HDAC inhibitors downregulate MRP2 expression in multidrug resistant cancer cells: Implication for chemosensitization

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Abstract. Although histone deacetylase (HDAC) inhibitors are emerging as a promising class of cancer chemotherapeutic agents, their effects on multidrug resistance (MDR) are poorly understood. In this study, we investigated whether HDAC inhibitors overcome MDR phenotype. HDAC inhibitors suppress the growth of both MDR positive cancer cells KBV20C and its parental cells KB with similar potencies. In parallel, histone acetylation and p21 WAF1 expression by the HDAC inhibitors were similarly increased in both cell types, indicating that these HDAC inhibitors are poor substrates of ABC drug transporters and effective in MDR cancer cells. In addition, multidrug resistance protein 2 (MRP2) expression is selectively attenuated by HDAC inhibitors, especially SAHA and TSA, in KBV20C cells, whereas MDR1 and BCRP expressions are not affected. This downregulation of MRP2 contributes to increase in paclitaxel-induced G2/M arrest and apoptosis, which might be due to intracellular accumulation of paclitaxel. Collectively, our data provide a molecular rationale for the application of HDAC inhibitors to overcome MDR in cancer cells.

Introduction

Multidrug resistance (MDR), by which cancer cells are resistant to many structurally and functionally unrelated anticancer drugs, is a significant impediment in cancer chemotherapy (1,2). The MDR phenotype is often due to over-expression of ATP binding cassette (ABC) multidrug transporters, such as P-glycoprotein (P-gp; ABCB1), BCRP (ABCG2), and multidrug resistance proteins (MRPs). P-gp, a 170-kDa transmembrane glycoprotein, is the first human ABC transporter whose cDNA was cloned and characterized based on its ability to confer a multidrug-resistance phenotype to cancer cells (3). MRP1 (ABCC1) and MRP2 (ABCC2) are identified as multidrug-resistance genes and associated with transport of glutathione conjugates of many toxic compounds (4,5). Breast cancer resistance protein (BCRP; ABCG2) is a ubiquitous ABC transmembrane protein that plays a significant role in absorption, distribution, and elimination of its substrate drugs. It also confers resistance in cancer cells to a variety of cancer chemotherapeutic agents such as mitoxantrone, topotecan, and methotrexate (6). These ABC transporters are able to reduce the intracellular concentrations of taxanes and vinca alkaloids to a nontoxic level, which confer MDR phenotype to cancer cells. In addition, over-expression of these ABC transporters has been closely correlated with poor prognosis for various human cancers (2,7).

Recently, histone deacetylase (HDAC) inhibitors are appreciated as one of emerging class of anticancer drugs, because HDAC inhibitors promote accumulation of acetylated histones and subsequent induction of a specific pre-programmed set of genes which are required for growth arrest, differentiation and apoptosis. Several HDAC inhibitors, such as vorinostat (suberolanilide hydroxamic acid, SAHA) (8,9), MS275 (10,11), phenylbutyrate (12) and depsipeptide FK228 (13,14), have shown potent anti-tumor activities and are currently in Phase I or II clinical trials. Among them, vorinostat (Zolinza, Merck & Co., Inc.) has been approved for the treatment of cutaneous T-cell lymphoma (15).

Recent reports demonstrate that HDAC inhibitor treatment increases the expression of P-gp, and subsequent induction of MDR phenotype, which might limit clinical application of HDAC inhibitors (16-19). However, the effects of HDAC inhibitors on MDR cancer cells are poorly investigated. In this study, we investigate the effects of various HDAC inhibitors on MDR cancer cells. Our data show that HDAC inhibitors suppress cell growth with similar potencies in both MDR-positive and -negative cancer cells, which is accompanied by an increase in p21WAF1 expression and an accumulation of acetylated histones.
of acetylated histone. In addition, treatment with HDAC inhibitors, especially SAHA and TSA, leads to a specific downregulation of MRP2, but not MDR1 and BCRP, which is necessary for sensitization of the cancer cells against paclitaxel.

Materials and methods

Cell culture and cytotoxicity test. Human nasopharyngeal epidermal carcinoma cell line KB was grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). The MDR subclone KBV20C cells were maintained in the medium containing additional 20 nM vincristine (Sigma Chemical, St. Louis, MO) as described previously (20). Paclitaxel, TSA, valproic acid and sodium butyrate were obtained from Sigma, and apicidin, SAHA and MS275 were form Calbiochem (San Diego, CA). Cytotoxicity was determined by the MTS assay (Promega, Madison, WI) according to the manufacturer's instructions.

