HDACs class II-selective inhibition alters nuclear receptor-dependent differentiation

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Abstract

Epigenetic deregulation contributes to diseases including cancer, neurodegeneration, osteodystrophy, cardiovascular defects, and obesity. For this reason, several inhibitors for histone deacetylases (HDACs) are being validated as novel anti-cancer drugs in clinical studies and display important anti-proliferative activities. While most inhibitors act on both class I, II, and IV HDACs, evidence is accumulating that class I is directly involved in regulation of cell growth and death, whereas class II members regulate differentiation processes, such as muscle and neuronal differentiation. Here, we show that the novel class II-selective inhibitor MC1568 interferes with the RAR- and peroxisome proliferator-activated receptor γ (PPARγ)-mediated differentiation-inducing signaling pathways. In F9 cells, this inhibitor specifically blocks endodermal differentiation despite not affecting retinoic acid-induced maturation of promyelocytic NB4 cells. In 3T3-L1 cells, MC1568 attenuates PPARγ-induced adipogenesis, while the class I-selective MS275 blocked adipogenesis completely thus revealing a different mode of action and/or target profile of the two classes of HDACs. Using in vivo reporting PPRE-Luc mice, we find that MC1568 impairs PPARγ signaling mostly in the heart and adipose tissues. These results illustrate how HDAC functions can be dissected by selective inhibitors.

Journal of Molecular Endocrinology (2010) 45, 219–228

Introduction

In the past years, epigenetic therapies have come of age. The finding that histone deacetylases (HDACs) are more often expressed in tumor cells gave credit to the application of HDAC inhibitors (HDACIs) to target cancer cells, without affecting the normal ones. In humans, HDACs class I – the RPD3/HDA1 family – comprises HDAC1, HDAC2, HDAC3, and HDAC8, whereas class IIa includes HDAC4, HDAC5, HDAC7, and HDAC9, and class IIb includes HDAC6 and HDAC10. Members of class I are homologous to yeast RPD3, while class II HDACs are related to the yeast HDA1. HDAC11, which shows homology to both RPD3 and HDA1, has been allocated to the separate class IV. The third HDAC class includes sirtuins, characterized by NAD+ dependent activity, differently from the other HDACs. With their ability to deacetylate histones, HDACs compact chromatin, thus regulating heterochromatin formation and maintenance. In addition, many HDACs have ‘non-histone’ targets, such as p53 (Bode & Dong 2004), NFKB (Chen et al. 2002, Furia et al. 2002), several nuclear receptors (NRs; Wang et al. 2001), and cofactors (Rodgers et al. 2005). In this context, it is interesting to note that acetylation modulates NR binding to chromatin and/or to factors involved in mediating NR functions. The implication of HDACs in cancer etiology and therapy has been revealed in both solid tumors and leukemias (Monneret 2005, Nebbioso et al. 2009). For example, suberoylanilide hydroxamic acid (SAHA), a class I–II HDACI, has been approved in 2006 by the FDA for the treatment of cutaneous T-cell lymphomas.

The HDACI MC1568 selectively inhibits the HDAC classes IIa and b (Mai et al. 2005, Nebbioso et al. 2009).
Class II HDACs appear to be mainly involved in the regulation of differentiation, such as myogenesis (Lu et al. 2000), neuronal differentiation (Chawla et al. 2003), and osteogenesis (Hug 2004). Mechanistically, class IIa members may compete with the histone deacetyltransferase p300 for direct binding to the myocyte enhancer factor 2 (MEF2), thus potentially modulating myocyte differentiation. Moreover, HDAC4 null mice display skeletal defects possibly linked to myocyte enhancer factor 2 (MEF2), thus potentially acetyltransferase p300 for direct binding to the class IIa members may compete with the histone deacetylases (Chang et al. 2004, McKinsey & Olson 2004, 2005). The class IIb HDAC6 displays distinct functionality, as its inhibition stimulates tubulin acetylation and influences cell motility (Hubbert et al. 2002, Palazzo et al. 2003, Zhang et al. 2003).

