sterile PBS. The HDAC assay was carried out using a labeled [^{3}H]histone

H4 peptide linked to streptavidin agarose beads, according to the supplier instructions (Upstate), as described by Mai et al. (2006).

Determination of H3 and α-Tubulin Acetylation. The effects of compounds 9 and 24 on histone H3 and α-tubulin acetylation were determined by Western blot analysis of protein extracts prepared from U937 cells that had been exposed to increasing compound concentrations for 16 h. For determination of α-tubulin acetylation, 25 μg of total protein extracts were separated on a 10% polyacrylamide gel, blotted, and probed by Western blot with antibodies specific for acetylated α-tubulin (dilution 1:500; Sigma) and total extracellular signal-regulated kinases (dilution, 1:1000; Santa Cruz Biotechnology), as loading control. For quantification of histone H3 acetylation, 100 μg of total protein extracts were separated on a 15% polyacrylamide gel, blotted, and analyzed by Western blot with antibodies specific either for the acetylated (dilution, 1:1000; Upstate) or total form (Abcam, loading control) of histone H3.

Statistical Analysis. Statistical analysis was obtained with the Origin 5.0 software for Windows (OriginLab Corp., Northampton, MA).

Results

Screening of HDACI as Inducer of Reporter Genes under the Control of Synthetic $^{A}\gamma$- and β-Globin Promoters. Increasing concentrations (0.02–20 μM) of the compounds described in Fig. 1 were tested for their ability to affect the luciferase activity driven by either the $^{A}\gamma$- or β-globin promoter expressed by GM979 cells incubated with each compound for at least 4 days. Under the conditions of the assay, GM979 cells have a doubling time of 48 h; therefore, this experimental design allowed the cells to proliferate at least twice in the presence of the HDACI, maximizing the likelihood that a compound would affect chromatin configuration. The results obtained are summarized in Table 1 as follows: 1) maximal induction, or repression, on the activity of the $^{A}\gamma$- and β-driven reporters; 2) maximal alteration in $^{A}\gamma$-F/($^{A}\gamma$-F + 2β-R)-reporter activity ratio; and 3) lower con-

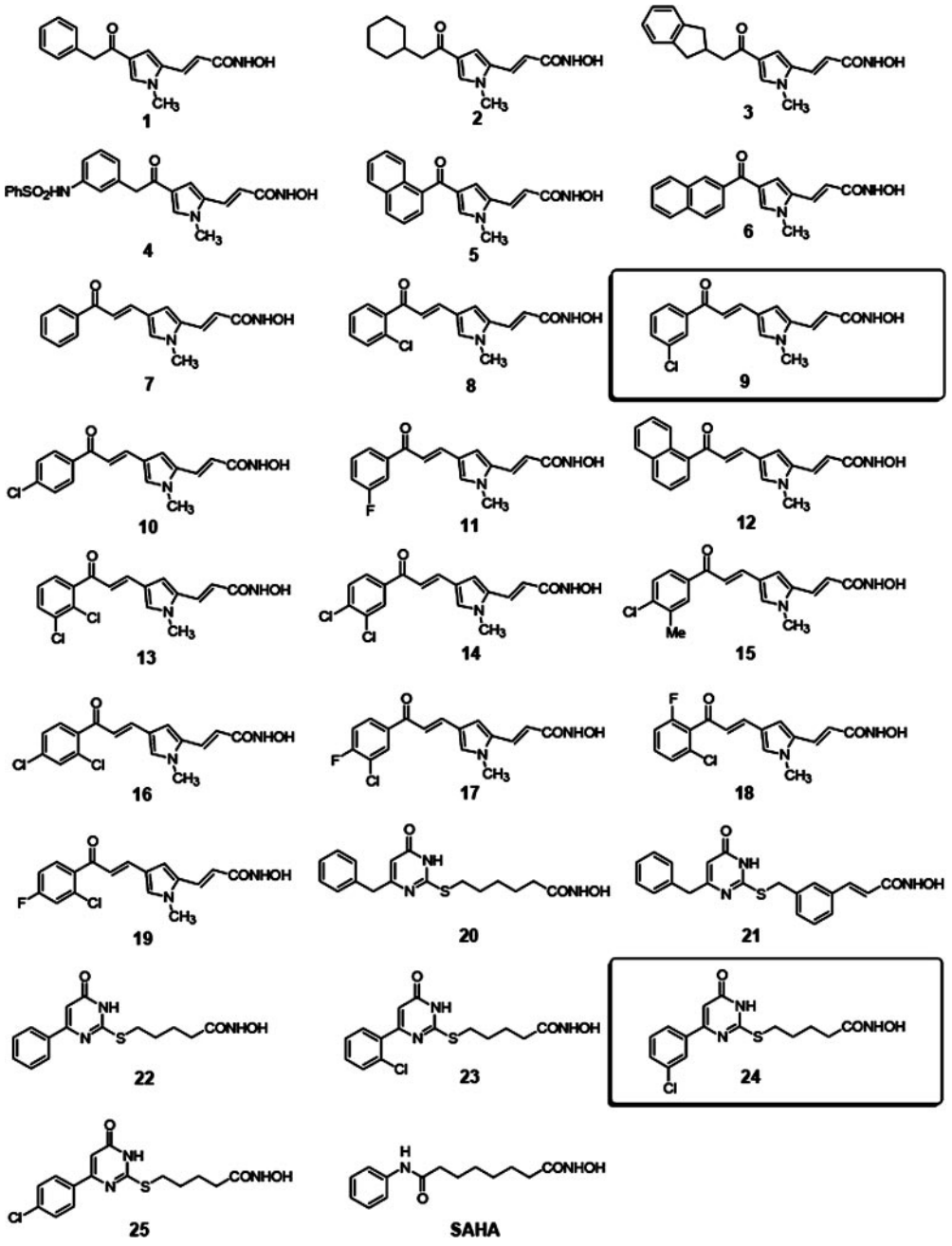

Fig. 1. Chemical structures of the HDACIs included in the library and analyzed in this study. The library contains previously reported and newly synthesized HDACIs identified using the maize HDACs inhibitory assay (see Supplemental Data). These compounds belong to three different chemical classes: aroyl pyrrolyl hydroxyamides (APHAs) (1–6), aryloxopropenylpyrrolyl hydroxyamides (7–19), and uracil-based hydroxyamides (UBHAs) (20–25). The rectangles highlight the two compounds (9 and 24) most extensively analyzed in this study. The chemical structure of SAHA (vorinostat) is included for comparison.

centration of the compound, which induced the maximal alteration. The number of cells present in the culture by the end of the assay was also recorded and expressed as number of cell duplications, as a measure of toxicity. GM979 cells were incubated in parallel cultures with SAHA and with the vehicle (DMSO) as positive and negative controls, respectively.

As in the studies summarized earlier (Cao et al., 2004), no relationship was observed between chemical structure of a compound and its effect on the activity of the $^{A}\gamma$- and β-driven reporters. For example, among the three isomers 8, 9, and 10, two, 8 and 9, induced the $^{A}\gamma$-driven reporter, whereas the third, compound 10, did not. Among all of the HDACIs tested, compounds 7, 8, 9, 11, 22, and 23 were all more effective than SAHA in inducing expression of the $^{A}\gamma$-driven reporter, with maximal activity between 0.2 and 2 μM (Table 1). Some of them (7, 8, 11, 22, and 23) also induced the β-driven reporter activity. As a consequence, they had little or no effect on the $^{A}\gamma$-F/($^{A}\gamma$-F + 2β-R) reporter activity ratio. It is noteworthy that compound 9 preferentially induced the activity of the $^{A}\gamma$-driven reporter and increased the $^{A}\gamma$-F/($^{A}\gamma$-F + 2β-R)-reporter activity ratio 2-fold more efficiently than SAHA (Table 1). Among all the HDACIs investigated, compound 24 had the "unique" ability to selectively increase the β-driven reporter activity.

