Context-selective death of acute myeloid leukemia cells triggered by the novel hybrid retinoid-

HDAC inhibitor MC2392

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ABSTRACT

HDAC inhibitors (HDACi) are widely used in the clinic to sensitize tumorigenic cells for treatment with other anticancer compounds. The major drawback of HDACi is the broad inhibition of the plethora of HDAC-containing complexes. In acute promyelocytic leukemia (APL), repression by the PML-RARα oncofusion protein is mediated by an HDAC-containing complex that can be dissociated by pharmacological doses of all trans retinoic acid (ATRA) inducing differentiation and cell death at the expense of side effects and recurrence. We hypothesized that the context-specific close physical proximity of a retinoid and HDACi binding protein in the repressive PML-RARα-HDAC complex may permit selective targeting by a hybrid molecule of ATRA with a 2-aminoanilide tail of the HDAC inhibitor MS-275, yielding MC2392. We show that MC2392 elicits weak ATRA and essentially no HDACi activity in vitro or in vivo. Genome-wide epigenetic analyses revealed that in NB4 cells expressing PML-RARα, MC2392 induces changes in H3 acetylation at a small subset of PML-RARα binding sites. RNA-seq reveals that MC2392 alters expression of a number of stress-responsive and apoptotic genes. Concordantly, MC2392 induced rapid and massive, caspase 8-dependent cell death accompanied by RIP1 induction and ROS production. Solid and leukemic tumors are not affected by MC2392, but expression of PML-RARα conveys efficient MC2392-induced cell death. Our data suggest a model in which MC2392 binds to the RARα moiety and selectively inhibits the HDACs resident in the repressive complex responsible for the transcriptional impairment in APLs. Our findings provide proof-of-principle of the concept of a context-dependent targeted therapy.
Introduction

Acute promyelocytic leukemia (APL) is characterized by the presence of blasts blocked at the promyelocytic stage of myeloid differentiation and by the fusion protein of RARα with the promyelocytic leukemia gene (PML) in more than 95% of the cases (1, 2). Despite that the majority of APLs carry this t(15;17) translocation, rare alternative RARα-containing fusion proteins have been described (3-5). In the treatment of APL, the efficacy of pharmacologic doses of ATRA is due to its ability to release the HDAC-containing repressive complexes bound to PML-RARα (6) and to recruit the multi-subunit HAT complex on RARE. Besides having this desired therapeutic effect on APL blasts, ATRA binds to and activates the nuclear receptors (RARα, β or γ) that bind to specific DNA responsive elements (RARE) (7, 8) thereby affect a variety of biological processes in essential all cell types such as cell proliferation, differentiation and apoptosis (9, 10).

The PML-RARα mediated epigenetic block of gene transcription (11, 12) can also be overcome by HDAC inhibitors (HDACi) allowing cells to re-start differentiation or pro-apoptotic events (13). Activation of differentiation programs, inhibition of the cell cycle and eventually induction of apoptosis are among the key antitumor activities of HDACi in cancer therapy (7, 11, 14-16). HDACi cause enzymatic inhibition and/or the release of HDACs from repressive complexes permitting HAT recruitment, histone acetylation, chromatin decondensation and, ultimately, transcription activation of tumor-suppressor genes (TSG) or other genes crucial for the normal functioning of the cells (7). The major drawback of HDACi in treatment is the broad inhibition of the plethora of HDAC-containing complexes.

MS-275/Entinostat is one of the most potent and widely used HDACi with micromolar affinity for class I HDACs and with selectivity for HDAC1, HDAC3 and HDAC8 (17-19). MS-275 promotes differentiation, apoptosis and inhibits the proliferation of multiple cancer cell lines (20). The effects of MS-275 have been examined in human leukemia and lymphoma cells (U937, HL-60, K562, and Jurkat). When administered at a low concentration (e.g., 1 μM), MS-275 exhibited potent
antiproliferative activity inducing p21\(^{CIP1/WAF1}\)-mediated growth arrest and expression of
differentiation markers (CD11b) in U937 cells (14, 21). However, at higher concentrations (e.g., 5
\(\mu M\)), MS-275 potently induced cell death and a very early increase in death receptor signaling (22)
as well as in reactive oxygen species (ROS), followed by the loss of mitochondrial membrane
potential and cytosolic release of cytochrome c (23).

The use of HDACi in combination with other anticancer agents (epi-drugs such as 5-aza-2’-
deoxycytidine and retinoic acid) is one way forward to a promising application against cancer. In
patients with leukemia, and particularly in the elderly, clinical studies combining ATRA treatment
with HDACi are in progress (24). For example, Valproic acid (VPA) has for many years been and
still is used as an anti-epileptic drug and inhibits preferably class I HDACs although in the high
micromolar to millimolar range. Moreover, even though there were a lot of concerns regarding toxic
side effects of HDACi in the clinical setting due to the roles of HDACs in multiples pathways, until
now clinical trials mostly showed manageable side effects (25). The combination of ATRA with an
HDACi could result in improved anti-tumorigenic activity (24).

