Histone Deacetylase Inhibitor Induction of P-Glycoprotein Transcription Requires Both Histone Deacetylase 1 Dissociation and Recruitment of CAAT/Enhancer Binding Protein β and pCAF to the Promoter Region

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Abstract

Although histone deacetylase (HDAC) inhibitors are appreciated as a promising class of anticancer drugs, recent reports show that P-glycoprotein (P-gp) is induced by HDAC inhibitor treatment in cancer cells, resulting in multidrug resistance of cancer cells to other chemotherapeutic agents. In this study, we investigated the molecular mechanism of HDAC inhibitor induction of P-gp expression. HDAC inhibitor treatment causes cell type–specific induction of P-gp expression without changes in the CpG methylation status of the promoter region. In addition, our data show that HDAC inhibitor does not alter the DNA binding activity of Sp1 but facilitates both the recruitment of a coactivator complex that includes CAAT/enhancer binding protein β and pCAF and the dissociation of the repressive complex, HDAC1, to the Sp1 binding region. Subsequently, the hyperacetylated histone H3 becomes enriched in the promoter region, leading to RNA polymerase II recruitment to activate P-gp gene transcription. Furthermore, specific down-regulation of HDAC1, but not HDAC2, by RNA silencing was enough to induce P-gp expression in HeLa cells, strongly supporting the essential role of HDAC1 in HDAC inhibitor induction of P-gp. Concomitantly, cell type–specific induction of P-gp expression seems to be dependent on phosphatidylinositol 3-kinase activity. Taken together, our findings show that HDAC inhibitor treatment leads to an increase in P-gp expression through dynamic changes in chromatin structure and transcription factor association within the promoter region. (Mol Cancer Res 2009;7(5):735–44)

Introduction

It has been well shown that histone deacetylase (HDAC) inhibitors alter the transcription of many tumor-associated genes and exhibit therapeutic activity against a variety of human malignancies, leading to phenotypic changes in the tumor, including growth arrest, morphologic reversion, differentiation, and apoptosis (1–6). It is also generally accepted that more active chromatin regions are associated with histone hyperacetylation and recruitment of histone acetyltransferases; histone deacetylation, which is associated with recruitment of HDACs, often restores these genomically active regions to a more repressed and condensed chromatin state. Thus, HDAC inhibitors are emerging as a promising class of chemotherapeutic agents against cancer. Several HDAC inhibitors, such as vorinostat [suberoylanilide hydroxamic acid (SAHA); refs. 7, 8], MS-275 (9, 10), phenylbutyrate (11), and depsipeptide FK228 (12–14), have shown potent antitumor activities and are currently in phase I and II clinical trials. Recently, vorinostat (Zolinza, Merck & Co., Inc.) was approved for the treatment of cutaneous T-cell lymphoma.

Multidrug resistance (MDR), in which cells are resistant to many structurally and functionally unrelated drugs, is a serious obstacle to cancer chemotherapy (15). The MDR phenotype is often due to overexpression of drug efflux pumps in the plasma membranes of cancer cells. Overexpression of P-glycoprotein (P-gp), an integral membrane protein encoded by the ABCB1 (MDR1) gene, is likely to be both a predominant contributor to this MDR phenotype and a marker for poor prognosis of several human cancers (16, 17). P-gp functions as a drug efflux pump that actively transports drugs from the inside to the outside of cells and causes a defect in the intracellular accumulation of drugs necessary for killing of cancer cells (16, 18, 19). Several reports show that P-gp expression can be silenced by epigenetic modifications involving methylation of CpG islands in the promoter region (20–24). Hypermethylation of CpG dinucleotides in the P-gp gene promoter region are associated with low expression of P-gp in various cell lines and tissues from patients (21, 23–25). This silencing can be reversed by treatment with a DNA methylation inhibitor (20, 21). In addition, accumulating evidence shows that HDAC inhibitors increase the expression of P-gp in cancer cells (26, 27), as well as peripheral blood mononuclear cells from patients (28), leading to chemoresistance in clinical applications and possibly limiting the clinical use of HDAC inhibitors. Recently, our group reported that apicidin, a HDAC inhibitor with broad-spectrum antiproliferative activity against various cancer cell
lines (3), induces the expression of P-gp in HeLa cells, leading to paclitaxel chemoresistance (29). However, the exact molecular mechanism for P-gp induction by apicidin is poorly understood. To extend on our previous study, we investigated potential mechanisms of apicidin-induced P-gp expression by analyzing epigenetic modifications of the promoter region. Our data provide novel evidence that apicidin treatment induces transcription of the P-gp gene by promoting active chromatin structures on the P-gp promoter region, which are associated with both HDAC1 dissociation and recruitment of CAAT/enhancer binding protein β (C/EBPβ) and pCAF but not changes in CpG methylation status.

