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Toxicities and beneficial protection of H_2S donors based on non-steroidal anti-inflammatory drugs

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Abstract: On the basis of our previous work, the H_2S donors based on non-steroidal anti-inflammatory drugs (NSAIDs) were further evaluated in following aspects, animal blood and urine analysis, liver and kidney toxicities, gastrointestinal protection, anti-hypertension and myocardial protection. The testing results showed: after successive administration of the compound for 14 days, the numbers of white blood cells in the blood of rats reduced, and protein and leukocytes appeared in urine; and 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 kidney was obvious. However, compared with the NSAIDs as precursors, the tested compounds displayed much lower side effects, especially, for the gastrointestinal mucosa of rats, there has hardly side-effect; moreover, all three compounds decreased blood pressures in spontaneously hypertensive rats in concentration dependent manner even though this antihypertensive effect was weaker than those of nifedipine and captopril. In addition, three compounds protected H9c2 cells from injure through anti-oxidation pathway; and they improved myocardial injury in spontaneously hypertensive rats. Compound **2** is the derivative of ibuprofen and has lower toxicity than ibuprofen to rat cardiomyocytes. So possibly, it will be a better substitute of ibuprofen for patients due to lower cardiotoxicity.

Keywords: NSAIDs; H_2S donors; gastrointestinal protection; anti-hypertension; myocardial protection

Introduction

Endogenous H_2S is the third endogenous gas signal molecule after nitric oxide (NO) and carbon monoxide (CO), and plays an important physiological role in the body.[1-4] H_2S significantly reduced lipopolysaccharide-induced accumulation of

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neutrophils in tissues and organs (liver and lung), reduced tumor necrosis factor-α (TNF-α) and plasma interleukin-1β (IL-1β) Level, and increased plasma IL-10 levels, thereby inhibiting inflammatory response.[5] In the cardiovascular system, H_2S caused vasodilation and lowered blood pressure by promoting K_{ATP} channels open;[6] meanwhile, it produced myocardial protection through activating protein kinase C (PKC) and myocardium K_{ATP} channels. [7]

Since H_2S is toxic gas and its dosage is not easy to control, it cannot be directly applied to the clinic. Therefore, using H_2S donors is simple and convenient way to replace H_2S. The earliest H_2S donors were sulfide salts, such as Na_2S, CaS, they released H_2S too fast under physiological conditions, which was not beneficial for its function. GYY4137 was found as a novel water-soluble H_2S releaser, and it displayed anti-shock, anti-tumor, antithrombotic and other functions. [8-11] After that, many H_2S donors were discovered and synthesized, such as desipramine (ADTOH) derivatives [12], 4-hydroxythiobenzamide (TBZ) derivatives[13], alpha-lipoic acid derivatives, and so on.

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to treat pain, inflammation and fever in clinic. [14] However, NSAIDs have many side effects, such as gastrointestinal bleeding, ulcers, high blood pressure, and increased risk of myocardial infarction. [15-17] Recently, H_2S donor on the basic of naproxen (ATB-346) was reported to be a good inhibitor of COX-2, moreover, it significantly reduced the gastrointestinal damage compared with naproxen.[18] Later, our group designed and synthesized a series of H_2S donors modified by traditional NSAIDs. The testing results showed all the compounds had good anti-inflammatory activity, lower cytotoxicity and reproductive toxicity, which suggests these compounds may be a kind of more potential anti-inflammatory drugs with lower toxicity. However, as we know, traditional NSAIDs cause myocardiac injure and blood pressure rising after administrated over a long period time. H_2S has anti-hypertension effect and myocardial protection, the NSAIDs modified by H_2S donors possibly counter or compensate for these side effects caused by NSAIDs.

Based on our previous work, [19-20] in this paper, we further evaluate these compounds from gastrointestinal protection, antihypertensive and myocardial protection. By which, we hope to obtain useful data and information to comprehensively evaluate NSAIDs-based H_2S donors whether they are more beneficial in clinic.

Results and discussion

1. Synthesis and characterization of compounds

All three compounds were prepared according to the method we used before. [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 are mild, the yields are high. All

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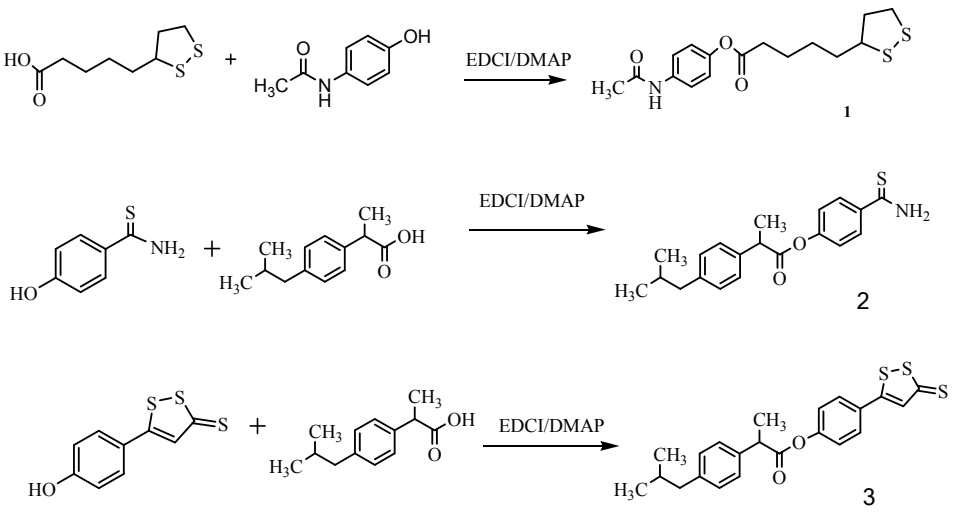
three target compounds were obtained by separation and purification using column chromatography.

The spectra of three compounds were consistent with the expected. In 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; whereas the characteristic absorption for C=S for compound **3** appears at 1136 cm^{-1}. In the ^{1}H-NMR spectrum of compound **1**, the chemical shift of hydrogen atoms in CH_3CO is at 2.14 ppm; for compound **2**, the signals of two hydrogen atoms in $CONH_2$ 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 trisulfide ring appears at around 7.40 ppm.



Scheme 1 Synthetic route and structures of compounds **1**−**3**

2. H_2S release of the compounds

The H_2S release ability of the compounds was examined using the methylene blue (MB^+) method. The MB^+ method is according to the reaction between zinc acetate and H_2S to form zinc sulfide in the presence of acid and ferric ion, and then reacting with N, N-dimethyl-1,4-phenylenediamine sulfate to form methylene blue. The absorbance at 670 nm was measured to quantify H_2S. Because the compounds were slower H_2S releasers, we used the methylene blue method to detect the ability of all compounds to release H_2S in the presence of TECP or cysteine.

The test results are shown in **Fig.1**. The release half-lives of the three compounds are all less than 30 min, and the release rates are roughly consistent with the first-order kinetics. This suggests they are all rapid H_2S releasers. The release half-life of

compound **1** is 14.1 min and its maximum release amount is 46.0 μM. For compound **2**, its H_2S release profile is shown in **Fig.1** (b); its half-life is 20.1 min and maximum release amount is 20.9 μM. Significantly, the H_2S release ability of compound **2** is weaker than that of compound **1**. **Fig.1** (c) is the H_2S release curve of compound **3**, its half-life is 1.4 min, the maximum release is 13.8μM. In contrast, its H_2S release ability is the weakest among the three compounds, but its rate is the fastest.