Cell cycle analysis. KBV20C cells were treated with indicated concentration of paclitaxel and/or HDAC inhibitors for 24 h. Cells were harvested, washed twice with ice-cold PBS and fixed in 70% ethanol at -20°C. Cells were washed with PBS, incubated with 100 μg/ml RNase A at 37°C for 30 min, stained with 25 μg/ml propidium iodide (Sigma) and analyzed with flow cytometry.

Apoptosis assays. After treatment of KBV20C cells with HDAC inhibitors and/or paclitaxel for 24 h, cells were harvested, and the extent of apoptosis was determined by flow cytometric analysis using Annexin V-FITC apoptosis detection kit (BD Biosciences, San Diego, CA), according to the manufacturer's description. And, PARP cleavage was analyzed by immunoblot analysis.

Histone isolation. The cells were harvested with PBS and washed with RSB buffer (10 mM Tris-Cl, 10 mM NaCl, 3 mM MgCl₂). Then the cells were homogenized with RSB buffer containing 0.5% NP-40 using loose-fitting glass homogenizer (10 strokes). After centrifuging at 1000 x g for 10 min, the pellet was resuspended in 0.8 M HCl solution containing 5 mM MgCl₂, and then incubated for 20 min on ice. Histone fractions were precipitated by adding 50% trichloroacetic acid and collected by centrifuging at 12,000 x g for 20 min at 4°C. The histone fractions were boiled in Laemmli sample buffer for 3 min, and were resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

Immunoblot analysis. Cells were lysed with 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 15 mM sodium pyrophosphate, 30 mM p-nitrophenyl phosphate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40 for 20 min at 4°C and centrifuged at 15,000 x g for 15 min at 4°C. Cell lysates were boiled in Laemmli sample buffer for 5 min, and 30 μg of protein was subjected to SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes. The membranes were blocked for 30 min in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% (w/v) dry skim milk powder and incubated overnight with primary antibodies to p21<sup>WAF1</sup>, PARP (Santa Cruz Biotechnology, Santa Cruz, CA), Acetyl-histone H3, Histone H3, and α-tubulin (Cell Signaling, Beverly, MA). The membranes were then washed with TBS-0.1% Tween-20, incubated for 1 h with a secondary antibody, and visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences, IL).

RT-PCR. Total RNA was extracted using the easy-BLUETM total RNA extraction kit (iNTRON Biotechnology, Sungnam, Korea), and the integrity of the RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was used as a template for each reverse transcriptase (RT)-mediated PCR (RT-PCR) reaction using the ImProm-II Reverse Transcription System (Promega) and Taq polymerase (Solgent, Daejeon, Korea). The PCR primer sets were 5′-CCCATCATTTGCAATAGGAGG-3′ and 5′-GGTCTAAACTTCTGCTCTGA-3′ for P-pg; 5′-ATGTCAGTGGGGATATACCCAG-3′ and 5′-GAAGACTGAACCTCCTTTTCTCTTTCT-3′ for MRP1; 5′-ACAGAGGCTGTTGGGACCAC-3′ and 5′-ACCATTACCTTTGCTAGTGGCT-3′ for MRP2; 5′-CAGATGTACAGTTCCAGAGGAT-3′ and 5′-CAGTCCAGAGCAGTTCG-3′ for BCRP; 5′-CTCATGGACACGTCCTGACCAC-3′ and 5′-CTGTCCTTACCACCTTCGATGTC-3′ for GAPDH.

Results

Growth inhibition of MDR-positive cancer cells by HDAC inhibitors. In order to evaluate the therapeutic potential of HDAC inhibitors on MDR cancer cells, we first determined the effects of various HDAC inhibitors on the proliferation of MDR positive cells KBV20C. Since KBV20C cells are more resistant to chemotherapeutic agents through over-expression of ABC transporter genes, the cells are widely used for studying MDR. As seen in Table I, KBV20C cells are more resistant to paclitaxel and vincristine, compared to parental line KB cells (up to 360- and 715-fold, respectively). Most of HDAC inhibitors (TSA, SAHA, MS275, valproic acid, and sodium butyrate) showed similar cytotoxicities against both KB and KBV20C cells (Table I), indicating that these HDAC inhibitors might be poor substrates of ABC drug efflux pumps, and be sufficiently accumulated into cancer cells to exert their growth inhibition effects. However, effect of apicidin was distinguished from the other HDAC inhibitor effects. The growth inhibitory effect of apicidin against KBV20C cells was weaker, comparing to KB cells; the IC<sub>50</sub> value was shifted from 0.42 μM (in KB cells) to 5.75 μM (in KBV20C cells) (Table I). Taken together, these results suggest that most of HDAC inhibitors except apicidin inhibit the growth of both MDR-positive and -negative cells with similar potencies.