Results

Cell Type–Specific Expression of P-gp by HDAC Inhibitors

Although HDAC inhibitors are widely appreciated as a new class of antitumor agents, recent reports have shown that HDAC inhibitors can induce resistance to other chemotherapeutic agents via induction of the drug efflux pump P-gp (26, 27, 29). In this study, we aimed to investigate the molecular mechanism by which HDAC inhibitor induces P-gp expression. We first analyzed the changes in P-gp expression mediated by the HDAC inhibitor apicidin in various cancer cell lines. Following treatment with apicidin for 24 hours, P-gp mRNA expression increased in human cervical cancer cells (HeLa and SiHa lines) and in a colon cancer cell line (DLD-1) but not in human glioblastoma cells (A172 and U87 lines) or human oral cancer cells (KB line; Fig. 1A). These results indicate that short-term exposure to apicidin is sufficient to increase the P-gp expression in a cell type–specific manner. To further confirm this cell type–specific expression of P-gp, we examined whether apicidin could stimulate the activity of the P-gp gene promoter in cells transiently transfected with a 1.57-kb wild-type P-gp promoter-luciferase fusion plasmid, pMDR1-1571-Luc. As shown in Fig. 1B, the luciferase activity was increased in a time-dependent manner up to ∼98-fold by treating HeLa cells with apicidin. However, the luciferase activity in KB cells was not affected by apicidin (Fig. 1B), indicating cell type–specific apicidin-induced P-gp expression. Next, to determine whether this induction of P-gp by apicidin was mediated through inhibition of HDAC activity, we examined the effect of various HDAC inhibitors, including SAHA, trichostatin A (TSA), MS-275, sodium butyrate, and valproic acid, on P-gp expression in HeLa and KB cells. All of the tested HDAC inhibitors led to a dramatic increase in P-gp expression in HeLa cells, but not KB cells (Fig. 1C), in agreement with the data presented in Fig. 1A and B supporting the cell type–specific induction of P-gp by apicidin, implying that it is possible that most, if not all, HDAC inhibitors induce the expression of P-gp in a cell type–specific manner.

Apicidin Induction of P-gp Does Not Require Changes in the CpG Methylation Status of the P-gp Promoter Region