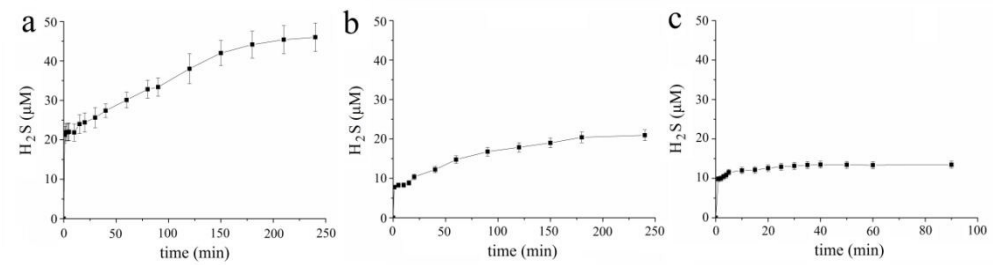


Fig. 1 H_2S release curves of the compounds, a for **1**; b for **2**; and c for **3**

3. Toxicity of the compounds after successive administration

Since the three compounds did not show toxicity to the mice as model in the acute toxicity test, we selected rats to further evaluate their toxicity after successive administration. Each compound was administered repeatedly 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 shown in **Table 1**. Seen from **Table 1**, some blood indexes are abnormality: the numbers of leukocyte significantly decreased compared with the blank control; the platelet numbers decreased too much. The platelets are only 194, 161, 114×10^9/L after rats treated with compound **1**, **2** and **3**, respectively, while the control is 670×10^9/L. However, the numbers of red blood cell and hemoglobin are within the normal range. This shows that the compounds have a certain impact on the blood system.

Table 1 Results of blood test after rat successive administration 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

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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）	135	119	136	138
Platelet (10^9/L)	194	161	114	670

The urine test results of rats are shown in **Table 2**. Some indexes are abnormality. Compared with the control group, the urine pH value decreased, and both urobilinogen and bilirubin levels increased. Among three compounds, compound **3** led to urobilinogen and bilirubin in rat urine increasing beyond 50-fold as high as the level of the control. Protein was detected in the urine of the three test rats, and the content was over 3 μmol•L^{-1}. Moreover, leukocytes were found in urine of compound **2** and **3** test groups. This suggests three compounds possibly injured glomeruli of kidney in some degree, further have an effect on rat kidney function.

Table 2 Results of urine test after rat successive administration after 14 days

Test items	Compd **1**	Compd **2**	Compd **3**	Blank Control
Urobilinogen（μmol/L）	3.3	16	66	1
Bilirubin（μmol/L）	12	17	50	10
Ketone body（mmol/L）	4	Neg	0.5	4
Occult blood(cacells/μL)	80	Neg	Neg	10
protein(g/L)	3.2	3.0	3.0	0
Nitrite	Neg	Neg	+	9
leukocyte(cacells/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

In order to make out the degree of damage of the test compounds to the rat liver and kidney, we do a liver and kidney biopsy through HE staining. Under microscopy, for the control group, the hepatic lobule was structurally intact, the hepatocytes were arranged neatly, and no obvious gap was observed between the cells; the renal tissue was stained deep and the glomerular structure was intact. After administration of 10 mg•kg^{-1} of compound in rats by intraperitoneal injection for 14 days, compound **2**

group: hepatic lobule structure was intact, central venous congestion, hepatic sinus congestion was obvious, hepatocytes were swollen and visible gaps were observed, cytoplasm was lightly stained; for kidney section, the small ball was intact, the cells of the proximal convoluted tubule were swollen and visible gaps were visible, the cytoplasm was loose, lightly stained, and some renal tubular epithelial cells were necrotic. As for compound **3** group: the liver tissue damage was similar to that of compound **2** group, the renal tissue was congested and the cells were swollen, the cytoplasm was loose, and the light staining was significantly lighter. This indicates that compounds **2** and **3** have a certain degree of damage to the physiological structure of rat liver and kidney tissue. In contrast, the damage caused by compound **2** was severer to rat kidney than that of compound **3**.

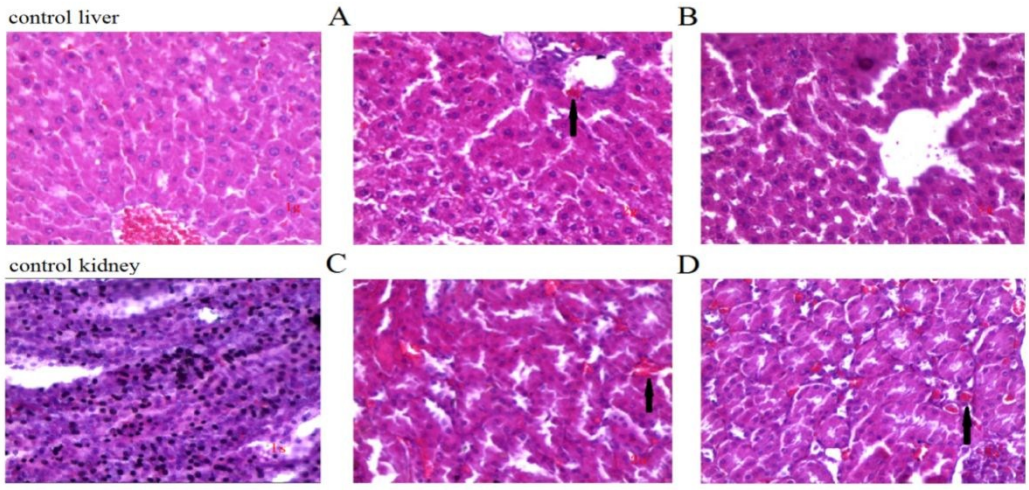

Fig.2 Optical microscopy morphological images of rat liver (A and B) and kidney tissues (C and D) after successive administration compound for 14d. Kidney section: A for compound **2** and B for compound **3**; kidney section: C for compound **2** and D for **3**. Black arrows indicate tissue congestion.

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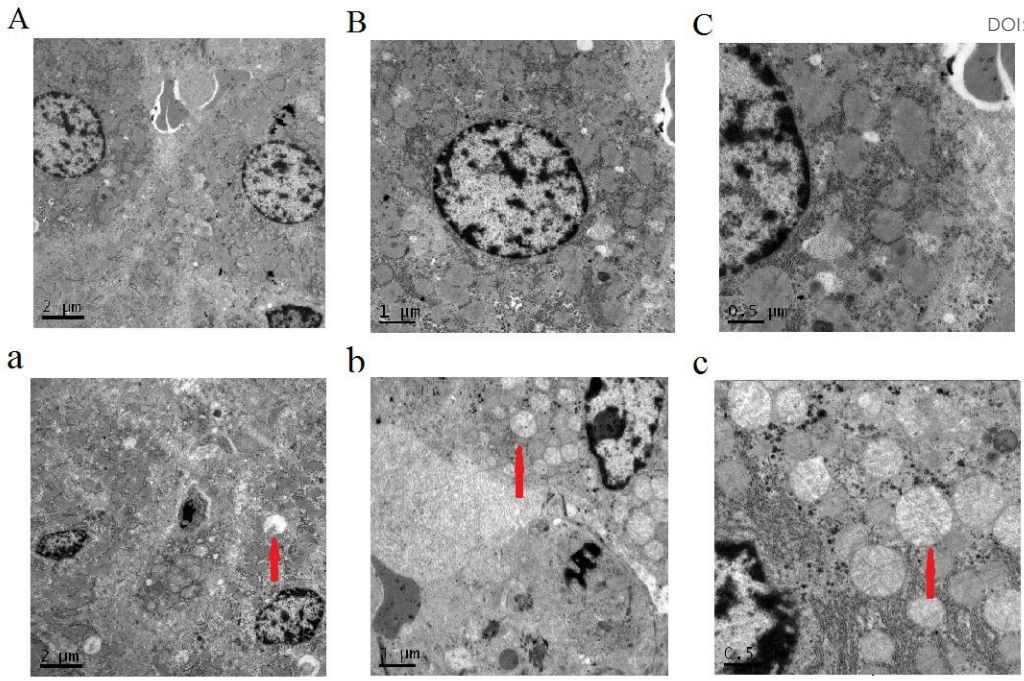

Fig.3 TEM morphological images of rat liver after successive administration compound for 14d. A, B, and C were the control group; a, b, and c for compound **2** group (6000, 10000, 20000-fold, respectively). The arrows in Fig.3a and 3b indicate an increase in lipid droplets.

