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Increased MDR1 Transporter Expression in Human Brain Endothelial Cells Through Enhanced Histone Acetylation and Activation of Aryl Hydrocarbon Receptor Signaling

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Abstract

Multidrug resistance protein 1 (MDR1, *ABCB1*, P-glycoprotein) is a critical efflux transporter that extrudes chemicals from the blood–brain barrier (BBB) and limits neuronal exposure to xenobiotics. Prior studies in malignant cells demonstrated that MDR1 expression can be altered by inhibition of histone deacetylases (HDAC), enzymes that modify histone structure and influence transcription factor binding to DNA. Here, we sought to identify the mechanisms responsible for the up-regulation of MDR1 by HDAC inhibitors in human BBB cells. Immortalized human brain capillary endothelial (hCMEC/D3) cells were treated with HDAC inhibitors and assessed for MDR1 expression and function. Of the HDAC inhibitors profiled, valproic acid (VPA), apicidin, and suberoylanilide hydroxamic acid (SAHA) increased MDR1 mRNA and protein levels by 30–200%, which corresponded with reduced intracellular accumulation of the MDR1 substrate rhodamine 123. Interestingly, induction of MDR1 mRNA by HDAC inhibitors mirrored increases in the expression of the aryl hydrocarbon receptor (AHR) and its target gene cytochrome P450 1A1. To explore the role of AHR in HDAC inhibitor-mediated regulation of MDR1, a pharmacological activator (β-naphthoflavone, βNF) and inhibitor (CH-223191, CH) of AHR were tested. The induction of MDR1 in cells treated with SAHA was amplified by βNF and attenuated by CH. Furthermore, SAHA increased the binding of acetylated histone H3K9/K14 and AHR proteins to regions of the *MDR1* promoter that contain AHR response elements. In conclusion, HDAC inhibitors up-regulate the expression and activity of the MDR1 transporter in human brain endothelial cells by increasing histone acetylation and facilitating AHR binding at the *MDR1* promoter.

Keywords MDR1 · HDAC · Transport · Blood–brain barrier · Aryl hydrocarbon receptor

Introduction

The exchange of xenobiotics between the circulation and brain is restricted by the blood–brain barrier (BBB). The BBB possesses tight junctions, few fenestrations, and efflux transporters that work in a coordinated fashion to limit the entry of chemicals into the brain. In particular, the multidrug resistance protein 1 (MDR1, *ABCB1*, P-glycoprotein) is a critical efflux transporter located at the apical surface of capillary endothelial cells that limits the accumulation of xenobiotics in the brain [1, 2]. MDR1 transports structurally diverse chemicals including anticancer agents, pesticides, antipsychotic drugs, and analgesics [3, 4]. As a result, MDR1 plays a critical role in determining the efficacy or toxicity of chemicals in the brain [5, 6]. For example, brain concentrations of the MDR1 substrate, morphine, are elevated in knockout mice lacking the *Mdr1a/1b* ortholog genes [6]. Similarly, in humans, a genetic polymorphism in *MDR1* that results in transporter loss-of-function has been associated with more

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significant adverse events following treatment with morphine [5]. Consequently, MDR1 is important in regulating xenobiotic disposition and responses in the brain.

The expression of MDR1 is tightly controlled through multiple transcriptional and translational mechanisms. The *MDR1* promoter contains multiple response elements that can interact with a variety of transcription factors. Nuclear transcription factor Y (NF-Y), Sp1, and Sp3 interact with response elements, including an inverted CCAAT box (Y-box) and GC boxes, in the *MDR1* promoter. Likewise, response elements for xenobiotic-activated transcription factors, such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AHR), are located near the transcriptional start site of the *MDR1* gene [7–18]. Collectively, multiple signaling pathways work in a coordinated fashion to control the basal and inducible expression of MDR1 in the BBB.

Recently, histone acetylation has gained attention as a potential epigenetic mechanism for regulating MDR1 transcription. The acetylation of histones loosens their interaction with DNA by neutralizing the positive charge in tail regions and reducing affinity to the negatively charged DNA. As a result, histones as well as transcription factors gain greater access to DNA often resulting in the activation of gene expression [19–21]. Histone deacetylases (HDACs) control the acetylation status of histones [22, 23]. There are four classes of HDACs: class I (HDACs 1, 2, 3, 8), class IIA (HDACs 4, 5, 7, 9) and IIB (HDACs 6, 10), and class IV (HDAC 11), which are Zn^{2+}-dependent enzymes, and class III, which are Zn^{2+}-independent sirtuin enzymes [24–26]. Inhibitors of HDAC enzymes fall into different chemical classes as outlined in Table 1 [25, 27]. HDAC inhibitors, including valproic acid (VPA), suberoylanilide hydroxamic acid (SAHA; Zolinza®), and romidepsin (Istodax®), have been approved by the US FDA for a variety of clinical indications, including the treatment of seizure disorders (VPA) and cancer (SAHA and romidepsin) [28–31]. One consequence of HDAC inhibition is altered expression and/or activity of the MDR1 transporter in cancer cells [13, 32–34]. Treatment of H69WT human small cell lung carcinoma cells with trichostatin A (TSA, 330 nM) increased the mRNA expression of MDR1 as well as the binding of acetylated histone H3 and H4 proteins at the *MDR1* promoter [33]. Furthermore, genetic knockdown of HDACs 1 and 2 using siRNA in cancer cells also enhanced MDR1 expression [34]. Moreover, recent studies showed that SAHA and TSA could induce MDR1 in human choriocarcinoma cells via HDAC2 inhibition [32, 35]. Collectively, these studies in cancer cells point to an epigenetic mechanism for regulating MDR1 expression through modulation of histone acetylation.

To date, no studies have systematically addressed the ability of HDAC inhibitors to regulate MDR1 expression and activity in the BBB. Therefore, the purpose of this study was to evaluate the effects of six different HDAC inhibitors, VPA, sodium butyrate (NaB), romidepsin, apicidin, SAHA, and TSA, on the expression and functional activity of the MDR1 transporter in an in vitro model of the human BBB. These six HDAC inhibitors were selected based on differences in chemical structure and HDAC targets (Table 1). We hypothesized that disruption of HDAC activity in human brain endothelial cells using pharmacological inhibitors would increase histone acetylation and up-regulate MDR1 expression and function and that modulation of transcriptional regulators, in particular AHR, could be responsible for transporter induction.

Materials and Methods

Chemicals and Reagents

VPA, NaB, apicidin, and SAHA were purchased from Sigma-Aldrich (St. Louis, MO). Romidepsin and TSA were purchased from Selleck Chemicals (Houston, TX) and Wako Chemicals USA, Inc. (Richmond, VA), respectively. All other chemicals were purchased from Sigma-Aldrich unless otherwise specified.

hCMEC/D3 Cell Culture

Human brain capillary endothelial cells (hCMEC/D3), obtained from Dr. Babette Weksler [36–38], were grown in EBM-2 basal medium (Lonza, Inc., Walkersville, MD) supplemented

Table 1 Classification of HDAC inhibitors [25]

HDAC inhibitor class	HDAC targets	Example compounds	Potency range[a]
Short-chain fatty acids	Classes I and IIA	• Valproic acid (VPA) • Sodium butyrate (NaB)	mM
Cyclic peptides	Class I	• Romidepsin (Romi) • Apicidin (Api)	nM
Hydroxamic acids	Classes I and II	• Suberoylanilide hydroxamic acid (SAHA) • Trichostatin A (TSA)	nM–μM

[a] This potency range represents general IC_{50} values (50% inhibitory concentrations) for purified HDAC proteins as determined by HDAC activity assays.

with 5% characterized fetal bovine serum (GE Healthcare Life Sciences, Logan, UT), 1% penicillin–streptomycin (Life Technologies, Carlsbad, CA), 1.4 μM hydrocortisone, 5 μg/mL ascorbic acid, 1% chemically defined lipid concentrate (Life Technologies), 10 mM HEPES (Life Technologies), and 1 ng/mL basic fibroblast growth factor. Cell culture dishes and plates were coated with rat collagen I (Trevigen, Gaithersburg, MD) at 5 μg/cm^2. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

Cell Viability Assay

To test the cytotoxicity of HDAC inhibitors, hCMEC/D3 cells were seeded in 96-well plates at a density of 8000 cells per well, allowed to attach overnight, and then treated for 24 h with increasing concentrations of one of following treatments (n = 5–10): vehicle, VPA, NaB, apicidin, romidepsin, SAHA, or TSA. The vehicles consisted of sterile, distilled water for VPA and NaB and dimethyl sulfoxide (DMSO) for other chemicals. The final concentration of DMSO was 0.1%. Cell viability was measured using the AlamarBlue® assay (Life Technologies) according to the manufacturer's protocol.

Cell Treatments

To screen a panel of HDAC inhibitors for their ability to regulate MDR1 expression, hCMEC/D3 cells were seeded on six-well plates at a density of 250,000 cells per well. On the following day, cells were incubated in media containing one of the following treatments (n = 3–6): vehicle, 5 mM VPA, 0.25 mM NaB, 1 nM romidepsin, 0.5 μM apicidin, 10 μM SAHA, or 0.25 μM TSA. For select HDAC inhibitors, concentration–response experiments were also performed. After 12 and 24 h of treatment, cells were collected and further processed to isolate total RNA and protein as described below.

