HDAC-class II specific inhibition involves HDAC proteasome-dependent degradation mediated by RANBP2

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ABSTRACT

Discovered for their ability to deacetylate histones and repress transcription, HDACs are a promising target for therapy of human diseases. The class II HDACs are mainly involved in developmental and differentiation processes, such as myogenesis. We report here that class I and class II HDAC inhibitors such as SAHA or the class II selective inhibitor MC1568 induce down-regulation of class II HDACs in human cells. In particular, both SAHA and MC1568 induce HDAC 4 down-regulation by increasing its specific sumoylation followed by activation of proteasomal pathways of degradation. Sumoylation that corresponds to HDAC 4 nuclear localization results in a transient increase of the HDAC 4 repressive action on target genes such as RAR α and TNFα. The HDAC 4 degradation that follows to its sumoylation results in gene target activation. Silencing of the RANBP2 E3 ligase reverses HDAC 4 repression by blocking its own sumoylation. These findings identify a crosstalk occurring between acetylation, deacetylation and sumoylation pathways and suggest that class II specific HDAC inhibitors may affect different epigenetic pathways.

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1. Introduction

Chromatin represents a signal transducer that regulates genome functions translating upstream signals into either transient or permanent (and heritable) information. A large variety of incoming signals induce complex patterns of enzyme-catalyzed modifications such as phosphorylation, acetylation, ubiquitination, methylation, ADP-ribosylation of DNA and/or histones. Chromatin-associated non-histone proteins, signaling complexes and nuclear machineries are also substrates for the above enzymes, but the functions of these modifications are still largely elusive [1–4].

Emerging evidence suggests that epigenetic alterations of DNA and chromatin are causally linked to oncogenesis and tumor progression, and that chromatin regulators represent important targets for the development of novel anti-cancer drugs [5–18]. This is true for both the effectors of chromatin changes (e.g. histone and DNA modifiers), and upstream signaling pathways.

Structural and/or functional alterations of histone acetyltransferases/deacetylases (HATs or HDACs) have been documented in cancer: aberrant chromatin recruitment of HDACs by various oncopgenic fusion proteins (PML-RAR, PLZF-RAR, AML1-ETO, TEL-AML1, and Bcl6) missense mutations, rearrangements or inactivation of CBP or p300 (HATs) by oncogenic viral proteins [19–22]. In the best-documented cases, deregulation of HATs or HDACs result in abnormal transcriptional regulation of target genes relevant to the transformation process [23,24].

At the present, there are four classes of HDAC enzymes known. Class I deacetylases include HDACs 1, 2, 3, and 8, related to yeast RPD3 deacetylase; class II deacetylases that include those homologous to yeast HDA1, are divided into two subclasses, Ila (HDAC 4, 5, 7 and 9 and its splice variant MTR) and Iib (HDAC 6, 10) [25–28]. HDAC 11 (class IV) contains conserved residues in the catalytic core region shared by both class I and II enzymes [29]. Like class I HDACs, members of the class II family are also found in complexes with SMRT/N-CoR co-repressors. Indeed, it has been suggested that deacetylase activity of HDAC 4 and 5 may arise from the class I HDAC 3 in SMRT/N-CoR containing HDAC complexes. In contrast to class I HDACs, which are found almost exclusively in the nucleus, class II enzymes are uniquely regulated by nucleus–cytoplasm shuttling [30–32]. Whereas class Ia HDACs are frequently considered redundant, it is likely that further studies may reveal discrete biological functions specific for each family member. Indeed, class II HDACs specifically interact with members of the human myocyte enhancer factor 2 (MEF2) family of transcription factors and with the 14-3-3 chaperone proteins in the control of myocyte differentiation [33,34]. Whereas both class I and II HDACs share homologies in their catalytic domain, class III HDACs are homologous to the yeast SIR2 and do not show homologies to class I and II HDACs.

