ISSN: 2476 - 2415

Research Article

International Journal of Clinical & Experimental Dermatology

The Anti-Hypoxia Protective Effect of WYY026B by Interacting with Hemoglobin in Red Blood Cells

Yuying Zhang[1,2], Pingping Zhang[2], Xin Zhang[1*], Ye Liu[2*]

[1]*Department of Maternal, Child and Adolescent Health, School of Public Health, Tianjin Medical University, No. 22 Qixiangtai Road, Tianjin 300070, China*

[2]*Beijing Honghui Meditech Co., Ltd, No. 50 Huatuo Road, CBP Daxing, Beijing 102600, China*

[*]Corresponding Author
Xin Zhang, Department of Maternal, Child and Adolescent Health, School of Public Health, Tianjin Medical University, No. 22 Qixiangtai Road, Tianjin 300070, China.
Ye Liu, Beijing Honghui Meditech Co., Ltd, No. 50 Huatuo Road, CBP Daxing, Beijing 102600, China.

Submitted: 31 Jan 2023; **Accepted:** 06 Feb 2023; **Published:** 20 Apr 2023

Citation: Zhang, Y., Zhang, P., Zhang, X., Liu, Y. (2023). The Anti-Hypoxia Protective Effect of WYY026B by Interacting with Hemoglobin in Red Blood Cells. *Int J Clin Expl Dermatol, 8*(2), 16-23.

Abstract

Background

Exposure to hypoxia may experience damage to the heart, brain, lungs and other organs due to the low inspired O2. The improvement of endurance of human in resisting hypoxia and reducing hypoxia-induced organ damage is an urgent problem to be solved. WYY026B is a honokiol derivatives by structural modification, and exhibits very potent pharmacological activities and medicinal properties in brain ischemia/reperfusion (I/R) injury.

Methods

We used hypoxia model cerebral I/R model to identify the anti-hypoxia effect of WYY026B. High performance liquid chromatograph and immunofluorescence assay were used to detect the relationship between WYY026B and Red blood cells (RBCs). Interaction between WYY026B and hemoglobin (Hb) were validated by surface plasmon resonance assay (SPR).

Results

We demonstrated that WYY026B increased arterial oxygen saturation (SaO2) and arterial partial pressure of oxygen under hypoxia stress to improve the hypoxia tolerance. Blood gas analysis showed that WYY026B significantly decreased cervical venous SaO2 and increased arteriovenous oxygen partial pressure difference in I/R model. Nissl′s staining indicated that WYY026B significantly reduced the degree of hypoxia in brain tissue. A series of tests in vitro revealed that WYY026B could combine with RBCs. SPR assays showed that WYY026B interacted with Hb.

Conclusion

Overall, WYY026B maybe a new drug to enhance the ability of Hb to carry oxygen and to protect brain from the hypoxia injury.

Keywords: hemoglobin, WYY026B, hypoxia, oxygen-carrying capacity.

Introduction

At present, many inhabitants still live in high altitude areas at >3,000 m elevation, and an increasing number of people have travelled to high-altitude areas, who have the potential risk of developing mountain sickness, high-altitude pulmonary edema, high-altitude cerebral edema due to exposure to a hypobaric hypoxia environment [1,2]. Exposure to hypoxia may experience damage to the heart, brain, lungs and other organs due to the low inspired O2 [3,4]. Stroke and acute myocardial infarction (AMI) have become two of the leading causes of death and disability in the world [5-7]. The energy crisis and oxidative stress are the primary causes of neuronal death and brain damage or permanent damage to the myocardium due to the hypoxic-ischemic injury [8]. Therefore, the improvement of the endurance of humans in resisting hypoxia and reducing hypoxia-induced organ damage is an urgent problem to be solved.

Researchers have established some animal models of hypoxia and

found many anti-hypoxia drugs through a lot of experimental studies [9,10]. Acetazolamide, a potent carbonic anhydrase (CA) inhibitor, is the most commonly used and best-studied agent for the amelioration of acute mountain sickness [11-13]. Rhodiola rosea L. has long been used as traditional medicines which can improve the anti-hypoxia ability of the body, relieve hypoxia response, and reduce tissue damage caused by hypoxia(Li et al. 2021). However, existing drugs cannot meet the need for effective prevention and treatment of hypoxia. It is still of great significance to find safe and effective anti-hypoxia drugs.

Current researches demonstrate that red blood cells (RBCs) are designated carriers of oxygen transport to various body tissues. Increasing the oxygen affinity of hemoglobin (Hb) in RBCs enhances physiological tolerance to hypoxia and is a promising approach for treating a range of diseases caused by hypoxia [14]. A variety of small molecules have been identified that increase the oxygen affinity of Hb [15-18]. GBT440, a novel anti-polymerization and anti-sickling agent, increases Hb oxygen affinity and reverses in vitro sickling of previously sickled red blood cells under hypoxic conditions [19,20]. GBT1118, a structural analog of GBT440, bonded covalently and reversibly via an imine intermediate to the NH2-terminal valine of the Hb-chain and allosterically increases intracellular Hb affinity for O2 to preserved O2 delivery to tissues [16]. TD-1, a novel allosteric effector of Hb induced a greater increase in oxygen affinity of human Hb [21].