As sign of toxicity, the number of cells present by the end of the assay in the presence of the different compounds was measured and the number of cell duplications that had occurred during the assay calculated and presented in Table 1. Because this number is an exponent, 0 means that the number of cells remained constant (i.e., the number of cells that died was equal to that of cells that proliferated), a negative number means that the number of cells that died was higher than that of cells that proliferated (toxicity), and a positive number means that the number of cells that proliferated was higher than that of cells that died. The interpretation that differences in positive numbers are indication of toxicity is debatable. In fact, it may be related to differences in cell cycle length as a result of increased time spent to reprogram the cell expression profile in response to the HDACI. With the exception of compounds 11 and 23, none of the HDACIs included in this study, including SAHA, decreased the number of GM979 cells alive by the end of the assay below input values (Table 1), an indication of their low toxicity in this cell system.

Activity of HDACI on the Expression of the Endogenous Globin Genes in Primary Erythroblasts Obtained in Vitro from Healthy Donors. Human erythroblasts obtained in human erythroblast massive amplification (HEMA) cultures express maximal and ontogenetically correct level of γ- and β-globin mRNA after 3 to 4 days of EPO-induced maturation (Di Baldassarre et al., 2007). Therefore, to evaluate the effects of HDACI on the expression of the endogenous globin genes, proerythroblasts were generated in vitro from healthy donors and allowed to mature in the presence of selected compounds for 4 days. By the end of the 4 days, erythroblasts were harvested and counted (as indication of toxicity), and mRNA and protein were extracted for quantitative RT-PCR and HbF determination, respectively. The compounds included in the assay were represented by compounds 9 and 24, for their respective selectivity as $^{A}\gamma$- and β-promoter inducers in GM979 cells. At least another member of each class (compounds 11, 23, and 25), with comparable IC_{50} values in the HDAC inhibition screening, as control of specificity, and SAHA, for comparison, were also analyzed. Compound 24 was included in the analyses because its selec-

TABLE 1

Reporter-inducer activity of compounds 1–25 on GM979 cells

Standard deviations are within 10% and are not reported for clarity. The number of cell duplications was calculated according to the formula: number of duplications at day x = $\log_2$ (cell number at day x/cell number at day 0).

Compound	Concentration	γ-F	β-R	$\gamma/(\gamma+2\beta)$	Number of Cell Duplications
	μM		*% of control*		
1	2	423.3	210.2	198.7	0.64 ± 0.17
2	0.6	499.5	279.6	177.1	0.69 ± 0.17
3	2	514.4	529	99	0.19 ± 0.17
4	6	25.6	134.6	19.4	2.36 ± 0.17
5	20	167.6	242.3	68.5	2.34 ± 0.17
6	6	179.5	178	100.3	0.96 ± 0.17
7	2	937.7	544.4	173.8	0.34 ± 0.17
8	2	266.5	350.8	77.5	2.42 ± 0.17
9	2	897.9	338.5	264.8	1.07 ± 0.17
10	0.2	78.6	94.8	83.7	1.9
11	2	255.7	207.6	122.9	−0.11 ± 0.17
12	Toxic	N.D.	N.D.	N.D.	N.D.
13	0.02	95.5	83.0	114.0	2.0
14	0.2	100.2	98.5	102.4	2.2
15	0.2	125.0	113.3	112.0	2.1
16	0.2	167.7	115.6	142.8	2.0
17	0.2	107.2	120.1	90.0	1.0
18	0.2	114.8	112.0	102.0	1.4
19	0.2	99.5	98.1	100.0	1.2
20	2	85.8	131.8	65.3	1.79
21	0.2	181.3	178.9	101.3	0.20
22	2	261.8	272.5	96.1	0.29
23	2	268.6	253.7	101.3	−0.70
24	2	117.7	234.8	50.3	0
25	0.2	173.0	267.8	64.7	1.94
SAHA	0.2	189.1	153.1	123.2	1.29
DMSO	1% (v/v)	100	100	100	2.4 ± 0.17

N.D., not determined.

tivity as a β-promoter inducer suggested to us that it would represent a negative control. Each HDACI was used at the minimal concentration that was maximally effective (either 0.2 or 2 μM) on GM979 cells.

As expected (Cao, 2004), SAHA (0.2–2 μM) increased by 10-fold (from 0.05 to 0.20–0.39) the γ/(γ+β) ratio expressed by normal erythroblasts (Fig. 2A). Among the tested compounds, 9 and 25 all significantly increased the γ/(γ+β) expression ratio above background, with efficiency either comparable (compound 9) or 30% lower (compound 25) than SAHA. In contradiction to results obtained in GM979 cells, compound 24 also increased the γ/(γ+β) ratios. Furthermore, this compound significantly, although modestly (by 50%), increased the amount of HbF contained in normal erythroblasts by the end of the culture (Fig. 2B).

To detail the mechanism that mediated the increase in γ/(γ+β) expression ratio in normal cells exposed to these compounds, a second set of experiments determined their concentration/response curve on the expression levels of γ- and β-globin mRNA. The effects of compounds 11, 23, and 24 on γ- and β-globin expression remained modest up to concentrations of 3 μM (results not shown). In contrast, both compounds 9 and 24 transiently affected (induced or suppressed, respectively), expression of the endogenous β-globin at 0.02 μM and significantly increased, by 2-fold, the expression of γ-globin, with maximal effects at concentrations of 2 μM (compound 9) and 0.2 μM (compound 24), respectively. As a result, they also increased the γ/(γ+β) expression ratio (Fig. 3). However, the increase was clearly due to different mechanisms. In the case of the compound 9, the increase in expression ratio was due to the different magnitude of the increments of γ-globin and β-globin mRNA. In the case of compound 24, it was due to a combination of γ-globin mRNA increase and β-globin mRNA decrease. As a result, although the two compounds induced similar increases in the level of γ-globin mRNA (≈ from 2.0 to 3.5 $2^{-\Delta C_t}$), compound 24 was 3 times more active than compound 9 in increasing the γ/(γ+β) ratio (from ≈ 0.05 to 0.30 and 0.10, respectively) (Fig. 3). It is noteworthy that compound 24 is the one that increased the HbF content per cell up to detectable levels (Fig. 2B). Neither compound 9 nor compound 24 consistently affected the levels of α-globin and AHSP, included in the analysis as control of specificity, expressed by normal erythroblasts (results not shown).