Here, we exploited the synergy between HDACi and ATRA by generating and testing a single
hybrid molecule, named MC2392 on APL cells. Genome-wide epigenetic analysis revealed that
MC2392 is a weak retinoid. In contrast to the genome wide effects of MS-275 on histone H3
acetylation, MC2392 induces acetylation at only a small subset of PML-RAR\(\alpha\) binding sites in NB4
cells line. RNA-seq analysis showed that MC2392 alters the expression of a number of stress-
responsive and apoptotic genes differently from ATRA. Importantly, the hybrid compound acts in a
context and PML-RAR\(\alpha\) fusion protein-dependent fashion to induce rapid and massive cell death,
RIP1 induction and ROS production. We propose a model in which this hybrid compound binds to
the RAR\(\alpha\) via its retinoid-moiety and selectively inhibits the HDACs contained in the repressive
complex via the MS-275 part. Taken together, MC2392 is a promising candidate for apoptosis-
based therapy of APL, representing a new and effective, *single hybrid* drug able to modulate
multiple cell death pathways. Our study convincingly shows that the targeting of multiple signaling pathways by a single hybrid drug is a feasible and attractive paradigm for new cancer therapies.

**Material and Methods**

**Cells.** NB4, LnCap and U937 were purchased by DSMZ and ATCC respectively. Cell lines have been tested and authenticated following manufacture’s instruction. NB4, NB4-R4 (26, 27), U937, U937 PML/RAR, U937-PLZF/RAR Zn inducible (28, 29) and LnCap cell lines were grown at 37°C in air and 5% CO2 in RPMI 1640 medium (EUROCLONE), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma), 1% L-glutamine, 1% ampicillin/streptomycin and 0.1% gentamicin (SIGMA). Cells were kept at the constant concentration of 200000 cells per milliliter of culture medium. HeLa cell line, was grown at 37°C in air and 5% CO2 in Dulbecco’s Modified Eagle Medium (GIBCO) with 10% FBS, 1% L-glutamine, 1% ampicillin/streptomycin and 0.1% gentamicin.

**Chemicals.** ATRA and MS-275 were dissolved in ethanol; MC2392, MC2677 and MC2678 were dissolved in DMSO and used at 1 µM and 5 µM respectively. SAHA (Merck), resveratrol, and EX-527 (Alexis) were dissolved in DMSO and used at 5, 100, and 5 µM, respectively.

**Synthesis of MC2392, MC2677, MC2678.** See Supplementary Materials and Methods.

**Total Protein, Histone extraction and western blot.** See Supplementary Materials and Methods.

**Colony assay.** Colony assay was carried out as in (22).

**Cell cycle and differentiation analyses.** See Supplementary Materials and Methods.

**Caspase-3, -8 and -9 assays.** Caspase activity was detected within living cells using B-BRIDGE Kits supplied with cell-permeable fluorescent substrates. The fluorescent substrates for caspase-3, -8 and -9 were FAM-DEVD-FMK, FAM-LETD-FMK, FAM-LEHD-FMK, respectively. Cells were washed twice in cold PBS and incubated for 1h in ice with the corresponding substrates, as recommended by suppliers. Cells were analyzed using Cell Quest software applied to a FACScalibur (BD). Experiments were performed in duplicate and values expressed in mean ± SD.
Caspase 2 assay. Caspase-2 activity was detected within whole living cells, using Caspase-2 Fluorometric Kit following supplier’s instructions (R&D). The plate was incubated at 37°C for 1h and Fluorescence quantified with a TECAN M200 station at a 400-505 nm wavelength. Results are expressed as fold increase in caspase activity.

Analysis of mitochondrial membrane potential. Detection of the changes in mitochondrial membrane potential (ΔΨm) was performed using the MITOPT™ JC-1 kit (Immunochemistry Technologies) following the supplier’s suggestions and analyzed by flow cytometry with Cell Quest Pro software.

ROS detection. NB4 cells were resuspended in pre-warmed PBS 1X, containing 5 μM of DCF-DA (dichlorodihydrofluorescein diacetate) probe and incubated at 37°C for 20 minutes. Cells were analyzed after washing, using the Cell Quest software applied to a FACScalibur (BD). H2O2 was used as positive control.

RNA extraction, reverse transcription (RT) and quantitative PCR in real time (RT-PCR). See Supplementary Materials and Methods.

Immunoprecipitation. See Supplementary Materials and Methods.

HDAC assay. HDAC assays have been performed as in (30). Specifically, samples immunoprecipitated with anti-PML-RAR or with IgG were pooled respectively to homogenize all samples. The Fluor de Lys Substrate was incubated (1h) with the immunoprecipitated PML-RAR (in presence or absence of SAHA or MC2392 5 μM) and fluorescence was quantified with a TECAN M200 station.

SIRT assay. See Supplementary Materials and Methods.

Transfections and luciferase assay. See Supplementary Materials and Methods.

IkB alpha ELISA assay. Assay was performed after 48h from the treatment with ATRA and MC2392 in NB4 cells following supplier’s instructions (Active Motif).

