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Toxicities and beneficial protection of H₂S donors based on nonsteroidal anti-inflammatory drugs†

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On the basis of our previous study, the H₂S donors based on nonsteroidal anti-inflammatory drugs (NSAIDs) were further evaluated with regard to the following aspects: animal blood and urine analyses, liver and kidney toxicities, gastrointestinal protection, anti-hypertension, and myocardial protection. The test results showed that after the successive administration of the compound for 14 days, the number of white blood cells in the blood of rats reduced, and protein and leukocytes appeared in urine; further, α -lipoic-acid-acetaminophen ester (1) and ibuprofen-ADTOH ester (2) had a certain effect on the physiological tissue and function of rat liver, and their side-effects on the kidneys were obvious. However, when compared with NSAIDs as the precursors, the tested compounds displayed much fewer side-effects; particularly, for the gastrointestinal mucosa of rats, there were hardly any side-effects. Moreover, all the three compounds decreased blood pressures in spontaneously hypertensive rats in a concentration-dependent manner, even though this antihypertensive effect was weaker than those induced by nifedipine and captopril. In addition, three compounds protected H9c2 cells from injury via an antioxidation pathway; and they improved myocardial injury in spontaneously hypertensive rats. Compound 2 is the derivative of ibuprofen and has lower toxicity to rat cardiomyocytes than ibuprofen. Therefore, it may become a better substitute of ibuprofen in patients due to its lower cardiotoxicity.

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Introduction

Hydrogen sulfide (H₂S) is the third endogenous gas signal molecule after nitric oxide (NO) and carbon monoxide (CO), and it plays an important physiological role in the body.^{1–4} H₂S significantly reduces the lipopolysaccharide-induced accumulation of neutrophils in tissues and organs (liver and lung), reduces tumor necrosis factor- α (TNF- α) and plasma interleukin-1 β (IL-1 β) levels, and increases plasma IL-10 levels, thereby inhibiting inflammatory responses.⁵ In the cardiovascular system, H₂S causes vasodilation and lowered blood pressure by promoting K_{ATP} channels to open;⁶ meanwhile, it produces myocardial protection through activating protein kinase C (PKC) and myocardium K_{ATP} channels.⁷

Since H₂S is a toxic gas and its dosage is not easy to control, it cannot be directly applied in clinics. Therefore, using H₂S donors is a simple and convenient way to replace H₂S. The earliest H₂S donors were sulfide salts, such as Na₂S and

CaS, but they release H₂S too fast under physiological conditions, which is not beneficial toward their applications. GYY4137 was found to be a novel water-soluble H₂S releaser, and it exhibits antishock, antitumor, antithrombotic, and other functions.^{8–11} After that, many H₂S donors have been discovered and synthesized, such as desipramine (ADTOH) derivatives,¹² 4-hydroxythiobenzamide (TBZ) derivatives,¹³ alpha-lipoic acid derivatives, and so on.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been widely used to treat pain, inflammation, and fever in clinics.¹⁴ However, NSAIDs have many side-effects, such as gastrointestinal bleeding, ulcers, high blood pressure, and increased risk of myocardial infarctions.^{15–17} Recently, H₂S donors based on naproxen (ATB-346) have been reported to be good inhibitors of COX-2; moreover, they can significantly reduce gastrointestinal damage when compared with naproxen.¹⁸ Later, our group designed and synthesized a series of H₂S donors modified by traditional NSAIDs. The test results showed that all the compounds had worthwhile anti-inflammatory activities, lower cytotoxicities, and lower reproductive toxicities, which suggests that these compounds may have higher potential toward anti-inflammatory drugs with lower toxicities. However, as we know, traditional NSAIDs cause myocardial injury and increase in blood pressures after administration over a long period time. H₂S has an anti-

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hypertension effect and myocardial protection effect; NSAIDs modified by H₂S donors can possibly counter or compensate for these side-effects caused by NSAIDs.

Based on our previous study,^{19,20} in this study, we further evaluate these compounds with regard to gastrointestinal protection, antihypertensive effect, and myocardial protection. Therefore, we hope to obtain useful data and information to comprehensively evaluate NSAIDs-based H₂S donors to confirm their benefits in clinics.

Results and discussion

1. Synthesis and characterization of compounds

All the three compounds were prepared according to the method used earlier.¹⁹ Compound 1 was synthesized by the reaction of α -lipoic acid and acetaminophen, and compounds 2 and 3 were prepared by TBZ and ADTOH reacting with ibuprofen, respectively (Scheme 1). The reaction conditions were mild and the yields were high. All the three target compounds were obtained by separation and purification using column chromatography.

The spectra of the three compounds were consistent with the expected spectra. In the IR spectra, there are strong absorption peaks in the range of 1728–1766 cm⁻¹, which are characteristic absorptions of C=O in the ester group. For compound 1, the absorption for the C–S bond appears at 604 cm⁻¹; for compound 2, the strong absorption at 1152 cm⁻¹ belongs to C=S; the characteristic absorption for C=S for compound 3 appears at 1136 cm⁻¹. In the ¹H-NMR spectrum of compound 1, the chemical shift in the hydrogen atoms in CH₃CO is at 2.14 ppm; for compound 2, the signals of two hydrogen atoms in CONH₂ occur in the range of 9.52–9.58 and 9.90–9.95 ppm. As for compound 3, the signal of the hydrogen atom in the trisulfide ring appears at around 7.40 ppm.

