Neuroprotective effect of Indobufen against pyroptosis following cerebral ischemia-reperfusion injury both in vivo and in vitro

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Abstract

Background: Indobufen is a new generation of antiplatelet agents and has been shown to have antithrombotic effects in animal models. However, the efficacy of Indobufen on cerebral ischemia/reperfusion (I/R) injury and its mechanisms remain to be investigated.

Methods: In this study, the efficacy of Indobufen with both pre- and post-treatment on rats suffering middle cerebral artery occlusion/reperfusion (MCAO/R) was investigated. Furthermore, human umbilical vein endothelial cells (HUVECs) were cultured and underwent oxygen glucose deprivation/reoxygenation (OGD/R) injury for in vitro studies. Relationship between Indobufen and pyroptosis associated NF-κB/Caspase-1/GSDMD pathway was preliminarily discussed.

Results: The pharmacodynamic tests revealed that Indobufen ameliorated I/R injury by decreasing the platelet aggregation, infarct size, brain edema and neurologic impairment in rats and rescuing cell apoptosis/pyroptosis in HUVECs. The underlying mechanisms were probably related to pyroptosis suppression by regulating the NF-κB/Caspase-1/GSDMD pathway. Conclusion: Overall, these studies indicates that Indobufen exerts protective and therapeutic effects against I/R injury by pyroptosis suppression via downregulating NF-κB/Caspase-1/GSDMD pathway.

1. Background

Stroke is characterized by high morbidity and disability rate, and is also one of the deadliest diseases globally [1]. However, only a limited number of strategies are now available for ischemic stroke [2, 3]. Therefore, the prevention and treatment of stroke has important clinical value. Indobufen (lab no. K3920, Figure 1), with a chemical structure of 2-[p-(1-oxo-2-isoindoline) phenyl] butyrate, is a new generation of antiplatelet aggregation drugs that reversely inhibits platelets, cycoperxygenase and reduces the production of thromboxane A2 (TXA2) [4]. We have previously reported that Indobufen could inhibit the coagulation process and reduce thrombosis [5], so we speculated that Indobufen may also have therapeutic and preventive effects against ischemia/reperfusion (I/R) injury.

Ischemic stroke is attributed to the blockage of brain blood circulation, which leads to the accumulation of hypoxia and toxic substances such as inflammatory cytokines, as well as the death of brain cells, and may induce subsequent neurobehavioral deficits in stroke survivors. There are three classical forms of brain cell death: necrosis, apoptosis and pyroptosis, among which pyroptosis, also known as Caspase-1 dependent cell death, is recently a hot topic [6]. Caspase-1 is activated by the inflammasome NLRP3 after receiving the signal of NF-κB nuclear translocation, followed by the activation of inflammatory cytokines IL-1β and IL-18 into active forms, which play important roles in the maintenance and development of inflammatory responses [7]. In addition, Caspase-1 also specifically cleaves Gasdermin D protein (GSDMD) into carboxy-terminal Gasdermin-c (GSDMD-C) and amino-terminal Gasdermin-n (GSDMD-NT), where GSDMD-NT is associated with cell membrane rupture [8]. In this study, we investigated by prophylactic and therapeutic administration the effects of Indobufen on I/R injury as well as its anti-
apoptosis/apoptosis efficacy in rat brain and HUVECs cells, conjecturing that the molecular mechanisms involved signal regulation of NF-κB/GSDMD/NLRP3 both in vivo and in vitro. This study may provide us new insights into the effects of Indobufen on I/R injury and suggest Indobufen as a potential strategy for ischemic stroke.