Inhibition of Human Class I (HDAC1) and Class IIa (HDAC4) HDACs and Deacetylation of Histone H3 and α-Tubulin in U937 Cells and of Histone H4 in Primary Proerythroblasts. HDACI library was based on the assumption that the maize HD2, although structurally different from mammalian HDACs, was considered a predictor model for mammalian class I HDACs (Brosch et al., 1996b), whereas HD1-B (Lechner et al., 2000) and HD1-A (Brosch et al., 1996a) were considered homologous to mammalian class I and II HDACs, respectively. More recent data call into question the validity for human enzymes of class selectivity identified with the maize HDAC isoforms (Mai et al., 2006). Mammalian HDACs, in fact, are organized within the cells in complexes with other HDACs and DNA-binding proteins. In these complexes, one of the HDAC isoforms (usually of class I) exerts the catalytic function, whereas the other (usually of class IIa or b) acts as regulator of the catalytic enzyme (Verdin et al., 2003; Minucci and Pelicci, 2006). Therefore, to clarify the class selectivity of compounds 9 and 24 for human HDACs, we performed inhibitory assays against the enzymatic activity of HDAC complexes purified by immunoprecipitation with antibodies specific for human HDAC1 (class I) and HDAC4 (class IIa) (Table 2). SAHA was used as control. Compound 24, which was potent but not selective in maize, inhibited at least as efficiently as SAHA both class I and class IIa human HDACs (IC_{50} = 0.2 μM in both cases). On the other hand, compound 9 was weak (10-fold less efficient than SAHA) but class IIa-selective. Therefore, in both cases, there was a good correlation between inhibition of maize and human HDAC class isoforms.

To clarify whether the HDAC inhibitory activity exerted by the compounds in vitro did correspond to increased histone acetylation in vivo, we measured the levels of acetylation of histone H3 and α-tubulin in U937 cells incubated with increasing concentration of compounds (Fig. 4). Again, SAHA was included as a control. The acetylation levels of α-tubulin were analyzed as indicator of functional inhibition of HDAC6, a class IIb HDAC isoform (Haggarty et al., 2003). In

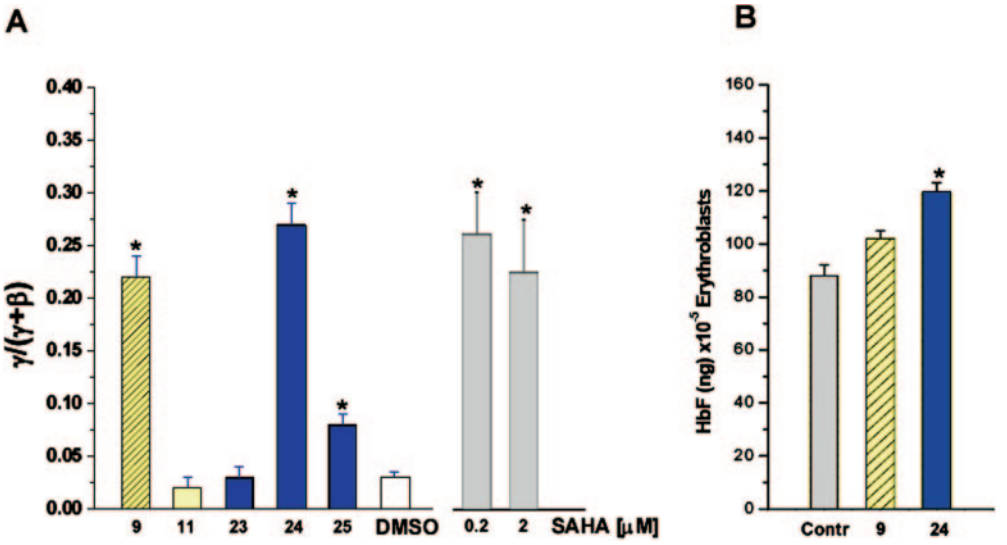

Fig. 2. Effects of selected HDACIs on the γ/(γ+β) mRNA ratio (A) and on the HbF content (B) expressed by primary erythroblasts obtained from healthy donors. Proerythroblasts were obtained in HEMA and were induced to mature for 4 days with EPO in the presence or in the absence of the indicated HDACI. HDACIs were used at the minimal concentration that was effective on GM979 cells and presented in Table 1. These concentrations are either 0.2 μM (compounds 9 and 25) or 2 μM (compounds 11, 23, and 24). Results obtained in parallel cultures incubated with SAHA (0.2–2 μM) are presented for comparison. Data are presented as mean (± S.D.) of five to six separate assays, each with a different donor, performed in triplicate. The γ/(γ+β) mRNA ratios observed in cultures with or without DMSO are 0.03 ± 0.005 and 0.023 ± 0.004, respectively. Values statistically different from untreated controls are indicated by * ($p < 0.5$) and ** ($p < 0.01$).

