Novel H2S Donor Proglumide-ADT-OH Protects HUVECs From ox-LDL-Induced Injury Through NF-κB and JAK/SATA Pathway

Xuelan Ou  
North Sichuan Medical College [Search North Sichuan Medical College]: North Sichuan Medical University

Chunyan Yang  
North Sichuan Medical College [Search North Sichuan Medical College]: North Sichuan Medical University

Chunlei Yu  
North Sichuan Medical College

Shipeng Zhang  
North Sichuan Medical College [Search North Sichuan Medical College]: North Sichuan Medical University

Rong Huang  
North Sichuan Medical College [Search North Sichuan Medical College]: North Sichuan Medical University

Chuan Chen  
North Sichuan Medical College [Search North Sichuan Medical College]: North Sichuan Medical University

Tianqin Xia  
North Sichuan Medical College [Search North Sichuan Medical College]: North Sichuan Medical University

Chunyang Zhou (zhouchunyang@nsmc.edu.cn)  
North Sichuan Medical College  https://orcid.org/0000-0002-9916-742X

Research

Keywords: hydrogen sulfide donor, atherosclerosis, NF-κB, JAK/STAT, proglumide-(5-(4-Hydroxyphenyl)-3H-1, 2-dithiole-3-thione) (P-A)

Posted Date: September 8th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-72333/v1
Novel H₂S donor proglumide-ADT-OH protects HUVECs from ox-LDL-induced injury through NF-κB and JAK/SATA pathway

Xuelan Ou, Chunyan Yang, Chunlei Yu, Shipeng Zhang, Rong Huang, Chuan Chen, Tianqin Xia, Chunyang Zhou

*Institute of Materia Medica, School of Pharmacy, North Sichuan Medical College, Nanchong 637100, Sichuan, China

Short Running Title: P-A protects HUVECs from ox-LDL-induced injury

*Correspondence author:

Chunyang Zhou
Institute of Materia Medica, School of Pharmacy
North Sichuan Medical College
Nanchong 637100, Sichuan, China

Tel:86-0817-3373323, Fax: 0817-2242761, E-mail: zhouchunyang@nsmc.edu.cn.

Number of Tables: 4
Number of Figures: 5
Word Count: 2500
Keywords

hydrogen sulfide donor, atherosclerosis, NF-κB, JAK/STAT, proglumide-(5-(4-Hydroxyphenyl)-3H-1, 2-dithiole-3-thione) (P-A)
Abstract

Introduction: As a gaseous me dilator, hydrogen sulfide (H$_2$S) has many physiological effects and pathological effects in atherosclerosis. In recent years, many exogenous H$_2$S donors have been synthesized to study atherosclerosis diseases.

Methods: Proglumide-(5-(4-Hydroxyphenyl)-3H-1, 2-dithiole-3-thione) (P-A) was synthesized as a H$_2$S donor. The protective effect and mechanism of P-A on HUVEC that injured by ox-LDL was detected.

Results: The HUEVCs was affected by 100μmol/L P-A for 24 hours, the release of H$_2$S was the largest. After 100μmol/L P-A acted on HUVEC damage model for 12h, the cell proliferation activity was the best. The results showed that P-A can down regulate the expression of p-NF-κBp65 protein and reduce the amount of TNF-α and IL-6 and promote the formation of IL-10 by inhibiting the NF-κB pathway, and also induce the expression of superoxide dismutase (SOD) to protect HUVEC from ox-LDL injury. P-A can also regulate JAK/STAT pathway to reduce the expression of p-JAK2 protein and reduce the production of IL-6 and TNF-α.

Conclusion: P-A has protective effect on HUVEC injured by ox-LDL, and the protective mechanism is related to the regulation of JAK/STAT pathway and NF-κB pathway.
Introduction

Atherosclerosis is a common cardiovascular disease caused by the interaction of environmental factors and genetic factors. The main manifestations of atherosclerosis include the lipid deposition of the intima, the infiltration of monocytes and macrophages, the formation of foam cells and fat veins, and the formation of fibrous plates that caused by the migration and proliferation of vascular smooth muscle cells (VSMCs), which causes the hardening of the vascular wall and the stenosis of the functional cavity and the formation of thrombus[1].