Several studies have suggested that enhanced P-gp expression in human malignancies following chemotherapy may be attributable to demethylation of the MDR1 gene promoter (21, 24). Thus, we examined the CpG methylation status of the P-gp promoter region (26 CpG dinucleotides between −341 and +262) using bisulfite genomic sequencing (Fig. 2A). The P-gp promoter region was hypermethylated in HeLa cells (Fig. 2B, left), in which P-gp is transcriptionally silent. Unexpectedly, however, apicidin treatment did not affect the CpG methylation status of the P-gp promoter (Fig. 2B, right), although P-gp was expressed in this condition. Additionally, apicidin did not alter the methylation status of the P-gp promoter region in P-gp nonexpressing KB cells (data not shown). These results indicate that apicidin induction of P-gp does not require changes in CpG methylation of the P-gp promoter region. To further confirm this notion, we determined the effect of the DNA demethylating agent 5-aza-2-deoxycytidine (AzadC) on apicidin induction of P-gp. Pretreatment of HeLa cells with AzadC for 24 hours did not alter the basal or apicidin-mediated up-regulation of P-gp following a 24-hour incubation (Fig. 2C), supporting the idea that apicidin induction of P-gp expression does not result from changes in the CpG methylation status of the promoter region. Next, we examined the global status of histone acetylation in apicidin-treated cells. We found that apicidin treatment led to an accumulation of acetylated histone H3 in HeLa cells in a dose-dependent manner (Fig. 2D). In addition, this histone hyperacetylation was attributed to demethylation of the MDR1gene promoter (21, 24). In this study, we aimed to investigate the molecular mechanism by which HDAC inhibitor induces P-gp expression. We first analyzed the changes in P-gp expression mediated by the HDAC inhibitor apicidin in various cancer cell lines. Following treatment with apicidin for 24 hours, P-gp mRNA expression increased in human cervical cancer cells (HeLa and SiHa lines) and in a colon cancer cell line (DLD-1) but not in human glioblastoma cells (A172 and U87 lines) or human oral cancer cells (KB line; Fig. 1A). These results indicate that short-term exposure to apicidin is sufficient to increase the P-gp expression in a cell type–specific manner. To further confirm this cell type–specific expression of P-gp, we examined whether apicidin could stimulate the activity of the P-gp gene promoter in cells transiently transfected with a 1.57-kb wild-type P-gp promoter-luciferase fusion plasmid, pMDR1-1571-Luc. As shown in Fig. 1B, the luciferase activity was increased in a time-dependent manner up to ∼98-fold by treating HeLa cells with apicidin. However, the luciferase activity in KB cells was not affected by apicidin (Fig. 1B), indicating cell type–specific apicidin-induced P-gp expression. Next, to determine whether this induction of P-gp by apicidin was mediated through inhibition of HDAC activity, we examined the effect of various HDAC inhibitors, including SAHA, trichostatin A (TSA), MS-275, sodium butyrate, and valproic acid, on P-gp expression in HeLa and KB cells. All of the tested HDAC inhibitors led to a dramatic increase in P-gp expression in HeLa cells, but not KB cells (Fig. 1C), in agreement with the data presented in Fig. 1A and B supporting the cell type–specific induction of P-gp by apicidin, implying that it is possible that most, if not all, HDAC inhibitors induce the expression of P-gp in a cell type–specific manner.
similarly detected in P-gp nonexpressing KB cells (Fig. 2D), indicating that apicidin-induced global histone acetylation levels are not required for P-gp induction, but some changes in the chromatin structure of the P-gp promoter region may be required for the induction.

Involvement of the Sp1 Transcription Factor in Apicidin Induction of P-gp

Accumulating evidence shows that HDAC inhibitors, such as apicidin and TSA, up-regulate the transcription of a set of Sp1-dependent genes, including p21WAF1/Cip1, cyclooxygenase-1, telomerase reverse transcriptase, and cyclin E (30-34). Therefore, we examined whether apicidin induction of P-gp is mediated by Sp1 transcription factors using the pharmacologic inhibitor mithramycin, which interferes with the binding of Sp1 family transcription factors to GC-rich promoters (35). As shown in Fig. 3, mithramycin pretreatment dramatically inhibited apicidin induction of P-gp mRNA and protein levels, as well as the reporter gene activity, strongly supporting the idea that apicidin induction of P-gp is specifically controlled by an Sp1 transcription factor–dependent mechanism.

To further confirm the requirement of Sp1 transcription factor for apicidin induction of P-gp expression, we next examined the functionality of putative regions for P-gp promoter activation using pMDR1 deletion and mutation constructs (Fig. 4). It has been shown that several cis-acting elements are involved in regulating P-gp promoter activity. These motifs include a distinct GC box, an inverted CCAATA box (Y-box), a putative activator protein-1 (AP-1) site, and C/EBPβ binding site (Fig. 4A; ref. 36). A series of 5′ deletion constructs of the P-gp promoter were transiently transfected into HeLa cells, and luciferase activities following apicidin or TSA treatment were measured. As shown in Fig. 4B, deletion of the distal promoter sequences to –223 bp from the transcription start site led to about a 1.5-fold increase in the luciferase activities in response to apicidin or TSA, suggesting the presence of a repressor binding site within the approximate region from –1,571 to –223. Further deletion of sequences to either –138 or –79 bp from the transcription start site (lacking C/EBPβ or AP-1 binding sites, respectively) did not affect activation of the reporter gene activities in response to either apicidin or TSA treatment. However, deletion of sequences from –79 to –43 (lacking the Y-box and GC box, responsible for NF-Y and Sp1 transcription factor binding, respectively) dramatically reduced HDAC inhibitor activation of the reporter gene activities (Fig. 4B), indicating that HDAC inhibitor activation of P-gp promoter activity requires both NF-Y box and Sp1 transcription factors but not C/EBPβ or AP-1. This result was further strengthened by the observations that HDAC inhibitor-induced reporter gene activities were significantly attenuated when the cells were...
transfected with a P-gp promoter expression construct containing the mutant version of either the Y-box or GC box but not the C/EBPβ and AP-1 mutant promoters (Fig. 4C).