Both class I and II HDACs are involved in the regulation of transcription by NRs such as ERz (ESR1), RARz, or peroxisome proliferator-activated receptor γ (PPARγ). The binding of HDACs to NR-recruited co-repressors, such as SMRT and NCOR, is thought to mediate the repression of target genes seen in the absence of agonists. The multi-subunit complex formed by the co-repressors HDAC4 or HDAC5 and HDAC3 has been studied extensively in several cellular systems (Karagianni & Wong 2007).

Here, we report on the effects of the selective inhibition of class II HDAC activity in three models of NR-regulated differentiation. The first comprises the F9 mouse embryonic carcinoma cells, derived from an experimentally induced teratocarcinoma (Berstine et al. 1973). Upon treatment with all-trans retinoic acid (ATRA), F9 cells differentiate into endodermal-type cells in monolayer cultures. By various criteria, these cells have been shown to correspond to parietal endodermal cells. The efficiency of differentiation of parietal endoderm-like cells can be stimulated by adding dibutyryl cyclic AMP to the culture medium. F9 cells can also differentiate into visceral endoderm-type cells in the presence of retinoic acid under non-adherent condition. The second model comprises NB4 cells, a prototypic model for human acute promyelocytic leukemia (Altucci & Gronemeyer 2001). The third is the murine 3T3-L1 adipogenesis model. The master regulator of this latter process is the NR PPARγ, the absence of which totally abrogates differentiation (Liao et al. 2007). Finally, in order to reveal the action of MC1568 in vivo, we have used engineered PPARγ ‘reporter’ mice to investigate the possible regulation of PPARγ signaling by class II HDACs. Altogether our results reveal an as yet unrecognized activity spectrum of class II HDACs, as MC1568 inhibits F9 cell endodermal, but not NB4 promyelocytic, differentiation by retinooids and attenuates PPARγ activity both in cell lines and in vivo.

Materials and methods

Ligands and chemicals

MS275 (Bayer-Schering) was dissolved in ethanol and used at 5 μM; MC1568 HPLC purified was synthesized as described (Mai et al. 2005, Nebbioso et al. 2009), dissolved in DMSO, and used at 1, 5 or 10 μM as indicated. Troglitazone was a gift of Bristol-Myers-Squibb, rosiglitazone (BRL 49653) was a gift of Novo Nordisk (Rome, Italy); dibutyryl cAMP, ATRA, dexamethasone, and insulin were obtained from Sigma.

Cell lines and cultures

F9 cells were maintained in DMEM supplemented with 10% FCS and 1 mg/l gentamicin and 2 mM glutamine. The F9 cells periodically were grown in bacteria plates like aggregates for 2–3 days and after put in plates coated with 0.1% gelatin. The 3T3-L1 cells were propagated and differentiated using a differentiation cocktail consisting of isobutylmethylxanthine, dexamethasone, and insulin (MDI) as previously described (Nielsen et al. 2008, Kim et al. 2009). From the second day post-confluence and throughout the differentiation period of 8 days, the cells were subjected to either DMSO, 5 or 10 μM MC1568, or 5 μM MS275. For the experiments where the three differentiation media were compared, the 3T3-L1 mouse fibroblasts were kept as follows: i) no induction: at post-confluence and throughout the differentiation period of 8 days, the cells were incubated with DMSO, 5 or 10 μM MC1568, or 5 μM MS275. Medium was renewed every second day. ii) Induction with troglitazone: at post-confluence and throughout the differentiation period of 8 days, the cells were induced with 5 μM troglitazone, 5 μM MC1568, or both. iii) Induction by rosiglitazone: at post-confluence and throughout the differentiation period of 8 days, the cells were induced with 1 μM rosiglitazone and either DMSO, 5 or 10 μM MC1568, or 5 μM MS275, or 125 nM trichostatin A (TSA). Medium was renewed every second day. iv) Induction by rosiglitazone and dexamethasone: at post-confluence, the cells received 1 μM of rosiglitazone and 390 ng/ml dexamethasone dissolved in abs. EtOH. Throughout the differentiation period of 8 days, the cells were incubated with 1 μM of rosiglitazone and either DMSO, 5 or 10 μM MC1568, 5 μM MS275, or 125 nM trichostatin A (TSA). Medium was renewed every second day.