2. Methods

2.1 Animals and Treatments

Male SPF Sprague–Dawley (SD) rats (250–300g) were purchased from Qinglongshan Animal Farm of Nanjing, China (Production License No. SCXX (su) 2018-0019; SCXK (zhe) 2019-0002). All animals were housed under a 12 hour light-dark cycle at temperature of 22-26°C in a room of 40-70% humidity. Rats were fasted for 12 hours before the surgery. Rats were randomly divided into 4 groups: (1) sham operation group; (2) MCAO/R model group; (3) Indobufen (20 mg/kg) group; (4) Aspirin (10 mg/kg) group. The in vivo trial was divided into 5 day prophylactic administration (5-day tests) and 15 day therapeutic administration (15-day tests). In the 5-day prophylactic administration experiment, oral administration was given for 5 days, and surgery was performed 30min after the 5th-day administration. In the 15-day treatment administration experiment, the drugs were administered 3 hours after the operation, once a day for 15 days. All rats were kept at a relative humidity of 55 ± 5% (at 25 ± 2 °C) in compliance with institutional guidelines of China Pharmaceutical University and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All experiments were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University (SYXK (Su) 2016-0011).

2.2 Drug administration

Indobufen (Huadong medicine Co., LTD) and Aspirin (Shanghai yuanye Bio-Technology Co., LTD) were both dissolved in 0.5% sodium carboxymethyl cellulose (CMC-Na). Drugs were administered intragastrically with a volume of 0.5mL/100g body weight. Sham and model group were given the same volume of 0.5% CMC-Na.

2.3 In vivo MCAO/R establishment

MCAO/R injury was established as previously described after anesthetization with 3% isoflurane [9] with 2h of ischemia followed by reperfusion. After the operation, the rats with successful surgery according to the behavioral scoring were retained. All efforts were made to minimize animal suffering and reduce the number of animals used. Five rats died from anesthesia and ten died from intracranial hemorrhage, which were excluded in the analysis.

2.4 Neurological defect scoring, cylinder test and postural reflex test

One days following MCAO/R, the neurological function of the rats was scored from 0 to 4 according to Bederson's method [10]. For the limb-use asymmetry test and postural reflex test, the rats were tested as
previous reported [2]. In 5-day tests: neurological scoring was conducted at 24 h after reperfusion. In 15-day tests: cylinder test and posture reflex test were carried out 30 min after drug administration as shown in Figure 2B.

2.5 Measurement of infarct size

In the 5-day tests, rats were sacrificed by cervical dislocation 24h after the MCAO surgery and in the 15-day tests, rats were sacrificed by cervical dislocation 30min after the last drug-administration. 2,3,5-Triphenyltetrazolium chloride (TTC, Sangon Biotech CO., LTD) staining were used to measure brain infarct size [11]. The percentage of infarction was calculated as following: Infarct rate (%) = (left hemisphere area – right white brain infarction area)/left hemisphere area × 100%.

2.6 Evaluation of brain edema

Brain sections were weighed right after TTC staining to obtain wet weight. Then the brain was placed in an oven at 110 °C for 24 h to dry to constant weight to obtain dry weight, and the brain water content was calculated: Brain water content (%) = (1 – dry weight/wet weight) × 100% [12].

2.7 Morris water maze (MWM)

In the 15-day tests, Morris Water Maze evaluation was conducted as mentioned before [13, 14]. The escape latency and swimming path were recorded along the first five days and percentage of time spent in the quadrant IV and number of crossing platform was measured in probe trial of the fifth day of MWM.

2.8 Primary cell culture of HUVECs

Primary cultures of Human umbilical vein endothelial (HUVEC) were purchased from ScienCell Research Laboratories, Inc (Cat. #8000). Endothelial cell medium (ECM, ScienCell Research Laboratories, Inc, Cat. #1001) containing 5% heat-inactivated fetal bovine serum (FBS) with 100 U/mL penicillin and 100 μg/mL streptomycin was used and cells was placed in a humidified atmosphere with 5% CO₂ at 37 °C. The medium was changed every 2 days.