Our researches developed a new series of honokiol derivatives by structural modification, and some exhibited very potent pharmacological activities and medicinal properties in brain ischemia/reperfusion (I/R) injury [22-26]. In this study, we aimed to discuss WYY026B, a honokiol derivatives, with the functional contribution under hypoxic and ischemic conditions and identify its potential relationship with Hb for future clinical development as anti-hypoxia protectant.

Material and methods

Compounds

WYY026B, WYY026B reference compound and WYY026B-biotin were synthesized at Beijing Honghui Medical Technology Co., Ltd, Beijing (China).

Hypoxia experiments in rats

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Tianjin Medical University and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague Dawley (SD) rats (220-240 g) were obtained from SiPeiFu (Beijing) Biotechnology Co., Ltd. Free access of standard diet and tap water was provided to animals.

The rats were placed in a closed device, and the O2 concentration in the device was monitored and controlled by an oxygen control instrument (Nanjing Xinfei Analytical Instrument Manufacturing Co., Ltd, XF-669, China), so that the O2 concentration in the device was reduced to 11%. Blood was taken from the femoral artery at 24 h before hypoxia treatment and 6 h after under hypoxia condition for arterial blood gas analysis by blood gas analyzer (Abbott Laboratories, i-STAT300, USA). NS as control group and WYY026B was administered (i.v.) at 0.25mg/kg and 1 mg/kg before hypoxia treatment (n = 4 for each group).

The blood oxygen saturation (SaO_2) and partial pressure of oxygen (PaO_2) of Jugular venous blood after cerebral I/R injury

The cerebral I/R injury model in Sprague Dawley (SD) rats were prepared as previously described [27,28]. Briefly, animals were anaesthetized with chloral hydrate (10%, 350 mg/kg, i.p.). Body temperature was maintained at 37°C with a heated surgery pad, fixed in the supine position. The right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were surgically exposed, and then a siliconized nylon filament with blunted tip was introduced into the ICA through an incision in the external carotid artery and gently advanced 20±2 mm for rats until the tip reached the origin of the middle cerebral artery. At the same time, the animals were administered with WYY026B (i.v., 0.25 mg/kg, 250 μL/250 g body weight) or NS (i.v., 250 μL/250 g body weight). After 2 h occlusion, the filament was gently removed to restore blood flow. Before surgery, 1 hour of ischemia and 0.5 hours of reperfusion, femoral artery blood and cervical venous blood were collected for further assays. Blood was analyzed by blood gas analyzer (Abbott Laboratories, i-STAT300, USA).

Nissl´s staining

Histology staining was performed on 3 m paraffin sections of the rat brain tissue. Nissl´s staining was carried out according to the manufacturer's protocols (Wuhan servicebio technology Co., Ltd, China). The staining was digitized with a Nikon orthotopic light microscope (Nikon Eclipse E100 and NIKON DS-U3 imaging system).

Isolation of red blood cells from rats

Whole blood (about 1 mL) was collected from orbital plexus of healthy SD rats (about 200 g), and the blood was added to a 1.5 mL centrifuge tube premixed with heparin sodium. The blood was gently shaken and centrifuged for 4 min at 4℃ (4800 rpm). The upper clear plasma and lower hematocrit were separated. Hematocrit (500 μL) was added to 500 μL NS was mixed and centrifuged for 4 min at 4℃ (4800 rpm). Washing was repeated 2 times, and about 500 μL hematocrit was obtained.

The content of WYY026B was determined by HPLC

The content of WYY026B in the supernatant was determined by HPLC (high performance liquid chromatograph, Agilent 1260 Series, USA) performed on an Agilent ZORBAX SB-C18 (4.6 × 250 mm, 5 μm) column and peak detection at 212 nm with Agilent Technologics UV detector. Mobile phase A: 20 mM sodium dihydrogen phosphate (pH 3.0); mobile phase B: acetonitrile; flow rate: 1 mL/min; column temperature: 35°C; injection volume: 40 μL.

Immunofluorescence assay of RBCs

RBCs 100 µL were incubated with 1 mL of the WYY026B-biotin solution (200 µg/mL) at 37°C for 10 min, then washed with NS for 3 times. The washed RBCs were centrifuged each time for 4 min at 4°C (2000 rpm), and the supernatant was removed. The RBCs were fixed in 1% paraformaldehyde solution for 15 min. Add 0.01% Triton X-100 permeable solution 100 µL to the RBCs at room temperature for 5 min, then washed with NS for 3 times. The RBCs were incubated with 400 µL 3% BSA blocking solution at room temperature for 1 h. The RBCs were incubated in the anti-Biotin-FITC antibody solution (1:100, Sigma Aldrich, F4024, USA) at room temperature avoid light for 1 h, then washed with NS for 3 times. The RBCs were incubated in the DiR solution (40 µg/mL, PerkinElmer, 125964, USA) at room temperature avoid light for 20 min, then washed with NS for 3 times. Images were obtained and analyzed using confocal laser microscope (Leica, SP8, Germany).

Surface plasmon resonance assay

Surface plasmon resonance (SPR) analysis was conducted with Open SPR instrument (Nicoyalife, Canada). WYY026B was diluted in running buffer PBS containing 1% DMSO at concentrations ranging from about 0.781 M to 12.5 µM. Analytes were injected through reference and active channels at a flow rate of 20 µL/min. The association and dissociation times were 240 and 360 s. The kinetic parameters of the binding reactions were calculated and analyzed by using Trace Drawer software (Ridgeview Instruments AB, The Kingdom of Sweden).