To assess the effects of the AHR modulators, hCMEC/D3 cells were seeded on six-well plates at a density of 350,000 cells per well. On the following day, cells (n = 3–4) were incubated in media containing one of the following treatments: vehicle (DMSO), 5 μM β-naphthoflavone (βNF, AHR activator), 5 μM CH-223191 (CH, AHR inhibitor), or 10 μM SAHA (HDAC inhibitor) in the presence or absence of either 5 μM βNF or 5 μM CH. Total RNA was isolated after 6- and 12-h treatments whereas protein was obtained after 24- and 36-h treatments.

RNA Extraction, Reverse Transcription, and Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated from the cells using RNAzol® RT reagent (Sigma-Aldrich) following the manufacturer's instructions. RNA purity and concentration were assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL). Complimentary DNA (cDNA) was obtained from total RNA using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA). Expression of *MDR1 (ABCB1)*, *BCRP (ABCG2)*, *MRPs (ABCCs)*, *AHR*, *CYP1A1*, and different *HDAC* genes was analyzed by qPCR. Specific forward and reverse primers (Integrated DNA Technologies, Coralville, IA) for each gene were added to 1 μg of cDNA from each sample, and then amplified products were detected using SYBR Green (Applied Biosystems). Sequences of the primers are listed in Supplemental Table 1. qPCR was performed in a 384-well plate format using the ViiA™ 7 real-time PCR instrument (Applied Biosystems). Ct values were converted to delta delta Ct values by comparing to beta$_2$-microglobulin (β2M), which was used as a reference gene.

Western Blot Analysis

Vehicle- and chemical-treated hCMEC/D3 cells were lysed in cell lysis buffer containing 20 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1% protease inhibitor cocktail, and then transferred to microcentrifuge tubes. Tubes were centrifuged for 10 min at 500 *g* and supernatants collected. Protein concentrations were determined using the Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Cell lysates (20 μg protein/well) were loaded on NuPAGE™ 4–12% Bis-Tris Midi Gel (Life Technologies) and then separated by SDS-PAGE electrophoresis. Proteins were then transferred overnight at 4 °C to Immobilon®-FL polyvinylidene fluoride transfer membranes (Millipore, Billerica, MA). Membranes were blocked for 1 h in 5% non-fat dry milk in phosphate-buffer saline (PBS) with 0.5% Tween-20 (PBS-T). Blocking was followed by incubation at room temperature for 3 h with the following primary antibodies diluted in 2% non-fat dry milk at 1:1000 concentration: MDR1 (C219, NB600-1036; Novus Biologicals, Littleton, CO), BCRP (BXP-53, ALX-801-036; Enzo Life Sciences, Inc., Farmingdale, NY), acetylated histone H3 lysine residues 9 and 14 (H3K9/14) (9677S; Cell Signaling Technology, Danvers, MA), and alpha-tubulin (T6199; Sigma-Aldrich). After washing in PBS-T, membranes were incubated with species-appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich). Proteins were detected using SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific) and a FluorChem E imager (Protein Simple, Santa Clara, CA). Protein expression was semi-quantified using AlphaView SA version 3.4.0 (Protein Simple) and normalized to alpha-tubulin.

Rhodamine 123 Accumulation Assay

Cells were treated with vehicle, VPA (5 mM), apicidin (0.5 μM), or SAHA (10 μM) for 24 h and then re-seeded in

a 96-well plate to undergo transporter efflux assays as described in a previous protocol [39]. Briefly, hCMEC/D3 cells ($n = 4$) were incubated for 30 min in medium containing the MDR1 fluorescent substrate rhodamine 123 (7.5 μM) in the presence or absence of the functional inhibitor, verapamil (100 μM) (*uptake phase*). Cells were then washed, centrifuged, resuspended, and incubated for 2 h in substrate-free medium in the presence or absence of the inhibitors (*efflux phase*). At the end of the efflux phase, cells were washed and re-suspended in cold PBS and evaluated for retention of rhodamine 123. Intracellular fluorescence intensity was quantified in relative fluorescence units (RFU) using the Cellometer Vision cell counter (Nexcelom Bioscience LLC, Lawrence, MA) with the filter cube VB-595-502 (excitation/emission, 525 nm/595 nm). The average for each treatment group was determined by the average fluorescence values of four independent samples.

Nuclear and Cytoplasmic Protein Extraction

Cells were seeded on 100 mm dishes at a density of 2,000,000 cells per dish and allowed to attach for 24 h. On the following day, cells were incubated in media containing vehicle or SAHA (10 μM). After 24 h, cells were harvested with trypsin-EDTA (Life Technologies) and centrifuged to obtain a cell pellet. The pellet was washed with PBS and underwent nuclear and cytoplasmic extraction using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Protein concentrations of the extracts were determined using BCA protein assay kit (Thermo Scientific), and western blot analysis was conducted as described previously with the following primary antibodies at 1:1000 concentration: MDR1 (C219, NB600-1036; Novus Biologicals), AHR (D5S6H, 83200S; Cell Signaling Technology), histone H3 (D2B12, 4620S; Cell Signaling Technology), and acetylated histone H3K9/K14 (9677S; Cell Signaling Technology).

Chromatin Immunoprecipitation (ChIP)-qPCR

Cells were seeded on 150-mm dishes at a density of 5,000,000 cells per dish and allowed to attach and grow for 24 h. On the following day, cells were incubated in the media containing vehicle or SAHA (10 μM). After 24 h, cells were washed, and ChIP was performed using SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads) (Cell Signaling Technology) according to the manufacturer's protocol. Briefly, cells were crosslinked in 1% formaldehyde for 10 min and the crosslinking was stopped with 0.125 M glycine. Crosslinked cells were washed and lysed to isolate nuclear pellets. Pelleted nuclei were digested by micrococcal nuclease to obtain DNA fragments of 150 to 600 bp, as confirmed by agarose gel electrophoresis (Supplemental Fig. 3). Immunoprecipitation was performed using the following antibodies: acetylated histone H3K9/H3K14 (1:50, 9677S; Cell Signaling Technology), AHR (1:50, D5S6H, 83200S; Cell Signaling Technology), histone H3 (1:50, D2B12, 4620S; Cell Signaling Technology), or normal rabbit IgG (1:333, 2729S; Cell Signaling Technology) which served as a negative control. Immunoprecipitated DNA was purified and quantified by qPCR using specific primers for GAPDH exon 1 (5516S; Cell Signaling Technology), α-satellite repeats (4486S; Cell Signaling Technology), and proximal regions of the *MDR1*/*ABCB1* promoter (P1, P2, P3, P4, and P5). Sequences of the primers are listed in Supplemental Table 1. Amplified products were detected using SYBR Green (Applied Biosystems). Fold enrichment of the immunoprecipitation was calculated by comparing the PCR products of the immunoprecipitated samples to values obtained for input DNA.

Statistical Analysis

GraphPad Prism v5© was used for statistical analysis (GraphPad Software, La Jolla, CA). Differences between groups were compared using a two-tailed Student's *t* test, one-way analysis of variance (ANOVA), or two-way ANOVA as appropriate for the number of comparisons and variables. Post hoc Tukey's and Bonferroni tests were performed for one-way and two-way ANOVA, respectively. Correlations and *R* values were calculated using two-tailed Pearson's correlation test. Statistical significance was set at $p < 0.05$.

Results

Viability of hCMEC/D3 Cells after Treatment with HDAC Inhibitors

The ability of HDAC inhibitors to alter hCMEC/D3 cell viability was evaluated using the AlamarBlue® assay. hCMEC/D3 cells were treated with increasing concentrations of each HDAC inhibitor for 24 h (Supplemental Fig. 1). Ranges of HDAC inhibitor concentrations were selected based on previous in vitro studies [6, 33, 34, 40–45]. For all HDAC inhibitors, the highest concentration that did not reduce cell viability or increase cell detachment was selected to perform further experiments. Therefore, 5 mM VPA, 0.25 mM NaB, 1 nM romidepsin, 0.5 μM apicidin, 10 μM SAHA, and 0.25 μM TSA were used for subsequent studies.

MDR1 Transporter Expression Is Increased after HDAC Inhibition

To determine whether brain endothelial MDR1 expression was altered by HDAC inhibition, hCMEC/D3 cells were treated with one of six different HDAC inhibitors and analyzed for

changes in the mRNA expression of MDR1 at 12 and 24 h (Fig. 1a). At 12 h, four HDAC inhibitors (VPA, apicidin, SAHA, and TSA) increased MDR1 mRNA levels between 30% and 200%. Up-regulation of MDR1 mRNA by HDAC inhibitors was generally attenuated by 24 h, with the exception of apicidin. Western blotting revealed that HDAC inhibitors increased acetylated histone H3K9/14 protein levels by up to 330%, which confirmed the ability of the majority of chemicals to modify histone protein status. Similar to mRNA expression, inhibition of HDACs by VPA, apicidin, TSA, and SAHA for 24 h up-regulated MDR1 protein expression (Fig. 1b).

HDAC inhibitors were also shown to alter the mRNA levels of other BBB transporters (Supplemental Fig. 2). For example, breast cancer resistance protein (BCRP, *ABCG2*) mRNA expression was significantly induced by all HDAC inhibitors at both 12 and 24 h by up to 220%. In contrast, the mRNA expression of other efflux transporters including multidrug resistance-associated proteins (MRPs, *ABCC*s) 1, 3, and 4 was moderately down-regulated after exposure to HDAC inhibitors for 12 h and/or 24 h.

