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PII: S0223-5234(17)30458-0

DOI: 10.1016/j.ejmech.2017.06.012

Reference: EJMECH 9507

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 12 March 2017

Revised Date: 4 June 2017

Accepted Date: 6 June 2017

Please cite this article as: M. Li, J. Li, T. Zhang, Q. Zhao, J. Cheng, B. Liu, Z. Wang, L. Zhao, C. Wang, Syntheses, toxicities and anti-inflammation of $H_2S$-donors based on non-steroidal anti-inflammatory drugs, *European Journal of Medicinal Chemistry* (2017), doi: 10.1016/j.ejmech.2017.06.012.

# Syntheses, toxicities and anti-inflammation of $H_2S$-donors based on non-steroidal anti-inflammatory drugs

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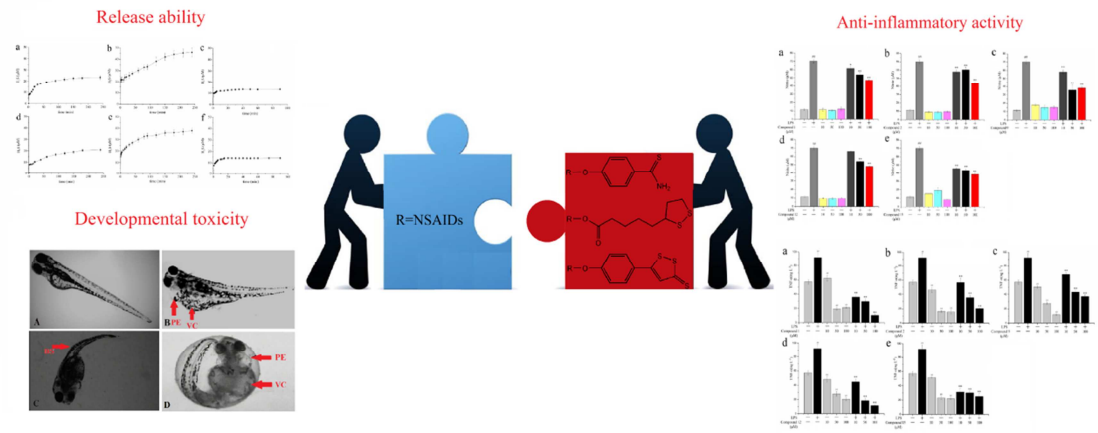
*(1 Institute of Medicinal Chemistry, School of Pharmacy of Lanzhou University; 2 GLP Lab centre, School of Basic medicine of Lanzhou University; 3 School of Stomatology of Lanzhou University, Lanzhou 730000, China)*

## Abstract

Three series of CORMs $H_2S$-donors based on NSAIDs were evaluated from toxicities and anti-inflammatory activities, and the synergistic effect of $H_2S$ and NSAIDs was preliminary explored.



# Syntheses, toxicities and anti-inflammation of $H_2S$-donors based on non-steroidal anti-inflammatory drugs

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*(1 Institute of Medicinal Chemistry, School of Pharmacy of Lanzhou University; 2 GLP Lab centre, School of Basic medicine of Lanzhou University；3 School of Stomatology of Lanzhou University, Lanzhou 730000, China)*

**Abstract** Three series of $H_2S$ donors based on NSAIDs were synthesized and characterized by $^1$H-NMR, IR and ESI-HRMS. The $H_2S$-release abilities of all compounds were evaluated in the presence of TECP or cysteine. The results show all compounds were fast $H_2S$-releasers, and their half-lives were in range of 0–20 min. Under the same condition, $H_2S$ released from compound **9** was more than any other compounds. In cytotoxicity aspect, all compounds but **1** and **2** displayed much lower toxicities to both LO2 and HepG2 cell lines, and the $IC_{50}$ values of most compounds were over 800μM. Compounds **1** and **2** had a stronger anti-proliferative activity to both cell lines, but they displayed lower toxicities to LO2 than to HepG2. Based on the cytotoxicity, the developmental toxicities of the compounds were assessed using zebrafish embryos. The results show all tested compounds **2**, **9** and **15** had effects on the mortality, hatching rate and spontaneous movements of zebrafish embryos, and caused embryos teratogenesis; and the compounds had dose-dependent toxicities to both embryonic and larval zebrafish. In addition, all compounds had a better anti-inflammatory activity. In the test of anti-inflammatory activities, the tested compounds all reduced the levels of intracellular nitrite and pro-inflammatory cytokines (TNF-α，COX-2)，increased the levels of anti- inflammatory cytokines (IL-10, HO-1). All these suggest these $H_2S$ donors based on NSAIDs have a potential to be a candidate medicine.

**Keywords:** $H_2S$-donors; NSAIDs; toxicity; anti-inflammatory

## 1. Introduction

* Corresponding author. Tel./fax: +86 9318915686
E-mail address: zhaoqy@lzu.edu.cn (Q. Zhao).

Hydrogen sulfide ($H_2S$) has been recognized as a new endogenous gaseous transmitter like nitric oxide NO and CO [1-4]. Many tests showed $H_2S$ affected several organ systems and biological processes in the human body, for example, inhibiting the inflammatory response[5-7], significantly reducing the accumulation of lipopolysaccharide induced neutrophils in the tissues and organs (liver and lung), reducing tumor necrosis factor-α (TNF-α) and plasma interleukin-1 (IL-1β) levels, and increasing plasma IL-10 levels [8]. In vascular, $H_2S$ is an important endogenous vascular dilation factor as a gaseous opener of KATP channels [9], and inhibits the proliferation of vascular smooth muscle through reducing the activity of mitogen activated protein kinase [10]. $H_2S$ also has an effect of nerve regulation by regulating the dynamic balance of neuronal $Ca^{2+}$ and intracellular pH homeostasis [11, 12].

