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The histone deacetylase inhibitor tubacin mitigates endothelial dysfunction by up-regulating the expression of endothelial nitric oxide synthase

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Endothelial nitric oxide (NO) synthase (eNOS) plays a critical role in the maintenance of blood vessel homeostasis. Recent findings suggest that cytoskeletal dynamics play an essential role in regulating eNOS expression and activation. Here, we sought to test whether modulation of cytoskeletal dynamics through pharmacological regulation of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation affects eNOS expression and endothelial function *in vitro* and *in vivo*. We found that tubulin acetylation inducer (tubacin), a compound that appears to selectively inhibit HDAC6 activity, dramatically increased eNOS expression in several different endothelial cell lines, as determined by both immunoblotting and NO production assays. Mechanistically, we found that these effects were not mediated by tubacin's inhibitory effect on HDAC6 activity, but rather were due to its ability to stabilize eNOS mRNA transcripts. Consistent with these findings, tubacin also inhibited proinflammatory cytokine-induced degradation of eNOS transcripts and impairment of endothelium-dependent relaxation in the mouse aorta. Furthermore, we found that tubacin-induced up-regulation in eNOS expression *in vivo* is associated with improved endothelial function in diabetic *db/db* mice and with a marked attenuation of ischemic brain injury in a murine stroke model. Our findings indicate that tubacin exhibits potent eNOS-inducing effects and suggest that this compound might be useful for the prevention or management of endothelial dysfunction-associated cardiovascular diseases.

Decreased endothelial nitric oxide synthase (eNOS)³ expression and activity have been implicated in the pathogenesis of a wide range of cardiovascular conditions, including atherosclerosis, essential hypertension, stroke, and coronary restenosis (1, 2). In healthy blood vessels, the expression of eNOS is tightly regulated through complex mechanisms, involving transcriptional, post-transcriptional, and post-translational controls (3). Although eNOS levels fluctuate very little under normal conditions, transcript and protein levels can vary considerably in response to different types of endogenous or exogenous exposures (4). For instance, the pro-inflammatory agonists TNF α , lipopolysaccharide (LPS), and oxidized low-density lipoprotein (LDL) down-regulate eNOS expression in endothelial cells (5, 6), whereas the steroid hormone estrogen and HMG-CoA reductase inhibitors act as potent stimulators of eNOS expression (7–9). Notably, in many of these scenarios, the fluctuation in eNOS levels is due to factors that either shorten or prolong the half-life of eNOS mRNA.

Recently, a growing body of evidence suggests that the composition of cytoskeletal components can influence the stability of eNOS mRNA (10). For example, Laufs and others showed that the half-life of eNOS transcripts can be significantly prolonged by physically disrupting the interactions of actin filaments (11, 12). Moreover, Xue *et al.* (13) showed that eNOS transcripts physically interact with microtubule proteins and that altering the structure of microtubules can by itself influence eNOS levels and NO production in endothelial cells (11, 14). Taken together, these findings suggest that pharmacological strategies targeting microtubule pro-

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This article contains Fig. S1.

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³ The abbreviations used are: eNOS, endothelial nitric oxide synthase; NOS, nitric oxide synthase; HDAC, histone deacetylase; PTBP1, polypyrimidine tract-binding protein 1; qRT-PCR, quantitative real-time PCR; HUVECs, human umbilical vein endothelial cells; MCAO, middle cerebral artery occlusion; TNF α , tumor necrosis factor α ; Ach, acetylcholine; BBMVECs, bovine brain microvascular endothelial cells; eEF1A, translation elongation factor 1- α 1; BAECs, bovine aortic endothelial cells; LPS, lipopolysaccharide; LDL, low-density lipoprotein; HMG, 3-hydroxy-3-methylglutaryl; EC, endothelial cell; L-NAME, N^ω-nitro-L-arginine methyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TTC, 2,3,5-triphenyltetrazolium chloride; ANOVA, analysis of variance.

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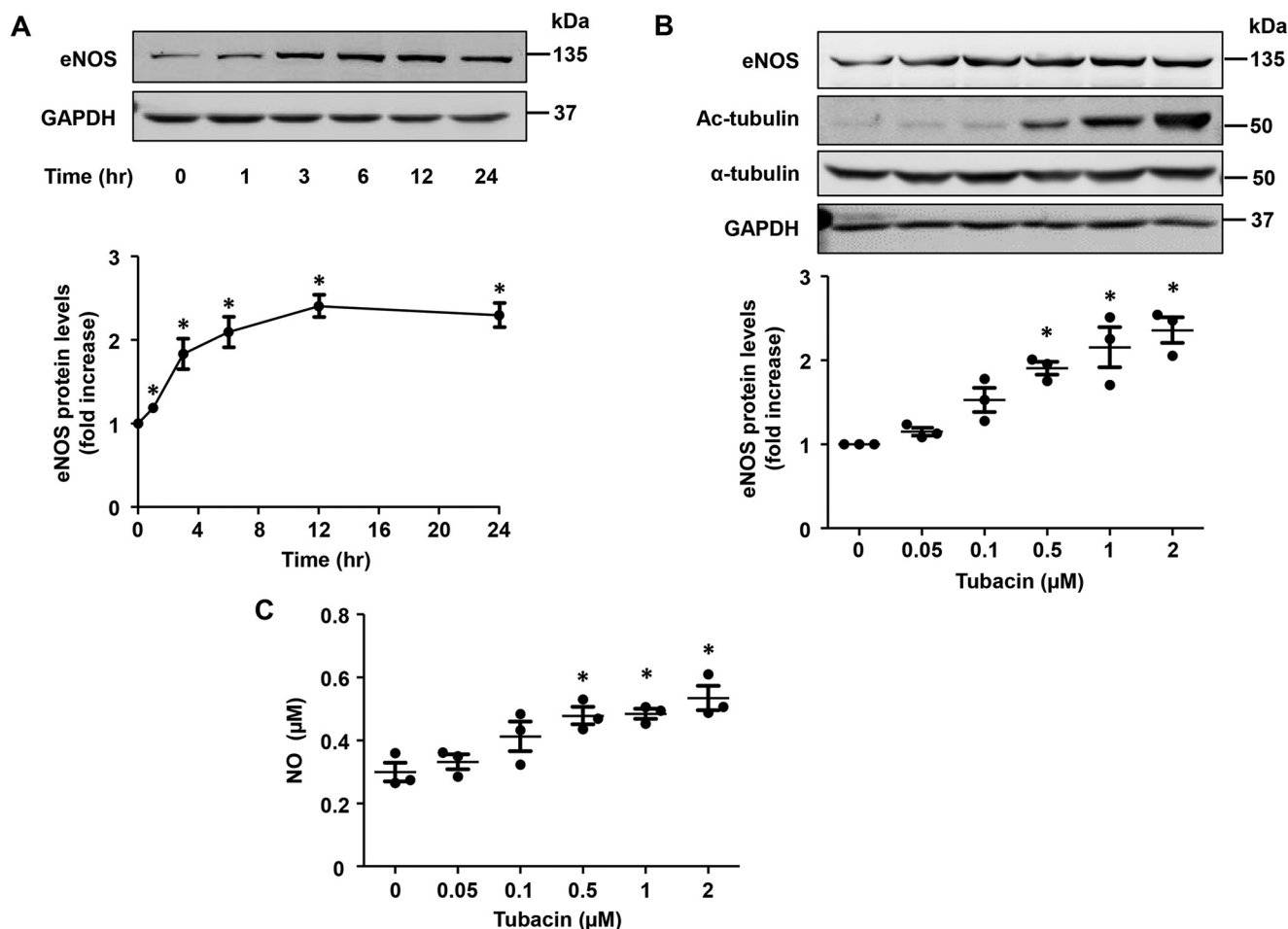


Figure 1. Tubacin increases eNOS expression and NO generation in HUVECs. A, eNOS expression in HUVECs treated with 1 $\mu\text{mol/liter}$ tubacin for different lengths of time. Protein expression was quantified by densitometry analysis. Results are mean \pm S.E. (error bars), $n = 3$; *, $p < 0.05$ versus 0 h, one-way ANOVA with Tukey's post-test. B, eNOS expression in HUVECs exposed to varying concentrations of tubacin for 12 h. Protein expression was quantified by densitometric analysis; mean \pm S.E., $n = 3$; *, $p < 0.05$ versus 0 $\mu\text{mol/liter}$, one-way ANOVA with Tukey's post-test. C, NO levels in supernatant of HUVECs treated with varying concentrations of tubacin for 24 h. Results are mean \pm S.E., $n = 3$; *, $p < 0.05$ versus 0 $\mu\text{mol/liter}$, one-way ANOVA with Tukey's post-test.

teins could be employed for manipulating eNOS levels in cells.