Statistical analysis

Data were analyzed with GraphPad Prism software. All values were expressed as the mean ± SD. Statistical analyses were carried out by Student t-test. A value of $P<0.05$ was considered statistically significant.

Results

Effect of WYY026B on the oxygen-carrying capacity of blood in rats under hypoxic stress

In order to determine the anti-hypoxia effect of WYY026B, we tested the oxygen carrying capacity of blood in rats. Arterial oxygen parameters were detected after 6 h under hypoxia condition (oxygen content, 11%). The blood oxygen saturation (SaO_2) and partial pressure of oxygen (PaO_2) of the control before hypoxia group and WYY026B before hypoxia group were used as the reference value for comparison. The results showed that there was a marked drop in SaO_2 and PaO_2, and RR was speeded up significantly after hypoxia treatment. PaO_2 and SaO_2 were increased markedly after administered (i.v.) with WYY026B after hypoxia group compared with control after hypoxia group (Fig. 1A and 1B). These results showed that WYY026B improved the oxygen-carrying capacity of blood in rats in hypoxia.

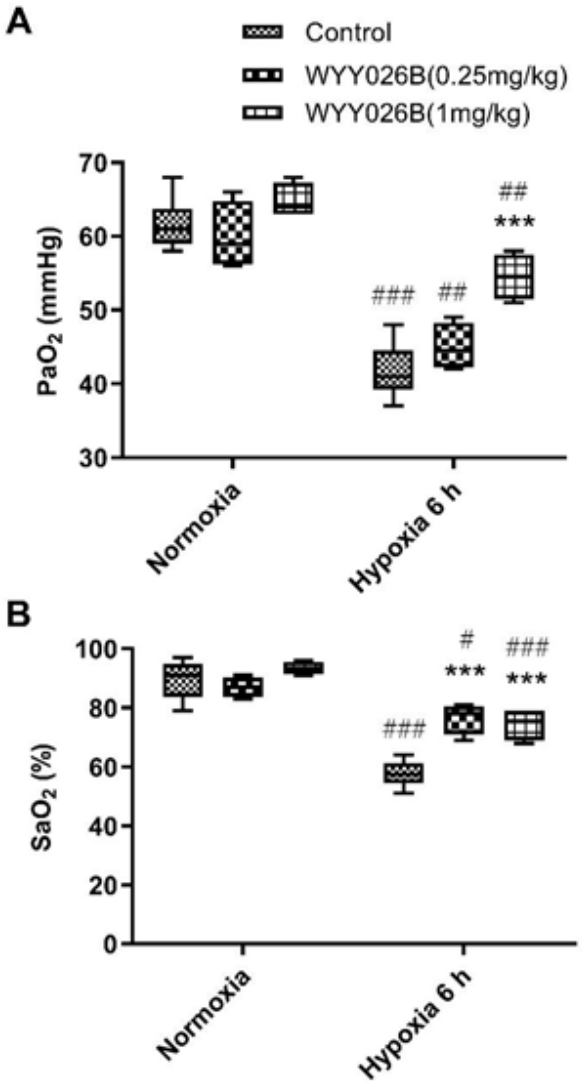

Figure 1: Effect of WYY026B on the oxygen-carrying capacity of blood in rats under hypoxic stress. Arterial oxygen parameters were detected at 24 h before hypoxia treatment and 6 h after under hypoxia condition (oxygen content, 11%). WYY026B was administered (i.v.) at 0.25 mg/kg and 1 mg/kg before hypoxia treatment. Control group: NS. (A) PaO2; (B) SaO2. Data are reported as the mean ± SD. Student t-test: ** $P<0.01$ versus control group after hypoxia. # $P<0.05$, ## $P<0.01$, ### $P<0.001$ versus normoxia group.

Anti-hypoxia effect of WYY026B in cerebral I/R model

In order to study the anti-hypoxia effect of WYY026B, we tested PaO_2 and SaO_2 in the femoral artery and cervical venous of rats subjected to 2 h ischemia following 0.5 h reperfusion. NS as vehicle, or WYY026B at 0.25 mg/kg were immediately administered (i.v.) after ischemia. WYY026B significantly decreased cervical venous PaO_2 and SaO_2 under cerebral ischemia and hypoxia (Fig. 2A and 2B). Under the condition of hypoxia, the oxygen partial pressure gradient (arteriovenous oxygen partial pressure differ-

ence, Fig. 2C) was increased, which promoted the diffusion of O2 molecules to tissue cells. We also investigated the potential therapeutic effects of WYY026B on I/R injury with the rat tMCAO model subjected to 2 h ischemia with following reperfusion for 24 h. WYY026B at 0.25 mg/kg was administered immediately (i.v.) after ischemia. Nissl's staining showed that WYY026B prevented apoptosis to protect brain tissue (Fig. 3). All of this implied that WYY026B can improve the hypoxia tolerance of rats.