Enhanced MDR1 Expression Leads to Increased Functional Activity Following HDAC Inhibition

To determine whether enhanced MDR1 protein expression corresponded with increases in transporter activity, a fluorescent dye accumulation assay was performed. hCMEC/D3 cells were treated with vehicle, VPA, apicidin, or SAHA for 24 h and then evaluated for the intracellular accumulation of the fluorescent substrate, rhodamine 123 (Fig. 1c). Cells treated with VPA, apicidin, and SAHA exhibited reduced accumulation of rhodamine by 20% to 30%, indicating increased efflux function by MDR1. This enhanced activity was reversed by co-incubation with verapamil, a specific MDR1 inhibitor [46]. These data indicate that the reduced retention of rhodamine 123 after 24-h exposure to VPA, apicidin, and SAHA was due to the up-regulation of MDR1 expression.

MDR1 Up-Regulation Can Be Achieved at Lower Concentrations of HDAC Inhibitors

To determine whether lower concentrations of HDAC inhibitors could also alter MDR1 expression, hCMEC/D3 cells were treated with vehicle, VPA (1, 2, 5 mM), apicidin (0.1, 0.25, 0.5 μM), or SAHA (2.5, 5, 10 μM) and analyzed for MDR1 mRNA and protein levels at 12 and 24 h, respectively. Even the lowest tested concentrations of apicidin and SAHA significantly increased MDR1 mRNA (Fig. 2a). Interestingly, the magnitude of induction was largely similar at each concentration of apicidin or SAHA. On the other hand, the magnitude of MDR1 mRNA up-regulation by VPA was concentration dependent. Similar to mRNA expression, the protein levels of MDR1 were similarly induced by 24 h at all tested

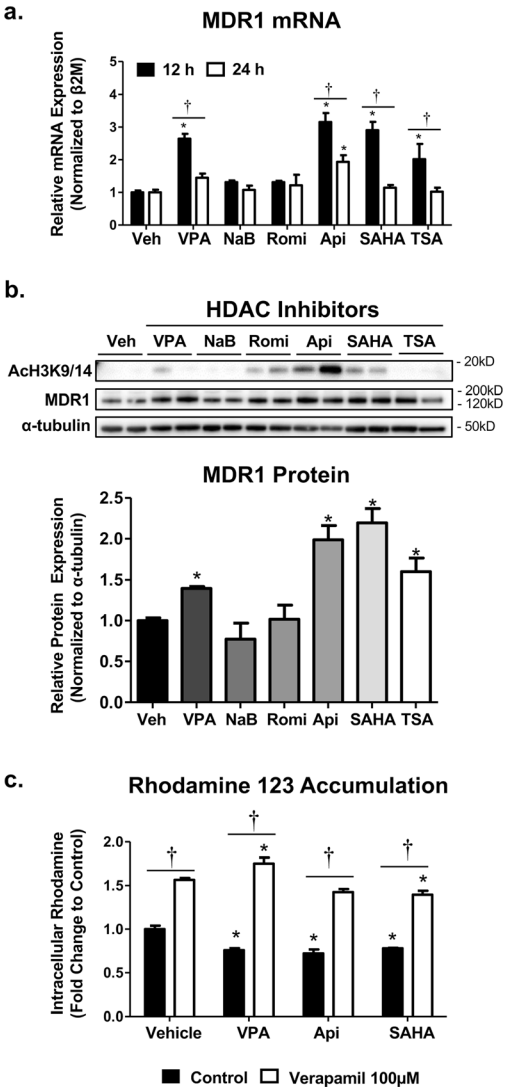


Fig. 1 MDR1 expression and function in human brain microvascular endothelial cells treated with HDAC inhibitors. (**a**) hCMEC/D3 cells (n = 3–6) were treated with vehicle (veh) or six HDAC inhibitors (5 mM VPA, 0.25 mM NaB, 1 nM romidepsin (Romi), 0.5 μM apicidin (Api), 10 μM SAHA, or 0.25 μM TSA) for 12 h (black) or 24 h (white) and analyzed for mRNA expression of MDR1. Data were normalized to beta$_2$-microglobulin and presented as mean ± SEM. Data were analyzed by one-way ANOVA between treatments within each time point (*), and by two-way ANOVA to compare between the two time points (†), with statistical significance at $p < 0.05$. (**b**) hCMEC/D3 cells (n = 3–4) were treated with vehicle or six HDAC inhibitors for 24 h and analyzed for protein expression of acetylated histone H3K9/14 or MDR1 by western blot analysis followed by densitometry to semi-quantify protein levels. Alpha-tubulin (α-tubulin) was used as a loading control. Data are presented as mean ± SEM and analyzed by one-way ANOVA. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle- and HDAC inhibitor-treated cells. (**c**) MDR1 function after exposure to HDAC inhibitors was assessed by measuring the cellular accumulation of a fluorescent MDR1 substrate, rhodamine 123 (7.5 μM), in the presence or absence of the MDR1 inhibitor, verapamil (100 μM), using the Nexcelom Cellometer Vision (n = 4). Intracellular fluorescence was quantified as mean relative fluorescence intensity. The bar graph is presented with mean ± SEM as analyzed by two-way ANOVA to compare each treatment group to vehicle (*) and within each treatment group (†), with statistical significance at $p < 0.05$

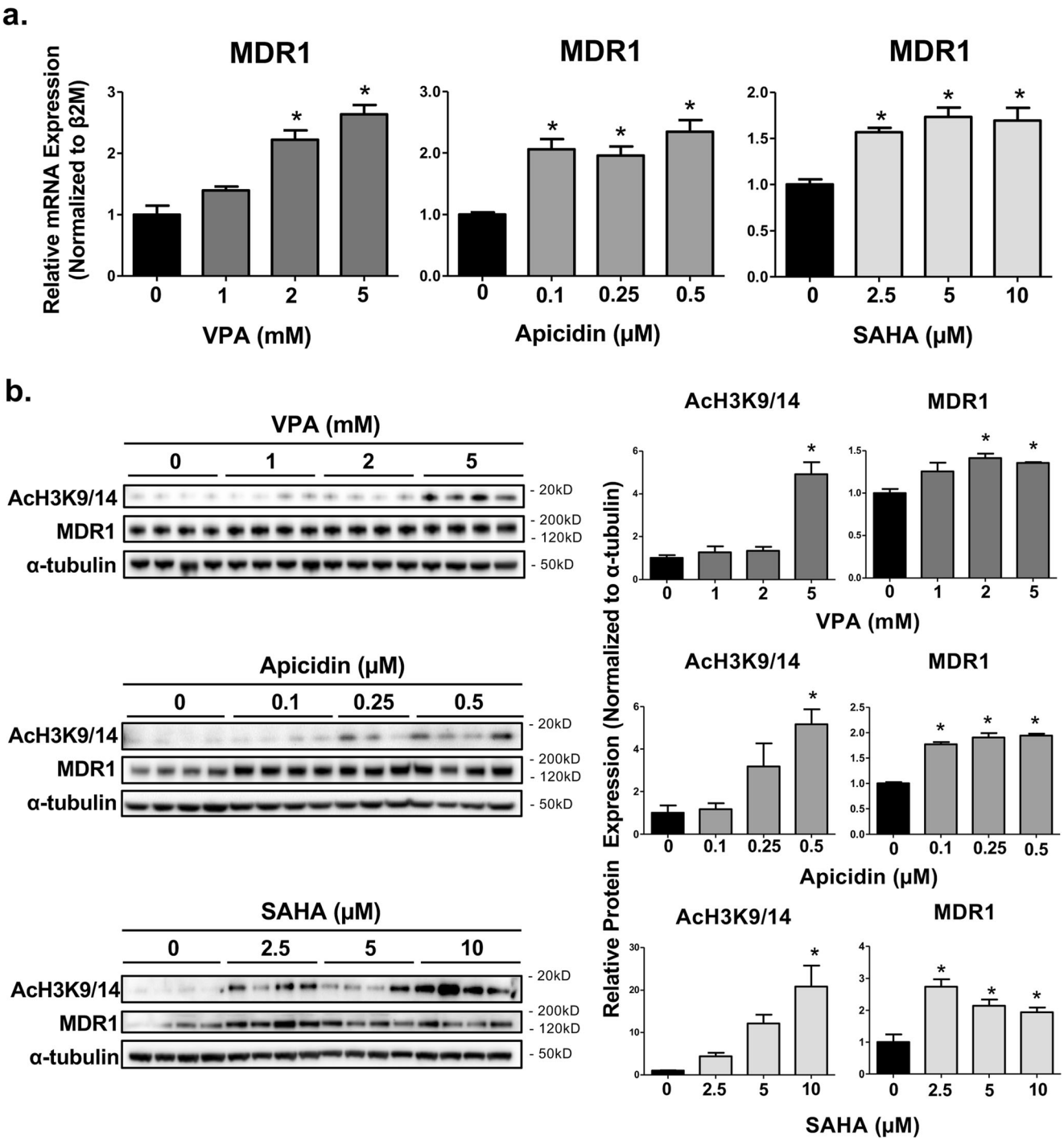


Fig. 2 Concentration-dependent regulation of MDR1 expression in human brain microvascular endothelial cells treated with HDAC inhibitors. hCMEC/D3 cells ($n = 3$–6) were treated with vehicle or increasing concentrations of VPA, apicidin, or SAHA for 12 h and 24 h and analyzed for MDR1 mRNA (**a**) and protein (**b**), respectively. Data were normalized to beta$_2$-microglobulin for mRNA and alpha-tubulin (α-tubulin) for protein, and presented as mean ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle- and HDAC inhibitor-treated cells

concentrations of VPA, apicidin, and SAHA (Fig. 2b). While the magnitude of MDR1 protein up-regulation was largely similar across all concentrations, the extent of total histone H3 acetylation by VPA, apicidin, and SAHA increased in a concentration-dependent manner.