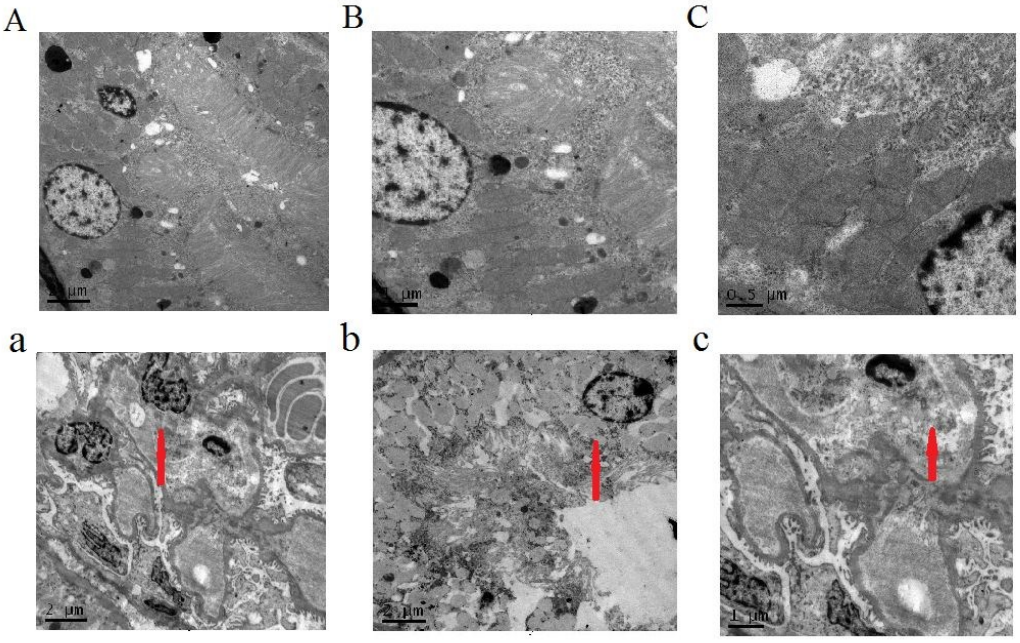

Fig. 4 TEM morphological images of rat kidney after successive administration compound for 14d. A, B, and C were the control group; a, b, and c for compound **2** group (6000, 10000, 20000-fold, respectively). The arrow in Fig.4a indicates swelling of glomerular endothelial cells; the arrow in Fig.4b indicates swelling of glomerular endothelial cells, and stromal hyperplasia; the arrow in Fig.4c indicates a large amount of exudation (precipitate) in the glomerulus.

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In order to further understand the reasons of liver and kidney dysfunction, we observed the liver and kidney tissues of the test rats by transmission electron microscopy. The results are shown in **Fig.3** and **4**. After 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, the stromal hyperplasia, and a large amount of exudation in the glomerulus. This indicates that compound **2** gave rise to the liver and kidney cells of the rat greatly damaged, which further affect their biological functions.

4. Gastrointestinal protection of the compounds

In order to figure out whether the compound as H_2S donor has protective effect on the gastrointestinal tract, the rats were given a continuous dose of 10 mg•kg^{-1} compound **2** for 14 days, the compound precursor ibuprofen as a positive control (ibuprofen: daily dose of 6.1mg•kg^{-1}; Note: clinical adult dose does not exceed 5.0mg•kg^{-1}). Gastrointestinal tissues were collected and further examined under microscopy. The results are shown in **Fig.**5. After HE staining, the gastrointestinal tissues were observed by optical microscopy. The control group: the gastric mucosal cells were arranged neatly, 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).

Compared with the blank group, the ibuprofen group (positive control) showed hyperemia and inflammatory cell infiltration in the gastric mucosa, and gastric mucosal cells swelled (**Fig**.5A); intestinal mucosa became hyperemia, inflammatory cell infiltration and intestinal mucosal edema (**Fig**.5C). This indicates ibuprofen has serious side effects to gastrointestinal, especially for the patients needing for long-term medication. In contrast, in compound **2** group, gastric congestion symptoms were significantly reduced compared with ibuprofen group, and no gastric cell swelling occurred (**Fig**.5B); intestinal mucosal congestion symptoms were also significantly reduced (**Fig**.5D). This indicates that compound **2** has a certain improvement effect on 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, and in compound **2**, the carboxyl group was transformed into ester, which reducing the irritation to the gastrointestinal tract. Compound **2** is a pro-drug that acts individually through esterase hydrolysis in the blood. At the same time, compound **2** not only improved the gastrointestinal side effects of ibuprofen, but also changed the absorption, distribution and metabolism of ibuprofen in *vivo*.

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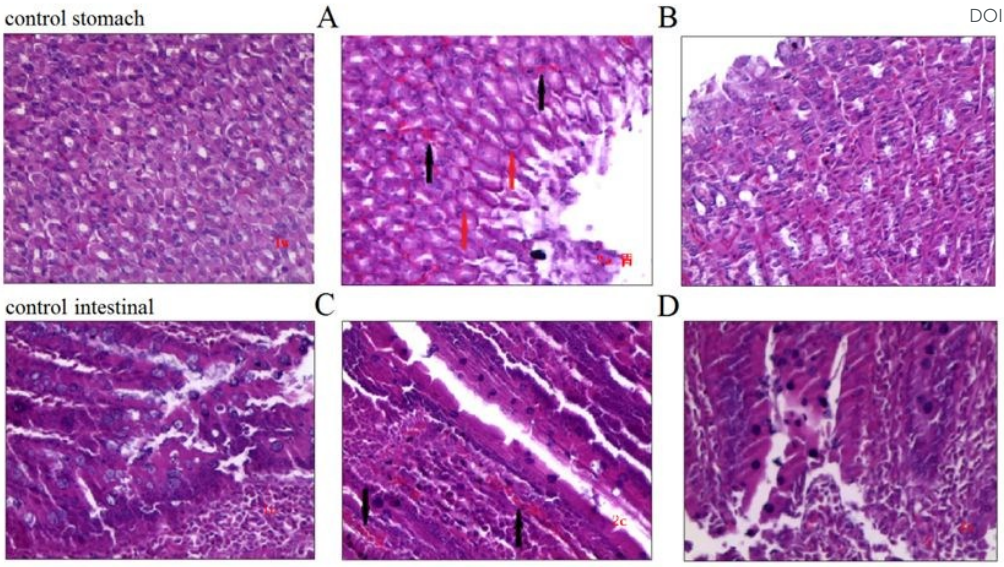

Fig. 5 Optical microscopy morphological images of rat stomach (A and B) and intestinal tissues (C and D) after successive administration compound for 14d. Stomach sections: A for ibuprofen group; B for compound **2** group; intestinal sections: C for ibuprofen group; D for compound **2** group. Black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

5. The anti-hypertension of the compound

In order to confirm the anti-hypertension of the compounds as H_2S donors, we selected spontaneously hypertensive rats (SHRs) as a model of hypertension, and the test compound was administered by intraperitoneal injection; anifedipine and captopril were used as positive control. Blood pressure was measured by a rat blood pressure monitor.

The test results showed compound **1** displayed a good antihypertensive effect, and it was concentration dependent. When compound **1** was 10, 20, 40 mg•kg^{-1}, the mean minimum systolic blood pressure of the SHR was 139.1, 127.6, and 115.3 mmHg, respectively (**Fig**. 6a). The statistical difference was significant between different concentrations, $p<0.01$; the duration of blood pressure was longer and about 60 min, this is because compound **1** has a strong H_2S release capacity and a longer release half-life. Compound **2** has a similar antihypertensive effect as compound **1**, but it has a shorter duration (**Fig**. 6b). Compound **3** is a norepine trisulfide compound. Under the same condition, the antihypertensive effect of compound **3** was weaker than those of the two compounds. The average minimum systolic blood pressure at three concentrations was 142.9, 136.7, and 135.1 mmHg, respectively (**Fig**. 6-c). This may be related to less H_2S released from compound **3**.

