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Research Article

Chemically distinct HDAC inhibitors prevent adipose conversion of subcutaneous human white preadipocytes at an early stage of the differentiation program

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ABSTRACT

The present study investigated the effect of HDAC inhibitors on the differentiation of human subcutaneous white adipocytes. Results showed that trichostatin A, suberoylanilide hydroxamic acid, valproic acid and MS-275 inhibited triglyceride accumulation, GPDH activity and FABP4 protein expression in adipocytes, as well as leptin and VEGF release, while cells retained a fibroblast-like morphology. HDAC inhibitors exerted their antiadipogenic effect without inducing apoptosis or affecting cell viability and number, while 1–2 log unit higher concentrations were mostly required to exert an antiproliferative effect or to reduce LDH activity. A brief exposure to HDAC inhibitors at the beginning of the differentiation program was sufficient to observe the antiadipogenic effect while differentiation restarted after compound withdrawal and further exposure to inducers of differentiation demonstrating reversibility of the events. HDAC inhibitors hyperacetylated histone H4, but only hydroxamate-based compounds produced a massive acetylation of α-tubulin, indicating that this latter event is not required to prevent adipose conversion. HDAC inhibitors induced a significant reduction of the expression of the transcription factor C/EBPα, an early marker of differentiation, and a diminution of fibronectin immunoreactivity was also observed. In conclusion, HDAC inhibitors from different chemical classes potently inhibited human adipose conversion at an early stage of the differentiation program.

Introduction

The adipose tissue consists of highly specialized cells, the adipocytes, whose function is to control energy storage in the form of lipids [1]. During periods of excessive caloric intake, energy is stored in the form of triacylglycerol by the process of lipogenesis, but when the organism needs energy this reserve is mobilized by the process of lipolysis. These two biochemical processes are tightly controlled and determine the rate of lipid storage in adipocytes but predominance of the lipogenic state leads to hypertrophy and hyperplasia of adipocytes in the adipose tissue associated with obesity. Adipocytes can also function as endocrine cells by secreting several adipokines which are involved not only in adipose tissue metabolism in an autocrine and paracrine manner but also in regulating whole-body energy metabolism, immune response, vasculature and others. By consequence, obesity is a well-known

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Abbreviations: C/EBPα, CCAAT/enhancer-binding protein-α; FABP4/aP2, adipose specific fatty acid binding protein; FCS, foetal calf serum; GPDH, Sn-glycerol-3-phosphate dehydrogenase; HBSS, Hank's balanced salt solution; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; LDH, lactate dehydrogenase; NaBu, sodium butyrate; PBS, phosphate buffered saline; SAHA, suberoylanilide hydroxamic acid; TSA, trichostatin A; VEGF, vascular endothelial growth factor; VPA, valproic acid

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risk factor for metabolic perturbations such as diabetes, coronary artery disease and hypertension and some kinds of cancer [2,3,4].

Both the number and size of adipocytes determine the overall adipose tissue mass. New adipocytes arise through adipogenesis, a complex process by which nondifferentiated preadipocytes residing in adipose tissue are triggered to undergo differentiation into adipocytes. Adipocyte differentiation has been studied in vitro by using preadipocyte cell lines, such as mouse 3T3-L1 and 3T3-F442A cell lines, and primary rodents and human stromal–vascular cell cultures derived from various fat depots. Preadipocytes acquire morphological and biochemical characteristics of mature adipocytes through coordinated structural and functional changes [5,6]. Proliferating undifferentiated preadipocytes are morphologically similar to fibroblasts. When the adipogenic program initiates, changes in cell morphology and in components of cytoskeleton and extracellular matrix allow cells to convert to a spherical shape and to accumulate lipid droplets. At a molecular level, differentiation is a well-organized program characterized by loss of preadipocyte markers and sequential expression of general and adipocyte-specific genes, encoding for both early and late markers of differentiation involved in creating and maintaining the adipocyte phenotype. Changes in the pattern of gene expression that occur in the progression of adipocyte differentiation are achieved by coordinated expression and action of transcription factors, including members of the CCAAT/enhancer binding protein (C/EBP) family. Differentiation is accompanied by dramatic increases in the fat cell-specific expression of receptors and signaling molecules, of proteins involved in the transport and metabolism of lipids and carbohydrates, i.e., adipocyte fatty acid binding protein (FABP4, aP2), and of several lipid-metabolizing enzymes including glycerol-3-phosphate dehydrogenase (GPDH). Levels of these proteins and/or mRNAs increase several fold during the terminal phase of differentiation, and adipocytes exhibit marked increases in de novo lipogenesis. Mature adipocytes also release adipokines including leptin that mainly acts on the hypothalamus to regulate energy metabolism by controlling both energy expenditure and food intake and VEGF, an angiogenic factor which plays an important role in adipose tissue development [7].

Covalent modification through acetylation and deacetylation is an important regulatory mechanism of protein structure/function and a key determinant of gene expression. Acetylation and deacetylation are reversible processes controlled by competing enzymatic activities, histone acetyltransferases (HATs) and histone deacetylases (HDACs). The acetylation state of target proteins, including nucleosomal histones, has been shown to result from a subtil equilibrium between addition and removal of acetyl group on specific lysine residues by these two enzymes. HDACs are a family of 11 enzymes capable to deacetylate and regulate the function of both histone and nonhistone proteins involved in cell cycle progression, differentiation and apoptosis [8]. HDACs inhibitors induced either cell cycle arrest, differentiation and/or cell death, blocked cellular invasion and decreased angiogenesis reversing the transformed phenotype of tumor cells in vitro and in animal models, at doses that cause little or no toxicity [9,10]. These effects have been correlated to the accumulation of acetylated histones and consequent changes in gene expression, and both induction and repression of genes have been linked to inhibition of HDAC activity. HDAC inhibitors are currently tested in clinical trials for the treatment of a wide range of solid and haematological tumors, SAHA (vorinostat, Zolinza) being approved by Food and Drug Administration for the treatment of the advanced primary cutaneous T-cell lymphoma.

Recent studies have demonstrated that HDAC inhibitors, in addition to their anticancer effects, modulate gene expression and differentiation in normal cells and determine the cell fate for both embryonic and adult stem cells, modulate inflammatory responses and exert antifibrogenic effects. Indeed treatment with HDAC inhibitors leads to increased hepatic and pancreatic cell determination [11,12], acceleration of gut epithelial differentiation [13] and inhibition of osteoclast-like cells formation [14]. Moreover, HDAC inhibitors have been reported to regulate the differentiation of preadipocytes although the role of HDACs during adipogenesis is somewhat controversial. Indeed, two groups showed that HDAC inhibitors suppress adipogenic gene expression and adipose conversion in rodents [15,16]. By contrast, more recent studies have highlighted a requirement for reduced HDAC activity to activate the transcription of adipogenic genes while inhibition of HDAC activity resulted in stimulation of adipocyte differentiation [17,18].

The aim of the present study was to evaluate the effect of increasing concentrations of HDAC inhibitors on the proliferation and the differentiation of human subcutaneous white adipocytes. We used HDAC inhibitors from both natural sources (TSA, NaBu) or synthetically developed (SAHA, MS-275, VPA). These agents, classified on the basis of their chemical structure, inhibit the enzymatic activity of HDACs with varying efficiency and specificity. While the hydroxamate-based inhibitors, TSA and SAHA, are pan-HDAC inhibitors, the short chain fatty acid, VPA, is a class I selective inhibitor and the benzamide compound MS-275 is selective towards only a subset of class I HDACs [19]. Cell proliferation was evaluated using the Alamar blue assay or by direct cell counting. Measurement of both intracellular and released LDH activity has been used as a parameter to evaluate the specificity and the potential cytotoxicity of compounds tested, respectively, while DNA fragmentation was used as an index of apoptosis. Long term effect of compounds on differentiation was evaluated by determining the accumulation of triglycerides, the activity of the cellular lipogenic enzyme GPDH, the expression of FABP4 and the release of the adipokines leptin and VEGF, while short-term effect was evaluated by measuring the acetylation of target proteins and the expression of both C/EBPα and fibronectin. Both reversibility and time-dependency of drug action were also investigated.

Materials and methods

Cryopreserved subcutaneous human white preadipocytes (HwprA), normal human dermal fibroblasts (NHDF) and NHDF culture reagents have been obtained from Cambrex Bioscience (Verviers, Belgium). Preadipocytes obtained from three healthy donors (donor 12081-lot 4F0572 male, 50 years BMI = 29; donor 12945 lot 5F0178 female, 55 years BMI = 27; donor 12936 lot 5F0199 female, 57 years) were used in this study. When $n = 4$, additional experiments with cells from donor 12936 have been included. Preadipocyte and adipocyte culture medium and supplements have been purchased from Promocell (Heidelberg, Germany). Cell culture flasks were from Falcon, tissue culture treated 96-well view plates were from Perkin Elmer (Life and Analytical Sciences, Monza, Italy) and 96-well black special optics with clear flat

bottom polystyrene plates were from Corning life Sciences (Schiphol-Rijk, The Netherlands). Anti-acetylated histone H4 rabbit polyclonal IgG was from Upstate Biotechnology (Lake Placid, NY); acetylated α-tubulin monoclonal antibody (clone 6-11B-1), anti-C/EBPα (14AA) rabbit polyclonal IgG and goat normal serum were from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-FABP4 rabbit polyclonal IgG was from Cayman Chemical (Ann Harbor, MI). Highly cross-adsorbed Alexa Fluor 488- and 546-labelled secondary antibodies and Image-iT™ FX signal enhancer (blocking solution 1) were from Molecular Probes (Eugene, OR). All the other chemicals reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. TSA, VPA and NaBu were from Calbiochem (Merck Chemicals, Darmstadt, Germany) and Alamar blue [modified 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Biosource International (Camarillo, CA). MS-275 (*N*-(2-aminophenyl)4-[*N*-(pyridine-3-yl-methoxycarbonyl)aminomethyl]benzamide) and vorinostat (suberoylanilide hydroxamic acid, SAHA) were synthesized at the Chemistry Department of Menarini Ricerche S.p.A. (Pomezia, Italy).