2.9 Oxygen-glucose deprivation followed by recovery (OGD/R)

HUVEC cells were randomly divided into groups below: (1) control; (2) OGD/R; (3) Indobufen (200 μM) (4) Aspirin (100μM);. All groups were incubated for 24 h with medium containing different drugs. Then HUVEC cells were subjected to hypoxic conditions (95% N₂, 5% CO₂) with no-glucose DMEM for 4 h, followed by reoxygenation for 2 h [15].

2.10. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

Cell viability was determined by MTT assay (Wanleibio, Shenyang, China; LOT#16B109). The optical density (OD) value at 570nm was measured to calculate the cell viability as follows: Cell viability (%) =
(OD value in the administration group-OD value in the solvent control group)/ (OD value in the blank group-OD value in the solvent control group) ×100%.

2.11. ELISA assay

The concentrations of 6-keto-PGF\textsubscript{1α} and TXB\textsubscript{2} in the brain tissue homogenates and culture cell supernatants were assessed by ELISA (Nanjing Jiancheng Bioengineering Institute) according to manufacturer's instructions. The levels were normalized to cell protein concentrations.

2.12. Real-time PCR

Changes in mRNA levels of IL-18, IL-1β and NLRP3 were detected in brain tissues in the 5-day pretreatment groups, and real-time PCR was performed as described previously [16]. Quantitative PCR (Mastercycler ep realplex, Eppendorf) was performed in the presence of SYBR Green I. Expression of IL-18, IL-1β and NLRP3 was normalized to β-actin analyzed by 2\textsuperscript{-ΔΔCt}. The primers (Sangon Biotech Co., LTD., Shanghai, China) were as follows:

Rat IL-18-F: ACCGCAGTAATACGGAGCAT; R: GTCTGGGATTCGTTGGCTGT

Rat IL-1β-F: AGGCTGACAGACCCAAAG; R: CTCCACGGGCAAGACATAGG

Rat NLRP3-F: GTGGAGATCCTAGGTTTCTCT; R: CAGGATCTCATTCTCTTGGATC

Rat-β-actin-F: AGACCTTCAACACCCCAAG; R: CACGATTTCCCTCTCAGC

2.13. Immunofluorescence (IF) assay

At 24 h after MCAO/R in the 5-day pretreatment groups, the rats were anesthetized, 4% paraformaldehyde was perfused through the heart, and brain tissue was quickly removed and fixed in 4% paraformaldehyde. Brain samples were performed on fixed frozen ultrathin sections (Leica CM3050s, Germany) as previously described [17]. As for in vitro experiments, HUVEC cells were fixed with 4% paraformaldehyde for 20 min after OGD/R treatment. Thereafter, brain sections or culture cells were incubated with rabbit antibody NF-κB p65 (1:200, Cell Signaling Technology), rabbit antibody NLRP3 (1:100, proteintech, Wuhan, China; CAT#17168-1-AP) or rabbit antibody Caspase-1 (1:100, proteintech, Wuhan, China; CAT#22915-1-AP) at 4 °C overnight. After being rinsed with 0.3% Triton-X PBS (PBST), cells were incubated with goat anti-rabbit IgG/Cy3 antibody (1:200; Bioss, Beijing, China; CAT#bs-0295G-Cy3) or goat anti-rabbit IgG (H+L) (1:200, proteintech, Wuhan, China; CAT#SA00013-4) in dark for 2h, and then stained with 4',6-diamidino-2-phenylindole (DAPI, Beyotime Institute of Biotechnology, Shanghai) for 30 min. Images were captured with a fluorescence microscope (Nikon Ts2R, Japan).

2.14. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and GSDMD co-staining
The pyroptosis cells emerged from rat MCAO/R or HUVECs OGD/R were detected by TUNEL staining and immunofluorescence assay. The brain slides or HUVEC cells were incubated with GSDMD rabbit antibody (1:100, abbexa, Houston, TX, USA; CAT#abx136074) overnight at 4 °C. Subsequently, Coralite594-conjugated goat anti-rabbit IgG (H+L) (1:200, proteintech, Wuhan, China; CAT#SA00013-4) was incubated for 2 h. TUNEL staining (Keygenbiotech, Nanjing, China; CAT#KGA7073) were processed followed with DAPI staining for 30 min and photograph by a fluorescence microscope (Nikon Ts2R, Japan).

