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4-Hexylresorcinol Inhibits Class I Histone Deacetylases in Human Umbilical Cord Endothelial Cells

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Featured Application: Histone deacetylase inhibitor has broad spectrum therapeutic merits. This study demonstrated that 4-hexylresorcinol was a novel histone deacetylase inhibitor.

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Abstract: Histone deacetylases (HDACs) are key enzymes for post-translational modification and influence on various cellular activities. Thus, HDACs are associated with many diseases and their inhibitors have clinical significance. Here, 4-Hexylresorcinol (4HR) was studied as an inhibitor for class I HDACs using the HDAC inhibitor (HDACi) Trichostatin-A as a positive control. The 4HR was administered 1–100 μ M to human umbilical endothelial cells (HUVECs) and the HDAC expression and activity were examined. The 4HR decreased the expression level of HDAC1, 3, 4, and 5 in a time and dose-dependent manner. The 4HR also increased acetylated lysine and decreased HDAC activity significantly ($p < 0.05$). Collectively, 4HR was a new class I HDAC inhibitor that reduced the expression and activity of HDAC in HUVECs.

Keywords: 4-hexylresorcinol; histone deacetylase; mitochondria; ATP

1. Introduction

Histone deacetylase (HDAC) is an enzyme for removing the acetyl group from lysine in the protein [1]. Many types of proteins have lysine as their amino acid. Adding or removing the acetyl group from lysine is a post-translational modification of the protein and affects protein conformation and function [2]. DNA has a strong negative charge because of phosphate groups. Acetylated histone loses its positive charge and relaxes the binding between histone and DNA. Accordingly, acetylation and deacetylation of histone can regulate specific gene transcription [1,2]. Except for histone, the function of the other proteins is also influenced by HDAC as a post-translational modification process [2]. There are several types of HDACs in 4 classes (Table 1). Their intracellular localizations are various according to their functional demands. The main localizations for HDACs are the nucleus and mitochondria [1].

Considering its profound implications in cellular function, class I HDACs are involved in many types of physiological processes and diseases such as cardiovascular disease, autoimmune disease, musculoskeletal disease, and malignancy [1,3,4]. HDAC1 regulates gene expression and its expression is elevated in transformed cells [5]. HDAC2 is overexpressed

in pancreatic cancer [6]. The overexpression of HDAC2 is also observed in pathologic cardiomegaly [7]. A representative broad-spectrum HDAC inhibitor (HDACi)-trichostatin A (TSA) inhibits cardiac hypertrophy [8]. TSA is also used for the treatment of established cardiac hypertrophy [9]. The administration of HDACi is helpful for muscle regeneration in muscle dystrophy [10]. Valproic acid was originally developed as an anti-convulsant and HDACi. Valproic acid improves nerve regeneration and motor function in the damaged rat model [11]. TSA treats rheumatism by suppressing the expression level of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) [12]. Butyrate is an organic chemical of bacterial origin and HDACi [13]. The administration of butyrate reduces pain in epileptic rats [13] and inhibits the proliferation of colorectal cancer cells [4].

Table 1. Classification of HDAC.

Class	Members
I	HDAC1, 2, 3, 8
IIA	HDAC4, 5, 7, 9
IIB	HDAC6, 10
III	SIRT1, 2, 3, 4, 5, 6, 7
IV	HDAC11

HDAC: histone deacetylase, SIRT: sirtuin.