2. H₂S release from compounds

The H₂S-release abilities of the compounds were examined using the methylene blue (MB⁺) method. The MB⁺ method in-

volves the reaction between zinc acetate and H₂S to form zinc sulfide in the presence of acid and ferric ions and then reacting with *N,N*-dimethyl-1,4-phenylenediamine sulfate to form methylene blue. The absorbance at 670 nm was measured to quantify H₂S. Because the compounds were slower H₂S releasers, we used the MB⁺ method to detect the ability of all the compounds to release H₂S in the presence of TECP or cysteine.

The test results are shown in Fig. 1. The release half-lives of all the three compounds are less than 30 min, and the release rates are roughly consistent with the first-order kinetics. This suggests that all of them are rapid H₂S releasers. The release half-life of compound 1 is 14.1 min and its maximum release amount is 46.0 μ M. For compound 2, its H₂S-release profile is shown in Fig. 1b; its half-life is 20.1 min and maximum release amount is 20.9 μ M. Significantly, the H₂S-release ability of compound 2 is weaker than that of compound 1. Fig. 1c shows the H₂S-release curve of compound 3; its half-life is 1.4 min and the maximum release is 13.8 μ M. In contrast, its H₂S-release ability is the weakest among all the three compounds, but its rate is the fastest.

3. Toxicity of compounds after successive administration

Since the three compounds did not show toxicity toward the mice model in the acute toxicity test, we selected rats to further evaluate their toxicity after successive administration. Each compound was repeatedly administered by intraperitoneal injection at a dose of 0.01 mmol kg⁻¹ for 14 days. The urine was collected and the rats were sacrificed 14 days later. Blood and liver, kidney, and other major organs were collected and further examined. The blood test results are listed in Table 1. From Table 1, it is evident that certain blood indexes are abnormal: the numbers of leukocytes significantly decreased when compared with the blank control; the platelet numbers decreased considerably. The platelets are only 194, 161, and 114 $\times 10^9$ /L after rats were treated with compounds 1, 2, and 3, respectively, while that for the

Scheme 1 Synthesis route and structures of compounds 1–3.

Fig. 1 H₂S-Release curves of the compounds: a for 1; b for 2; and c for 3.

control is $670 \times 10^9/L$. However, the numbers of red blood cells and hemoglobin are within the normal range. This shows that the compounds have a certain impact on the blood system.

The urine test results of rats are shown in Table 2. Certain indexes are abnormal. When compared with the control group, the urine pH value decreased, and both urobilinogen and bilirubin levels increased. Among the three compounds, compound 3 led to urobilinogen and bilirubin increases in rat urine, more than 50-fold higher than that in the control. Protein was detected in the urine of the three test rats, and the content was over $3 \mu\text{mol L}^{-1}$. Moreover, leukocytes were found in the urine samples of the test groups comprising compounds 2 and 3. This suggests that the three compounds possibly injured the glomeruli of the kidneys to a certain degree, thereby having an effect on the kidney functions of the rats.

In order to determine the degree of damage to the rat liver and kidney by the compounds, we undertook liver and kidney biopsies by means of H&E staining. Under a microscope, for the control group, the hepatic lobule was viewed to be structurally intact; the hepatocytes were arranged neatly, and no obvious gap was observed between the cells. The renal tissue was deeply stained and the glomerular structure was intact. After the administration of 10 mg kg^{-1} of compound in rats by intraperitoneal injection for 14 days (Fig. 2), for the compound 2 group, the hepatic lobular structure was intact,

central venous congestion and hepatic sinus congestion were obvious, hepatocytes were swollen and visible gaps were observed, cytoplasm was lightly stained; with regard to the kidney, the small ball was intact, cells of the proximal convoluted tubule were swollen and visible gaps were observed, cytoplasm was loose and lightly stained, and certain renal tubular epithelial cells were necrotic. With regard to the compound 3 group, the liver tissue damage was similar to that observed in the compound 2 group (Fig. 2); further, the renal tissue was congested and the cells were swollen, cytoplasm was loose, and light staining was significantly lighter. This indicates that compounds 2 and 3 have a certain degree of damage to the physiological structure of the liver and kidney tissues in the rats. In contrast, the damage caused by compound 2 was severer to rat kidneys than that induced by compound 3.

In order to further understand the reasons for liver and kidney dysfunctions, we observed the liver and kidney tissues of the tested rats under a transmission electron microscope. The results are shown in Fig. 3 and 4. After being treated with compound 2, the rat liver cells had abnormal nuclear morphology, increased heterochromatin, chromatin edge collection, and increased lipid droplets in the cytoplasm. The glomerular endothelial cells were swollen, stromal hyperplasia was present, and a large amount of exudation was observed in the glomerulus. This indicates that compound 2 induced a large amount of damage to the liver

Table 1 Results of blood tests after successive administration in rats for 14 days

Test items	Compd 1	Compd 2	Compd 3	Blank control
Leukocyte ($10^9/L$)	2.89	1.72	3.27	6.88
Neutrophil ratio (%)	31.7	44.4	35.9	54.7
Lymphocyte ratio (%)	64.2	52.8	60.4	42.1
Monocyte ratio (%)	1.3	1.8	0.9	0.5
Eosinophil ratio (%)	2.6	1	2.6	1.8
Basophilic ratio (%)	0.2	0	0.2	2.2
Red blood cell ($10^{12}/L$)	6.08	5.5	5.9	6.83
Hemoglobin (g L^{-1})	135	119	136	138
Platelet ($10^9/L$)	194	161	114	670