ChIP and ChIP-seq. NB4 cells were treated for 4, 6, 24 and 48h with 1 μM ATRA, 5 μM MS-275 and 5 μM MC2392. Chromatin was harvested as described previously (31). ChIP experiments were
performed using the H3K9K14ac (Diagenode), Nrf2, c-Fos and c-Jun (Santa Cruz) antibodies. ChIPed DNA was analyzed by real time qPCR with specific primers (Biolegio, see Supplementary Methods) using the 2x SYBR Green mix (BIORAD) in a MyiQ thermocycler (BIORAD). Primers amplifying myoglobin were used as negative control. In addition, ChIPed DNA was prepared for sequencing and sequenced according to the manufacturer’s instructions (Illumina) in Supplementary Materials and Methods and essentially as already described (32, 33).

**Peak Detection, Clustering analysis and Motif Search** are detailed in the Supplementary Materials and Methods.

**RNA-sequencing.** See Supplementary Materials and Methods.

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**Results**

**MC2392 inhibits HDAC activity of the PML-RARα complex and maintains retinoid activity.**

We set out to exploit the possibility to combine the active parts of the HDACi MS-275 and the retinoid, ATRA, yielding MC2392 (Fig. 1A). To explore the activities of this hybrid compound, U937 cells were treated for 24h with 5 μM MC2392, its parent compound MS-275 (Entinostat) a class I inhibitor and the class I/II HDAC inhibitor SAHA (Vorinostat). In contrast to the reference compounds, MC2392 did not result in a net increase of histone acetylation (Fig. 1B). Moreover MC2392 did not induce p21 protein expression (Fig. 1B) corroborating that at this concentration the molecule has reduced or no HDACi activity as compared to MS-275. MC2392 exerted only minimal (if any) inhibitory action on HDAC1 and HDAC4, nor did it display SIRT1 modulating activities *in vitro* (Fig. 1C). Our premise for the design of MC2392 was that the close physical proximity of a retinoid (PML-RARα) and an HDACi binding moiety (HDAC1 and/or -4) could convey context-specific inhibitory activity by a hybrid molecule spanning the physical distance. To investigate this assertion, PML-RARα was immunoprecipitated from HeLa cells after transient
transfection with PML-RARα and assayed for HDACi activity. Indeed, MC2392 inhibits the HDAC(s) resident in the PML-RARα repressive complex similarly to SAHA (Fig. 1D).

To investigate whether MC2392 acts as a retinoid, its potential to activate the ATRA-responsive luciferase reporter (RARE3tkluc) was first tested in transient transfected HeLa that expresses endogenous RAR and RXR. MC2392 activates RARE3tkluc weakly compared to ATRA (Fig. 1E).

Consistent with these observations, quantitative real-time PCR in NB4 cells showed a similar low response to MC2392 for the RNA expression of specific genes known to be retinoid modulated (34, 35) such as HOXA1, IRF1 and, to a lesser extent, TNFα, (Supplementary Fig. 1A) as compared to ATRA suggesting that MC2392 still maintains at least part of its retinoid activity. Finally, western blot analyses of PML-RARα following MC2392 treatment showed rapid degradation of PML-RARα in NB4 cells (Fig. 1F) as has been reported for ATRA (36, 37). Taken together the results show that MC2392 acts as a weak retinoid when compared to ATRA implying that it binds to RARα, activates RARα signaling as well as induces the degradation of PML-RARα (Fig. 1F). We tentatively conclude that MC2392 is an HDACi in the context of the PML-RARα-HDAC repressive complex.

**MC2392 induces local H3 acetylation.**

We showed that the HDACi activity of MC2392 is indeed apparent in the context of the PML-RARα-contained HDAC complexes implying that acetylation maybe altered at PML-RARα genomic binding sites. To examine whether MC2392 induces local epigenetic alterations, ChIP-seq profiling was performed using the H3K9K14ac antibody in NB4 cells treated with ATRA, MS-275, MC2392 or solvent, DMSO. In DMSO treated cells, H3K9K14 acetylation appeared in the typical sharp narrow peaks. Treatment with the HDACi MS-275 had a dramatic effect on the genomic acetylation landscape: H3K9K14ac peaks were dampened and spreading of acetylation occurred (Fig. 2A). In line with the absence of global HDACi activity (Fig. 1B), MC2392 did not affect the overall distribution of H3K9K14ac. Genomic annotation of the peaks called by MACS (38) with a
P-value of $10^{-6}$ (32) revealed a small percentage of H3K9K14ac binding sites at promoter regions (8.74%) while the major portion of the peaks were located in the gene body (52.11%) or intergenic (34.19%) (Fig. 2B). Importantly, MC2392 did induce low but reproducible local changes in acetylation at some PML-RARα binding sites such as at RARβ promoter or TGM2, ICAM1 gene (Fig. 2C). The RARβ is avidly transcriptionally induced by ATRA and expectedly H3 acetylation is induced at promoter region. These data suggest specificity and selectivity of MC2392 towards at least some PML-RARα binding sites and reinforced the similar, although weaker, retinoid activity of MC2392 as compared to ATRA (Fig. 1E, Supplementary Fig. 1A).