It is now known that post-translational modifications play an important role in regulating the stability of microtubules (15–17). Based on this understanding, we hypothesized that manipulating post-translational modifications of tubulin might have an effect on eNOS levels in endothelial cells. To test this hypothesis, we focused on the enzyme histone deacetylase 6 (HDAC6), which serves as a potent microtubule-associated tubulin deacetylase (18, 19), and pharmacological inhibition of this enzyme is known to regulate acetylation of tubulin and the stability of microtubules in various types of cells (20, 21). Further, HDAC6 knockout mice are not only viable but also have normal blood vessel development (22), suggesting that selective inhibition of HDAC6 might be safe when delivered systemically (20, 23). In this study, we investigated the biological effects of tubacin, a compound with selective HDAC6-inhibitory activity, on the expression of eNOS in endothelial cells as well as examined the effects of this compound on NO production and blood vessel functions in mice.

Results

Tubacin increases eNOS expression in endothelial cells

To investigate the role of HDAC6 in eNOS expression, we treated HUVECs with tubacin, a compound discovered from screening over 7000 compounds with an inhibitory effect on tubulin deacetylation (24, 25). As shown in Fig. 1A, we found that tubacin dramatically increased eNOS expression in HUVECs, displaying a maximal effect at 12 h after first exposure. Further, we found that tubacin also increased eNOS expression in a dose-dependent manner, exhibiting an EC_{50} of ~ 0.3 $\mu\text{mol/liter}$ and showing a maximal effect at a concentration of 1–2 $\mu\text{mol/liter}$. Consistent with the ability to induce eNOS expression, we found that NO levels were also markedly increased in the supernatant of tubacin-exposed cells (Fig. 1, B and C). As expected, tubacin also appeared to inhibit deacetylase activity in our cells, as evidenced by a marked increase in acetylated tubulin levels. Next, to assess whether the above findings were specific to HUVECs, we examined the effects of tubacin on eNOS expression in other EC populations. As shown in Fig. S1, we found that tubacin induced a similar dose-de-

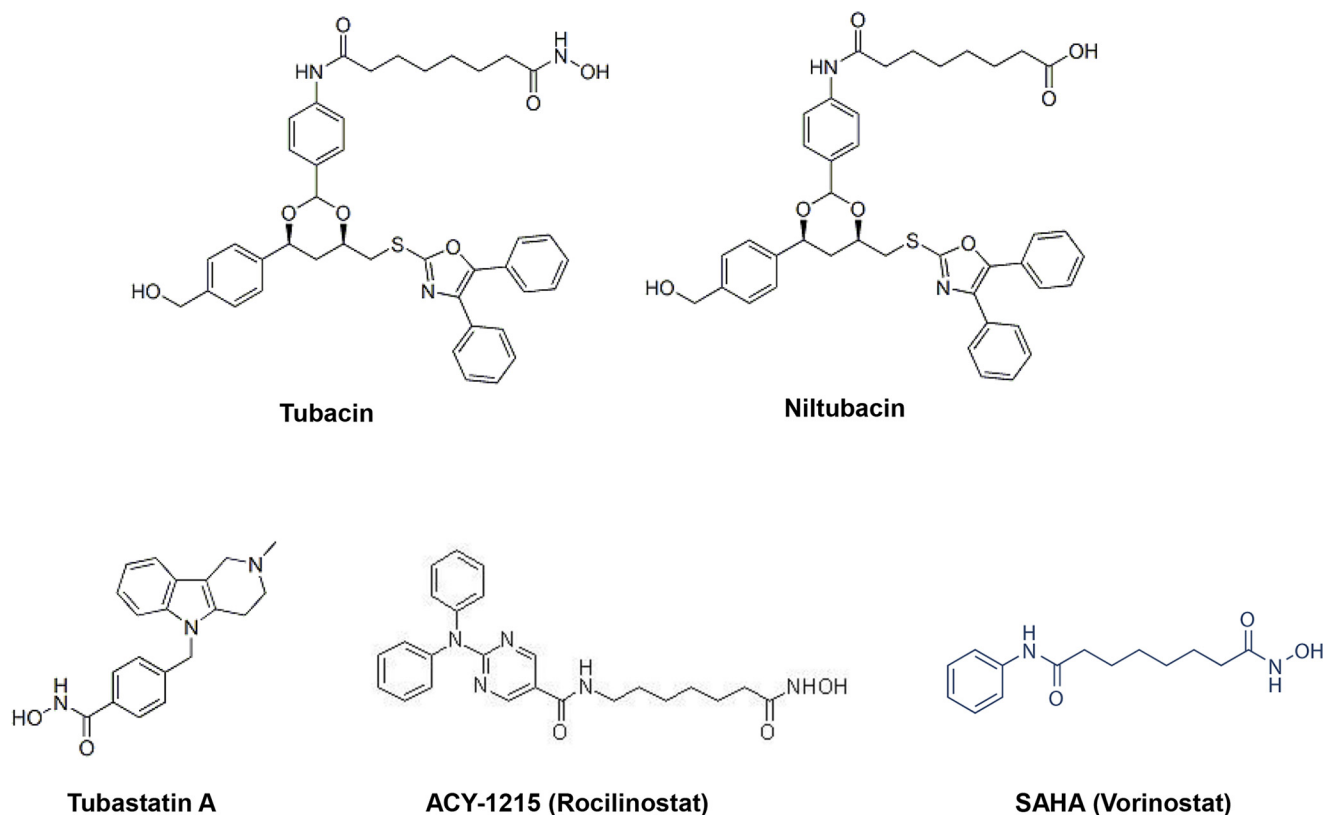


Figure 2. Chemical structure of different HDAC6 inhibitors. Tubacin is a highly selective and reversible HDAC6 inhibitor that permeates the cell membrane. Tubastatin A and rocilinostat (ACY-1215) are also selective HDAC6 inhibitors. SAHA is a pan-HDAC inhibitor. Niltubacin, which retains the bulk of the chemical structure of tubacin, but lacks HDAC6-inhibitory activity, is normally used as a negative control for studies with tubacin.

pendent increase in eNOS expression in both bovine aortic endothelial cells (BAECs) and primary bovine brain microvascular endothelial cells (BBMVECs), illustrating the reproducibility of findings across ECs from different species and vascular beds.

Tubacin induces eNOS expression through HDAC6-independent mechanisms

Recently, several different HDAC6 inhibitors have been discovered (20). To assess whether the effects of tubacin on eNOS expression were specific to this compound, we exposed HUVECs to several other HDAC6 inhibitors (Fig. 2), including tubastatin A (selective HDAC6 inhibitor), ACY-1215 (Rocilinostat) (selective HDAC6 inhibitor), and SAHA (vorinostat) (pan-HDAC inhibitor). We also exposed HUVECs to niltubacin, a compound that is structurally similar to tubacin but lacks any known HDAC6-inhibitory activity (24). As shown in Fig. 3, we found that other HDAC6 inhibitors had little or inhibitory effect on eNOS expression in our cells, although each clearly inhibited deacetylase activity, as evidenced by the marked increase in acetylated tubulin levels. In fact, eNOS expression and NO production were actually reduced in response to tubastatin A and SAHA, but not affected by ACY-1215, suggesting that effects of tubacin on eNOS might be independent of actions on HDAC6. Consistent with this line of reasoning, we found that niltubacin, a compound that lacks HDAC6-inhibitory activity, also dramatically increased eNOS expression in our cells. Next, to confirm that tubacin mediates its effect through HDAC6-independent mechanisms, we performed siRNA-mediated knockdown of the *HDAC6* gene and examined

the effects on eNOS expression. As expected, siRNA knockdown of HDAC6 markedly reduced mRNA and protein levels of HDAC6 in our cells (Fig. 4, A and C). Furthermore, we found that tubacin still readily increased eNOS mRNA and protein levels in HDAC6-deficient cells (Fig. 4, B–E), supporting the notion that tubacin augments eNOS expression largely through HDAC6-independent mechanisms.