Resorcinolic lipid is an organic chemical of bacterial origin [14]. The substance 4-Hexylresorcinol (4HR) is a synthetic resorcinolic lipid and has been developed as an antiseptic agent [14]. The 4HR suppresses the respiration of micro-organisms and induces dormancy [15]. The long alkyl group of 4HR binds to tyrosinase effectively and inhibits enzyme activity [16]. Accordingly, 4HR can be used to prevent melanosis of food [16]. As it is considered as a food additive, toxicological studies have been widely done. In these studies, anticancer effects have been found [17,18]. Based on these results, the detailed anticancer mechanisms of 4HR have been investigated [19,20]. The 4HR suppresses the nuclear factor- κ B (NF- κ B) pathway by inhibiting transglutaminase-2 (TG-2) in oral cancer cells [19,21]. Unlike oral cancer cells, 4HR administration reduces the formation of foreign body giant cells in the silk-based graft material [22] and suppresses TNF- α expression in a deep burn [23]. Recently, 4HR has been identified as a potent M2 macrophage polarizing agent [24]. Transforming growth factor- β 1 (TGF- β 1) is increased by 4HR, while decreasing TNF- α expression in RAW263.7 cells [25], and the expression level of vascular endothelial cell growth factor (VEGF) is increased by 4HR via the TGF- β 1 mediated pathway in human umbilical endothelial cells (HUVECs) [26]. During investigation of the mechanism of TGF- β 1 expression by 4HR administration, we found that the broad function of 4HR might be similar to that of HDACi. The 4HR has a hydrophobic alkyl group [14] and the length of the alkyl group is similar to that of the functional group in other HDACi [27]. Though 4HR does not have an acetyl group at the end of the hexyl group, hydroxyl-benzene can do the role as a cap group. Therefore, the possibility of HDACi for 4HR is highly probable.

The purposes of this study were to clarify 4HR as Class I HDACi in HUVECs. Class I HDAC (HDAC1 and 3) and class IIA HDAC (HDAC4 and 5) expression levels and acetylated lysine level were evaluated by Western blot. For comparison purposes, TSA was treated and the expression levels of HDAC1, 3, 4, and 5 and Ac-lys were investigated. The enzymatic activity of Class I HDAC was also investigated after 4HR administration.

2. Materials and Methods

2.1. HUVEC Culture

HUVECs (Lonza, Walkersville, MD, USA) were cultured as previously described in our publications [28,29]. The medium was endothelial cell growth medium-2 (Clonetics, Lonza). Cells were cultured in CO₂ incubator. To prevent mycoplasma contamination, tests were done on a regular basis.

2.2. Western Blot and HDAC Inhibitory Assay

When HUVECs were grown, approximately 70% confluent, the cells were treated with 1, 10, and 100 μM 4HR for 2, 8, or 24 h; control cells were treated with 1 mL of normal saline. TSA known HDACi was purchased from Sigma-Aldrich (CAT#: T8552, St. Louis, MO, USA). TSA inhibits HDAC1, HDAC4, and HDAC6 and IC_{50} is 6 nM, 38 nM, and 8.6 nM, respectively [27]. Accordingly, 1, 10, and 100 nM TSA were applied for 24 h. According to the datasheet from the manufacturer, IC_{50} for TSA was announced as 20 nM. The expression of TGF- β 1 and apoptosis-inducing factor (AIF) was increased by 4HR administration [26,28]. The expression changes of TGF- β 1 and AIF after 20 nM TSA application were also investigated. Cultured cells were harvested with protein lysis buffer (PRO-PREP, iNtRON Biotechnology Inc., Sungnam, Korea) and underwent Western blotting for HDAC1, HDAC3, HDAC4, HDAC5, and Ac-lys. Antibodies for HDACs, TGF- β 1, AIF, and Ac-lys were purchased from SantaCruz Biotechnology (Santa Cruz, CA, USA). The quantification of the proteins was done as described previously [28,29].

To assess the effect of 4HR administration on HDAC enzyme activity, 1, 10, and 100 μM of 4HR were applied and cellular lysates were collected after 2, 8, and 24 h. A subsequent procedure was done with a commercially available kit (CAT: ab156064, Abcam, Cambridge, UK). This kit can measure the activity of HDAC1, 2, 3, and 8 (Class I HDAC) according to the product datasheet. After the preparation of samples, HDAC assay buffer and substrate were placed into the reaction wells. Inhibitor and developer were added to wells and mixed thoroughly. The prepared samples were added to each well and incubated for 20 min at room temperature. Then, stop solution was added and incubated for 10 min at room temperature. The fluorescence intensity was measured by plate reader.