Mutations in HDAC genes have not been identified in human malignancies, but multiple associations between HDACs and well-
characterized oncogenes or tumor suppressor genes have been documented. Gene silencing by HDACs has been also identified in the pathogenesis of certain leukemias [20,22] and in some types of non-Hodgkin’s lymphomas. Indeed in acute promyelocytic leukemia (APL), the fusion protein PML-RAR-α recruits HDAC complexes to repress transcription of RAR-regulated genes, causing a maturation block in the myeloid cell line. Also in the t(8;21) acute myeloid leukemia (AML), the chromosomal translocation produces an AML1-ETO fusion protein that plays as potent dominant transcriptional repressor through recruitment of HDAC complexes. HDAC inhibition can relieve ETO-mediated transcriptional repression and induced differentiation of the AML1-ETO cells.

It is now well-established that certain epigenetic drugs (“epi-drugs”; e.g. HDAC inhibitors) [35,36] and NR ligands (e.g. ret(Ω)nikoids) are potent cancer-therapeutic and cancer-preventive agents [7–9,37–43]. Recent studies have shown an unexpected convergence of the two signaling paradigms: both activate the TNF-related apoptosis-inducing ligand (TRAIL) pathway and TRAIL activation is, at least in part, the cause of the tumor-selective action of HDACi’s and retinoids [35–37,41,42,44,45]. HDAC inhibitors and demethylating agents are in multiple clinical trials to assess their activity on a variety of hematopoietic and solid malignancies. Drugs that interfere with the activity of DNA methyltransferases (demethylating agents) display anti-tumor effects in vitro and synergize with HDACi [15,35,46–48]. A growing series of molecules has been designed to inhibit HDAC and other epigenetic activities either globally or more specifically [49]. Indeed, chemically diverse agents have been discovered, including the suberoylanilide hydroxamic acid (SAHA) [50,51]. SAHA binds directly to the HDAC catalytic site, inhibits its enzymatic activity [52] and exerts anti-proliferative and/or pro-apoptotic effects in transformed cells [8]. SAHA is bio-available and has objective evidence of anti-tumor activity with a favourable side effect profile in phase I and II clinical trials [53–55]. All these observations strongly support the emergence of a novel perspective in the development of cancer-therapeutic drugs that target chromatin modifiers through various routes with the aim of exploiting more cancer-selective signaling pathways.

In the present manuscript, we studied the biological activity of pan and class specific HDAC inhibitors in different cancer models identifying a mechanism of HDAC 4 degradation mediated by SAHA or by the class II selective inhibitor MC1568. We found that both SAHA and the class II HDAC inhibitor MC1568 mediate HDAC 4 proteasomal degradation in MCF7 breast cancer cells, by inducing its nuclear localization and sumoylation mediated by the activity of the E3 ligase RANBP2.

2. Materials and methods

2.1. Chemicals

SAHA (Alexis Biochemicals) was prepared in 100% DMSO at the concentration of 5 × 10⁻⁴M, stored at −20 °C and used at the dilution of 1:1000. VPA (Sigma) was used at the final concentration of 10⁻³ M; MS275 (Schering AG) was initially dissolved in 100% DMSO at the concentration of 5 × 10⁻² M; then a stock solution at the concentration of 5 × 10⁻⁴ M was prepared in 10% DMSO, 90% ethanol and used at the dilution of 1:1000 in the experiments. The class specific compound, MC1568 [49], was resuspended in DMSO and used at the final concentration of 5 × 10⁻⁶ M. The cell-permeant proteasome inhibitors, MG-132 and lactacystin, were purchased from Alexis Biochemicals. The caspase inhibitor ZVAD was purchased from RD.

2.2. Cell cultures

Human breast carcinoma cell lines (ZR-75.1 and MCF-7), colon cancer (Caco-2) and osteosarcoma (U2OS) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM—Sigma); prostate cancer (LnCap) and leukemia (U937) cell lines were grown in RPMI1640 medium (Sigma). All cells were supplemented with 10% foetal bovine serum, 2 mM glutamine and 100 U/ml penicillin/streptomycin. All cell lines were maintained at 37 °C in a humidified atmosphere of 95% air, 5% CO₂. COS cells were cultured as previously reported [56].