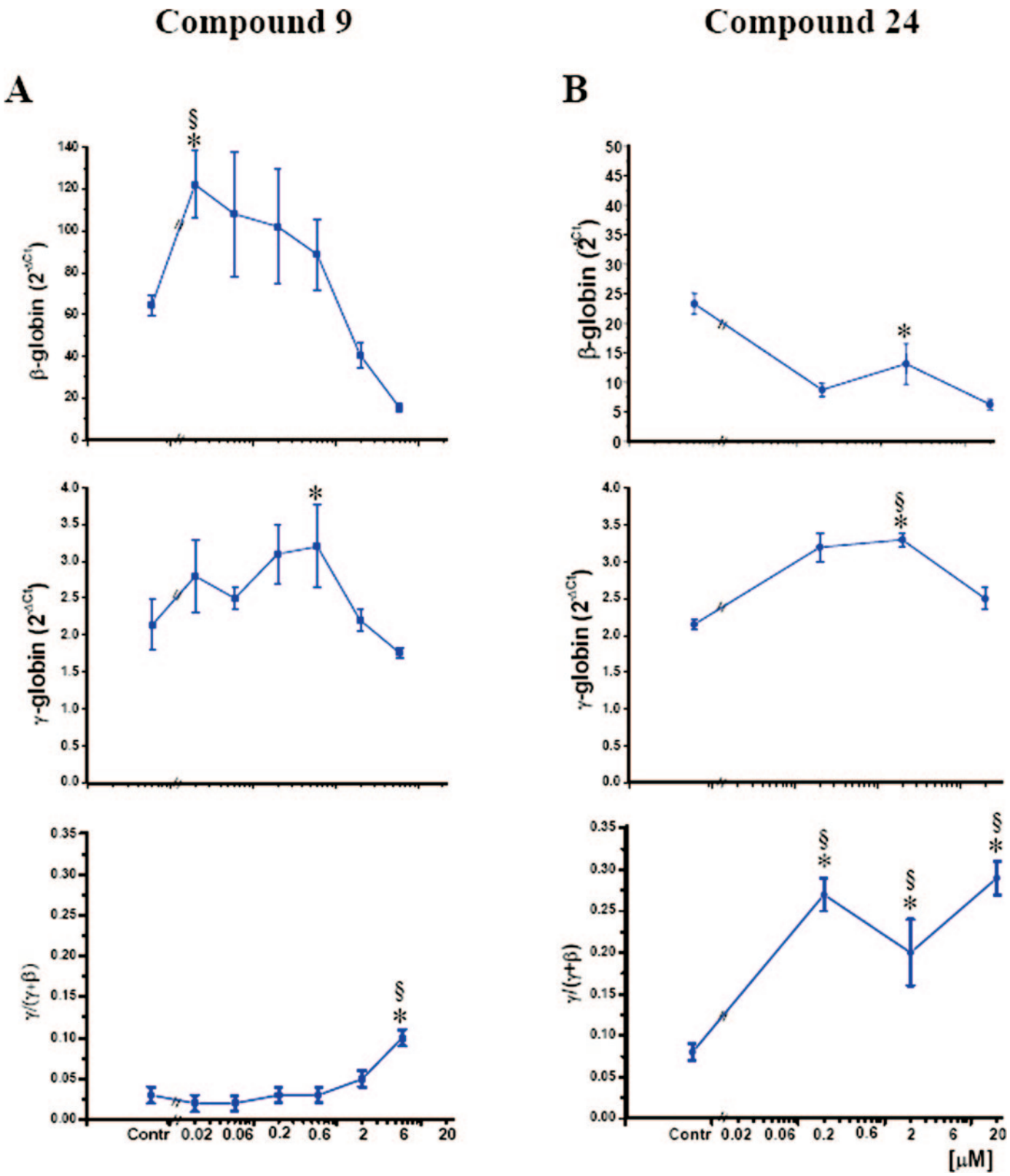

Fig. 3. Concentration/response curve for compounds 9 (A) and 24 (B) on the levels of β- and γ-globin mRNA and on the γ/(γ+β) ratio, expressed by normal human erythroblasts. Cells incubated with DMSO were used as negative control (Contr). Results are presented as mean (± S.D.) of a single experiment performed in triplicate and are representative of those observed in at least five to six experiments, each with a different donor. § and ∗ indicate values statistically different ($p < 0.05$) from control by paired t test and analysis of variance, respectively. See the legend to Fig. 2 for further details. In the experiment presented in B, untreated cells expressed levels of β and γ globin mRNA of 23.3 ± 1.8 versus 2.2 ± 0.1, respectively. The values expressed by untreated cells in the experiment presented in A were not measured.

these experiments, compounds 9 and 24 induced similar levels of α-tubulin acetylation, levels that were slightly lower than those induced by SAHA. Therefore, compounds 9 and 24, which had a clear different activity on class IIa HDAC (Table 2), exerted similar levels of inhibition on the class IIb isoform (Fig. 4). Differential inhibition on class IIb and IIa enzymes has been already reported for other molecules with HDACI activity (Mai et al., 2006) and is consistent with the distinctive chemical structure of the two classes of HDAC isoforms (Minucci and Pelicci, 2006). In addition, compound 9 was less efficient than SAHA, and compound 24 was as efficient as SAHA in inducing histone H3 acetylation. The differences observed between compounds 9 and 24 in histone H3 acetylation inducing activity in vivo are consistent with their potency as inhibitors of class IIa HDAC in vitro (see Table 2). It is also possible, however, that the difference between compounds 9 and 24 in histone acetylation was because the compounds had been used at equimolar rather than equitoxic concentrations.

As an indication that the induction of HbF synthesis exerted by the compounds in primary erythroblasts was mediated by increased levels of histone acetylation, we compared by flow cytometry the acetylation state of histone H4 in normal erythroblasts that had matured in the absence or in the presence of compounds 9 and 24 (Fig. 5). The acetylation state of histone H3 was not analyzed because of low abundance of this protein in primary cells does not allow its evaluation by flow cytometry. A clear increase above background (AFU = 523 versus 170) in the acetylation state of histone H4 was observed in cells incubated with compound 9. A small, but detectable, increase in acetylation levels of histone H4 was also observed in cells incubated with compound 24 (256 versus 170).

The fact that HDACI had increased the levels of H4 acetylation in primary erythroblasts is proof that they had inhibited HDAC activity in these cells. Two recent publications have demonstrated that increases in the levels of hystone H4 acetylation, obtained either through HDAC inhibition (Fathallah et al., 2007) or through activation of the p38 MAPK signaling (Aerbajinai et al., 2007), are responsible for the increased γ-globin expression induced in adult erythroblasts by butyrate and thalidomide, respectively. It is conceivable, therefore, that the increased levels of H4 acetylation were directly responsible for the effects of compounds 9 and 24 on γ-globin expression observed in this study.

TABLE 2

Comparison of the inhibitory activity of compounds 9 and 24 and SAHA on human class I (HDAC1) and II (HDAC4) HDAC isoforms purified from human cell lines

Enzymatic activities are expressed as percentage of the control activity exerted by the enzyme alone.

Compound	Class I (HDAC1)	Class IIa (HDAC4)
	%	%
SAHA		
0.2 μM	52.2 ± 1.4	66.6 ± 1.6
2.0 μM	15.5 ± 1.4	33.8 ± 1.1
9		
0.2 μM	108.2 ± 7.1	94.3 ± 0.6
0.6 μM	116.0 ± 2.8	98.1 ± 0.3
2.0 μM	113.3 ± 4.3	89.8 ± 0.7
6.0 μM	107.5 ± 3.9	78.0 ± 0.2
20 μM	102.8 ± 2.1	41.8 ± 0.8
24		
0.2 μM	55.1 ± 0.7	70.3 ± 0.7
0.6 μM	29.8 ± 1.6	43.5 ± 0.01
2.0 μM	14.2 ± 0.9	30.8 ± 0.6
6.0 μM	9.8 ± 0.07	18.4 ± 0.8
20 μM	8.5 ± 0.05	17.0 ± 0.6

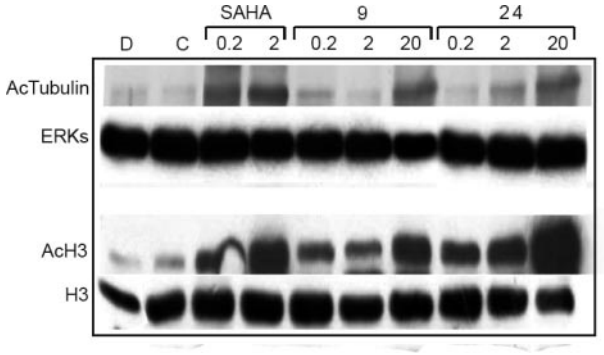

Fig. 4. Effects of compounds 9 and 24 on the levels of H3 and α-tubulin acetylation in U937 cells. U937 cells were incubated either with increasing concentration of SAHA (positive control) and of compounds 9 and 24 (0.2–20 μM in all the cases), as indicated. Untreated cells (C) and cells incubated with DMSO alone (D) were analyzed as negative controls.