Fluorimetric human recombinant HDAC1 and HDAC4 in vitro assays

HDAC1 and 4 assays were performed as previously described (Lahm et al. 2007, Nebbioso et al. 2009). For HDAC4, the non-histone substrate ‘trifluoroacetyl lysine’ was used as in Lahm et al. (2007).
Oil red O staining

This assay was performed following standard procedures. 3T3-L1 cells were fixed with 3-7% formaldehyde for 10 min and then stained with oil red O for 1 h followed by washing with 70% methanol and water.

NBT assay

For the NBT reduction assay, 500 μl medium containing 1×106 cells were mixed with 500 μl of 0.2% NBT and 200 ng 12-O-tetradecanoylphorbol-13-acetate (Sigma). After incubation for 30 min at 37 °C, the liquid was discarded, cells and formazan deposits were lysed by 500 μl of lysis buffer (50% dimethylformamide and 20% SDS, pH 7.4), and optical density was measured on a spectrophotometer at 570 nm.

RT-PCR, real-time PCR, and primers

Total RNA was extracted from 3T3-L1 cells and F9 cells (Trizol). Two micrograms of total RNA were reverse transcribed using superscript VILO (Invitrogen). For RT-PCR, real-time PCR, and primers

Use of the PPRE-Luc transgenic mouse

The PPRE-Luc transgenic mouse has been previously described (Ciana et al. 2007, Biserni et al. 2008); in this reporter mouse model, luciferase expression is modulated by PPAR ligands and can be considered a surrogate marker for PPAR transcriptional activation.

Luciferase enzyme assay

Mice were euthanized and dissected, and tissues were homogenized in 200 μl of 100 mM KPO4 lysis buffer (pH 7.8 containing 1 mM dithiothreitol, 4 mM EGTA, 10 mM imidazole, 0.5 mM EDTA, and 0.7 mM phenylmethylsulfonyl
fluoride), three cycles of freezing–thawing, and 30 min of minifuge centrifugation (Eppendorf, Hamburg, Germany) at maximum speed. Supernatants containing luciferase were collected, and protein concentrations were determined by Bradford’s assay. Luciferase enzymatic activity was measured by a commercial kit (Luciferase assay system, Promega) according to the supplier’s instructions. Light intensity was measured with a luminometer (Veritas, Promega) over 10 s time periods and expressed as relative light units per µg protein (RLU/µg protein).

Results

The class II HDACi MC1568 blocks differentiation in a cell-specific manner

Supporting the notion that class II HDACs may function primarily during specific cell physiological events, several class II HDACs interact with factors involved in cell differentiation (Verdin et al. 2003, Yang & Gregoire 2005). To assess directly the role of class II HDACs on cell differentiation, we investigated the effect of the class II-selective HDACi MC1568 (Fig. 1A) in well-established models, namely primitive or parietal endodermal differentiation (Rochette-Egly & Chambon 2001), NB4 promyelocytic maturation (Altucci & Gronemeyer 2001), and adipogenesis (Rangwala & Lazar 2000). Note that the complete characterization of the inhibitory action of the MC1568 has been previously reported (Nebbioso et al. 2009).

Exposure of F9 embryonal carcinoma cells to ATRA induced primitive endodermal differentiation that was blocked by MC1568 as revealed by cell morphology (Fig. 1B, top panel) and confirmed by the lack of induction of the differentiation marker collagen IV (Fig. 1C and D). The same differentiation block was observed for parietal endodermal differentiation induced by ATRA and cAMP (Fig. 1B, bottom panel). Indeed, the de novo induction of thrombomodulin (Weiler-Guettler et al. 1992) and the enhanced expression of SPARC, collagen IV, and laminin B1 were all impaired in the presence of MC1568 (Fig. 1C and D).