H$_2$S is a novel gas transmitter and has important physiological functions in atherosclerotic lesions[2]. The deficiency of H$_2$S in vivo may be related to the early development of atherosclerotic lesions. On the contrary, an appropriate amount of hydrogen sulfide is helpful to delay atherosclerosis[3]. Thus far, more and more exogenous H$_2$S donors have been created, including 5-(4-Hydroxyphenyl)-3H-1, 2-dithiole-3-thione (ADT-OH)[4]. ADT-OH is one of the most widely studied slow-releasing H$_2$S donors.

The NF-кB plays an important role in inflammatory response, immune response and cell growth and development[5]. H$_2$S could decrease the production of TNF-α and IL-1$\beta$ as well as leukocyte adhesion to the endothelium by inhibiting the activation of NF-кB[6]. Meanwhile, in the early atherosclerosis development process, TNF-α, IL-1$\beta$, IL-6 and IL-10 is closely related to the activation of JAK/STAT signaling pathway which is a signal transduction pathway that can be stimulated by cytokine and participates in the signal transduction and regulation process of various inflammatory and anti-
inflammatory factors[7]. Proglumide could reduce the release of cytokines and inflammatory mediators by inhibiting the activation of NF-κB pathway in acute pancreatitis. Considering that one of the pathogenesis of atherosclerosis disease is related to the inflammation and the anti-inflammatory effect of proglumide, we combined proglumide with ADT-OH to create proglumide-(5-(4-Hydroxyphenyl)-3H-1, 2-dithiole-3-thione) (P-A). In this study, we proved that P-A is a novel slow-releasing H₂S donor and shows anti-atherosclerotic effect on the HUVECs injured model by inhibiting the activation of JAK/STAT pathway and NF-κB pathway.

Materials and Methods

Synthesis of P-A

5-(4-methoxyphenyl)-1, 2-dithiole-3-thione (ADT) (Reference Number: 150127002, Yansheng Biotechnology, Shanghai, China). Proglumide (Reference Number: 6620-60-6, Ziqi Biotechnology, Shanghai, China). Anhydrous pyridine hydrochloride (sigma, USA).

Step1: synthesis of ADT-OH (Supplementary Figure S1, Supplementary Table S1). After the product were cooled to 25°C, 1mol/L HCL solution was added to dissolve the product and then the solution was filtered. After the filtrate is removed, the product is washed to neutral by distilled water. And then vacuum filtration, the residue was retained. The ADT-OH was obtained by recrystallization of anhydrous ethanol. The productivity of ADT-OH was 86.32%, with a total of 9.1382 g.
Step 2: synthesis of P-A (Figure 2A, Supplementary Table S2). Dichloromethane (DCM), N, N-Dimethyl-4-pyridinamine (DMAP), sodium hydroxide (NaOH), N, N'-dicyclohexylcarbodiimide (DCC) are from sigma in USA. After the reaction was stopped, a small amount of NaOH solution (1mol/L) was added to the reaction termination system until the color of the pH test paper displayed 7. After vacuum filtration for 2-3 times, filter the residue and filtrate. The filtrate is added to water in the separation funnel and layered, the water layer was removed and the dichloromethane layer was retained. The residual water in the dichloromethane layer was removed by adding anhydrous sodium sulfate. After vacuum filtration 2-3 times, the residue was filtered and the filtrate was left. The products were obtained after drying with rotary evaporator at 50°C. The product is dissolved in anhydrous ethanol. The product was recrystallized in the refrigerator at -20°C for one night. After vacuum filtration for 2-3 times the product was retained. The oil pump is evacuated to remove the product water and organic solvent to obtain P-A.