SAHA Increases Histone Acetylation at the MDR1 Promoter

SAHA was selected as a prototypical HDAC inhibitor for use in subsequent studies that investigated mechanisms

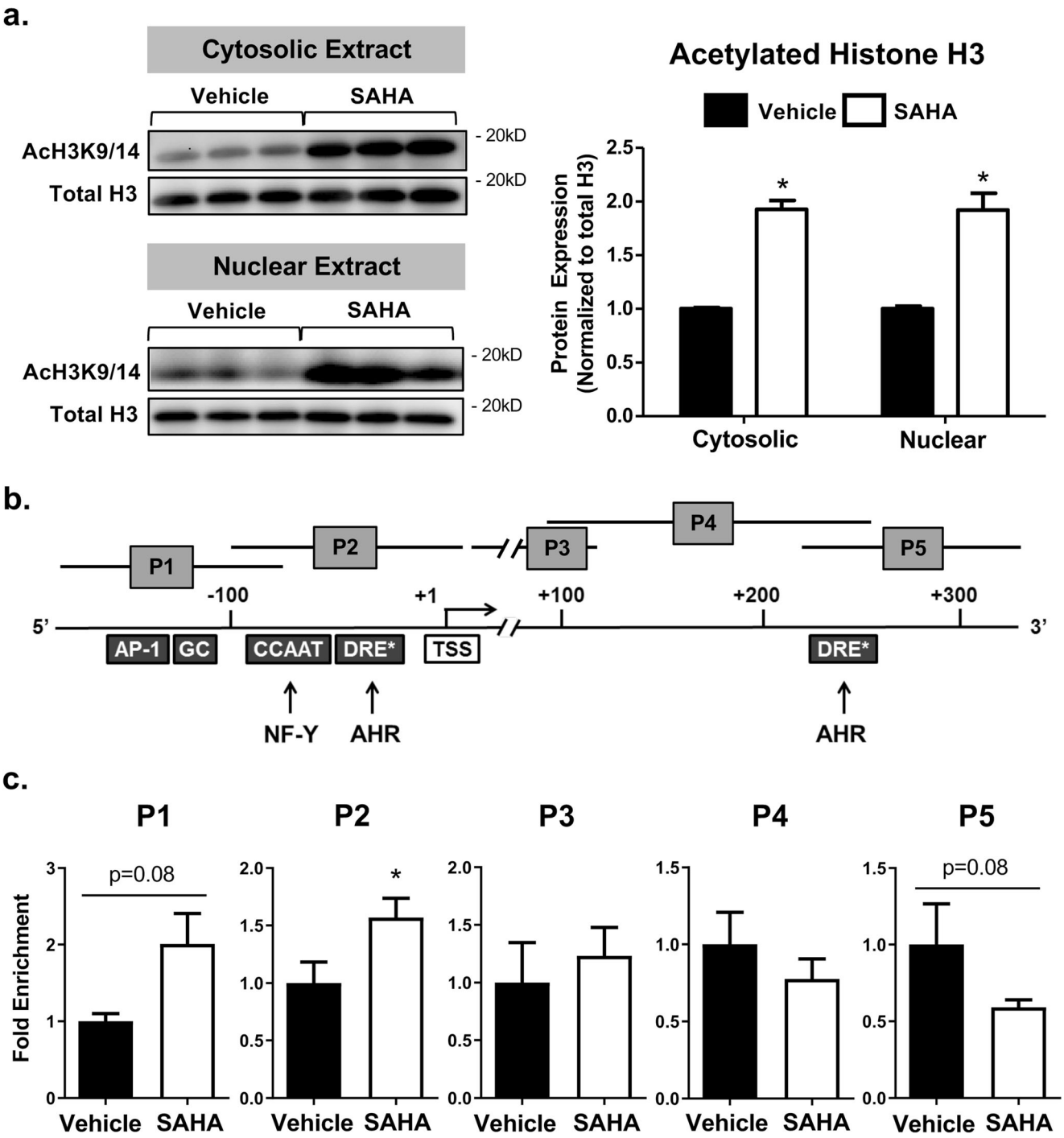
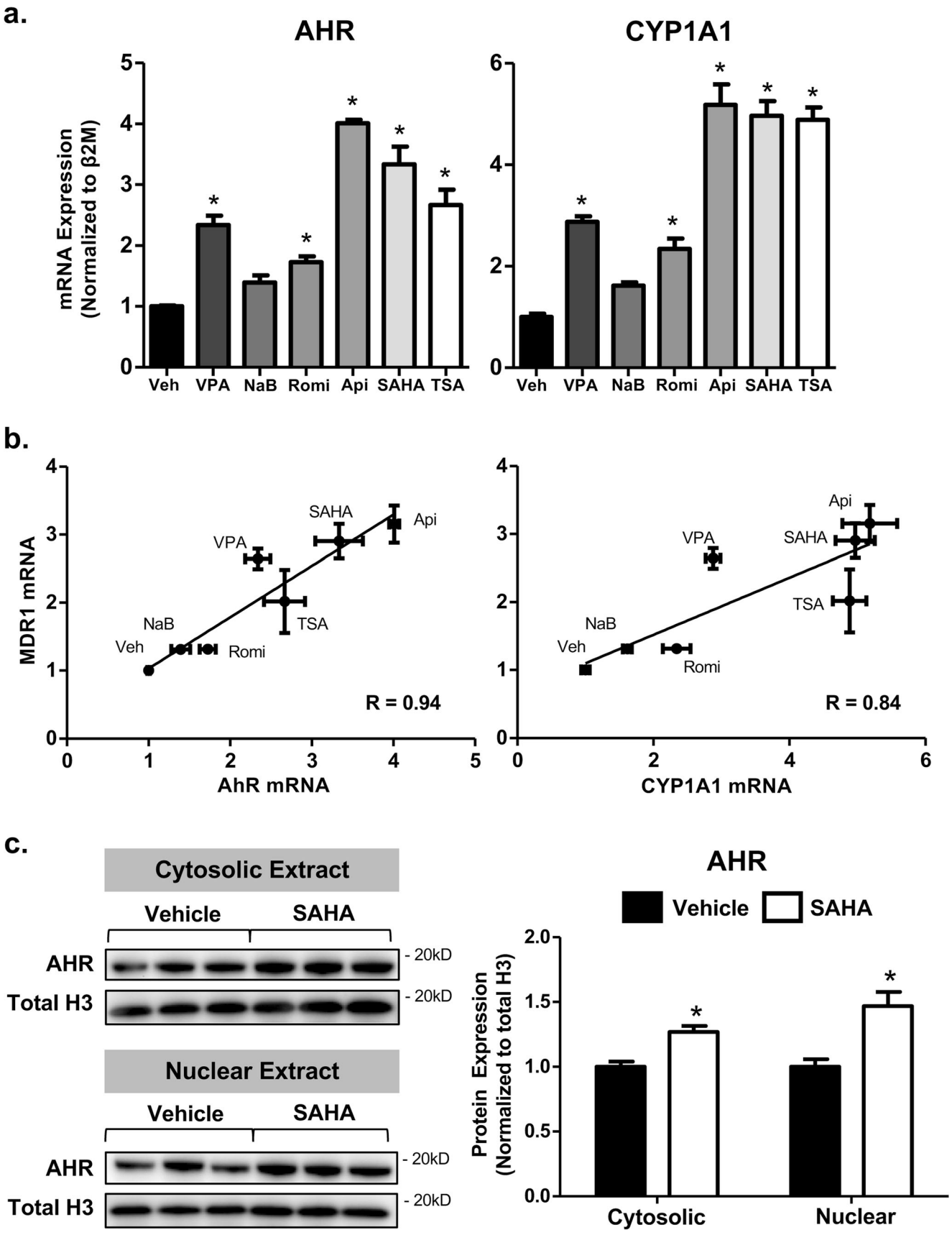


Fig. 3 The enrichment of acetylated histone H3 proteins and DNA binding in human brain microvascular endothelial cells treated with SAHA. (**a**) hCMEC/D3 cells ($n = 3–10$) were treated with vehicle or 10 μM SAHA for 24 h and cytosolic and nuclear extracts were collected. The relative protein expression of acetylated histone H3K9/14 in each compartment was analyzed by western blot analysis followed by densitometry to semi-quantify protein levels. Total histone H3 was used as a loading control. Data are presented as mean ± SEM and analyzed by a two-tailed Student's *t* test compared to the vehicle control (*) for each compartment with statistical significance at $p < 0.05$. (**b**) The locations of different response elements and transcription factor binding sites at human *MDR1/ABCB1* gene (NC_000007.14) promoter region. DRE, dioxin response element; TSS, transcription start site; AHR, aryl hydrocarbon receptor. (**c**) hCMEC/D3 cells ($n = 3–6$) were treated with vehicle or 10 μM SAHA for 24 h and analyzed for relative histone H3K9/14 acetylation at different regions of the *MDR1/ABCB1* gene promoter. Data collected from qPCR amplification of ChIP samples were presented as mean ± SEM. Data were analyzed by two-tailed Student's *t* test between treatment groups with statistical significance at $p < 0.05$. The graph titles correspond to the labels in (**b**)


a.
AHR
CYP1A1
mRNA Expression (Normalized to β2M)
Veh VPA NaB Romi Api SAHA TSA
b.
MDR1 mRNA
AhR mRNA
R = 0.94
CYP1A1 mRNA
R = 0.84
c.
Cytosolic Extract
Vehicle SAHA
AHR
Total H3
- 20kD
Nuclear Extract
Protein Expression (Normalized to total H3)
Cytosolic
Nuclear