$H_2S$ is not used directly in clinic because of uncontrollable dose and high toxicity, $H_2S$ donors is a potential substitute to provide suitable $H_2S$. NaSH can release $H_2S$, and it was reported it inhibited aspirin-induced leukocyte adherence in rat mesenteric venules and reduced leukocyte infiltration in a carrageenan-induced air pouch model of inflammation [13]. However, NaSH relases $H_2S$ too fast to be used. Lawesson's reagent is the first to use as $H_2S$ donor, which it spontaneously released $H_2S$ in aqueous solution, but because its water solubility is poor to limit its further application [14]. In 2008, based on the structure of Lawesson's reagent, Moore synthesized a novel water-soluble $H_2S$ releaser GYY4137 [15], and studies showed GYY4137 has the function of anti-shock, anti-tumor, anti-thrombosis and other effects [16-18]. After that, more and more novel $H_2S$ donors appeared, such as dithiothione derivatives ADTOH [19], thiobenzamide derivatives [20], S-acetylthosulfide compounds [21], α-thioctic acid, thioamino acids compounds [22]. All these $H_2S$ donors can release $H_2S$ under special condition.

Non-steroidal anti-inflammatory drugs (NSAIDs) have a very wide range of applications all over the world, which are basically used for the treatment of pain, inflammation and fever, the mechanism of action is the inhibition of the cyclooxygenase enzymes [23]. However, current available NSAIDs have a lot of side effects, like gastrointestinal hemorrhage, ulceration and kidney failure [24-26]. Considering $H_2S$ anti-inflammatory activity and the mechanism of action which is completely different from NSAIDs, the researchers combined $H_2S$ donors and NSAIDs together to get a kind of new molecules. In 2007, Wallace blended $H_2S$ donor (ADTOH) with diclofenac to obtain a new compound ATB-337, and the pharmacological tests showed ATB-337 not only decreased the adhesion of leukocyte, but increased the activity of anti-inflammatory and reduced the side effects of diclofenac [27]. In 2010, ATB-346 was synthesized by combination $H_2S$ donor with

naproxen, the tested results showed ATB-346 inhibited COX-2 activity more effectively than naproxen, and distinctly reduced the gastrointestinal injury [28].

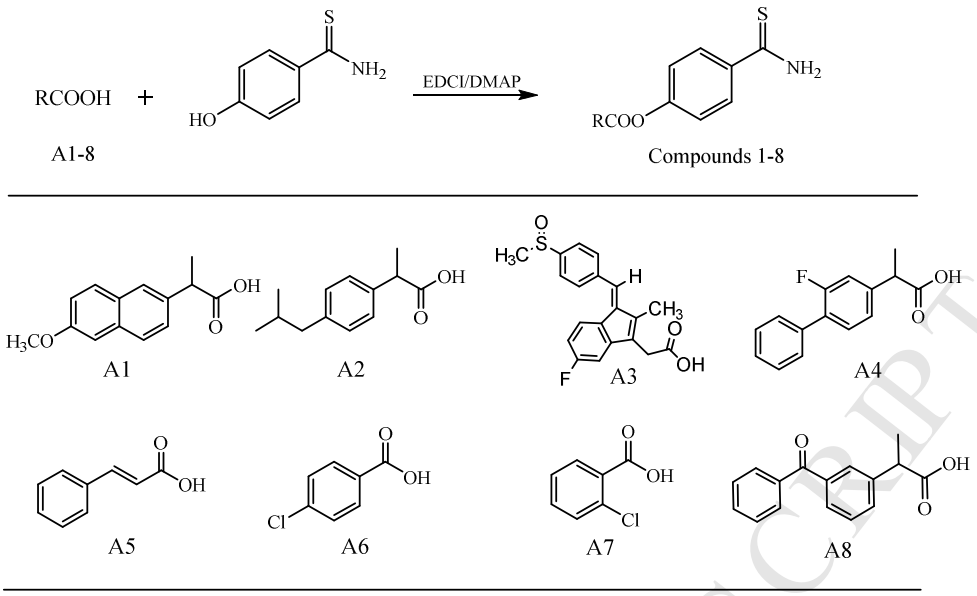
As a strategy for drug design [29-31], we want to know other anti-inflammatory drugs combined with $H_2S$ donors whether also improve their activities and decrease their side effects. In addition, several special compounds which $H_2S$ donors modified NSAIDs showed better activities and lower side effects, but we still do not know whether they can be used in clinic [32]. After all, they are new active compounds, and there are not enough data about preclinical studies. Especially, the toxicity is crucial element to determinate them to investigate further or give up.

Therefore, in this paper, we synthesized 15 compounds based on three kinds of exogenous $H_2S$ release donors: 4-hydroxythiobenzamide, ADTOH, α-thioctic acid. And we assessed their cytotoxicities using both LO2 cells and HepG2 cells, and genetic toxicities using zebrafish embryos. Meanwhile, their anti-inflammatory activities were evaluated by measuring nitrite content and four cytokines in macrophage RAW264.7. By which, we hope to obtain some information about real $H_2S$-releasing molecules fit for clinical application.