Detection of H$_2$S releasing

Human umbilical vein endothelial cells (HUVECs) were incubated in 12-well plates, and four microporous filtering films of 0.22µm were adhered to the inner side of the 12-well plates of each hole to set up the filter membrane adsorption device. After 500µL 1% (g/100mL) zinc acetate (Kelong, Chengdu, China) solution was added to each filter membrane, the P-A solutions were added in HUVECs. The filter membranes were collected after P-A acted on HUVEC for 1, 3, 6, 12, 24, 48h, respectively. Then the filter membrane was soaked in 2.5mL ultrapure water. The release of H$_2$S was detected
by methylene blue spectrophotometry at 670 nm, and the Na$_2$S standard curve was
drawn according to the OD.

**Establishment of ox-LDL induced HUVECs injured model**

HUVECs were damaged by 80µg/mL ox-LDL for 24h. The oil red O staining method
was used to judge whether the HUVEC had been damaged.

**CCK-8 assay**

HUVECs were seeded in 96-well plates and cultured for overnight at 37°C. HUVECs
were induced by 80µg/mL ox-LDL for 24h, then the P-A were acted on HUVECs. Then
Cell proliferation was detected by CCK8 kits (Boster Biotechnology, Chengdu, China)
according to the manufacturer’s protocol. Absorbance was determined at the 450nm by
enzyme-linked immunosorbent assay reader.

**ELISA assay of IL-6, IL-10 and TNF-α**

After the HUVECs were injured for 24h by 80µg/mL ox-LDL in 12-well plates, the
HUVECs were treated with 100µmol/L P-A for 12h. The supernatant from each well
was collected and used to detect the secretion of IL-6, IL-10 and TNF-α by ELISA
assay with commercial ELISA kits of IL-6, IL-10 and TNF-α (Boster Biotechnology,
Chengdu, China) according to the manufacturer’s protocol. In the AG490and PDTC
pre-treated assay, AG490 and PDTC were added in HUVECs respectively for 1h before
80µg/mL ox-LDL induced HUVEC for 24h. All other methods were the same as
described above.

**Determination of intracellular SOD**

After injured by 80µg/mL ox-LDL for 24h, HUVECs were treated with 100µmol/L P-
A for 12h. Then HUVECs were collected to lysis at 4°C in RIPA buffer. The lysate was clarified by centrifugation at 12000 rpm for 15 min at 4°C. Protein concentration of HUVEC lysate was determined by BCA assay kits (Yiyuan Biotechnology, Guangzhou, China). The activity of intracellular SOD was determined by SOD assay kits (Yiyuan Biotechnology, Guangzhou, China) according to the manufacturer’s protocol.

**Protein expression by western blotting**

p-NF-κBp65, p-JAK2, p-STAT3, NF-κBp65, JAK2, STAT3 antibody and goat anti-rabbit IgG were from Cell Signaling Technology, China. The total protein was extracted from HUVECs according to the standard procedures. Protein samples (40μg) were separated by 10% SDS-PAGE and then transferred into PVDF membranes. The membrane was blocked with 5% nonfat dry milk solutions. After washing the PVDF membranes with TBST, the PVDF membranes were incubated overnight at 4°C with the above antibody, respectively. It was followed by secondary antibody for 2h with goat anti-rabbit IgG. After washing, the membrane was developed with ECL kit and detected with VILBER Fusion FX5 system.

**Statistical analysis**

All data were analyzed with Graph Pad Prism5 and were presented as the mean ± SD. For all tests, P<0.05 was considered statistically significant.

**Results**

**P-A was synthesized successfully**

AS proved by $^1$H-NMR (Table 1), MS (Figure 1B) and HPLC (Figure 1C), P-A was
successfully synthesized and used for subsequent experiments.

**P-A is a slow-releasing H$_2$S donor**

The H$_2$S productivity of P-A was analyzed in HUEVCs, and we found that the release of H$_2$S from P-A increased in a time and concentration dependent manner, generally. However, the release rate decreased after the incubation time reached 24h or the concentration reached 100μmol/L. (Table 2, Figure 2).

