

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 (PPAR γ)-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 PPAR γ -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 PPAR γ signaling mostly in the heart and adipose tissues. These results illustrate how HDAC functions can be dissected by selective inhibitors.

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Introduction

In the past years, epigenetic therapies have come of age. The finding that histone deacetylases (HDACs) often are more 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), NF κ B (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.* 2005). 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 acetyltransferase 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 altered Runx2 action (Vega *et al.* 2004), while knock-out of HDAC5 or HDAC9 displays cardiac hypertrophy (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 ER α (ESR1), RAR α , or peroxisome proliferator-activated receptor γ (PPAR γ). 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 PPAR γ , 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 PPAR γ 'reporter' mice to investigate the possible regulation of PPAR γ 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 retinoids and attenuates PPAR γ 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 μ M; 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 μ M 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/1 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 μ M MC1568, or 5 μ M 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 μ M MC1568, or 5 μ M 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 μ M troglitazone, 5 μ M MC1568, or both. iii) Induction by rosiglitazone: at post-confluence and throughout the differentiation period of 8 days, the cells were incubated with 1 μ M of rosiglitazone and either DMSO, 5 or 10 μ M MC1568, 5 μ M 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 μ M of rosiglitazone and 390 ng/ml dexamethasone dissolved in abs. EtOH. Throughout the differentiation period of 8 days, the cells were induced with 1 μ M of rosiglitazone and either DMSO, 5 or 10 μ M MC1568, 5 μ M MS275, or 125 nM 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 1×10^6 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 amplification, we used the following primers: laminin B1, forward (5'-ACAACACCAAAGGCCTGAAC-3') and reverse (5'-TGCCAGTAGCCAGGAAGACT-3'); collagen IV, forward (5'-ACAACAGARGACCCACTGTG-3') and reverse (5'-GTGTGCATCACGAAGGAATA-3'); *SPARC*, forward (5'-GTCCACACTGAGCTGGC-3') and reverse (5'-AAGCACAGAGTCTGGGTGAGTG-3'); adiponectin, forward (5'-AGGGTGAGACAGGAGATGTTGG-AAT-3') and reverse (5'-GCCAGTAAATGTAGAG-TCGTTGACGT-3'); *A-FABP* (aP2), forward (5'-AACACC-GAGATTTTCCTTCAAACCTG-3') and reverse (5'-TCACG-CCTTTCATAACACATTCCA-3'); actin, forward (5'-GAC-GGCCAGGTCATCACTAT-3') and reverse (5'-CCACCG-ATCCACACAGAGTA-3'); *PPAR γ* , forward (5'-TCTCTCCG-TAATGGAAGACC-3') and reverse (5'-GCATTATGAGACA-TCCCCAC-3'); thrombomodulin, forward (5'-TGGAGCA-TGAGTGCTTCGC-3') and reverse (5'-GGTGTGTAGGT-ACTAGAGA-3'); *GAPDH*, forward (5'-TCAACGGGAAG-CCCATCACCA-3') and reverse (5'-ACGGAAGGCCATGC-CAGTGA-3'). Real-time PCR was performed with the following primers: *TFIIB*, forward (5'-GTTCTGCTCCAA-CCTTTGCGCT-3') and reverse (5'-TGTGTAGCTGCCATC-TGCACTT-3'); *A-FABP* (aP2), forward (5'-CTG GCGGTGG-AATTCGAT-3') and reverse (5'-GCTCTTCACCTTCCT-GTCGTCT-3'); adiponectin, forward (5'-TGACTGCA-ACTACCCATAGC-3') and reverse (5'-TTAATGAACAAGT-GAGTACACG-3'); leptin, forward (5'-TGTGCTGCAGATA-GCCAATGA-3') and reverse (5'-AGATGGAGGAGGTCTC-GGAGA-3'); *C/EBP α* , forward (5'-CAAGAACAGCAACGAG-TACCG-3') and reverse (5'-GTCACTGGTCAACTCCAGCA-C-3'); actin, forward (5'-GACGGCCAGGTCATCACTAT-3') and reverse (5'-CCACCGATCCACACAGAGTA-3'); laminin B1, forward (5'-ACAACACCAAAGGCCTGAAC-3') and

reverse (5'-TGCCAGTAGCCAGGAAGACT-3'); *SPARC*, forward (5'-GAGGAGGTGGTGGCTGACAA-3') and reverse (5'-CACCTTGCCATGTTTGCAAT-3'); collagen IV, forward (5'-ACAACAGAGACCCACTGTG-3') and reverse (5'-GTGTGCATCACGAAGGAATA-3'); *PPAR γ* , forward (5'-TTTTCAAGGGTGCCAGTTTC-3') and reverse (5'-AATCCTTGCCCTCTGAGAT-3'); thrombomodulin, forward (5'-TGGAGCATGAGTGCTTCGC-3') and reverse (5'-GGTGTGTAGGTACTAGAGA-3').