Tubacin increases eNOS expression by enhancing eNOS mRNA stability

Because eNOS mRNA levels were significantly increased following tubacin exposure, we hypothesized that this might relate to an increase in gene transcription. To test this, we next transfected COS-7 cells with a luciferase reporter plasmid linked to the eNOS promoter (F1: –1600 nucleotides from the transcription start site) (26). As shown in Fig. 5, we found that transcription of eNOS was readily increased in cells exposed to simvastatin, which has been shown to increase eNOS promoter activity (27). In contrast, tubacin had little to no effect on gene transcription of eNOS in our cells (Fig. 5A). Because post-transcriptional mechanisms can also have an influence on eNOS transcript levels, we next examined whether tubacin acts to prolong the half-life of eNOS mRNA in our cells. To test this, we inhibited gene transcription with actinomycin D and measured transcript levels at various time points after tubacin exposure. As shown in Fig. 5B, we found that treatment with tubacin had a dramatic effect on eNOS mRNA stability, as demonstrated by a doubling of its half-life from 12 to 24 h. Further, we confirmed these findings using a unique reporter

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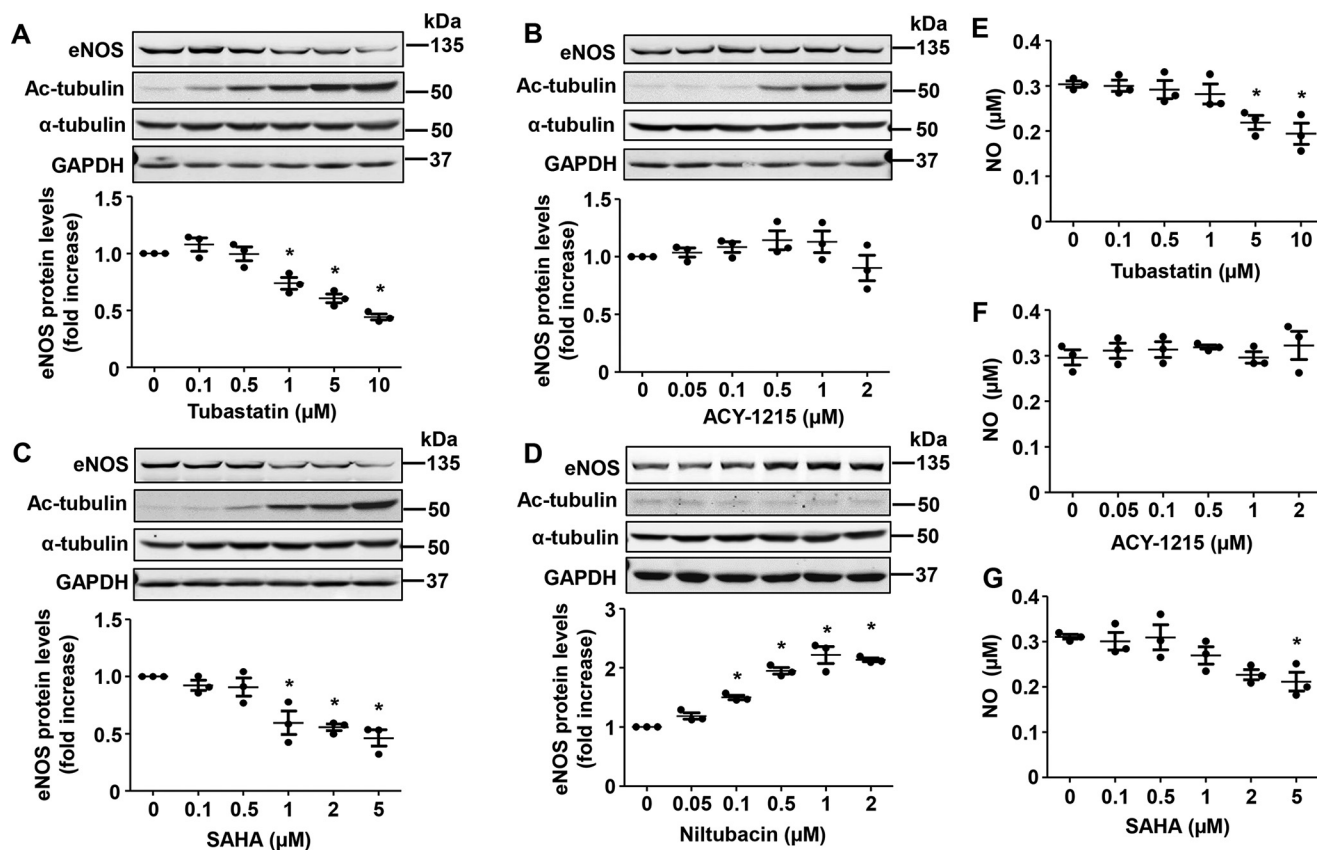


Figure 3. The effects of different HDAC inhibitors on eNOS expression in HUVECs. Protein levels of eNOS, acetylated tubulin (*Ac-tubulin*), total tubulin, and GAPDH in HUVECs treated with tubastatin (A), ACY-1215 (B), SAHA (C), or niltubacin (D) for 24 h. Representative images of Western blots (top) and densitometry measures (bottom) are shown. Results are mean \pm S.E. (error bars), $n = 3$; *, $p < 0.05$ versus $0 \mu\text{mol/liter}$, one-way ANOVA with Tukey's post-test. E–G, NO levels in the supernatant of HUVECs treated with varying concentrations of tubastatin, ACY-1215, or SAHA for 24 h. Results are mean \pm S.E., $n = 3$; *, $p < 0.05$ versus $0 \mu\text{M}$, one-way ANOVA with Tukey's post-test.

system in which the luciferase gene was linked to the 3'-UTR of eNOS (Fig. 5C) as we reported previously (28).

In recent work, we reported that the cytoplasmic proteins eEF1A and PTBP1 play a crucial role in regulating the stability of eNOS mRNA in ECs (28, 29). To assess whether tubacin alters the expression of these proteins, we compared levels of eEF1A and PTBP1 between control and tubacin-exposed cells. Although tubacin was found to have little to no effect on total protein levels of either protein (Fig. 5D), we detected a substantial reduction in cytoplasmic levels of PTBP1 in our cells (Fig. 5E). Because the binding of cytoplasmic PTBP1 to the eNOS 3'-UTR is known to facilitate mRNA degradation (28), we hypothesized that tubacin might also act to reduce this protein-transcript interaction. To test this, we immunoprecipitated PTBP1 from the cytoplasmic fraction of control and tubacin-exposed cells and quantified eNOS transcript levels in this protein fraction. Consistent with our proposed hypothesis, we found that eNOS transcript levels were markedly reduced in PTBP1 immunocomplexes after tubacin exposure (Fig. 5F).

Tubacin ameliorates TNF α -induced endothelial dysfunction through increasing eNOS expression

The pro-inflammatory cytokine TNF α is known to cause endothelial cell dysfunction, in part, by targeting eNOS mRNA for degradation (28, 29). With this in mind, we next sought to determine whether tubacin can mitigate eNOS mRNA degra-

dation in response to TNF α exposure. Consistent with previous reports (28, 29), we found that eNOS expression and NO production were dramatically reduced in TNF α -exposed cells (20 ng/ml) and this was associated with a decrease in eNOS mRNA stability. Conversely, we found that eNOS expression, NO production, and the half-life of eNOS transcripts were markedly increased in cells treated with tubacin (Fig. 6, A–C). Because down-regulation of eNOS expression is known to contribute to endothelial dysfunction in various model systems, we next sought to determine whether augmenting eNOS expression with tubacin could restore vascular function in TNF α -exposed blood vessels. After confirming that tubacin ($1 \mu\text{mol/liter}$) can restore eNOS expression in the isolated mouse aorta after TNF α exposure (20 ng/ml), we examined whether this associated with improved acetylcholine (ACh)-mediated vasorelaxation. As shown in Fig. 6D, we found that treatment with tubacin largely reversed TNF α -induced endothelial dysfunction in isolated aorta segments. As expected, tubacin did not have an effect on vasodilatory responses to sodium nitroprusside (Fig. 6E), supporting the notion that tubacin's effects are mediated specifically through augmenting eNOS expression.