Table 2 Results of urine tests after successive administration in rats after 14 days

Test items	Compd 1	Compd 2	Compd 3	Blank control
Urobilinogen ($\mu\text{mol L}^{-1}$)	3.3	16	66	1
Bilirubin ($\mu\text{mol L}^{-1}$)	12	17	50	10
Ketone body (mmol L^{-1})	4	Neg	0.5	4
Occult blood (caccells per μL)	80	Neg	Neg	10
Protein (g L^{-1})	3.2	3.0	3.0	0
Nitrite	Neg	Neg	+	9
Leukocyte (caccells per μL)	Neg	70	500	10
Glucose	Neg	Neg	Neg	0
Proportion	1.005	1.005	1.005	1.01
PH	7.5	7.5	7.5	8.4
V _C	Neg	Neg	Neg	10

Fig. 2 Optical microscopy morphological images of rat (A and B) liver and (C and D) kidney tissues after successive administration of the compound for 14 days. Kidney section: A for compound 2 and B for compound 3; kidney section: C for compound 2 and D for 3. Black arrows indicate tissue congestion.

Fig. 3 TEM morphological images of rat liver after the successive administration of the compound for 14 days. A–C denote the control group; a–c for the compound 2 group (6000-, 10 000-, and 20 000-fold, respectively). The arrows in Fig. 3a and b indicate an increase in lipid droplets.

and kidney cells of the rats, which further affected their biological functions.

4. Gastrointestinal protection by the compounds

In order to determine whether the compounds as H_2S donors have any protective effect on the gastrointestinal tract, the rats were given a continuous dose of 10 mg kg^{-1} of compound 2 for 14 days; the compound precursor of ibuprofen was given as a positive control (ibuprofen: daily dose of 6.1 mg kg^{-1} ; note: clinical adult dose does not exceed 5.0 mg kg^{-1}). Gastrointestinal tissues were collected and further examined under a microscope. The results are shown in Fig. 5. After H&E staining, the gastrointestinal tissues were observed by an optical microscope. For the control

group, the gastric mucosal cells were neatly arranged and the gastric mucosa showed no obvious hyperemia (Fig. 5: control stomach); intestinal mucosa showed no hyperemia, inflammatory cell infiltration, and intestinal mucosal edema (Fig. 5: control intestinal).

When compared with the blank group, the ibuprofen group (positive control) showed hyperemia and inflammatory cell infiltration in the gastric mucosa, as well as swelling of the gastric mucosal cells (Fig. 5A); the intestinal mucosa exhibited hyperemia, inflammatory cell infiltration, and intestinal mucosal edema (Fig. 5C). This indicates ibuprofen has serious side-effects on the gastrointestinal tract, particularly in patients needing long-term medication. In contrast, in the compound 2 group, gastric congestion symptoms were significantly reduced when compared with the ibuprofen

Fig. 4 TEM morphological images of rat kidney after the successive administration of the compound for 14 days. A–C for the control group; a–c for the compound 2 group (6000-, 10 000-, and 20 000-fold, respectively). The arrow in Fig. 4a indicates the swelling of the glomerular endothelial cells; the arrow in Fig. 4b indicates the swelling of the glomerular endothelial cells and stromal hyperplasia; the arrow in Fig. 4c indicates a large amount of exudation (precipitate) in the glomerulus.

Fig. 5 Optical microscopy morphological images of rat (A and B) stomach and (C and D) intestinal tissues after successive administration of the compound for 14 days. Stomach sections: A for ibuprofen; B for compound 2. Intestinal sections: C for ibuprofen; D for compound 2. Black arrows indicate tissue congestion and red arrows indicate swelling of the gastric cells.

group and no gastric cell swelling was visible (Fig. 5B); intestinal mucosal congestion symptoms were also significantly reduced (Fig. 5D). This indicates that compound 2 has a certain improvement effect on the gastrointestinal side-effects caused by NSAIDs.

It is to be noted that all the side effects of ibuprofen on the gastrointestinal tract are caused by the free carboxyl group in its molecule; in compound 2, the carboxyl group was transformed into an ester, which reduces irritation to the gastrointestinal tract. Compound 2 is a prodrug that individually acts through esterase hydrolysis in the blood. At the same time, compound 2 not only improves the gastrointesti-

nal side-effects of ibuprofen, but also changes the absorption, distribution, and metabolism of ibuprofen *in vivo*.

5. Anti-hypertension by the compound

In order to confirm the anti-hypertension effect of the compounds as H₂S donors, we selected spontaneously hypertensive rats (SHRs) as the model of hypertension, and the test compound was administered by intraperitoneal injection; nifedipine and captopril were used as the positive control (Fig. 6 and 7). Blood pressure was measured by a rat blood pressure monitor.