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. For optical imaging studies, mice were first anesthetized using an s.c. injection of 50 µl ketamine–xylazine solution composed of 78% ketamine (Ketavet 50, Intervet, Peschiera Borromeo, Italy), 15% xylazine (Rompun 2% solution, Bayer), and 7% water, and then received an i.p. injection of 25 mg/kg D-luciferin (25 mg/kg; Promega); to obtain a uniform biodistribution of the substrate, CCD camera detection was performed 20 min after luciferin injection (Ciana *et al.* 2003, 2007). Bioluminescence measurements were done with a Night Owl imaging unit (Berthold Technologies, Bad Wildbad, Germany) consisting of a Peltier cooled charge-coupled device slow-scan camera equipped with a 25 mmf/0·95 lens. Images were generated by a Night Owl LB981 image processor and transferred via video cable to a peripheral component interconnect frame grabber using WinLight32 software (Berthold Technologies). To lower the background activity of endogenous PPAR activities due to dietary intake of lipids, experimental mice were fed solely during the night (Ciana *et al.* 2007), and photon emission was measured at 1700 h. In chronic studies, 50 mg/kg MCI568 and 5 mg/kg of rosiglitazone or vehicle (water solution of 0·5% carbosimetilcellulose) were administered by gavage once a day at 0900 h, for a total of 7 days starting from day 1. In co-treatment, MCI568 was administered 30 min before rosiglitazone. Photon emission was measured in chest and abdomen; photon emission from these areas is due mainly to liver and intestine as demonstrated in preliminary experiments (data not shown and Ciana *et al.* (2007)).

Luciferase enzymatic assay

Mice were euthanized and dissected, and tissues were immediately frozen on ice. Protein extracts were homogenized in 200 µl of 100 mM KPO₄ lysis buffer (pH 7·8 containing 1 mM dithiothreitol, 4 mM EGTA, 4 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 PPAR γ -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 PPAR γ expression by MC1568 (Fig. 2C). Interestingly, the class I-selective HDACI MS275 blocks PPAR γ 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 p2 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 p2, adiponectin, and C/EBP α 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 PPAR γ 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,