Tubacin enhances endothelium-dependent vasorelaxation in murine aorta

To examine whether tubacin mediates similar effects *in vivo*, we next treated mice with a one-time intraperitoneal injection

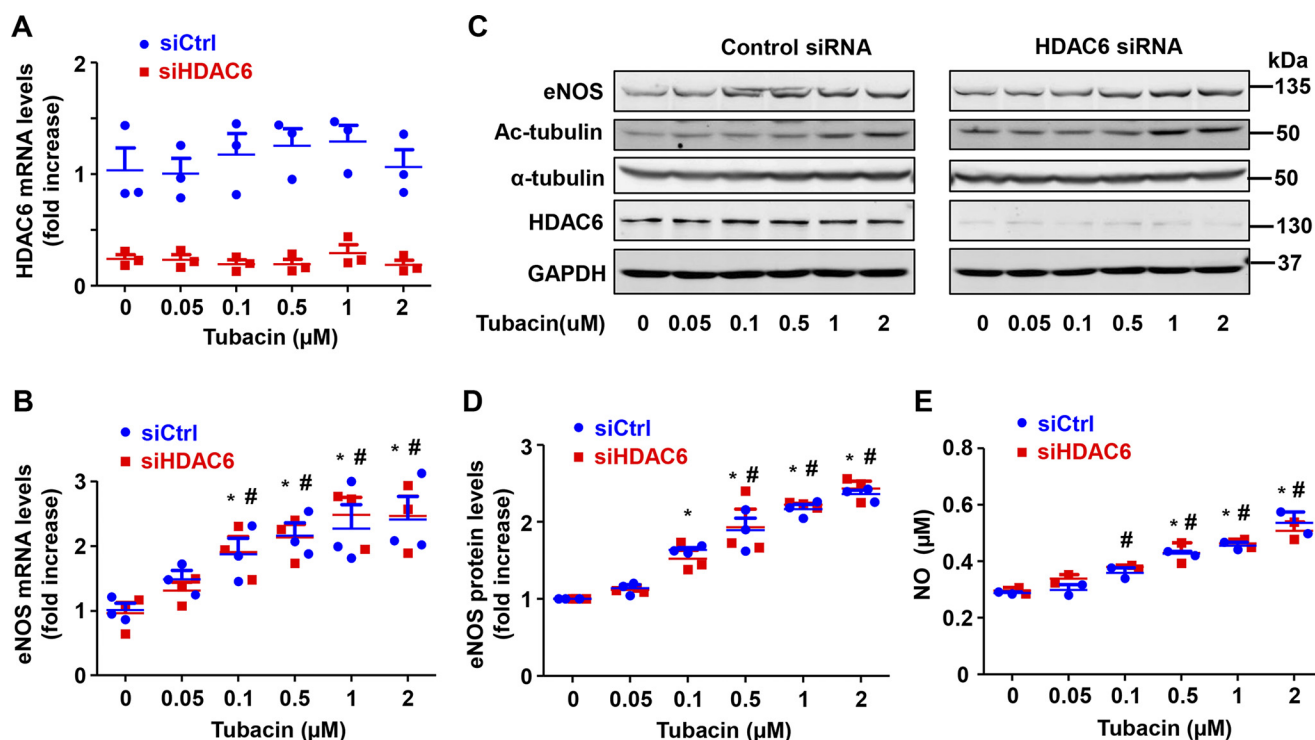


Figure 4. Tubacin-induced eNOS expression occurs independent of HDAC6 inhibition. *A* and *B*, transcript levels for HDAC6 or eNOS in control and HDAC6-deficient cells exposed to varying concentrations of tubacin for 6 h. *C*, protein levels for eNOS, acetylated α -tubulin, total α -tubulin, HDAC6, and GAPDH in control and HDAC6-deficient cells exposed to different concentrations of tubacin for 24 h. *D*, densitometry measurements of eNOS and HDAC6 in control and HDAC6-deficient cells exposed to different concentrations of tubacin for 24 h. *E*, NO levels in supernatants of HUVECs exposed to 0–2 μ mol/liter tubacin for 24 h. Results are mean \pm S.E. (error bars), $n = 3$; *, $p < 0.05$ versus 0-h control siRNA, one-way ANOVA with Tukey's post-test; #, $p < 0.05$ versus 0-h HDAC6 siRNA, one-way ANOVA with Tukey's post-test.

of tubacin (5 mg/kg) or vehicle control and examined eNOS expression in the mouse aorta. As shown in Fig. 7 (*A* and *B*), we found that both mRNA and protein levels for eNOS were significantly increased in the aorta of tubacin-treated mice. Moreover, this was associated with a substantial reduction in cytoplasmic levels of PTBP1 (Fig. 7*C*) and a weak, but significant, increase in Ach-induced vasorelaxation (Fig. 7*D*). Importantly, in the presence of a NOS inhibitor (*N*^G-nitro-L-arginine methyl ester) or NO donor sodium nitroprusside, tubacin's effects on Ach-mediated vasorelaxation were largely abolished (Fig. 7, *D* and *E*), indicating that tubacin mediates its effects on blood vessel tone via eNOS-dependent mechanisms.

To determine whether tubacin is able to attenuate endothelial dysfunction in disease, we employed *db/db* mice, which are known to manifest an impaired endothelial function at 4 months of age (30). Using these mice, we found that treatment with tubacin (5 mg/kg/day, intraperitoneally, 1 week) significantly improved endothelium-dependent vasorelaxation to ACh in aortas of *db/db* mice (Fig. 8*A*) and the expression of eNOS, as determined by Western blotting (Fig. 8*B*). Consistent with a previous report (31), Ach-mediated vasorelaxation in the aorta of eNOS knockout mice was significantly impaired, which was not affected by tubacin treatment (Fig. 8*C*), further illustrating the vasoprotective effects of tubacin *in vivo* through eNOS-dependent mechanisms.

Tubacin attenuates cerebral injury in mice

Finally, because NO is known to have potent neuroprotective and anti-apoptotic properties (11, 32), we hypothesized that

tubacin might reduce brain injury in a cerebral ischemia model. To this end, we employed the well-established mouse middle cerebral artery occlusion (MCAO) model to assess the effects of tubacin on ischemia-induced brain injury. As shown in Fig. 9, we found that pretreatment with tubacin (5 mg/kg) significantly reduced cerebral infarct size and the severity of cortical edema at 24 h after arterial occlusion (Fig. 9, *A* and *B*) when compared with controls. Consistent with a reduction in brain injury, we found that functional neurological scores were also significantly improved in tubacin-treated mice (Fig. 9*C*). Moreover, these findings were associated with a marked increase in eNOS expression in the ischemic penumbra at 24 h after MCAO (Fig. 9*D*), supporting the concept that tubacin mediates its effects by augmenting eNOS expression.

Discussion

In this study, we show that tubacin acts to increase eNOS expression and NO production *in vitro* and *in vivo*. However, in contrast to previous reports, we found that tubacin does not mediate its effects through inhibiting HDAC6 activity but rather through stabilizing eNOS mRNA. Because many pathological conditions are known to decrease eNOS expression in endothelial cells by reducing mRNA stability (5, 6, 28, 29), identification of tubacin as a potent eNOS enhancer suggests that tubacin or its analogues might be effective in ameliorating systemic vascular complications of disease. Along these lines, we found that tubacin ameliorated endothelial dysfunction in a mouse diabetic model and reduced the size of cerebral infarct-