While these observations supported the hypothesis that class II-selective HDAC inhibition antagonized retinoic acid signaling, no such effects were seen in another retinoid-induced cellular differentiation system. Indeed, in stark contrast to F9 cells, the ATRA-induced maturation of NB4 promyelocytic leukemia cells was entirely unaffected by the HDAC class II-selective inhibitor (Fig. 1E) after treatment for 96 h. Thus, retinoic acid signaling apparently involves class II HDAC action in a cell-specific manner, possibly due to differential expression of HDACs and/or different functional role of HDACs in the retinoic acid signaling pathway.

HDAC class II inhibition decreases PPARγ-dependent adipogenesis, while class I inhibitors block adipogenesis completely

Complex transcriptional hierarchies govern the changes and maintenance of cell morphology and gene expression associated with adipogenesis (Chen et al. 2005). Transcription factors form cross-regulatory circuits and act in concert with epigenetic programs (Yoo & Jones 2006) that can be altered by HDACIs (Lagace & Nachtigal 2004, Qiao et al. 2006). Interestingly, our data indicate that class II HDACs are involved in the regulation of adipogenesis, as MC1568 inhibits the adipogenic activity of troglitazone in 3T3L1 pre-adipocytes, as verified by oil red O staining (Fig. 2A) and induction of differentiation markers (Fig. 2B). Most likely, this effect results from the attenuation of troglitazone-induced PPARγ expression by MC1568 (Fig. 2C). Interestingly, the class I-selective HDACi MS275 blocks PPARγ induction (Fig. 2C) and adipogenesis (Fig. 2D) completely. In contrast to MS275 (Fig. 2D), even at higher concentration equal to 10 µM, complete differentiation medium can partially override the adipogenesis block imposed by MC1568 (Fig. 2D, lower panel). The activation of the aP2 and adiponectin differentiation markers is blocked by MC1568 after induction of adipogenesis with troglitazone or rosiglitazone (Fig. 3A), whereas the activation of these same targets is unaffected in the presence of complete differentiation medium or the combination of rosiglitazone and dexamethasone (Fig. 3B–C). Note that both troglitazone and rosiglitazone have been reported to similarly induce 3T3L1 adipocyte differentiation (Huang et al. 2006). Moreover, we note that while the differentiation markers aP2, adiponectin, and C/EBPz are unaffected by exposure to MC1568 (Fig. 3B, C, and E), the expression of leptin is significantly down-regulated (Fig. 3D) in both rosiglitazone and MDI-induced differentiation of 3T3-L1 cells. Thus, HDAC class II inhibitors seem to interfere with a subset of gene programs associated with adipogenesis.

HDAC class II inhibition exerts organ-selective effects on PPARγ signaling in vivo

Given that MC1568 attenuated adipogenesis in vitro in pro-adipogenic 3T3L1 cells, the possible effect of the drug was assessed in the PPRE-Luc PPAR reporter mouse model in vivo with the idea of exploring a possible anti-obesity activity. In this reporter, mouse luciferase expression is activated in response to PPAR agonists in cognate target organs (Ciana et al. 2007,
Figure 1 The class II-selective HDAC inhibitor MC1568 blocks retinoic acid-induced F9 cell differentiation, but not promyelocytic NB4 cell maturation. (A) *In vitro* human recombinant HDAC1 and HDAC4 assays with or without MC1568 or SAHA used at 5 µM; for HDAC4 assay, the specific trifluoroacetyl lysine substrate has been used; (B) morphological analysis of F9 cells upon treatment with MC1568 in combination with ATRA and cAMP at the indicated time; (C) RT-PCR and qPCR of molecular differentiation markers in F9 cells treated as indicated; (D) RT-PCR of collagen IV and laminin B1 in F9 cells treated as indicated; (E) NBT differentiation assay carried out in NB4 cells treated as described.