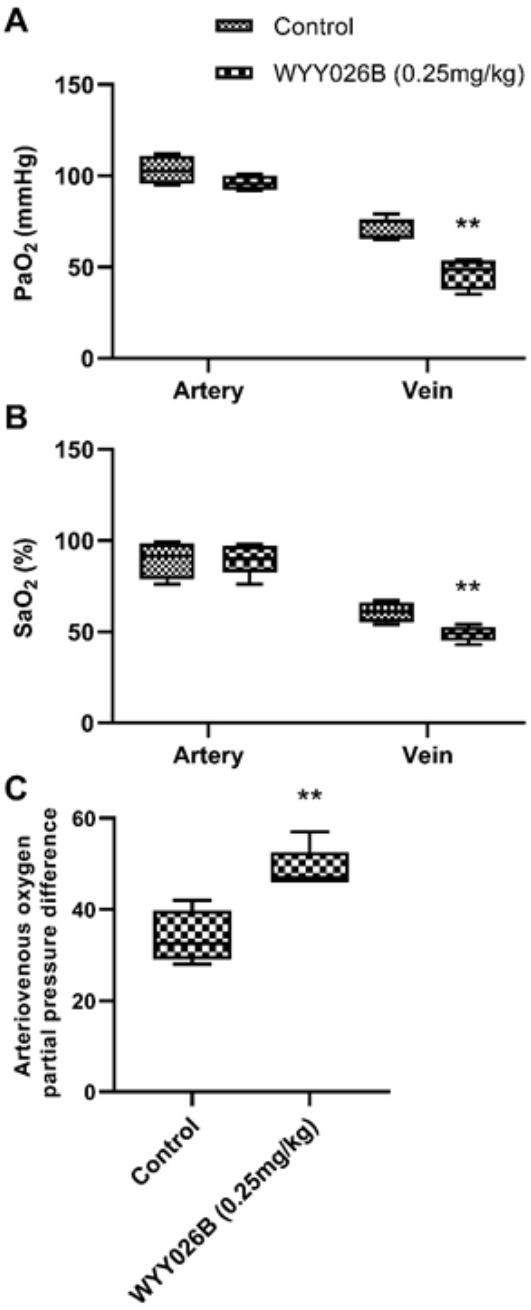

Figure 2: WYY026B increases the hypoxia tolerance of brain in cerebral I/R rat model. (A) The PaO2 of femoral arterial and cervical venous blood after 2.5 h of cerebral I/R injury in rats by blood gas analysis (n=4/5). (B) The SaO2 of femoral arterial and cervical venous blood after 2.5 h of cerebral I/R injury in rats by blood gas analysis (n=4/5). (C) The arteriovenous oxygen partial pressure difference between femoral artery and cervical venous after 2.5 h of cerebral I/R injury in rats (n=4/5). Data are reported as the mean ± SD. Student t-test: ** P<0.01 versus control group.

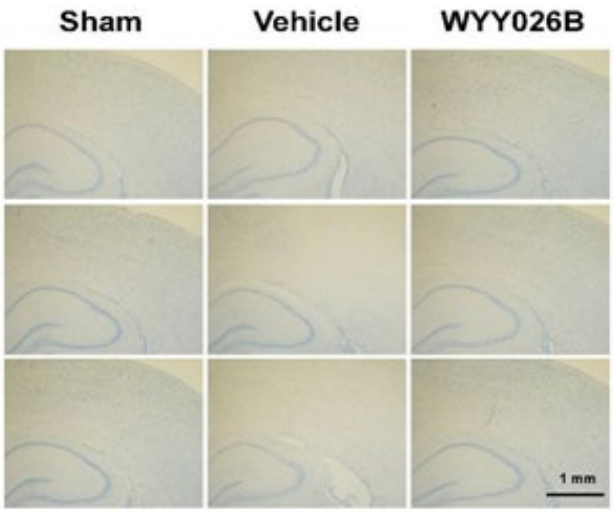

Figure 3: The anti-apoptosis effect of WYY026B on cerebral I/R injury in rats. Representative pictures of Nissl's staining in the ischemic brain after 24 h of cerebral I/R (scale bar, 1 mm).

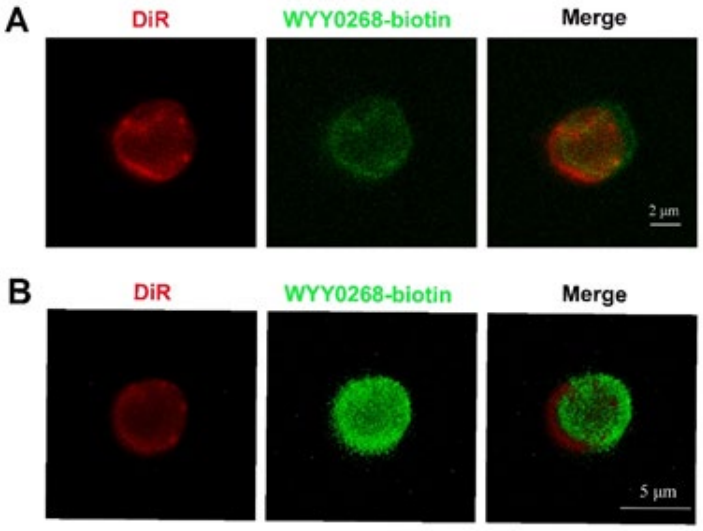

Figure 4: The localization of WYY026B after interaction with RBCs. Monoclonal anti-Biotin-FITC antibody was used for immunofluorescence staining of RBCs incubated with WYY026B-Biotin. At the same time, RBCs were labeled with DiR fluorescent marker. (A) The fluorescence was observed from the 2D perspective. Scale bar: 2 μm. (B) The fluorescence was observed by 3D perspective from the frontal view of RBCs. Scale bar: 5 μm.

