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# Inhibition of class IIa histone deacetylase activity by gallic acid, sulforaphane, TMP269, and panobinostat

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ABSTRACT

Histone deacetylase (HDAC) inhibitors are gaining increasing attention as potential therapeutics for cardiovascular diseases as well as cancer.

We recently reported that the class II HDAC inhibitor, MC1568, and the phytochemical, gallic acid, lowered high blood pressure in mouse models of hypertension. We hypothesized that class II HDACs may be involved in the regulation of hypertension. The aim of this study was to determine and compare the effects of well-known HDAC inhibitors (TMP269, panobinostat, and MC1568), phytochemicals (gallic acid, sulforaphane, and piceatannol), and anti-hypertensive drugs (losartan, carvedilol, and furosemide) on activities of class IIa HDACs (HDAC4, 5, 7, and 9).

The selective class IIa HDAC inhibitor, TMP269, and the pan-HDAC inhibitor, panobinostat, but not MC1568, clearly inhibited class IIa HDAC activities. Among the three phytochemicals, gallic acid showed remarkable inhibition, whereas sulforaphane presented mild inhibition of class IIa HDACs. Piceatannol inhibited only HDAC7 activity. As expected, the anti-hypertensive drugs losartan, carvedilol, and furosemide did not affect the activity of any class IIa HDAC.

In addition, we evaluated the inhibitory effect of several compounds on the activity of class l HDACs (HDAC1, 2, 3, and 8) and class IIb HDAC (HDAC6). MC1568 did not affect the activities of HDAC1, HDAC2, and HDAC3, but it reduced the activity of HDAC8 at concentrations of 1 and 10 μM. Gallic acid weakly inhibited HDAC1 and HDAC6 activities, but strongly inhibited HDAC8 activity with effectiveness comparable to that of trichostatin A. Inhibition of HDAC2 activity by sulforaphane was stronger than that by piceatnnaol.

These results indicated that gallic acid is a powerful dietary inhibitor of HDAC8 and class IIa/b HDAC activities. Sulforaphane may also be used as a dietary inhibitor of HDAC2 and class IIa HDAC. Our findings suggest that the class II HDAC inhibitor, MC1568, does not inhibit class IIa HDAC, but inhibits HDAC8.

## 1. Introduction

Histone deacetylases (HDACs) are a family of enzymes that remove acetyl groups from ε-*N*-acetyl lysine amino acid on a histone and many non-histone proteins including p53, signal transducers and activators of transcription (STAT3), E2F1, heat shock protein 90 (Hsp90), and NF-κB [1]. Deacetylation by HDACs causes chromatin compaction and transcription repression, whereas acetylation of histones facilitates chromatin access and induces activation of gene transcription.

HDACs are divided into four classes depending on structure. Class I HDACs include HDAC1, HDAC2, HDAC3, and HDAC8 which are mainly distributed in the nucleus. Class IIa HDACs include HDAC4, HDAC5, HDAC7, and HDAC9 which can translocate from the nucleus to the cytoplasm. Class IIb HDACs include HDAC6 and HDAC10. HDAC11 is a class IV HDAC. These HDACs are all zinc-dependent. HDAC expression and activity are dysregulated in various diseases including asthma [2], chronic obstructive pulmonary disease (COPD) [3], cancer [4], cardiac hypertrophy [5], and neurodegenerative and psychological disorders [6]. Thus, HDAC inhibitors could be a potential therapeutic target for many diseases.

To date, vorinostat (SAHA), romidepsin (depsipeptide), panobinostat (LBH589), and belinostat (PXD101) HDAC inhibitors have been

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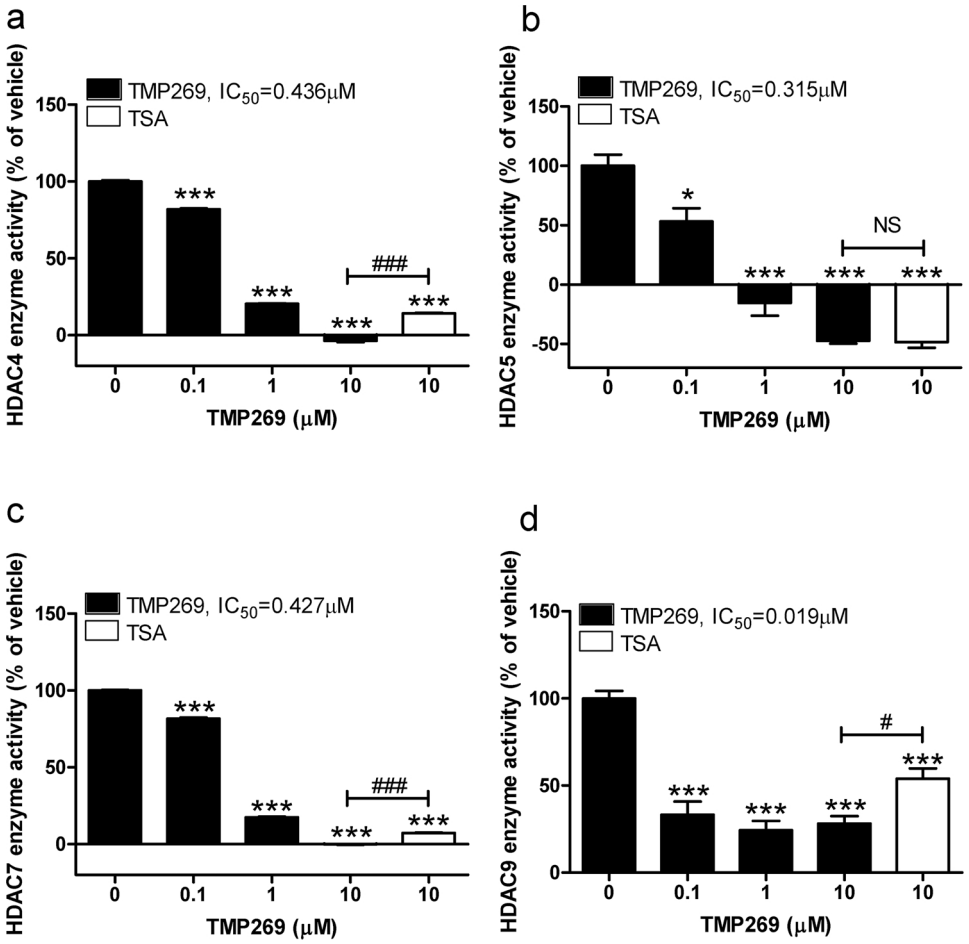


**Fig. 1.** TMP269 inhibits HDAC4, HDAC5, HDAC7, and HDAC9 enzyme activities.
a–d, HDAC4 (a), HDAC5 (b), HDAC7 (c), and HDAC9 (d) enzyme activities were measured in the cell-free system. TMP269 was tested at different concentrations (0.1–10 μM). TSA (10 μM) was used as the reference compound. * $p < 0.05$ and *** $p < 0.001$ versus vehicle group. # $p < 0.05$ and ### $p < 0.001$ versus TSA-treated group. NS indicates not significant. Data represent the means ± SE of at least three independent experiments.