2.3. Transfections

The MCF7 cells were transfected with lipofectamine (GIBCO) as indicated by suppliers. Briefly, MCF7 cells have been plated at 90% confluence and transfected with 4 µg of plasmids (pSuper-RanBP2 and pSuper-EMPTY, kindly provided by M. Fornerod, The Netherlands Cancer Institute, NL). For the HDAC 4 knockdown experiments, MCF7 cells were transfected with 50 nM of siRNA directed against HDAC 4 (S18839) or with the negative control siRNA (AM4611), both purchased by Ambion. After 48 h, RNA was extracted. Cos1 cells plated at a density of 10⁶ cells per well of 24-well plates were transfected by calcium phosphate method [56]. Precipitates contained: 100 ng of RARE-tk-luc or TNF-luc or ΔNF-kB-TNF-luc (kindly provided by D. Joyce, University of Western Australia, AU); 20 ng of hRARα or 20 ng (1×), 40 ng (2×) and 160 ng (4×), 200 ng (5×) of HDAC 4 siRNA plasmid (kindly provided by E. Jaffray, University of St. Andrews, UK) and 50 ng of cytomegalovirus-α-galactosidase (CMV-α-Gal, used as an internal control to normalize variations in the transfection efficiency). The total quantity of DNA was adjusted at 1 µg with pBluescript. Luciferase were measured using standard protocols with the TECAN infinite 200. For the experiments shown in Fig. 4D (bottom panel), MCF7 cells have been ‘nucleofected’ with the HDAC 4 siRNA plasmid (kindly provided by E. Jaffray, University of St. Andrews, UK) as indicated by suppliers (AMAXA). Briefly, the cells have been harvested by trypsinization and counted. The required number of cells (2 × 10⁶ cells/siRNAtransfection) was centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in the Cell Line Nucleofector™ Solution V to a final concentration of 2 × 10⁶ cells/100 µl. 1 µg of DNA was added and the sample was transferred into a cuvette, inserted into the cuvette holder selecting the program P-20 (for high transfection efficiency). Transfection efficiency was measured as about 75%.

2.4. FACS analysis of apoptosis and cell cycle

Adherent and supernatant cells were harvested, and cells resuspended in staining solution containing RNase A, propidium iodide (50 µg/ml), sodium citrate (0.1%), NP40 (0.1%) in PBS 1X for 30 min in the darkness. Cell cycle distribution was assessed with a FACScalibur flow cytometer (Becton Dickinson) and 10,000 cells were analyzed by ModFit version 3 Technology (Verity) and Cell Quest (Becton Dickinson). Apoptosis was quantified by propidium iodide/Annexin V double staining (Roche) according to standard procedures [41].

2.5. Western blotting analysis and antibodies

For immunoblotting, cells were lysed (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP40, 10 µM NaF, 0.1 mM Na₃VO₄), 40 µg/ml PMSF and protease inhibitors (SIGMA). Insoluble material was removed by centrifugation, and protein concentration was determined by Bio-Rad assay (BioRad). Lysates (30 µg protein) were loaded onto SDS-PAGE minigels and proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and recognized by specific antibodies. The antibodies against HDAC 1 (1:1000), HDAC 2 (1:1000), HDAC 3 (1:100), HDAC 5 (1:500) were purchased from Alexis Biochemicals. The HDAC 4 (1:500) antibody was purchased from SIGMA and the anti-HDAC 7 (1:1000) antibody from Cell Signalling Technology. The α-tubulin (1:1000) and anti-His antibodies were acquired from SIGMA; p21 Cip1/Waf1 (1:500) from BD Transduction Laboratories. Primary antibodies were detected using horseradish peroxidase-linked anti-mouse or anti-rabbit (Amersham Biosciences),
2.6. Rnase protection assay

The Ribonuclease (Rnase) Protection Assay was performed according to standard procedures (Pharmingen, San Diego, California) and as reported previously [41]. For hybridization 6 µg of total RNA were used and from 6 to 8×10^5 cpm/µl of labeling probe. These fragments were recovered by ethanol precipitation and analyzed by electrophoresis on a sequencing gel (5% of urea-polyacrylamide-bis-acrylamide). The gel, after the drying process, was placed in contact with a autoradiografic film (Hyperfilm-MP, Amersham). The exposure time was O.N. The presence of the target mRNA in the samples is shown in Fig. 1.
revealed by the appearance of an appropriately sized fragment of the probe. Probes used for specific HDAC detection are available upon request.