The test results showed that compound 1 exhibited a good antihypertensive effect, and it was concentration-dependent. When compound 1 was administered at 10, 20, and 40 mg kg⁻¹, the mean minimum systolic blood pressures of the SHR were 139.1, 127.6, and 115.3 mmHg, respectively (Fig. 6a). The statistical difference was significant between the different concentrations ($p < 0.01$); the duration of blood pressure was longer at about 60 min; this is because compound 1 has strong H₂S-release capacity and longer release half-life. Compound 2 has a similar antihypertensive effect as that of compound 1, but it has a shorter duration (Fig. 6b). Compound 3 is a norepinephrine trisulfide compound. Under the same conditions, the antihypertensive effect of compound 3 was weaker than those of the two compounds. The average minimum systolic blood pressures at the three concentrations were 142.9, 136.7, and 135.1 mmHg, respectively (Fig. 6c). This may be related to the less H₂S amount released from compound 3.

The effect of the compounds as H₂S donors on the diastolic blood pressures of SHR was similar to that on the systolic blood pressures. Compound 2 had the strongest effect on reducing the diastolic blood pressure among the three compounds (Fig. 8b), and it also was significantly concentration-dependent, but the duration of the diastolic blood pressure reduction was shorter. The effect of compound 1 on re-

ducing the diastolic blood pressure was also concentration-dependent and had the longest duration (Fig. 8a), which may be related to the strong H₂S-release ability and longer release half-life of compound 1. When compound 1 concentrations were 10, 20, and 40 mg kg⁻¹, the minimum diastolic blood pressures for the SHR were 113.8, 110.9, and 99.4 mmHg, respectively. Compound 3 had the weakest effect on reducing the diastolic blood pressure, and the concentration dependence was not obvious. When compound 3 concentrations were 10, 20, and 40 mg kg⁻¹, the minimum diastolic blood pressures of the SHR were 116.3, 115.1, and 112.4 mmHg, respectively (Fig. 8c).

However, at the same concentration (10 mg kg⁻¹), regardless of the systolic or diastolic blood pressures, the antihypertension effect of the compound was weaker than those at similar concentrations of nifedipine and captopril, and the duration of blood pressure was shorter than that observed for nifedipine and captopril (Fig. 7 and 9); the compounds with higher concentrations lowered the blood pressure of SHR to the blood pressure level of normal WKY rats. During the hypotensive process, the antihypertensive effect of the compound was related to the release ability of H₂S. Compound 1 with strong H₂S-release ability displayed a stronger antihypertensive effect, which indicates that the antihypertensive effect of compound 1 was possibly mediated by H₂S.

Fig. 6 Effect of compound concentration on systolic blood pressures in SHR. Here, a is for compound 1; b for compound 2, and c for compound 3. Each column represents the mean $\pm$ SD values of four independent experiments.

Fig. 7 Effect of compound on the systolic blood pressures in SHRs. Here, a is for compound 1; b for compound 2; c for compound 3. Each column represents the mean $\pm$ SD values of four independent experiments.

From the above results, it is evident that the compounds as H_2S donors can reduce the systolic and diastolic blood pressures of SHRs in a concentration-dependent manner. In the process of modulating the blood pressure, the effects of the compounds on the systolic and diastolic blood pressures are related to the H_2S -release ability. Compound 1 with a higher H_2S -release ability showed a stronger antihypertensive effect, indicating that the antihypertensive effect of the compound may be mediated by H_2S .

6. Protective effect of compounds as H_2S donors on myocardium

6.1 Protection of compounds against cardiomyocyte H9c2.

To confirm the effect of the compounds as H_2S donors on myocardial protection, we used H9c2 cell lines as the test model. The cell viability of the H9c2 cells was measured under test conditions and then the myocardial protection of the compound was determined. The cell tests were divided into a blank group, H_2O_2 group, control group (acetaminophen or ibuprofen), and compound- H_2O_2 group. The damage models involving light, medium, and heavy damage were obtained only through the cardiomyocyte treated with H_2O_2 for 1 h, 4 h, and 8 h, respectively.

From Fig. 10, in the range of 10–80 μM , it is evident that the tested compounds had no effect on the cardiomyocyte survival rate when compared with the blank control. This indicates that the three compounds were less toxic and the results of myocardial protection against the cells in this concentration range are meaningful.

In the subsequent tests, the compounds displayed protective effects on cell damage. From Fig. 11(A–C), after treatment with H_2O_2 (400 μM) for 1 h, it is evident that the survival rate of H9c2 cells was 82.3%; in the presence of compound 1, 2, or 3, the cell survival rates increased in a dose-dependent manner; when the doses for all the compounds were 80 μM , the survival rates of the cells increased by 13.3%, 11.9%, and 9.4%, respectively. We found that the compounds exhibited different activities in relation with the degree of cell injury. When the H9c2 cells were treated for more time, the survival rates of the cells marginally increased even in the presence of the compounds at the same concentrations. When H9c2 cells were treated with H_2O_2 for 8 h, compound 1 induced a cell viability increase by 8.6%, while compounds 2 and 3 induced increases by less than 5.0%. Obviously, when the myocardial cells were slightly injured, the protective effect of the compound on the cardiomyocyte was significant; and the cells were seriously damaged; the

Fig. 8 Effect of compound concentration on the diastolic blood pressure in SHRs. Here, a is for compound 1; b for compound 2; c for compound 3. Each column represents the mean $\pm$ SD values of four independent experiments.

activity of the compound is low because the damaged cells are too severe to repair. In comparison, compound 1 has stronger activity, which can be attributed to the larger maximum release of H_2S .

To understand the mechanism of the myocardial protection of compounds as H_2S donors, we measured the SOD and MDA levels in H9c2 cells.