Culture and differentiation of human subcutaneous white preadipocytes

Human subcutaneous white preadipocytes passage 1 were used for experiments without subculturing. Cells were seeded at a density of 55,000/cm^2 in tissue culture treated 96-well view plates in preadipocyte growth medium and maintained in an humidified atmosphere of 5% CO_2–95% air at 37 °C. After 48 h, cells were transferred in differentiation medium containing 4.5% FCS, d-biotin 8 μg/ml, insulin 0.5 μg/ml, dexamethasone 400 ng/ml, isobutylmethylxanthine 44 μg/ml, L-thyroxine 9 ng/ml and ciglitazone 3 μg/ml. To induce partial differentiation (e.g., ~50% of cells containing visible lipid droplets), confluent cultures were maintained in this medium for the first 3 days while full differentiation was obtained by exposing preadipocytes to differentiation medium for 6 days [20]. After the induction period, differentiating cells are transferred into adipocyte nutrition medium containing FCS 3%, d-biotin 8 μg/ml, insulin 0.5 μg/ml and dexamethasone 400 ng/ml, until assay, typically on days 10 and 11. Post-confluence determination of cell number was achieved by trypsinizing cell monolayers and counting the cell suspension with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). Unless specified, HDAC inhibitors were added at the initiation of the differentiation program and maintained in culture medium until assay. VPA and NaBu were dissolved in aqueous buffer while SAHA, TSA and MS-275 were first dissolved in 100% DMSO. Finally, compounds were diluted in culture medium in order to achieve the final test concentration. The final vehicle concentration in each well did not exceed 1%.

Culture of NHDFs

NHDFs were routinely grown as monolayers in growth medium with medium change every day at 37 °C in an atmosphere of 5% CO_2/95% air and 90% relative humidity according to the supplier's instructions. When 70–80% confluency was reached, cells were subcultured using a solution of trypsin/EDTA and plated at a density of 3.5×10^3 cells/cm^2 into new flasks for maintenance. Cells were split twice weekly and used between passage 2 and 5.

Cell proliferation assay

Both preadipocytes and NHDFs were seeded on 96-well special optics microplates at a density of 6000 cells/well and transferred after 18 h in fresh medium supplemented with the indicated concentration of HDAC inhibitor. Plates were then incubated at 37 °C in an atmosphere of 5% CO_2/95% air and 90% relative humidity for 4 days. Cell number was then measured using the Alamar blue assay which is based on detection of cellular metabolic activity. Each well was added a 10% volume of diluted Alamar blue and microplates were incubated for 4 h in a CO_2 incubator at 37 °C with orbital shaking. Cell number was quantified by fluorescence measurement at 560 nm (excitation) and 590 nm (emission) wavelengths in a luminescence spectrometer (Perkin Elmer, LS-50B) equipped with a microplate reader. Blank fluorescence did not exceed an intensity of 10 AU. Data were acquired by the well plate reader program of the Flwinlab software (Perkin Elmer). Percent inhibition of proliferation was determined by comparison of treated cells to control untreated cells with the background fluorescence without cells subtracted out.

Quantification of intracellular triglyceride content

Preadipocytes were induced to differentiate in the absence or presence of HDAC inhibitors at the indicated concentration. Intracellular triglyceride content was measured using a commercially available colorimetric assay that quantifies the glycerol content of the samples (triglyceride assay kit Zen-Bio, Inc., Research Triangle Park, NC). This assay involves the enzymatic hydrolysis of triglycerides by lipases to free fatty acid and glycerol, and the reaction of the glycerol moiety with a substrate to produce a red dye. Absorbance was quantified at 540 nm using a Biotek Ceres UV900 microplate reader and triglyceride concentration in each sample was determined based on a standard curve according to the manufacturer's instructions.

Determination of GPDH activity

GPDH activity was determined spectrophotometrically by measuring the disappearance of NADH during GPDH-catalyzed reduction of dihydroxyacetone phosphate. At days 10 and 11, cells induced to differentiate in the absence (control) or presence of HDAC inhibitors at the indicated concentrations (treated cells) were washed twice with PBS, then enzyme extraction buffer was added to induce cell lysis, and cell extracts were collected by scraping. After centrifugation at 10,000 rpm for 5 min at 4 °C, GPDH specific activity in the supernatant was measured using a GPDH Activity Assay Kit (Takara Bio, Inc., Shiga, Japan). Decreases in absorbance at 340 nm were determined as a function of time using a Biotek Ceres UV900 microplate reader. Changes in absorbance per min were obtained by analyzing the linear part of the kinetics curves using a linear regression fitting, and the quality of the fit was determined by evaluating the coefficient of determination ($r^2 > 0.9$).

Determination of LDH activity

LDH activity was measured either in cell supernatants or in cell extracts of both control and HDAC inhibitor treated cells. Release of LDH in the culture medium through membrane leakage was

measured as an indicator of cytotoxicity while intracellular LDH activity was used as an index of HDAC inhibitor selectivity. Cell supernatants were withdrawn and LDH activity was determined using the cytotoxicity LDH kit as described by ROCHE Diagnostics (Roche Molecular Biochemicals, Mannheim, Germany). The test is based on the colorimetric determination of formazan formed in a reaction mixture containing NAD+, lactate, the tetrazolium salt INT, diaphorase and cell lysates. The negative control was fresh HBSS HEPES, pH 7.4, and the positive control was a total cellular extract obtained by treating control cells with Triton X100 2% in HBSS HEPES, pH 7.4. The percentage of cytotoxicity was calculated relative to the LDH activity released in the media as a percentage of the total LDH activity in both cells and media. Intracellular LDH activity was determined in extracts as obtained in GPDH assays, by measuring changes in absorbance as a function of time using a Biotek Ceres UV900 microplate reader. LDH activity was calculated by linear regression analysis of the kinetic curves in order to get coefficients of determination, r^2, of at least 0.9.

Apoptosis analysis

The terminal deoxynucleotidyl transferase (TdT)-mediated dNTP nick end labelling (TUNEL) method was used for the detection of cells undergoing apoptosis. We used the fluorescent-TdT enzyme DNA Fragmentation Labeling Kit, FragEL, from Calbiochem (Oncogene Research Products, Cambridge, MA). In this method, TdT binds to exposed 3′-OH ends of DNA fragments generated in apoptotic cells in order to add fluorescein-(un)labelled dNTPs and the staining of the cells was performed according to the manufacturer's recommended procedure. Cells that stained positive produce an intense fluorescent signal when excited with fluorescein filters, whereas the total cell population was counterstained with Hoechst 33258. Data are reported as the percentage of TUNEL-positive cells determined by dividing the number of TUNEL staining cells by the total number of cells (Hoechst-stained cells).

Measurement of adipokine release by ELISA

Leptin and VEGF were measured in cell culture media of differentiating human preadipocytes using commercially available ELISA kits. The cells were treated with vehicle or HDAC inhibitors at the indicated concentration and for the indicated period of time. Media were collected, centrifuged to remove cell debris, and stored at −80° until assay. Amounts of leptin and VEGF protein in supernatants were quantified by using colorimetric sandwich enzyme immunoassays conducted in 96-well microplates according to the manufacturer's instructions (human leptin Quantikine ELISA kits from R&D Systems, Abingdon, UK, and human VEGF ELISA kit from Oncogene Research products, San Diego, CA).

Indirect immunofluorescence staining procedures and quantitative image analysis

Immunofluorescence studies were used to assess the expression and distribution of acetylated α-tubulin, acetylated histone H4 and fibronectin, and to detect proteins involved in adipose conversion (FABP-4 and C/EBPα). Cells were cultured on 96-well special optics microplates and exposed to HDAC inhibitors (TSA, SAHA and MS-275 all at 1 μM, VPA 1 mM) for 6 h (acetylation assay) or for 2–6 days (FABP4, C/EBP-α and fibronectin detection). Cells were washed twice with PBS, then fixed for 15 min in 4% paraformaldehyde in PBS, permeabilized with methanol for 5 min at −20 °C, and incubated for 30 min in blocking solution 1 then for further 30 min in 10% goat normal serum/PBS to neutralize non-specific binding sites. Thereafter, cells were incubated or coincubated, for double-labeling experiments involving visualization of acetylated proteins, overnight at 4 °C with primary antibody at the appropriate dilution. The following day, treated cells were exposed for 1 h at room temperature in the dark to the appropriate fluorochrome-conjugated secondary antibody at a dilution of 1:400. To visualize nuclei, cells were counterstained with 1 μg/ml bisbenzimide (Hoechst 33258; Fluka). Primary antibodies were prepared and diluted in PBS plus 0.15% goat normal serum; rabbit polyclonal anti-acetylated histone H4, anti-acetylated α-tubulin and anti-fibronectin monoclonal antibodies were diluted 1:100; rabbit polyclonal anti-C/EBPα was diluted 1:30; and rabbit polyclonal anti-FABP4 was diluted 1:50. Rabbit polyclonal anti-acetylated histone H4 antibody and anti-acetylated α-tubulin monoclonal antibody were used in combination followed by labelling with a mixture of Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse IgG. In the case of C/EBPα, FABP4 and fibronectin immunostaining, the secondary antibodies were Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 546 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse IgG, respectively. Specificity tests were performed by omission of primary antibodies from staining and use of preimmune serum instead of the first antiserum. When pairs of antibodies were used, control experiments were performed in which one of the primary antibodies was omitted. In all cases, omission of the primary antibody resulted in total elimination of fluorescent signals. Images were acquired from a Leitz inverted fluorescence microscope equipped with a CCD camera (Infinity, Lumenera Corporation, Ottawa, Canada). Computerized image analysis was performed on fluorescence images collected with identical settings using the Nikon Image analysis software (NIS elements). The cell imaging system determines the labeling area, mean fluorescence intensities and integral fluorescence signals. Fluorescence intensity signals were normalized to the number of cells. We analyzed multiple non-overlapping microscopic fields from independent experiments for a total of more than 1500 cells scored for each treatment.

Data analysis

All the data are expressed as mean ± SEM and plotted using the program GraphPad Prism, version 4 (GraphPad Software, San Diego, CA). Percent effect was calculated relative to a vehicle treated control. The mean concentration required to inhibit cell growth or differentiation by 50% (IC_{50} value) was calculated from nonlinear regression analysis of individual concentration response curves using a four-parameters logistic equation [$Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10\text{^}((\text{LogEC}_{50} - X) * \text{HillSlope}))$]. The smooth curves shown in the figures were derived by fitting the mean data. Statistical analysis by two-tailed Student's *t*-test for unpaired data or by means of analysis of variance (one-way or two-way ANOVA), followed by a multiple comparisons test (Bonferroni's or Dunnett's post-hoc tests), was made by using the computer program InStat for Macintosh (GraphPad software) or the program GraphPad Prism. A value of $p < 0.05$ was considered statistically significant.