2.7. Immunoprecipitation assay (IP)

Cell extracts were prepared from pellets of ZR-75.1 cells to 80% confluence in 150-mm tissue culture plates. Proteins were extracted in buffer containing NP-40 (0.5%), Tris–HCl pH 8.0 (20 mM), 150 mM NaCl, PMSF (1 mM), Glycerol 10%, EDTA (1 mM) and 1× Complete protease inhibitor mixture (SIGMA) for 20 min on ice. Cell debris was removed by centrifugation for 10 min 12,000×g and the soluble material was incubated with the antibodies against HDAC 4 (Sigma) and HDAC 1 (Abcam) at the recommended concentrations over night at 4 °C. The immune-complexes were precipitated with Sepharose-protein A/G Plus (Santa Cruz Biotechnology) for an additional 2 h at 4 °C. After four washes in lysis buffer and two in PBS, the proteins bound to beads were used for HDAC assays or resuspended in SDS-PAGE sample buffer and analyzed by Western blotting to verify specific IP.

2.8. HDAC assay

We analyzed the HDAC activity (Upstate) in samples immunoprecipitated with HDAC 4, HDAC 1 and with non-specific IgG as negative control. HDAC assays have been performed in the absence or presence of HDAC inhibitors at the indicated concentrations.

2.9. RT-qPCR

Total RNA was prepared using the TRIZOL reagent (Invitrogen) and reverse-transcribed with oligo-(dT) primer and Superscript II Reverse Transcriptase (Invitrogen). For the qPCR a commercial kit (Qiagen) and the following primers were used: for IRF1: forward: GCAGGCCCCT-GACTCCACGAC; reverse: TGGACACTCGACTGCTCAA; for TNFα: forward: CTTTGGTCTGTTAGGAGAC; reverse: CAGAGGAAAGAGGTTCACCCAG; for RANBP2: forward: GTGACGAAACCAGCAAGC; reverse: TCCAACCCGGCCCTCCTCC; for GAPDH: forward: GTGACTGTTGACCTGACCT; reverse: AGGGGAGATTCAGTGTGGTG; for HDAC 4: forward: TTGGCCCGGATGTGTTGCT; reverse: TCCCGAGGTCGCAAATGG.

2.10. Chromatin IP

ChIP assays have been carried out as previously described [57].

2.11. Nuclear–cytoplasm extraction

MCF7 cells were washed with ice-cold sterilized PBS 1X with Phosphatase Inhibitor Buffer (0.5 ml PIB/10 ml PBS) (PIB 125 mM NaF, 250 mM β-glycerophosphate, 250 mM para-nitrophenyl phosphate (PNPP), 25 mM NaVO3). Later 10 ml ice-cold PBS/PIB were added and the cells were scraped, transferred into a pre-chilled 15 ml tube and spun at 300×g for 5 min at 4 °C. The pellet was resuspended in 1 ml ice-cold Hypotonic Buffer HB pH 7.5 (20 mM HEPES pH 7.5, 5 mM NaF, 10 µM Na2MoO4, 0.1 mM EDTA). The cells were incubated on ice for 15 min. Later 50 µl 10% Nonidet P-40 (0.5% final) was added and the tube vortexed vigorously for 10 s. The homogenate was centrifuged for 30 s at 4 °C. The supernatant (cytoplasmic fraction) was removed and stored at −80 °C. The nuclear pellet was resuspended in 50 µl Lysis Buffer AM1 (Active Motif) and incubated at 4 °C for 30 min on a shaking platform. After a centrifugation for 10 min at 14,000×g at 4 °C the supernatant (nuclear extract) was aliquoted and stored at −80 °C to avoid freeze/thaw cycles. The protein concentration of the extract was determined by using a Bradford-based assay.