2.15. Western blot analysis

Equivalent protein samples (50 μg) of brain tissues or HUVEC cells were separated by 10% SDS/PAGE gel electrophoresis. Following primary antibodies were incubated: rabbit antibody NF-κB p65 (1:1000, Cell Signaling Technology), rabbit antibody Caspase-1 p20 (1:500, Wanleibio, Shenyang, China), mouse antibody β-actin (1:1000, proteintech, Wuhan), and rabbit antibody Histone H3 (1:1000, proteintech, Wuhan, China) and membranes were visualized via chemiluminescence.

2.16. Statistical analysis

The data in this study were expressed as mean ± SD. Kruskal-Wallis test was used for behavioral tests, the number of crosses in the probe test in quadrant IV of MWM. The remaining data were analyzed by one-way ANOVA followed by Bonferroni test post hoc test for equal variance data and Dunnett 3 post hoc test for unequal variance by IBM SPSS 25.0 software. Differences were considered significant when p-values were smaller than 0.05. Photoshop 2020, Image J and GraphPad Prism Version 8.0 were used for statistical analysis.

3. Results

3.1 Pre-treatment with Indobufen alleviates cerebral ischemic injury in MCAO/R rats

MCAO was conducted to mimic human cerebral ischemic injury in rats [18]. The diagram in Fig. 2 showed timeline of in vivo studies. In 5-day tests, 24 h after MCAO/R, TTC staining showed that infarcted white zone in MCAO/R model group was apparent (Fig. 2C) and held 32.73±1.83% (Fig.2D) of the brain, which marked the successful establishment of MCAO. Quantification showed that Aspirin (10 mg/kg, 20.20±2.55%) exhibited an significant reduction in the infarct size (P < 0.01, Fig. 2D), with Indobufen (20 mg/kg, 15.03 ± 2.20%) exhibiting better effects on infarction alleviation (P < 0.01, Fig. 2C). Similarly, Indobufen (20 mg/kg, 79.31 ± 0.64%) and Aspirin (10 mg/kg, 79.38 ± 1.12%) significantly reduced the water content compared with model group (P < 0.05, Fig. 2E). Indobufen (20 mg/kg, 1.75 ± 0.71) and Aspirin (10 mg/kg, 1.625 ± 0.74) showed an apparent decrease on the neurological score (P < 0.05, Fig. 2F). TXB₂ and 6-keto-PGF₁α levels in the model group both increased versus sham group (P<0.01, Fig. 2G). Indobufen group showed obvious reduction in TXB₂ content compared with MCAO/R group (P<0.01, Fig. 2G) while Aspirin exhibited no apparent difference. The 6-keto-PGF₁α level in both the Indobufen and Aspirin groups had no significant difference versus the MCAO/R group.
3.2 Post-treatment with Indobufen promotes exercising, learning and memory ability in MCAO/R rats

Therapeutic effects of 15-day post-treatment of Indobufen with the rat MCAO/R model was also investigated. As in Fig. 2B, TTC staining were conducted and representative photographs was shown in Fig.3A. Indobufen (20 mg/kg) and Aspirin (10 mg/kg) group showed a marked decrease in infarct size (P < 0.01, Fig. 3B).

Representative swimming tracks in MWM of rats on the 2nd day on visible platform were presented in Fig. 3C. Other results of MWM were in Fig. 3D-F. The MCAO/R group had an apparently longer escape latency than the sham group (Day10, Day11, Day13: P < 0.01; Day12, Day14: P < 0.01, Fig. 3D). Compared with model group, escape latency of Indobufen and Aspirin groups were significantly reduced in the visible platform trial and escape latency of Indobufen group was significantly reduced in the invisible trial (Fig. 3C). In the probe trial, model group had fewer numbers of platform crossings (P < 0.01) compared with sham group, while Indobufen group increased the crossing numbers (P < 0.01, Fig. 3E). Time spent in the target quadrant also showed an increase in Indobufen group (P < 0.01, Fig. 3F).