Results

Antiproliferative activity of HDAC inhibitors on subcutaneous human white preadipocytes

HDAC inhibitors were tested for their ability to inhibit the proliferation of subcutaneous human white preadipocytes using the Alamar blue growth inhibition assay. Results showed that TSA, SAHA and MS-275 prevented cell growth in a concentration-dependent manner although high concentrations are required to achieve a maximal effect, with 98.2 ± 0.3, 95.2 ± 0.3 and 97.5 ± 0.3% inhibition at 10, 100 and 100 μM, respectively, the rank order of potency being TSA > SAHA = MS-275 (Fig. 1A, Table 1). The IC_{50} values necessary to inhibit the proliferation of human preadipocytes are close to that obtained with NHDFs, other human normal fibroblast-like cells (Fig. 1B, Table 1). In both cell cultures, VPA exhibited only partial effects at mM concentrations.

Effect of long term treatment with HDAC inhibitors on markers of adipose conversion

To assess the importance of HDACs in adipocyte differentiation, HDAC inhibitors at concentrations comprised between 0.1 nM and 3 mM were added to confluent preadipocytes at the initiation of the differentiation program. Treatment was continued until differentiation was almost complete in control conditions as assessed by microscopic observation (typically 10–11 days), then both intracellular triglyceride content and GPDH activity were determined. Results showed that the number of oil droplets was reduced in cells exposed to HDAC inhibitors but was abundant in control differentiated adipocytes. Moreover, HDAC inhibitor treated cells displayed a fibroblast-like morphology (Fig. 2A). Consistent with the appearance of the formed oil droplets, HDAC inhibitors reduced intracellular triglyceride levels and GPDH activity in a concentration-dependent manner (Figs. 2B and C). The hydroxamate-based HDAC inhibitors, TSA and SAHA, and to a lesser extent the benzamide inhibitor MS-275 are potent compounds as compared to the short chain fatty acid inhibitor VPA, with inhibitory potencies in the nanomolar range and a maximally effective concentration at 1 μM (Table 1). Similarly to VPA, the short chain fatty acid NaBu 0.5 mM inhibited GPDH activity and triglyceride accumulation by 74 ± 2% and 66 ± 3%, respectively.

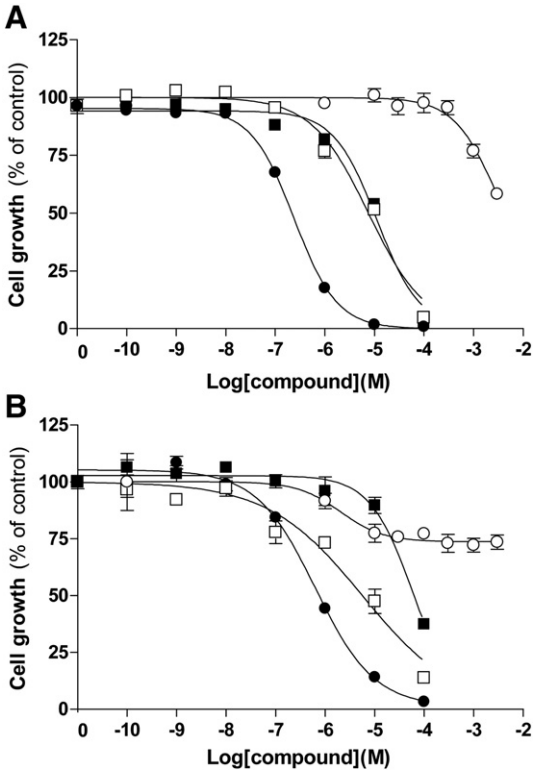

Fig. 1 – Inhibition of human subcutaneous white preadipocyte (A) and normal dermal fibroblast (B) growth by HDAC inhibitors. Cells were seeded at low density on 96-well culture plates in respective growth medium and, after 18 h, they were incubated with increasing concentrations of TSA (●), SAHA (□), MS-275 (■) and VPA (○). Following a 4-day incubation period the number of viable cells was evaluated using the metabolic stain, Alamar blue. Data are presented as a percentage of untreated 96 h control and expressed as mean ± SEM of separate determinations (n = 4).

The effect of HDAC inhibitors on the expression of the lipid binding protein FABP4 was investigated by immunofluorescence analysis on day 6. These studies revealed that immunostaining of FABP4 was strongly reduced by exposure of the cells to MS-275 1 μM and almost abolished by SAHA 1 μM and VPA 1 mM (87.1 ± 3.5%, 98.1 ± 0.6% and 98.8 ± 0.4% inhibition, respectively, Figs. 2A and D).

Effect of HDAC inhibitors on LDH activity, cell number, and apoptosis

The activity of intracellular LDH was determined on day 11 in control cells and in cells exposed to various concentrations of HDAC inhibitors in order to evaluate their selectivity versus another metabolic enzyme. Results showed that TSA, SAHA and MS-275 at concentrations that completely inhibited GPDH activity and triglyceride accumulation had little or no effect on intracellular LDH activity (Fig. 3A, Table 1). At 10 μM concentration, about 50% of LDH activity was still present. By difference, VPA inhibited intracellular LDH activity in a concentration range similar to that necessary to inhibit adipose conversion. Moreover LDH release into the extracellular medium, as an index of cell viability, and cell number have been determined at the end of the induction period. As shown in Figs. 3B and C, TSA, SAHA and VPA did not induce cell lysis since LDH release was 95 ± 21%, 93 ± 19% and 89 ± 6% of control, respectively, and did not alter cell number that was 90 ± 5%, 93 ± 5% and 94 ± 5% of control, respectively. Consistent with these results, 48 h treatment with HDAC inhibitors did not enhance DNA fragmentation that, in control cells, concerned only a very small portion of the cell population. Indeed TUNEL-positive cells, as a measure of apoptosis, were 2.6 ± 0.8%, 3.3 ± 0.5%, 2.7 ± 0.6% and 3.5 ± 0.5% in control and in the presence of TSA, SAHA and VPA, respectively (Fig. 3D).

Time-dependency and reversibility of HDAC inhibitors effects on adipose conversion

To test whether the time of addition of HDAC inhibitors and the duration of treatment could have an influence on adipocyte differentiation, human white preadipocytes were cultured in conditions that induced partial differentiation in the presence of TSA

Table 1 – Summary data of the effect of HDAC inhibitors on human subcutaneous white preadipocyte proliferation and adipose conversion.

HDAC inhibitor	Antiproliferative effect IC_{50} (μM)		Antiadipogenic effect IC_{50} (μM)		
	NHDF	HwprA	Triglyceride accumulation	GPDH activity	LDH activity
TSA	0.6 ± 0.1	0.24 ± 0.01	0.017 ± 0.003	0.023 ± 0.006	67 ± 2% inhibition [a]
SAHA	5.6 ± 1.7	8.1 ± 0.8	0.018 ± 0.004	0.043 ± 0.006	54 ± 1% inhibition [a]fs
MS-275	56 ± 6	11.1 ± 0.6	0.043 ± 0.007	0.29 ± 0.034	48 ± 2% inhibition [a]
VPA	27 ± 4% inhibition [b]	42 ± 2% inhibition [b]	275 ± 28	131 ± 4	232 ± 13

[a] At 10 μM.
[b] At 3.3 mM.