◀ **Fig. 4** AHR expression and activity in human brain microvascular endothelial cells treated with HDAC inhibitors. (**a**) hCMEC/D3 cells ($n = 3–6$) were treated with vehicle (Veh) or six HDAC inhibitors (5 mM VPA, 0.25 mM NaB, 1 nM romidepsin (Romi), 0.5 μM apicidin (Api), 10 μM SAHA, or 0.25 μM TSA) for 12 h and analyzed for mRNA expression of AHR and CYP1A1. Data were normalized to beta$_2$-microglobulin and presented as mean ± SEM. Data were analyzed by one-way ANOVA compared to the vehicle control (*) with statistical significance at $p < 0.05$. (**b**) Correlation between changes in the expression of MDR1 and AHR and CYP1A1 mRNA levels after 12-h treatment with HDAC inhibitors was analyzed by Pearson's correlation test. (**c**) hCMEC/D3 cells ($n = 3–7$) were treated with vehicle or 10 μM SAHA for 24 h and cytosolic and nuclear extracts were collected. The relative protein expression of AHR in each compartment was analyzed by western blot analysis followed by densitometry to semi-quantify protein levels. Total histone H3 was used as a loading control. Data are presented as mean ± SEM and analyzed by two-tailed Student's *t* test compared to the vehicle control (*) for each compartment with statistical significance at $p < 0.05$

responsible for MDR1 up-regulation. To determine whether HDAC inhibitors altered histone acetylation at the *MDR1* promoter in a manner that facilitates *MDR1* gene activation, we quantified the enrichment of acetylated histone H3K9/K14 proteins in cytoplasmic and nuclear fractions of hCMEC/D3 cells treated with SAHA (Fig. 3a). Basal expression of acetylated histone H3 K9/K14 proteins was detectable in cytosolic and nuclear fractions from vehicle-treated cells. Treatment of hCMEC/D3 cells with SAHA increased acetylated histone H3 K9/K14 proteins by 100% in both nuclear and cytosolic fractions.

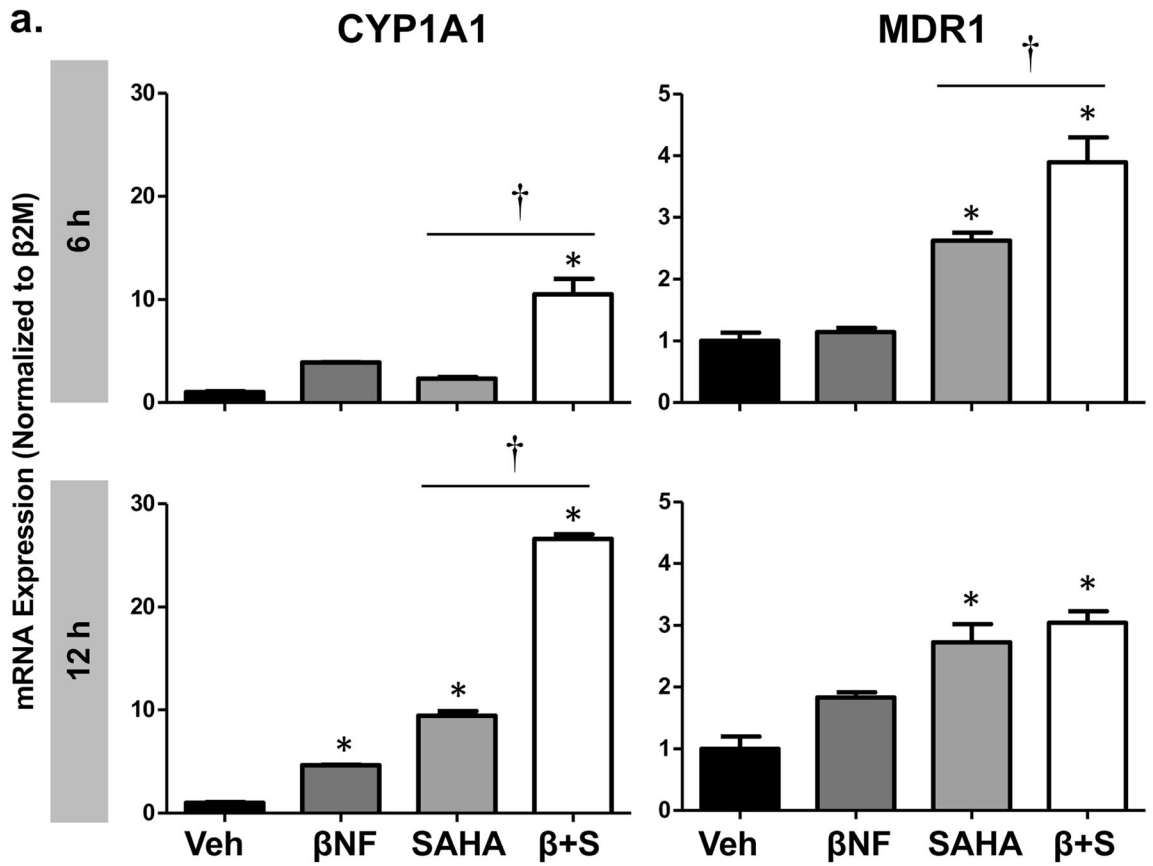
To investigate whether enrichment of acetylated histones in the nuclear fraction of hCMEC/D3 cells treated with SAHA enabled the induction of MDR1 mRNA, chromatin immunoprecipitation (ChIP) was performed using an antibody that recognizes acetylation of H3K9/14 and qPCR amplification of different segments of the *MDR1* promoter. Five different regions spanning the *MDR1* transcriptional start site (− 182 to + 339 bp), namely, P1, P2, P3, P4, and P5, were selected based on previous studies highlighting the importance of these regions for efficient transcription of *MDR1* (Fig. 3b). Prior data demonstrated the presence of key transcription factor binding sites within these regions and the critical role of corresponding response elements to mediate induction of MDR1 expression [8, 11, 14, 18, 47]. Treatment of hCMEC/D3 cells with SAHA for 24 h increased the enrichment of acetylated H3K9/14 protein binding at positions P1 and P2 (Fig. 3c). In particular, binding of acetylated H3K9/14 protein was significantly increased by 50% within the P2 region where a putative dioxin response element (DRE) for AHR, a known regulator of MDR1 transporter, is located. Interestingly, P4 and P5 regions, which also include AHR-like DRE elements, suggested trends of reduced DNA binding by acetylated histone H3K9/14 proteins.

AHR Signaling Modulates HDAC Inhibitor-Mediated Induction of the MDR1 Transporter

The results in Fig. 3 indicated that the transcriptional activation of MDR1 involves enhanced binding of acetylated histone proteins in the region of the *MDR1* promoter that contains AHR-like DRE elements. In hCMEC/D3 cells treated with a panel of HDAC inhibitors, we found a correlation between induction of mRNA levels of MDR1 and AHR as well as cytochrome P450 1A1 (CYP1A1), a target gene of AHR (Fig. 4a, b). These data demonstrate increased expression and activity of AHR after HDAC inhibition. Furthermore, nuclear extracts of hCMEC/D3 cells treated with SAHA for 24 h exhibited significant enrichment of AHR protein (Fig. 4c), suggesting that AHR may play a transcriptional role in regulating *MDR1* gene expression following exposure to HDAC inhibitors. It is important to note that the mRNA expression of other xenobiotic-activated MDR1 regulators, PXR and CAR, was barely detectable in hCMEC/D3 cells, as previously noted (Supplemental Table 2) [48]. Therefore, subsequent experiments investigated the role of AHR in HDAC inhibitor-mediated regulation of MDR1.

The effects of AHR activation on MDR1 expression in hCMEC/D3 cells were assessed using the pharmacological AHR agonist, β-naphthoflavone (βNF, 5 μM), in combination with SAHA (Fig. 5). Activation of AHR signaling by βNF was confirmed by a 290–370% increase of CYP1A1 mRNA at 6 and 12 h (Fig. 5a). Likewise, treatment with SAHA enhanced CYP1A1 mRNA levels by 135–845% at 6 and 12 h which was further elevated to 910–2660% of control levels following combination with βNF. At 6 h, MDR1 mRNA expression was significantly increased 170% by SAHA and further up-regulated to 300% following combined treatment with SAHA and βNF. Notably, βNF on its own had no significant effect on MDR1 mRNA expression at either time point although there was a trend for 80% increase at 12 h. However, by 12 h, the augmented induction of MDR1 mRNA in the cells treated with SAHA and βNF was no longer observed. Enhanced MDR1 expression by AHR activation and HDAC inhibition was also observed at the protein level (Fig. 5b). After 24 h, treatment of hCEMC/D3 cells with βNF alone did not affect MDR1 protein expression while SAHA significantly up-regulated levels by 47% as expected. Importantly, the addition of βNF to SAHA further increased MDR1 protein expression by 85% compared to vehicle-treated cells.