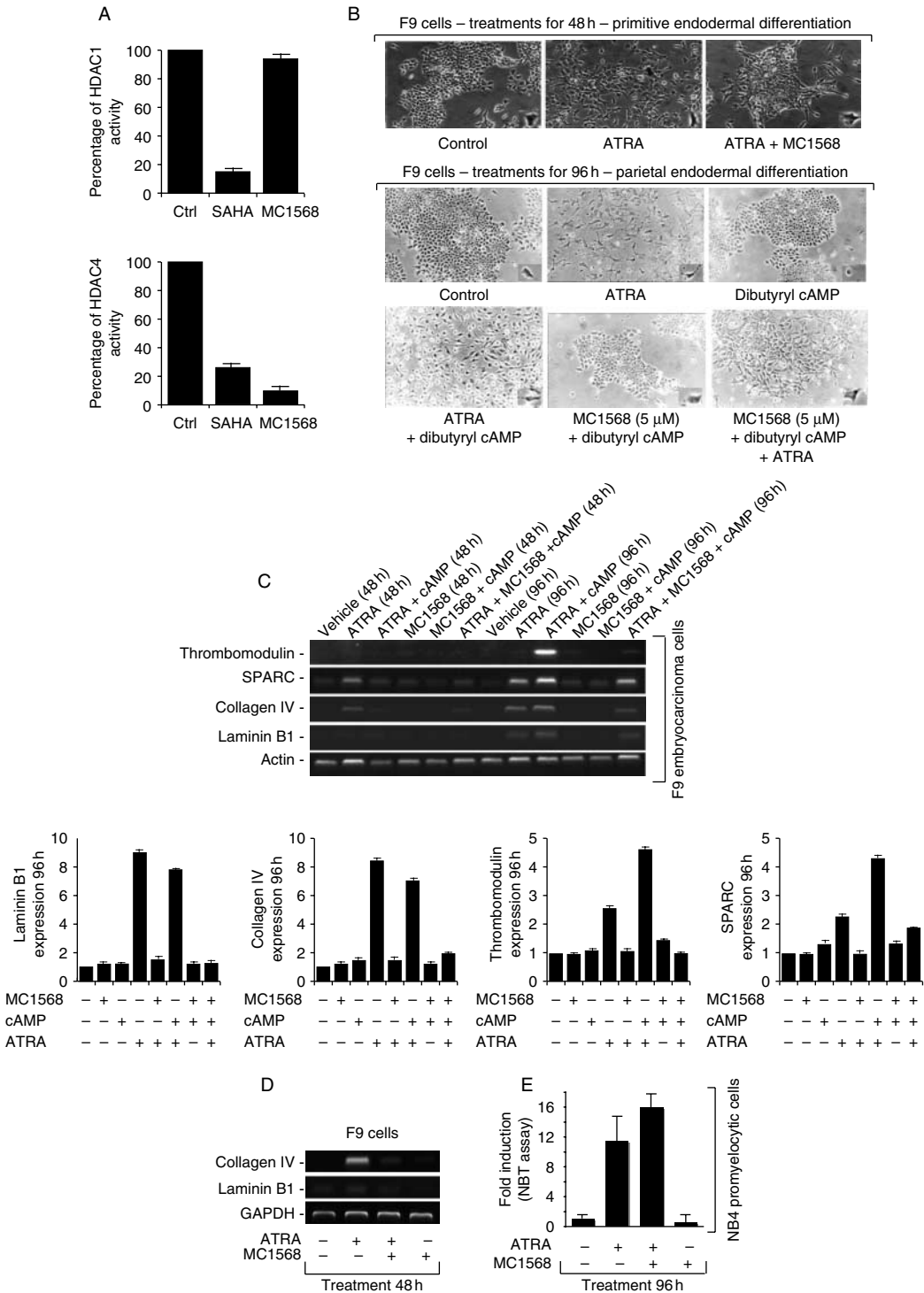


Figure 1 The class II-selective HDAC inhibitor MC1568 blocks retinoic acid-induced F9 cell differentiation, but not promyelocytic NB4 cell maturation. (A) *In vitro* human recombinant HDAC1 and HDAC4 assays with or without MC1568 or SAHA used at 5 μM; for HDAC4 assay, the specific trifluoroacetyl lysine 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.