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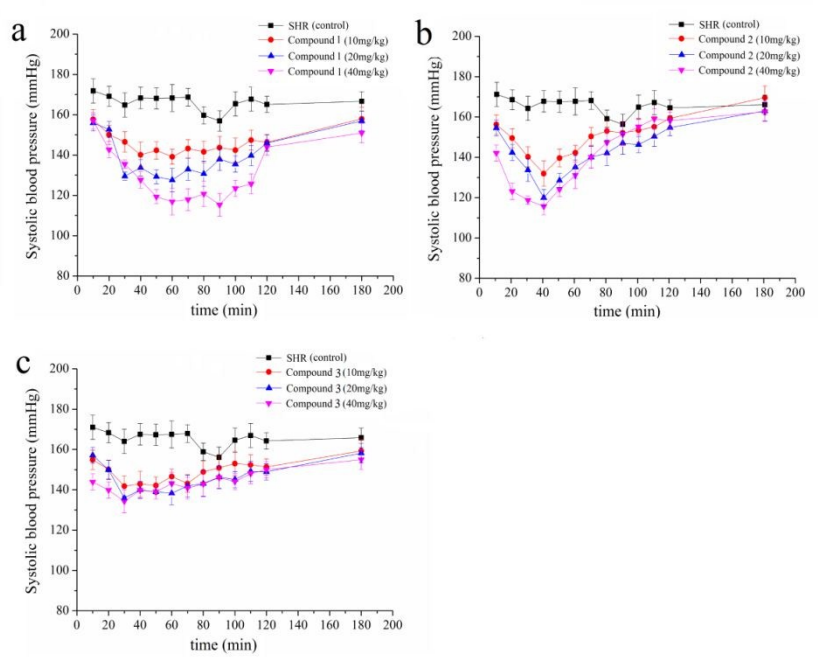

Fig. 6 Effect of compound concentration on systolic blood pressure in SHR rats. a for compound **1**; b for compound **2**, and c for compound **3**. Each column represents the mean ± SD of four independent experiments.

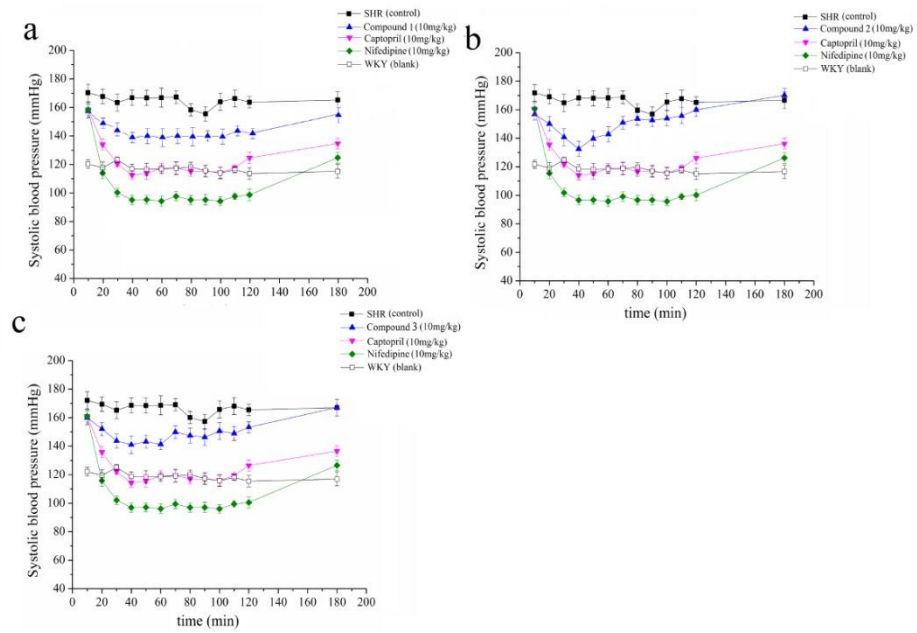

Fig.7 Effect of compound on systolic blood pressure in SHR rats. a for compound **1**; b for compound **2**; c for compound **3**, and each column represents the mean ± SD of four independent experiments.

The effect of the compounds as H_2S donors on the diastolic blood pressure of SHRs was similar to that of systolic blood pressure. Compound **2** had the strongest

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effect on reducing diastolic blood pressure among three compounds (**Fig.** 8b), and it also had a significant concentration-dependent, but the duration of diastolic blood pressure reduction was shorter. The effect of compound **1** on reducing diastolic blood pressure was also concentration-dependent and had the longest duration (**Fig.** 8a), which may be related to the strong H_2S release ability and longer release half-life of compound **1**. When compound **1** was at 10, 20 and 40 mg•kg^{-1}, the minimum diastolic blood pressures of SHRs were 113.8, 110.9 and 99.4 mmHg, respectively. Compound **3** had the weakest effect on reducing diastolic blood pressure, and the concentration dependence was not obvious. When compound **3** was 10, 20 and 40 mg•kg^{-1}, the minimum diastolic blood pressure of SHRs was 116.3, 115.1, 112.4 mmHg, respectively (**Fig**.8c).

However, at the same concentration (10 mg•kg^{-1}), regardless of systolic or diastolic blood pressure, the anti-hypertension of the compound was weaker than the same concentration of nifedipine and captopril, and the duration of blood pressure was shorter than that of nifedipine and captopril (**Fig**. 9); the compounds with higher concentration lowered the blood pressure of SHRs to the blood pressure level of normal WKY rats. During the hypotensive process, the antihypertensive effect of the compound is related to the release ability of H_2S. Compound **1** with strong H_2S releasing ability displayed a stronger antihypertensive effect, which indicates that the antihypertensive effect of compound **1** was possibly mediated by H_2S.

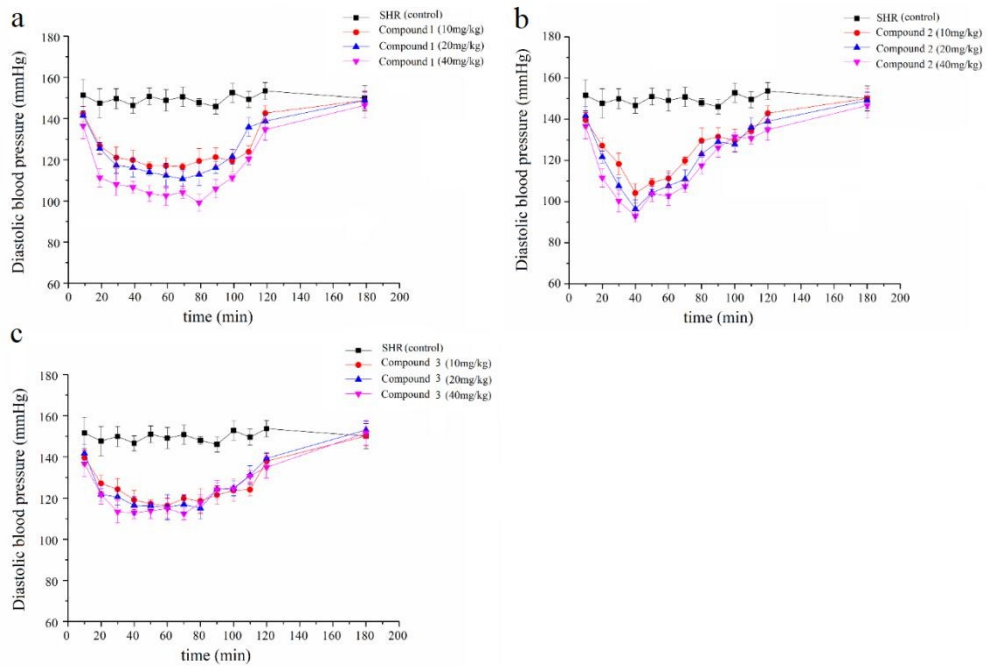

Fig. 8 Effect of compound concentration on diastolic blood pressure in SHR rats: a for compound **1**; b for compound **2**; c for compound **3**. Each column represents the mean ± SD of four independent experiments.