approved by the United States Food and Drug Administration (FDA) for the treatment of cutaneous T cell lymphoma (CTCL) and peripheral T cell lymphoma (PTCL). Chidamide is approved in China for the treatment of PTCL. HDAC inhibitors are divided into four classes: hydroxamate, cyclic peptide, benzamide, and aliphatic acids. Hydroxamate includes trichostatin A (TSA), vorinostat, panobinostat, and belinostat. TSA is the most intensively studied pan-HDAC inhibitor. Depsipeptide belongs to the cyclic peptide class. MS-275 (etinostat) and MGCD0103 (mocetinostat) are benzamides which generally target class I HDACs. Valproic acid, sodium butyrate, and phenyl butyrate are aliphatic acids. Zinc-dependent HDAC inhibitors have common pharmacophores composed of cap group, linker, and zinc-binding domain.

Hypertension is a major leading risk factor in cardiovascular diseases. In hypertension, aortic stiffness is usually increased and vascular smooth muscle cells (VSMCs) contribute to vascular stiffness [7]. Therefore, we used VSMCs to test the degree of acetylation of histones in this study. Lemon et al. reported HDAC6 catalytic activity induced in deoxycorticosterone acetate (DOCA)-salt hypertensive rats [8]. We have demonstrated increased HDAC6 and HDAC8 activities in DOCA-salt hypertensive rats. Interestingly, valproic acid treatment inhibited both enzyme activities in DOCA-salt hypertension [9]. Recently, we have identified that tubastatin A, a selective HDAC6 inhibitor, did not affect high blood pressure in angiotensin II-induced hypertensive mice, implying that HDAC6 enzyme activity is not associated with the development of hypertension [10]. MC1568 is a class IIa/b HDAC inhibitor. We have demonstrated that MC1568 lowers angiotensin II-induced hypertension [11]. This result suggests that class IIa HDACs might have a critical role in hypertension.

Gallic acid is a trihydroxybenzoic acid found in many plants. Especially, black tea has high amounts of gallic acid [12]. Piceatannol is a metabolite of resveratrol and is found in red wine. Sulforaphane is organosulfur compound found in broccoli sprouts. Sulforaphane has been reported to inhibit HDAC activity in human colorectal and prostate cancer cells [13,14].

Gallic acid, piceatannol, and sulforaphane have been shown to negatively regulate hypertrophy [15–17]. Gallic acid was reported to reduce hyperglycemia-induced cytokine secretion and NF-κB activity in human monocytes (THP-1 cells) through downregulation of histone acetyltransferase and upregulation of HDAC2, indicating that gallic acid has a potential for the treatment of diabetes [18]. Furthermore, we have demonstrated that gallic acid reduces hypertension in spontaneously hypertensive rats [19] and in mice with *N*-nitro-L-arginine methyl ester-induced hypertension [20]. However, the inhibitory HDAC enzyme activity of phytochemicals, including gallic acid, piceatannol, and sulforaphane, has not yet been investigated in a cell-free system.

Here, we examined the class IIa HDAC enzyme activity of HDAC inhibitors, phytochemicals, and anti-hypertensive agents in a cell-free system. We found that gallic acid mildly inhibited HDAC1, HDAC4, and HDAC6. Gallic acid showed strong suppression of HDAC5, HDAC7, HDAC8, and HDAC9 enzyme activities. Sulforaphane attenuated class IIa HDAC and HDAC2 enzyme activities. TMP269 and panobinostat completely inhibited class IIa HDACs. Piceatannol inhibited HDAC7 enzyme activity. We demonstrate that gallic acid is a new dietary inhibitor of class IIa/b HDACs as well as HDAC8.

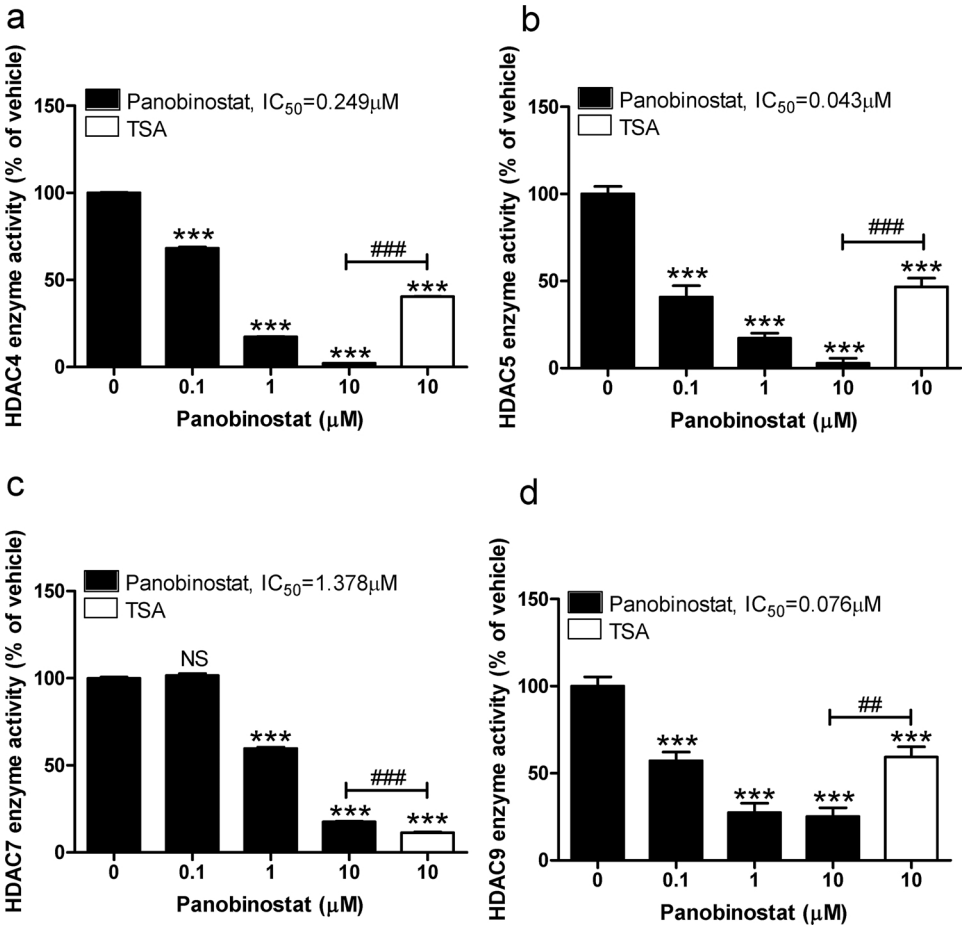


**Fig. 2.** Panobinostat inhibits HDAC4, HDAC5, HDAC7, and HDAC9 enzyme activities.
a-d, HDAC4 (a), HDAC5 (b), HDAC7 (c), and HDAC9 (d) enzyme activities were measured in the cell-free system. Panobinostat was tested at different concentrations (0.1–10 μM). TSA (10 μM) was used as the reference compound. *** $p < 0.001$ versus vehicle group. ## $p < 0.01$ and ### $p < 0.001$ versus TSA-treated group. NS indicates not significant. Data represent the means ± SE of at least three independent experiments.