**P-A reliefs ox-LDL induced HUVECs injury**

To test the protective effect of P-A on vein endothelial cells, we established the ox-LDL injured HUVECs in vitro model. After the HUVECs were induced by 80μg/mL ox-LDL for 24h, oil red O staining showed that a large number of red dye particles appeared in the cells (Figure 3A & B). This phenomenon indicated that HUVECs had formed damage which causing the oil red O enter into the cell and dissolve in the lipid. Then we treated the ox-LDL injured HUVECs with P-A, as shown in figure 3 C & D, the cell proliferation activity increased with time in a concentration dependent manner in24h..When the concentration of P-A reached to 200 mol/L, the declined cell viability indicated that P-A produces cytotoxicity at very high concentration above 100mol/L. (Figure 3C). After 100μmol/L P-A acted on HUVECs damage model for 12h, the cell proliferation activity was the best (Figure 3D).

**P-A regulates the expression of IL-6, IL-10, TNF-α and SOD**

Compared with normal cell control group, the amount of IL-6, TNF-α, IL-10 in the HUVEC injury model group increased significantly (P<0.01). As shown in Table 3, compared with the HUVEC damage model group, the secretion of IL-6 and TNF-α
reduced significantly after treated with P-A as well as positive control NaHS and ADT-OH (P<0.01), while the secretion of IL-10 increased significantly (P<0.01).

SOD can regulate the level of superoxide anion in the vascular wall, and alleviate the oxidative damage of oxygen free radicals to endothelial cells, as well as protect endothelial cells from atherosclerosis[8], therefore we analyzed the SOD level after P-A treatment. As shown in Figure 4, compared with normal cell control group, the activity of SOD in the HUVEC injury model group decreased significantly (P<0.01). Compared with the HUVEC damage model group, the activity of SOD in the NaHS group, P-A group and proglumide group increased significantly (P<0.01).

**P-A regulates expression of IL-6, IL-10 and TNF-α through NF-κB and JAK/SATA pathway**

Compared with P-A group, the expression of p-NF-κBp65 protein increased in the PDTC+P-A group (Figure 5A, P<0.05); the expression of p-NF-κBp65 protein reduced and the expression of p-JAK2 protein as well as the expression of p-STAT3 protein increased in NaHS group (Figure 5A-C); the expression of p-JAK2 protein increased in the AG490+P-A group(Figure 5B, P<0.01). Compared with NaHS group, the expression of p-NF-κBp65 protein increased in the PDTC+ NaHS group (Figure 5A, P<0.05); the expression of p-JAK2 protein increased in the AG490+P-A group (Figure 5B, P<0.01).

As shown in Table 4, compared with NaHS group, the amount of IL-6 and TNF-α increased significantly as well as the amount of IL-10 reduced significantly in the PDTC+ NaHS group in the PDTC+NaHS group (P<0.05). Compared with P-A group,
the amount of IL-6 increased significantly and the amount of IL-10 reduced significantly in the PDTC+P-A group and AG490+P-A group (P<0.05); the amount of TNF-α increased significantly in the PDTC+P-A group (P<0.05). Compared with Proglumide group, the amount of IL-10 reduced significantly in the PDTC+Proglumide group (P<0.05). Compared with ADT-OH group, the amount of IL-6 increased significantly (P<0.05).

Discussion

The current research on H$_2$S presents the trend of cross disciplinary research in pharmacology, physiology, chemistry, biology, materials science and so on[9]. In addition to the endogenous H$_2$S and the traditional hydrogen sulfide donor NaHS, more and more exogenous hydrogen sulfide donor[10], and some sulfur compounds extracted from natural plants have also been widely studied[11]. In this study, a novel hydrogen sulfide donor P-A was successfully synthesized as proved by $^1$H-NMR and MS. The demethylation reaction of ADT is the key in the whole synthesis reaction. The purity of the P-A will be affected by the purity of the ADT-OH. The addition of DCC in this system can activate carboxyl. After the reaction was stopped, adding NaOH solutions to the reaction system to pH 7 can wash out some acidic by-products, and can also adjust the reaction system pH to the neutral to avoid the degradation of the products. The chemical synthesis method in this experiment is simple and the reaction conditions are mild, while the post-processing is also simple. The product is easy to be purified through recrystallization. The study shows that the synthetic method in this experiment
can be used to obtain the target product P-A, which also provides valuable reference for the synthesis of other H₂S donors in the future.