Interaction between WYY026B and Hb were validated by SPR assay

To investigate the effect of WYY026B on affinity of Hb-O2 by binding to Hb. The interaction of WYY026B and Hb was evaluated by biomolecular interaction analysis with SPR. The kinetics oμf the binding reaction was determined by injecting different concentrations of WYY026B over a human Hb (Sigma, USA) immobilized on the chip surface. The data showed that the equilibrium dissociation constants (KD) was 2.22 μM according to the obtained association and dissociation rates (Fig. 5). In this study, we determinate that WYY026B can combine with the Hb, thus improving the oxygen carrying capacity of blood to resist hypoxia injury.

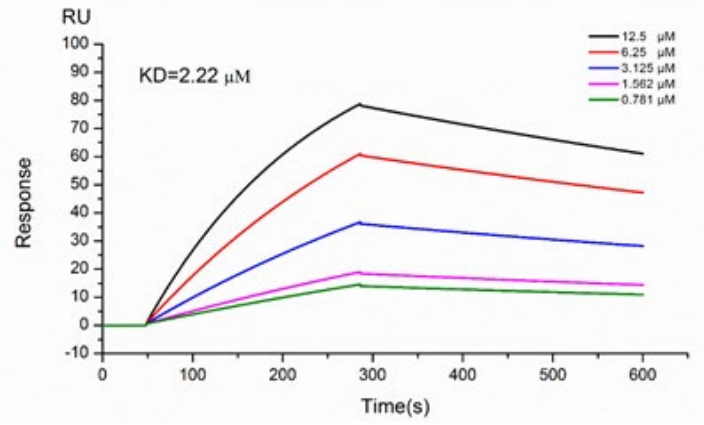

Figure 5: Characterization of the affinity between WYY026B and Hb, which was immobilized on a CM5 sensor chip, based on the SPR assay. The KD value was labeled in the corresponding curves.

WYY026B interacts with RBCs

In order to study the relationship between WYY026B and various components in blood, WYY026B was mixed with whole blood, plasma or RBCs of rats and incubated in water bath at 37°C for 10 min. NS has no blood component as a control. After incubation, the samples of each group were treated to obtain the supernatant. High performance liquid chromatograph (HPLC) was used to detect the drug content in the supernatant (Supplementary Fig. 1). According to the WYY026B content in the supernatant, the loss ratio of the WYY026B was calculated which was only 1.05% in plasma, but 75% in whole blood and 80% in RBCs (Table 1). It was speculated that the content change of WYY026B was mainly caused by RBCs after contact with blood.

To further investigate the interaction relationship between WYY026B and RBCs. WYY026B (0.2 mg/mL) was mixed with RBCs of rats and incubated in water bath at 37°C for 10 min, 30 min and 60 min. After incubation, the samples of each group were treated to obtain the supernatant. HPLC was used to detect the drug content in the supernatant (Table 2 and Supplementary Fig. 2). Accordingly, the retention amount of WYY026B in supernatant decreased. At 60 min, no free drugs could be detected in supernatant. This suggested that all of WYY026B were acted upon by RBCs at 60 min.

Table 1: The interaction relationship between WYY026B with blood components

Blood component	Blood component volume (μL)	WYY026B dosage (mg)	Area under curve (mAU*s)	WYY026B content in the supernatant (μg)	Amount of loss (μg)	loss ratio (%)
Plasma	100	0.2	1270.1	197.9	2.1	1.05
RBCs	100	0.2	258.4	39.3	160.3	80
Whole blood	200	0.1	164.6	25.0	75.0	75
NS	/	0.2	5262.5	200	0	0

WYY026B was mixed with whole blood, plasma or RBCs of rats and incubated in water bath at 37°C for 10 min. NS has no blood component as a control. HPLC was used to detect the drug content in the supernatant, in terms of Area under curve.

Table 2: The interaction relationship between WYY026B and RBCs]

Action time (min)	WYY026B content in the supernatant (μg)	WYY026B content in RBCs (μg)
10	35.94±0.226	164.02±0.001
30	14.47±0.095	185.51±0.022
60	0±0.000	200±0.000

WYY026B was mixed with RBCs of rats and incubated in water bath at 37°C for 10 min,.30 min and 60 min. HPLC was used to detect the drug content in the supernatant, in terms of Area under curve. Data are reported as the mean ± SD, n = 3 for each group.

The effect of drug dosage on the interaction of WYY026B to RBCs To explore the effect of drug dosage on the interaction of WYY026B to RBCs, dosage were set at 20、50、100、200、250、260、270、280 μg/mL. Different doses of WYY026B were mixed with RBCs of rats and incubated in water bath at 37°C for 10 min. After incubation, the samples of each group were treated to obtain the supernatant. HPLC was used to detect the drug content in the supernatant (Table 3 and Supplementary Fig. 3). There was a saturation trend, when the dosage of WYY026B was 250 μg/mL. The slope value of WYY026B action showed an inflection point (from 0.83 to 0.48), and tended to be stable afterwards.