## 2. Materials and methods

### 2.1. Reagents

TMP269 (#S7324, 99% purity), panobinostat (#S1030, 99.7% purity), losartan (#S1359, 99.7% purity), carvedilol (#S1831, 99.5% purity), and furosemide (#S1603, 99.4% purity) were purchased from Selleckchem (Burlington, NC, USA); MC1568 (#sc362767, > 95% purity) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA); piceatannol (#FC5001, > 95% purity) was purchased from Futurechem (Seoul, Korea); sulforaphane (#S4441, > 90% purity) and gallic acid (#G7384, 97.5–102.5% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Fluorogenic HDAC4, HDAC5, HDAC7, and HDAC9 enzyme activities

HDAC enzyme activity determination was carried out using fluorogenic HDAC4 (#50064, BPS Bioscience), HDAC5 (#50065, BPS Bioscience), HDAC7 (#50067, BPS Bioscience), and HDAC9 (#50069, BPS Bioscience) enzyme assay kits according to the manufacturer's instructions. TSA was used as the reference compound. Purified diluted HDAC4, HDAC5, HDAC7, or HDAC9 enzymes were incubated with various HDAC inhibitors, phytochemicals, or anti-hypertensive drugs at various concentrations at 37 °C for 30 min. To test the activity of HDAC inhibitors and anti-hypertensive drugs, 0.1, 1, 10 μM concentrations were used, whereas phytochemicals were used at higher concentrations (1, 10, 100 μM). Phytochemicals are regarded as natural compounds from plants that are highly safe for use. Thus, we used phytochemicals at higher concentrations than HDAC inhibitors and anti-hypertensive drugs.

Each HDAC enzyme activity was measured using a fluorometer (Spectra Max GEMINI XPS; Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 350 nm and 460 nm, respectively.

### 2.3. Viability assay

Primary vascular smooth muscle cells (VSMCs) were plated in wells of 24-well plates, serum-starved overnight, and then treated with the indicated concentrations of TMP269, TSA, and gallic acid for 24 h. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

### 2.4. Cell cultures

Primary VSMCs were enzymatically isolated from aortas of Sprague-Dawley rats as previously described [11]. The VSMCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and a low concentration of glucose. VSMCs were used at passages 5 to 7.

### 2.5. Western blot analysis

Cells were harvested and lysed with RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 50 mM Tris–HCl pH 7.5, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM $Na_3VO_4$, and 5 mM NaF) containing a protease inhibitor cocktail (Calbiochem, EMD Millipore, Billerica, MA, USA). Proteins were transferred to polyvinylidene difluoride (PVDF) membrane and probed with the indicated antibodies: anti-acetyl Histone H3 (# 9677, Cell Signaling Technology, Danvers, MA, USA), anti-acetyl Histone H4 (#9672, Cell Signaling Technology), anti-acetyl tubulin (#5335, Cell

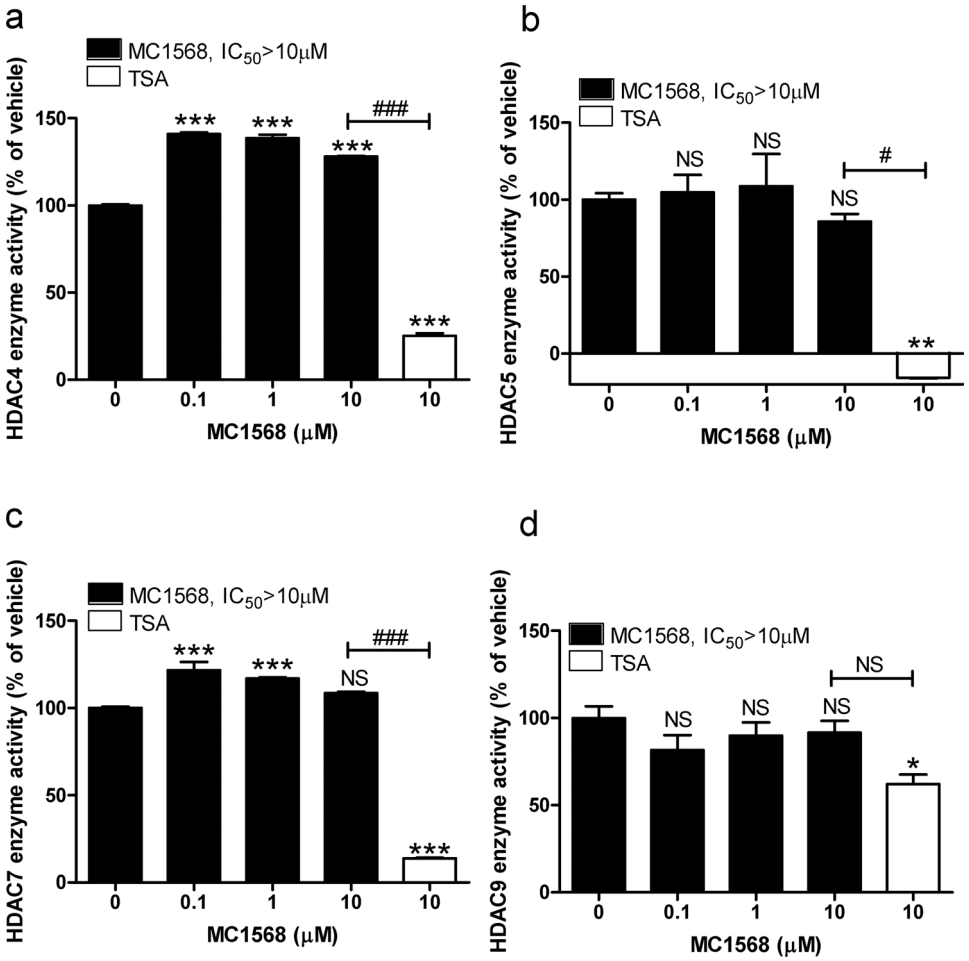


**Fig. 3.** MC1568 does not inhibit HDAC4, HDAC5, HDAC7, and HDAC9 enzyme activities.
a–d, HDAC4 (a), HDAC5 (b), HDAC7 (c), and HDAC9 (d) enzyme activities were measured in the cell-free system. MC1568 was tested at different concentrations (0.1–10 μM). TSA (10 μM) was used as the reference compound. * $p < 0.05$ and *** $p < 0.001$ versus vehicle group. # $p < 0.05$ and ### $p < 0.001$ versus TSA-treated group. NS indicates not significant. Data represent the means ± SE of at least three independent experiments.