**Apicidin Induction of P-gp Requires the Dissociation of HDAC1 and the Recruitment of pCAF and C/EBPβ onto the Promoter Region**

We next determined the effect of apicidin on the association of the transcriptional machinery with the MDR1 promoter region using a chromatin immunoprecipitation (ChIP) assay (Fig. 5A). Because our previous data indicated that the Sp1 transcription factor was required for apicidin activation of P-gp promoter activity (Figs. 3 and 4), the binding status of the Sp1 transcription factor to the P-gp promoter region was determined. Unexpectedly, however, apicidin treatment of HeLa cells did not affect the binding of Sp1 transcription factor to the promoter region of the P-gp gene (Fig. 5B), suggesting that whereas apicidin activation of P-gp promoter activity requires Sp1 binding, apicidin treatment does not result in Sp1 binding to the P-gp promoter. This result is supported by the previous observation that the Sp1 transcription factor constitutively associates with its DNA binding sites on the p21WAF1/Cip1 promoter and the association is not altered by HDAC inhibitor treatment (33, 37). Next, we examined the binding of transcriptional coactivators or corepressors to the P-gp promoter. The binding of the transcriptional coactivators C/EBPβ and pCAF were dramatically increased by treatment with apicidin in HeLa cells; however, the transcriptional corepressor HDAC1 was significantly dissociated from the promoter region by apicidin treatment, whereas HDAC2 was not (Fig. 5B). In parallel, hyperacetylation and K4 trimethylation of histone H3, which are well-known active gene markers (38), were observed in apicidin-treated HeLa cells (Fig. 5B), indicating that apicidin treatment induces hyperacetylation of the histones associated with the P-gp promoter region through the association of pCAF and C/EBPβ as well as the dissociation of HDAC1. Concomitantly, RNA polymerase II (Pol II) binding was dramatically increased in the promoter region following treatment with apicidin in HeLa cells (Fig. 5B), which likely triggers the observed increase in P-gp gene transcription. However, these dynamic changes in chromatin structure and transcriptional machinery associations were not detected in KB cells. Treatment of KB cells with apicidin caused specific recruitment of HDAC2 and dissociation of C/EBPβ and pCAF from the P-gp promoter region, leading to hypoacetylation and K4 demethylation of histone H3 (Fig. 5B). These changes allowed neither the recruitment of Pol II to the promoter region (Fig. 5B) nor the induction of P-gp expression (Fig. 1). Taken together, our results suggest that apicidin induces dynamic changes in the chromatin structure and transcriptional machinery associations with the P-gp promoter region in a cell type–specific manner, which results in transcription of the P-gp gene. To better clarify the role of HDAC1 in apicidin induction of P-gp expression in HeLa cells, specific small interfering RNAs (siRNA) for either HDAC1 or HDAC2 were transfected into HeLa cells. Transfection of HDAC1 and HDAC2 siRNAs successfully reduced endogenous expression of HDAC1 and HDAC2, respectively (Fig. 5C). The reduction of the HDAC1 mRNA level was sufficient to induce P-gp mRNA and protein production; however, HDAC2 knockdown was not (Fig. 5C). These results strongly support that the dissociation of HDAC1 from the P-gp promoter region is necessary for apicidin induction of P-gp expression in HeLa cells.