As shown in Fig. 12, when the H9c2 cardiomyocytes were treated with only H_2O_2 for 24 h, the level of SOD in the cells was significantly decreased; however, after the cells were incubated with the compounds under investigation, the SOD levels were significantly increased. Accordingly, the MDA levels decreased in a concentration-dependent manner. Therefore, we speculate that the myocardial protection of the compound possibly results from the antioxidants in the H_2S donors. The positive controls of acetaminophen and ibuprofen exhibited weak antioxidant activity, which suggests that the antioxidant effect of the compound is mediated through H_2S released from the compound.

During the testing process, the cells were stained by using the Giemsa stain and the H9c2 cell morphology was observed under a microscope. In the blank group, the cells were long spindle-shaped and were adherent and firm; however, in the H_2O_2 group, the cells exhibited an irregular shape, and certain cells contracted and became round. With an increase in

the H_2O_2 intervention time, the number of deformed cells increased (Fig. 13d). However, in the compound- H_2O_2 group, the number of cells significantly reduced; moreover, with an increase in the concentration of compound 1, the repair effect was more pronounced (Fig. 13l). Meanwhile, we can see that acetaminophen as the control also showed antioxidation activity due to the presence of the phenolic hydroxyl group (Fig. 13g and h). In contrast, at similar concentrations, compound 1 exhibited better activity than acetaminophen because the H_2S released from compound 1 also has an anti-oxidation effect. This is consistent with earlier conclusions.

6.2 Myocardial protective effect of target molecules on hypertensive rats. Compounds with H_2S -release abilities have displayed H9c2 cardiomyocytes protection to a certain degree, but we need to confirm if they can also induce myocardial remodeling and protect cardiac functions in animals. For this reason, hypertensive rats were administered the tested compounds at a dose of 10 mg kg^{-1} per day by intraperitoneal injection for 14 consecutive days; ibuprofen was used as the positive control. Then, the myocardial tissues were removed and observed after H&E staining under a light microscope. From Fig. 14, it is evident that for the positive control group, the myocardial fibers of the rats were neatly arranged and coarser, muscle bundle gaps increased to varying degrees when compared with those in the blank control, and certain

Fig. 9 Effect of the compound on the diastolic blood pressure in SHRs. Here, a is for compound 1; b for compound 2; c for compound 3. Each column represents the mean $\pm$ SD values of four independent experiments.

Fig. 10 H9c2 cell viability after treatment with the compound for 24 h; A for compound 1, B for compound 2, C for compound 3.

myocardial interstitial collagen fibers were visible. However, for the compound 1 group, the myocardial fibers of the rats were neatly arranged and the muscle bundle gap was slightly reduced when compared with that in the ibuprofen group; the collagen fibers were not significantly stained in the interstitium. Myocardial fibers are significantly thinner, which indicates that compound 1 has lower toxicity than that of the positive control. As for the compound 2 group, myocardial fibers were neatly arranged and the muscle bundle gap was significantly reduced. Further, the thickening of the myocardial fibers have a certain improvement effect when compared

with the ibuprofen group, indicating that compound 2, as a nonsteroidal anti-inflammatory drug modified as a H_2S donor, has lower side-effects than the original compound, namely, ibuprofen.

In order to make the injury of myocardial cells more visible, myocardial tissues were observed under TEM. As shown in Fig. 15, in the positive control group, the rat myocardial interstitial vascular tissues swelled, myocardial fibers loosened, myocardial fibrous submucosal tissues swelled, and myocardial fibers melted; meanwhile, a myocardial contraction band formed, myocardial disk

Fig. 11 Effect of compound concentration on the survival rate of H9c2 cells, A, B, C are compound 1, 2, 3 after one hour of treatment; D, E, F are compound 1, 2, 3 after four hour of treatment; G, H, I are compound 1, 2, 3 after eight hour of treatment; each column represents the mean $\pm$ SD values of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

dissociated, and mitochondrial disk swelled. The mitochondria were damaged, causing energy production, release, and transmission disorders, which led to ventricular pumping dysfunction. However, for the compound 2 group, the myofibrils of the rats were substantially arranged in a neat manner when compared with the control group; no obvious muscle fiber dissolution, no significant swelling of the mitochondria, and no myocardial disk dissociation were observed.

This result indicates that ibuprofen has more toxicity toward rat cardiomyocytes than that of compound 2. Compound 2 is the derivative of ibuprofen and has worthwhile anti-inflammatory activity like ibuprofen. It can be assumed

that compound 2 can become a better substitute of ibuprofen for patients due to the former's lower cardiotoxicity.

Conclusion

In the presence of cysteine or TECP, all the three compounds were rapid H₂S releasers. After successive administration for 14 days, the tested compounds reduced white blood cells in rats, and proteins and white blood cells were visible in urine samples. Under a microscope, the structures of the kidney tissues were observed to be abnormal to a certain degree. This indicates that the compounds have side-effects on the kidney of rats and can be the case for patients with long-term

Fig. 12 Effect of compound on SOD and MDA contents in H9c2 cells. A, B, and C are the SOD levels of compounds 1, 2, and 3 after one hour of treatment, and D, E, and F are the MDA levels of compounds 1, 2, and 3 after one hour of treatment. Each column represents the mean $\pm$ SD values of four independent experiments. * $P < 0.05$, ** $P < 0.01$.