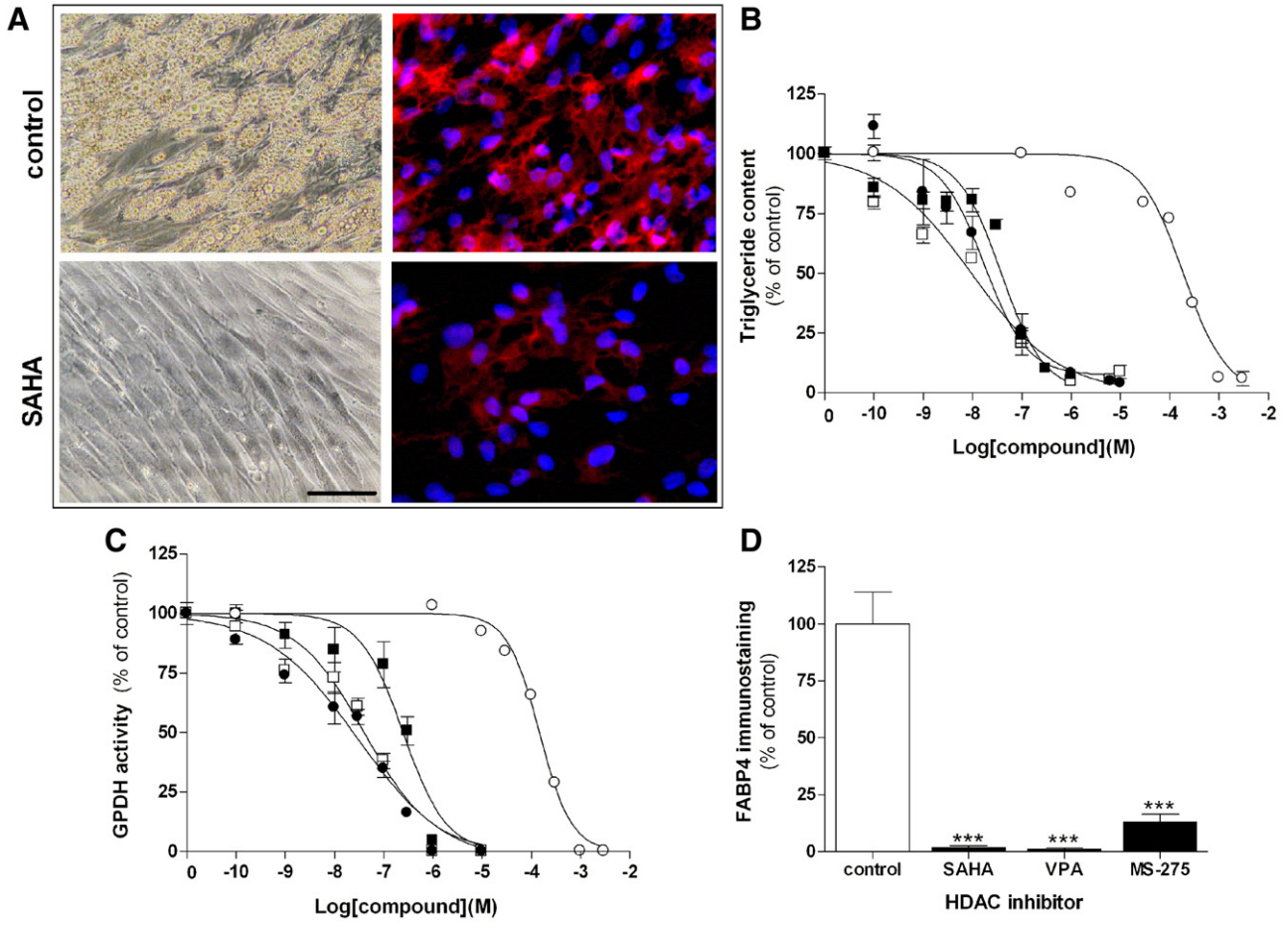

Fig. 2 – Concentration-dependent effect of HDAC inhibitors on human subcutaneous white preadipocytes differentiation. (A) Phase-contrast microscopy pictures showing HDAC inhibitor (SAHA 1 μM) treated cells as compared to control differentiated cells, and representative images from immunofluorescence staining experiments showing cytoplasmic FABP4 (Alexa 546 red label) in cells exposed to vehicle or SAHA, and counterstaining of nuclei with Hoechst 33258 (blue label). Scale bar, 50 μm. To quantify the effect of compounds on adipose conversion cells were induced to differentiate in the presence of increasing concentrations of TSA (●), SAHA (□), MS-275 (■) and VPA (○) and intracellular triglyceride content (B) and GPDH activity (C) were measured and presented as percentage of control, untreated cells. Inhibition of FABP4 expression by the HDAC inhibitors (full columns) SAHA (1 μM), VPA (1 mM) and MS-275 (1 μM) is shown in (D) as compared to control (empty column). Data are expressed as mean ± SEM of separate determinations ($n = 4$). Differences from control cells are reported as ***, $p < 0.001$ (one-way ANOVA followed by Dunnett's post-hoc test).

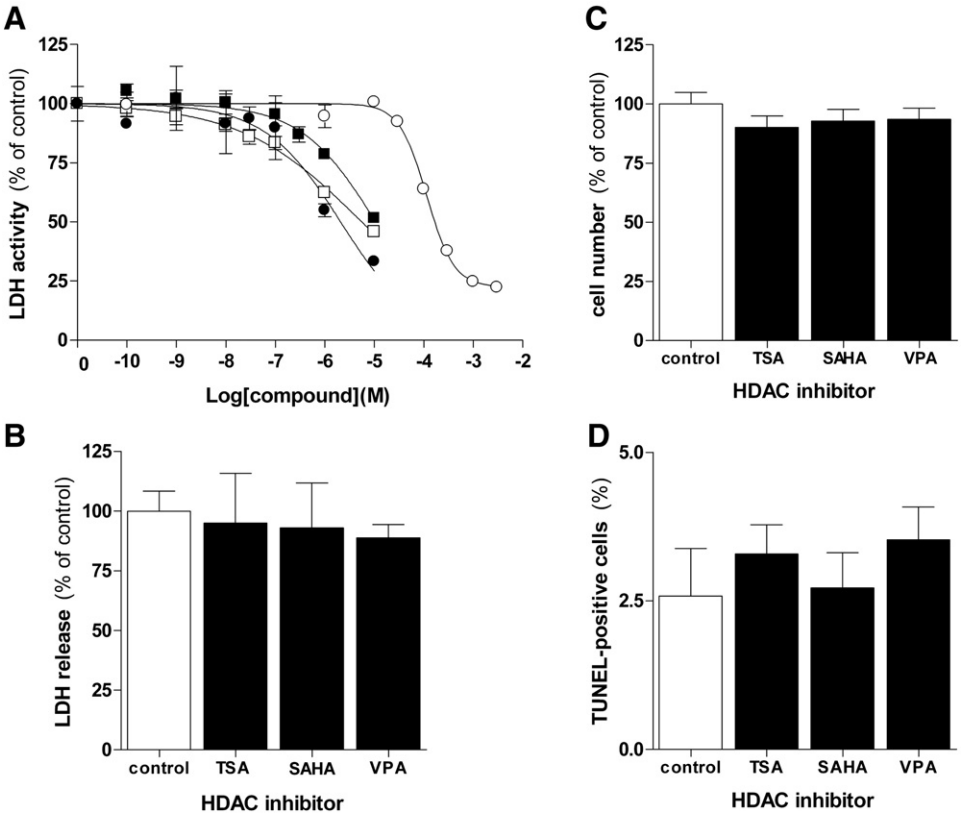

Fig. 3 – Effect of HDAC inhibitors on LDH activity, cell number, and apoptosis. (A) Cells were induced to differentiate in the presence of increasing concentrations of TSA (●), SAHA (□), MS-275 (■) and VPA (○) and intracellular LDH activity was determined as compared to control. Released LDH activity in cell supernatants (B) and relative number of cells (C) after treatment with TSA (1 μM), SAHA (1 μM) and VPA (1 mM) were compared to control. In (D) are reported the data from apoptosis assay showing the percentage of TUNEL-positive cells in control conditions or after treatment with HDAC inhibitors. Data are expressed as mean ± SEM of separate determinations ($n = 4$). Differences between treated and control cells in section (B), (C) and (D) were not significant, $p > 0.05$ (one-way ANOVA followed by Dunnett's post-hoc test).

(0.2 μM), SAHA (1 μM) and VPA (1 mM) for different time intervals after initiation of differentiation. Data showed that lipid accumulation and GPDH activity were dependent on the time of treatment with HDAC inhibitors (Figs. 4, 5). Indeed, most of the inhibitory effect of HDAC inhibitors is exerted between day 0 and day 3 of differentiation and persisted after compound withdrawal although increasing the duration of treatment up to day 6 resulted in a more pronounced effect on both triglyceride content and GPDH activity for VPA (Figs. 4A and C) and on GPDH activity for TSA (Fig. 4C). Initiating treatment after induction of differentiation reduced their efficacy and the later cells are exposed to the compound (interval of treatment from days 3–6 and days 6–10) the less is the inhibitory effect observed (Fig. 4B).

To investigate the reversibility of HDAC inhibitory effect, preadipocytes were incubated in the absence or in the presence of TSA, SAHA and VPA in conditions that induced partial differentiation. On day 6, pharmacological treatment was interrupted and cells were exposed to nutrition medium or again to differentiation medium until full differentiation was obtained in control cells. Results showed that the inhibitory effect of HDAC inhibitors persisted for several days after their removal when cells are maintained in nutrition medium while addition of differentiation medium on day 6 produced an increase in triglyceride content and GPDH activity in both control and treated cells (Figs. 5A and B). The increase in GPDH activity and triglyceride content is accompanied by an increase in the number of oil droplets in both control and HDAC inhibitor treated cells (Fig. 5C), these latter cells having lost their fibroblastic morphology.

Effect of HDAC inhibitors on adipokine secretion

The effect of HDAC inhibitors on adipokine release was investigated by measuring the amount of leptin and VEGF in cell supernatants. Results showed that the antiadipogenic effect of HDAC inhibitors is accompanied by a drastic inhibition of the release of both adipokines. Indeed, continuous exposure of differentiating preadipocytes to TSA, SAHA or VPA resulted in a marked reduction of leptin secretion on day 10. As observed before for lipid accumulation, HDAC inhibitors retained their efficacy in preventing leptin release when applied to preadipocytes for a shorter period of time, from day 0 to day 6 and from day 0 to day 3 after induction of differentiation (Fig. 6A). Similarly, treating the cells from day 0 to day 3 with SAHA or VPA impaired the 6-fold increase of VEGF secretion that accompanied differentiation, the inhibitory effect of HDAC inhibitors being observable on both day 6 and day 10 after induction of differentiation (Fig. 6B).

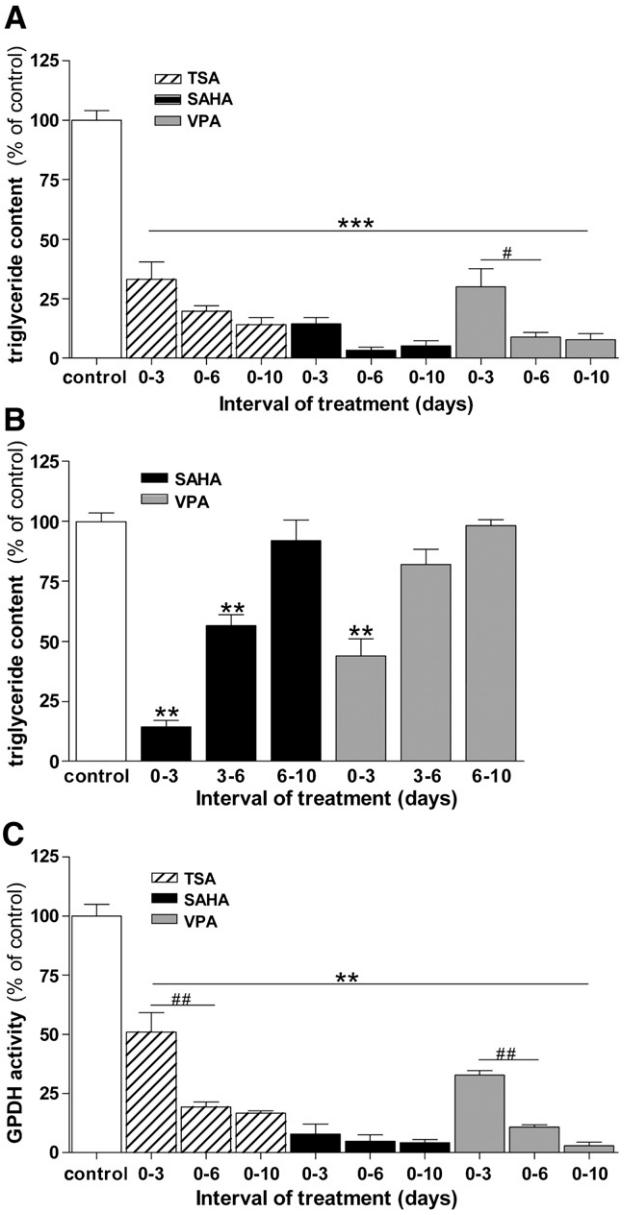

Fig. 4 – Time-dependency of HDAC inhibitor antiadipogenic effect. Cells were treated with TSA (0.2 μM), SAHA (1 μM) and VPA (1 mM) for the indicated time interval in conditions that promote partial differentiation. Intracellular triglyceride content (A and B) and GPDH activity (C) were reported as percent of control. Differences are reported as **, $p < 0.01$ and ***, $p < 0.001$ versus control; #, $p < 0.05$ and ##, $p < 0.01$ versus previous time interval (two-way ANOVA followed by Bonferroni's post-hoc test, $n = 4$).