Changes in histone acetylation levels and p21<sup>WAF1</sup> expression by HDAC inhibitors in KBV20C cells. It has been well demonstrated that HDAC inhibitors induces p21<sup>WAF1</sup> gene expression through histone hyperacetylation of its promoter region, leading to cell cycle arrest (21-23). We next examined whether
HDAC inhibitor could induce p21\textsuperscript{WAF1} gene expression as well as an accumulation of acetylated histone in MDR cancer cells. Treatment with HDAC inhibitors led to a dose-dependent increase in p21\textsuperscript{WAF1} expression as well as acetylated histone H3 in KB cells (Figs. 1 and 2). Similar expression patterns by HDAC inhibitors (SAHA, TSA and MS275) were observed in KBV20C cells. However, the apicidin effect in KBV20C cells was weaker than in KB cells. An accumulation of acetylated histone H3 in KB cells appeared at 0.3 μM of apicidin and reached maximal accumulation at 1 μM, while the accumulation in KBV20C cells observed at 1 μM, and reached maximal accumulation at 10 μM (Fig. 1). In addition, apicidin induction of p21\textsuperscript{WAF1} expression in KBV20C cells required much higher concentration (Fig. 2). These results strongly support the notion that HDAC inhibitors (SAHA, TSA and MS275) are poor substrates of ABC drug efflux pumps.

Specific downregulation of MRP2 by HDAC inhibitors. We next examined the effect of HDAC inhibitors on the expression of ABC transporter genes. The expression levels of MDR1, MRP1, MRP2 and BCRP were analyzed by RT-PCR. Among them, MDR1, MRP2 and BCRP were highly expressed in KBV20C cells, which might be responsible for MDR phenotype, however, MRP1 was not expressed (Fig. 3). The expression of MDR1 and BCRP was not affected by HDAC inhibitors (SAHA, TSA, MS275 and apicidin), while MRP2 expression was dose-dependently downregulated by HDAC inhibitors, especially by SAHA and TSA (Fig. 3A). Treatment with 1 μM SAHA led to >50% decrease in MRP2 expression, and >90% decrease was observed at 3 μM concentration (Fig. 3A). The effect of TSA was comparable to SAHA’s effect. However, MS275 and apicidin slightly decreased the MRP2 expression at 3 μM concentration (Fig. 3B).

Table I. Growth inhibition by various HDAC inhibitors in KB and KBV20C cells.\textsuperscript{a}

<table>
<thead>
<tr>
<th>HDAC inhibitors</th>
<th>IC\textsubscript{50} (μM) KB</th>
<th>IC\textsubscript{50} (μM) KBV20C</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>0.004</td>
<td>1.44</td>
<td>360</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.008</td>
<td>5.72</td>
<td>715</td>
</tr>
<tr>
<td>Apicidin</td>
<td>0.42</td>
<td>5.75</td>
<td>14.7</td>
</tr>
<tr>
<td>TSA</td>
<td>0.13</td>
<td>0.10</td>
<td>0.78</td>
</tr>
<tr>
<td>SAHA</td>
<td>0.23</td>
<td>0.15</td>
<td>0.65</td>
</tr>
<tr>
<td>MS275</td>
<td>0.18</td>
<td>0.28</td>
<td>1.56</td>
</tr>
<tr>
<td>Valproic acid\textsuperscript{b}</td>
<td>18.6</td>
<td>36.1</td>
<td>1.94</td>
</tr>
<tr>
<td>Sod. butyrate\textsuperscript{b}</td>
<td>19.9</td>
<td>25.2</td>
<td>1.26</td>
</tr>
</tbody>
</table>

\textsuperscript{a}KB and KBV20C cells were seeded at a density of 5x10\textsuperscript{3}/well in 96-well plates and treated with various concentrations of vincristine, paclitaxel, and HDAC inhibitors (apicidin, MS275, SAHA, TSA, valproic acid, and sodium butyrate) for 48 h. Cell viability was determined using the MTS assay. \textsuperscript{b}IC\textsubscript{50} values of these cells are millimolar (mM) concentrations.

Concomitantly, MRP2 protein level was decreased by SAHA treatment but not by MS275 (Fig. 3C). These results indicate that MRP2 expression is specifically downregulated by
SAHA or TSA and this downregulation may affect MDR phenotype.