## 2. Results and discussion

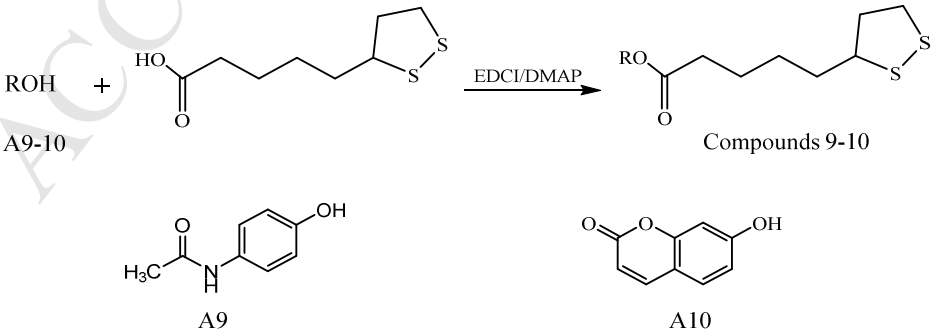
### 2.1 Syntheses characterization and properties

Compounds **1-15** were synthesized by the reaction of three kinds of parent molecules and aromatic organic acids A**1-15** [33]. These acids have been used as anti-inflammatory drugs in clinic. The structures and synthetic routes are shown in schemes 1, 2 and 3. The reaction conditions are warm, and the yields are high. The testing samples were obtained by the separation and purification using column chromatography. Among three parent compounds, ADTOH was obtained by treatment of 5-(4-methoxyphenyl)-3H-1, 2-dithiole-3-thione (ADT-$OCH_3$) with pyridine hydrochloride [34].

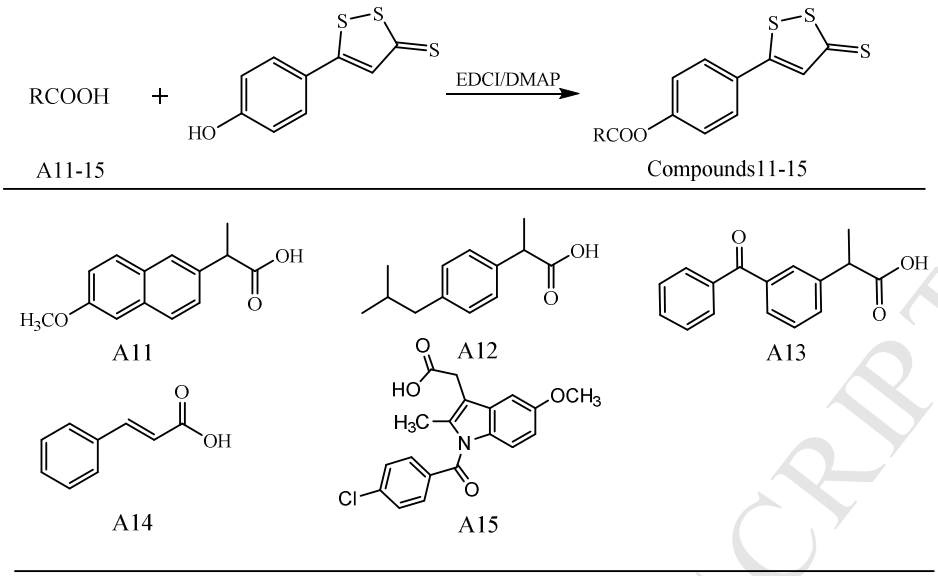



**Scheme 1** Synthetic route for the target compounds **1-8**

The spectra of the compounds correspond with the expected. In IR spectra of all the compounds, there has a strong absorption peak at in range of 1728-1766 $cm^{-1}$, which is the characteristic absorption of ester C=O. For compounds **1-8**, a strong peak appeared in 1138-1181$cm^{-1}$ is the absorption bands for C=S; As for compounds **9-10**, the signals of C-S bond appeared 604-624$cm^{-1}$; whereas the characteristic absorptions of C=S in compounds **11-15** appeared in the range of 1120-1160$cm^{-1}$. In the $^{1}$H-NMR spectra of compounds **1-8**, the signals of two protons in $CONH_2$ were observed at about 9.56 and 9.93ppm as two singlet peaks. For compound **9**, the signal appeared at 2.14ppm as singlet belongs to the protons of $CH_3CO$. Compound **10** has α, β – unsaturated lactone structure, the signals of α-H and β-H appeared at 6.40ppm and 7.70ppm as doublet, respectively. For every compound among **11-15**, a singlet at about 7.40ppm was the signal of the proton in trithionum ring.




**Scheme 2** Synthetic route for the target compounds **9-10**




**Scheme 3** Synthetic route for the target compounds **10-15**

Compounds **1-10** are pale yellow solid, compounds **11-15** are red solid or oil. All the compounds are insoluble in water, and easily dissolve in organic solvents, like DMSO, methanol, THF, and so on. All the compounds are stable in air at room temperature, and do not change even for several weeks. In their solution, pH values affect their stability; for compounds **11-15**, they hydrolyze easily with pH increasing.

### 2.2 $H_2S$ releasing measurement

$H_2S$-release capability of the donors was measured using the methylene blue ($MB^+$) method [35]. The $MB^+$ method is based on the reaction of zinc acetate with $H_2S$ to form zinc sulfide, which reacting with N, N-dimethyl-1,4-phenylenediamine sulfate to generate methylene blue in the presence of ferric ions under acid medium condition. The $H_2S$ is quantified by measuring the absorbance of the solution at 670nm.