Table 3: The effect of drug dosage on the interaction of WYY026B to RBCs

WYY026B concentration (μg/mL)	WYY026B content in the supernatant (μg)	WYY026B content in RBCs (μg)	Slope value
20	0	20	1.00
50	0.84	49.26	0.98
100	7.05	92.95	0.87
200	36.54	163.45	0.71
250	44.86	205.14	0.83
260	50.03	209.97	0.48
270	62.23	207.76	-0.22
280	67.12	212.87	0.51

Different doses at 20、50、100、200、250、260、270、280 μg/mL of WYY026B were mixed with RBCs of rats and incubated in water bath at 37°C for 10 min. After incubation, the samples of each group were treated to obtain the supernatant. HPLC was used to detect the drug content in the supernatant, in terms of Area under curve.

The localization of WYY026B after interaction with RBCs

In order to intuitively observe the localization of WYY026B and RBCs after interaction, WYY026B and RBCs were labeled with fluorescence respectively, and then observed the site of action between the WYY026B and RBCs by fluorescence copolymerization technology. WYY026B were labeled by biotin. Monoclonal anti-Biotin-FITC antibody was used for immunofluorescence staining of RBCs incubated with WYY026B-Biotin. At the same time, RBCs were labeled with DiR fluorescent marker. Confocal fluorescence microscopy showed that WYY026B co-located with RBCs (Fig. 4). The fluorescence was observed from the 2D and 3D perspective. The presence of WYY026B on the surface of RBCs could be observed from the frontal view of RBCs, and the presence of WYY026B in the interior of RBCs could be observed from the lateral view of RBCs.

Discussion

Hypoxia can occur in many extreme environments including plateau, diving, aerospace, and high intensity exercise, and may occur during many diseases including acute cerebral ischemia, myocardial infarction and serious hypotension (Nikinmaa 2020) [29,30]. The heart, brain, lungs are extremely sensitive to different forms of hypoxia [31-33].Therefore, an anti-hypoxia protectant for improving tissue damage due to a lack of oxygen or obstacles to the use of oxygen has become an important are [9]. In the norm baric hypoxia assessment, insufficient oxygen supply severely reduced the intracellular oxygen partial pressure and oxyhemoglobin saturation, while WYY026B increased markedly SaO2 and PaO2. In the focal cerebral I/R assessment, WYY026B significantly reduced the degree of hypoxia in brain tissue and increased arteriovenous oxygen partial pressure difference. Hence, WYY026B have the function of improving anoxia tolerance.

RBCs act as the major media for transporting oxygen in the blood. In this study, a series of studies were conducted on the in vitro reaction mode of WYY026B with red blood cells to confirm that WYY026B and red blood cells have obvious effects. Through the study of the reaction between WYY026B and blood components, it was concluded that the content of WYY026B remained unchanged after the reaction with plasma in the supernatant, and WYY026B is substantially reduced after contact with whole blood or RBCs. Through the study of the combination of WYY026B and RBCs, it was found that the effect of WYY026B and RBCs increased with the increase of the time and dosage, and there was a saturation trend. Further, it was found that WYY026B co-located with RBCs determined by fluorescence labeling. The results in the present study suggest that WYY026B can enter into the RBCs to improve the oxygen carrying capacity of blood.

The oxygen-carrying capacity of Hb in RBCs is the key to tissue oxygen demand [34,35]. WYY026B significantly increased cervical venous SaO2 under cerebral ischemia and hypoxia of mice and rats. This implied that WYY026B can improve the hypoxia tolerance of animal model. The interaction of WYY026B and Hb evaluated by biomolecular interaction analysis with SPR to indicate the effect of WYY026B on affinity of Hb-O2 by binding to Hb. What is not enough is that we need to further explore how

WYY026B regulates the oxygen affinity of Hb and its influence on the structure of oxygenated Hb.

In addition to high altitude hypoxia, many diseases cause the body's tissues to be exposed to low oxygen, such as ischemic stroke and acute myocardial ischemia. WYY026B significantly decreased cervical venous SaO2 under cerebral ischemia and hypoxia. WYY026B improves the oxygen utilization of brain tissue and increases the ability of brain tissue to withstand hypoxia.

WYY026B, one of the honokiol derivatives, has the function with improving the oxygen-carrying capacity of blood in rats under hypoxia. WYY026B significantly reduced the degree of hypoxia in brain tissue in brain I/R injury animal model. We identified the potential relationship between WYY026B and Hb for future clinical development as anti-hypoxia protectant.

Acknowledgements

This work was supported by R&D Foundation of Beijing Honghui Meditech Co., Ltd.

Author Contributions

YYZ conceived and designed research. YYZ and PPZ conducted experiments. YYZ and XZ analyzed data. YYZ and YL wrote the manuscript. All authors read and approved the manuscript.

Data availability

HPLC spectrum of WYY026B (Supplementary Fig. 1 to 3) are available from the corresponding author on request.

Declarations

Conflict of interest

The authors declare no competing interests.