Downregulation of MRP2 increases the sensitivity of KBV20C cells to paclitaxel. It is known that paclitaxel induces cell cycle arrest at mitotic stage via disrupting micro-tubule function and has apoptotic potential (24,25). In addition, recent reports show that MRP2 transports taxanes and confers substantial resistance against paclitaxel (26). We thus examined whether downregulation of MRP2 by SAHA could affect the actions of paclitaxel. We first analyzed the cell cycle progression in the presence of SAHA and/or paclitaxel. For this experiment, we chose the minimal concentration of paclitaxel at which the cell cycle of KBV20C cells was not affected (Fig. 4). Single treatment with SAHA or MS275 led to an increase in the cell population at G0/G1 phase (Fig. 4), which seemed to be mediated by inhibition of CDKs through p21WAF1 induction. However, paclitaxel in combination with SAHA led to an increase in G2/M phase cells, but not with MS275 (Fig. 4), which was well correlated with the previous observations that MRP2 is specifically downregulated by SAHA but not by MS275. These results imply that specific downregulation of MRP2 by SAHA leads to an increase in intracellular accumulation of paclitaxel which is responsible for mitotic arrest. To confirm this notion, we examined the effect of SAHA on the paclitaxel-induced apoptosis. As shown in Fig. 5, treatment with SAHA or MS275 alone did not increase apoptosis, whereas combination treatment with SAHA and paclitaxel synergistically increased apoptosis, as evidenced by the increase in Annexin V-positive cells and PARP cleavage. These results strongly support the notion that SAHA-mediated
downregulation of MRP2 contributes to the sensitization of cancer cells to paclitaxel.

Discussion

Chemotherapy is the most effective treatment for metastatic cancers. The effectiveness of chemotherapy, however, is seriously limited by MDR which is mainly due to the over-expression of ABC drug transporters (1, 2). ABC drug transporters function as drug efflux pumps which actively transport anticancer drugs from the inside to the outside of cancer cells and decrease the intracellular accumulation of anticancer drugs inside cancer cells necessary for cytotoxic activity. Therefore, novel agents which can inhibit the drug transporter function of ABC drug transporters or its expression have the potential to overcome the MDR phenotype by enhancing intracellular accumulation of anticancer drugs. The present study shows that HDAC inhibitors, especially SAHA and TSA, specifically downregulate the expression of MRP2 in MDR cancer cell lines, but not MDR1 and BCRP expressions (Fig. 3). In addition, this downregulation of MRP2 is closely associated with synergistic increase in paclitaxel-induced G2/M arrest and apoptosis (Figs. 4 and 5). These findings imply that HDAC inhibitors, especially SAHA, might be useful for MDR cancer treatment. At present time we cannot explain the exact molecular mechanism for the specific downregulation of MRP2. Recent report shows that DNA methyltransferase 1 is downregulated by treatment with HDAC inhibitor, resulting from the formation of transcriptional repressive complex on the promoter region (27). So, it is possible that similar repressive complexes might be specifically recruited to the MRP2 promoter region by HDAC inhibitors, which is in need of future study. Our observation that apicidin (HDAC 2 and 3 inhibitor) and MS275 (HDAC 1, 2, 3, and 9 inhibitor) do not downregulate MDR2 in contrast to the pan-HDAC inhibitors TSA and SAHA might point to class II HDACs as potential targets in mediating the MRP2 downregulation.

Recent accumulating evidences strongly suggests that HDAC inhibitors are a novel class of anticancer drugs. HDAC inhibitors induce a specific pre-programmed set of genes which are required for growth arrest, differentiation and apoptosis, resulting from dynamic changes in chromatin structures by accumulation of acetylated histones (21, 22, 28). Indeed, our data show that treatment with HDAC inhibitors shows a dramatic increase in p21WAF1 expression in MDR cancer cells (Fig. 2), which is accompanied by histone hyper-acetylation (Fig. 1). Concomitantly, HDAC inhibitor treatment led to a cell cycle arrest at G0/G1 phase in MDR cancer cells (Fig. 4), which might be attributable to p21WAF1 over-expression by HDAC inhibitors (Fig. 2). In addition, HDAC inhibitors show significant growth inhibition in MDR-positive cancer cells (Table I). Interestingly, all of these HDAC inhibitor effects are observed in both MDR-negative and -positive cells with similar potencies. These results strongly indicate that HDAC inhibitors are poor substrates of ABC drug transporters, which allow their intracellular accumulation for the pharmacological actions in MDR cancer cells. It is interesting that the only HDAC inhibitor harboring a cyclic tetrapeptide structure (apicidin) displays relative resistance in KBV20C cells in contrast to the hydroxamic acids (TSA and SAHA) and short chain fatty acids (valproic acid and butyrate), indicating that apicidin might be a partial substrate of ABC drug transporters.

In conclusion, the present study shows that HDAC inhibitors, especially SAHA, are poor substrates of ABC drug transporters and specifically downregulate MRP2 expression in MDR cancer cells, implying a possible application of HDAC inhibitors for MDR cancer treatment. These findings show the therapeutic potentials of HDAC inhibitors against MDR cancer and provide a molecular rationale for a novel application of HDAC inhibitors to overcome MDR in cancer.

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References