Fig. 13 Morphology of H9c2 cells under different conditions. Here, a, e, and i are blank groups; b–d for H₂O₂ treatment for 1 h, 4 h, and 8 h, respectively; f–h for acetaminophen treatment for 1 h, 4 h, and 8 h, respectively; j–l for compound 1 treatment for 1 h, 4 h, and 8 h, respectively.

medications. However, when compared with ibuprofen, the compounds had fewer side-effects. Secondly, when compared with ibuprofen, the side-effects on the gastrointestinal tract were significantly alleviated; there were no symptoms such as

gastric mucosa and intestinal mucosal hyperemia. Thirdly, the tested compounds as H₂S donors have a good concentration-dependent anti-hypertension effect on SHR even though the antihypertensive effect is weaker than those

Fig. 14 Optical microscopy morphological images of rat myocardial tissues. Control group: ibuprofen; A is for compound 1; B for compound 2. The black arrow indicates the muscle bundle gap and red arrow indicates myocardial fibers.

Fig. 15 TEM images of the myocardial tissues of rats after the administration of the tested compound for 14 days. A–C are for the positive control group; a–c for the compound 2 group (6000 $\times$, 10 000 $\times$, and 20 000 $\times$ -fold, respectively). The arrow in Fig. 15a indicates the swelling of cardiomyocytes. The arrow in Fig. 15B indicates the dissolution of cardiomyocytes. The arrow in Fig. 15C indicates the swelling of mitochondria.

induced by nifedipine and captopril. Fourthly, the tested compounds displayed a protective effect on H9c2 cardiomyocytes, and this effect was achieved by reducing the intracellular SOD and MDA levels; these compounds have a protective effect on the myocardium tissues of rat. Among the three compounds, compound 2 has lower toxicity to rat cardiomyocytes than that by ibuprofen. Compound 2 is the derivative of ibuprofen and can be a better substitute of ibuprofen for patients due to its lower cardiotoxicity.

Experimental part

1. Experimental instruments and materials

AVANCE III 400 M NMR instrument; maXis4G TOF mass spectrometer (Bruker, Germany); Nicolet NEXUS 360 FT-IR infrared spectrometer (Nicolet, USA); LAMBDA 25 UV-visible spectrometer (PerkinElmer, USA); CO₂ incubator; microplate reader (Thermo Varioskan Flash 3000); Beckman-Coulter LX20 fully automated biochemical analyzer; JEM-1230 transmission electron microscope.

BP-300A automatic large mouse noninvasive blood pressure detector (Chengdu Taimeng Software Co., Ltd.). Captopril, purity 98%, supplied by Beijing Bailingwei Technology Co., Ltd.; product number: 237181. Nifedipine, purity 98%,

supplied by Beijing Bailingwei Technology Co., Ltd.; product number: 307429. Other chemical reagents used in the experiment were analytically pure. All the reagents used were dried.

The experimental animals were purchased from the GLP Laboratory of Experimental Animal Center of Lanzhou University, and the experimental animal certificates are SCXK (Gan) 2009-0004; Wistar-Kyoto (WKY) rats; clean male spontaneously hypertensive rats (SHRs), Zhou Ling, 8 weeks, weight is 300 ± 25 g. Rats were housed in cages, one rat per cage, kept at room temperature (23 ± 2 °C, relative humidity of 60–65%, free access to drinking water, and all the animals were kept for 7 days before the experiment. All the animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Lanzhou University and Experiments were approved by the Animal Ethics Committee.

2. Synthesis of H₂S donors

The synthesis of compound 1 has been described elsewhere.^{21,22} Briefly, 206 mg (1 mmol) α -lipoic acid was dissolved in 10 mL dried dichloromethane, and 227 mg (1.1 mmol) DCC was added and stirred at room temperature for 30 min. Then, 150 mg (1 mmol) of acetaminophen and 18 mg (0.15 mmol) of DMAP were added and stirred at room temperature for 3 h. The precipitate was removed by filtration,

and the filtrate was washed three times with water, 5% acetic acid solution, and then dried over anhydrous sodium sulfate overnight. The solvent was evaporated under reduced pressure and the crude material was applied to column chromatography (CH_2Cl_2 - CH_3OH = 30:1) to yield 103 mg of a pale yellow solid. IR (KBr, cm^{-1}): 1751 (v, C=O), 1663 (v, C=O), 604 (w, C-S). ^1H NMR (DMSO-d_6 , ppm): δ 1.55 (m, 2H, dithiolane- C-CH_2), 1.76 (m, 4H, dithiolane- CH_2 , O=CC-CH_2), 1.93 (m, 1H, 5H-dithiolane), 2.14 (s, 3H, CH_3), 2.47 (m, 1H, 3H-dithiolane), 2.50 (t, J = 6.0 Hz, 2H, 4H-dithiolane), 3.09 (m, 2H, O=C-CH_2), 3.60 (m, 1H, 5H-dithiolane), 7.00 (d, J = 8.8 Hz, 2H, Ar-H), 7.47 (d, J = 8.8 Hz, 2H, Ar-H), 7.53 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 24.4, 24.6, 28.5, 33.8, 34.5, 38.6, 39.7, 40.4, 56.5, 120.3, 122.3, 137.4, 146.1, 168.7, 172.3. HRMS (m/z): calcd. for $\text{C}_{16}\text{H}_{21}\text{NO}_3\text{S}_2\text{Na}$ [$\text{M} + \text{Na}$] $^+$: 362.0861; found 362.0857.