Acetylation of histone H4 and α-tubulin in the presence of HDAC inhibitors

To detect changes in the acetylation of both histone and nonhistone proteins human preadipocytes were exposed to TSA, SAHA, MS-275, 1 μM each, and VPA 1 mM in differentiation medium and quantitative immunocytochemistry analysis was performed after 6 h. As shown in Figs. 7A and C, the basal level of acetylated histone H4 immunostaining is increased by 4- to 5-fold in the presence of TSA, SAHA and VPA and by 2-fold in the presence of MS-275 indicating that treatment of preadipocytes with these compounds strongly inhibited HDAC activity. As shown in Figs. 7B and C, acetylated α-tubulin positive cells were undetectable under control conditions. However, the hydroxamate-based HDAC inhibitors, TSA and SAHA, strongly induced the acetylation of α-tubulin while VPA and MS-275 were without effect. These latter compounds failed to stimulate acetylated α-tubulin immunostaining even at 24 and 48 h contact time. Fig. 7C showed that, in the presence of TSA and SAHA, acetylation of histone H4 and α-tubulin concerned most of the cells, and it also showed the cytoplasmic distribution of acetylated α-tubulin immunoreactivity at difference of the nuclear localisation of acetylated histone H4.

Effect of short-term treatment with HDAC inhibitors on C/EBPα and fibronectin expression

Human adipocyte differentiation is activated by the coordinate action of transcription factors and C/EBPα played a crucial role in this process. To determine the effect of HDAC inhibitors on early markers of adipocyte differentiation, preadipocytes were induced to differentiate in the presence of HDAC inhibitors and the expression of C/EBPα was evaluated by immunocytochemical analysis on days 2 and 6 after induction of differentiation. After 48 h treatment with HDAC inhibitors in differentiation medium, nuclear C/EBPα immunostaining was significantly reduced, with SAHA and TSA (1 μM), VPA (1 mM) and MS-275 (1 μM) displaying a similar effect ($50 \pm 4\%$, $58 \pm 4\%$, $57 \pm 3\%$ and $51 \pm 5\%$ of inhibition, respectively) (Figs. 8A and C). The effect of the HDAC inhibitors was still persistent on day 6 after induction of differentiation with $50 \pm 2\%$, $28 \pm 5\%$ and $33 \pm 7\%$ of inhibition in the presence of SAHA, VPA and MS-275, respectively ($p < 0.01$, $n = 3$).

HDAC inhibitor treated preadipocytes maintained a fibroblast-like morphology while control cells rapidly round up and accumulate lipid droplets (Figs. 2A and 5C). To assess if HDAC inhibitor treatment is accompanied by alterations in the extracellular matrix components as reported recently for TSA in human skin fibroblasts [27], we evaluated fibronectin level in preadipocytes exposed to maximally effective concentrations of SAHA, TSA, VPA and MS-275. Results showed that fibronectin immunostaining was significantly reduced by TSA (1 μM), VPA (1 mM) and MS-275 (1 μM) to $50 \pm 2\%$, $65 \pm 5\%$ and $65 \pm 6\%$, respectively (Figs. 8B and C). An effect of SAHA on fibronectin expression was not observed although treated preadipocytes retained their fibroblast-like morphology (Figs. 2A and 5D).

Conclusion

The present study was designed to evaluate the effect of HDAC inhibitors, representative of different chemical classes of compounds, on both proliferation and differentiation of subcutaneous human white preadipocytes. We report here that HDAC inhibitors exerted an antiproliferative effect on human preadipocytes in a range of concentrations close to that effective on other non-tumoral fibroblast-like cells, NHDFs, but higher than those reported to inhibit the growth of tumor cells extending the

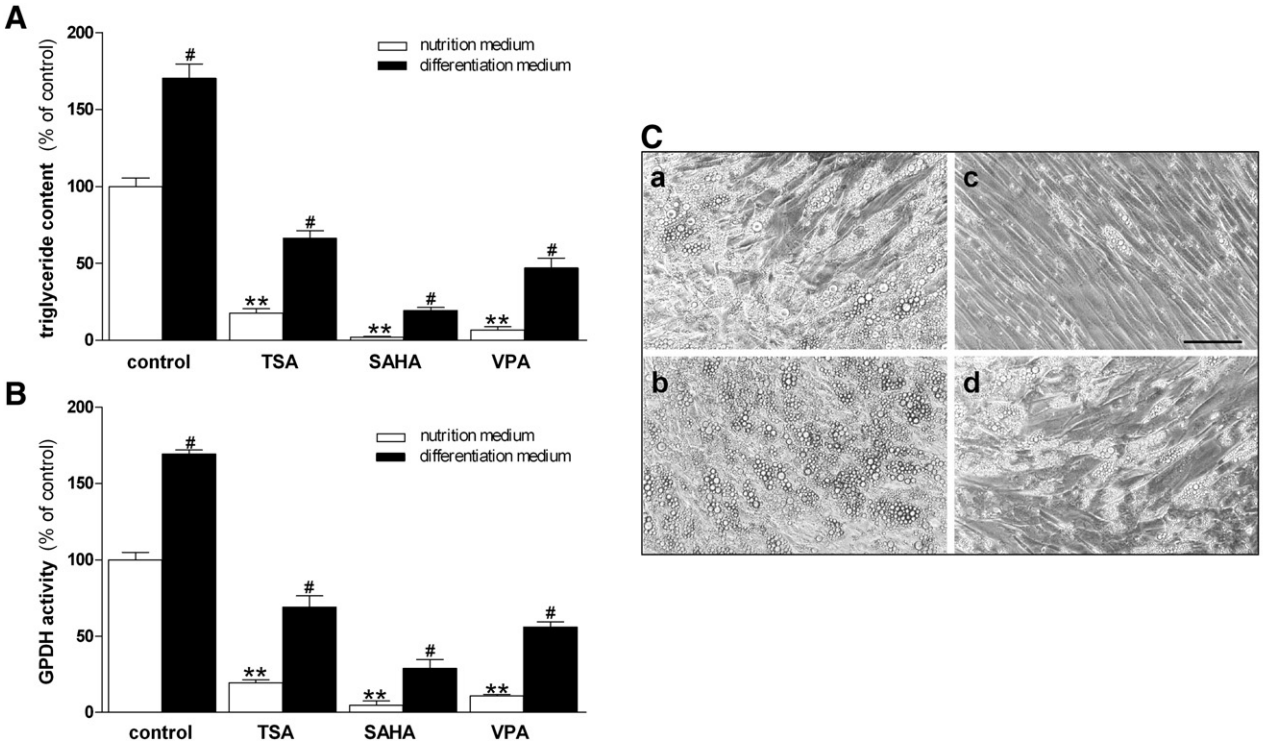

Fig. 5 – Reversibility of HDAC inhibitor antiadipogenic effect. Cells were treated with HDAC inhibitors (TSA 0.2 μM, SAHA 1 μM, VPA 1 mM) in conditions that promote partial differentiation until day 6, then exposed to nutrition (empty columns) or differentiation (full columns) medium. Intracellular triglyceride content (A) and GPDH activity (B) were expressed as percent of control in nutrition medium. Differences from control nutrition medium are indicated as **, $p < 0.01$; differences between nutrition and differentiation medium are reported as #, $p < 0.001$ (two-way ANOVA followed by Bonferroni's post-hoc test, $n = 4$). (C) Phase-contrast microscopy pictures showing preadipocytes treated with vehicle (a and b) or SAHA 1 μM (c and d) for 6 days in conditions that promote partial differentiation. Cells were then exposed to vehicle (a and c) or to inducers of differentiation (b and d). Scale bar, 50 μm (a–d).

previous observations on the relative resistance of normal cells to the cell death inducing properties of HDAC inhibitors [21,22]. Indeed, HDAC inhibitors impaired preadipocyte growth at concentrations ranging from 0.1 to 10 μM for TSA and from 1 to 100 μM for SAHA and MS-275 while only a weak effect was observed at mM concentrations for VPA. When added to confluent human preadipocytes, HDAC inhibitors suppressed in a concentration-dependent manner the induction of late markers of adipocyte differentiation as determined by the drastic reduction of triglyceride accumulation, GPDH activity, FABP4 expression and adipokine release and cells maintained a fibroblast-like morphology. In this case, HDAC inhibitors resulted to be potent compounds since prevention of lipid storage was observed at concentrations ranging from 1 to 300 nM. These data confirmed that HDAC inhibitors may exert not only a stimulatory effect on cell differentiation, as described for tumor cells and some normal cells, but also an inhibitory effect [14]. HDAC inhibitors may also exhibit opposing effect like on neural progenitor cells with induction and suppression of neuronal and glial differentiation, respectively [23], and on mesenchymal stem cells with enhancement of osteogenic differentiation and inhibition of adipogenic differentiation [24].

Inhibition of adipose conversion is not an effect restricted to a particular class of compounds but is observed with broad spectrum HDAC inhibitors, the hydroxamic acids TSA and SAHA, and with compounds having a restricted HDAC specificity (MS-275, VPA and NaBu) [19]. A similar action exhibited by chemically distinct compounds suggested that the antiadipogenic effect was mediated by their ability to inhibit HDAC, possibly of the class I HDACs, and to increase target proteins acetylation. Indeed, TSA and SAHA induced both nuclear histone H4 and cytoplasmic α-tubulin acetylation while MS-275 and VPA induced histone H4 hyperacetylation only. Although a recent study reported a stimulatory effect of VPA on α-tubulin acetylation in thyroid cancer cell lines, no effect was observed in our conditions [25]. These data suggested that acetylation of this structural protein is not necessary to the antiadipogenic effect of HDAC inhibitors and that acetylation of lysine residues in nucleosomal histones plays a major contribution.