In both the cylinder test and posture reflex test, Indobufen groups showed a significantly difference (Cylinder test: P < 0.01; Posture reflex test: Day 2, 10,15: P <0.01, Day5: P <0.05) compared with model group, and particulary showed better effects than Aspirin in cylinder test (Day10 and 15, P <0.01), which suggested that Indobufen (20 mg/kg) has an obvious effect on behavioral imporvement (Fig. 3G-H).

3.3. Indobufen reduces cerebral pyroptosis and regulates NF-κB/Caspase-1/GSDMD expressions in vivo

To evaluate the protective effects of Indobufen on cerebral cell survival especially pyroptosis against I/R injury, 24 h after MCAO/R, pyroptosis related components Caspase-1, NLRP3 and NF-κB-p65 was measured through IF in the brains of rats in 5-day tests. GSDMD, a hallmark of pyroptosis, was stained along with TUNEL assay to distinguish cerebral pyroptosis cells (Fig. 4-5). Fig. 4A-B showed the ipsilateral cortex with Caspase-1 or NLRP3 costainging with DAPI. It was obvious that MCAO/R group showed more apparent Caspase-1/NLRP3 expression compared with sham group, while Aspirin and Indobufen groups exhibited similar expression as sham group. In Fig. 5A, B showed the ipsilateral cortex with NF-κB costainging with DAPI or GSDMD costaining with TUNEL and DAPI. It was also apparent to see that MCAO/R group showed more nuclear import of NF-κB compared with sham group, while Aspirin and lindobufen groups exhibited similar level of nuclear import as sham group. Results from Fig. 5B showed that GSDMD-positive TUNEL cells were markedly reduced in both the pretreated groups, indicating the protective effect of Indobufen against cerebral cell pyroptosis (Fig. 5C).

During stroke associated pyroptosis, Caspase-1 plays a central role, which, activated by inflammasome NLRP3 following the signal from NF-κB nuclear transporation, cleaves the inactive pro-IL-18 and pro-IL-1β into mature inflammatory cytokines IL-18 and IL-1β, mediating pyroptosis and also promoting GSDMD-NT expression, leading to cell membrane rupture [19]. To explore whether Indobufen inhibits cerebral pyroptosis through NF-κB/Caspase-1/GSDMD signal, western blot and PCR were used to examine the expressions of Caspase-1, IL-18, IL-1β, NF-κB-p65 at 24 h after MCAO/R. The results revealed
that IL-18, IL-1β, NLRP3 mRNA, Caspase-1 protein expression and NF-κB-p65 nuclear import were all significantly enhanced in model group versus sham group (P < 0.01, Fig. 4C-E, 5C-F), indicating that pyroptosis was markedly activated after I/R injury, while Indobufen (20 mg/kg) exhibited an reverse effect on pyroptosis activation (P < 0.01, Fig. 4C-E, 5C-F), which was similar to Aspirin (10mg/kg), identical to the results of IF.

3.4 Indobufen protects OGD/R induced cell injury in HUVEC cells

HUVEC cells were used to determine the effect of Indobufen on cell viability using the MTT assay. As shown in (Fig. 6A-B), compared with the control group, the HUVEC cells subjected to OGD for 4h and reperfusion for 2h exhibited a decrease in cell viability, and this effect was potently reversed by pretreatment of Indobufen (200μM) and Aspirin (100μM). Moreover, consistent with the in vivo results, Indobufen showed similar effects on TXA2/PGI2 balance. (Fig. 6C).