Not like allyldisulfide, allyltrisulfide, thiobenzamides and GYY 4137 [36-38], ADTOH and its derivatives were much slower $H_2S$-releasing molecules, and even some of them were not release $H_2S$ at all under the same conditions. But recently, Hasegawa reported ADTOH released $H_2S$ in the presence of TCEP (a water-soluble phosphine reducing agent) [39]. We found TECP also promoted compounds **1-8** to release $H_2S$. For compounds **9-10**, TECP did not active α-thioctic acid to release $H_2S$, we chose cysteine as activator to measure $H_2S$ release abilities of them, which have been reported in several papers [20]. Therefore, the ability of $H_2S$ release was evaluated in a $MB^+$-based aqueous assay in the presence of TECP or cysteine.

**Table 1 $H_2S$-releasing half-lives and $C_{max}$ of the compounds at 40μM**

| Compound | $t_{1/2}$(min) | $H_2S$ $C_{max}$ (μM) | Compound | $t_{1/2}$ (min) | $H_2S$ $C_{max}$ (μM) |
|---|---|---|---|---|---|
| Thiobenzamide [a] | 0.9 | 106.1 | α-thioctic [b] | 118.1 | 28.4 |
| **1** | 13.4 | 23.5 | **9** | 14.1 | 46.0 |
| **2** | 20.1 | 20.9 | **10** | 4.6 | 37.9 |
| **3** | 3.1 | 24.0 | ADTOH [a] | 3.9 | 14.1 |
| **4** | 3.3 | 23.5 | **11** | 3.1 | 11.3 |
| **5** | 4.4 | 17.7 | **12** | 1.4 | 13.8 |
| **6** | 14.4 | 21.9 | **13** | 3.4 | 14.2 |
| **7** | 7.1 | 18.2 | **14** | 1.9 | 9.5 |
| **8** | 9.8 | 16.3 | **15** | 1.8 | 14.0 |

a, $t_{1/2}$ and $H_2S$ concentration of their derivatives and themselves were measured in the presence of TECP; b, $t_{1/2}$ and $H_2S$ concentration of its derivatives and itself were measured in the presence of cys.

In the process of measurement, a solution of the $MB^+$ cocktail was prepared and subsequently treated with a solution of TECP with the compounds at 37.5 °C. To evaluate both the total amount of $H_2S$ released and the rate of $H_2S$ release, $H_2S$ amount released from the compound was measured every 5 min, then described the curve about $H_2S$ releasing amount vs time.

The concentrations of $H_2S$ which released from the tested compounds were calculated according to the $Na_2S$ standard curve (SI-Fig.1). Fig. 1 is the representative release curves about the $H_2S$ concentration to time. The testing results demonstrate all compounds released $H_2S$ under the promotion of appropriate activator. They were all fast $H_2S$ releasing agents, and the half-lives of most compounds were all less than 20 min (Table 1). However, interestingly, each releasing characteristics was not distinctly different from each other. Fig. 1-a and Fig. 1-d are the release curves of 4-hydroxythiobenzamide derivatives, compounds **1** and **2**, their release rates of $H_2S$ accorded with the first-order kinetics. The $H_2S$ max amount released from compounds **1** and **2** were only 23.5μM, 20.9μM respectively, while the $H_2S$ amount from 4-hydroxythiobenzamide arrived at 106.1μM. Compared with the parent compound, the derivatives released $H_2S$ amount decreasing sharply, and their half lives of $H_2S$ release were longer than that of parent 4-hydroxythiobenzamide. Possibly, this is caused by **A1-8** structural fragments, because the big steric hindrance disturbs $H_2S$

release, and the larger the structure of the fragment, the stronger the inhibition of $H_2S$-releasing.

Compounds **9** and **10** are derivatives of α-thioctic acid. Seen from Fig.1-b, compound **9** was also fast $H_2S$ releaser in the presence of L-cysteine, and it released $H_2S$ up to 46.0μM while α-thioctic acid released 28.4μM under the same condition. We inferred **A9** fragment (acetaminophen) promoted $H_2S$ release of $H_2S$ in some degree. In the curve of α-thioctic acid (seen SI-Fig. 2-b), there were two rapid release periods, we thought the two sulfur atoms in the α-thioctic acid were transformed $H_2S$ stepwise. The max amount of $H_2S$-release from compound **10** was 37.9μM. Compared with the parent compound α-thioctic acid, fragments A**9** and A**10** promoted $H_2S$-release in some degree, the half-releasing time of $H_2S$ was shorter than that of α-thioctic acid.

Compounds **11-15** belong to the derivatives of ADTOH. Fig. 1-f shows compound **15** was fast $H_2S$ releasing agents under the promotion of TECP, and its release rate of $H_2S$ accorded with the first-order kinetics; the $H_2S$ max amount was 14.0μM, in contrast, it was almost the same as that from ADTOH 14.1μM. Similarly, the max amount of $H_2S$ from compounds **12** and **13** were 13.8μM and 14.2μM, respectively, which shows the fragments **A11-15** did not affect $H_2S$ release. Possibly, like ADTOH parent compound, ADTOH derivatives do not release $H_2S$ until after lactones ring hydrolysis.