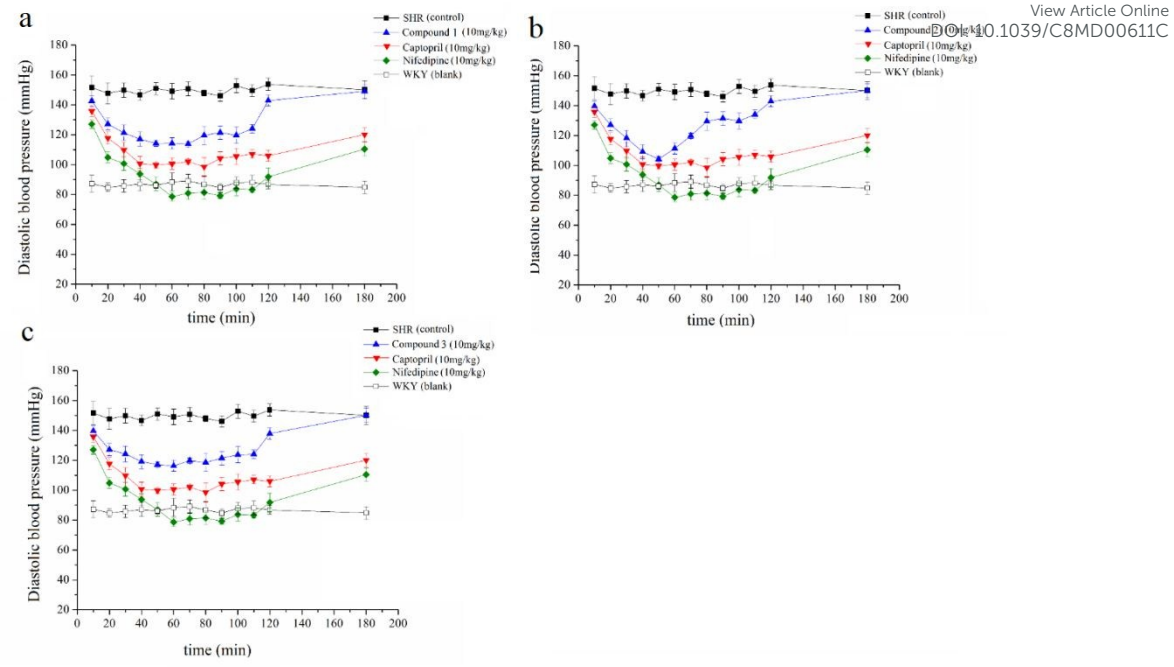

Fig. 9 Effect of compound on diastolic blood pressure in SHR rats: a for compound **1**; b for compound **2**; c for compound **3**. Each column represents the mean ± SD of four independent experiments.

It can be seen from the above results that the compounds as H_2S donors can reduce the systolic blood pressure and diastolic blood pressure of SHRs in a concentration-dependent manner. In the process of modulating blood pressure, the effects of compounds on systolic blood pressure and diastolic blood pressure are related to H_2S release ability. Compound **1** with strong 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 the compounds as H_2S donors on myocardium

6.1 Protection of the 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 determined the myocardial protection of the compound. The cell tests were divided into blank group, H_2O_2 group, control group (acetaminophen or ibuprofen), compound-H_2O_2 group. The damage models of light, medium and heavy were obtained only through the cardiomyocyte treated with H_2O_2 for 1h, 4h and 8h respectively.

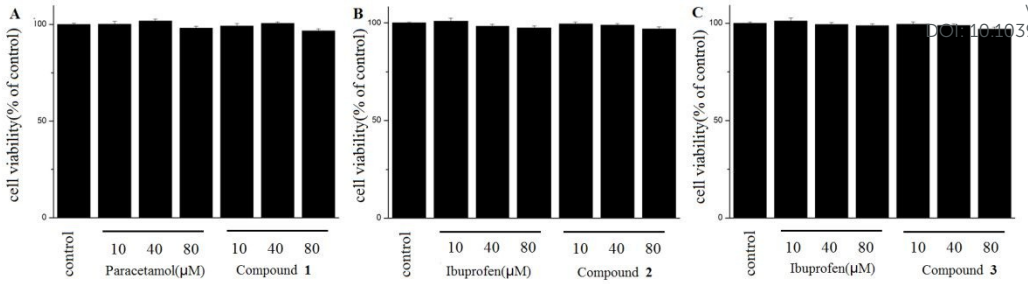

Fig. 10 H9c2 cell viability after treated with compound for 24 h

Seen from **Fig.** 10, in the range of 10-80 μM, the tested compounds had no effect on cardiomyocyte survival rate compared with the blank control. This indicates the three compounds were less toxic and the results of myocardial protection against the cells in this concentration range will be meaningful.

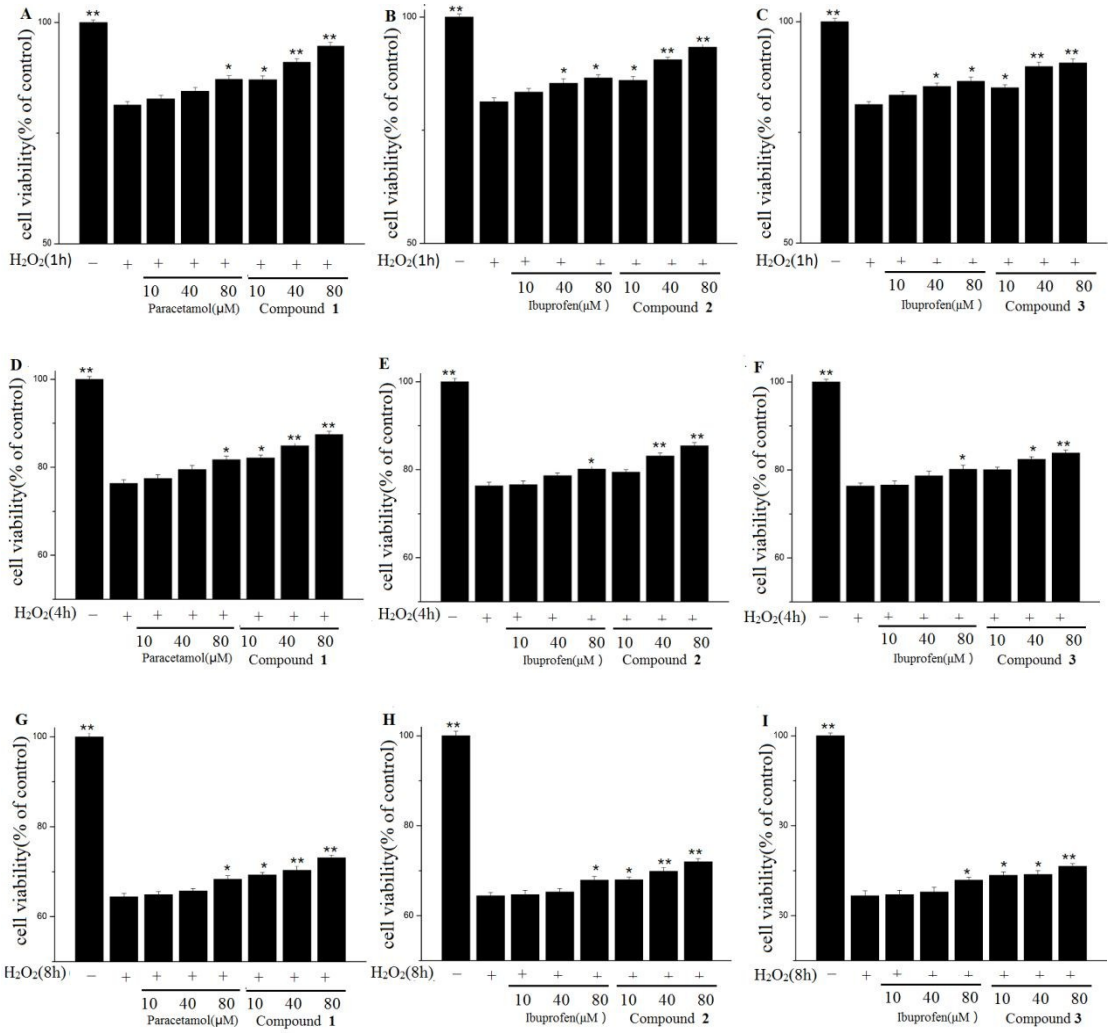

Fig. 11 Effect of compound concentration on the survival rate of H9c2 cells. Each column represents the mean ± SD of three independent experiments, *P <0.05, **P <0.01

In following tests, the compounds displayed protective effects on cell damage. Seen from **Fig.** 11 (A, B and C), after treatment with H_2O_2 of 400 μM for 1h, the survival rate of H9c2 cells was 82.3%; in the presence of compound **1** or **2** or **3**, the cell survival rates increased in dose-dependent manner; and when they all were 80 μM, the survival rates of the cells increased by 13.3%, 11.9% and 9.4%, respectively. We found the compound displayed a different activity with the cell injure degree. When the H9c2 cells treated for more time, the survival rates of the cells increased little even in the presence of the compounds at the same concentration. When H9c2 cells treated with H_2O_2 for 8 h, compound **1** made cell viability increase by 8.6%, while compounds **2** and **3** less than 5.0%. Obviously, when the myocardial cells injured slightly, the protective effect of compound on cardiomyocyte is significant; and the cell damaged seriously, the activity of the compound is low, this is because the cell damaged too severe to repair. In comparison, compound **1** has a stronger activity, which possibly resulting from the larger maximum release of H_2S.