The antiadipogenic activity of HDAC inhibitors is exerted at concentrations that are 1 to 2 log unit lower than those required to inhibit preadipocyte growth or to inhibit the activity of another metabolic enzyme, LDH, that was observed at μM concentrations, only. Moreover, the antiadipogenic effect of HDAC inhibitors was not associated to alterations of either cell number or cell viability, as assessed by LDH release, or induction of apoptosis. Under control conditions, preadipocytes undergo differentiation with only a negligible fraction of the cell population presenting signs of DNA fragmentation, as an indicator of cell apoptosis. Although HDAC inhibitors have been found to trigger cell death by

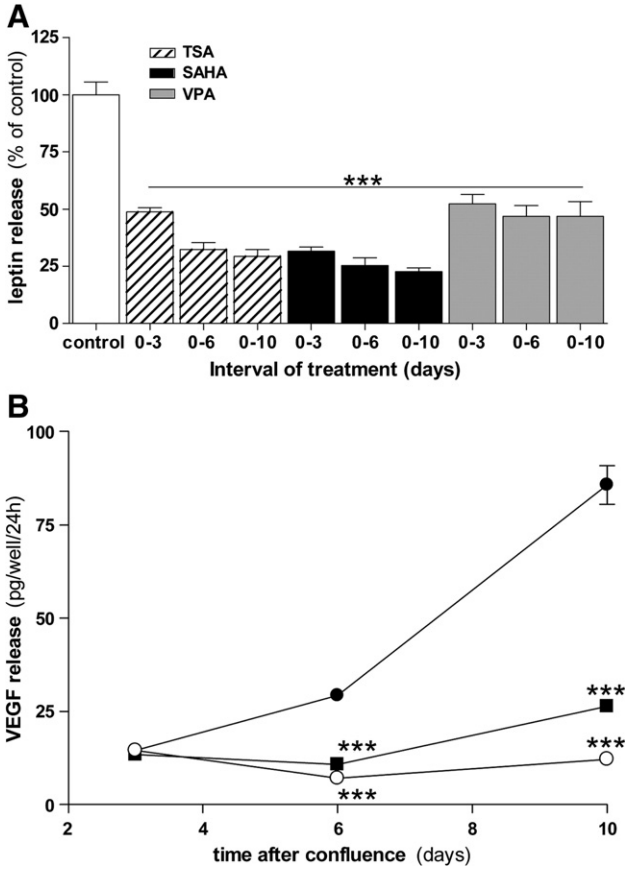

Fig. 6 – Effect of HDAC inhibitors on adipokine release. (A) Leptin release in cell supernatants after treatment with TSA (0.2 μM), SAHA (1 μM) and VPA (0.5 mM) for the indicated time interval ($n = 4$). (B) To evaluate the effect of HDAC inhibitors on the release of VEGF, cells were treated from day 0 to day 3 after confluence with SAHA (1 μM, ○) and VPA (0.5 mM, ■) or vehicle (●) and the amount of VEGF in cell supernatants was determined at the indicated time. Data obtained with HDAC inhibitor treated cells are significantly different from control, *** $p < 0.001$. Differences between successive time intervals are not significant (two-way ANOVA followed by Bonferroni's post-hoc test, $n = 4$).

altering both mitochondrial and death receptor pathways and increasing proapoptotic signals [9,10], no significant increase in apoptotic index was observed in cells cultured in the presence of HDAC inhibitors at concentrations that completely prevent adipose conversion. The results presented provided evidence that inhibition of differentiation by HDAC inhibitors is therefore not related to their cytostatic, proapoptotic or antiproliferative potential, and was specific for the expression of markers of the adipocyte differentiation program. These data confirm the relative resistance (lower sensitivity) of normal cells to cell death whatever they are proliferating or growth arrested while displaying a good sensitivity to the differentiating properties of HDAC inhibitors. These findings also suggested that the phenotype response and the sensitivity to a HDAC inhibitor is determined by the cell context although the molecular basis for the differential effects of these compounds in transformed cells, as compared to normal cells, in distinct cell types or developmental stages has to be defined [26].

Results showed both time-dependency and reversibility of HDAC inhibitor antiadipogenic effect. Indeed HDAC inhibitors prevented the progression to a mature phenotype as measured by triglyceride accumulation, GPDH activity and adipokine release when present during the first 3 days after induction of differentiation. The antiadipogenic capacity of HDAC inhibitors diminished as cells go through the differentiation program indicating that their action is mediated by the inhibition of a critical step at an early phase of the differentiation process. The reversibility of HDAC inhibitors effect was demonstrated by the fact that GPDH activity, lipid droplets and triglyceride accumulation returned to increase after the interruption of the pharmacological treatment and further exposure to inducers of differentiation. These data are compatible with the reversible nature of compounds used, that block HDAC activity by chelating Zn^{2+} in the active site, and with the reversible nature of histone acetylation status, that is the result of removal (by HDAC) and transfer (by HAT) of acetyl groups on lysine residues. Although restoration of HDAC activity after removal of the drug plays an essential role to induce a sequence of events leading to the differentiation program, it is not sufficient to promote an increment in triglyceride content and GPDH activity comparable to that observed in control cells, especially for SAHA. The molecular basis for partial recovery of the differentiation potential after HDAC inhibitor treatment has to be defined. Data also suggest that presumably histone hyperacetylation by HDAC inhibitors plays a unique central role in controlling cell fate at the beginning of the differentiation program and that this covalent modification has not produced events leading to permanent changes in chromatin conformation. Our findings support the model of cascade regulation of terminal adipocyte differentiation where the sequential expression of a number of specific genes, including those encoding for transcription factors, controls the conversion of preadipocytes into fully functional adipocytes [5,6]. Precocious and sequential action of specific proteins is necessary and/or sufficient to turn on the differentiation program and, inversely, preventing the expression and/or function of these regulatory proteins, in our case by HDAC inhibitors, blocked terminal differentiation.

One of the possible target of HDAC inhibitors at the beginning of the differentiation program is the transcription factor C/EBPα which is known to be required for adipogenesis. Indeed a significant reduction of C/EBPα protein level was observed on day 2 of the differentiation program in cells exposed to HDAC inhibitors from the different chemical classes and the inhibitory effect was still persistent on day 6. During the same period of time, there was a significant reduction in the level of fibronectin by most of the HDAC inhibitors used, an effect recently described for TSA in human skin fibroblasts [27]. Alteration of extracellular matrix protein level may be correlated to the difference in morphology that distinguished HDAC inhibitor treated preadipocytes from control cells early during the differentiation program. These data are also indicative of a pleiotropic effect of HDAC inhibitors.

A partial inhibition of C/EBPα at the early stages of preadipocyte differentiation could explain, at least in part, the consequent impairment of the late stages of differentiation and lipid filling in HDAC inhibitor treated cells, although it must be considered that several pathways may be involved in the regulation of the differentiation process. That C/EBPα is a major target of HDAC inhibitors action in differentiating preadipocytes has been previously reported in 3T3-L1 cells although data are contradictory showing both inhibition [15,16] and induction [28] of expression

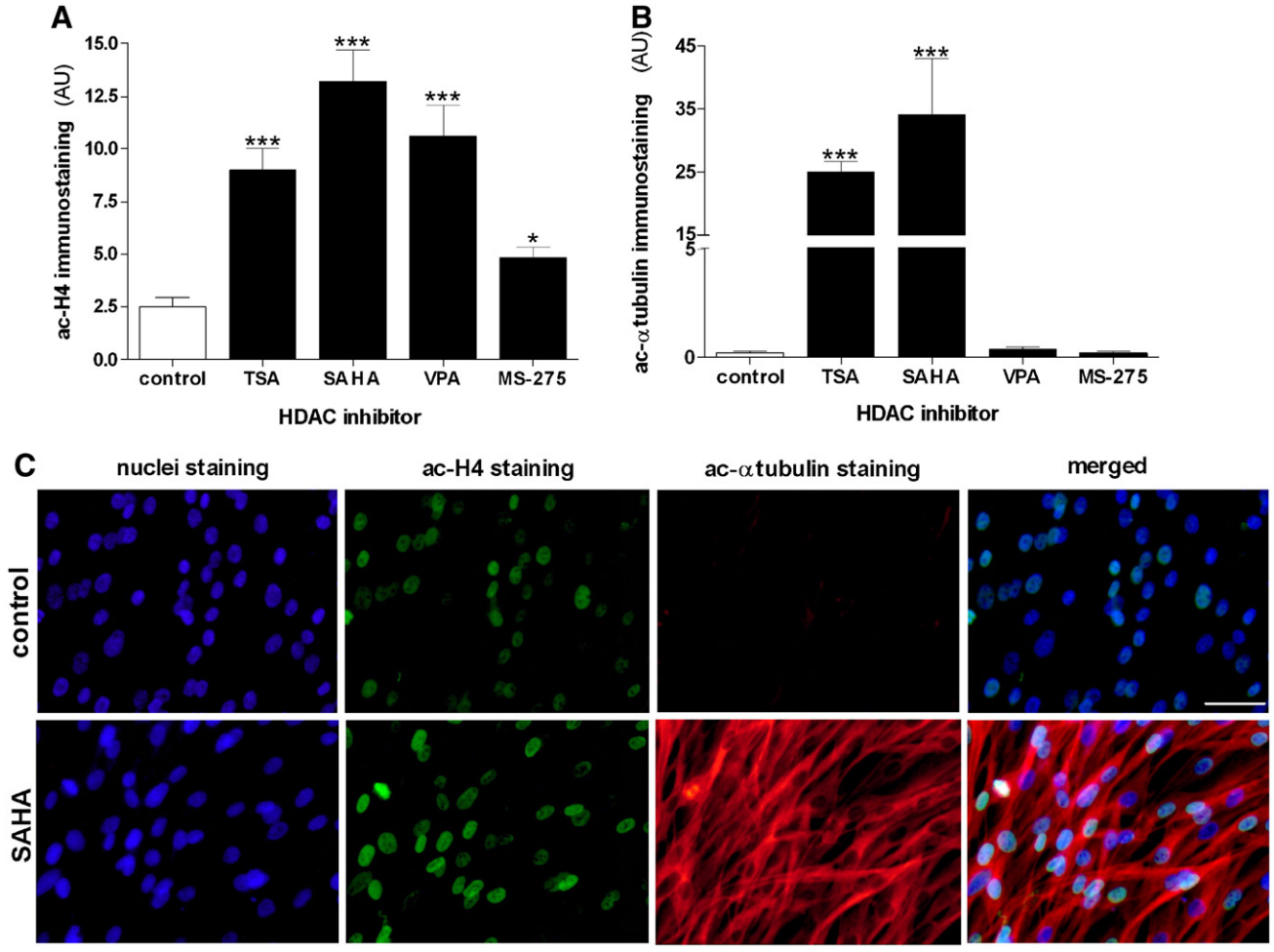

Fig. 7 – Effect of HDAC inhibitors on the acetylation of target proteins. Relative acetylation level of histone H4 (A) ($n = 4$) and α-tubulin (B) ($n = 3$) in human preadipocytes exposed for 6 h to differentiation medium and HDAC inhibitors (full columns) (TSA, SAHA, MS-275, 1 μM each, and VPA 1 mM) as compared to control cells (empty column). Data are expressed as mean ± SEM of separate determinations. Differences from control cells are indicated as *, $p < 0.05$, or ***, $p < 0.001$ (one-way ANOVA followed by Dunnett's post-hoc test). (C) Representative images from double immunofluorescence staining experiments showing nuclear histone H4 (Alexa 488 green label) and cytoplasmic α-tubulin (Alexa 546 red label) acetylation in cells exposed to vehicle or SAHA, and counterstaining of nuclei with Hoechst 33258 (blue label). Scale bar, 50 μm.