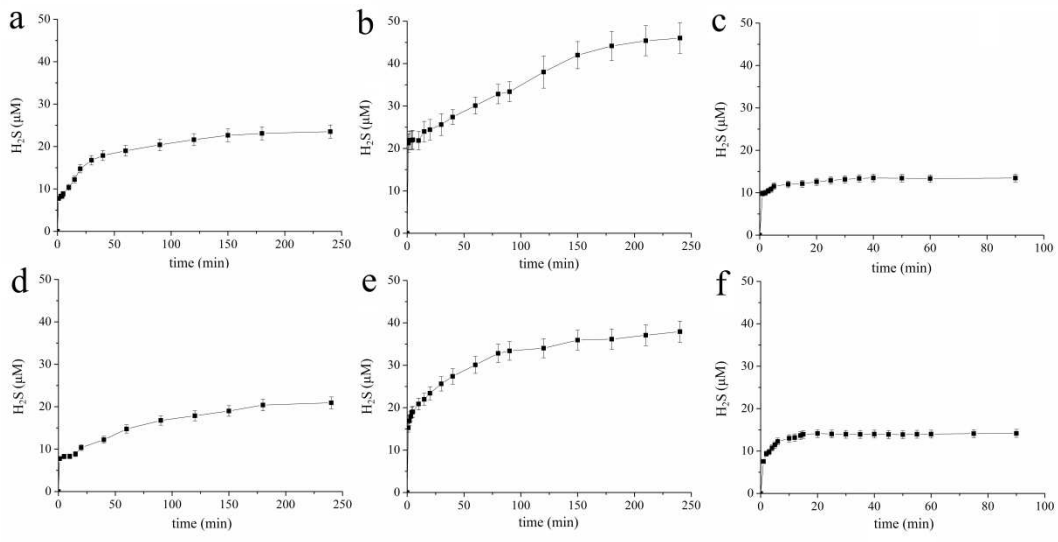



**Fig.1** $H_2S$-releasing curve of compounds, a for compound **1**, b for compound **9**, c for compound **12**, d for compound **2**, e for compound **10**, f for compound **15**. Each bar represents the mean ± SD of four independent experiments.

### 2.3 Cytotoxicities

Compounds **1-15** were assessed for toxicities against LO2 cell lines (normal liver cell) and HepG2 cell lines (liver tumor cell) using MTT assay. Stock solutions of the tested compounds in DMSO (8000 μM) were prepared freshly immediately prior to testing. Initially, cells were seeded in a 96 well plate about $10^5$cells/well, then let adhere overnight. The cells were then dosed with 12.5 μM, 25μM, 50μM, 100μM, 200μM, 400μM and 800μM tested compound respectively, and cell survival relative to control was assessed after 24 h.

The results are shown in Table 2. Compounds **1-8** are the derivatives of 4-Hydroxythiobenzamide; and their $IC_{50}$ values for HepG2 cells were over 1000μM except those of compounds **1**, **2** and **5**; among eight compounds, compound **2** showed the strongest toxicity to HepG2, and its $IC_{50}$ value was 89.3μM; as for LO2 cell lines, all eight compounds but **2** showed lower toxicities, and their $IC_{50}$ values were over 800μM. In contrast, the toxicities of the compounds to HepG2 were slightly stronger than those of LO2. As a whole, except for compound **2**, all seven compounds showed almost no toxicities to both LO2 and HepG2 cell lines.

For compounds **9-10** which are the derivatives of α-thioctic acid, their $IC_{50}$ values were above 900μM against both LO2 and HepG2 cell lines. Similarly, two compounds displayed slightly stronger toxicities to HepG2 cell lines than to LO2 cell lines. As we expected, compounds **11-15** also showed low toxicities, their $IC_{50}$ values to HepG2 were beyond 800μM and to LO2 over 300μM; and just like compounds **9** and **10**, they displayed a bit stronger toxicities to LO2 cell lines than to HepG2 cell lines also.

**Table 2 $IC_{50}$ values of all the compounds**

| Compound | $IC_{50}$(μM) | | Compound | $IC_{50}$(μM) | |
|---|---|---|---|---|---|
| | HepG2 | LO2 | | HepG2 | LO2 |
| **1** | 227.6 | 3614.9 | **9** | 965.8 | 1792.1 |
| **2** | 89.3 | 221.0 | **10** | 916.8 | 2541.4 |
| **3** | 1080.2 | 1480.7 | **11** | 1118.6 | 1042.7 |
| **4** | 1683.1 | >800 | **12** | 922.7 | 889.3 |
| **5** | 410.0 | >800 | **13** | 1277.0 | 543.8 |
| **6** | 1058.1 | >800 | **14** | 1076.7 | 536.5 |
| **7** | 1058.1 | 1091.1 | **15** | 3294.4 | 337.2 |
| **8** | 1073.4 | 1177.3 | | | |

In summary, the toxicities of the series compounds are low to both normal liver cells LO2 and tumor cells HepG2, so that these compounds do not harm liver severely in the range of tested dose.

### 2.4. Toxicity of zebrafish embryos

#### 2.4.1 Compound-induced mortality and hatchability of zebrafish

To further evaluate the toxicities of the compounds, we chose zebrafish embryos as test subjects; and in order to get the information as comprehensive as about the toxicities on the development, the hatchability and the embryos alive were measured at 24, 48, 72, 96 and 120 hpf in the presence of tested compounds, respectively. As shown in Fig. 2, DMF as solvent showed toxicity to zebrafish embryo in some degree. For every tested compound, the rate of embryo alive had slight changes in the period of 120hpf, and the percents of embryos alive decreased gradually with the concentrations increasing; clearly it was dose-dependent. For compound **2**, there was almost no change in the mortality after treated embryo with 5μM and 10μM, and the percents of embryos alive were about 60%. Similarly, compound **9** also decreased the percent of embryos alive at the same concentration compared to the control group; the percents were 51% and 41% after 120 hpf when its concentrations were 10 and 20 μM respectively. In contrast, compound **9** has slightly stronger toxicity to zebrafish embryos than compound **2**. As for compound **15**, its harmful effect on embryos was in the middle of compound **2** and compound **9** (SI-Fig. 4-a) under the same condition.