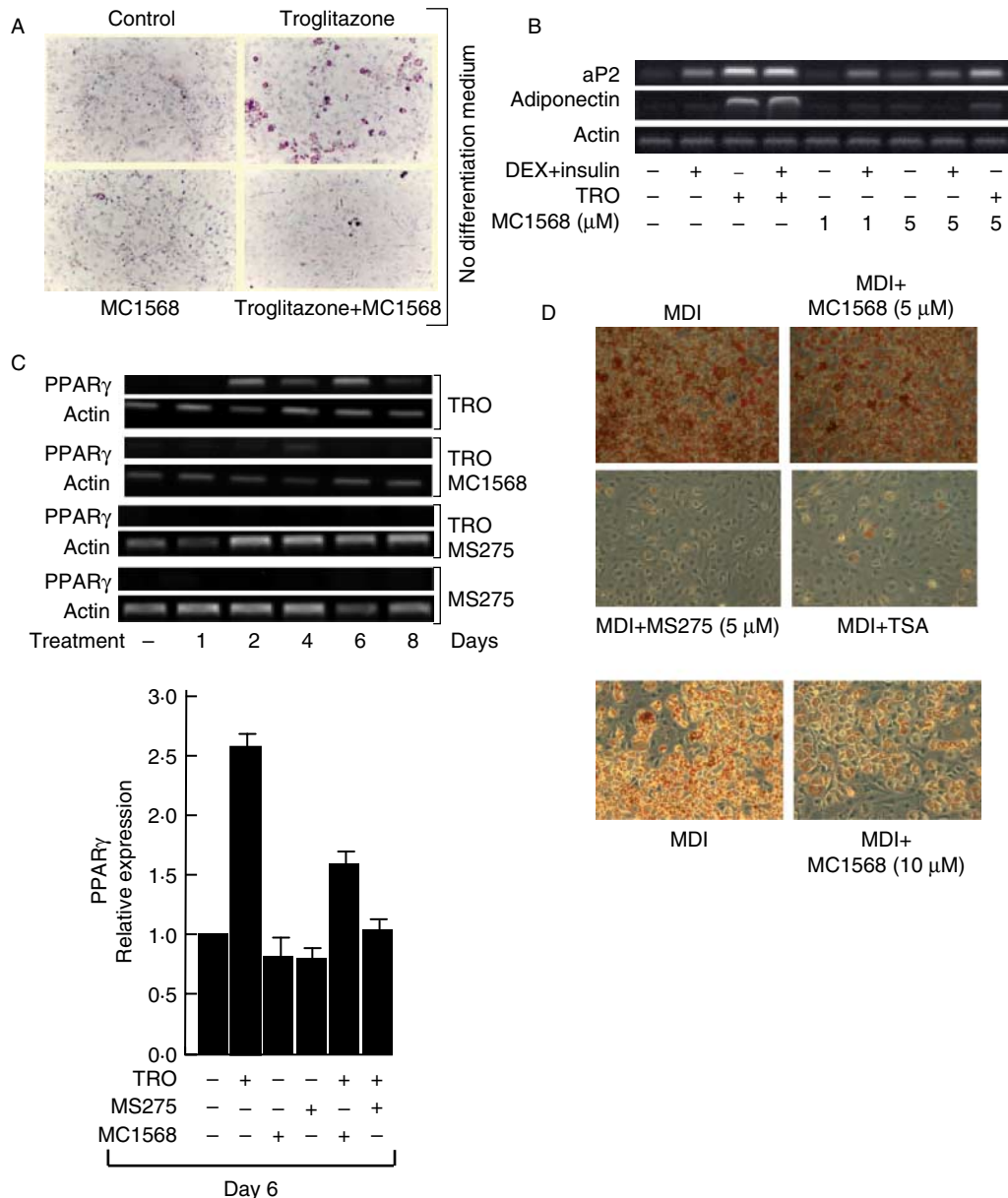


Figure 2 MC1568 attenuates TZD-induced PPAR γ induction and adipogenesis of 3T3-L1 cells, while pan-HDAC inhibitors block differentiation completely. (A) Morphological analysis obtained with oil red O staining and (B) RT-PCR of molecular differentiation markers in 3T3-L1 cells treated as indicated; (C) RT-PCR of PPAR γ expression in the 3T3-L1 cells treated as indicated; (D) morphological analysis obtained with oil red O staining of 3T3-L1 cells in complete differentiation medium and treated with TSA at 125 nM and with MS275 or MC1568 at 5 or 10 μ M. TRO, troglitazone; DEX, dexamethasone; MDI, differentiation cocktail consisting of isobutylmethylxanthine, dexamethasone, and insulin.

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 PPAR α and 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*

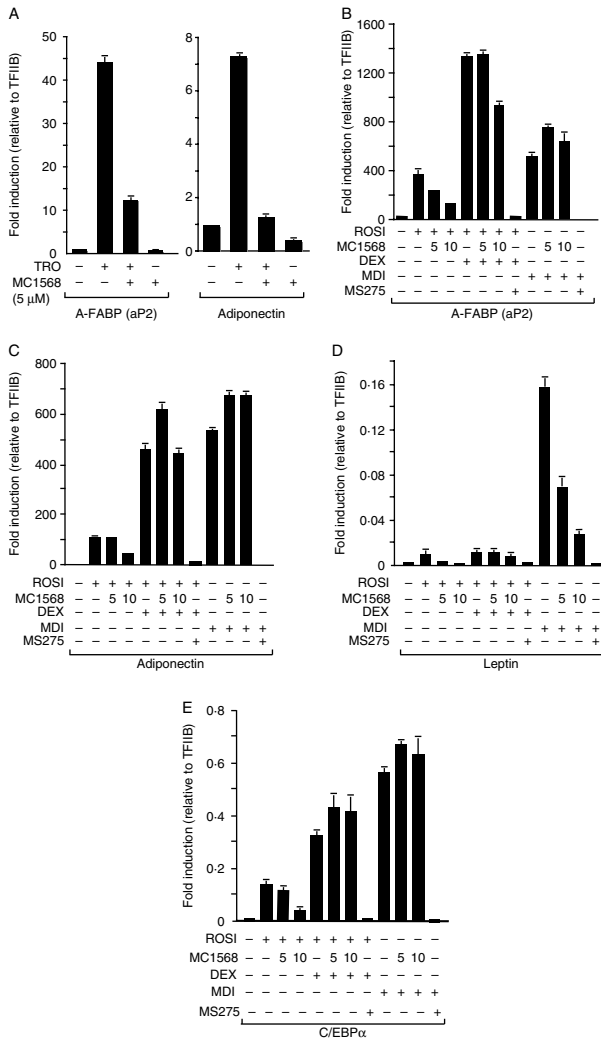


Figure 3 Expression levels of adipogenesis markers determined by real-time PCR. (A) A-FABP/aP2 and adiponectin expression levels relative to those of TFIIIB by RT-qPCR in 3T3-L1 cells after the indicated treatments in the absence of MDI; (B–E) A-FABP/aP2 (B), adiponectin (C), leptin (D), and C/EBP α (E) expression levels measured relative to those of TFIIIB by RT-qPCR in 3T3-L1 cells after the indicated treatments in the presence of MDI. ROS1, rosiglitazone; TRO, troglitazone; DEX, dexamethazone.