Fig. 2. HDAC expression analysis after treatment with 5 µM SAHA. (A) Western blot analysis for the indicated HDACs after treatment with 5 µM SAHA in ZR75.1 and U2OS cells; (B) RPA analysis of HDAC 1, 2, 3, 4, 5, 6, 8 after treatment at 37 h with 5 µM SAHA; (C) Western blot analysis for HDAC 4 expression in MCF7 cells after treatment with SAHA in the presence or absence of MG132 and Lactacystin at the indicated time points; (D) Immunoprecipitation of HDAC 4 and detection with Sumo-1 and HDAC 4 in MCF7 cells after the indicated treatment with 5 µM SAHA and MC1568.
3. Results

3.1. The HDAC inhibitor SAHA induces a G2/M cell cycle block in cancer cell lines of different origin

We investigated the effects of the class I–II HDAC inhibitor SAHA (Merck) on cell cycle arrest and apoptosis in cancer cell lines as models of different types of human cancer. To this aim, some epithelial cancer cell lines such as breast (ZR75.1, MCF-7), prostate (LnCap) and intestine (Caco-2) cancer cells, together with the osteosarcoma cell line U2OS and the acute myeloid leukemia U937 cell line have been treated with 5 µM SAHA at the indicated time course (Fig. 1A) and cell cycle phases have been analyzed. Although with some differences of sensitivity (being the hematopoietic cells the most sensitive), all cancer cell lines tested responded to SAHA stimulation with a G2-M block of cell cycle and with the induction of apoptosis (Fig. 1B). Both apoptosis and G2-M block resulted to be dose-dependent given that lower concentrations of SAHA (1 and 2.5 µM) did not reach the same induction of programmed cell death and resulted into a G1 block (data not shown) instead of a G2/M of cell cycle. Moreover, in all the tested cancer cells, p21WAF1/Cip1 induction was present upon SAHA stimulation (Fig. 1C) thus confirming that p21 is a general target of the activity of SAHA and that SAHA treatment had an impact at the molecular level. Note that lower concentrations of SAHA able to induce a G1 but not a G2/M block were still able to induce p21WAF1/Cip1 (data not shown).

3.2. SAHA alters the expression levels of class II HDACs

In parallel to cell cycle and apoptosis assays, we verified the HDAC expression levels by Western blot analysis in two different cell lines, as model of epithelial and connective cancers. To this aim, we used the ZR75.1, as model of epithelial cancer, and the U2OS, as model of sarcoma. As shown in Fig. 2A, HDAC 1, 2, 3 were very abundant and not altered by SAHA treatment, whereas HDAC 4, 5 and 7, all members of the class IIa HDACs, were generally expressed to a lower extent and down-regulated by SAHA. The effect of HDAC 4, 5 and 7 down-regulation appeared to be dose-dependent being delayed with lower concentrations of SAHA (data not shown). The U937 cells taken as a model of hematopoietic cancer minimally expressed class II HDACs and did not express at all class II HDAC 4 (data not shown and [58]).

3.3. SAHA-dependent class II HDAC’s down-regulation is proteasome mediated

We reasoned that the effect of SAHA on class II HDACs might result from a transcriptional down-regulation. To test this hypothesis, we performed multiple RNase protection assays (RPA) to check HDAC 1, 2, 3, 4, 5, 6, 8 and 8 mRNA expression in ZR75.1 breast cancer cells. As shown in Fig. 2B, we did not detect any decrease in the mRNA levels of these HDACs when compared to house keeping genes. HDAC 1, 2, 3 were more abundant than the other HDACs and HDAC 5 mRNA was even up-regulated upon SAHA treatment. RPA analysis carried out in U2OS cells fully confirmed these results (data not shown). Note that HDAC 7 mRNA expression levels have been evaluated separately by quantitative PCR and did not display any change (data not shown). As a second step, we postulated that SAHA down-regulation of class II HDACs might be due to the inhibition of class II HDACs, reflecting a situation in which a decreased class II HDAC expression level, accounts for the specific enzymatic inhibition. To verify whether this effect represents a general mechanism or a prerogative of SAHA, we tested the activities of pan (SAHA), class I selective inhibitors (MS275, VPA) and the recently identified class II inhibitor (MC1568) [49] on HDAC 1 and HDAC 4 immunoprecipitated from MCF7 cells (Fig. 3A). Whereas SAHA clearly inhibits HDAC 1 and 4 activity, VPA and MS275 inhibit HDAC 1, but not HDAC 4 that is instead blocked by the MC1568 inhibitor. Given that MC1568 is able to induce HDAC 4 down-regulation in MCF7 cells (Fig. 3B) whereas VPA and MS275 do not influence HDAC 4 expression levels (Fig. 3B), we concluded that HDAC 4 is sumoylated upon SAHA treatment (Fig. 2D). Sumo 1 is immunoprecipitated with HDAC 4 in MCF7 cells upon treatment with SAHA or with MC1568, a class II specific HDAC inhibitor, thus indicating that sumoylation is involved in HDAC 4 deregulation (Fig. 2D).