Signaling Technology), and anti-β-actin (sc-47778, Santa Cruz Biotechnology). Horseradish peroxidase (HRP)-conjugated secondary antibody was used and protein was detected using a chemiluminescent HRP substrate (Immobilon Western WBKLS0500; Millipore, Billerica, MA, USA). Protein expression levels were quantified by Bio-ID software (Vilber Lourmat, Eberhardzell, Germany).

### 2.6. Statistics

Statistical analyses were performed with one-way ANOVA followed by a Bonferroni post hoc test using the GraphPad Prism software (version 5.0; Graphpad, La Jolla, CA, USA). Numbers of experiments are shown in the figure legends.

## 3. Results

### 3.1. TMP269 and panobinostat, but not MC1568, inhibit class IIa HDAC enzyme activities

TMP269 is a selective class IIa HDAC inhibitor [21]. To identify whether TMP269 inhibits class IIa HDAC enzyme activity, we analyzed HDAC4, HDAC5, HDAC7, and HDAC9 activity in the cell-free system. As shown in Fig. 1, TMP269 inhibited the activities of all class IIa HDACs (HDAC4, 5, 7, 9) in a dose-dependent manner. In addition, TMP269 had a stronger inhibitory effect than TSA at the same concentration (10 μM).

Panobinostat (LBH589) is a pan HDAC inhibitor that has been approved by the U.S. FDA [22]. Like TMP269, panobinostat inhibited enzymatic activities of HDAC4, HDAC5, HDAC7, and HDAC9 (Fig. 2). Interestingly, panobinostat treatment at a high concentration (10 μM) showed a stronger inhibition effect of HDAC4, HDAC5, and HDAC9 than TSA (Fig. 2a, b, and d). Panobinostat inhibited HDAC7 in a dose-dependent manner (Fig. 2c).

MC1568 is a class II HDAC inhibitor [23]. But, we observed that MC1568 did not inhibit all class IIa HDACs enzymatic activities at up to 10 μM concentration (Fig. 3). Even MC1568 increased HDAC4 and HDAC7 enzyme activities at a lower concentration (Fig. 3a and c). We examined class I HDAC enzyme activities of MC1568. MC1568 did not affect the enzyme activities of HDAC1, HDAC2, and HDAC3 (Supplementary Fig. S1a–c). However, MC1568 significantly reduced HDAC8 enzyme activity (Supplementary Fig. S1d).

### 3.2. Gallic acid inhibits HDAC8 and class IIa/b HDAC enzyme activities

We recently reported that gallic acid attenuates $N^G$-nitro-L-arginine methyl ester-induced hypertension [20]. We investigated whether gallic acid can affect class IIa HDAC enzymatic activities. Gallic acid dose-dependently inhibited HDAC4, HDAC5, HDAC7, and HDAC9 enzymatic activities (Fig. 4a–d). Gallic acid showed good inhibition of HDAC5 and HDAC9 enzyme activities comparable to TSA (Fig. 4b and d). We also observed that a high concentration of gallic acid inhibited HDAC6 activity (Supplementary Fig. S2). Next, we determined class I HDAC

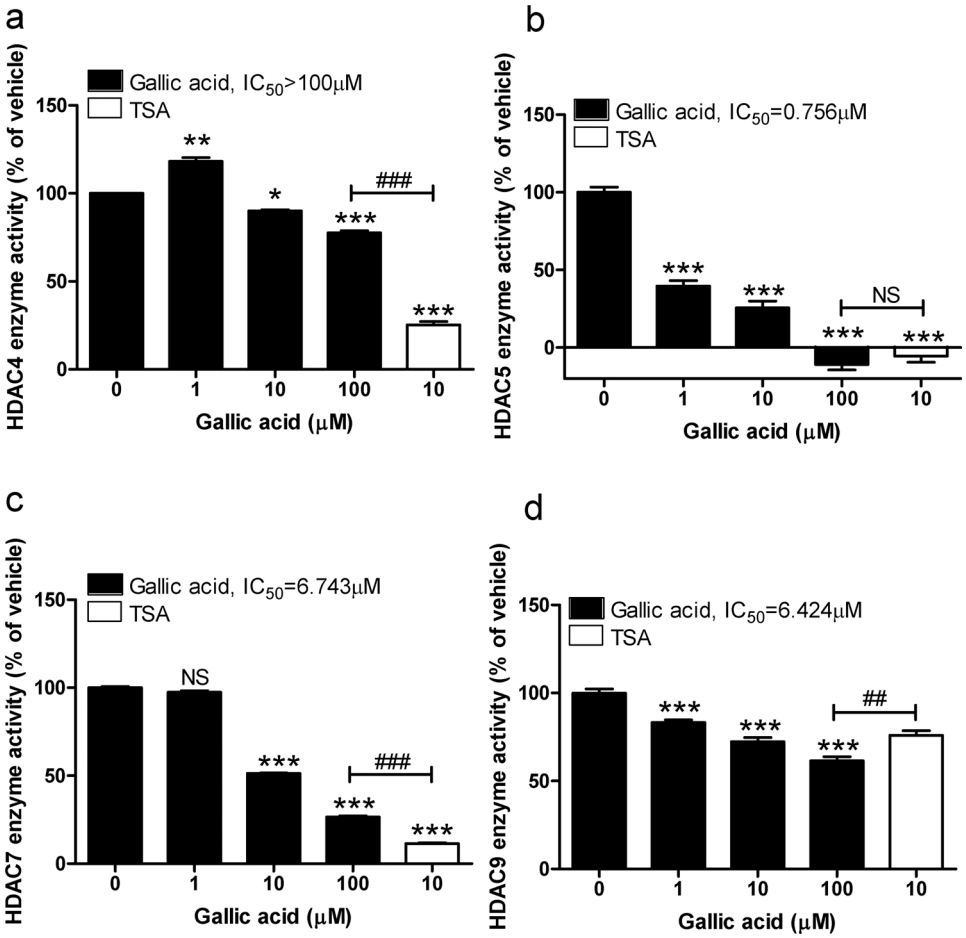


**Fig. 4.** Gallic acid inhibits HDAC4, HDAC5, HDAC7, and HDAC9 enzyme activities.
a–d, HDAC4 (a), HDAC5 (b), HDAC7 (c), and HDAC9 (d) enzyme activities were measured in the cell-free system. Gallic acid was tested at different concentrations (1–100 μM). TSA was used as the reference compound. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus vehicle group. ## $p < 0.01$ and ### $p < 0.001$ versus TSA-treated group. NS indicates not significant. Data represent the means ± SE of at least three independent experiments.

activities of gallic acid. Gallic acid inhibited HDAC1 activity at a high concentration (10 μM) (Supplementary Fig. 3a). Gallic acid did not inhibit enzyme activity of HDAC2 and 3, which are class I HDACs (Supplementary Fig. 3b and c). Interestingly, gallic acid effectively inhibited HDAC8 enzyme activity (Supplementary Fig. S3d).