The normal zebrafish embryos had a hatching period from 48 to 72 hpf. In the process of hatching, some compounds may harm severely embryo development because they inhibit key enzymes which promote embryo to develop. The hatching rate of embryos is inversely proportional to the toxicity of compound. Seen from Fig. 3, for compounds **2** and **9**, the hatching rates of embryos were in accord with the alive embryos percents; at the low concentration (0.5 and 1.0μM), the hatching rate was no significant change compared with that of the control (DMF) group, while in 5.0, 10 and 20 μM-treated groups, their hatchability of the embryos decreased steeply with the concentration increasing. But for compound **15**, when the concentration was not up to 5μM until it showed inhibitory effects on the embryos to hatch (SI-Fig. 4-b). This demonstrates these compounds have toxicities to zebrafish embryos on the development in other aspect.

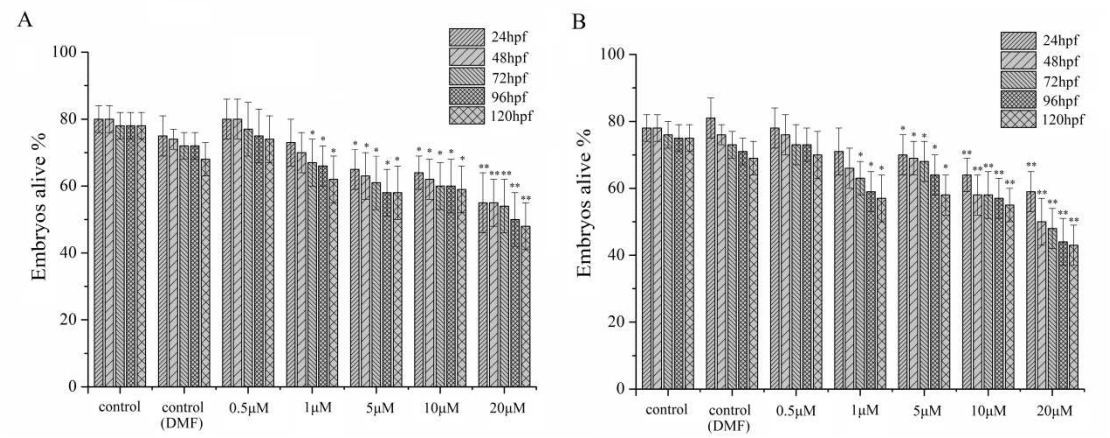



**Fig. 2** Effects of the compounds on zebrafish embryos alive. A: for compound **2**. B: for compound **9**. *P＜0.05 and **P＜0.01 compared to control (DMF). Data shown represents three independent experiments. Values represent the mean ± SD of three replicates. DMF was used as a solvent control and had no effect on experiment of zebrafish in the dilution used (1:1000) compared with control.

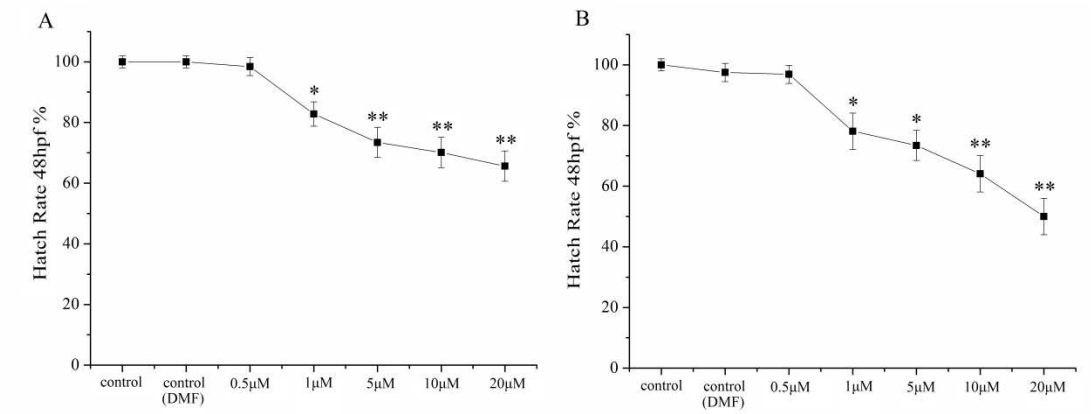



**Fig. 3** Hatch rate of compounds at 48 hpf (n=100). A: for compound **2**. B: for compound **9**. *P＜0.05 and **P＜0.01 compared to control (DMF). Data shown represents three independent experiments. Values represent the mean ± SD of three replicates. DMF was used as a solvent control and had no effect on experiment of zebrafish in the dilution used (1:1000) compared with control.

### 2.4.2 Effect of compound on zebrafish embryonic spontaneous movement

To evaluate the toxicities of the compounds on the nerves of zebrafish, the embryonic spontaneous movement frequency (1min) was measured at 24 hpf. As shown in Fig. 4, as a whole, at low concentration (0.5μM), the tested compounds did not show significantly toxicity compared to the control (DMF); but at higher concentration, the frequencies reduced with the compound concentration increasing. For compound **2**, the spontaneous movement frequencies of 1.0, 5.0, 10 and 20 μM –treated group were about 11.0 times and lower than that of the control group obviously (15.0 times). As for compound **9**, it did not show toxicity till to 1.0 μM;

however, the spontaneous movement frequencies of 5.0, 10 and 20 μM-treated groups were lower than that of the control group (15.9 times), and they were 14.1, 13.5 and 12.2 times, respectively. This provided the evidence to prove the high toxicity of higher doses of compound **9**. Therefore, we draw a conclusion that the compounds have embryonic toxicity in another aspect.