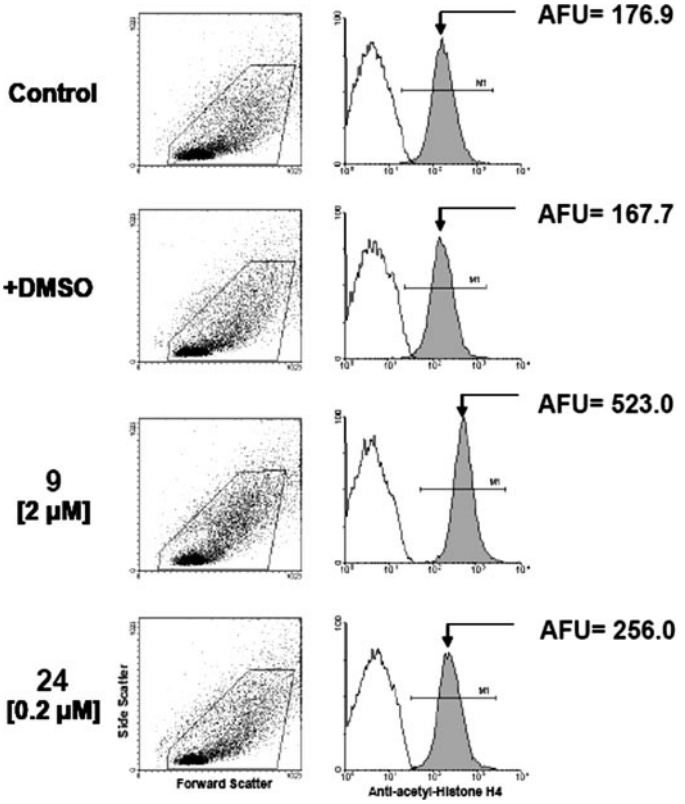

Fig. 5. Effects of compounds 9 and 24 on the level of histone H4 acetylation in primary erythroblasts obtained from healthy donors. Proerythroblasts obtained in HEMA were cultured with EPO alone (control, top panel) or with EPO plus DMSO (vehicle, negative control), or compound 9 or 24, as indicated. After 4 days, the cells were harvested and the levels of H4 acetylation measured by flow cytometry. The forward and side scatter plots present the gate used for the fluorescence analyses presented in the histograms on the right. The white and gray area correspond to the fluorescence intensity expressed by cells labeled with an irrelevant antibody or with the anti-acetyl-histone H4, respectively. The average fluorescence intensity (AFU, in arbitrary units) expressed by cells incubated with the anti-acetyl-histone H4 is reported on the right, and is proportional to the acetylation state of histone H4 in the cell population analyzed (Ronzoni et al., 2005).

Comparison of in Vitro Differentiation of Normal and β^0 Thalassemic Erythroblasts. It has been reported already that progenitor cells present in the blood from healthy donors, as well as those present in the blood from patients with β^0 thalassemia, generate high numbers of erythroblasts in ex vivo expansion cultures (Fibach et al., 1989; Migliaccio et al., 2002). However, the differences between the in vitro maturation of normal and β^0 thalassemic erythroblasts are still poorly defined. For this reason, a pilot study compared number, morphology, and level of globin gene expression of erythroblasts obtained in vitro from healthy and β^0 thalassemic donors.

Under HEMA conditions, mononuclear blood cells from patients with β^0 thalassemia generated, in 10 to 12 days, a proerythroblast population that, although slightly lower in number (-fold increase with respect to day 0 = 0.52 ± 0.22 versus 1.71 ± 0.84 in β^0 thalassemic and normal cultures, respectively, $p < 0.01$), was equivalent in morphology to that generated by the corresponding normal cells (Fig. 6). However, whereas normal pro-erythroblasts transferred in cultures containing only EPO matured in 4 days up to the stage of orthochromatic erythroblasts (Fig. 6A), those obtained from patients with β^0 thalassemia matured poorly, remaining large in size, with large nuclei and poorly condensed chromatin (Fig. 6B). Such retarded maturation was reflected by increased proliferation. In fact, normal proerythroblasts have limited proliferative capacity and proliferate only one to three times before initiating terminal maturation. Some of them will undergo apoptosis. As a result of the balance between the two processes, the number of cells after 4 days of maturation culture remain similar to input (-fold increase = 0.85 ± 0.28). In contrast, in the corresponding maturation cultures seeded with β^0 thalassemic proerythroblasts, the number of cells increased by ~2-fold (-fold increase = 1.99 ± 0.93) by day 4. Although the difference from the 2-fold increase is not statistically significant, this result indicates that β^0 thalassemic proerythroblasts proliferate more and/or die less than normal cells when exposed to EPO alone.

Differences were also observed in the levels of globin gene expressed by normal and β^0 thalassemic erythroblasts after 4 days of maturation culture in EPO alone. AHSP was included in this analyses because of its abundant expression in erythroid cells (Kihm et al., 2002) and because of the function of its product to bind free α-chains, stabilizing their structure and limiting their ability to participate in chemical reactions that generate reactive oxygen species (Feng et al., 2004). Normal orthochromatic erythroblasts expressed high levels of α- and β-globin ($2^{-\Delta C_t}$ in the 10–100 range for both) and relatively low levels of γ-globin and AHSP ($2^{-\Delta C_t}$ in the 1–10 range) (Fig. 6). The levels of α- and β-globin expressed by normal cells obtained from different donors were very similar. In contrast, the donor variability in γ-globin and AHSP expression was as high as 10-fold (see the corresponding SD in Fig. 6A). As expected, β^0 thalassemic erythroblasts expressed levels of α- and γ-globin not statistically different from those expressed by the corresponding cells obtained from healthy donors, but significantly less β-globin than normal cells ($2^{-\Delta C_t}$ in the order of magnitude of 1–10). We were surprised to find, however, that β^0 thalassemic cells expressed significantly more AHSP (by 1 log) than normal cells (Fig. 6). With the exception of AHSP, the subject-to-subject variability in globin genes expression in β^0 thalassemic erythroblasts was much wider than that observed with the corresponding cells from healthy donors (Fig. 6B). More specifically, the difference in β-globin expression among erythroblasts obtained from different patients was so wide that, in the case of two patients, it was only 10-fold lower than normal.

Compounds 9 and 24 Restore the Impaired in Vitro Maturation of β^0 Thalassemic Erythroblasts. In a last set of experiments, compounds 9 and 24 were tested for their ability to restore the impaired maturation expressed in vitro by erythroblasts obtained from patients with β^0 thalassemia.

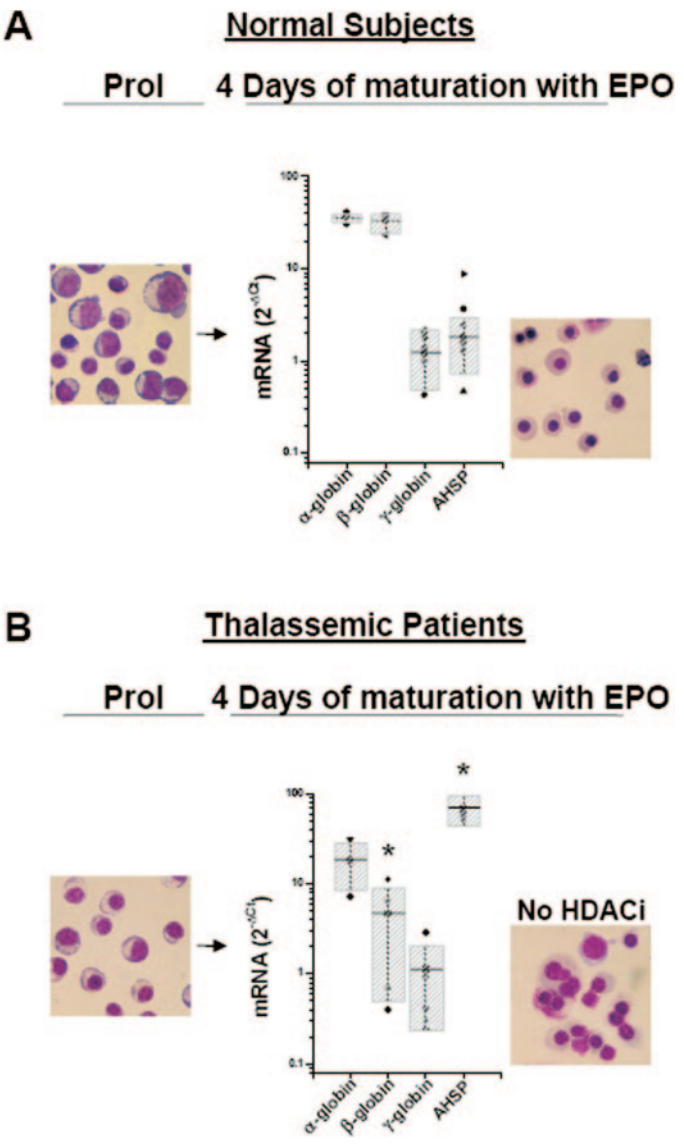

Fig. 6. Comparison of the in vitro maturation of primary pro-erythroblasts obtained from healthy donors (A) or from patients with β^0 thalassemia (B), as indicated. Pro-erythroblasts were obtained in the proliferative phase of HEMA (prol) and induced to mature for 4 days with EPO. Cell morphology before (Prol) and after maturation was analyzed by May-Grunwald staining (original magnification, 40×). Expression levels of α-, β- and γ-globin, and AHSP, in cells at the end of the maturation culture were analyzed by quantitative RT-PCR and expressed as $2^{-\Delta C_t}$. Data obtained with a total of three to seven healthy donors and five patients with thalassemia (each donor is represented by a different symbol) are presented. The straight line and the shaded area indicate the mean (± S.D.) obtained in all the experiments. Expression levels in β^0 thalassemic erythroblasts statistically different ($p < 0.01$) from those of normal cells are indicated by *.