### 3.3. Piceatannol attenuates HDAC7 enzyme activity

We next tested the effect of piceatannol on class IIa HDACs enzymatic activities. As shown in Fig. 5, all concentrations of piceatannol increased the enzyme activity of HDAC4. Especially, HDAC5 enzyme activity was highly enhanced at the piceatannol concentration of 100 μM (Fig. 5b). However, piceatannol reduced enzymatic activity of HDAC7 in a dose-dependent manner (Fig. 5c). Like HDAC5, piceatannol significantly increased HDAC9 enzyme activity at the high concentration (Fig. 5d). Among the class I HDACs, we determined that HDAC2 enzyme activity was decreased at the high concentration (100 μM) of piceatannol (Supplementary Fig. S4a).

### 3.4. Sulforaphane decreases HDAC2 and class IIa HDAC enzyme activity

We also investigated the effect of sulforaphane on class IIa HDAC enzymatic activities. As shown in Fig. 6a–c, sulforaphane showed an opposite pattern for HDAC4, HDAC5, and HDAC7. A low concentration (1 μM) of sulforaphane increased the enzyme activities of HDAC4, HDAC5, and HDAC7, whereas a high concentration (100 μM) decreased the enzyme activities of HDAC4, HDAC5, and HDAC7. However, sulforaphane strongly reduced HDAC9 enzyme activity at all concentrations (Fig. 6d). We observed that sulforaphane also inhibited HDAC2 enzyme activity (Supplementary Fig. S4b).

### 3.5. Losartan, carvedilol, and furosemide do not inhibit enzymatic activities of class IIa HDACs

Clinically, angiotensin II receptor blocker (ARB), β-blocker, and diuretics are usually used to treat hypertension. To determine whether anti-hypertensive drugs can inhibit class IIa HDAC enzymatic activities, we assayed the drugs in the cell-free system. Losartan, an ARB, increased the enzyme activities of HDAC4, HDAC5, and HDAC7 (Fig. 7a–c). However, losartan attenuated HDAC9 enzyme activity at a high concentration (10 μM), like TSA (Fig. 7d).

Like losartan, the β-blocker carvedilol considerably increased HDAC4, HDAC5, and HDAC7 enzymatic activities (Supplementary Fig. S5a–c). In addition, losartan did not affect HDAC9 enzyme activity (Supplementary Fig. S5d).

Furosemide, a loop diuretic, is used to treat hypertension and heart failure. Furosemide did not affect enzymatic activities of HDAC4 and HDAC5 (Supplementary Fig. S6a and b). Furthermore, furosemide markedly increased HDAC7 enzyme activity (Supplementary Fig. S6c). Furosemide did not affect HDAC9 enzyme activity (Supplementary Fig. S6d).

### 3.6. Gallic acid does not increase acetylation of histone H3 and H4, as well as tubulin

Considering the inhibition of class IIa HDACs by gallic acid, we hypothesized that gallic acid can acetylate histone proteins. To explore the hypothesis, we performed western blot analysis. As shown in Fig. 8a–d, gallic acid did not affect the acetylation of histone H3 and H4. In addition, gallic acid had no influence on acetylation of tubulin. TSA is a potent pan HDAC inhibitor. TSA increased the acetylation of

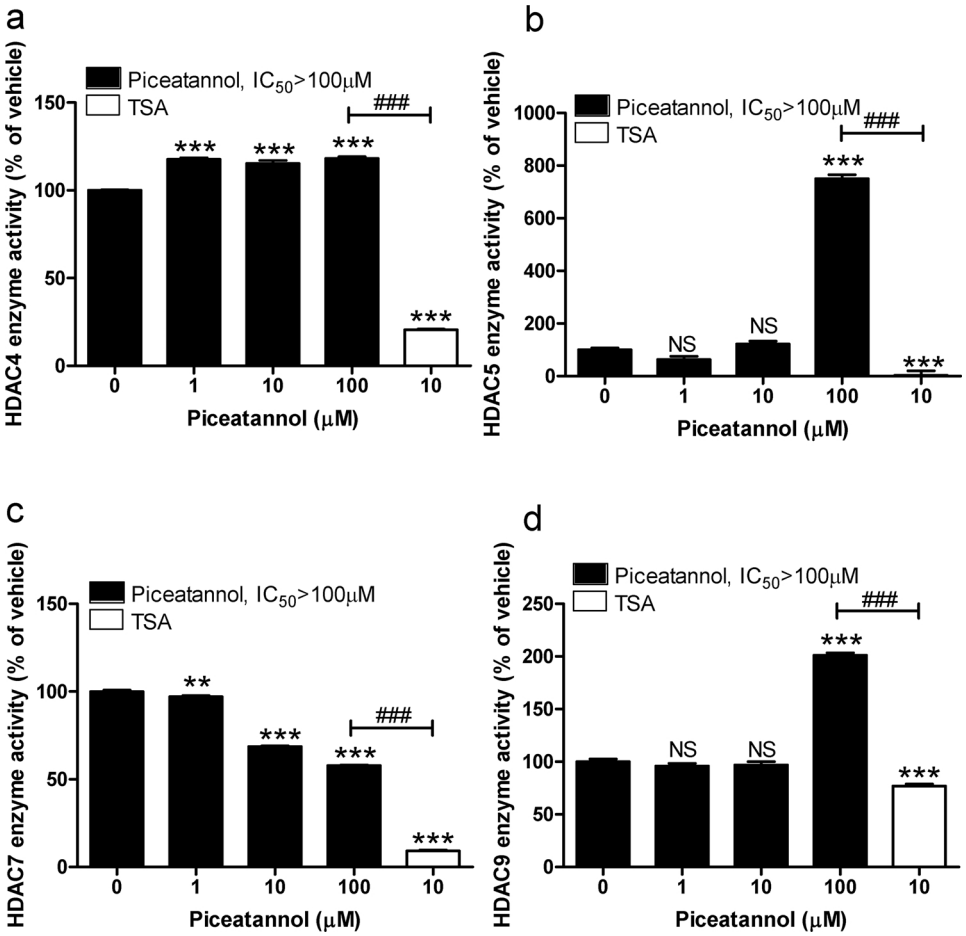


**Fig. 5.** Piceatannol attenuates HDAC7 enzyme activity.
a–d, HDAC4 (a), HDAC5 (b), HDAC7 (c), and HDAC9 (d) enzyme activities were measured in the cell-free system. Piceatannol was tested at different concentrations (1–100 μM). TSA was used as the reference compound. ** $p < 0.01$ and *** $p < 0.001$ versus vehicle group. ### $p < 0.001$ versus TSA-treated group. NS indicates not significant. Data represent the means ± SE of at least three independent experiments.

histone H3/H4 and tubulin (Fig. 8a–d). Tubastatin A is a HDAC6 selective inhibitor and can acetylate tubulin. As expected, tubastatin A did not acetylate histone but acetylated tubulin (Fig. 8d). TMP269, a class IIa HDAC selective inhibitor, did not affect the acetylation of histone and tubulin proteins (Fig. 8a–d).