With the increase amount of P-A used for treating HUVECs, the release of hydrogen sulfide and the cell proliferation gradually increased. However, excessive P-A has cytotoxic effect on cells, the proliferation and release of hydrogen sulfide was inhibited.

Vascular endothelial cells, smooth muscle cells and macrophages can secrete interleukin at different stages of inflammation[12]. In our study, we found that intracellular triglyceride and cholesterol metabolism disorder cause lipid aggregation to damage endothelial cells after the HUVEC was injured by ox-LDL, and the inflammatory reaction started at the same time, as well as the secretion of inflammatory factors TNF-α, IL-6 and anti-inflammatory factor IL-10 increased. IL-6 can cause chronic inflammation and magnify acute inflammatory response to some extent, and promote the release of some chemokines and reactive oxygen species to participate in and further aggravate the atherosclerosis process[13]. TNF-α is present in atherosclerotic plaques, which stimulates the production of inflammatory factors and directly promotes the development of inflammation. IL-10 has the functions of anti-inflammatory for the atherosclerosis disease[14].

The release of TNF-α, IL-6 and IL-10 can activate NF-κB to promote the production of inflammatory factors such as IL-6 and IL-8, that further aggravate the inflammatory reaction in the atherosclerosis process[15, 16]. Our study shows that P-A and ADT-OH can significantly reduce the secretion of IL-6 and TNF-α in the HUVEC damage model.

It is indicated that P-A reduces the secretion of IL-6 and TNF-α, which is related to the
The structure of ADT-OH. P-A and proglumide can significantly increase the secretion of IL-10 in the HUVEC damage model. It is indicated that P-A increases the secretion of IL-10, which is related to the structure of proglumide.

H$_2$S can inhibit the expression of intercellular adhesion molecule-1 mediated by NF-$\kappa$B pathway in HUEVC, and induce the expression of SOD in endothelial cells at the same time[17]. SOD can regulate the level of superoxide anion in the vascular wall, and alleviate the oxidative damage of oxygen free radicals to endothelial cells, as well as protect endothelial cells from atherosclerosis[8]. We found that P-A can significantly increase the activity of SOD in the experimental ox-LDL affected HUVECs, which is related to the structure of proglumide.

As the main transcription factor of the inflammatory response, NF-$\kappa$B can be activated by IL-6, TNF-$\alpha$, CRP, and so on, which participates in the whole process of atherosclerosis[18, 19]. JAK/STAT signal transduction pathway is activated after the JAK2 phosphorylation. Inhibition of JAK2 activity can inhibit STAT3 phosphorylation so that inhibiting the production of IL-6, IL-8, TNF-$\alpha$ and other inflammatory factors. Blocking the JAK/STAT signal pathway can effectively prevent the occurrence and aggravation of atherosclerosis diseases[20]. Our study found that P-A can downregulate the expression of p-NF-$\kappa$Bp65 protein and reduce the production of TNF-$\alpha$ and IL-6 and promote the formation of IL-10 by inhibiting the NF-$\kappa$B pathway, and also induce the expression of SOD in HUVEC damage model to protect HUVEC from ox-LDL. P-A can also regulate JAK/STAT signal transduction pathway to reduce the expression of p-JAK2 protein and reduce the production of TNF-$\alpha$ and IL-6.
However, there is no direct evidence showing that P-A protect HUVEC from ox-LDL damage only through the NF-κB pathway and JAK/STAT signaling pathway. Other associated signaling pathways may also play important roles in protecting HUVECs from ox-LDL damage. Based on the existing basis, the further study of the P-A is needed to find the downstream targets and genes of NF-κB pathway and JAK/STAT signaling pathway protect HUVECs, as well as the receptors of IL-6, IL-10 and TNF-α mediated by NF-κB signaling pathway and JAK/STAT signaling pathway, and other related signaling pathways and indicators.

In conclusion, P-A has the protective effect for experimental ox-LDL affected HUVEC, and the protective mechanism is related to the regulation of JAK/STAT pathway and NF-κB pathway to some extent. What’s more, our study provides direct evidence that JAK/STAT pathway and NF-κB pathway participate in the atherosclerosis process.