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

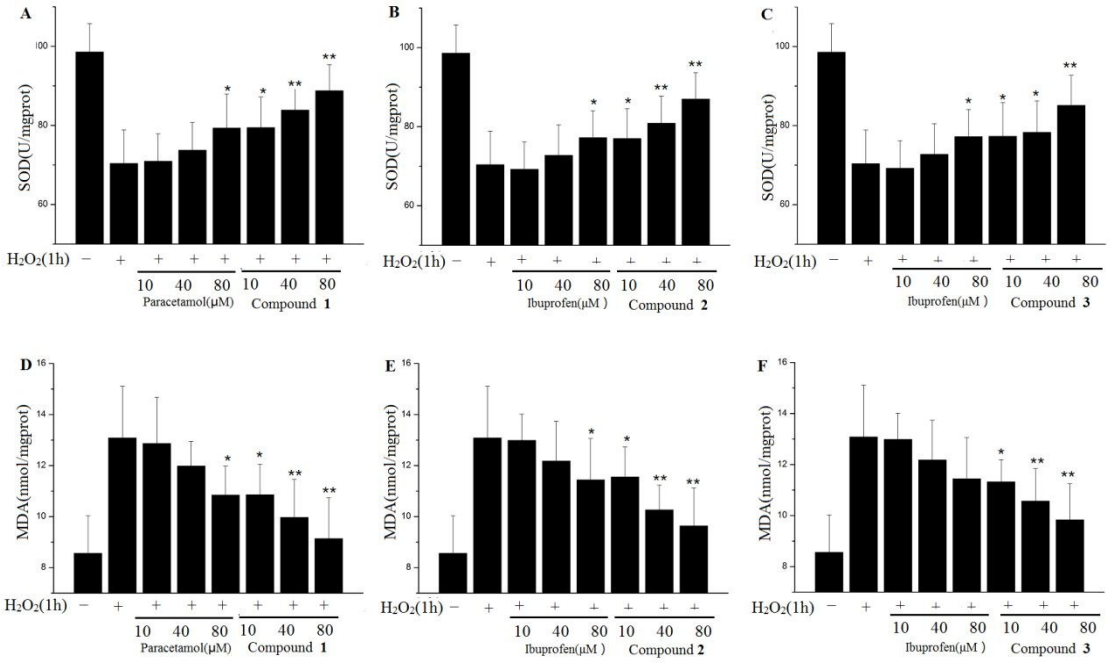

Fig. 12 Effect of compound on SOD and MDA content in H9c2 cells, each column representing the mean ± SD of four independent experiments; *P < 0.05, **P < 0.01.

As shown in **Fig.** 12, when the H9c2 cardiomyocytes were treated with only H_2O_2 for 24h, the level of SOD in cells was significantly decreased, but after the cells incubated with the tested compounds, the levels of SOD were significantly increased. Accordingly, MDA levels decreased in concentration-dependent manner. Therefore, we speculate the myocardial protection of the compound possibly results from the antioxidant of the H_2S donors. The positive control acetaminophen and ibuprofen

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displayed weak antioxidant activity, which it suggests the antioxidant effect of the compound is mediated through H_2S released from the compound.

In the process of test, the cells were stained by giemsa and observe the H9c2 cell morphology under microscope. In the blank group, the cells were long spindle-shaped, and adherent firm cell; but in H_2O_2 group, the cells showed an irregular shape, and some cells contracted and became round. With the prolongation of H_2O_2 intervention time, the number of deformed cells increased (**Fig.** 13-d). But in compound-H_2O_2 group, the number of cells reduced significantly; moreover, with the concentration of compound **1**, the repair effect was more pronounced (**Fig.** 13-l). Meanwhile, we see acetaminophen as a control also showed anti-oxidation activity due to phenolic hydroxyl group (**Fig.** 13g-h). In contrast, under the same concentration, compound **1** displayed better activity than acetaminophen did, just because H_2S released from compound **1** also has anti-oxidation activity. This is consistent with the previous conclusions.

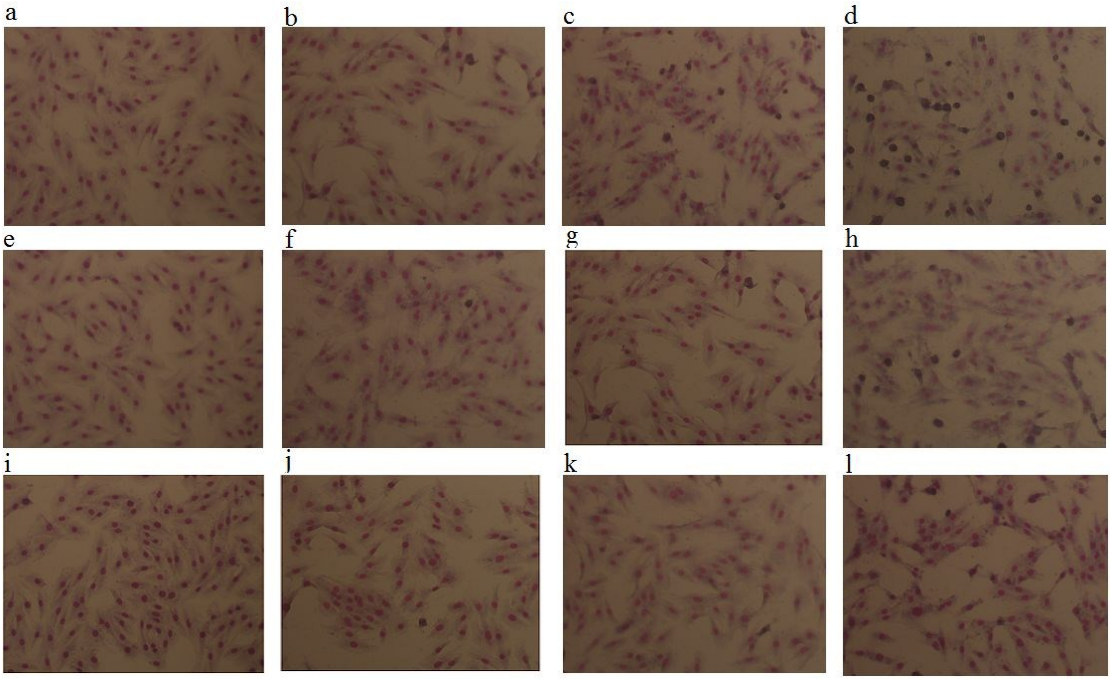

Fig. 13 Morphology of H9c2 cells under all kinds conditions: a, e, and i are blank groups; b, c, and d for H_2O_2 treated for 1h, 4h, and 8h, respectively; f, g, and h for acetaminophen treated for 1h, 4h, and 8h, respectively; j, k, and l for compound **1** treated for 1h, 4h, and 8h, respectively.