## 4. Discussion

### *4.1. Relevance of HDAC expression and its activity in cardiac hypertrophy and hypertension*

HDAC inhibitors have been developed to treat solid tumors and cutaneous T cell lymphoma. More recently, they have shown beneficial effects in a variety of diseases, including neurodegenerative disease, cardiac hypertrophy, fibrosis, and hypertension. The activation of HDAC2 is required to induce cardiac hypertrophy [5]. HDAC6 and HDAC8 enzymatic activities are increased in chronic systemic hypertension [9]. A recent study implicated HDAC6 in pulmonary arterial hypertension [24]. Our group has demonstrated that MC1568, a class II HDAC inhibitor, attenuates hypertension and arterial remodeling in an angiotensin II-infusion model [11]. Another study reported that HDAC4 mediates hypertension via vascular inflammation in spontaneously hypertensive rats [25]. We assume that class IIa and IIb HDACs may be implicated in the development of hypertension. However, except for reports on the increase in HDAC6 activity in chronic hypertension, no studies have explored the association between HDAC activity with arterial hypertension [8,9]. Thus, HDAC activity should be investigated in an *in vivo* model of arterial hypertension in the near future.

### *4.2. Role of phytochemicals in cardiac hypertrophy and hypertension*

We have also revealed that gallic acid lowers high blood pressure in animal models of hypertension [19,20]. Sulforaphane also improves blood pressure in spontaneously hypertensive stroke-prone rats [26]. Moreover, we reported that gallic acid, sulforaphane, piceatannol suppress cardiac hypertrophy [15–17]. Sulforaphane is a dietary HDAC inhibitor [27]. However, the HDAC inhibitory activity of gallic acid remains unknown. Consequently, we decided to test whether phytochemicals, including gallic acid, piceatannol, and sulforaphane, can inhibit class IIa HDACs.

### *4.3. Activity of HDAC inhibitors, phytochemicals, and anti-hypertensive drugs in cell free system*

In the present study, we aimed to determine and compare the effects of well-known HDAC inhibitors (TMP269, panobinostat, and MC1568), phytochemicals (gallic acid, sulforaphane, and piceatannol), and anti-hypertensive drugs (losartan, carvedilol, and furosemide) on activities of class IIa HDACs (HDAC4, 5, 7, and 9).

First, we measured the enzyme activities of class IIa HDACs (HDAC4, 5, 7, and 9) using TMP269, panobinostat, and MC1568 HDAC inhibitor. TMP269 and MC1568 are class IIa HDAC inhibitors. As expected, TMP269 definitely inhibited HDAC4, HDAC5, HDAC7, and HDAC9 enzyme activities in the cell-free system. However, MC1568 did not inhibit the activities of most of the class IIa HDACs. Rather, MC1568 increased HDAC4 and HDAC7 enzyme activities. Interestingly, MC1568 significantly suppressed HDAC8 enzyme activity. Fleming et al. designated MC1568 as a class IIa selective HDAC inhibitor, but MC1568 did not inhibit HDAC4 activity in their Fig. S2 [28]. The title of the paper

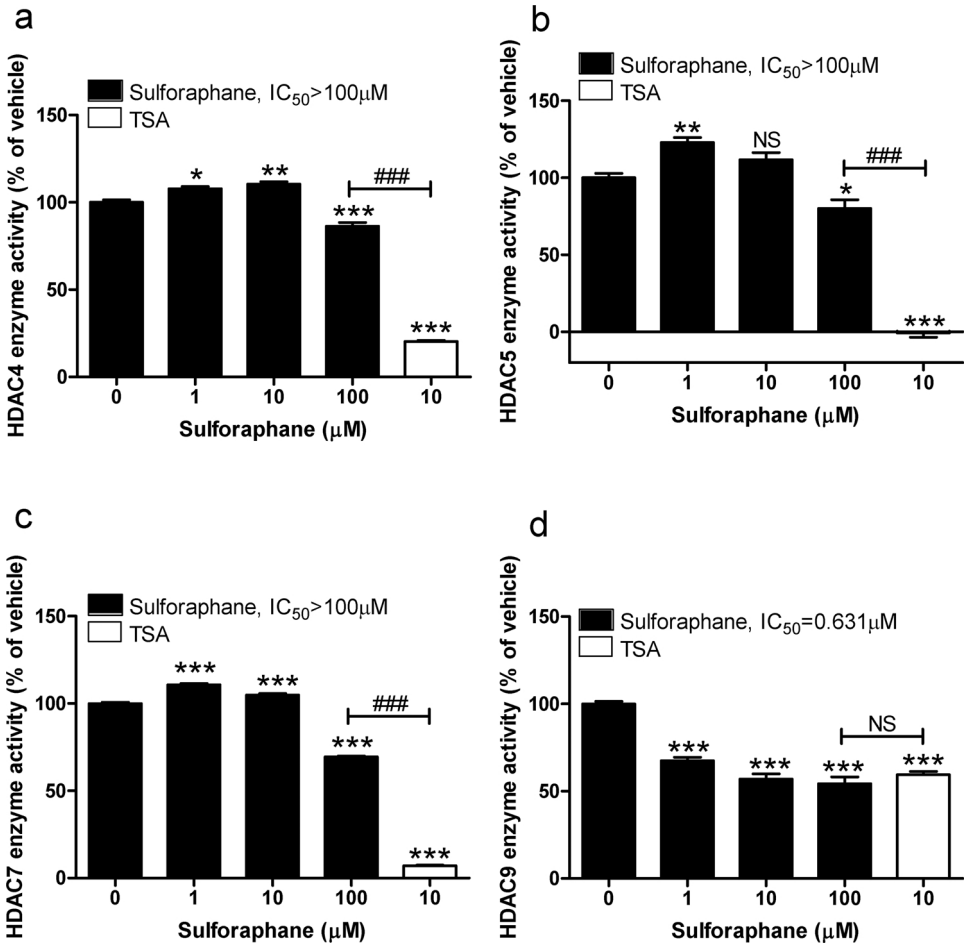


**Fig. 6.** Sulforaphane attenuates HDAC4, HDAC5, HDAC7, and HDAC9 enzyme activities.
a–d, HDAC4 (a), HDAC5 (b), HDAC7 (c), and HDAC9 (d) enzyme activities were measured in the cell-free system. Sulforaphane was tested at different concentration (1–100 μM). TSA was used as the reference compound. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus vehicle group. ### $p < 0.001$ versus TSA-treated group. NS indicates not significant. Data represent the means ± SE of at least three independent experiments.

misleadingly identified MC1568 as a class IIa HDAC inhibitor. This result is in accordance with our present enzyme activity. Panobinostat is a pan-HDAC inhibitor. In the present study, it definitely suppressed class IIa HDACs.