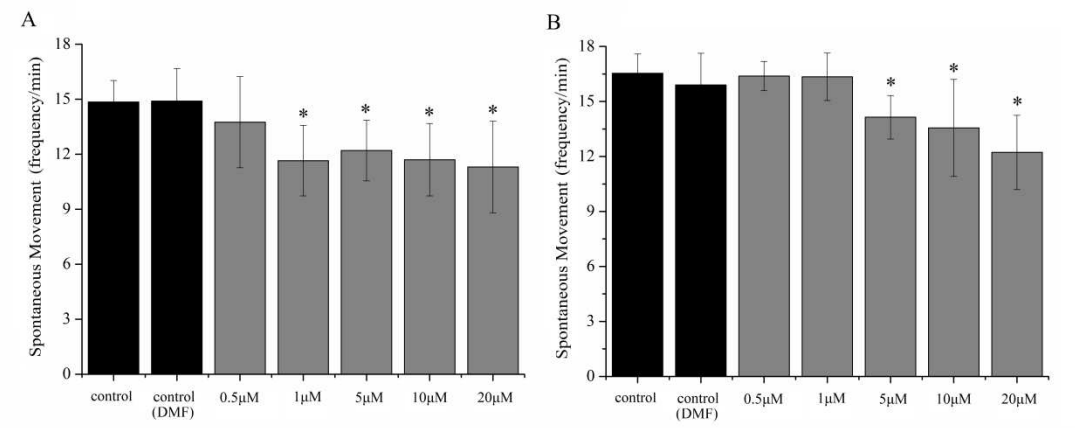



**Fig. 4** Effects of the compounds on zebrafish spontaneous movement at 24 hpf (n=20);*P＜0.05 compared with control (DMF). Data shown represents three independent experiments. Values represent the mean ± SD of three replicates. DMF was used as a solvent control and had no effect on experiment of zebrafish in the dilution used (1:1000) compared with control.

### 2.4.3 Compound-induced malformation of embryos

The zebrafish were exposed to 0.5 μM tested compound from 24 to 96 hpf and the malformation were observed at 96 hpf. We found at low concentration (0.5μM), the tested compounds did not make the embryos malformed compared to the control (DMF); but when their concentrations rose up, every compound displayed different toxicity to embryos. Compound **2** induced remarkably zebrafish embryos malformation. The malformation rate increased with the concentration rising, which shows that the malformation rate of zebrafish is dependent on concentration. When its concentration was up to 20μM, the malformation was approximately 5% (Fig. 5A). For compounds **9** and **15**, they also led to malformation of zebrafish, but their rates of malformation only 3 and 2% at most in the range of 0.5-20 μM (Fig. 5B, SI-Fig. 4-c). Several malformation patterns (including pericardial edema, vitelline cyst and bent spine) were observed (Fig. 6). These observations showed that pericardial edema and vitelline cyst were the typical malformations in the embryos induced by compound **2**.

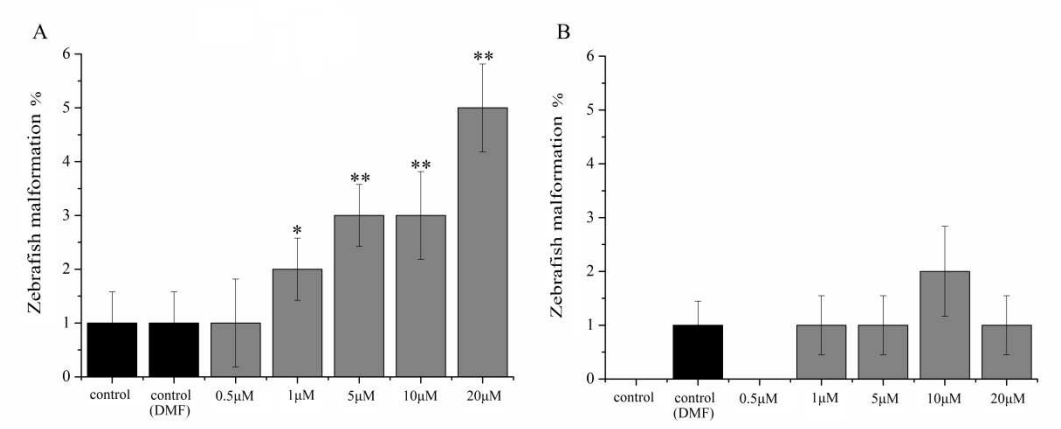



**Fig. 5** Malformation rate of zebrafish embryos exposed to compounds **2** and **9** (n=100). A: for compound **2**. B: for compound **9**.*P＜0.05 and **P＜0.01 compared to control (DMF). Data shown represents three independent experiments. Values represent the mean ± SD of three replicates. Control (DMF): DMF was used as a solvent control and had no effect on experiment of zebrafish in the dilution used (1:1000) compared with control.