To further elucidate the role of AHR in mediating the induction of MDR1 in hCMEC/D3 cells treated with HDAC inhibitors, we assessed whether inhibition of AHR using CH-223191 (CH, 5 μM) altered the expression of MDR1 in cells co-treated with SAHA (Fig. 6). Induction of CYP1A1 expression by SAHA at 6 h (82%) and 12 h (540%) was attenuated by co-treatment with CH confirming that AHR


a.
CYP1A1
MDR1
6 h
12 h
mRNA Expression (Normalized to β2M)
Veh
βNF
SAHA
β+S
b.
Vehicle
βNF
SAHA
βNF+SAHA
AcH3K9/14
MDR1
α-tubulin
- 20kD
- 200kD
- 120kD
- 50kD
MDR1
Protein Expression (Normalized to α-tubulin)

◀ **Fig. 5** MDR1 expression in human brain microvascular endothelial cells treated with SAHA and an AHR activator. (**a**) hCMEC/D3 cells ($n = 3$) were treated with vehicle (Veh), AHR activator (βNF, β-naphthoflavone 5 μM), and/or 10 μM SAHA for 6 h or 12 h. Total RNA was isolated and analyzed for mRNA expression of MDR1 and CYP1A1, the positive control, by qPCR. Data were normalized to beta$_2$-microglobulin and presented as mean ± SEM. Data were analyzed by one-way ANOVA compared to the vehicle control (*), and between SAHA and β + S (βNF + SAHA) (†) with statistical significance at $p < 0.05$. (**b**) hCMEC/D3 cells ($n = 3$) were treated with Veh, βNF 5 μM, and/or 10 μM SAHA for 24 h and analyzed for protein expression of acetylated histone H3K9/14 or MDR1 by western blot analysis followed by densitometry to semi-quantify protein levels. Alpha-tubulin (α-tubulin) was used as a loading control. Data are presented as mean ± SEM and analyzed by one-way ANOVA compared to the vehicle control (*), and between SAHA and β + S (†) with statistical significance at $p < 0.05$

signaling was reduced by CH (Fig. 6a). At 6 h, CH did not alter the up-regulation of MDR1 mRNA by SAHA. However, by 12 h, CH significantly suppressed SAHA-mediated induction of MDR1 (175% in SAHA cells; 120% in SAHA + CH cells) similar to that observed for CYP1A1 mRNA. Likewise, at 36 h, CH largely prevented SAHA-mediated induction of MDR1 protein (Fig. 6b).

HDAC Inhibition by SAHA Increases AHR Binding to the MDR1 Promoter

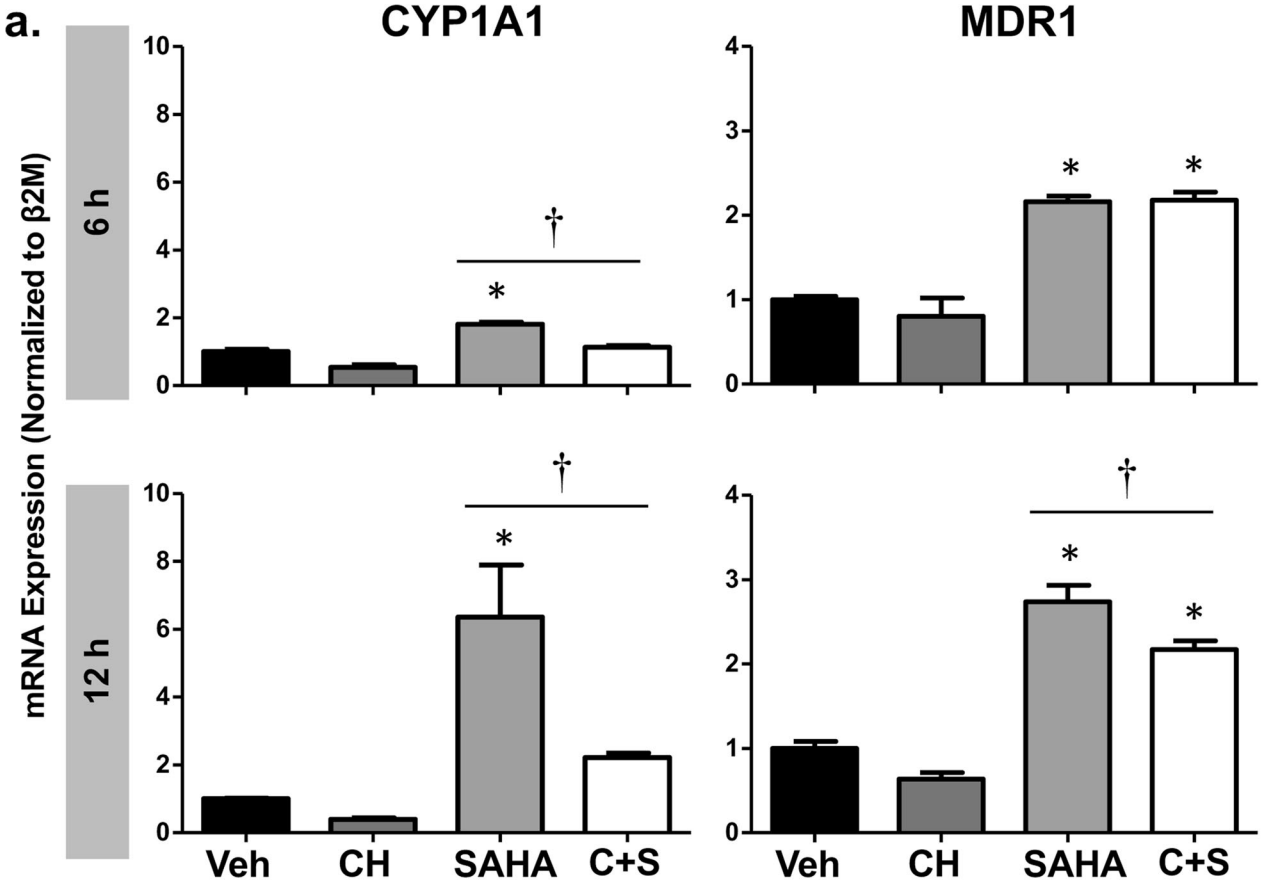
To investigate the interaction of AHR at the *MDR1* gene, we performed a ChIP assay to assess the binding of AHR proteins to *MDR1* promoter regions, P1 through P5. Of these regions, P2, P4, and P5 contain putative AHR response elements as illustrated in Fig. 7a. Treatment of hCMEC/D3 cells with SAHA for 24 h significantly increased AHR binding between 50% and 100% at the P2, P4, and P5 regions (Fig. 7b). Of these regions, P2 also exhibited significant histone H3K9/14 acetylation as aforementioned (Fig. 3c). The promoter of *CYP1A1*, a positive control gene, also exhibited significantly higher enrichment of AHR binding after SAHA treatment (Supplemental Fig. 4). On the contrary, the enrichment of AHR did not change at the P1 and P3 regions of the *MDR1* promoter where no AHR response elements are found (Fig. 7b).

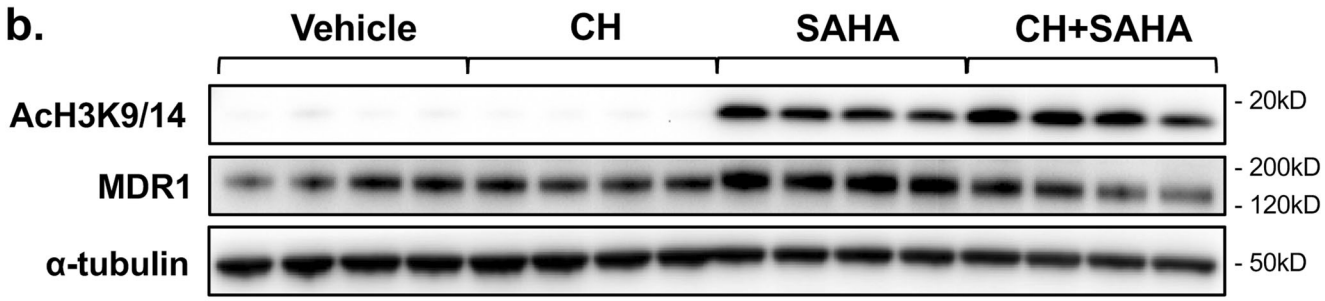
Discussion

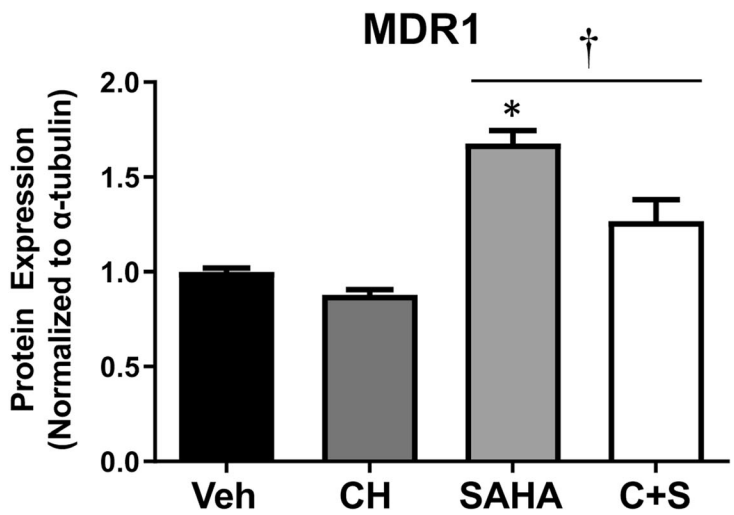
MDR1, which is tightly regulated by multiple mechanisms, confers protection to the brain by preventing the passage of numerous xenobiotics into the parenchyma [2–4]. Previous studies have identified several nuclear receptors and transcription factors as regulators of MDR1 [43, 49–52]. Recent studies have revealed the ability of HDAC inhibitors to up-regulate the expression and function of MDR1 in various cancer cell lines. This induction occurs via histone acetylation at gene promoters, demonstrating a novel role for epigenetic modifications to control transporter expression [33, 44, 53]. Since HDAC inhibitors have clinical utility in treating various brain diseases including epilepsy and glioblastoma, it is important to evaluate the effects of HDAC inhibitors on transporters at the BBB. Therefore, the purpose of this study was to investigate the ability of pharmacological inhibitors of HDAC enzymes to regulate the expression and function of the MDR1 transporter in human brain endothelial cells. Our findings demonstrated that (1) HDAC inhibitors increased the mRNA and protein expression of MDR1 in hCMEC/D3 cells, an in vitro model of the human BBB; (2) up-regulated expression translated into increased functional activity of MDR1; and (3) enhanced histone acetylation and activation of AHR signaling are involved in MDR1 up-regulation.