Second, we determined class IIa HDACs activities of three phytochemicals, including gallic acid, piceatannol, and sulforaphane, in the cell-free system. We observed that gallic acid significantly inhibited class IIa HDACs. Especially, gallic acid remarkably suppressed HDAC5 enzyme activity. Furthermore, gallic acid showed weak inhibition of HDAC1 and HDAC6. Of note, gallic acid effectively inhibited HDAC8 enzyme activity. Gallic acid did not affect the HDAC2 and HDAC3 class I HDACs. Here, we for the first time demonstrate that gallic acid is a dietary class IIa/b HDACs inhibitor as well as an inhibitor of HDAC8. Our group has reported that piceatannol attenuates cardiac hypertrophy and renal fibrosis using in vivo models [17,29]. In a model of unilateral ureteral obstruction (UUO), piceatannol attenuated renal fibrosis through the downregulation of HDAC4 and HDAC5. However, in the present study, piceatannol did not inhibit enzymatic activities of HDAC4 and HDAC5 in the cell-free system. Rather, piceatannol inhibited HDAC7 enzyme activity. In the study of UUO, we did not determine HDAC7 expression. Sulforaphane is a well-known phytochemical with anticancer activity [30,31]. We previously reported that sulforaphane suppresses cardiac hypertrophy [15] and restenosis [32]. Sulforaphane is regarded as a dietary HDAC inhibitor [13]. However, total HDAC enzyme activity was measured and each HDAC enzyme activity was not measured [33]. The present study showed that sulforaphane acts as a mild class IIa HDAC inhibitor at 100 μM. Unlike gallic acid and piceatannol, sulforaphane had relatively good inhibition of HDAC2 enzyme activity. Sulforaphane-cysteine, a metabolite of sulforaphane, inhibited HDAC activity in the cell free assay [33]. Simulation studies showed that similar to trichostatin A, sulforaphane-cysteine was highly competent for the HDAC active site. We assumed that gallic acid may bind to the HDAC active site to inhibit its activity.

Third, we evaluated class IIa HDAC activity using three clinically-relevant anti-hypertensive drugs. Losartan is an angiotensin II receptor antagonist. Carvedilol is a beta blocker used to treat hypertension and heart failure. Furosemide, a loop diuretic, is used to treat hypertension and edema. Three different acting anti-hypertensive drugs did not inhibit the enzymatic activities of HDAC4, HDAC5, and HDAC7. In particular, losartan showed weak inhibition of HDAC9 enzyme activity that was comparable to TSA.

### 4.4. Acetylation of histone proteins by TSA, but not by gallic acid

Finally, we assessed whether gallic acid can increase the acetylation of histone protein. Unexpectedly, gallic acid could not acetylate histone H3 and H4 protein. TMP269 did not increase the acetylation of histones. However, the pan HDAC inhibitor TSA effectively increased acetylation of histone H3 and H4, as well as tubulin. Tubulin is a downstream target of the HDAC6 microtubule-associated deacetylase [34]. In the present study, we used the HDAC6 selective inhibitor tubastatin A to identify the acetylation of tubulin. Tubastatin A increased acetylated tubulin comparably to TSA. Considering the anti-hypertensive effect of gallic acid in vivo, the gallic acid-mediated class IIa HDAC

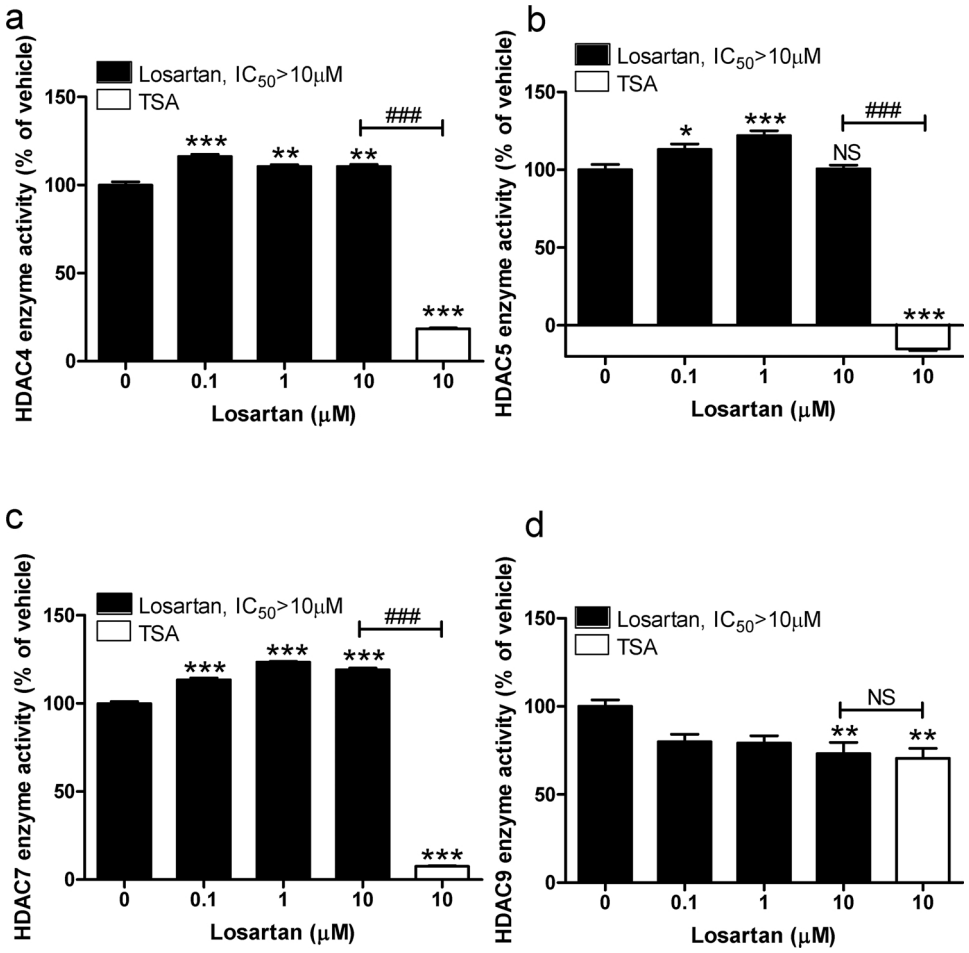


**Fig. 7.** Losartan attenuates HDAC9 enzyme activity.
a–d, HDAC4 (a), HDAC5 (b), HDAC7 (c), and HDAC9 (d) enzyme activities were measured in the cell-free system. Losartan was tested at different concentrations (1–100 μM). TSA was used as the reference compound. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus vehicle group. ### $p < 0.001$ versus TSA-treated group. NS indicates not significant. Data represent the means ± SE of at least three independent experiments.

enzyme inhibition could be closely implicated with the regulation of hypertension. Of course, losartan, carvedilol, and furosemide did not act on most class IIa HDAC enzyme activities. They control hypertension via different regulatory mechanisms. However, if phytochemicals have anti-hypertensive characteristics, they could be very useful to treat or prevent hypertension. Like gallic acid, the chemical class IIa HDAC inhibitor TMP269 needs to be examined concerning its blood pressure lowering effect in a hypertensive animal model.