**Apicidin Induction of P-gp Requires the Phosphatidylinositol 3-Kinase Signaling Pathway**

To delineate a possible mechanism for the cell type–specific modification of the chromatin structure, we next examined the signaling pathway required for apicidin induction of P-gp in HeLa cells. Treatment of HeLa cells with U0126, SP600125, or SB203580 did not affect apicidin stimulation of P-gp promoter activity (Fig. 6A). However, specific inhibition of the phosphatidylinositol 3-kinase (PI3K) signaling pathway with LY294002 caused an attenuation of the P-gp promoter activity (Fig. 6A), which was accompanied by a down-regulation of mRNA and protein levels (Fig. 6B), indicating that apicidin induction of P-gp expression requires PI3K signaling pathway but not the extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, or p38 pathway. In addition, apicidin treatment led to an increase in the phosphorylation level of Akt, a downstream target of PI3K, in HeLa cells, but not in KB cells (Fig. 6C), which is in accordance with the cell type–specific induction of the chromatin structure modification. To further confirm the involvement of PI3K signaling pathway in apicidin induction of P-gp, we examined the association of the transcriptional machinery on the MDR1 promoter region in the presence...
of LY294002. As expected, inhibition of PI3K activity with LY294002 disrupted not only the dissociation of HDAC1 but also the recruitment of C/EBPβ on the promoter region (Fig. 6D). Concomitantly, the Pol II recruitment was dramatically abolished by inhibiting PI3K activity (Fig. 6D). These results strongly suggest that differential activation of PI3K by apicidin is one of the possible mechanisms for cell type–specific induction of P-gp expression. Because Sp1 phosphorylation is critical for its transcriptional activity (39, 40), we next examined the possibility of Sp1 phosphorylation by apicidin. As shown in Fig. 6E, Sp1 phosphorylation was dramatically increased by apicidin treatment, which was strongly inhibited by inhibiting PI3K signaling pathway. In addition, apicidin treatment led to an increase in interaction of pCAF with Sp1, which was dependent on phosphorylation status of Sp1 (Fig. 6E). Taken together, our data suggest that apicidin induces PI3K-dependent phosphorylation of Sp1, which is required for the release of HDAC1 from the P-gp promoter and the recruitment of pCAF and Pol II to the P-gp promoter region, leading to a subsequent induction of P-gp expression.

Discussion

In the present study, we show that HDAC inhibitor-induced P-gp expression is cell type selective, as evidenced by the fact that treatment with HDAC inhibitor induces the expression of P-gp in HeLa, SiHa, and DLD-1 cells but not in A172, U87, or KB cells (Fig. 1). Several reports have shown that P-gp expression in cancer cells is governed by epigenetic changes in the promoter region. Hypermethylation of the CpG dinucleotides within its promoter region is closely associated with low expression of P-gp in various cell lines and tissues from patients, which is reversed by treatment with a DNA methylation

![FIGURE 4. Mutational analysis of apicidin-induced P-gp promoter activity. A. Schematic diagram of the P-gp promoter. B. 5' deletion analysis of the P-gp promoter. The indicated plasmids shown on the left, pMDR1-1571, pMDR1-223, pMDR1-138, pMDR1-79, pMDR1-71, pMDR1-43, and pMDR1-7, were transiently transfected into HeLa cells and luciferase activities were analyzed after treatment with 1 μmol/L apicidin or 0.5 μmol/L TSA for 24 h. Columns, mean of three independent experiments; bars, SD. C. Mutation analysis of the P-gp promoter. Four different mutant constructs, shown on the left, were transiently transfected into HeLa cells and apicidin- or TSA-induced luciferase activities were analyzed. Black boxes represent the area of mutation.](image-url)
inhibitor (20, 21). Indeed, CpG dinucleotides within the P-gp promoter region in both HeLa and KB cells were hypermethylated (Fig. 2), and concomitantly, P-gp expression was low (Fig. 1). However, although P-gp expression was observed in HeLa cells, apicidin treatment did not alter the CpG methylation status of the promoter region in either cell line (Fig. 2). These results indicate that apicidin induction of P-gp expression requires mechanism(s) other than DNA methylation regulation. Here, we strongly suggest that dynamic changes in the chromatin structures on the P-gp promoter region are one of the possible molecular mechanisms for cell type–specific expression of P-gp by apicidin. Treatment of HeLa cells with apicidin facilitates the dissociation of HDAC1 and the recruitment of C/EBPβ and pCAF to the P-gp promoter region, which subsequently increases the level of the active gene markers, hyperacetylated histone H3 and K4-trimethylated H3, which accompany P-gp expression (Fig. 5). However, these changes were not observed in KB cells in which P-gp was not induced by apicidin. This finding suggests that alteration of histone modifications induced by apicidin might be responsible for the cell type–specific induction of P-gp expression. Although our data in Fig. 4 indicate that the C/EBPβ binding site is not involved in HDAC inhibitor-mediated P-gp expression, C/EBPβ is still recruited to the P-gp promoter region, as evidenced by ChIP analysis (Fig. 5). It is possible that C/EBPβ might be recruited to the inverted CCAATA box (Y-box) on the P-gp promoter region because C/EBPβ can transactivate the P-gp gene in human cancer cells by interaction with the Y-box and not with the C/EBPβ binding site (22, 36).