3.5. Indobufen reduces pyroptosis and regulates NF-κB/Caspase-1/GSDMD expressions in vitro

To investigate whether Indobufen also exerts protective efficacy on pyroptosis via the NF-κB/Caspase-1/GSDMD signaling pathway in vitro, the pyroptosis-associated cellular proteins, including Caspase-1, NLRP3, NF-κB and GSDMD-NT in HUVEC cells were examined with IF and western blot. The results showed that OGD/R exposure enhanced the Caspase-1, NLRP3, GSDMD protein expressions, and induced NF-κB nuclear import. IF results showed that Indobufen (200 μM) exhibited a reverse effect on Caspase-1/NLRP3/GSDMD activation (Fig. 7A-D). Typical bands of protein expression also revealed that Indobufen had reverse influence against the Caspase-1 up-regulation and NF-κB nuclear transportation (Caspase-1: P < 0.05; NF-κB P < 0.01, Fig. 7E-I), which was in accordance with the results in the in vivo experiments.

4. Discussion

In this research, we first examined the neuroprotective effects of Indobufen against cerebral I/R injury and proved its possible mechanism of reducing pyroptosis by inhibiting NF-κB/Caspase-1/GSDMD signal pathway.

Our previous studies showed that Indobufen had antithrombotic and anticoagulant effects on rats [5]. Therefore, we speculated that Indobufen may be able to treat cerebral ischemia reperfusion injury. In MCAO/R induced I/R injury, exacerbate damage from numerous inflammatory responses occurs, leading to cell injury and eventually programed cell death, in which pyroptosis is dependent on an inflammatory response and a hot topic recently [20]. In vivo pharmacodynamic tests with pretreatment or post-treatment were managed to assess the efficacy of Indobufen on I/R injury. Aspirin was used as the positive drug for its common use in the clinic [21, 22]. In the pre-treatment test, Indobufen significantly alleviated I/R injury by reducing infarct area and water edema 24 hours after MCAO/R treatment, and Indobufen could adjust the balance of TXA2 and PGI2 expression after I/R injury both in vivo and in vitro, preliminarily proving its anti-thrombotic ability. In post-treatment tests, the assessment of motor function,
learning and memory ability 24 hours to 15 days after reperfusion showed that the I/R injury was significantly reduced in rats treated with Indobufen, and Indobufen significantly improved spatial cognition and motor function in rats over a longer period of time. In vitro, we simulated I/R injury with HUVEC cells by OGD/R, and Indobufen pretreatment reduced OGD/R-induced cytotoxicity and cellular morphological changes, and increased cell viability. The reduction of I/R injury and the improvement of motor, memory and learning abilities are all closely related to the reduction of brain cell injury and death by Indobufen. Recently, more and more studies have shown that cell pyroptosis, also known as inflammatory death, can be caused by cerebral ischemia-reperfusion injury [23]. A hallmark of pyroptosis is GSDMD-NT-dependent membrane rupture due to Caspase-1 activation, which, unlike apoptosis, allows the release of intracellular pathogens such as pro-inflammatory mediators (e.g., IL-1β and IL-18) [24]. Caspase-1 is stimulated by the typical signal of NF-κB-p65 entry into the nucleus and is activated by canonical inflammasomes such as NLRP3, which promotes brain tissue damage [25]. Therefore, in order to determine the role of Indobufen against cerebral ischemia and cell pyroptosis, IF, PCR, western blot method were conducted 24 hours after MCAO/R to detect the Indobufen in vivo and in vitro NF-κB/Caspase-1/GSDMD gene and protein expression in composition of pyroptosis signaling pathways, and using TUNEL co-staining with GSDMD-NT to measure the apoptosis/pyroptosis levels in the 5-day pretreatment rats. The results showed that Indobufen significantly reduced the mRNA expression levels of IL-1β, IL-18 and NLRP3 in the rat brain, reducing the entry of NF-κB into the nucleus, and apparently inhibited the expression levels of Caspase-1 and NLRP3, thus lowering the number of pyroptosis cells exhibiting certain anti-apoptosis/pyroptosis effects. Consistent with the results in vivo, Indobufen had a strong anti-pyroptosis effect on OGD/R-induced cell death by potently decreasing the pyroptosis signal of NF-κB/Caspase-1/GSDMD in HUVEC cell culture. In addition, TUNEL+ GSDMD-NT co-staining also confirmed this anti-pyroptosis effect in vitro. However, since this experiment was only a preliminary study of the relationship between Indobufen and pyroptosis related pathway, further studies are needed on whether Indobufen directly inhibits pyroptosis signals and whether it further activates the downstream signals.