### 4.5. Possible regulatory mechanism of gallic acid in hypertension

To the best of our knowledge, this study is the first to determine class I and IIa/b HDAC activities in cell free systems. In particular, we showed that gallic acid is a newly identified inhibitor of HDAC8 and class IIa/b HDACs. It is necessary to identify novel downstream targets involved in the development of hypertension. Our group [19] and Kang et al. [35] reported that gallic acid regulates hypertension through the regulation of vasorelaxation or oxidative stress. In our preliminary data, we also observed that class I HDAC inhibitor (MS275) and HDAC8 selective inhibitor (PCI34051) reduces vasoconstriction in isometric tension experiment (data not shown). As mentioned above, gallic acid strongly inhibited HDAC8 activity in cell-free system. In this regard, gallic acid and HDAC8 seems to be related to the hypertension through the regulation of vasoconstriction-relaxation response. Hypertension accompanies inflammatory response. Some studies demonstrated that deletion or pharmacological inhibitor of HDAC6 and HDAC9 enhances regulatory T cell (Treg) number and function [36–38]. Considering the relevance of class II HDACs in the inflammatory disease and autoimmune disorders, anti-hypertensive effect of gallic acid may be also attributed to the improvement in the function of Treg cells through inhibition of class IIa/b HDACs.

In summary, we provide the first evidence that gallic acid efficiently inhibits HDAC8 and class IIa/b HDAC enzyme activities in a cell-free system. Furthermore, piceatannol selectively inhibits the class II HDAC7. Sulforaphane weakly inhibits HDAC2 and class IIa HDAC enzymatic activities. Considering the evidence of the anti-hypertensive effects of gallic acid and sulforaphane, it is worthwhile to search for new natural class IIa HDAC inhibitors in cell-free systems.

## Conflict of interest

None.

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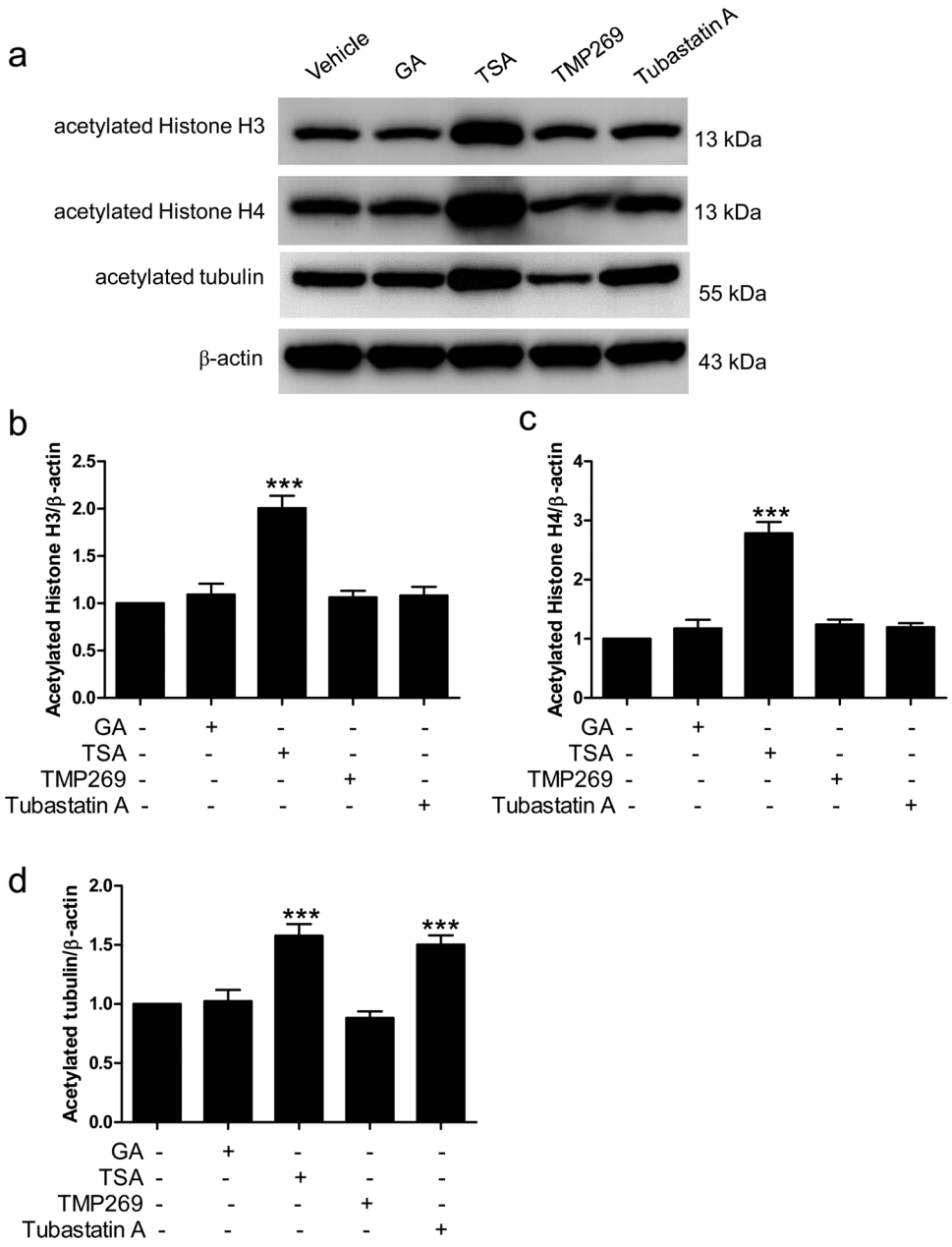


**Fig. 8.** Gallic acid does not increase acetylation of Histone H3 and H4, as well as Tubulin.
a, Representative immunoblots of VSMCs treated with vehicle, gallic acid (GA), TSA, TMP269, or tubastatin A for 45 min. Acetylated Histone H3, acetylated Histone H4, acetylated tubulin, and β-actin was used. b–d, Proteins were quantified by densitometry. *** $p < 0.001$ versus vehicle group.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.02.071.

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