By morphological criteria, both compounds 9 and 24 restored the in vitro maturation of β^0 thalassemic erythroblasts as orthochromatic cells became readily detectable in the cultures (Fig. 7). By expression analysis, β^0 thalassemic erythroblasts that matured in the presence of these compounds expressed significantly lower levels of β-globin (by 10-fold) and of AHSP (by 2- to 10-fold). In particular, the levels of AHSP expressed by β^0 thalassemic cells exposed to compound 9 became no longer statistically different from those expressed by normal erythroblasts obtained from healthy donors (Fig. 6 and 7).

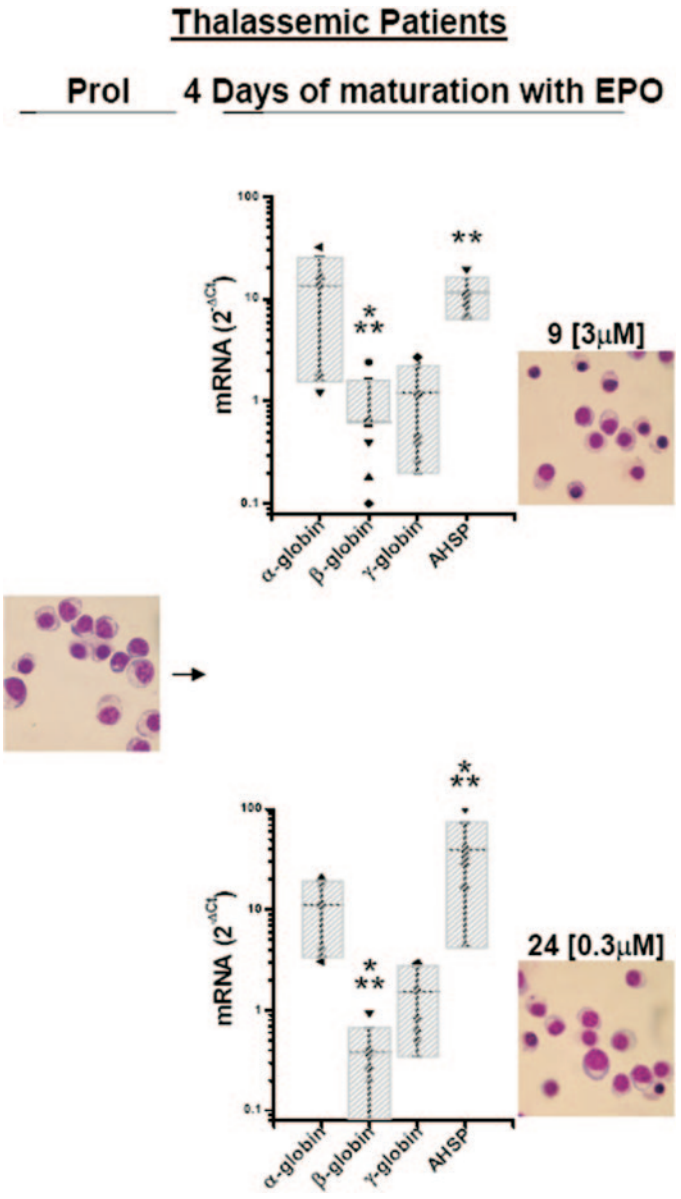

Fig. 7. Compounds 9 (top) and 24 (bottom) both restore the impaired in vitro maturation of proerythroblasts from patients with β^0 thalassemia. The morphology of the cells before (Prol) and after the 4 days of maturation in the presence of EPO plus each compound was analyzed by May-Grunwald staining (original magnification, 40×). Expression levels of α-, β-, and γ-globin, and AHSP, in cells cultured for 4 days in the presence of the compounds were analyzed by quantitative RT-PCR and expressed as $2^{-\Delta Ct}$. Data obtained with a total of five patients with β^0 thalassemia (each donor is represented by a different symbol, the same as in Fig. 6B) are presented. The levels of mRNA expressed by cells treated with DMSO alone are presented as control and ranged from 91% (γ-globin) to 107% (α-globin) of those expressed by untreated cells and presented in Fig. 6. The straight line and the shaded area indicate the mean (± S.D.) obtained in all the experiments. Values statistically different from those expressed by untreated normal and β^0 thalassemic erythroblasts, and presented in Fig. 6, are indicated by * and by **, respectively.

On average, neither compound 9 nor compound 24 affected the $\gamma/(\gamma+\beta)$ expression ratio in the β^0 thalassemic erythroblasts (Fig. 7 and results not shown). However, as shown by the detailed concentration/response curves on gene expression presented in Fig. 8, in the case of two patients with β^0 thalassemia, those whose cells expressed the highest baseline levels of β-globin, both compounds increased the $\gamma/(\gamma+\beta)$ ratio by 4- to 6-fold. Compound 9 did not significantly affect γ-globin expression but decreased that of β-globin (by 4-fold) in both patients. Compound 24 decreased β-globin expression (by 4-fold) and significantly increased γ-globin expression (by 2-fold) in both patients. In contrast with the results on normal cells, compound 9 decreased α-globin (by 4-fold) in one patient (Fig. 8A). As a result, the α/non-α expression ratio was not affected by the compound in one patient and was increased (by 20-fold) in the other one. Compound 24 had opposite effects on α-globin expression in the two patients: it increased it in one patient (the same who responded to compound 9) and decreased it in the other (Fig. 8B). Consequently, the α/non-α expression ratio was significantly increased (by 4-fold) in one patient and was not affected in the other one.

Toxicity Exerted by Compounds 9 and 24 in Cultures of Normal and β^0 Thalassemic Human Erythroblasts. Last but not least, Fig. 9 compares the toxicity exerted by compounds 9 and 24 in maturation cultures of normal and β^0 thalassemic erythroblasts. In cultures of normal cells, both compounds 9 and 24 decreased the number of cells alive by the end of the maturation culture less than SAHA, almost at all the concentrations tested. Therefore, both HDACI were at least no more toxic than SAHA at the concentrations (0.2 and 2 μM) found to be active as γ-globin inducer in normal erythroblasts. On the other hand, both compounds 9 and 24 were far less toxic in cultures of β^0 thalassemic erythroblasts than they were in those of normal cells. Compound 24 had no effect on the cell number over the wide range of concentrations tested, whereas compound 9 exerted a 50% inhibitory activity at the concentration (3 μM) that was effective as $\gamma/(\gamma+\beta)$ inducer in these cells.