Six different HDAC inhibitors were tested including three FDA-approved drugs, VPA, romidepsin, and SAHA, as well as NaB, apicidin, and TSA. HDAC inhibitors are classified based upon chemical properties, which enable the targeting of different HDAC isoforms (Table 1) [25]. Across classes, HDAC inhibitors differ in their potency. Whereas short-chain fatty acids such as VPA and NaB are weak HDAC inhibitors typically effective at millimolar concentrations, hydroxamic acids (SAHA and TSA) and cyclic peptides (romidepsin and apicidin) are more potent, eliciting effects at nanomolar to micromolar concentrations [27]. In the current study, HDAC inhibitors displayed differential abilities to regulate MDR1 expression in hCMEC/D3 cells. Generally, short-chain fatty acids were less potent and/or effective at increasing HDAC acetylation and inducing MDR1 expression compared to other HDAC inhibitors. This observation likely results from the relatively weaker inhibition of HDACs as indicated by minimal changes in acetylated histone H3 level.

On the other hand, apicidin, a potent HDAC inhibitor that specifically targets class I HDACs, was effective in inducing both mRNA and protein levels of MDR1. Interestingly, romidepsin, also a potent HDAC inhibitor, which increased MDR1 mRNA expression and MDR1 promoter acetylation in acute promyelocytic leukemia NB4 cells [54], did not alter MDR1 mRNA or protein levels in hCMEC/D3 cells despite detectable histone H3 acetylation. It is possible that the romidepsin-mediated changes of MDR1 expression are concentration and time specific. Alternatively, this disconnect may be due to (1) different specificity of targeting HDAC isoforms between romidepsin and apicidin; or (2) the involvement of an alternative (non-histone), cell-specific mechanism that is not altered by romidepsin. In contrast, although TSA significantly induced MDR1 mRNA level, it did not affect histone acetylation status as observed with SAHA, which targets the same isoforms of HDACs. It is important to note that histone H3 acetylation is just one type of epigenetic modification and the possibility exists that other epigenetic events such as H4 acetylation may be involved. A study by Tabe and


a.
CYP1A1
MDR1
6 h
12 h
mRNA Expression (Normalized to β2M)
Veh
CH
SAHA
C+S


b.
Vehicle
CH
SAHA
CH+SAHA
AcH3K9/14
MDR1
α-tubulin
20kD
200kD
120kD
50kD


MDR1
Protein Expression
(Normalized to α-tubulin)
Veh
CH
SAHA
C+S

◀ **Fig. 6** MDR1 expression in human brain microvascular endothelial cells treated with SAHA and an AHR inhibitor. (**a**) hCMEC/D3 cells ($n = 3$–4) were treated with vehicle (Veh), AHR inhibitor (CH, CH-223191 5 μM), and/or 10 μM SAHA for 6 h or 12 h. Total RNA was isolated at the end of the treatment and analyzed for mRNA expression of MDR1 and CYP1A1, the positive control, by qPCR. Data were normalized to beta$_2$-microglobulin and presented as mean ± SEM. Data were analyzed by one-way ANOVA compared to the vehicle control (*), and between SAHA and C + S (CH + SAHA) (†) with statistical significance at $p < 0.05$. (**b**) hCMEC/D3 cells ($n = 4$) were treated with Veh, CH 5 μM, and/or 10 μM SAHA for 36 h and analyzed for protein expression of acetylated histone H3K9/14 or MDR1 by western blot analysis followed by densitometry to semi-quantify protein levels. Alpha-tubulin (α-tubulin) was used as a loading control. Data are presented as mean ± SEM and analyzed by one-way ANOVA compared to the vehicle control (*), and between SAHA and C + S (†) with statistical significance at $p < 0.05$

coworkers (2006) showed that romidepsin increased the acetylation of H4, but not H3K9, at the CCAAT box of *MDR1* promoter in NB4 acute promyelocytic leukemia cells [54]. Taken together, these data suggest that HDAC inhibitors can elicit highly specific patterns of histone acetylation.

The most likely mechanism for induction of MDR1 following treatment of hCMEC/D3 cells with HDAC inhibitors is through the binding of acetylated histones at the *MDR1* promoter and subsequent gene activation due to an open, accessible DNA conformation [55–58]. In fact, we observed a significant increase in the protein levels of acetylated histone H3 in nuclear fractions of hCMEC/D3 cells treated with SAHA. Further analyses showed that these acetylated histone H3 proteins were highly enriched at the upstream region of the *MDR1* promoter (− 182 to + 8 bp relative to the TSS) which contains several response elements interacting with key transcription factors such as Sp1, Sp3, AP-1, and NF-Y [11–13, 15, 18, 59]. In particular, significant enrichment of histone H3 K9/K14 binding was observed between − 100 and + 8 bp where an inverted CCAAT box (Y-box) and a potential DRE are located [11, 14, 18]. This finding was significant because it further supports that (1) Y-box, which was identified as the critical element for the induction of MDR1 by TSA and apicidin in cancer cells [13, 53], is an important sequence for the activity of HDAC inhibitors; and (2) AHR plays a role in inducing MDR1 transcription. Our results further demonstrated that there was a correlation between AHR and MDR1 mRNA upon HDAC inhibition, suggesting the possible involvement of AHR signaling. Enhanced histone acetylation at a DRE suggests that there is an increased accessibility for AHR to interact with this response element to facilitate *MDR1* gene activation.

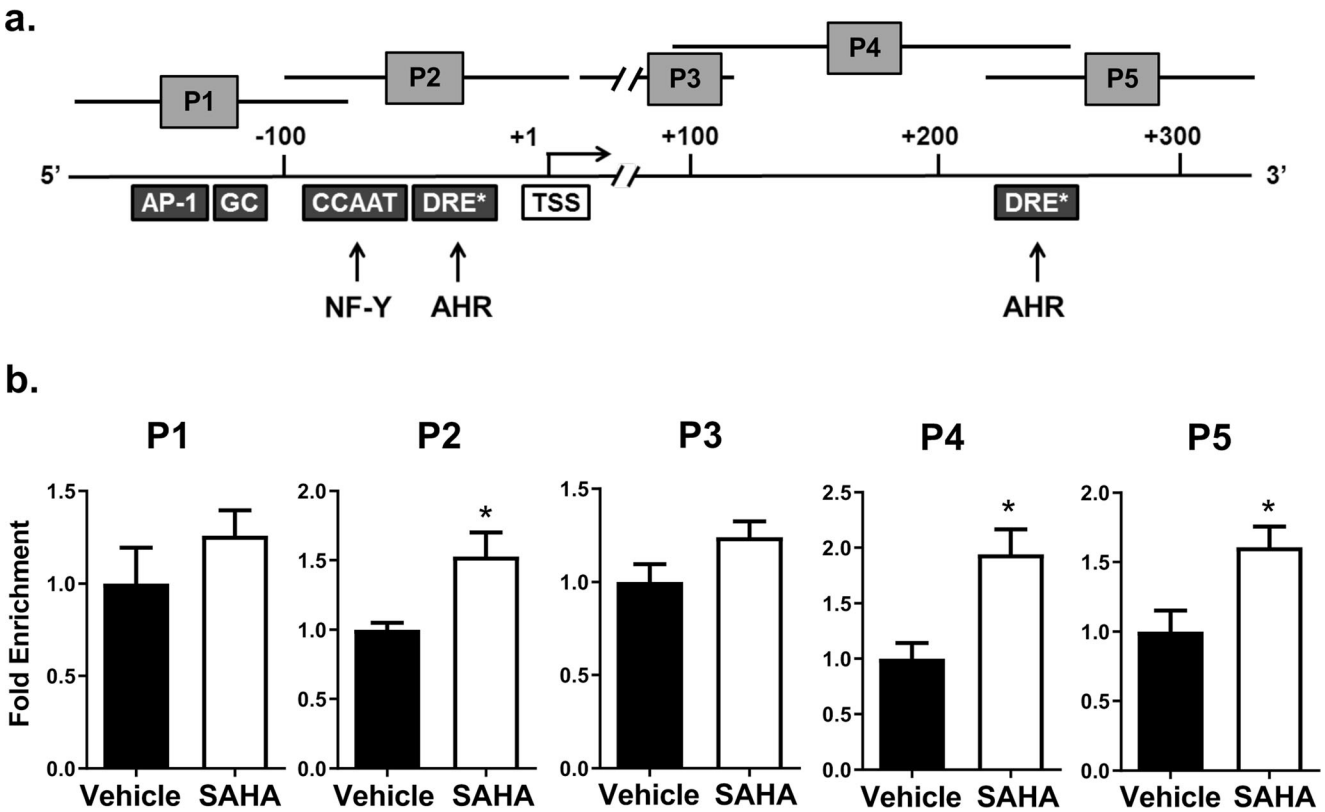


Fig. 7 Relative aryl hydrocarbon receptor binding at different regions of the *MDR1/ABCB1* gene promoter in human brain microvascular endothelial cells treated with SAHA. hCMEC/D3 cells ($n = 3$–10) were treated with vehicle or 10 μM SAHA for 24 h and analyzed for relative AHR binding at different regions of the *MDR1/ABCB1* gene promoter (**a**). Data collected from qPCR amplification of ChIP samples were presented as mean ± SEM (**b**). Data were analyzed by two-tailed Student's *t* test between treatment groups with statistical significance (*) at $p < 0.05$. The graph titles correspond to the labels in (**a**)