3. Results

3.1. HR Decreased HDAC Expression and Increased Ac-Lys

The administration of 1 to 100 μM 4HR decreased the expression of HDAC1, 3, 4, and 5 in HUVECs (Figure 1a). If HDAC activity is reduced, acetylated lysine (Ac-lysine) will be increased [1]. Accordingly, the administration of 1 to 100 μM 4HR increased the expression of Ac-lysine in HUVECs (Figure 1b). TSA is known as broad-spectrum HDACi [30]. The expression changes of HDACs and Ac-lysine after administration of TSA were examined to compare them to those of 4HR administration. The administration of TSA decreased the expression of HDAC1, 3, 4, and 5 in HUVECs (Figure 2a). The administration of 1 to 100 nM TSA increased the expression of Ac-lysine in HUVECs (Figure 2b).

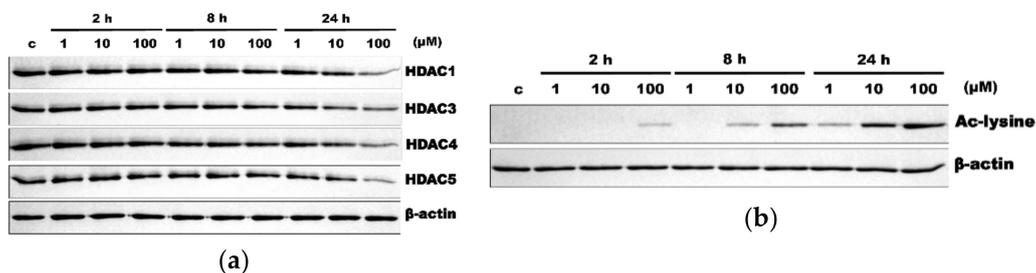


Figure 1. Western blot analysis. (a) The administration of 4HR decreased the expression of HDACs in HUVECs. The densitometric measurements are shown in Figure S1. (b) The administration of 4HR increased the expression of Ac-lysine in HUVECs. The densitometric measurements are shown in Figure S2.

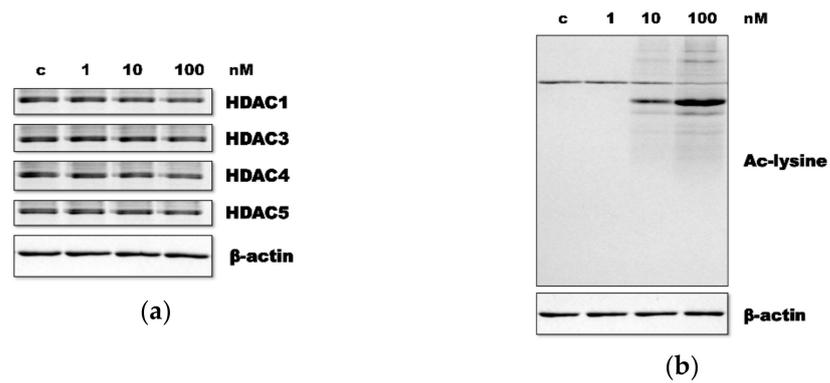


Figure 2. Western blot analysis. (a) The administration of TSA slightly decreased the expression of HDACs in HUVECs. (b) The administration of TSA increased the expression of Ac-lysine in HUVECs.

The administration of 20 nM TSA did not significantly change the expression of TGF-β1 in HUVECs (Figure 3a). The administration of 20 nM TSA increased the expression of AIF in HUVECs (Figure 3b).



Figure 3. Western blot analysis after 20 nM TSA application. (a) The administration of 20 nM TSA did not increase the expression of TGF-β1 prominently in HUVECs. (b) The administration of 4HR increased the expression of AIF in HUVECs.

3.2. HR Inhibited Class I HDAC Activity in HUVECs

The administration of 1 to 100 μM 4HR decreased the enzyme activity of HDACs in HUVECs (Figure 4). HDAC enzyme activity was decreased significantly in 10 and 100 μM 4HR administration at 8 h and 24 h ($p < 0.05$).