To substantiate our findings we examined the 1000 most significantly changed acetylation peaks after ATRA induction in PML-RARα/RXR binding regions. The intensity plots covering the region of 10 kb up- and downstream for cells treated with ATRA and MC2392 show acetylation increases following MC2392 treatment although to a lower level and after longer treatment times as compared to ATRA (Fig. 2D): a vivid increase is already apparent after 4h of ATRA treatment whereas a similar response amplitude is reach only after 48h of MC2392 treatment.

The normalized tag numbers (in treated and untreated samples) were counted for all binding regions and clustered using k-means. This analysis identified 20 clusters (Supplementary Fig. 1B), amongst which in particular cluster 2 and cluster 9 showed H3K9K14ac peaks that responded similarly to ATRA for 4h and 24h, and to MC2392 for 24h and 48h though with a strong timely delay (Fig. 3A). Moreover, Gene Ontology (GO) analysis revealed that the regulated genes in cluster 2 are functionally linked to leukocyte differentiation (Supplementary Fig. 1C). The overlap between H3K9K14ac peaks in cluster 2 and PML-RARα binding sites (33) revealed a common set of 191 loci suggesting that indeed a subset of H3K9K14ac peaks are associated with PML-RARα/RXR. The overlap between cluster 9 H3K9K14ac peaks and PML-RARα binding sites revealed a common set of 183 regions, suggesting that also here a subset of induced H3K9K14ac peaks are associated with PML-RARα/RXR (Fig. 3B, top).
Recent studies analyzing the genome-wide PML-RARα binding sites not only identified DR2 and DR5 elements as the primary PML-RARα response elements but also regions containing DR1, DR3 and DR4 motifs (33, 39). We found that both the DR1 and DR2 motifs are enriched within the cluster 2 and 9 binding sites (Fig. 3B, middle) whereas DR3, DR4 and DR5 motifs are not enriched. In addition, a de novo motif search identified the DR1 element in the 191 H3K9K14ac peaks of the cluster 2 that are regulated by MC2392 (Fig. 3B, bottom). This suggest that a proper configuration, i.e. spatial position of the PML-RARα with respect to the HDAC(s) in the repressive complex is an important prerequisite for MC2392 to physically bridge the RAR and HDAC(s) moieties and consequently cause inhibition of HDAC activity.

MC2392, but not ATRA, induces H3K9K14 acetylation at pro-apoptotic genes.

Our analysis also revealed cluster 8 consisting of 774 regulated genes that showed an opposite epigenetic response to MC2392 as compared to ATRA treatment (Fig. 3C) that is induction by MC2392 and reduction by ATRA. The assignment of genes in this cluster to biological functions (Fig. 3C, bottom) revealed enrichment for regulation of cell death. De novo motif search showed enrichment of the NF-E2-related factor-2 (NFE2L2, Nrf2) and Fos (AP1) response element each present at about 10% of H3K9K14ac peaks (Fig. 3D). Note that the stress-responsive transcription factors Nrf2 is reported to play a role in protection against oxidative stress-induced cellular damage. ChIP experiments coupled with qPCR showed enrichment of Nrf2 over specific responsive element (ARE) of target genes after MC2392 treatment, confirming an early Nrf2-mediated transcriptional response during oxidant-induced cell death, differently to ATRA which reduces the binding of Nrf2 to the ARE enhancer region (Supplementary Fig. 1D).

Finally, we found an increase in transcription in the target genes that encode antioxidant proteins or enzymes to buffer the intracellular redox activities, such as \textit{FTH1}, \textit{GCLM}, \textit{GCLC}, \textit{NQO} and \textit{HMOX, STK17A}. Conversely, ATRA inhibits/reduces the expression of these Nrf2 target genes (Supplementary Fig. 2A).
**MC2392 and ATRA induce expression of apoptotic genes at transcriptional level.**

To analyze the expression changes induced by MC2392, we performed transcriptome profiling on MC2392 and ATRA-treated NB4 cells. The gene expression values from RNA-seq data were calculated by quantifying the number of sequence tags for each genes using Genomatix (40, 41). Scatter plots show the general differences of gene expression of MC2392 versus ATRA after 24 and 48h (Supplementary Fig. 2B). Moreover, functional annotation clustering of differentially expressed genes by Gene Ontology (GO; PANTHER) analysis revealed that genes up-regulated in response to MC2392 are enriched for terms associated with protein transport, response to stress and apoptosis induction. Moreover ATRA up-regulated genes are enriched for terms associated mostly to protein transport and metabolic processes (Supplementary Fig. 2C). The hierarchical clustering analysis supports these data (Supplementary Fig. 2D).

Moreover, MC2392 down-regulated genes are enriched for terms associated with metabolic processes and pathway analysis showed the decreased expression of components that drive cell growth, proliferation and cell communication such as JAK/STAT and integrin pathway (data not shown).