Although the specific molecular mechanism for the cell type–specific modification of the chromatin structure by the HDAC inhibitor is not completely defined, it is quite likely that each cell type may adopt different signaling pathways. Recent reports show that phosphorylation of Sp1 by PI3K/protein kinase C (PKC)ζ is critical for TSA-activated luteinizing hormone receptor expression (39) and PI3K/PKCε is required for apicidin induction of p21 WAF1/Cip1 (33). In addition, PI3K/PKCζ-mediated Sp1 phosphorylation causes a release of the repressive complex, HDAC1 and HDAC2, from the luteinizing hormone receptor gene promoter, leading to an increase in luteinizing hormone receptor expression (39). Similarly, our data show that HDAC inhibitor-mediated induction of P-gp requires dissociation of HDAC1 from the P-gp promoter region in HeLa cells (Fig. 5). In addition, we show that apicidin induction of P-gp is abrogated by treatment with the PI3K inhibitor LY294002 in HeLa cells, and Akt (downstream target of PI3K) is selectively phosphorylated/activated by apicidin treatment in HeLa cells but not KB cells (Fig. 6). Taken together, it is possible to hypothesize that the Sp1 transcription factor might be regulated through a cell type–specific activation of
PI3K/PKCζ (or PKCζ) in response to a HDAC inhibitor, leading to dynamic changes in the chromatin structure of the P-gp promoter region. Indeed, our data show that apicidin treatment leads to a PI3K-dependent increase in Sp1 phosphorylation, leading to the release of corepressor HDAC1 from the promoter region and the recruitment of coactivators including C/EBPβ and pCAF, and Pol II (Fig. 6). In addition, apicidin induction of P-gp seems to require PKCζ signaling pathway because PKCζ phosphorylation is observed in apicidin-treated HeLa cells but not in KB cells, and inhibition of PKCζ activity with its dominant-negative mutant leads to a decrease in the P-gp expression (Supplementary Data).

Previous reports have suggested a therapeutic potential for HDAC inhibitors by elucidating the mechanism of how HDAC inhibitors induce cell cycle arrest or apoptosis (3, 4, 30, 32, 33). Nevertheless, recent reports have cautiously warned about the clinical application of HDAC inhibitors because HDAC inhibitors can induce MDR via P-gp induction (27, 29). Here, we provide a novel molecular mechanism for cell type–specific expression of P-gp by the HDAC inhibitor apicidin. Apicidin treatment causes both a dissociation of the repressive complex HDAC1 from the Sp1 binding region of the P-gp promoter and a concomitant recruitment of the coactivator complex that includes C/EBPβ and pCAF, leading to subsequent changes in histone H3 hyperacetylation and H3 K4 trimethylation. These dynamic changes in the chromatin structures facilitate a recruitment of Pol II to the promoter region for activation of P-gp gene transcription. This cell type–specific induction of