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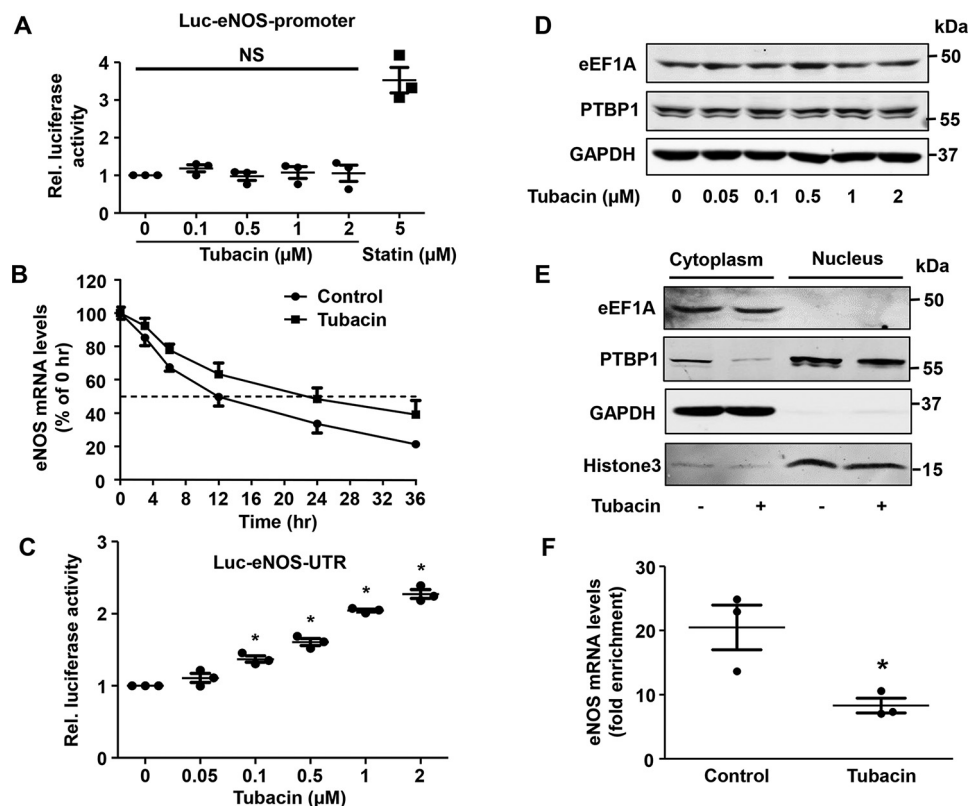


Figure 5. Tubacin increases eNOS mRNA stability in HUVECs. *A*, relative luciferase activity in COS-7 cells treated with tubacin or 5 μM /liter simvastatin for 12 h. Cell lysates were assayed for luciferase activities. Results are mean \pm S.E. (*error bars*), $n = 3$. *B*, eNOS transcript levels in HUVECs treated with or without 1 μM tubacin for 3 h. eNOS mRNA stability was determined by exposing cells to 5 μM actinomycin D. Results are mean \pm S.E., $n = 3$. *C*, relative luciferase activity in COS-7 cells treated with different concentrations of tubacin. Results are mean \pm S.E., $n = 3$; * $p < 0.05$ versus 0 μM /liter, one-way ANOVA with Tukey's post-test. *D*, the protein levels of PTBP1 and eEF1A in HUVECs treated with different concentrations of tubacin for 24 h. *E*, PTBP1 and eEF1A levels in the cytoplasmic or nuclear fraction of HUVECs treated with either vehicle or 1 μM /liter tubacin for 24 h. GAPDH was used as a cytoplasmic marker, and histone 3 was used as a nuclear marker. *F*, eNOS mRNA levels in the anti-PTBP1 immunoprecipitates were quantified by real-time PCR of control and tubacin-treated cells. Data are shown as enrichment over the control IgG antibody. Results are mean \pm S.E., $n = 3$; * $p < 0.05$ versus control, unpaired Student's *t* test.

tion in mice after transient occlusion of the middle cerebral artery.

The interplay between histone deacetylases (HDACs) and histone acetyltransferases differentially regulates the acetylation status of histone and nonhistone proteins and regulates gene expression in various ways (33, 34). In addition to regulating gene expression, HDACs are also involved in a number of other important biological pathways, including those involved in growth, proliferation, and differentiation of cells (35, 36). Indeed, in the vessel wall, several HDACs have been identified to regulate eNOS through both transcriptional and post-translational mechanisms (37, 38). For example, at post-translational levels, both HDAC1 and HDAC3 have been shown to induce lysine deacetylation of eNOS, thereby inhibiting eNOS activity (38). Furthermore, treatment of endothelial cells with pan-HDAC inhibitors, such as trichostatin A and butyric acid, has been shown to decrease eNOS expression at post-transcriptional levels through yet unidentified mechanisms (37). Consistent with the previous report (37), we found that tubastatin A (selective HDAC6 inhibitor) and SAHA (pan-HDAC inhibitor) reduced eNOS protein expression in HUVECs. Recent studies have demonstrated that modulation of the endothelial actin cytoskeleton represents a major mechanism in regulating eNOS expression and mRNA stability (39, 40). Among 18 HDACs in mammals, HDAC6 is a unique member of the type II

HDACs that has been shown to regulate cytoskeleton dynamics by acting as a specific α -tubulin deacetylase (18, 22), which prompted us to investigate whether inhibition of HDAC6 could alter eNOS gene expression through modulating cytoskeleton dynamics in endothelial cells. In the present study, we treated endothelial cells with three structurally unrelated inhibitors of HDAC6 and found that only tubacin potently increased eNOS expression, despite all three inhibitors exhibiting inhibitory effects on α -tubulin deacetylation. Furthermore, we found that niltubacin, an inactive tubacin analog without inhibitory effect on HDAC6, also increased eNOS protein expression in a dose-dependent manner, with no changes on the acetylated levels of α -tubulin. Most importantly, our experiments performed in HDAC6 knockdown cells demonstrated that eNOS expression increased by tubacin was largely, if not wholly, independent of HDAC6, as tubacin increased eNOS expression to a similar extent in control siRNA- and HDAC6 siRNA-transfected cells. Together, these results identified tubacin as a unique compound that potently increases eNOS expression and NO production through mechanisms independent of its effects on HDAC6.

Endothelially derived NO is a critical mediator of vascular integrity (41). Although the expression of eNOS is regulated at multiple levels, it has been increasingly appreciated that post-transcriptional regulation plays an important role (40). Our