Statement of Ethics

There is no human or animal studies were conducted in this research.

Conflict of Interest Statement

The authors declared no potential conflicts of interest with respect to the research, authorship and publication of this article.

Funding Sources

This work was funded by the Science and Technology Project of Nanchong
Author Contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. XO, SZ, RH, CC, and TX conducted the experiments; CY, CYU and CZ wrote the manuscript.

Reference

1. Taleb, S., Inflammation in atherosclerosis. Arch Cardiovasc Dis, 2016. 109(12): p. 708-715.
2. Gimbrone, M.A. and G. García-Cardeña, Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. Circulation Research, 2016. 118(4): p. 620-636.
3. Li, H., et al., The interaction of estrogen and CSE/H2S pathway in the development of atherosclerosis. Am J Physiol Heart Circ Physiol, 2017. 312(3): p. H406-H414.
4. Wei, X., et al., Hydrogen Sulfide Inhalation Improves Neurological Outcome via NF-kappaB-Mediated Inflammatory Pathway in a Rat Model of Cardiac Arrest and Resuscitation. Cell Physiol Biochem, 2015. 36(4): p. 1527-38.
5. Wang, Z.J., et al., Atherosclerosis and the Hydrogen Sulfide Signaling Pathway - Therapeutic Approaches to Disease Prevention. Cell Physiol Biochem, 2017. 42(3): p. 859-875.
6. Song, D., et al., Selective inhibition of endothelial NF-kappaB signaling attenuates chronic intermittent hypoxia-induced atherosclerosis in mice. Atherosclerosis, 2018. 270: p. 68-75.
7. Wang, R., et al., Protein Inhibitor of Activated STAT3 Suppresses Oxidized LDL-
induced Cell Responses during Atherosclerosis in Apolipoprotein E-deficient Mice. Sci Rep, 2016. 6: p. 36790.

8. Liu, M., et al., The Essential Role of Pin1 via NF-kappaB Signaling in Vascular Inflammation and Atherosclerosis in ApoE(-/-) Mice. Int J Mol Sci, 2017. 18(6).

9. Testai, L., et al., The novel H2S-donor 4-carboxyphenyl isothiocyanate promotes cardioprotective effects against ischemia/reperfusion injury through activation of mitoKATP channels and reduction of oxidative stress. Pharmacol Res, 2016. 113(Pt A): p. 290-299.

10. Hasegawa, U. and A.J. van der Vlies, Design and synthesis of polymeric hydrogen sulfide donors. Bioconjug Chem, 2014. 25(7): p. 1290-300.

11. Wang, Y., et al., Synthesis and Biological Evaluation of Danshensu and Tetramethylpyrazine Conjugates as Cardioprotective Agents. Chem Pharm Bull (Tokyo), 2017. 65(4): p. 381-388.

12. Fava, C. and M. Montagnana, Atherosclerosis Is an Inflammatory Disease which Lacks a Common Anti-inflammatory Therapy: How Human Genetics Can Help to This Issue. A Narrative Review. Front Pharmacol, 2018. 9: p. 55.

13. Akita, K., et al., An Interleukin-6 Receptor Antibody Suppresses Atherosclerosis in Atherogenic Mice. Front Cardiovasc Med, 2017. 4: p. 84.

14. Pfeiler, S. and N. Gerdes, Atherosclerosis: cell biology and lipoproteins - focus on anti-inflammatory therapies. Curr Opin Lipidol, 2018. 29(1): p. 53-55.

15. Libby, P., et al., Inflammation in atherosclerosis: transition from theory to practice. Circ J, 2010. 74(2): p. 213-20.

16. Yu, X.H., X.L. Zheng, and C.K. Tang, Nuclear Factor-kappaB Activation as a Pathological Mechanism of Lipid Metabolism and Atherosclerosis. Adv Clin Chem, 2015. 70: p. 1-30.

17. Wang, Y., et al., Macrophage mitochondrial oxidative stress promotes atherosclerosis and nuclear factor-kappaB-mediated inflammation in macrophages. Circ Res, 2014. 114(3): p. 421-33.