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Biserni et al. (2008). Using whole body optical imaging, 7 days of treatment resulted in only minor changes between the various treatment groups (Fig. 4A). Indeed, quantification of bioluminescence did not show any significant effect of the agonist (rosiglitazone) or MC1568 with a trend towards a decrease in the overall photon emission upon co-treatment with both molecules in the chest but not in the abdomen (Fig. 4B). The limited effect of treatments in whole body bioluminescence emission after 7 days of rosiglitazone treatment may be ascribed to the high background activity of PPARγ and PPARα/β/δ isoforms in the liver and intestine, the two most visible PPAR target organs in optical imaging experiments on PPRE-Luc mice. In contrast, ex vivo
quantification of individual organs revealed a strikingly different picture. While no significant drug effects on reporter gene activity were measured in the intestinal compartment, administration of rosiglitazone or MC1568 alone revealed a trend towards lower activities in the liver, and co-administration of the two drugs resulted in significantly decreased liver luciferase expression (Fig. 4C). Most strikingly luciferase activity in the other PPARγ target organs, heart, and perirenal adipose tissue was strongly induced by rosiglitazone, and this induction was completely abolished by co-treatment with MC1568 (Fig. 4C bottom panels). These results indicate that the class II HDACI MC1568 can antagonize rosiglitazone activity in PPARγ target organs in vivo.

**Discussion**

While two HDACIs (SAHA (vorinostat) and depsipeptide) are in use for cancer therapy and others are enrolled in clinical trials, the clinical indications for HDAC subtype-selective modulators – which conceptually should display a reduced spectrum of side effects – have still to be determined. Given the ability of HDAC1 to repress Myo D and class II HDACs to block MEF2 target gene expression, it could be predicted that pharmacological HDAC inhibition would stimulate muscle gene expression and thus enhance myogenesis. Paradoxically, the same inhibitors were later reported to modulate skeletal muscle differentiation in a stage-specific manner revealing that HDACIs have the potential to enhance myogenesis (Iezzi et al. 2002). Inhibitory effects of HDACIs were reported for adipocyte differentiation. Treatment with class I-selective or pan-HDACIs blocked the differentiation of 3T3-L1 cells (Kim et al. 2009) as confirmed in the present study.

That class II HDAC activity is required for some steps within the cascade of gene regulatory events that constitute a differentiation program is supported by our data on the retinoic acid induction of endodermal differentiation of F9 cells. Moreover, in studying the effects of class II HDACs in two different NR-dependent differentiation systems, we show that the class II-specific inhibitor MC1568 interferes with the transcriptional signaling of RARs as well as PPARγ. That we are able to interfere with retinoic acid-mediated endodermal differentiation in F9 cells by altering the transcriptional regulation of target genes such as the collagen IV but that retinoic acid-mediated maturation of NB4 promyelocytes is not affected indicates that class II HDACs and their cognate inhibitors can exert tissue-specific effects. This observation is in keeping with class II HDACs-restricted expression in selected organs and tissues, and a stage-specific requirement of certain HDACs or HDAC classes may also account for the divergent observations reported for pan-HDACs on myogenesis. Moreover, note that NB4 cells express limited amounts of selected class II HDACs. Selective HDAC class II inhibition blocks the (weak) differentiation of 3T3-L1 pre-adipocytes induced by troglitazone or rosiglitazone alone supposedly by inhibiting the induction of PPARγ expression. Notably, this block can be overridden by simultaneously activating several adipogenic pathways when using complete differentiation medium (MDI). Under such conditions, class II