5. Conclusion

Collectively, we observed that pre-treatment or post-treatment of Indobufen exerts anti-pyroptosis effects against cerebral I/R injury and is beneficial to long term memory, learning and cognitive recovery. The underlying mechanisms are probably related to its potential of down-regulating pyroptosis related NF-κB/Caspase-1/GSDMD signaling pathway. This study suggests that aside from anti-thrombotic and anti-coagulant effects, Indobufen may have neuroprotective efficacy in cerebral ischemic injury and is ideal as a new candidate drug for ischemic stroke in clinical trials.

Abbreviations

I/R: cerebral ischemia/reperfusion; MCAO/R: middle cerebral artery occlusion/reperfusion; HUVECs: human umbilical vein endothelial cells; OGD/R: oxygen glucose deprivation/reoxygenation; TXA2:
thromboxane A2; GSDMD: gasdermin D; CMC-Na: sodium carboxymethyl cellulose; TTC: 2,3,5-
Triphenyltetrazolium chloride; MWM: Morris Water Maze.

Declarations

Ethics declarations and consent to participate: All animal protocols were approved by the local-research
ethics review board of the Animal Ethics Committee of China Pharmaceutical University Health Science
Center.

Consent for publication: Not applicable.

Availability of data and materials: The data used to support the findings of this study are available from
the corresponding author upon request.

Competing interests: The authors declare no conflict of interest.

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Author Contributions: D.X. and F.L. were involved in collecting information and writing a draft manuscript;
K.H. and X.G. performed information consolidation and were involved in modifying article; Y.L. was
involved in selecting theme. All authors read and approved the final manuscript.

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**Figures**

![Chemical structures of Indobufen](image)

**Figure 1**

Chemical structures of Indobufen
Figure 2

Effects of Indobufen pretreatment on changes in infarct size, water content, neurological deficits and TXA2/PGI2 release after ischemic stroke injury. (A) Study of the protective effects of Indobufen administration in MCAO/R-induced pyroptosis in vivo. (B) Study of the therapeutic effects of Indobufen administration in MCAO/R-induced pyroptosis in vivo. (C) Representative images of cerebral infarct area after cerebral ischemia 24h after MCAO/R; (D) Quantification of cerebral infarct area (ANOVA followed by Bonferroni post hoc test); (E) Quantification of cerebral edema; (F) Neurological scoring statistics (Kruskal-Wallis test); (G). Effects of Indobufen on TXB2 and 6-keto-PGF1α in brain homogenate (ANOVA followed by Bonferroni post hoc test). Data are shown as mean ± SD, n=8. *P < 0.05, **P < 0.01 vs. MCAO/R; #P < 0.05, ##P < 0.01 vs. Sham.
Figure 3

Post-treatment of Indobufen promotes learning, memory and motor ability of MCAO/R rats. (A) Representative images of cerebral infarct area after cerebral ischemia 15 days after MCAO/R; (B) Quantification of cerebral infarct area (ANOVA followed by Bonferroni post hoc test); (C) Representative swimming tracks of MWM. (D) Escape latency to find the visible/non-visible platform (ANOVA followed by Bonferroni post hoc test). (E) Number of crosses in the probe test in quadrant IV (Kruskal-Wallis test). (F) Time spent in quadrant IV (ANOVA followed by Bonferroni post hoc test). (G-H) Postural reflex test and cylinder test (Kruskal-Wallis test). Data are shown as mean ±SD, n = 8. *P < 0.05, **P < 0.01 vs. Model; #P < .05, ##P < 0.01 vs. Sham.
Figure 4

A and B: Immunofluorescence images showing the expression of Caspase-1 and NLRP3 under different conditions: Sham, MCAO/R, Aspirin (10mg/kg), Indobufen (20mg/kg).