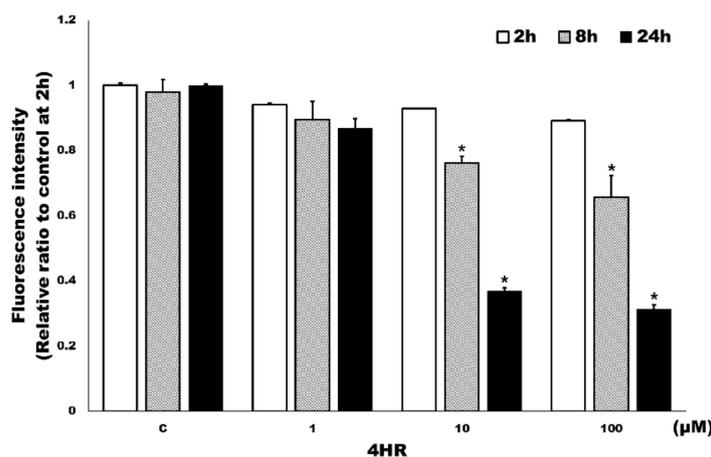


Figure 4. Enzyme inhibition assay. The administration of 4HR decreased HDAC enzyme activity (* $p < 0.05$ compared to the control group).

4. Discussion

In our previous study, 4HR had wide functions such as antimicrobial effects, anti-cancer effects, proangiogenic effects, anti-inflammatory effects, increasing calcification, and reducing metabolism. These divergent effects should be related to the broad spectrum regulation of cellular events. HDACs are key enzymes in post-translational modifications

for the epigenetic regulation of various cellular events. In this study, we found the 4HR's role as an HDACi and that 4HR decreased the expression level of HDAC1, 3, 4, and 5 in HUVECs. (Figure 1a). In addition, HDAC's enzyme activity was decreased by 4HR administration (Figure 4). With inhibition of HDAC expression and activity by 4HR, Ac-lys is increased dose and time-dependently by 4HR administration (Figure 1b). This inhibitory effect on HDACs was comparable to those of TSA (known HDACi) (Figure 2). To our best knowledge, this was the first study of 4HR as novel HDACi.

In our previous study, 4HR increased the expression level of AIF in HUVECs [28,29]. In this study, TSA increased the expression level of AIF in HUVECs, too (Figure 3b). However, increased AIF expression by TSA did not result in the increasing TGF- β 1 expression (Figure 3a). Though IC₅₀ of TSA is <38 nM, a decreased expression level of cyclin A was observed at 10 μ M TSA administration [27]. As 1–100 nM TSA was used in this study, the increasing TGF- β 1 expression might be observed in a much higher concentration of TSA. Interestingly, 0.3 μ M TSA administration increased TGF β type II receptor expression [31]. As the expression level of AIF was observed in 20 nM TSA application, it looked evident that expression level of TGF- β 1 might not be directly associated with AIF expression. Sp1 is a transcription factor for TGF- β 1 and its expression is increased by butyrate—another kind of HDACi [32]. Interestingly, 4HR also increases Sp1 expression in SCC-9 cells [20]. Therefore, increased TGF- β 1 expression by 4HR administration might be associated with its HDACi activity and subsequent promotion of Sp1 expression.

Every HDAC has a hydrophobic pocket as its enzymatic domain. The 4HR has a long alkyl group and is strongly hydrophobic [14]. Therefore, 4HR might be a decoy molecule which could block the hydrophobic enzymatic domain (Figure 5). TSA has a six carbon chain for inserting the hydrophobic pocket of HDAC (Figure 5) and its derivatives also have a similar structure [27]. The 4HR has a six carbon alkyl group, but does not have an acetyl group at the end of alkyl chain [14]. The binding affinity of 4HR on each HDAC might be influenced by its own structure. As 4HR does not have acetyl group, the binding affinity to HDAC might be weaker than TSA or its derivatives. Actually, TSA showed its HDACi activity at a much lower concentration than 4HR (Figures 1 and 2). Adding an acetyl or amine group to 4HR might be an interesting approach for novel HDACi development. In the search for novel HDACi, TSA derivatives having carboxylic acid group have 100 nM as IC₅₀, however, TSA derivatives having acetylated lysine have 31 μ M as IC₅₀ [27]. Hydroxyl-benzene group might do its role as cap (Figure 5). TSA inhibits angiogenesis by suppressing hypoxia-inducible factor-1 α (HIF1 α) [33]. However, 4HR increases angiogenesis via the HIF1 α -independent pathway [34]. Though 4HR is known as an antiseptic, the administration of 4HR on a microorganism reduces the metabolic rate and does the role as a chemical chaperone [14]. Accordingly, the surviving microorganism undergoes dormancy [15]. The microorganism in the state of dormancy extremely reduces its metabolic rate and resists environmental stress [15]. Based on these observations, 4HR in eukaryotes may be mimicking the action in the prokaryotes. Though cellular dormancy in eukaryote is hardly defined, the dormancy-inducing property of 4HR may be beneficial for cancer treatment. Interestingly, the anticancer effect of 4HR was identified during its toxicology study by National Toxicology Program (NTP) team [18].