**Figure 3** Expression levels of adipogenesis markers determined by real-time PCR. (A) A-FABP/aP2 and adiponectin expression levels relative to those of TFIIB by RT-qPCR in 3T3-L1 cells after the indicated treatments in the presence of MDI. (B–E) A-FABP/aP2 (B), adiponectin (C), leptin (D), and C/EBPα (E) expression levels measured relative to those of TFIIB by RT-qPCR in 3T3-L1 cells after the indicated treatments in the presence of MDI. ROS1, rosiglitazone; TRO, troglitazone; DEX, dexamethasone.
inhibition results only in an attenuation of the efficiency of adipogenesis as determined by oil red O staining, suggesting both a direct and indirect involvement of class II HDACs in PPARγ signaling. The influence of HDAC class II on PPARγ signaling – and as a consequence the modulation of PPARγ by HDACIs (Kim et al. 2009) – might be explained both as a consequence of the interaction of HDACs with NRs (Franco et al. 2003) and of the influence on PPARγ target genes. That MC1568 also inhibits HDAC6 might suggest a role for HDAC6 in these settings. Despite HDAC6 contribution cannot be excluded, its main cytoplasm localization (Valenzuela-Fernández et al. 2008) does not fully support the interference with the PPARγ signaling pathway. Interestingly enough, the impairment of PPARγ signaling is supported by experiments in vivo with PPRE-Luci reporter mice, further arguing for a role of class II HDACs in mediating PPARγ signaling. Indeed, only the PPARγ ‘target organs’, such as the heart and adipose tissues, displayed inhibited PPARγ activity when animals were co-exposed to MC1568 and rosiglitazone. We also noted that inhibition of class II HDACs in the PPRE-Luci reporter mice did not lead to any side effects or detrimental alterations after administration of MC1568 as a single agent. Although PPARγ is expressed at low levels in many different cell types, it is well established that PPARγ activation in adipose tissue and to some extent also in macrophages is of key importance for the insulin-sensitizing effects of thiazolidinediones (TZDs; Kahn et al. 2000, Hevener et al. 2007). However, PPARγ activation in other cell types may also contribute to the physiological effects of these TZDs including the side effects that limit the use of these drugs. Thus, treatment with TZDs such as rosiglitazone leads to edema in some patients (Nesto et al. 2003), and genetic experiments in mice indicate that the edema results from activation of PPARγ in the kidney epithelium (Zhang et al. 2005). Our observation that HDAC class II inhibition decreases TZD activation of PPARγ in cell culture as well as in vivo suggests that HDAC class II inhibition could potentially be used in combination with PPARγ agonists
to modulate PPARγ activation in a tissue-specific manner. This would require that these inhibitors preferentially interfere with the deleterious side effects rather than the many beneficial effects of TZDs.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This work was supported by the following EU projects: Epitron LS-HEC-CT2005-518417 (LA, HG, and AM); Apo-Sys HEALTH-F2-2007-206290 (LA and HG); ATLAS HEALTH-F4-2009-221952 (HG and LA); XTRA-NET LS-HEC-CT2005-018882 (HG and SM), by funds from the Associazione Italiana per la ricerca contro il cancro, AIRC (LA), the Fondazione ONLUS Luigi Califano (LA), PRIN 2008 (DT), and the Ligue Nationale Contre le Cancer (HG). FM was a French–Italian University PhD holder enrolled in the Vinci program.

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Bode AM & Dong Z 2004 Post-translational modification of p53 in LSHC-CT2005-5188417 (LA, HG, and AM); Apo-Sys www.endocrinology-journals.org Epitron. This work was supported by the following EU projects: X-TRA-NET F2-2007-200620 (LA and HG); ATLAS HEALTH-F4-2009-221952 (HG and LA); XTRA-NET LS-HEC-CT2005-018882 (HG and SM), by funds from the Associazione Italiana per la ricerca contro il cancro, AIRC (LA), the Fondazione ONLUS Luigi Califano (LA), PRIN 2008 (DT), and the Ligue Nationale Contre le Cancer (HG). FM was a French–Italian University PhD holder enrolled in the Vinci program.

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