depending on the final effect of HDAC inhibitors on adipose conversion. The importance of C/EBPα expression in human adipose conversion has been reported by the group of Tchkonia et al. [29] which demonstrated that adipogenesis, as determined by lipid accumulation, GPDH activity and aFABP level in different human adipose tissue depots, well correlated with early C/EBPα expression level. In addition, overexpression of C/EBPα in preadipocytes displaying low differentiating capacity enhanced differentiation and only cells that acquired lipid inclusions exhibited C/EBPα upregulation.

Our results are in agreement with previous reports showing that TSA and VPA inhibited adipogenesis in 3T3-L1 cells as evidenced by a marked reduction in lipid accumulation and expression of different sets of genes normally induced upon differentiation including C/EBPα and aP2 [15,16]. It was also observed that VPA inhibited lipid accumulation in human preadipocytes [16] and that NaBu suppressed the adipogenic differentiation and decreased the expression of PPARγ and lipoprotein lipase in human mesenchymal stem cells treated under conditions that promote adipogenic determination [24]. By contrast, other research groups obtained opposite results by demonstrating the stimulatory effect of distinct HDAC inhibitors on the adipocyte differentiation of 3T3-L1 cells [17,18,28,30]. Promotion of adipogenesis by NaBu was not only observed in 3T3-L1 cells [30] but was previously reported in subconfluent Swiss 3T3 cells [31]. Two groups have demonstrated the importance of HDAC1 in 3T3-L1 cell adipogenic program since HDAC1 silencing has a stimulatory effect on adipogenic gene expression and adipocyte differentiation and, inversely, HDAC1 overexpression attenuated adipocyte differentiation [17,28] while in another study, the proadipogenic activity of HDAC inhibitors has been attributed to inhibition of HDAC3 [30]. The discrepancies on the effect of HDAC inhibitors in 3T3 cells may be attributed, at least in part, to differences in experimental conditions. In fact, 2 or 4 days post-confluent cells have been used in experiments that demonstrate a negative effect of HDAC inhibitors on differentiation [15,16] while confluent or

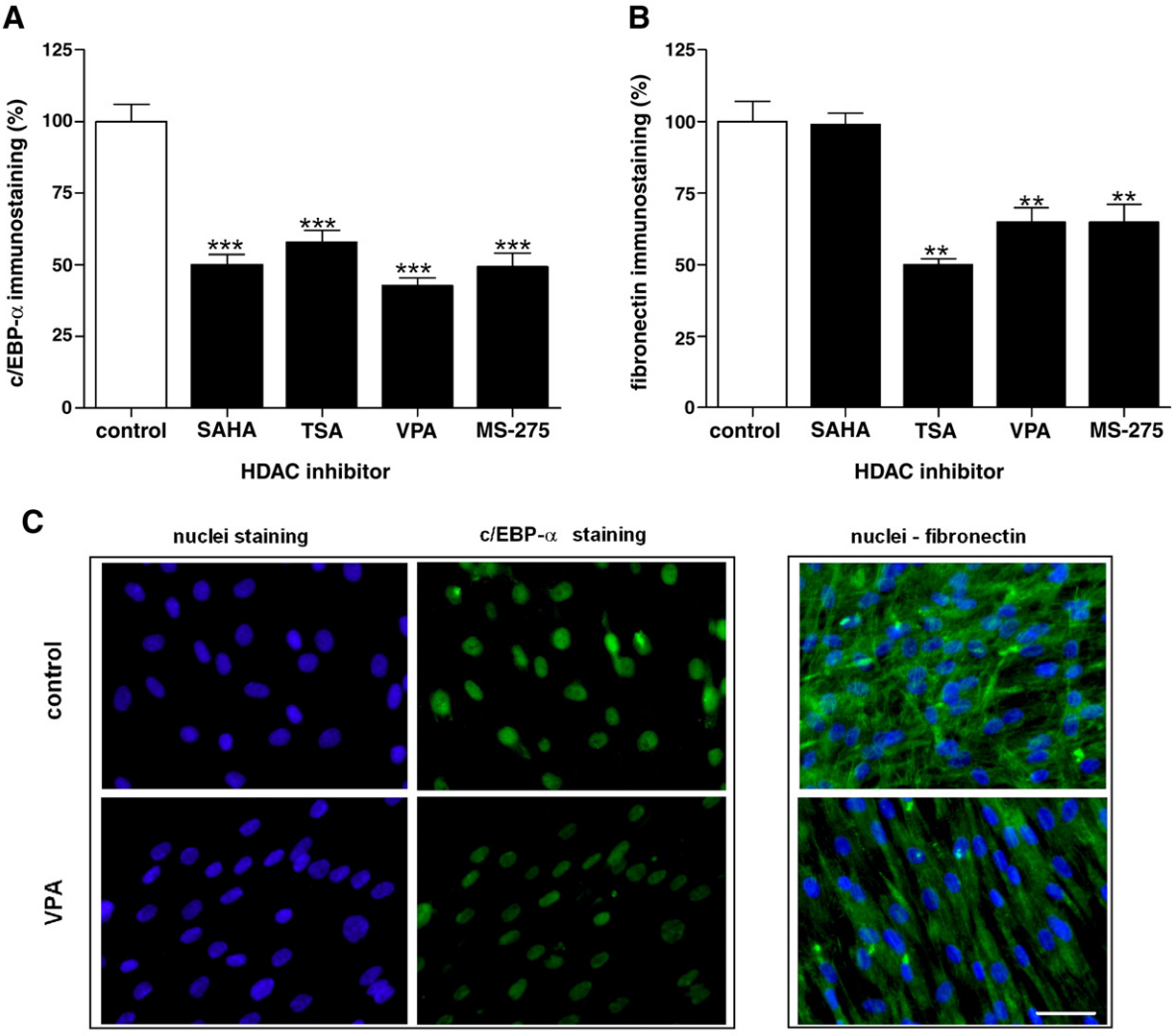

Fig. 8 – Expression level of C/EBPα and fibronectin after treatment with HDAC inhibitors. Human subcutaneous preadipocytes were exposed to differentiation medium for 2 days in the presence of vehicle (empty column) or the HDAC inhibitors (full columns) SAHA, TSA, MS-275 (1 μM each) and VPA (1 mM). The level of C/EBPα (A) and fibronectin (B) expression was determined by immunocytochemical analysis. Error bars represent SEM ($n = 4$ and $n = 3$, respectively). Differences from control cells are indicated as **, $p < 0.01$ or ***, $p < 0.001$ (one-way ANOVA followed by Dunnett's post-hoc test). (C) Representative images from immunofluorescence staining experiments showing nuclear C/EBPα and diffuse fibronectin network (Alexa 488 green label) in cells exposed to vehicle or VPA, and counterstaining of nuclei with Hoechst 33258 (blue label). Scale bar, 50 μm.

subconfluent cells have been used in those studies that evidenced a positive effect of HDAC inhibitors [17,18,28,30,31]. This observation is particularly important since 3T3 cells undergo post-confluent mitosis and cells may be found in a different state of the cell cycle when treated with differentiation medium and HDAC inhibitors. As mentioned before, proliferating and arrested cells may respond in a different way to HDAC inhibitors both quantitatively and qualitatively. Such hypothesis need experimental confirmation. The role of HDAC in 3T3-L1 cell differentiation seems, however, to be more complex as compared to human preadipocytes since both inhibitory (TSA, SAHA, apicidin) and stimulatory (NaBu) effects of HDAC inhibitors have been recently described on the same cell preparation, 2-day post-confluent 3T3-L1 cells, as assessed by measurement of oil Red O staining, triglyceride content and adipogenic genes expression including aP_2 and C/EBPα [32]. Overall observations suggest that the consequences of HDAC inhibition in rodent cell lines are most likely influenced by both culture conditions and the nature of HDAC inhibitor.

That data obtained with human preadipocytes that do not agree with those obtained in 3T3 cells may be not surprising since differentiation in humans differs in several aspects from that of rodent cells. First, human preadipocytes undergo terminal differentiation without the need to reach confluence and in the absence of clonal expansion [33]. Indeed, we did not find alteration of cell number in HDAC inhibitor treated post-confluent preadipocytes although Burton et al. [15] showed that prevention of 3T3-L1 differentiation by TSA was accompanied by inhibition of post-confluent mitosis. Second, induction of C/EBPα in human preadipocytes followed the addition of adipogenic stimulus whereas, in

the 3T3-L1 model, the transcription factors C/EBPδ and C/EBPβ initially accumulated being gradually replaced by C/EBPα that regulates terminal adipocyte differentiation [34,35]. Finally, in 3T3-L1 cells expression of adipokine may also be different, for example, resistin increased along adipogenesis, whereas in humans its expression decreased [36,37]. Therefore, HDAC inhibitors may exert differential effects in human and rodent preadipocytes that for several aspects may be considered as distinct cells.