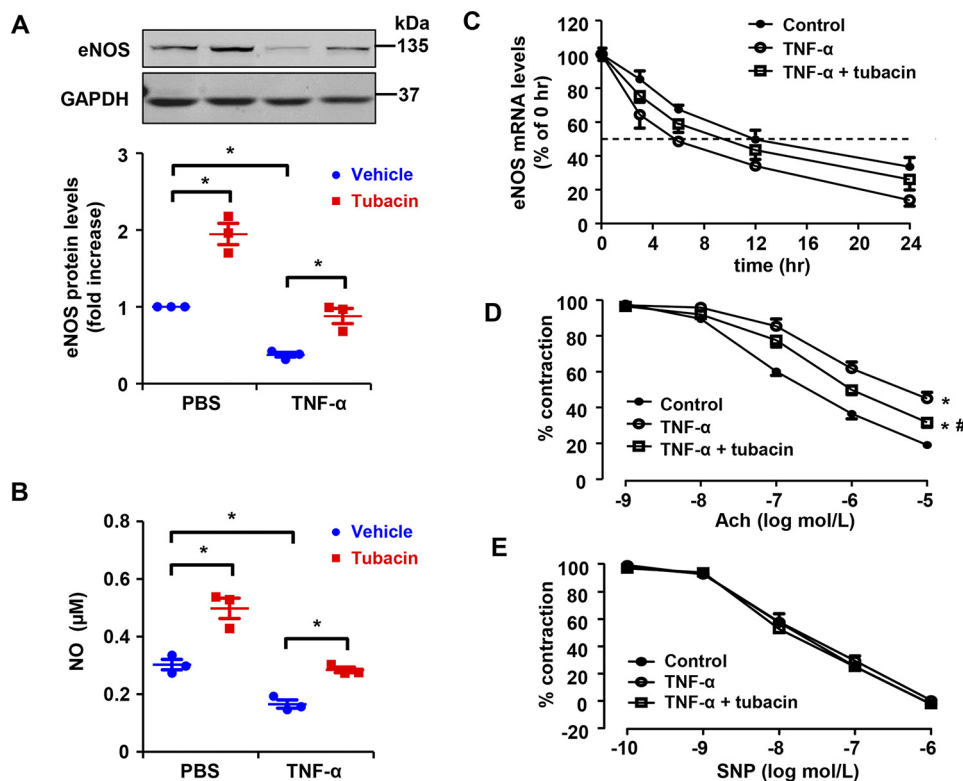


Figure 6. Tubacin inhibits TNF α -induced down-regulation of eNOS in HUVECs and ameliorates TNF α -induced endothelial dysfunction in the aorta of mice. A, protein levels of eNOS in HUVECs treated with or without TNF α (20 ng/ml) in the presence or absence of tubacin. Results are mean \pm S.E. (error bars), $n = 3$; *, $p < 0.05$, one-way ANOVA with Tukey's post-test. B, NO levels in the supernatant of HUVECs pretreated with 1 μ mol/liter tubacin for 1 h and then exposed to vehicle or 20 ng/ml TNF α for 24 h. Results are mean \pm S.E. $n = 3$; *, $p < 0.05$, one-way ANOVA with Tukey's post-test. C, eNOS mRNA half-life in control and tubacin-treated cells exposed to PBS versus TNF α . The data are representative of five independent experiments. D, Ach-induced endothelium-dependent relaxation in aortas treated with or without tubacin (1 μ mol/liter) for 24 h while also being exposed to control or TNF α . Endothelium-dependent vasorelaxation was determined by measuring Ach-induced relaxation in rings precontracted with phenylephrine. Endothelium-independent vasorelaxation was determined by measuring Ach-induced relaxation in rings precontracted with phenylephrine; $n = 8$; *, $p < 0.05$ versus control group at 10^{-5} mol/liter acetylcholine; #, $p < 0.05$ versus TNF α group at 10^{-5} mol/liter acetylcholine, one-way ANOVA with Tukey's post-test. E, endothelium-independent vasorelaxation to sodium nitroprusside (SNP) was examined.

laboratory has recently reported that PTBP1, also known as hnRNP1 (heterogeneous nuclear ribonucleoprotein 1), is an essential trans-acting factor that binds to eNOS 3'-UTR in the cytoplasm, leading to eNOS mRNA destabilization and impairment of endothelium-dependent vasorelaxation (28). In our study, we found that tubacin up-regulated eNOS expression, in part, through suppressing cytoplasmic PTBP1 levels in endothelial cells. At this point, the molecular mechanism underlying tubacin-mediated suppression of PTBP1 remains unknown. PTBP1 is a nucleocytoplasmic shuttling protein that has been shown to regulate RNA metabolism, including mRNA stability, translation, and localization (42, 43). For example, glucose stimulation of pancreatic beta cells can induce redistribution of PTBP1 from the nucleus to the cytoplasm, thus promoting PTBP1 binding to 3'-UTR of insulin to enhance mRNA stability (44). Interestingly, PTBP1 has been shown to bind to the mRNAs of actin and the focal adhesion proteins vinculin and α -actinin 4 to regulate cytoskeletal assembly (45, 46). Thus, it is tempting to speculate that tubacin may increase eNOS expression through at least two mechanisms, which include the disruption of PTBP1 binding to eNOS 3'-UTR and the inhibition of PTBP1-mediated cytoskeleton assembly. Further, it would be interesting to investigate whether tubacin can also regulate nucleocytoplasmic shuttling of PTBP1 by affecting its phos-

phorylation status, as reported previously in the neuronal PC12 cells (47).

Vascular NO production regulates cerebrovascular perfusion and protects against stroke by increasing collateral flow to the ischemic area (48, 49). In general, preclinical studies demonstrate that NO generated by the neuronal and inducible NOS after stroke is detrimental to neuronal survival (30), whereas eNOS and endothelial NO are neuroprotective (49). Mice lacking eNOS exhibit larger cerebral infarctions, and further inhibition of NOS activity by N^G -nitro-L-arginine methyl ester (L-NAME) increases infarct size (11, 48, 50). In contrast, up-regulation of eNOS by estrogen, statins, and Rho kinase inhibitors has consistently been shown to confer protection from ischemic stroke in mice (8, 51). Therefore, conditions that enhance eNOS activity could have beneficial effects on cerebrovascular disease. In our study, we show that treatment of mice with tubacin for only 2 days prior to ischemic injury markedly reduced infarct size and brain edema after MCAO. Furthermore, our findings suggest that vascular protective effects of tubacin might be mediated through its ability to increase eNOS expression. This was suggested based on finding elevated eNOS levels in the ischemic penumbra and our other findings showing that tubacin's effects on vessel relaxation were dependent on the expression of eNOS. Interestingly, the increased relaxing

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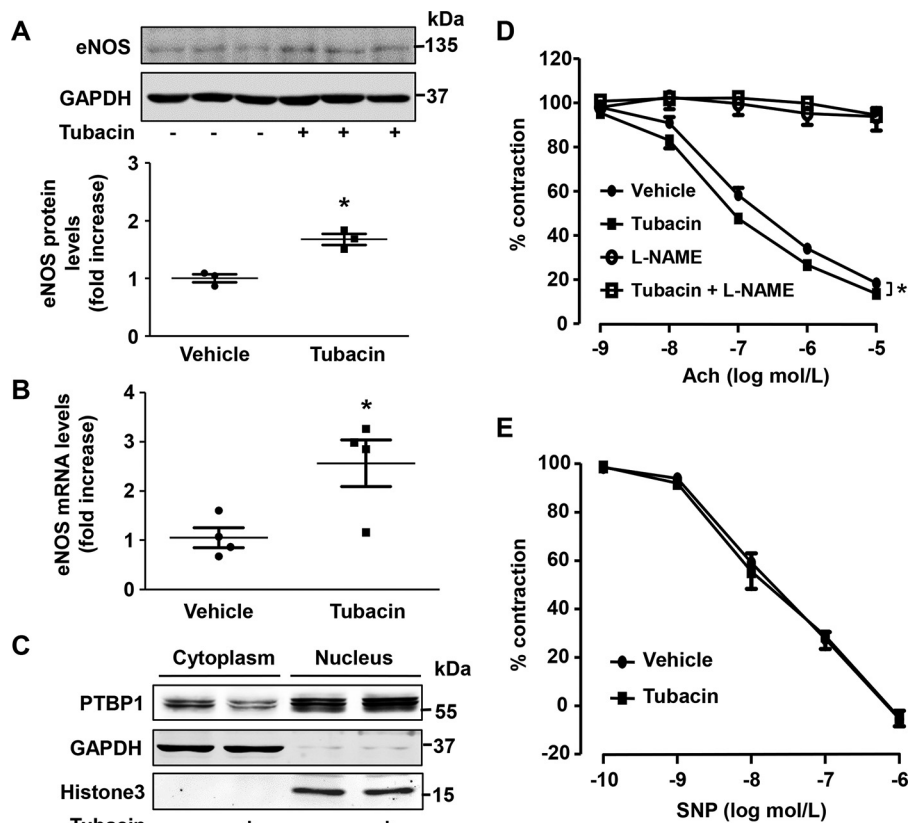


Figure 7. The effect of tubacin on eNOS expression and endothelium-dependent relaxation in mice. Mice were intraperitoneally injected with 5 mg/kg tubacin. 24 h after injection, the eNOS protein levels (A) and eNOS mRNA levels (B) in mouse aorta were measured by Western blotting and qRT-PCR, respectively. Results are mean \pm S.E. (error bars), $n = 3-4$; *, $p < 0.05$ versus control group, unpaired Student's t test. C, the levels of PTBP1 in cytosolic and nuclear subcellular fractions were determined by Western blotting in mouse aorta treated with or without tubacin for 24 h. GAPDH was used as a cytoplasmic marker, and histone 3 was used as a nuclear marker. D, representative traces of Ach-induced endothelium-dependent relaxations in aortas from mice treated with or without tubacin for 24 h. Endothelium-dependent vasorelaxation was determined by measuring Ach-induced relaxation in rings precontracted with phenylephrine; $n = 8$; *, $p < 0.05$ versus control group at 10^{-5} mol/liter acetylcholine, one-way ANOVA with Tukey's post-test. E, endothelium-independent vasorelaxation to sodium nitroprusside (SNP) was examined.