Analysis of the transcription level of the 148 genes associated with apoptosis (http://www.reactome.org) revealed main differences between ATRA and MC2392 (Supplementary Fig. 2E and Supplementary Materials) such as the fast induction of TRAF2, E2F1, DYNLL2 and DBNL following MC2392 but not ATRA treatment. Next, we selected several MC2392-modulated genes related to apoptosis and oxidative stress, which are also modulated in the H3K914ac levels (Fig. 3 and Supplementary Fig. 1) upon MC2392 but not ATRA treatment and examined transcription factor binding. This revealed an increased occupancy of c-Jun, c-Fos and NRF2 in a time- and MC2392-dependent manner (Supplementary Fig. 3), corroborating and extending our observations that the apoptotic action of the MC2392 is causally bound to the modulation of cell death and redox pathways in response to modulation of AP1 and NRF2 bindings and...
their downstream pathways. Taken together the transcriptome and histone H3 acetylome analysis strongly point to a differential effect of MC2392 as opposed to ATRA on apoptotic genes.

**MC2392 induces cell death in APL cells.**

Genome-wide epigenetic and transcriptome analysis have provided strong indications that MC2392 effects are distinct from those elicited by ATRA in particular on apoptotic genes. Therefore, we assessed the activity of MC2392 on NB4 proliferation, cell cycle progression, granulocytic differentiation and apoptosis induction in comparison to MS-275, SAHA, and ATRA (Fig. 4). Interestingly, analysis of NB4 clonogenicity in semisolid media upon MC2392 treatment (Fig. 4A, left) revealed a clear loss of clonogenic activity at 10^{-5} M. In addition, two MC2392 analogues that differ for the position of the amino group at the anilide ring (position 2, 3 and 4 in MC2392, MC2678, and MC2677, respectively) (42) were used. The MC2678 and -2677 compounds do not display HDAC inhibition (data not shown) and MC2677 has been previously described to inhibit the growth of a number of tumor cell lines including prostate, head and neck, squamous carcinoma and neuroblastoma (42, 43). In contrast to MC2392 and MS-275, MC2678 and MC2677 are unable to induce a proliferation arrest of NB4 cells (Fig. 4A, right). MC2392 increased the pre-G1 fraction (Fig. 4B), a measure of cell death induction but did not induce major changes in the cell cycle phases (Fig. 4C). MC2392 did also induce an increase of the CD11c levels to 24%, thus inducing maturation of APL cells similar to ATRA, MC2677 and MC2678 (Fig. 4D) but the MC2678 and MC2677 compounds did not induce apoptosis (Fig. 4B).

Induction of cell death by MC2392 is not dependent on differentiation as MC2392 readily induces cell death but not differentiation of the ATRA-resistant NB4 subclone, NB4R4 (26, 27) (Fig. 4E-F). Note that as a result of the acquired mutation in the ligand-binding domain, PML-RARα can bind ATRA but it is thought that the binding does not induce the conformational change needed to dissociate the repressive complex and recruit activating proteins (26). Thus, the cell death activity of MC2392 acts independently from maturation and is likely linked to the HDAC inhibition of the
resident repressive complexes. Taken together, our analysis shows that MC2392 indeed acts as a weak retinoid in line with the transcriptome and histone H3 acetylome data but clearly has acquired additional properties or such that were ‘masked’ in ATRA and MS-275. Moreover, our results corroborate and extend the rapid response to the apoptosis/cell death versus slow differentiation in response to MC2392 treatment.

**MC2392-induced cell death involves ROS and caspase-8.**

To gain insight into the mechanism(s) by which MC2392 initiates cell death, caspases-8, -9 and -3/7 activities in NB4 cells were analyzed. Similarly to SAHA, MC2392 notably induced caspase activation with caspase-8, -9 (initiator caspases), caspase-3/7 (effector caspases) (Fig. 5A-B) and mitochondrial potential activated in NB4 cells (Fig. 5C), thus indicating that the cell death induced by MC2392 is caspase dependent. MS-275 induced cell death is caspase-8 dependent and related to TRAIL induction (22). In order to identify the molecular pathway we evaluated the levels of known players of apoptosis, such as FAS, TRAIL, FADD, caspase-8 and -3/7, Bcl2 and Bid in NB4 cells after 24h and 48h treatment. As shown in Fig. 5D and Supplementary Fig. 3A, MC2392 induced the extrinsic apoptotic pathway, increased the expression of FasL, Fas and TRAIL similarly to what has been reported for MS-275 (22). MC2392 also induced degradation of Bid into the apoptotic factor t-Bid, mitochondrial depolarization (Fig. 5D) and Bcl2 down-regulation (Supplementary Fig. 4B).