3.4. The inhibitor MC1568 specifically regulates class II HDAC expression levels

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down-regulation is a phenomenon linked to the specific class II HDAC inhibition (MC1568) in these cells. In full support of this hypothesis, transfection of the specific sumo mutant HDAC 4-K559R in MCF7 cells fully abrogated HDAC 4 down-regulation by MC1568 thus confirming the role of HDAC 4 sumoylation in its degradation (Fig. 3C). Moreover, the addition of the pan-caspase inhibitor ZVAD to MCF7 cells treated with SAHA or MC1568 did not alter HDAC 4 down-regulation suggesting that this phenomenon occurs independently from apoptosis (Supplementary Fig. 1).

3.5. HDAC 4 sumoylation mediates its repressive capacities whereas HDAC 4 degradation results into a transcriptional activation stimuli

That HDAC 4 sumoylation regulates its enzymatic activity, improving transcriptional repression has been shown [60] although not fully confirmed [61]. Aiming to understand the role of HDAC 4 sumoylation and its degradation in our system, we transfected the HDAC 4 sumoylation mutant (K559R) in COS cells and tested its action on RARE-tk-luc and TNF-luc, transcriptional binding sites accounting for RARα and NF-κB activity. Both RARα and NF-κB are known to bind to HDAC 4 that regulates their transcriptional activity [62–64]. Interestingly, the HDAC 4 sumoylation mutant was able to induce activation of both RARE-tk-luc, and the TNF-luc in COS cells, thus suggesting that the repressive capacities of HDAC 4 on RARα and NF-KB (Fig. 4A, B) might be mediated by HDAC 4 sumoylation. Interestingly when a mutant for the NF-κB binding site (Dnf-Kb) to the TNF promoter was added, K559R-HDAC 4 was not able to induce TNF-luc activation (Fig. 4B), thus suggesting a NF-κB dependent activity of HDAC 4 repression. Since the K559R-HDAC 4 mutant induced both RARE-tk-luc and TNF-luc when transfected in COS cells
(Fig. 4A, B), we analyzed the expression levels of some endogenous RARα and NF-kB target genes, such as IRF1 and TNFα, respectively. Note, indeed, that HDAC 4 is normally present in the repressive complex of both RARα and NF-KB [62–64]. After 96 h of treatment with the MC1568, both IRF1 and TNFα were highly up-regulated, as a positive effect of the complete degradation of HDAC 4 (Fig. 4C, D). Accordingly, at the same time point, the IRF1 promoter analyzed by ChIP assay, showed recruitment of the RNA POL II, thus confirming the transcriptional activation of the gene (Fig. 4E). Finally, siRNAs against HDAC 4 (Fig. 4F) were able to up-regulate both TNFα and IRF1 when transfected in MCF7 cells (Fig. 4G–H), thus confirming the repressive role of HDAC 4 in our system and the activation in the absence of HDAC 4.

3.6. HDAC 4 sumoylation is mediated by RANBP2 E3 ligase activity and is able to repress HDAC 4 target genes

As here shown (Fig. 2D), we found that HDAC 4 was sumoylated after 48 h of treatment with 5 µM MC1568 or 5 µM SAHA. To strengthen the hypothesis of HDAC 4’s sumoylation repressive role, we assessed IRF1 and TNFα expression levels after 48 h of MC1568 treatment in MCF7 cells. Indeed, at this time point HDAC 4 is still expressed and sumoylated (Fig. 2D). Note that in MCF7 cells already after 40 h upon SAHA treatment, HDAC 4 translocated into the nuclei (Fig. 5D). Both IRF1 and TNFα were heavily repressed as shown by RT-qPCR in Fig. 5A, B and as RNA pol II recruitment on the IRF1 promoter (Fig. 5C). Nuclear localization of HDAC 4 has been reported to be...
involved in its sumoylation via the activity of RANBP2, a nucleoporin with suumo-E3-ligase action [60]. Thus, we hypothesized that RANBP2 might regulate the repressive activities of HDAC 4 in this system. RANBP2 knockdown in MCF7 cells by specific transfection a shRANBP2 (Fig. 5E–G) indicates that RANBP2 silencing is able on its own to activate both IRF1 and TNFα, showing the involvement of RANBP2 into the regulation of their expression levels (Fig. 5F lane 4, Fig. 5G, lane 4). Interestingly, RANBP2 knockdown was fully able to reverse the repression mediated by MC1568 (Fig. 5F, G) both on the IRF1 and TNFα, clearly indicating that MC1568 is enhancing HDAC 4 sumoylation through the activity of RANBP2 suumo-E3-ligase. How RANBP2 is than regulated by HDAC inhibitors in this system and whether RANBP2 might be a target of acetylation still needs to be defined.