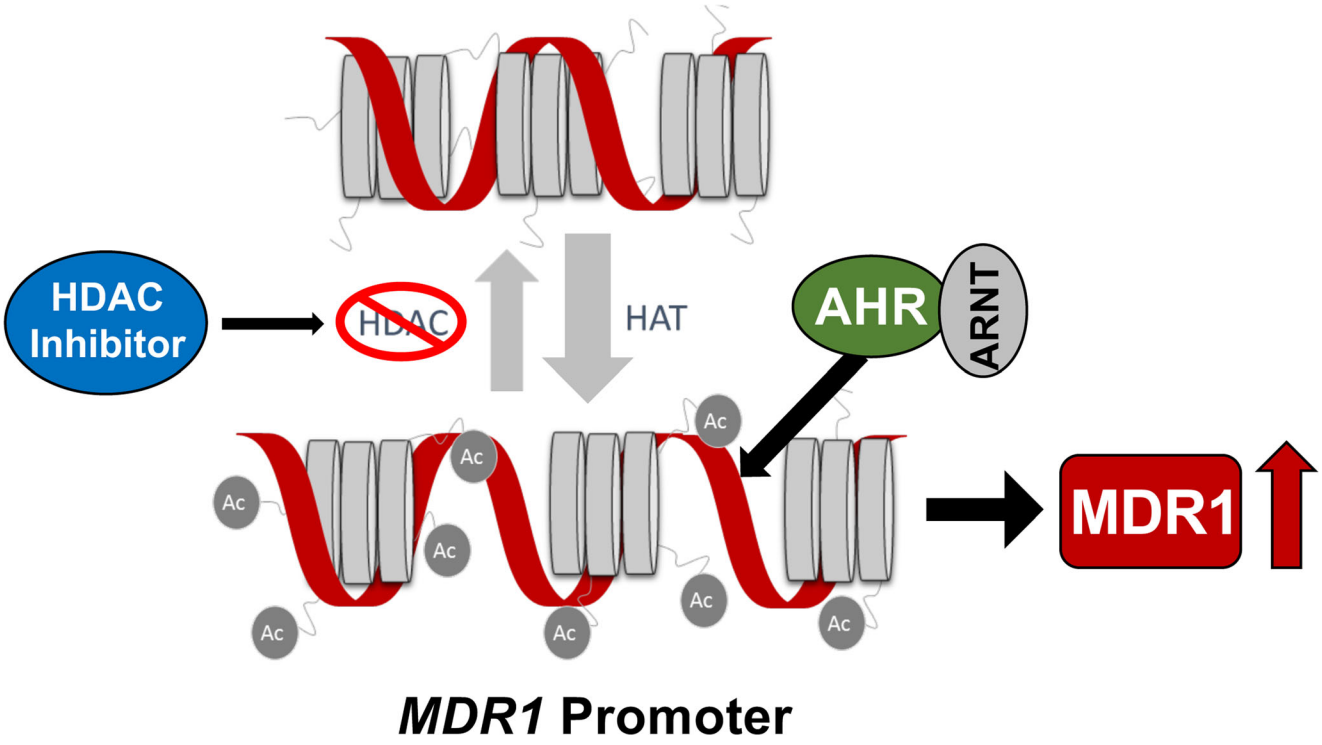


Fig. 8 Proposed mechanism of interaction between histone acetylation and AHR signaling in MDR1 regulation at the human blood–brain barrier. Pharmacological inhibition of HDACs promotes the acetylation of histones which consequently increases the accessibility of the *MDR1* promoter to AHR binding and transactivation of the *MDR1* gene

By comparison, histone H3 acetylation at the "P3" region of the *MDR1* promoter (spanning from + 11 to + 110 bp), which does not have transcription factor binding sites, did not significantly change. This indicates that the binding of acetylated histone H3 preferentially occurs at regions that interact with transcription factors. Interestingly, there was a trend of reduced binding of acetylated histone H3 proteins in the P4 and P5 regions (spanning from + 90 to + 339 bp) which also contains a DRE [14, 18]. However, it is possible that other markers of histone modification, such as acetylated histone H4, may have been enriched at these regions.

We performed further studies to investigate the importance of AHR activity in SAHA-mediated regulation of MDR1. First, we evaluated the effects of pharmacologic modulators of AHR activity on MDR1 induction by SAHA. βNF activation of AHR augmented MDR1 mRNA induced by SAHA at 6 h, suggesting that SAHA induces binding of acetylated histones at the *MDR1* promoter to increase the accessibility of DRE, facilitate the binding of the ligand-activated AHR, and induce *MDR1* gene activation. By comparison, the AHR inhibitor CH attenuated the induction of MDR1 mRNA and protein by SAHA. Assessment of nuclear lysates of SAHA-treated cells showed that there was indeed a significant increase in AHR expression in the nucleus, suggesting the possibility of enhanced AHR binding to target genes. ChIP analysis revealed enrichment of AHR binding at the P2 (− 100 to + 8 bp), P4 (+ 90 to + 245 bp), and P5 (+ 230 to + 338 bp) regions of the *MDR1* promoter which contain putative DREs [14, 18]. On the other hand, no significant changes were observed at the P1 (− 182 to − 76 bp) and P3 (+ 11 to + 110 bp) regions that do not possess AHR binding sites. These data point to a critical role of AHR in HDAC-mediated regulation of MDR1. Nonetheless, it is possible that additional mechanisms play a role. It is well known that the regulation of MDR1 is complex and involves the interaction of multiple pathways. As discussed above, the promoter regions with significant histone H3 acetylation after SAHA treatment contain multiple response elements. It is plausible that other transcription factors, such as Sp1 and Sp3, also work cooperatively with AHR to regulate the transcriptional activation of *MDR1* in response to HDAC inhibition.

Interestingly, HDAC inhibition did not result in global up-regulation of efflux transporters as MRP mRNAs were either unchanged or down-regulated, suggesting that MRP regulation by HDAC inhibitors may be mediated via different mechanisms. Differences in the organization of the *MRP* promoter relative to the *MDR1* promoter is a likely cause for divergent responses to HDAC inhibitors. To address this question, it would be important to assess the characteristics of *MRP* promoters, such as the methylation status, baseline histone acetylation level, as well as the types and locations of the transcription factor binding sites. Alternatively, suppression of

MRP expression may be compensatory response to the MDR1 up-regulation to maintain the overall transport properties of shared substrates.

Taken together, this study demonstrated that HDAC inhibitors, in particular VPA, apicidin, and SAHA, significantly up-regulated the mRNA and protein expression of MDR1 in a human model of the BBB. Enhanced functional activity of MDR1 after HDAC inhibition was indicated by reduced accumulation of its substrate rhodamine. Transporter up-regulation was associated with histone acetylation as shown by increased levels of acetylated histone H3. Our mechanistic studies showed that SAHA induces the nuclear enrichment and binding of acetylated histones and AHR to the *MDR1* promoter. Concurrent exposure to an AHR activator further enhanced SAHA-mediated induction of MDR1 expression while an AHR inhibitor moderately reduced MDR1 mRNA and protein levels induced by SAHA. These results suggest that inhibition of HDAC activity by pharmacological inhibitors promotes the binding of acetylated histones at the *MDR1* promoter to make the region more accessible for transcription factors, such as AHR, to bind and activate *MDR1* gene transcription. A proposed mechanism is illustrated in Fig. 8.

It is important to note that although the hCMEC/D3 cells are a representative model of the BBB, it does not possess all properties of intact brain microvessels. For example, we were unable to achieve sufficient transendothelial electrical resistance (TEER), a key feature of the BBB, when these cells were cultured as a monolayer on transwell inserts (data not shown). Prior studies showed that the co-culture of brain microvascular endothelial cells with supporting cells, such as the astrocytes and pericytes, can improve tight junction formation as measured by TEER [60, 61]. Implementing this co-culture system to test the effects of HDAC inhibitors on transporters may provide a more translational platform. In addition, it would be advantageous to assess the effects of HDAC inhibitors using other types of BBB models. A recent in vivo study in our laboratory showed that HDAC inhibitors (apicidin and valproic acid) caused brain region-specific up-regulation of Mdr1 in mice, with the most notable induction in the striatum [62]. Future studies are also needed to investigate the effects of HDAC inhibitors on MDR1-mediated efflux of pharmaceuticals and environmental chemicals using ex vivo mouse brain microvessels.

Collectively, our data suggest that the clinical administration of HDAC inhibitors may alter efflux properties at the BBB leading to reduced penetration of MDR1 substrates into the parenchyma. This may be relevant for (1) the concomitant administration of neuroactive drugs that are substrates of the MDR1 transporter and (2) the treatment of neurological conditions caused by the neurotoxicants cleared by MDR1. Expanding our understanding of the epigenetic regulation of MDR1 transporters can aid in identifying specific molecular targets that are important for maintaining efflux transport properties of the human BBB. HDAC and AHR pathways may represent novel molecular targets to modulate the BBB transporter activity to either increase the brain concentration of psychoactive drugs or decrease the penetration of toxicants implicated in neurodegeneration.

Authors' Contributions Participated in research design: D.Y., X.W., J.R.R., L.M.A.
Conducted experiments: D.Y., L.G., A.M.
Performed data analysis: D.Y., A.M.
Wrote or contributed to the writing of the manuscript: D.Y., J.R.R., L.M.A.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflicts of interest.

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