Next, NB4 cells were treated with MC2392 and H2O2 with and without the antioxidant N-acetylcysteine (NAC). The data suggest that MC2392 mediates a ROS-dependent cell death associated with the generation of reactive oxygen species (Fig. 5E and 5F). We further observed increased H2AX phosphorylation which is a hallmark of ongoing DNA damage as a result of increased reactive oxygen species (ROS) (44, 45). The response of APL cells to MC2392 includes the generation of ROS as a consequence of Bid cleavage, which triggers mitochondrial membrane permeabilization. As both caspase-2 and caspase-8 are capable of cleaving the pro-apoptotic Bid we set out to investigate whether caspase-2 or caspase-8 are responsible. MC2392 induced caspase-2
activation, which is fully blocked by the caspase-2 inhibitor Z-VDVAD-FMK (Fig. 6A). However, cell death mediated by MC2392 stayed unaltered (Fig. 6A, right). MC2392-mediated cell death is also not NOX-dependent, given that Apocynin - a NOX specific inhibitor - is not able to block apoptosis (Supplementary Fig. 3C). Furthermore, NF-kB is neither induced nor transcriptionally activated by MC2392 (Supplementary Fig. 4D-4E). In contrast, when the pan-caspase inhibitor ZVAD (data not shown) or the caspase-8 inhibitor IETD are used together with MC2392, NB4 cell death is fully abolished (Fig. 6B), strongly suggesting that the MC2392 mechanism of cell death is caspase 8-dependent. Surprisingly, necrostatin-1 (NEC-1), the known RIP1 kinase and necroptosis inhibitor, completely abrogated MC2392 cell death (Fig. 6C), thus suggesting a RIP1 mediated cell death. Consistent with these observations, analysis for RIPK1 expression showed both a strong up-regulation upon MC2392 and a full abrogation with NEC-1 (Fig. 6D). Finally, in line with our hypothesis, MC2392 induces cell death specifically in a PML-RARα but not in RARα or PML driven way (Fig. 4F and data not shown).

Taken together our data revealed that at the level of acetylation, MC2392 induces much fewer changes as compared to ATRA. Moreover, MS-275 resulted in genome wide dampening and broadening of all acetylation sites (Fig. 2A) whereas MC2392 affected acetylation only at a subset of PML-RARα sites (DR1-2 type). Finally, transcriptome analysis corroborated and extended that MC2392 affects apoptotic genes.

Discussion

Epigenetic modifications can be reversed by epi-drugs that are promising for antitumor therapy. In patients with leukemia, HDACi are widely used in combination with ATRA resulting in improved anti-tumorigenic activity. Here we identified and characterized the mechanism of action of MC2392, a compound merging the HDACi part of the well-known MS-275 to ATRA. When compared to both ATRA and MS-275, MC2392 displays similar as well as divergent characteristics. Despite being inactive as an HDACi standard in vitro biochemical assays, MC2392
specifically inhibits the HDACs contained in the PML-RARA repressive complex (Fig. 1D). MC2392 induces degradation of PML-RARA (Fig. 1E) and differentiation of NB4 cells (Fig. 4D). Genome-wide epigenetic analysis shows that MC2392 induces increased acetylation at a small subset of PML-RARA sites with preponderance for DR1 and -2 type (Fig. 3B) distinct from acetylation at PML-RARA sites induced by ATRA. This MC2392 characteristic is particularly interesting and classifies it as a novel class of drugs that need precise protein-protein ‘positioning’ to function. Moreover a specific configuration, i.e. spatial position of the PML-RARA with respect to the HDAC(s) in the repressive complex is likely an important prerequisite for MC2392 to bridge the RARA and HDAC(s) moieties and consequently inhibition of HDAC activity. The fact that MC2392 may represent the first RARA-driven HDACi is corroborated by i) its inactivity on human recombinant HDACs in vitro, ii) its inability to modify general histone and/or tubulin acetylation; iii) its selective HDACi action on PML-RARA complexes; iv) its enhanced ability to induce apoptosis in APL cells but not in other leukemia or solid cancer models. The fact that MC2392 induces apoptosis in NB4 cells and increases caspases-8, -2 and -9 activity suggests that MC2392 activate both mitochondrial and death receptor apoptotic pathways. Although MC2392 is able to activate the extrinsic pathway by inducing FAS and TRAIL, the death response to MC2392 includes also the generation of ROS as a possible consequence of the cleavage of Bid (Fig. 5D, F). Indeed, the cleavage of Bid in APL cells occurs concurrently with early generation of ROS, thereby contributing to cell death by promoting mitochondrial release of cytochrome c. For this reason, normally the death receptor pathway (extrinsic pathway) converges with the mitochondrial pathway (intrinsic pathway) through caspase-mediated cleavage and activation of Bid. Furthermore, both the induction of RIP1 by MC2392 and the abrogation of cell death operated by NEC-1, an inhibitor of RIP1, dictate a complex scenario in which, alternatively to apoptosis, necroptotic pathways may lead to mitochondrial depolarization and cell death of NB4 APL cells. Of note, both ATRA and MS-275 have been previously shown to function via TRAIL and FAS induction (16, 22). Moreover, the fact that the MC2392 caspase 8-dependent apoptosis can be
abrogated by NEC-1 supports a model in which mixed types of cell death can occur, displaying shared features of both necroptosis and apoptosis. Clearly, MC2392-induced apoptosis occurs in response to oxidative stress and is orchestrated by stress-responsive transcription factors. Nuclear translocation of Nrf2 and subsequent induction of its target genes via antioxidant stress response element (ARE) may function to buffer oxidative stress response. Moreover, MC2392 induces the modulation of a large number of stress-responsive genes (Supplementary Fig. 2A) and apoptotic genes (see Supplementary Materials and Methods). Finally, the evidence that apoptosis occurs in APL independently from an ‘classical’ ATRA response is suggested both by the MC2392 action in Zn-inducible PLZF-RARα expression in U937 cells (28, 29) as well as by its activity on NB4R4 cells (26, 27). Note that in both PLZF-RARα U937 cells and in NB4R4 cells ATRA signaling stays impaired upon treatment (27, 46, 47). Excitingly, given that MC2392 is not an HDACi in vitro, MC2392 is able to exert its HDAC inhibition only when driven by its ATRA moiety to PML-RARα and thereby co-localized to HDAC-containing repressive complexes (Fig. 4). In this way, MC2392 relieves the HDAC repressive complex and allows induction of cell death in ATRA responsive and in bona fide APL ATRA-resistant cells. Accordingly, MC2392 is not inducing apoptosis in solid tumor such as prostate or breast cancer (data not shown). Moreover, ChIP-seq profiles show that MC2392 induces changes in H3 acetylation at a small subset of PML-RARα/RXR binding regions but also in regions not regulated by ATRA (Fig. 2, 3). Unlike natural retinoids, MC2392 exerts distinct biological effects, inducing partial differentiation and promoting apoptosis, possibly similarly to As2O3. MC2392 displays a minimal or no cytotoxicity in other kind of tumor cells. Collectively these data underpin the specificity and selectivity of the MC2392. In conclusion, MC2392 represents the creation of a single chemical entity with dual action thus reflecting the concept of a laboratory-created ad hoc drug, which as a single drug can overcome resistance in cancer.