Discussion

We have identified new synthetic HDACI capable to affect expression of γ-driven and/or of β-driven reporter activity in GM979 cells. Three compounds (8, 9, and 11) increased expression of both $^A\gamma$- and β-driven reporter activities. The effects exerted on the two promoters were of different magnitude and occurred at different concentrations (Table 1). This allowed us to define a concentration window at which a compound was more potent as $^A\gamma$- than as β-driven reporter inducer (Table 1). Compound 9, in particular, was 2-fold more potent than SAHA as inducer of $\gamma/(\gamma+\beta)$ expression ratio. On the other hand, compound 24, exclusively affected expression of the β-driven reporter in this synthetic model of Hb switch (Table 1).

Selected compounds were then tested for their ability to modify expression of the γ- and β-globin genes in normal erythroblasts. Two of them, compounds 9 and 24, altered

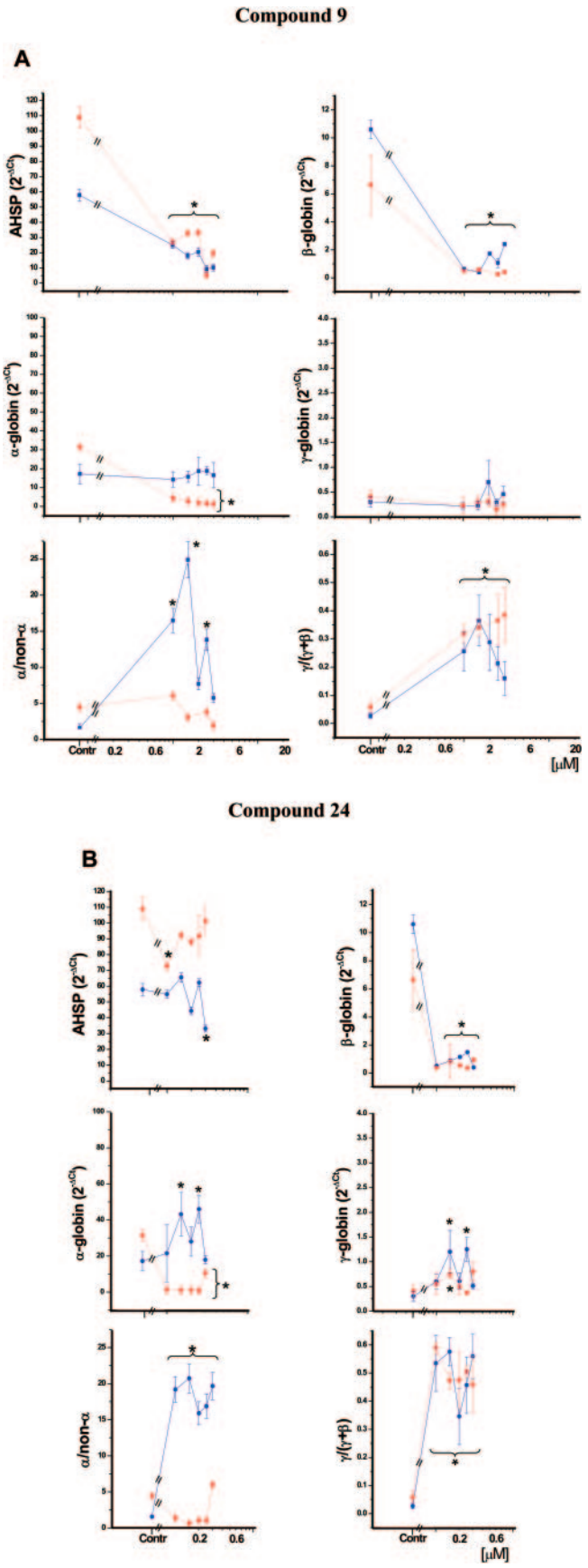

Fig. 8. Concentration/response curves of compounds 9 (A) and 24 (B) on the levels of α-, β-, and γ-globin, as well as AHSP, mRNA expressed by primary erythroblasts obtained from two separate patients with β^0 thalassemia (each color represents a different donor). The corresponding α/non-α and γ/γ+β ratios are reported on the bottom (see legend to Fig. 7 for more information). Results are presented as mean (± S.D.) of single experiments performed in triplicate. ∗ indicate values statistically different ($p < 0.05$) from controls.

expression of the endogenous globin genes, with no or minimal effects on that of α-globin and AHSP (Fig. 2, 3 and results not shown). Because maximal effects on γ- and β-globin expression were induced at different concentrations (Fig. 3) also, in this case, it was possible to identify a concentration window at which γ/(γ+β) was significantly increased (3–8-fold). It is noteworthy that the concentrations (0.2–3 μM) that mostly increased the γ/(γ+β) ratio in primary and in GM979 cells were the same. The synthetic double-reporter assay used in our laboratory only partially predicted the activity of a compound on the endogenous promoters in primary cells. In fact, two of the compounds (8 and 11) active on GM979 cells failed to induce the endogenous genes, whereas one compound (9) induced the β-driven reporter while suppressing expression of the endogenous β-globin gene. This might be because the GM979 cells, while thawed in our laboratory, had partially drifted from those established by Skarpidi et al. (2000).

There was no apparent relationship between class-selective inhibition of maize and human HDACs exerted by a compound (Supplemental Tables 2 and 3) and its efficacy as γ/(γ+β) inducer (Figs. 2 and 3). In fact, compound 11, which was the most class-II selective HDACI (selectivity ratio, 176), was inactive in the γ-globin gene inducing assay, whereas the related but less class II-selective (selectivity ratio, 71) compound 9 was a good γ/(γ+β) inducer (Figs. 2 and 3). On the other hand, compound 24 lacked class selectivity and was more potent than compound 9 as a γ/(γ+β) inducer. Compounds 9 and 24, however, exerted their effects through at least partially different mechanisms (preferential activation of γ- versus β-globin expression versus activation of γ- and inhibition of β-globin expression). The discovery that mammalian HDAC are assembled within the cells as multicomplexes of more than one isoenzyme, each one exerting a specific function, with many other DNA-binding proteins (Minucci and Pelicci, 2006) is rendering obsolete both the concept of class selectivity and the use of the maize enzyme assay as a screening method to predict pharmacologically relevant HDACI. Furthermore, it is likely that a specific HDAC complex regulates the globin gene locus. In this regard, it has been recently reported that the effect of butyrate on γ-globin expression is mediated by the class I HDAC3 (Mankidy et al., 2006), an enzyme part of the complex that include HDAC4 (Verdin et al., 2003). It is conceivable, then, that the identification of the specific HDAC complexes involved in the regulation of the globin locus, which may include but may not be limited to the HDAC3-HDAC4 complex, will finally allow the prediction of the chemical structure of the HDACI most effective as HbF inducer.

Orthochromatic erythroblasts obtained from healthy donors expressed high levels of AHSP and of globin genes (Fig. 6). We were surprised to find that the levels of AHSP expressed by erythroblasts obtained from different donors were different by 1-log (Fig. 6). Three-fold variability in AHSP expression has been recently described in reticulocytes obtained from different healthy subjects. This variability has

been ascribed to a T-homopolymer polymorphism in the putative gene promoter (T18 versus T15) (Lai et al., 2006). The higher (10-fold) variability observed here might be due to the fact that the T18 polymorphism affects most prominently AHSP expression at early than at late stages of maturation or to the existence, in the AHSP locus, of additional regulatory polymorphisms, still to be identified. Further studies will clarify this point.