Ethical approval

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Tianjin Medical University and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

References

1. Swenson, E. R. (2016). Pharmacology of acute mountain sickness: old drugs and newer thinking. Journal of Applied Physiology, 120(2), 204-215.
2. Kooman, J. P., Stenvinkel, P., Shiels, P. G., Feelisch, M., Canaud, B., & Kotanko, P. (2021). The oxygen cascade in patients treated with hemodialysis and native high-altitude dwellers: lessons from extreme physiology to benefit patients with end-stage renal disease. American Journal of Physiology-Renal Physiology, 320(3), F249-F261.
3. MacIntyre, N. R. (2014). Tissue hypoxia: implications for the respiratory clinician. Respiratory care, 59(10), 1590-1596.
4. Hou, Y., Wang, X., Chen, X., Zhang, J., Ai, X., Liang, Y., ... & Hu, Y. (2019). Establishment and evaluation of a simulated high altitude hypoxic brain injury model in SD rats. Molecular Medicine Reports, 19(4), 2758-2766.
5. Liu, M., Wu, B., Wang, W. Z., Lee, L. M., Zhang, S. H., & Kong, L. Z. (2007). Stroke in China: epidemiology, prevention, and management strategies. The Lancet Neurology, 6(5), 456-464.
6. Ibáñez, B., Heusch, G., Ovize, M., & Van de Werf, F. (2015). Evolving therapies for myocardial ischemia/reperfusion injury. Journal of the American College of Cardiology, 65(14), 1454-1471.
7. Neri, M., Riezzo, I., Pascale, N., Pomara, C., & Turillazzi, E. (2017). Ischemia/reperfusion injury following acute myocardial infarction: a critical issue for clinicians and forensic pathologists. Mediators of inflammation, 2017.
8. Liu, N., Zhang, X. L., Jiang, S. Y., Shi, J. H., Cui, J. H., Liu, X. L., ... & Shao, G. (2020). Neuroprotective mechanisms of DNA methyltransferase in a mouse hippocampal neuronal cell line after hypoxic preconditioning. Neural Regeneration Research, 15(12), 2362.
9. Berger, M. M., & Grocott, M. P. W. (2017). Facing acute hypoxia: from the mountains to critical care medicine. BJA: British Journal of Anaesthesia, 118(3), 283-286.
10. Dunham-Snary, K. J., Wu, D., Sykes, E. A., Thakrar, A., Parlow, L. R., Mewburn, J. D., ... & Archer, S. L. (2017). Hypoxic pulmonary vasoconstriction: from molecular mechanisms to medicine. Chest, 151(1), 181-192.
11. Leaf, D. E., & Goldfarb, D. S. (2007). Mechanisms of action of acetazolamide in the prophylaxis and treatment of acute mountain sickness. Journal of applied physiology.
12. Vagal, A. S., Leach, J. L., Fernandez-Ulloa, M., & Zuccarello, M. (2009). The acetazolamide challenge: techniques and applications in the evaluation of chronic cerebral ischemia. American journal of neuroradiology, 30(5), 876-884.
13. Bradbury, K. E., Yurkevicius, B. R., Mitchell, K. M., Coffman, K. E., Salgado, R. M., Fulco, C. S., ... & Charkoudian, N. (2020). Acetazolamide does not alter endurance exercise performance at 3,500-m altitude. Journal of Applied Physiology, 128(2), 390-396.
14. Safo, M. K., & Kato, G. J. (2014). Therapeutic strategies to alter the oxygen affinity of sickle hemoglobin. Hematology/Oncology Clinics, 28(2), 217-231.
15. Kong, H. L., Wang, J. P., Li, Z. Q., Zhao, S. M., Dong, J., & Zhang, W. W. (2009). Anti-hypoxic effect of ginsenoside Rbl on neonatal rat cardiomyocytes is mediated through the specific activation of glucose transporter-4 ex vivo. Acta Pharmacologica Sinica, 30(4), 396-403.
16. Dufu, K., Yalcin, O., Ao-Ieong, E. S., Hutchaleelala, A., Xu, Q., Li, Z., ... & Cabrales, P. (2017). GBT1118, a potent allosteric modifier of hemoglobin O2 affinity, increases tolerance to severe hypoxia in mice. American Journal of Physiology-Heart and Circulatory Physiology, 313(2), H381-H391.
17. Patel, M. P., Siu, V., Silva-Garcia, A., Xu, Q., Li, Z., & Oksenberg, D. (2018). Development and validation of an oxygen dissociation assay, a screening platform for discovering, and characterizing hemoglobin–oxygen affinity modifiers. Drug Design, Development and Therapy, 1599-1607.