The synthesis of compound 2 is described elsewhere.²³ Briefly, ibuprofen (206 mg, 1 mmol) was dissolved in 8 mL dried acetonitrile; EDCI (210 mg, 1.1 mmol) was added and stirred at room temperature for 30 min. Then, 4-hydroxythiobenzamide (153 mg, 1 mmol) and DMAP (18 mg, 0.15 mmol) were added to the reaction system, and the mixture was reacted at room temperature for 1 h. After the reaction was complete, the mixture was filtered, and the filtrate was evaporated under reduced pressure. The crude product was separated by column chromatography (CH_2Cl_2 - CH_3OH = 30:1) to give the product as a pale yellow solid, 145.8 mg, yield 43%. IR (KBr, cm^{-1}): 3374 (w, NH_2), 3281 (w, NH_2), 1741 (v, COO), 1152 (v, C=S). ^1H NMR (DMSO-d_6 , ppm): δ 0.86 (d, J = 6.4 Hz, 6H, $\text{C}(\text{CH}_3)_2$), 1.50 (d, J = 6.8 Hz, 3H, Ar- C-CH_3), 1.83 (m, 1H, Bn-CH), 2.44 (d, J = 7.2 Hz, 2H, Ar- CH_2), 4.07 (q, J = 7.2 Hz, 1H, Ar-CH), 7.07 (d, J = 8.4 Hz, 2H, Ar-H), 7.17 (d, J = 7.6 Hz, 2H, Ar-H), 7.21 (d, J = 8.0 Hz, 2H, Ar-H), 7.91 (d, J = 8.8 Hz, 2H, Ar-H), 9.54 (s, 1H, H of NH_2), 9.92 (s, 1H, H of NH_2). ^{13}C NMR (DMSO-d_6 , ppm): δ 19.2, 22.9, 30.3, 44.9, 121.6, 127.9, 129.5, 130.0, 137.8, 138.0, 140.8, 153.3, 173.3, 199.6. HRMS (m/z): calcd. for $\text{C}_{20}\text{H}_{23}\text{NO}_2\text{SNa}$ [$\text{M} + \text{Na}$] $^+$: 364.1347; found 364.1337.

The synthesis of compound 3 was similar to that of compound 2; 226 mg (1 mmol) of ADTOH was used in place of 4-hydroxythiobenzamide. The mobile phase in the crude product in the column chromatography was adjusted to chloroform:methanol = 50:1. Brownish yellow oily liquid, 180.0 mg, yield: 44.1%. IR (KBr, cm^{-1}): 1753 (s, COO), 1136 (v, C=S). ^1H NMR (DMSO-d_6 , ppm): δ 0.94 (d, J = 6.0 Hz, 6H, $\text{C}(\text{CH}_3)_2$), 1.65 (d, J = 8.0 Hz, 3H, Ar- C-CH_3), 1.90 (m, 1H, Ar- C-CH), 2.50 (d, 2H, Ar- CH_2), 3.98 (q, J = 8.0 Hz, 1H, Ar-CH), 7.18 (m, 4H, Ar-H), 7.31 (m, 2H, Ar-H), 7.40 (s, 1H, 4H-1, 2-dithiole-3-thione), 7.67 (d, J = 8.0 Hz, 2H, Ar-H). ^{13}C NMR (DMSO-d_6 , ppm): δ 18.9, 22.7, 30.1, 44.7, 123.3, 127.7, 129.2, 129.3, 129.8, 136.3, 137.7, 140.6, 172.9, 173.0, 215.9. HR-MS (m/z): calcd. for $\text{C}_{22}\text{H}_{22}\text{O}_2\text{S}_3\text{Na}$ [$\text{M} + \text{Na}$] $^+$: 437.0680; found 437.0701.

3. H_2S -Release detection

3.1 Na_2S standard curve plotting. A buffer solution of 20 mM at pH 7.4 was first prepared for 1 L. Then, 120.2 mg

$\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ was dissolved in a 100 mL volumetric bottle and 5 mM standard solution of Na_2S was prepared with pH 7.4 in a phosphate-buffered saline solution. Then, 50, 100, 200, 400, 600, 800, and 1000 μL of 5 mM Na_2S standard solutions were placed into corresponding 50 mL volumetric bottles and phosphate-buffered saline solution was added up to the scale line. The standard solution of Na_2S with the respective concentration gradients of 5, 10, 20, 40, 60, 80, and 150 mM were obtained. The gradient standard solution of 1 mL was added to a methylene blue (MB) mixture. The blank control was a mixture of MB with phosphoric acid buffer solution at pH 7.4 at 1 mL. After 20 min, the absorbance of the solution was measured at 670 nm by an ultraviolet-visible spectrophotometer (LAMBDA 25), and the standard curve between the absorbance and Na_2S concentration was plotted.

3.2 H_2S -Release detection of the compounds. Then, 75 μL solution of 40 mM compound was added to the volumetric flask, and TECP (1.0 mM) or L-cysteine (1.0 mM) was added as the promoter. Then, 2.0 mL of the above mixed solution was placed in a cuvette, and MB mixture was added. After 20 min, the absorbance was measured at a wavelength of 670 nm, and the H_2S concentration was calculated using a Na_2S calibration curve. Finally, the concentration *versus* time curve for the H_2S release was plotted.