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References


Figure legends

Figure 1. MC2392 selectively inhibits the HDAC-repressive complexes associated to PML-RARα in NB4 cells. A, structure of MS-275, ATRA and MC2392. B, acetylation evaluation of histone H3
by SAHA and MC2392 at 5 μM; p21 induction by MS-275 and MC2392 at 5 μM in U937 cells. Histone H1 or ERKs were used for loading control. C, HDAC1-4 and SIRT1 assay in presence of the indicated compounds. SAHA and EX-527 were used at 5 μM, Resveratrol at 100 μM. Data are expressed as % of activity compared to control. Error bars represent standard deviations (SD) based on three independent experiments. D, HDAC assay after immunoprecipitation of PML-RARα in HeLa cells overexpressing PML-RARα. Data are expressed as % of activity compared to control, error bars represent SD based on three independent experiments. E, activation of RARE-3tkluc after transfection in HeLa cells treated as indicated. pMAX-EGFP co-transfection was used as an internal control, error bars represent ST based on three independent experiments. F, Western blot analyses for PML-RARα in nuclei extracts in NB4 cells treated with ATRA 1 μM and MC2392 5 μM. HDAC2 was used as loading control.

Figure 2. MC2392 selectively regulates acetylation at some PML-RARα binding sites in NB4 cells. A, genomic tracks of random regions displaying ChIP-seq data for H3K9K14ac in NB4 cells after 24h of treatment with MS-275, ATRA and MC2392. B, distribution of the H3K9K14ac enrichment. Locations are divided in gene body (gene), upstream near (-5 kb to the transcription start site), intergenic (upstream far from -25 to -5 kb of the transcription start site, downstream far, end of untranslated region +5 to +25 kb, and distant (everything else)), downstream near (end of untranslated region to +5 kb). C, overview of the TGM2, ICAM1, RARβ, PML-RARα/RXR binding site in NB4 cells. D, tag density maps depicting the pattern of H3K9K14ac occupancy around (+ 10 and –10 kb) the PML-RARα/RXR sites. The intensity plot indicates the level of H3K9K14ac occupancy (log2 of tag density; see scale on the top) in a 500-bp window. The position of one example (TGM2) is indicated.

Figure 3. A proper PML-RARα spatial position is a prerequisite for MC2392 mediated HDAC inhibition at PML-RARα binding sites in NB4 cells. A, k-means clustering using a Pearson correlation metric and the log2 value of the tag density. Cluster 2 and cluster 9 of H3K9K14ac changes with typical and similar epigenetic responses to ATRA and to MC2392. The number of
genes in each cluster is indicated. B, Venn diagram representing the overlap of cluster 2 or 9 regulated genes and PML-RARα/RXR binding sites; analysis of the presence of DR motifs underlying the overlapped genes; response element for PPAR-RXRα identified as the predominant motif in the 191 genes of the cluster 2. Motif was identified by a de novo motif search. C, Cluster 8 of H3K9K14ac changes with different epigenetic responses to ATRA and to MC2392. The number of genes is indicated. Functional annotation clustering (GO) of the clustered genes. D, Response element for NFE2L2, FOS, AP1 identified like predominant motifs in the H3K9K14ac binding sites of the regulated genes of Cluster 8. Motifs were identified by a de novo motif search.