In this study, the HDACi activity of 4HR was much lower than that of TSA. As the function of HDAC is broad, its inhibition should be tailored to the intended purpose. The complete knock-out of HDAC1 and 3 is lethal in the embryonic period [35,36]. Pharmacological inhibition of HDAC has many benefits over genetic deletion such as reversible and incomplete inhibition [2]. The 4HR might be less potent for anticancer purposes than TSA. However, 4HR might have more advantages for wound healing acceleration or treatment of metabolic disease than TSA. Recently, a Chinese team found that 4HR administration increases bone mineral density in the osteoporosis animal model [37].

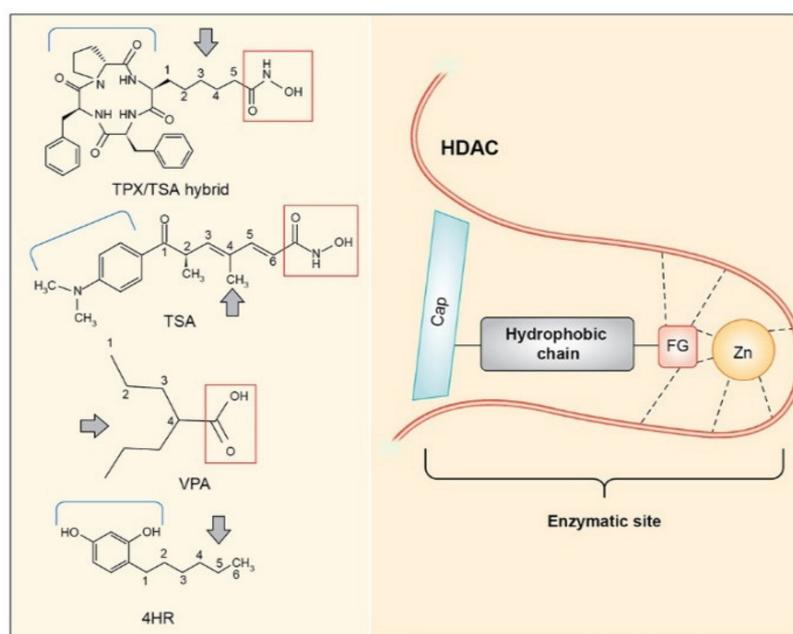


Figure 5. Model of 4HR action as a histone deacetylase inhibitor (HDACi). As the enzymatic site of HDACs is a deep hydrophobic pocket, HDACi has a hydrophobic group (arrows). The functional group (FG, red rectangle) allows hydrogen bonds (dotted lines) with zinc ions and surrounding amino acids. The cap (sky blue) is for blocking the pore to prevent the entrance of acetylated proteins. Trapoxin (TPX), trichostatin A (TSA) hybrid, and TSA have all three groups. Valproic acid (VPA) has a hydrophobic chain and functional group. The 4HR has a cap and hydrophobic chain.

5. Conclusions

Collectively, 4HR was found to be novel HDACi. The 4HR inhibited class I HDAC activity and their protein expression. Considering functional divergence of HDAC, the therapeutic effect of 4HR administration should be studied independently according to its target disease.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11083486/s1>, Figure S1: Relative expressions of histone deacetylase (HDAC) after 4-hexylresorcinol administration, Figure S2: Relative expressions of acetylated lysine after 4-hexylresorcinol administration.

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