18. Martelli, A., et al., Hydrogen sulphide: biopharmacological roles in the cardiovascular system and pharmaceutical perspectives. Curr Med Chem, 2012. 19(20):
19. Mitchell, J.P. and R.J. Carmody, *NF-kappaB and the Transcriptional Control of Inflammation*. Int Rev Cell Mol Biol, 2018. 335: p. 41-84.

20. Ortiz-Munoz, G., et al., *Suppressors of cytokine signaling modulate JAK/STAT-mediated cell responses during atherosclerosis*. Arterioscler Thromb Vasc Biol, 2009. 29(4): p. 525-31.

**Figure Legends**

**Figure 1.** P-A was successfully synthesized. (A) The method of synthesizing P-A. (B) MS of P-A. The molecular weight of P-A was 542 by MS. The molecular weight was 543, indicating that the combination of P-A and H. The molecular weight was 565, indicating that the combination of P-A and Na+. The molecular weight was 1107, indicating that the combination of 2 molecule of P-A and Na+. (C) P-A has higher purity and less impurity content, as well as a single peak was showed by HPLC.

**Figure 2.** HUVECs produce H$_2$S after P-A was added in cells. The release of H$_2$S of different concentrations P-A acted on HUVECs at different time. The release of H$_2$S was the largest after the HUVECs was affected by 100μmol/L P-A for 24 hours. (Mean ± SD, n=3).

**Figure 3.** The cell proliferation of different concentrations P-A acted on HUVEC at different time. (A) representative images of HUVECs. (B) representative images of ox-LDL induced HUVECs. After the HUVECs were induced by 80μg/mL ox-LDL for 24h, the cells had been damaged. (C)The cell proliferation activity increased with time in a concentration dependent manner in 0-24h. $^-$P<0.01 vs control; $^#$P<0.05 vs 25μmol/L; $^\Delta\Delta$P<0.01 vs 50μmol/L; &$^\&$P<0.01 vs 100μmol/L. (D) After 100μmol/L P-
A acted on HUVEC 12h, the cell proliferation activity was the largest. *P<0.05 vs 1h; **P<0.05 vs 3h; ^P<0.001 vs 6h; &P<0.01 vs 12h; δδδ P<0.001 vs 24h. (Mean ± SD, n=3).

**Figure 4.** Effect of P-A acted on the activity of SOD in HUVEC damage model. (Mean ±SD. n=4) **p<0.01 vs cell control; △△p<0.01 vs model; #p<0.05 vs P-A.

**Figure 5.** P-A regulates expression of IL-6, IL-10 and TNF-α through NF-κB and JAK/SATA pathway. (A) The expression of p-NF-κB p65 protein in HUVEC damage model., 1: cell control; 2: model; 3: NaHS; 4: P-A; 5: PDTC+NaHS; 6: PDTC+P-A. *p<0.05 **p<0.01 vs model; ^p<0.05 #p<0.01 vs P-A; δp<0.05 vs NaHS. Mean ± SD, n=3. (B)The expression of p-JAK2 protein in the HUVEC damage model 1: cell control; 2: model; 3: NaHS; 4: P-A; 5: AG490+NaHS; 6: AG490+P-A. (C)The expression of p-STAT3 protein in the HUVEC damage model 1: cell control; 2: model; 3: NaHS; 4: P-A; 5: AG490+NaHS; 6: AG490+P-A. **p<0.01 vs model; #p<0.01 vs P-A; △△p<0.01 vs NaHS. Mean ± SD, n=3.
Fig. 3

(A) and (B) show images of cells under different conditions. (A) is labeled with an arrow and (B) has a small black circle. The images represent the cell morphology and viability under various conditions.

(C) and (D) are bar graphs showing the relative cell viability over time. (C) plots the viability at 24 hours for different concentrations: control, 12.5, 25, 50, 100, and 200. (D) shows the viability at different time points: 1h, 3h, 6h, 12h, 24h, and 48h, with concentration 100 μmol/L. The graphs indicate significant changes in cell viability with statistical significance marked by symbols.