In conclusion, HDAC inhibitors from different chemical classes are potent inhibitors of adipose conversion in human subcutaneous preadipocytes. These compounds discriminate between proliferating and differentiating cells since prevention of adipogenesis is independent from their antiproliferative or proapoptotic effect, TSA, SAHA and MS-275 being also without effect on another metabolic enzyme, LDH. The antiadipogenic effect of HDAC inhibitors is reversible, more intense at an early stage of the differentiation program and correlated with the rapid accumulation of acetylated histones. Our study has clinical implications because HDAC inhibitors are currently developed as anticancer drugs and it suggests that during antitumoral therapy, HDAC inhibitors if delivered during a critical temporal window will impair energy storage in the adipocyte while maintaining the number of preadipocytes. After interruption of the pharmacological treatment, preadipocytes will reacquire the capacity to differentiate into adipocytes. This may have importance when treating obese patients since both the incidence of cancer and survival as well as the rate of cancer recurrence are under influence of body-mass index and weight gain, and account for overall increase death rates [38,39].

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REFERENCES

[1] E.D. Rosen, B.M. Spiegelman, Adipocytes as regulators of energy balance and glucose homeostasis, Nature 444 (2006) 847–853.
[2] A.H. Berg, P.E. Scherer, Adipose tissue, inflammation, and cardiovascular disease, Circ. Res. 96 (2005) 939–949.
[3] G. Fantuzzi, Adipose tissue, adipokines, and inflammation, J. Allergy Clin. Immunol. 115 (2005) 911–919.
[4] H.E. Bays, J.M. Gonzalez-Campoy, G.A. Bray, A.E. Kitabchi, D.A. Bergman, A.B. Schorr, H.W. Rodbard, R.R. Henry, Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity, Expert Rev. Cardiovasc. Ther. 6 (2008) 343–368.
[5] C.M. Smas, H.S. Sul, Control of adipocyte differentiation, Biochem. J. 309 (1995) 697–710.
[6] F.M. Gregoire, C.M. Smas, H.S. Sul, Understanding adipocyte differentiation, Physiol. Rev. 78 (1998) 783–809.
[7] K.P. Claffey, W.O. Wilkison, B.M. Spiegelman, Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathways, J. Biol. Chem. 267 (1992) 16317–16322.
[8] A.J.M. De Ruijter, A.H. Van Gennip, H.N. Caron, S. Kemp, A.B.P. Van Kuilenburg, Histone deacetylases (HDACs): characterization of the classical HDAC family, Biochem. J. 370 (2003) 737–749.
[9] J.E. Bolden, M.J. Peart, R.W. Johnstone, Anticancer activities of histone deacetylase inhibitors, Nat. Rev., Drug Discov. 5 (2006) 769–784.
[10] W.S. Xu, R.B. Parmigiani, P.A. Marks, Histone deacetylase inhibitors: molecular mechanisms of action, Oncogene 26 (2007) 5541–5552.
[11] T. Henkens, P. Papeleu, G. Elaut, M. Vinken, V. Rogiers, T. Vanhaecke, Trichostatin A, a critical factor in maintaining the functional differentiation of primary cultured rat hepatocytes, Toxicol. Appl. Pharmacol. 218 (2007) 64–71.
[12] C. Haumaitre, O. Lenoir, R. Scharfmann, Histone deacetylase inhibitors modify pancreatic cell fate determination and amplify endocrine progenitors, Mol. Cell. Biol. 28 (2008) 6373–6383.
[13] L. Tou, Q. Liu, R.A. Shivdasani, Regulation of mammalian epithelial differentiation and intestine development by class I histone deacetylases, Mol. Cell. Biol. 24 (2004) 3132–3139.
[14] M. Rahman, A. Kukita, T. Kukita, T. Shobuike, T. Nakamura, O. Kohashi, Two histone deacetylase inhibitors, trichostatin A and sodium butyrate, suppress differentiation into osteoclasts but not into macrophages, Blood 101 (2003) 3451–3459.
[15] G.R. Burton, R. Nagarajan, C.A. Peterson, R.E. McGehee, Microarray analysis of differentiation-specific gene expression during 3T3-L1 adipogenesis, Gene 31 (2004) 167–185.
[16] D.C. Lagace, M.W. Nachtigal, Inhibition of histone deacetylase activity by valproic acid blocks adipogenesis, J. Biol. Chem. 279 (2004) 18851–18860.
[17] E.J. Yoo, J-J. Chung, S.S. Choe, K.H. Kim, J.B. Kim, Down-regulation of histone deacetylases stimulates adipocyte differentiation, J. Biol. Chem. 281 (2006) 6608–6615.
[18] J.H. Lee, Diallyl disulfide accelerates adipogenesis in 3T3-L1 cells, Int. J. Mol. Med. 20 (2007) 59–64.
[19] N. Khan, M. Jeffers, S. Kumar, C. Hackett, F. Boldog, N. Khramtsov, X. Qian, E. Mills, S.C. Berghs, N. Carey, P.W. Finn, L.S. Collins, A. Tumber, J.W. Ritchie, P.B. Jensen, H.S. Lichenstein, M. Sehested, Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors, Biochem. J. 409 (2008) 581–589.
[20] S. Chung, K. LaPoint, K. Martinez, A. Kennedy, M.B Sandberg, M.K. McIntosh, Preadipocytes mediate LPS-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes, Endocrinology 147 (2006) 5340–5351.
[21] S. Armeanu, A. Pathil, S. Venturelli, P. Mascagni, T.S. Weiss, M. Gottlicher, M. Gregor, U.M. Lauer, M. Bitzer, Apoptosis on hepatoma cells but not on primary hepatocytes by histone deacetylase inhibitors valproate and ITF2357, J. Hepatol. 42 (2005) 210–217.
[22] P. Atadja, L. Gao, P. Kwon, N. Trogani, H. Walker, M. Hsu, L. Yeleswarapu, N. Chandramouli, L. Perez, R. Versace, A. Wu, L. Sambucetti, P. Lassota, D. Cohen, K. Bair, A. Wood, S. Remiszewski, Selective growth inhibition of tumor cells by a novel histone deacetylase inhibitor, NVP-LAQ824, Cancer Res. 64 (2004) 689–695.
[23] J. Hsieh, K. Nakashima, T. Kuwabara, E. Mejia, F.H. Gage, Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells, P.N.A.S. 101 (2004) 16659–16664.
[24] T.-H. Chen, W.-M. Chen, K.-H. Hsu, C.-D. Kuo, S.-C. Hung, Sodium butyrate activates ERK to regulate differentiation of mesenchymal stem cells, Biochem. Biophys. Res. Commun. 355 (2007) 913–918.
[25] M.G. Catalano, R. Poli, M. Pugliese, N. Fortunati, G. Boccuzzi, Valproic acid enhances tubulin acetylation and apoptotic activity of paclitaxel on anaplastic thyroid cancer cell lines, Endocr.-Relat. Cancer 14 (2007) 839–845.
[26] P. Papeleu, T. Vanhaecke, G. Elaut, M. Vinken, T. Henkens, S. Snykers, V. Rogiers, Differential effects of histone deacetylase inhibitors in tumor and normal cells—what is the toxicological relevance? Crit. Rev. Toxicol. 35 (2005) 363–378.
[27] C. Huber Lars, J.H.W. Distler, F. Moritz, H. Hemmatazad, T. Hauser, B.A. Michel, R.E. Gay, M. Matucci-Cerinic, S. Gay, O. Distler, A. Jüngel, Trichostatin A prevents the accumulation of extracellular matrix in a mouse model of bleomycin-induced skin fibrosis, Arthritis Rheum. 56 (2007) 2755–2764.

[28] N. Wiper-Bergeron, D. Wu, L. Pope, C. Schild-Poulter, R.J.G. Haché, Stimulation of preadipocyte differentiation by steroid through targeting of an HDAC1 complex, EMBO J. 22 (2003) 2135–2145.
[29] T. Tchkonia, N. Giorgadze, T. Pirtskhalava, Y. Tchoukalova, I. Karagiannides, R.A. Forse, M. DePonte, M. Stevenson, W. Guo, J. Han, G. Waloga, T.L. Lash, M.D. Jensen, J.L. Kirkland, Fat depot origin affects adipogenesis in primary cultured and cloned human preadipocytes, Am. J. Physiol., Regul. Integr. Comp. Physiol. 282 (2002) R1286–R1296.
[30] L. Fajas, V. Egler, R. Reiter, J. Hansen, K. Kristiansen, M-B. Debril, S. Miard, J. Auwerx, The retinoblastoma–histone deacetylase 3 complex inhibits PPARγ and adipocyte differentiation, Dev. Cell 3 (2002) 903–910.
[31] A. Toscani, D.R. Soprano, K.J. Soprano, Sodium butyrate in combination with insulin or dexamethasone can terminally differentiate actively proliferating Swiss 3T3 cells into adipocytes, J. Biol. Chem. 265 (1990) 5722–5730.
[32] S.N. Kim, H.Y. Choi, Y.K Kim, Regulation of adipocyte differentiation by histone deacetylase inhibitors, Arch. Pharm. Res. 32 (2009) 535–541.
[33] G. Entenmann, H. Hauner, Relationship between replication and differentiation in cultured human adipocyte precursor cells, Am. J. Physiol. 270 (1996) C1011–C1016.
[34] W-C. Yeh, Z. Cao, M. Classon, S.L. McKnight, Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins, Genes Dev. 9 (1995) 168–181.
[35] J.J. Tomlinson, A. Boudreau, D. Wu, E. Atlas, R.J.G. Haché, Modulation of early human preadipocyte differentiation by glucocorticoids, Endocrinology 147 (2006) 5284–5293.
[36] C.M. Steppan, S.T. Bailey, S. Bhat, E.J. Brown, R.R. Banerjee, C.M. Wright, H.R. Patel, R.S. Ahima, M.A. Lazar, The hormone resistin links obesity to diabetes, Nature 409 (2001) 307–312.
[37] J. Janke, S. Engeli, K. Gorzelniak, F.C. Luft, A.M. Sharma, Resistin gene expression in human adipocytes is not related to insulin resistance, Obes. Res. 10 (2002) 1–5.
[38] C.H. Kroenke, W.Y. Chen, B. Rosner, M.D. Holmes, Weight, weight gain, and survival after breast cancer diagnosis, J. Clin. Oncol. 23 (2005) 1370–1378.
[39] E.E. Calle, C. Rodriguez, K. Walker-Thurmond, M.J. Thun, Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults, N. Engl. J. Med. 348 (2003) 1625–1638.