4. Toxicity of compounds after repeated administration

In the dose range set, the mice did not show significant toxicity in a single dose. Therefore, we selected rats and studied the toxicity of the target molecule by multiple administrations. The target compound was dissolved in 10% 1,3-propanediol to prepare a standard solution of the compound and then normal WKY rats were administered a dose of 0.01 mol kg^{-1} by intraperitoneal injection; the control group was also administered by intraperitoneal injection. An equal volume of 10% 1,3-propanediol in blank (6 rats per group) was administered for 14 consecutive days during which rat urine was collected by using a rat metabolic cage. After the end of the administration, blood was taken through the eyeball, and the liver, kidney, and other tissues of the rat were collected: the tissue was immediately fixed with 4% glutaraldehyde solution. After sectioning, the tissue was observed *via* a transmission electron microscope, and the liver and kidney tissues of the rat were observed by H&E staining. The physiological condition determines if the target compound has an effect on the structure and function of the liver and kidney of the rat, and the urine and blood of the rat were analyzed by biochemical instruments to further evaluate the influence of the target compound on the physiological condition of the rat.

5. Gastrointestinal protection

WKY rats were divided into two groups (3 rats per group): control group and compound group. Rats were administered the tested compound for 14 days: each dose was 10 mg kg^{-1} . Ibuprofen served the control at a dose of 6.1 mg kg^{-1} . After anesthesia with ether, the rats were sacrificed by cervical

dislocation in the supine position. The rats were fixed on the surgical board with 70% alcohol disinfection of the abdominal skin; the midline incision of the abdomen was taken, fully exposing the organs inside the abdominal cavity. The stomach was removed. A section of the complete small intestine (about 10 cm) was taken at the proximal end of the ileocecal. The contents of the stomach and small intestine were removed by refrigerated saline. The following procedure was used: (1) fresh materials were cut into a small piece of 1.0 cm and immediately put into the fixing agent. (2) The fixed material was removed from the water in the tissue by ethanol; after being transparent to xylene, it was immersed in melted paraffin to be soaked and embedded. (3) A 5–10 μm slice was cut with a microtome, attached to a glass slide, dewaxed, and stained by H&E. (4) It was then observed under an optical microscope.

6. Measurement of blood pressure in hypertensive rats

Male SHR rats were randomly divided into 4 groups (6 rats per group): hypertension control group, captopril group (10 mg kg^{-1}), nifedipine group (10 mg kg^{-1}), and compound group (25, 50, and 100 mg kg^{-1}). Male WKY rats formed the normal blood pressure control group. All the compounds were administered *via* intraperitoneal injections. The solvent was 0.5% (w/v) mixed solution of sodium carboxymethyl cellulose and DMSO 3/1 (v/v). The SHR rats were administered at the daily dosage of 2 mL kg^{-1} (8 a.m. every day). The control and WKY groups were administered equal amounts of blank solvent. Blood pressures and heart rates of the rats were measured before administration and measured every 30 min after the administration for 6 h. The arterial systolic blood pressure (SBP), arterial diastolic blood pressure (DBP), and heart rate were recorded before and after administration. The following measurement method was used. When the rat was quiet and awake, it was fixed on a special holder; the rat tail was passed through the pressure cuff to the root. Immediately after the cuff, the pulse transducer was attached to the rat tail, and the ventral tibial artery was confirmed to be in good contact. When the rat pulse wave was stable, the measurement was initiated. Inflation pressure was subsequently applied: the blocking pressure was 250 mmHg (the pulse wave disappeared when the blocking pressure reached the systolic pressure level), and the pressure was slowly decompressed after the blocking pressure was maintained for 6 s. When the pulse wave appeared, the corresponding blood pressure at that time was defined as the arterial systolic pressure. The peak of the corresponding curve was the arterial diastolic pressure, and the system automatically displayed the heart rate. Each group was continuously measured 6 times, and the average value was taken.

7. Myocardial protective effect in SHR rats

After the end of the blood pressure measurement, the compound 1 group, compound 2 group, and hypertensive control group were sacrificed after anesthesia with ether. The rats were fixed in the supine position on the surgical plate, 70%

alcohol was used to disinfect the abdominal skin, a midline incision was taken, organs in the abdominal cavity were fully exposed, the heart was removed, and the tissue was immediately fixed with 4% glutaraldehyde solution. Then, the sections were observed *via* a transmission electron microscope, and the physiological condition of the rat myocardium was determined by H&E staining.

8. Cardiomyocyte H9c2 protection

The rat cardiac H9c2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS, 100 U mL^{-1} penicillin, and 100 $\mu\text{g mL}^{-1}$ streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 .

The cardiomyocytes grown to the log phase were collected and adjusted for cell concentration. The cells were seeded at a cell density of 5×10^4 cells per mL in a 96-well plate at 100 μL per well and cultured at 37 °C in a 5% CO_2 incubator for 8 h to adhere the cells. The cytotoxicity of each tested compound was firstly determined before the compounds were evaluated. The tests were divided into the blank group, control group (acetaminophen, ibuprofen), and compound group (10 μM , 40 μM , and 80 μM). The blank group only added cells without the drug or compound. The cell survival rate was measured after the cells were incubated with the drug or compound for 24 h. Using this, the cardioprotective effects of the tested compound were evaluated.

Conflicts of interest

The authors confirm that this article content has no conflict of interest.

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