4. Discussion

The role of HDACs in transcriptional repression and regulation suggests the therapeutic potential of HDAC inhibitors and, more in general, of epigenetic modifiers in cellular systems in which the HAT/HDAC’s balance is altered. Indeed, HDAC inhibitors have a promise for cancer therapy and a part of them are already in a clinical trial for different types of diseases. We have shown here that the class I and II HDAC inhibitor SAHA is a powerful inducer of growth arrest and apoptosis in multiple cancer cells (Fig. 1A and B). Indeed, in all cancer models used SAHA is able to induce the cell cycle inhibitor p21, a known target of the activity of HDAC inhibitors together with a dose-dependent G2-M cell cycle block (Fig. 1). Verifying the expression levels of the most known HDACs, we found that members of the class II HDACs 4, 5 and 7 had decreased expression upon SAHA treatment whereas the HDAC 1, 2, 3 were unaltered. Moreover, this regulation was similar in very different tumor types such as carcinomas and sarcomas indicating a possible general mechanism of regulation independent from the tissues examined. Those evidences prompt us to postulate that a mechanism for HDAC inhibition could lead to the decreased presence of the enzyme and, of consequence, to a decreased activity. In logical terms, we investigated if this type of regulation might be transcriptional or post-translational, finding that the decreased expression level of class II enzymes (such as HDAC 4) are due to a specific activation of protein degradation, independently from the mRNA levels. In agreement with these findings, it has been previously described a proteasome-dependent mechanism of HDAC 2 degradation in the presence of VPA [65]. Thereby, we postulated that SAHA induction of HDAC 4 down-regulation might be linked with its inhibitory activities. In this case, the presence/absence of the protein would be the direct link to enzymatic function. Following our hypothesis, SAHA might exert different levels of HDAC inhibition: it might interfere with class I function without altering the protein expression and, at the same time, destabilize the levels of class II HDACs with a proteasome-dependent mechanism. In line with these notions, the class II specific inhibitor MC1568, fully able to block HDAC 4 enzymatic function decreased HDAC 4 expression levels. This result not only confirmed that class II HDAC inhibition can be achieved by deregulation of the protein, but indicates that i) the proposed mechanism can represent a general feature for class II HDAC inhibition in tumours; ii) the contemporaneous inhibition of class I HDACs is not a necessary event to obtain class II HDAC inhibition; iii) the HDAC 4, 5 and 7 down-regulation may represent a mechanism to inhibit the enzymatic activity by chemically different HDAC inhibitors. In terms of function clearly HDAC 4 decrease leads to transcriptional activation of target genes (Fig. 4), thus suggesting an indirect activating role for the inhibitors used. Moreover, our data support a model in which HDAC 4 decrement is precisely regulated by RANBP2 sumoylation followed by proteasomal degradation of the enzyme (Fig. 5). This concept highlights the complex interplay of pathways that occur upon treatment of HDAC inhibitors in cancer cells indicating that a crosstalk between acetylation and sumoylation may take part into the molecular effects of HDAC inhibitors. Due to this interplay both repressive and activating effects on HDAC target genes can be identified thus identifying a scenario in which opposite HDAC roles are mediated by the sumoylation (repressive) and by the proteasomal degradation (activation) in function of the time of treatment. If these pathways are cancer cell specific or mediated by selective acetylation of non-histone targets stays to be defined.

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Appendix A. Supplementary data


References

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