6.2 Myocardial protective effect of target molecules on hypertensive rats

The compound as H_2S releasing molecule has displayed H9c2 cardiomyocytes protection in some degree, but we want to know whether it also can reverse the myocardial remodeling and protect the cardiac function for animal. For this reason, hypertensive rats were administrated the tested compounds at the dose of 10 $mg \bullet kg^{-1}$ per day by intraperitoneal injection for 14 consecutive days, ibuprofen as the positive control. Then the myocardial tissues were removed, and observed after HE staining under light microscopy. As seen in **Fig.** 14. The positive control group: the myocardial fibers of the rats were neatly arranged and coarser, the muscle bundle gaps

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were increased to varying degrees compared with the blank control, and some myocardial interstitial collagen fibers were visible. But compound **1** group: the myocardial fibers of the rats were arranged neatly, and the muscle bundle gap was slightly reduced compared with the ibuprofen group, and the collagen fibers were not significantly stained in the interstitium. Myocardial fibers are significantly thinner, which indicating compound **1** has lower toxicity than the positive control. As for compound **2** group: myocardial fibers were arranged neatly, and the muscle bundle gap was significantly reduced, and the thickening of the myocardial fibers have a certain improvement effect compared with the ibuprofen group, indicating that compound **2** as non-steroidal anti-inflammatory drug modified H_2S donor has lower side-effect than the original compound ibuprofen.

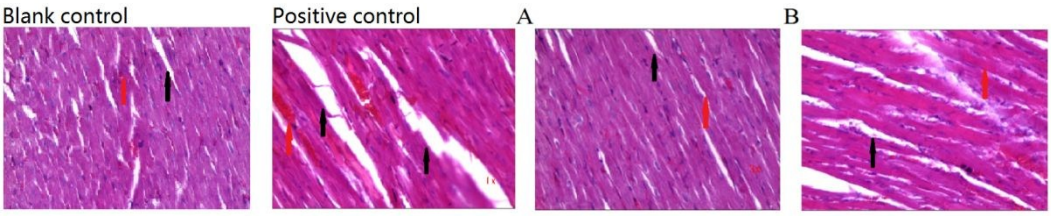

Fig. 14 Optical microscopy morphological images of rat myocardial tissues. The control group: ibuprofen; A for compound **1**; B for compound **2**; C for normal control group. The black arrow indicates the muscle bundle gap and the red arrow indicates the myocardial fiber.

In order to make the injury of myocardial cells more visible, myocardial tissues were observed under TEM. As shown in **Fig.** 15, the positive control group, the rat myocardial interstitial vascular swelled, myocardial fibers loosed, with myocardial fibrous submucosal swelling and myocardial fiber melting; meanwhile, myocardial contraction band formed, and myocardial disk dissociated and mitochondrial swelled. Mitochondria were damaged, causing energy production, release and transmission disorders, which led to ventricular pumping dysfunction. But for compound **2** group, the myofibrils of the rats were arranged substantially neatly compared with the control group, no obvious muscle fiber dissolution, no significant swelling of the mitochondria, and no myocardial disk dissociation.

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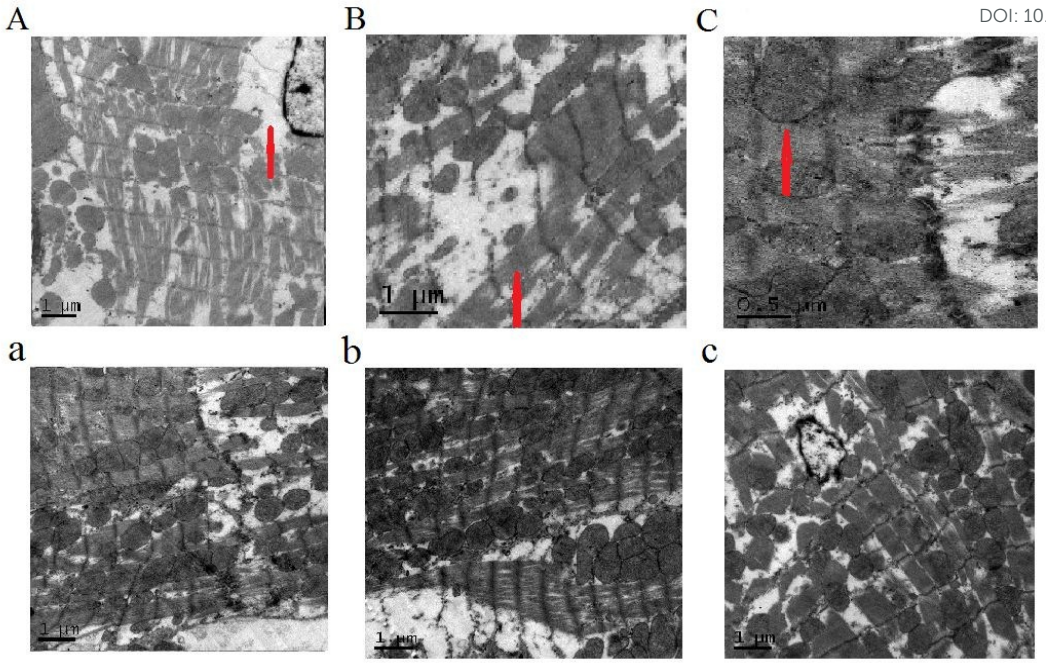

Fig. 15 TEM images of myocardial tissues of rats after administration of the tested compound for 14 days: A, B, and C for the positive control group; a, b, and c for compound **2** group (6000, 10000, 20000-fold); The arrow in **Fig**. 15a indicates swelling of cardiomyocytes; the arrow in **Fig.**15B indicates dissolution of cardiomyocytes; the arrow in **Fig.**15C indicates swelling of mitochondria.

This result indicates that ibuprofen has more toxicity to rat cardiomyocytes than compound **2**. Compound **2** is the derivative of ibuprofen, and has good anti-inflammatory activity like ibuprofen. Maybe, compound **2** will be a better substitute of ibuprofen for patients due to lower cardiotoxicity.

Conclusion

In the presence of cysteine or TECP, all three compounds were H_2S releasers rapidly. After successive administration for14 days, the test compound reduced the white blood cell number of rats, and the protein and white blood cells appeared in urine. Seen under the microscope, the structure of the kidney tissues were abnormal in some degree. This indicates that the compounds rat still have side-effect to kidney for the patients with long-term medication. However, compared with ibuprofen, their side effects were less. Secondly, compared with ibuprofen, the side effects of gastrointestinal tract significantly alleviated; and there were no symptoms such as gastric mucosa and intestinal mucosal hyperemia. Thirdly, the test compounds as H_2S donors have a good concentration-dependent anti-hypertension on SHRs even though its antihypertensive effect is weaker than nifedipine and captopril. Fourthly, the tested compound displayed protective effect on H9c2 cardiomyocytes, and its effect was achieved by reducing intracellular SOD and MDA levels, and the compounds have

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protective effect on the myocardium tissues of rat. Among three compounds, compound **2** has lower toxicity than ibuprofen to rat cardiomyocytes. Compound **2** is the derivative of ibuprofen and will be a better substitute of ibuprofen for patients due to lower cardiotoxicity.

Experimental part

1. Experimental instruments and materials

AVANCE III 400M NMR instrument; maXis4G TOF mass spectrometer (Brucker, Germany); Nicolet NEXUS 360 FT-IR infrared spectrometer (Nieolet, USA); Lambda25 UV-visible spectrometer (PerkinElmer, USA); CO_2 incubator; microplate reader (ThermoVarioskan Flash 3000) Beckman-Coulter LX-20 fully automated biochemical analyzer; JEM-1230 transmission electron microscope.

BP-300A automatic large mouse non-invasive 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 are analytically pure The reagents used were all dried.

The experimental animals were purchased at the GLP Laboratory of Experimental Animal Center of Lanzhou University, and the experimental animal certificate: SCXK (Gan) 2009-0004; WKY (Wistar-Kyoto) rat; Clean male spontaneously hypertensive rat (SHR), Zhou Ling, 8 weeks; weight is 300 ± 25g. Rats were housed in cages, one per cage, kept at room temperature (23 ± 2) °C, relative humidity of 60-65%, free access to drinking water, and all animals were kept for 7 days before the experiment. All 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 of agree.