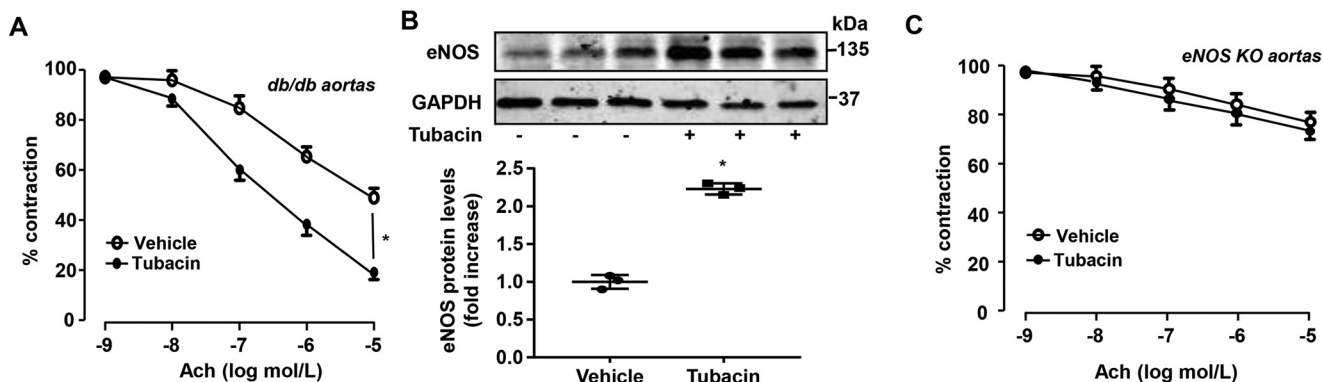


Figure 8. Tubacin improves endothelial function in diabetic mice. A, *db/db* mice received intraperitoneal injection of either vehicle or 5 mg/kg/day of tubacin for a week, and Ach-induced endothelium-dependent relaxation of aortic ring was determined. Results are mean \pm S.E. (error bars); $n = 8$; *, $p < 0.05$ versus vehicle at 10^{-5} mol/liter acetylcholine, one-way ANOVA with Tukey's post-test. B, *db/db* mice received intraperitoneal injection of either vehicle or 5 mg/kg/day of tubacin for a week. Expression of eNOS was determined by Western blotting. $n = 3$; *, $p < 0.05$ versus vehicle control group, unpaired Student's t test. C, eNOS knockout (KO) mice received intraperitoneal injection of vehicle or 5 mg/kg/day of tubacin for a week, and Ach-induced endothelium-dependent relaxation of aortic ring was determined. $n = 8$.

response to acetylcholine in normal mice is relatively weak. This is not surprising because eNOS gene expression and the resulting content of eNOS protein is not a limiting factor under normal conditions (*i.e.* in the absence of endothelial dysfunction). In fact, this effect is more remarkable in *db/db* mice, indicating that tubacin is capable of restoring altered eNOS expression under pathological conditions.

Several lines of evidence suggest that HDAC6 is essentially involved in the development of cancer and neurodegenerative disorders, such as Parkinson disease and Huntington disease (20, 23, 52, 53). Thus, HDAC6 has emerged as a promising therapeutic target for treatment of these diseases. Investigation of HDAC6 inhibitors on eNOS expression is not only important to identify eNOS regulators, but also allows us to predict

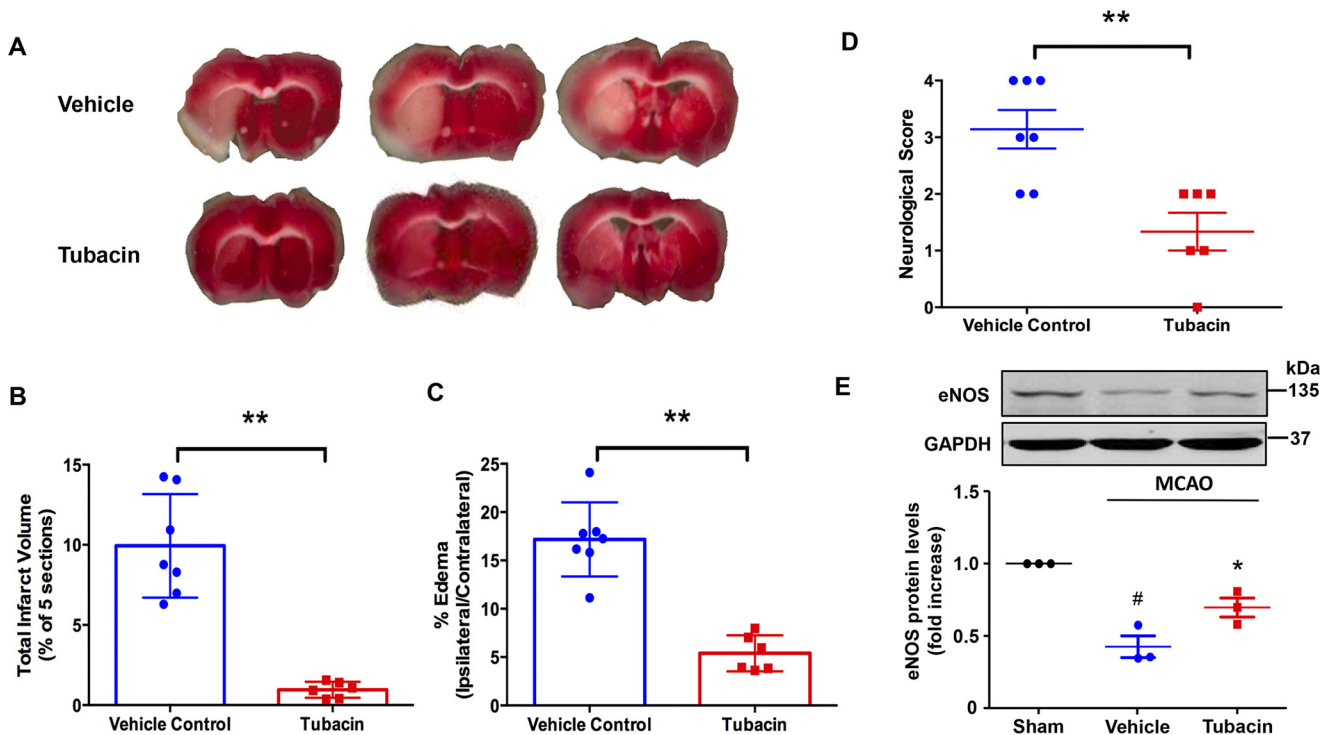


Figure 9. Tubacin reduces cerebral infarct size, improves neurological function, and increases eNOS expression in MCAO mice. *A*, representative TTC-stained cerebral sections of the MCAO model showing decrease infarct size (white area) in tubacin-treated mice as compared with the vehicle-treated group. *B* and *C*, quantitative measurement of infarct size and cerebral edema in control and tubacin-treated mice after MCAO. $n = 6-7$ for each group; **, $p < 0.01$, unpaired Student's *t* test. *D*, neurological deficit scores in control and tubacin-treated mice at 24 h after MCAO. Data are presented as mean \pm S.E. (error bars) $n = 6-7$ for each group; **, $p < 0.01$, unpaired Student's *t* test. *E*, eNOS protein levels in brain penumbra after reperfusion for 24 h. Densitometry measurements are depicted in a bar graph. Each bar represents the mean \pm S.E. $n = 3$; *, $p < 0.05$ compared with vehicle-treated group, one-way ANOVA with Tukey's post-test; #, $p < 0.05$ compared with the sham group, one-way ANOVA with Tukey's post-test.

potential side effects of these inhibitors in the cardiovascular system. Tubastatin was previously shown to have selectivity and potency similar to those of tubacin in inhibiting HDAC6 activity (54), but we found that each exerted opposite effects on eNOS expression. Moreover, we found that rocilinostat (ACY-1215), which is structurally similar to tubacin (55), had no effect on eNOS expression. In this regard, it is tempting to speculate that *in vivo* application of tubastatin may lead to cardiovascular complications through inhibiting eNOS activity, as compared with other inhibitors, such as tubacin and rocilinostat.

In conclusion, we provide compelling evidence that tubacin is a potent eNOS enhancer and can induce the production of NO both *in vitro* and *in vivo* as well as exert vascular protective effects in various clinical scenarios. Importantly, we found that these unique activities were independent of its HDAC6 inhibitor effects and related more to its ability to selectively increase eNOS mRNA stability. Despite its high lipophilicity and difficulties in synthesis, successful identification of tubacin as a promising vaso-protective agent suggests that further optimizing its structure for *in vivo* use might be worthwhile because of its potential as a treatment for a wide range of cardiovascular diseases.