**FIGURE 6.** Apicidin induction of P-gp requires PI3K signaling pathway. A. HeLa cells were transfected with pMDR1-1571-Luc for 24 h. After pretreatment with 10 μmol/L U0126, 20 μmol/L SB203580, 20 μmol/L SP600125, or 20 μmol/L LY294002 for 1 h, the cells were incubated for an additional 24 h following treatment with 1 μmol/L apicidin. Luciferase activity was determined as described in Materials and Methods. The activity was expressed as fold increase relative to the untreated control. B. After pretreatment with 20 μmol/L LY294002 for 1 h, the cells were incubated for an additional 24 h following treatment with 1 μmol/L apicidin. The expression levels of P-gp mRNA and protein were analyzed by real-time PCR and Western blot analysis, respectively. C. After treatment with 1 μmol/L apicidin in HeLa or KB cells for 24 h, the phosphorylation level of Akt was analyzed. D. HeLa cells were treated with 1 μmol/L apicidin for 24 h in the presence of 20 μmol/L LY294002. Changes in the transcription machinery associations with the promoter regions of P-gp were determined by quantitative ChIP analysis using specific antibodies as described in Materials and Methods. Columns, mean of three independent experiments; bars, SD. E. After treatment of HeLa cells with 1 μmol/L apicidin for 24 h in the presence of 20 μmol/L LY294002, cellular extracts were immunoprecipitated with Sp1 antibody. The immunocomplex was analyzed by Western blot with phosphoserine (p-Ser), Sp1, or pCAF antibodies.
P-gp expression by HDAC inhibitors is independent of changes in the DNA methylation status and dependent on PI3K activity, which will help to both better our understanding of the molecular mechanisms of MDR acquisition and aid in the development of a strategy to overcome MDR during cancer chemotherapy.

Materials and Methods

Cell Culture

HeLa, SiHa (human cervical cancer cell lines), DLD-1 (human colon cancer cell line), A172, U87 (human glioblastoma cell lines), and KB cells (human oral squamous cell carcinoma cell line) were cultured in 10% fetal bovine serum-DMEM (Invitrogen) and fetal bovine serum-RPMI 1640 (Invitrogen), respectively.

Reagents

Apicidin [cyclo(N-O-methyl-l-tryptophanyl-l-isoleucinyl-d-pipocetyl-l-2-amino-8-oxodecanoyl)] was prepared from the Fusarium sp. strain, KCTC 16677, according to a previously described method (41). TSA, sodium butyrate, valproic acid, and 5-aza-2-deoxycytidine were obtained from Sigma Chemical Co. and SAHA and MS-275 were purchased from Calbiochem.

Plasmids

The human wild-type P-gp promoter-luciferase fusion plasmid, pMDR1-1571-Luc, was generated by inserting a HindIII-BglII MDR1 promoter sequence (−1,571 to +287) into the luciferase vector, pLuc-MCS (Stratagene), between the HindIII and BglII sites. All human MDR1 promoter/luciferase deletion constructs were derived from pMDR1-1571-Luc and mutants from pMDR1-223-Luc. The human MDR1-Luc constructs pMDR1-1-223, pMDR1-1-38, pMDR1-1-79, pMDR1-1-71, pMDR1-1-43, and pMDR1-1-7 contain 510 bp (−223 to +287), 425 bp (−138 to +287), 366 bp (−79 to +287), 358 bp (−71 to +287), 330 bp (−71 to +287), and 294 bp (−7 to +287) of MDR1 promoter sequences, respectively (Fig. 4B). The mutant constructs pMDR1-1-223-mtAP-1 (MutA), pMDR1-1-223-mtY-box (MutY), and pMDR1-1-223-mtGC (MutG) were generated by site-directed mutagenesis using conditions recommended by the manufacturer (Invitrogen). Ten clones were selected and conversion of the cytosine to thymine was analyzed by DNA sequencing using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit.

Immunoblot Analysis

Cells were lysed by incubation in 50 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 20 mmol/L NaF, 1 mmol/L EDTA, 5 mmol/L EGTA, 15 mmol/L sodium pyrophosphate, 30 mmol/L p-nitrophenyl phosphate, 1 mmol/L benzamidine, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1% NP40 for 20 min at 4°C and centrifuged at 15,000 × g for 15 min at 4°C. Cell lysates were boiled in Laemmli sample buffer for 3 min and 30 μg protein was subjected to SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes. The membranes were blocked for 30 min in TBS containing 0.1% Tween 20 and 5% (w/v) dry skim milk powder and incubated overnight with primary antibodies against acetylated histone H3, phosphorylated Akt, Akt (Cell Signaling), P-gp, and actin (Santa Cruz Biotechnology). The membranes were then washed with TBS containing 0.1% Tween 20, incubated for 1 h with a secondary antibody, and visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences).