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 PPAR γ 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 PPAR γ target organs *in vivo*.

Discussion

While two HDACi (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 HDACi have the potential to enhance myogenesis (Iezzi *et al.* 2002). Inhibitory effects of HDACi were reported for adipocyte differentiation. Treatment with class I-selective or pan-HDACi 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 PPAR γ . 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-HDACi 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 PPAR γ expression. Notably, this block can be overridden by simultaneously activating several adipogenic pathways when using complete differentiation medium (MDI). Under such conditions, class II

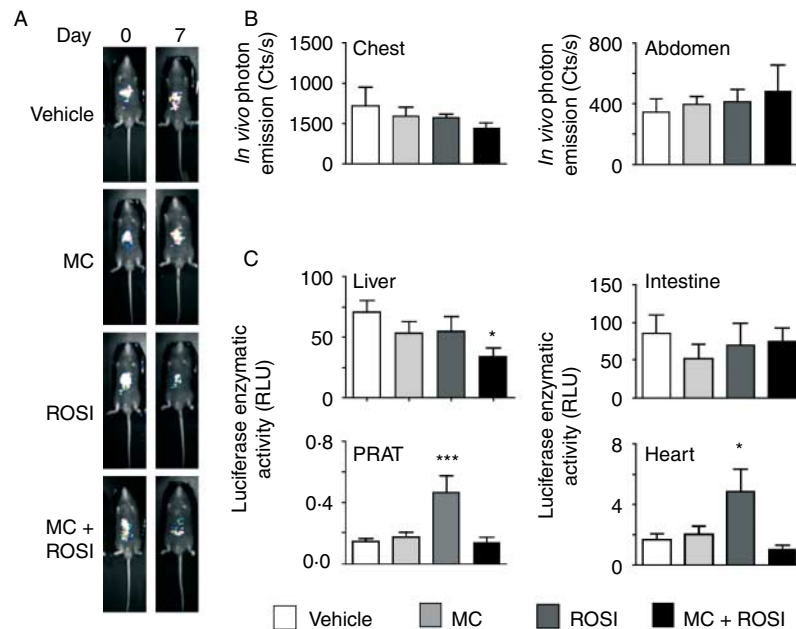


Figure 4 MC1568 antagonizes rosiglitazone-dependent induction of luciferase expression in adipose tissue and heart of the PPRE-Luc mouse. PPRE-Luc mice were treated by gavage daily with 5 mg/kg rosiglitazone (ROSI), 50 mg/kg MC1568 (MC), or vehicle (water solution of 0.5% carboxymethylcellulose); when co-administered, MC1568 was given 30 min before rosiglitazone for 7 days. Photon emission in chest and abdomen was measured at 1700 h at day 0 and at the end of the treatment (day 7). (A) Pictures of photon emission measured *in vivo* of a single, representative, individual at day 0 and 7. (B) Photon emission measured *in vivo* after 7 days treatment; bars represent the mean \pm s.e.m. (C) Luciferase contents from liver, intestine, perirenal adipose tissue (PRAT), and heart. Relative luciferase units (RLU, photon counts/ μ g of proteins) were determined by enzymatic assay as described in Materials and methods. Bars represent the mean RLU \pm s.e.m. * $P < 0.05$ and *** $P < 0.001$ versus vehicle-treated group.

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 PPAR γ signaling. The influence of HDAC class II on PPAR γ signaling – and as a consequence the modulation of PPAR γ by HDACs (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 PPAR γ 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 PPAR γ signaling pathway. Interestingly enough, the impairment of PPAR γ signaling is supported by experiments *in vivo* with PPRE-Luc reporter mice, further arguing for a role of class II HDACs in mediating PPAR γ signaling. Indeed, only the PPAR γ ‘target organs’, such as the heart and adipose tissues, displayed inhibited PPAR γ activity when animals were co-exposed to MC1568 and

rosiglitazone. We also noted that inhibition of class II HDACs in the PPRE-Luc reporter mice did not lead to any side effects or detrimental alterations after administration of MC1568 as a single agent. Although PPAR γ is expressed at low levels in many different cell types, it is well established that PPAR γ 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, PPAR γ 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 PPAR γ in the kidney epithelium (Zhang *et al.* 2005). Our observation that HDAC class II inhibition decreases TZD activation of PPAR γ in cell culture as well as *in vivo* suggests that HDAC class II inhibition could potentially be used in combination with PPAR γ agonists

to modulate PPAR γ 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.

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