2. Synthesis of H_2S donors

The synthesis of compound **1** is described in [22]. 206 mg (1 mmol) of α-lipoic acid was dissolved in 10 mL of dried dichloromethane, and 227 mg (1.1 mmol) of 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 3h. 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 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-

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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=6.0Hz, 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 $C_{16}H_{21}NO_3S_2Na$ $[M+Na]^+$: 362.0861; found 362.0857.

The synthesis of compound **2** is described in [23]. Ibuprofen (206 mg, 1 mmol) was dissolved in 8 mL of dried acetonitrile, EDCI (210 mg, 1.1 mmol) was added, and stirred at room temperature for 30 min. 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 completed, 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). ^{1}H NMR (DMSO-d_6, ppm) : δ 0.86 (d, J = 6.4 Hz, 6H, $C(CH_3)_2$), 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). 13C 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 $C_{20}H_{23}NO_2SNa$ $[M+Na]^+$: 364.1347; found 364.1337.

The synthesis of compound **3** was similar to that of compound **2**, and 226 mg (1 mmol) of ADTOH was used in place of the above 4-hydroxythiobenzamide; the mobile phase in the crude product 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, C $(CH_3)_2$), 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): δ18.9, 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 $C_{22}H_{22}O_2S_3Na$ $[M+Na]^+$: 437.0680; found 437.0701.

3. H_2S release detection

3.1 Na_2S standard curve plotting

A buffer solution of 20 mM pH 7.4 was first prepared for 1 L. Then the 120.2mg $Na_2S•9H_2O$ was dissolved in the 100mL volumetric bottle, and the 5 mM standard solution of Na_2S was prepared with the ph=7.4 phosphate buffer solution. 50, 100, 200, 400, 600, 800, 1000 μL of Na_2S standard solution of 5 mM were moved into 50mL volumetric bottle respectively, and added phosphate buffer solution up to the

scale line. The standard solution of Na_2S with a concentration gradient of 5, 10, 20, 40, 60, 80 and 150 mM were obtained. The gradient standard solution of 1mL was added to methylene blue (MB) mixture. The blank control was a mixture of methylene blue with phosphoric acid buffer solution of pH=7.4 of 1mL. 20 min later, the absorbance of the solution was measured at 670nm by ultraviolet visible spectrophotometer (lambda 25), and the standard curve between absorbance and Na_2S concentration was plotted.

3.2 H_2S release detection of the compounds

75 μL of 40 mM compound solution was added to the volumetric flask, and the promoter TECP (1.0 mM) or L-cysteine (1.0 mM) was added. Then 2.0 mL of the above mixed solution was moved into a cuvette, and methylene blue (MB^+) mixture was added. 20min later, 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 H_2S release curve 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 by intraperitoneal injection according to the dose of 0.01 $mol•kg^{-1}$, and the control group was given 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 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, and the tissue was immediately fixed with 4% glutaraldehyde solution. After sectioning, the tissue was irradiated by transmission electron microscopy, and the liver and kidney tissues of the rat were observed by HE staining. The physiological condition determines whether the target compound has an effect on the structure and function of the liver and kidney of the rat, and analyzes the urine and blood of the rat 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 each group): control group, compound group. Rats were administrated the tested compound for 14 days, each dose was 10 $mg•kg^{-1}$, and the control was ibuprofen. The dose was 6.1 $mg•kg^{-1}$. After anesthesia with ether, the rats were sacrificed by cervical dislocation and supine. The rats were fixed on the surgical board, with 70% alcohol disinfection of the abdominal skin, and taken the midline incision of the abdomen, fully exposing the organs of the abdominal cavity. The stomach was removed. A section of the complete small intestine about 10cm was taken at the proximal end of the ileocecal. The contents of

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the stomach and small intestine were removed by refrigerating saline. The procedure is as follows: 1. the fresh materials were cut into small piece of 1.0 cm and immediately put it into the fixing agent for fixing. 2. The fixed material was removed from the water in the tissue by ethanol, and after being transparent to xylene, it was immersed in the melted paraffin to be soaked and embedded. 3. A 5-10 μm slice was cut with a microtome, attached to a glass slide, dewaxed, stained, hematoxylin and eosin stained (HE stained). 4. Observed under an optical microscope.

6. Measurement of blood pressure in hypertensive rats

Male SHRs were randomly divided into 4 groups (6 rats in each group): hypertension control group, captopril group (10 mg•kg^{-1}), nifedipine group (10 mg•kg^{-1}), compound group (25, 50, 100 mg•kg^{-1}), male WKY rats were the normal blood pressure control group. All compounds were administered by intraperitoneal injection. The solvent was 0.5% (w/v) mixed solution of sodium carboxymethyl cellulose and DMSO 3/1(v/v), and the SHRs were administrated at the dosage per day was 2 mL•kg^{-1} at 8 am every day. The control group and the WKY group were administrated equal amount of blank solvent. Blood pressures and heart rates of the rats were measured before administration, and were measured every 30 minutes after administration for 6 hours. Arterial systolic blood pressure (SBP), arterial diastolic blood pressure (DBP) and heart rate were recorded before and after rat administration. Measurement method: When the rat was quiet and awake, fixed it on the special holder, and passed the rat tail through the pressure cuff to the root. Immediately after the cuff, bound the pulse transducer to the rat tail, and the ventral tibial artery was in good contact. When the rat pulse wave was stable, the measurement was started. The inflation pressure was 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 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 of SHRs

After the end of blood pressure measurement, the compound 1 group, the compound 2 group and the hypertensive control group were sacrificed after anesthesia with ether. The rat supine position was fixed on the surgical plate, 70% alcohol was used to disinfect the abdominal skin, the midline incision was taken, the organs in the abdominal cavity were fully exposed, the heart was removed and the heart was removed, and the tissue was immediately fixed with 4% glutaraldehyde solution. Then, the sections were irradiated by transmission electron microscopy, and the physiological condition of the rat myocardium was observed by HE staining.

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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} of penicillin, and 100 μgmL^{-1} of streptomycin at 37°C in a humidified atmosphere of 5% CO_2 .

The cardiomyocytes grown to log phase were collected and adjusted for cell concentration. The cells were seeded at a cell density of 5×10^4 cells/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 blank group, control group (acetaminophen, ibuprofen) and compound group (10μM, 40μM, 80μM). The blank group only added cells without drug or compound. The cell survival rate was measured after the cells were incubated with the drug or compound for 24 h. By which cardioprotective effects of tested compound was evaluated.

Conflict of Interest

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

Acknowledgements

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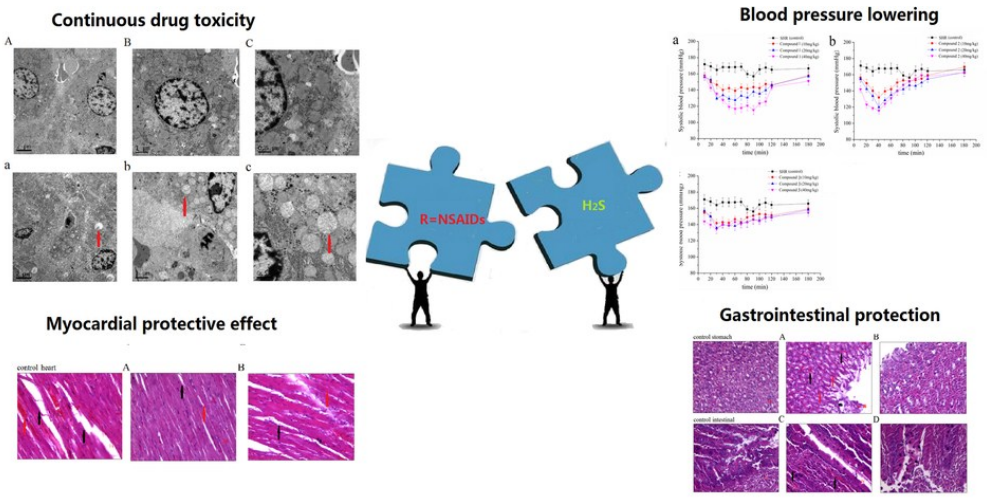

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