Immunoprecipitation

The 500 μg proteins from cell lysates were incubated with Sp1 antibody (Millipore Co.) overnight at 4°C. This was followed by addition of 40 μL protein A/G beads and incubation for additional 1 h. The beads were collected and washed thrice (3 min per wash) with lysis buffer. Bound proteins were eluted by boiling in Laemmli sample buffer, separated by SDS-PAGE, and subjected to immunoblot analysis.

Luciferase Assay

HeLa or KB cells were plated into 24-well plates at a density of 2 × 10⁵ per well and incubated for 24 h. For P-gp promoter analysis, the cells were transfected with 0.2 μg/well of reporter plasmids by using TransFast transfection reagent (Promega). After 24 h of transfection, the cells were treated with 1 μmol/L apicidin and the cell lysates were collected for the luciferase assay 24 h later. The luciferase activities of the cell lysates were measured according to the manufacturer’s recommendation (Promega).

siRNA Transfection

Stable RNA interference duplex (Invitrogen) was designed to silence HDAC1 (oligo ID: HSS104725) and HDAC2 (oligo ID: HSS104730). HeLa cells were transfected with 20 nmol/L control siRNA (12935-300), 20 nmol/L HDAC1, or HDAC2 stealth RNA interference using Lipofectamine 2000 (Invitrogen).

Bisulfite Genomic Sequencing

Genomic DNA was prepared from HeLa and KB cells and bisulfite conversion of genomic DNA was carried out following the protocol as described previously (42). Bisulfite-treated DNA was amplified with a primer set specific for the P-gp promoter region: 5′-ATGGCAATCCCGAGAAA-3′ and 5′-GACTTGCCAGGACTACA-3′. The PCR products were cloned into the pGEM-T vector according to the manufacturer’s recommendation (Promega). Immunoprecipitation

The 500 μg proteins from cell lysates were incubated with Sp1 antibody (Millipore Co.) overnight at 4°C. This was followed by addition of 40 μL protein A/G beads and incubation for additional 1 h. The beads were collected and washed thrice (3 min per wash) with lysis buffer. Bound proteins were eluted by boiling in Laemmli sample buffer, separated by SDS-PAGE, and subjected to immunoblot analysis.
Reverse Transcription-PCR

Total RNA was extracted using easy-BLUE Total RNA Extraction kit (iNtRON Biotechnology). The integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was used as a template for each reverse transcription-PCR by using the ImProm-II Reverse Transcription System (Promega) and Taq polymerase (Solgent). The primer sets for P-gp were 5′-CCCCATAGGAAACTTTCCAG-3′ and 5′-GGTCAACTTTCTTGGCTCTG-3′ and the primer sets for glyceraldehyde-3-phosphate dehydrogenase were 5′-CTCTAGACCATGTCATCGC-3′ and 5′-CTGCTTACACCTTTGATGTC-3′.

Quantitative Real-time PCR

Quantitative real-time PCR analysis was done using the Rotor-Gene RG-3000 (Corbett Research). Reactions were done in a 15 μL volume containing 7.5 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems), 0.6 μL of cDNA (corresponding to 60 ng of reverse-transcribed total RNA), and 5 pmol of each primer. PCR conditions consist of a 10-min hot start at 95°C followed by 40 cycles of 30 s at 95°C, 40 s at 58°C, and 20 s at 72°C. Data were analyzed using Rotor-Gene analysis software version 6.0 (Corbett Research).

ChIP Assay

ChIP assays were done using the Immunoprecipitation Assay kit (Upstate Biotechnology) according to the manufacturer’s instructions. Chromatin from 1 × 10⁶ HeLa cells was sheared by a sonicator was precleared with salmon sperm DNA–saturated protein G-Sepharose and then precipitated by antibodies to acetylated histone H3 (Cell Signaling), Sp1, pCAF, C/EBPβ, HDAC1, HDAC2, trimethyl K4 histone H3, and RNA Pol II (Abcam, Inc.). After immunoprecipitation, recovered chromatin fragments were subjected to PCR or quantitative real-time PCR using a primer set specific for the P-gp promoter: 5′-TGTTTCCAGTTTCTCCAG-3′ and 5′-AGA-TAACCACCTTTGAGTG-3′.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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