18. Ma, Y., Wu, Y., Xia, Z., Li, J., Li, X., Xu, P., ... & Xue, M. (2019). Anti-hypoxic molecular mechanisms of rhodiola crenulata extract in zebrafish as revealed by metabonomics. Frontiers in Pharmacology, 10, 1356.
19. Dufu, K., & Oksenberg, D. (2018). GBT440 reverses sickling of sickled red blood cells under hypoxic conditions in vitro. Hematology reports, 10(2), 7419.
20. Dufu, K., Patel, M., Oksenberg, D., & Cabrales, P. (2018). GBT440 improves red blood cell deformability and reduces viscosity of sickle cell blood under deoxygenated conditions. Clinical hemorheology and microcirculation, 70(1), 95-105.
21. Nakagawa, A., Lui, F. E., Wassaf, D., Yefidoff-Freedman, R., Casalena, D., Palmer, M. A., ... & Zapol, W. M. (2014). Identification of a small molecule that increases hemoglobin oxygen affinity and reduces SS erythrocyte sickling. ACS chemical biology, 9(10), 2318-2325.
22. Liou, K. T., Shen, Y. C., Chen, C. F., Tsao, C. M., & Tsai, S. K. (2003). Honokiol protects rat brain from focal cerebral ischemia–reperfusion injury by inhibiting neutrophil infiltration and reactive oxygen species production. Brain research, 992(2), 159-166.
23. Hu, Z., Bian, X., Liu, X., Zhu, Y., Zhang, X., Chen, S., ... & Wang, Y. (2013). Honokiol protects brain against ischemia–reperfusion injury in rats through disrupting PSD95–nNOS interaction. Brain research, 1491, 204-212.
24. Sulakhiya, K., Kumar, P., Jangra, A., Dwivedi, S., Hazarika, N. K., Baruah, C. C., & Lahkar, M. (2014). Honokiol abrogates lipopolysaccharide-induced depressive like behavior by impeding neuroinflammation and oxido-nitrosative stress in mice. European journal of pharmacology, 744, 124-131.
25. Talarek, S., Listos, J., Barreca, D., Tellone, E., Sureda, A., Nabavi, S. F., ... & Nabavi, S. M. (2017). N europrotective effects of honokiol: from chemistry to medicine. Biofactors, 43(6), 760-769.
26. Zhao S, Liu X, Zhu Y, Liu Y and Wang Y 2019 The proteomic study and the target discovery of W026B, a new compound with brain protective effect. Journal of Chinese Pharmaceutical Sciences 28 381-392.
27. Fluri, F., Schuhmann, M. K., & Kleinschnitz, C. (2015). Animal models of ischemic stroke and their application in clinical research. Drug design, development and therapy, 9, 3445.
28. Ma, R., Xie, Q., Li, Y., Chen, Z., Ren, M., Chen, H., ... & Wang, J. (2020). Animal models of cerebral ischemia: A review. Biomedicine & Pharmacotherapy, 131, 110686.
29. Ward, D. S., Karan, S. B., & Pandit, J. J. (2011). Hypoxia: developments in basic science, physiology and clinical studies. Anaesthesia, 66, 19-268.
30. Grimminger, J., Richter, M., Tello, K., Sommer, N., Gall, H., & Ghofrani, H. A. (2017). Thin air resulting in high pressure: mountain sickness and hypoxia-induced pulmonary hypertension. Canadian respiratory journal, 2017.
31. Nicolls, M. R., & Voelkel, N. F. (2007). Hypoxia and the lung: beyond hypoxic vasoconstriction. Antioxidants & redox signaling, 9(6), 741-743.
32. Sendoel, A., & Hengartner, M. O. (2014). Apoptotic cell death under hypoxia. Physiology, 29(3), 168-176.
33. Tasbihgou, S. R., Netkova, M., Kalmar, A. F., Doorduin, J., Struys, M. M., Schoemaker, R. G., & Absalom, A. R. (2018). Brain changes due to hypoxia during light anaesthesia can be prevented by deepening anaesthesia; a study in rats. PLoS One, 13(2), e0193062.
34. Lee, E. J., Woodske, M. E., Zou, B., & O'Donnell, C. P. (2009). Dynamic arterial blood gas analysis in conscious, unrestrained C57BL/6J mice during exposure to intermittent hypoxia. Journal of Applied Physiology, 107(1), 290-294.
35. Ahmed, M. H., Ghatge, M. S., & Safo, M. K. (2020). Hemoglobin: structure, function and allostery. Vertebrate and invertebrate respiratory proteins, lipoproteins and other body fluid proteins, 345-382.
36. Chiang, T., Messing, R. O., & Chou, W. H. (2011). Mouse model of middle cerebral artery occlusion. JoVE (Journal of Visualized Experiments), (48), e2761.
37. Li, S., Hafeez, A., Noorulla, F., Geng, X., Shao, G., Ren, C., ... & Ji, X. (2017). Preconditioning in neuroprotection: from hypoxia to ischemia. Progress in neurobiology, 157, 79-91.
38. Li, Y., Cai, M., Mao, G. X., Shu, Q. F., Liu, X. B., & Liu, X. L. (2021). Preclinical evidence and possible mechanisms of Rhodiola rosea L. and its components for ischemic stroke: a systematic review and meta-analysis. Frontiers in Pharmacology, 12, 736198.
39. HIF1α, H. (2020). Finally, a promising model for high-altitude pulmonary edema (HAPE)—A Mountaineers' Malady. Acta Physiologica, 229, e13472.
40. Sommer, C. J. (2017). Ischemic stroke: experimental models and reality. Acta neuropathologica, 133(2), 245-261.
41. Xu, G. G., Pagare, P. P., Ghatge, M. S., Safo, R. P., Gazi, A., Chen, Q., ... & Abdulmalik, O. (2017). Design, synthesis, and biological evaluation of ester and ether derivatives of antisickling agent 5-HMF for the treatment of sickle cell disease. Molecular pharmaceutics, 14(10), 3499-3511.