C: Relative gene expression levels of IL-18, IL-1β, and NLRP3 in Sham, MCAO/R, Aspirin (10mg/kg), and Indobufen (20mg/kg) groups. Significant differences are indicated by * and **.

D: Western blot analysis showing the expression of cleaved-caspase-1 at 20kD and β-actin at 42kD.

E: Relative expression levels of Caspase-1 in Sham, MCAO/R, Aspirin (10mg/kg), and Indobufen (20mg/kg) groups compared to β-actin. Significant differences are indicated by *.

Figure 4
In vivo Indobuefn reduces cerebral pyroptosis and regulates Caspase-1/IL-18/IL-1β/NLRP3 expressions. (A, B) Representative images showing Caspase-1 or NLRP3-positive cells in ipsilateral cortex; green-positive staining; blue-DAPI counterstaining of nuclei (×200 magnification, scale bar=50μm). (C) mRNA expression and quantification of key components in NF-κB/Caspase-1/GSDMD signaling pathway: IL-18, IL-1β, NLRP3; (D, E) Western blot analysis of Caspase-1 expression in the cortex (ANOVA followed by Bonferroni post hoc test). Data are shown as mean ± SD, n = 3. *P < 0.05, **P < 0.01 vs. Model; #P < 0.05, ##P < .01 vs. Sham.
In vivo Indobuefn reduces cerebral pyroptosis and regulates NF-κB/GSDMD expressions. (A) Representative images showing NF-κB-positive cells in ipsilateral cortex, red-positive staining; blue-DAPI counterstaining of nuclei (×200 magnification, scale bar = 50μm). (B) Representative images showing GSDMD/TUNEL-positive cells in ipsilateral cortex, green-TUNEL-positive staining; red-TUNEL-positive staining; blue-DAPI counterstaining of nuclei (×200 magnification, scale bar=50μm). (C, D) Western blot analysis of the ratio of NF-κB-p65 expression in the cortex (ANOVA followed by Bonferroni post hoc test). Data are shown as mean ± SD, n=3. *P < 0.05, **P < 0.01 vs. MCAO/R; #P < 0.05, ##P < 0.01 vs. Sham.
Figure 6

Effects of drugs pretreatment on OGD/R-induced cytotoxicity in cultured HUVEC cells. (A) Microscope images showing respective cell morphology of HUVEC cells caused by OGD 4 h and Reperfusion 2 h (×400 magnification, scale bar=50μm); (B) Cell viability (ANOVA followed by Bonferroni post hoc test); (C) Effects of Indobufen on TXB2 and 6-keto-PGF1α (ANOVA followed by Bonferroni post hoc test). Data are shown as mean ± SD, n=8. *P < 0.05, **P < 0.01 vs. OGD/R; #P < 0.05, ##P < 0.01 vs. Control
Figure 7
In vitro Indobuefn reduces HUVEC pyroptosis and regulates NF-κB/Caspase-1/GSDMD expressions after OGD/R. (A-D) The representative images of (A) Caspase-1, (B) NLRP3, (C) NF-κB, and (D) TUNEL/GSDMD in HUVEC cells were detected by IF staining (×200 magnification, scale bar=50μm). (E-I) Western blot analysis of the ratio of Caspase-1 and NF-κb-p65 expression in HUVEC cells. Data are shown as mean ± SD, n=3. *P < 0.05, **P < 0.01 vs. OGD/R; #P < 0.05, ##P < 0.01 vs. Control.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NC3RsARRIVEChecklist.pdf