The overall figure suggests an analysis of cell viability and morphology under specific conditions, highlighting the impact of concentration and time on cell behavior.
Supplementary materials

Supplementary Figure S1

Supplementary Figure 1. Reaction equation for the synthesis of ADT-OH compounds.

Supplementary Table S1

Supplementary Table S1. Synthesis of ADT-OH

| reagent           | $C_{10}H_8OS_3$(ADT) | $C_5H_6NCl$ |
|-------------------|----------------------|-------------|
| M(g/mol)          | 240.36               | 115.56      |
| m(g)              | 10.5853              | 50.01       |
| n(mol)            | 0.44                 | 0.4327      |
| melting point (°C)| 110                  | -41.6       |
| boiling point (°C)| —                    | 115.2       |
| solvent           | —                    | —           |
| reaction temperature (°C)| 215                   |
| reaction time(min) | 60                   |
### Supplementary Table S2

#### Supplementary table S2. Synthesis of P-A

| reagent    | M(g/mol) | m (g) | n(mmol) |
|------------|----------|-------|---------|
| Proglumide | 334.42   | 1.338 | 4       |
| ADT-OH     | 226.34   | 0.995 | 4.4     |
| DCC        | 206.33   | 0.992 | 4.8     |
| DAMP       | 0.066    |       |         |
| DCM        | 100ml    |       |         |

Reaction conditions: RT, 4h, N₂
P-A was successfully synthesized. (A) The method of synthesizing P-A. (B) MS of P-A. The molecular weight of P-A was 542 by MS. The molecular weight was 543, indicating that the combination of P-A and H. The molecular weight was 565, indicating that the combination of P-A and Na+. The molecular weight was 1107, indicating that the combination of 2 molecule of P-A and Na+. (C) P-A has higher purity and less impurity content, as well as a single peak was showed by HPLC.
HUVECs produce H2S after P-A was added in cells. The release of H2S of different concentrations P-A acted on HUVECs at different time. The release of H2S was the largest after the HUEVCs was affected by 100μmol/L P-A for 24 hours. (Mean ± SD, n=3).
The cell proliferation of different concentrations P-A acted on HUVEC at different time. (A) representative images of HUVECs. (B) representative images of ox-LDL induced HUVECs. After the HUVECs were induced by 80 μg/mL ox-LDL for 24h, the cells had been damaged. (C) The cell proliferation activity increased with time in a concentration dependent manner in 0-24h. ~P<0.01 vs control; ##P<0.05 vs 25 μmol/L; & P<0.01 vs 50 μmol/L; && P<0.01 vs 100 μmol/L. (D) After 100 μmol/L P18 A acted on HUVEC 12h, the cell proliferation activity was the largest. *P<0.05 vs 1h; ##P<0.05 vs 3h; δδδδ P<0.001 vs 6h; &&P<0.01 vs 12h; δδδδ P<0.001 vs 24h. (Mean ± SD, n=3).
Effect of P-A acted on the activity of SOD in HUVEC damage model. (Mean ±SD. n=4) **p<0.01 vs cell control; ⋆p<0.01 vs model; #p<0.05 vs P-A.
Fig. 5

P-A regulates expression of IL-6, IL-10 and TNF-α through NF-κB and JAK/SATA pathway. (A) The expression of p-NF-κB p65 protein in HUVEC damage model. 1: cell control; 2: model; 3: NaHS; 4: P-A; 5: PDTC+NaHS; 6: PDTC+P-A. *p<0.05 **p<0.01 vs model; #p<0.05 ##p<0.01 vs P-A; ฀ p<0.05 vs NaHS. Mean ± SD, n=3. (B) The expression of p-JAK2 protein in the HUVEC damage model 1: cell control; 2: model; 3: NaHS; 4: P-A; 5: AG490+NaHS; 6: AG490+P-A. **p<0.01 vs model; ##p<0.01 vs P-A. (C) The expression of p-STAT3 protein in the HUVEC damage model 1: cell control; 2: model; 3: NaHS; 4: P-A; 5: AG490+NaHS; 6: AG490+P-A. **p<0.01 vs model; ##p<0.01 vs P-A; ฀฀ p<0.01 vs NaHS. Mean ± SD, n=3.