Erythroblasts obtained from patients with β^0 thalassemia had a clearly abnormal maturation profile in response to EPO. β^0 thalassemic erythroblasts matured poorly, as documented by their morphology, and expressed levels of β-globin 1- to 5-log lower than normal (Fig. 7). We were surprised to find that β^0 thalassemic erythroblasts expressed levels of AHSP 10 times higher than normal. The reason for such high expression is unknown. Because expression of AHSP increases with erythroblast maturation (dos Santos et al., 2004), it is unlikely that such high levels are a reflection of retarded cell maturation. Instead, the significant ($p < 0.05$) linear correlation between the amount of AHSP and that of α-globin mRNA present in erythroblasts obtained from different patients (data not shown) suggests that it might result from an auto-regulatory loop, triggered by the concentration of uncoupled α-chains in the cytoplasm of these cells.

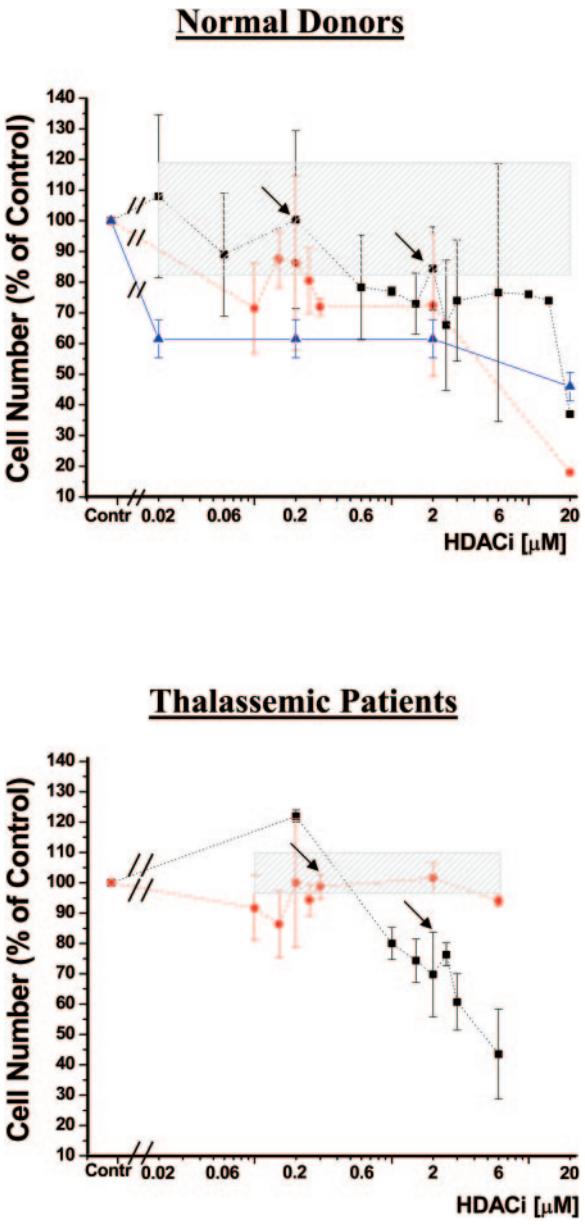

Fig. 9. Toxicity exerted by compounds 9 (squares) and 24 (circles) in cultures of normal (top) and β^0 thalassemic (bottom) erythroblasts, as indicated. Toxicity was evaluated based on the number of cells observed by the end (4 days) of the maturation culture. The number of cells observed in the presence of SAHA (triangles) and of DMSO (the vehicle, shaded area) is also reported, for comparison. Results are expressed as percentage of untreated control cells (100% = 0.85 ± 0.28 versus 1.99 ± 0.93 × 10^6 cells in cultures from healthy and β^0 thalassemic donors, respectively) and are presented as mean (± S.D.) of five to six separate assays. The arrows indicate the concentrations used in Figs. 2 and 7, respectively.

Compounds 9 and 24 both restored the morphological maturation of β^0 thalassemic erythroblasts (Fig. 7). The molecular mechanism used by each compound is not obvious. In fact, in only two patients of five analyzed was it possible to demonstrate that the improved maturation was associated with increased $\gamma/(\gamma+\beta)$ ratios (Fig. 8 and Supplemental Fig. 1). The alterations induced in these two patients were consistent with those induced in normal cells: compound 9 decreased β-globin expression, whereas compound 24 increased γ-globin expression and reduced β-globin expression. It is possible that similar modifications were induced by these compounds in erythroblasts from all the patients included in this study but went undetected because of the time point (4 days of culture) chosen for analysis. Heterogeneity of in vivo response of patients with β^0 thalassemia or sickle-cell anemia to treatment with the HDACI butyrate has been described previously (Perrine et al., 1993; Sher et al., 1995). It has been reported that such heterogeneity is retained by cells obtained from the same patients in vitro (Fathallah et al., 2007). Therefore, a comparison of the response to compounds 9 and 24 of erythroblasts obtained in vitro from patients who do or do not respond to butyrate might provide indications whether these compounds could be used for personalized therapies. These experiments will be performed in the near future.

A difference between the response to compounds 9 and 24 of normal and β^0 thalassemic erythroblasts was represented by the fact that in normal cells, compounds 9 and 24 did not affect α-globin and AHSP expression, whereas both of them reduced the expression of these genes in β^0 thalassemic erythroblasts: the reduction of α-globin expression was modest (by 5-fold) and donor-dependent; the reduction of AHSP expression was of greater magnitude (in the case of compound 9, it was reduced down to the levels observed in normal cells) and was donor-independent (Figs. 7 and 8 and Supplemental Fig. 1). It is tempting to interpret this last result as an indication that the level of AHSP expression was normalized because the concentration of free α-chains in the cytoplasm of the cells had been reduced. However, it is also possible that the effects of HDACI on AHSP expression are independent of their action on the β-globin locus. For example, in β^0 thalassemic erythroblasts, HDACI might interfere with the homeostatic regulatory loop, involving α-globin and AHSP, activated by the cells to compensate for the presence of defective β-chains. In other words, the effects of HDACI on AHSP regulation might function as modifier of the β thalassemic trait, at least in vitro. The hypothesis that AHSP might represent a gene modifier that, as the hereditary per-

sistence of fetal hemoglobin mutation, might ameliorate the phenotype of thalassemic patients was originally suggested by the observation that double AHSP^{null} β thalassemic mice have an exacerbated phenotype (Kong et al., 2004). Although not supported by clinical evidence so far (Viprakasit et al., 2004; Lai et al., 2006), this hypothesis is worthy of further investigation because it might lead to the identification of additional targets for the therapy of sickle-cell anemia and/or β thalassemia.

In conclusion, using a new assay based on primary erythroblasts, we have identified two new HDACIs, compounds 9 and 24, that specifically altered the levels of γ- and β-globin expressed by these cells increasing the $\gamma/(\gamma+\beta)$ ratio with limited donor-to-donor variability. One of the compounds (24) also increased the HbF content of normal cells. Both compounds restored the defective morphological maturation and increased the $\gamma/(\gamma+\beta)$ ratio expressed by β^0 thalassemic erythroblasts in vitro. Because of their low toxicity in all of the assays investigated, we suggest that compounds 9 and 24 might represent new candidates for pharmacological reactivation of HbF for the treatment of patients with β^0 thalassemia.

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