Experimental procedures

Cell culture

HUVECs were purchased from ATCC and cultured in EBM-2 Basal Medium (Lonza) supplemented with EGM-2 BulletKit (Lonza). COS-7 cells and BAECs were also purchased from ATCC but cultured in Dulbecco's modified Eagle's

medium. BBMECs were purchased from Lonza and grown in EBM-2 basal medium (Lonza) supplemented with EMVB SingleQuots (Lonza). In some studies, endothelial cells were exposed to tubacin (Tocris Bioscience), tubastatin A (Tocris Bioscience), ACY-1215 (Cayman Chemical), or vorinostat (Cayman Chemical). Niltubacin (Enzo Life Sciences) served as a negative control in HDAC6 inhibitor experiments.

Gene silencing with small interference RNA

Human HDAC6 and scrambled (Mission siRNA Universal Negative Control) siRNA were transfected into HUVECs using the Lipofectamine RNAiMAX transfection Reagent (Invitrogen) according to the manufacturer's recommendations.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the TRIzol reagent kit (Invitrogen). qRT-PCR analysis was performed as we described previously (56). Briefly, cDNA was synthesized from total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). qRT-PCR was performed using the MyiQTM single-color real-time PCR detection system (Bio-Rad) and Hot-Start-IT SYBR Green one-step qRT-PCR master mix kit (AB Science).

Luciferase assay

COS-7 cells were transfected with the firefly luciferase F1-eNOS-promoter reporter plasmid or firefly luciferase-eNOS-UTR reporter plasmid, together with control luciferase

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reporter plasmid (28, 29). 24 h after transfection, cells were incubated with tubacin or simvastatin (eNOS-inducing agent) for an additional 24 h. Cell lysates were then collected and assayed for luciferase activity using the Dual-Luciferase assay system (Promega).

Determination of eNOS mRNA stability

The effects of tubacin on eNOS mRNA stability were determined by comparing mRNA levels in control and actinomycin D (5 μM)-exposed cells. -Fold change in gene expression was calculated using the $2^{-\Delta\Delta\text{CT}}$ method with 18S rRNA as an internal control as described previously (28). The relative amount of eNOS mRNA at 0 h AcD was set at 100%.

Western blot analysis

Western blot analysis was performed as described previously (28). In brief, cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). Blots were incubated with diluted primary antibodies against histone 3 (Santa Cruz Biotechnology, Inc., 10809, 1:1000), GAPDH (Santa Cruz Biotechnology, 32233 and 25778, 1:1000), α -tubulin (Cell Signaling, 2144, 1:2000), acetylated α -tubulin (Cell Signaling, 3971, 1:1000), HDAC6 (Cell Signaling, 7558, 1:500) and eNOS (BD Biosciences, 610297, 1:500), PTBP1 (Abcam, ab5642, 1:1000), eEF1A (Thermo Fisher Scientific, PA5-17213, 1:1000) followed by either IRDye 700- or 800-labeled secondary antibodies (1:10,000, LI-COR, 926-32212, 926-68073, and 925-68074) and then were visualized on an Odyssey Imaging System (LI-COR).

Measurement of NO production

The NO production was assessed by quantifying nitrite and nitrate levels in the supernatant of cells. Nitrite (NO_2^-) and nitrate (NO_3^-) levels were determined using a chemiluminescence NO detector (Siever 280i NO Analyzer), as described previously (57).

Preparation of nuclear and cytoplasmic extracts

HUVECs were lysed in hypotonic buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl_2 , 100 units of RNase OUT (Invitrogen), protease inhibitor mixture (Roche Applied Science)) on ice for 15 min, and then 25 μl of detergent (10% Nonidet P-40) was added. The mixture was vortexed for 10 s, followed by centrifugation at 3000 rpm for 10 min at 4 °C. After centrifugation, the supernatant was removed and represents the cytoplasmic fraction for Western blotting and RNA Immunoprecipitation. Nuclear extract was suspended by Cell Extraction Buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 0.1% SDS mixture) with protease inhibitors, followed by centrifugation at 16,000 rpm for 30 min at 4 °C.

RNA immunoprecipitation

Cytoplasmic fraction was incubated with 4 μg of anti-PTBP1 mAb (Abcam) 2 h at 4 °C and 20 μl of Protein A/G (Santa Cruz Biotechnology) to perform immunoprecipitation. Immune complexes were then washed multiple times with hypotonic buffer, total RNA was extracted using the TRIzol reagent (Invit-

rogen), and eNOS mRNA levels were quantified by qRT-PCR (29).

Arterial ring preparation and vascular tension recording

Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health following protocols that were reviewed and approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University. All experiments were performed on adult (8–12-week-old) male C57BL/6J mice, male eNOS knockout mice (stock no. 002684, Jackson Laboratory, Bar Harbor, ME), and male BKS-*db/db* mice (stock no. 000642, Jackson Laboratory). All animals were euthanized by carbon dioxide. Experimental mice were randomized to receive either 5 mg/kg tubacin or DMSO by intraperitoneal injection. 24 h after injection, mice were sacrificed, and segments of the descending aorta were recovered. Aortic tissues were maintained in ice-cold Krebs–Henseleit buffer consisting of 118 mM NaCl, 25 mM NaHCO_3 , 4.5 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2.5 mM CaCl_2 , and 11 mM glucose. Loose fat and connective tissue were removed, and 2–3-mm aorta rings were isometrically mounted on a Multi-Wire Myograph System (DMT620M). Resting tension for each aortic ring was set at 4 millinewtons and maintained at this level throughout the experiment. During equilibration, the rings were exposed to Krebs–Henseleit buffer (replaced every 15 min) at 37 °C and continuously gassed with 95% O_2 , 5% CO_2 . After 2 h, rings were exposed to phenylephrine (1×10^{-6} M) to elicit contraction, followed by monitoring of endothelium-dependent vasorelaxation to acetylcholine (10^{-9} to 10^{-5} M) over time. eNOS-independent vasorelaxation was assessed by measuring the cumulative response to acetylcholine in rings pretreated with the eNOS inhibitor L-NAME (10^{-4} M). Endothelium-independent vasorelaxation was measured by assessing response to sodium nitroprusside (10^{-10} to 10^{-6} M). Data were analyzed using the Powerlab system (AD Instruments). Vasorelaxation was expressed as a percentage based on the percentage change from the precontracted state.

MCAO model

Both male and female mice (12 weeks old) were injected with either vehicle (DMSO) or tubacin (5 mg/kg, intraperitoneally) twice at 24 and 3 h, respectively, before surgical occlusion. Focal cerebral ischemia was induced by transient MCAO as previously described (58). Animals were randomly divided into three groups: the sham group, the MCAO with vehicle-treated group, and the MCAO with tubacin-treated group. In the tubacin-treated group, 5 mg/kg tubacin was intraperitoneally injected 3 h before MCAO. Mice were subjected to MCAO by transient right MCA occlusion (60 min) under isoflurane (3%) anesthesia, followed by 24 h of reperfusion. Body temperature was controlled at 37 °C. Occlusion and reperfusion were verified in each animal by laser speckle contrast imaging (Pericam PSI). All animals were euthanized by carbon dioxide. For the quantification of infarct size, brain tissue was collected at 24 h for standard 2,3,5-triphenyltetrazolium chloride (TTC) histology and digital image analysis of infarct volume. Neurological function was evaluated using a 0–4-point neurological score: 0 = no neuro-

logical dysfunction; 1 = failure to extend left forelimb fully when lifted by tail; 2 = circling to the contralateral side; 3 = falling to the left; 4 = no spontaneous walk or in a comatose state, or barrel rolling. All scores were performed while blinded to study groups.

Statistical analysis

All values are expressed as the mean \pm S.E. Comparisons between two groups were analyzed by *t* test, whereas comparisons between more than two groups were made using one-way ANOVA followed by Tukey's post-test. *p* < 0.05 was considered statistically significant. All statistical analyses were performed via GraphPad Prism version 5.

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