Figure 4. MC2392 induces PML-RARα-context specific apoptosis in APL cells. A, clonogenity assay in NB4 cells upon MC2392; proliferation curves in NB4 cells at the indicated time. B, pre-G1 fraction analysis of NB4 cells treated for 48h with the indicated compounds. C, cell cycle analysis of NB4 cells treated for 48h with the indicated compounds. MS-275, SAHA, MC2677 and MC2678 were used at 5 μM; ATRA was used at 1 μM. D, granulocytic differentiation in NB4 by the CD11c positive cells. PI positive cells have been excluded from the analysis. E, granulocytic differentiation by the CD11c positive cells in NB4R4 cells. PI positive cells, have been excluded from the analysis; pre-G1 fraction in NB4R4 cells treated with the indicated compounds. F, analysis of the pre-G1 fraction in U937 PML-RARα and PLZF-RARα cells after 24h and 48h of treatment with the indicated compounds in presence or absence of zinc. All experiments have been performed at least three times and error bars represent SD based on three independent experiments.

Figure 5. MC2392 leads to caspase activation and ROS production in NB4 cells. A, B, Caspase-8, -9 and -3/7 assay in NB4 cells treated 48h with the indicated compounds. C, mitochondrial membrane potential dissipation of NB4 treated with MS-275, SAHA and MC2392 used at 5 μM. D, Western blot analysis of apoptosis players in NB4 cells treated with ATRA and MC2392 for 24h and 48h. ERKs and tubulin have been used as control for equal protein loading. E, pre-G1 fraction in NB4 cells treated with the indicated compounds. F, analysis of ROS production in conditions as
in A. NAC was used 20 mM and H2O2 500 μM; Western blot analysis for phosphoH2AX(S139). Error bars shown in A, B, C, E, F represent SD based on three independent experiments.

Figure 6. MC2392-mediated apoptosis is caspase 8 dependent. A, Caspase-2 activity on NB4 cells treated for 48h with the indicated compounds; pre-G1 pick detection in identical settings; the caspase-2 inhibitor VDVAD was used at 100 μM. B, Caspase-8 activity on NB4 cells treated for 48h with the indicated compounds; pre-G1 pick detection in identical settings. The caspase-8 inhibitor IETD was used at 100 μM. C, pre-G1 fraction in NB4 cells treated with the indicated compounds; NEC-1 was used at 50 μM, D, Western blot analysis on RIP1 after 24h and 48h of treatment with the indicated compounds. Blot against ERKs represents control for equal protein loading. Error bars shown in A, B, C, represent SD based on three independent experiments.
Figure 2: 

A) Scale chr3: CpGIslands

CpG Islands (Islands < 300 Bases are Light Green)

UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics

CDCP1
CDCP1
TMEM158
LARS2
LARS2
LIMD1
AK026206
KIAA0851
SACM1L
SACM1L
SLC6A20
SLC6A20
LZTFL1
LZTFL1
CCR9
CCR9
CCR9
CCR9
CC3R
CC3R
CC3R
ATRA
MS_275
DMSO
MC2392
H3K9K14ac

B) Pie chart showing gene body, intergenic, downstream near, and upstream near regions.

- Gene body: 34.19%
- Intergenic: 52.11%
- Downstream near: 8.74%
- Upstream near: 4.94%

C) Diagram showing H3K9K14ac occupancy in PML-RARα/RXR binding sites.

D) Heatmap showing tag density with adjacent genes.

Tag density

0 220kb

H3K9K14ac occupancy in PML-RARα/RXR binding sites

TGM2

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De Bellis et al., Figure 6

(A) Graph showing caspase 2 activation (fold) and percent cells in pre-G1 for various treatments: Ctrl, SAHA, MC2392, Z-VDVAD, VDVAD+MC2392, Z-VDVAD, VDVAD+MC2392. The x-axis represents the treatments and the y-axis represents the caspase 2 activation fold and percent cells in pre-G1.

(B) Graph showing caspase 8 activation (fold) and percent cells in pre-G1 for various treatments: Ctrl, MC2392, SAHA, IETD, IETD+MC2392. The x-axis represents the treatments and the y-axis represents the caspase 8 activation fold and percent cells in pre-G1.

(C) Graph showing percent cells in pre-G1 for various treatments: Ctrl, MC2392, nec-1, MC2392+nec-1. The x-axis represents the treatments and the y-axis represents the percent cells in pre-G1.

(D) Western blot analysis showing RIPK1 and ERK1 expression levels over time (h): Ctr, MC2392, MC2392+nec-1. The x-axis represents the treatments and the y-axis represents the expression levels. The blots show bands for RIPK1 and ERK1 at different time points (48, 24 h) for each treatment.
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