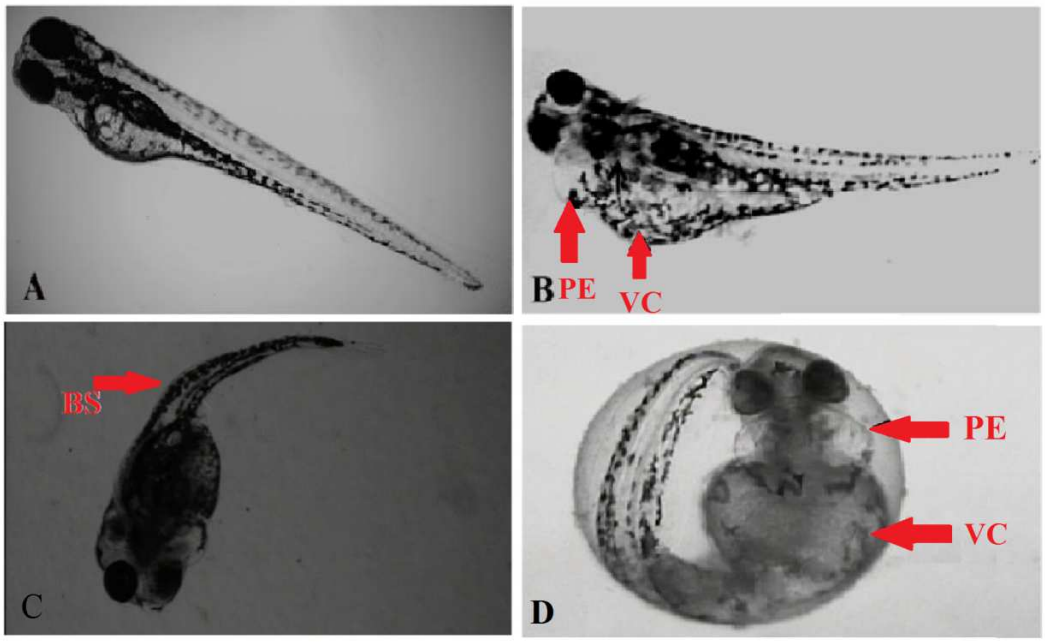



**Fig. 6** Malformation of zebrafish embryos exposed to 0.5 μM compound **2** (A) Normal larvae and (B-D) abnormal larvae. Malformations are indicated by red arrows. PE, pericardial edema; VC, vitelline cyst; BS, bent spine

## 2.5 Effects of compounds on zebrafish larval locomotor activity

The larval locomotor activities of the zebrafish during a 5 min period were recorded at 144 hpf, to determine whether complexes **2** and **9** exposure had a

persistent effect on larval behavior. In the visible light test, the total swimming distances decreased concentration-dependently. Seen from Fig. 7, compared to the controls (DMF), complex **2** and complex **9** at 10 μM treatments caused a significant decrease in the total swimming distance, while at the low concentration (0.5 and 1.0μM), the total distance have no significant differences with the control group. In contrast, compound **9** has stronger effect to the larval locomotor activities of the zebrafish than compound **2**. This trend is similar to embryo of the zebrafish for both compounds.

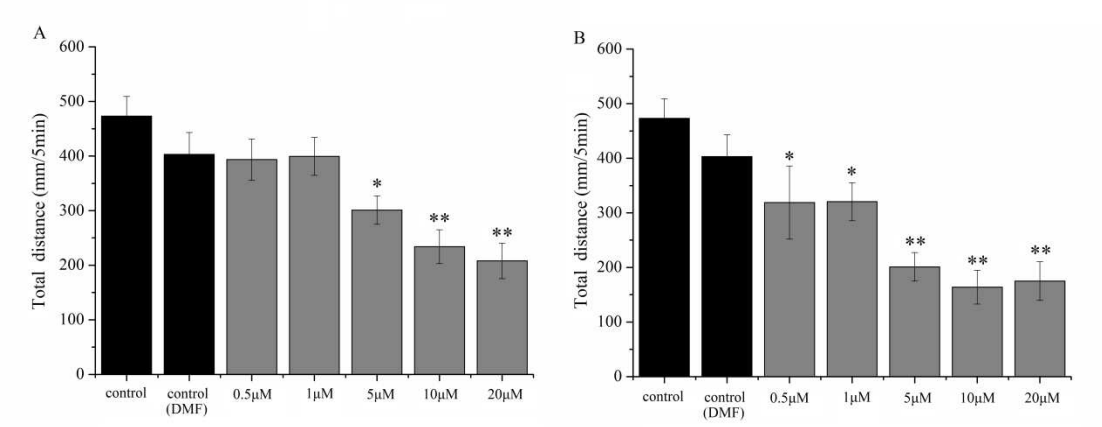



**Fig. 7** Effects of the compounds on zebrafish total distance of movement. A: for compound **2**. B: for compound 9.*P＜0.05 ** P＜0.01 compared with control (DMF). Data shown represents three independent experiments. Values represent the mean ± SD of three replicates. Control (DMF): DMF was used as a solvent control and had no effect on experiment of zebrafish in the dilution used (1:1000) compared with control.

Therefore, on the basis of testing data, we draw a conclusion that the compounds have toxicities to the zebrafish embryo and the larval, especially their concentrations are higher. The compounds will not be used at higher concentration, and not fit for the female in pregnancy even at low concentration.

## 2.6. Anti-inflammatory activities

### 2.6.1 Cell viability

In the process of anti-inflammatory test, we chose to use macrophages RAW264.7. In order to obtain more accurate results of anti-inflammatory experiments, eliminate the effects of cell death which the tested compounds give rise to on the results as can as possible, macrophages were exposed for 24 h to the compounds (10, 50, and 100μM) in the presence or absence of LPS (1μg $mL^{-1}$), then the cell viability was measured using MTT method. The results demonstrate compound **9** and compound **15**

had much lower toxicities to macrophages and did not kill them at all (Fig. 8), and for the other tested compounds (compounds **1**, **2** and **12**), macrophages viability rates was up to 70–110%, this suggests the compounds at the range of 10-100μM are acceptable and effective in anti-inflammatory tests.

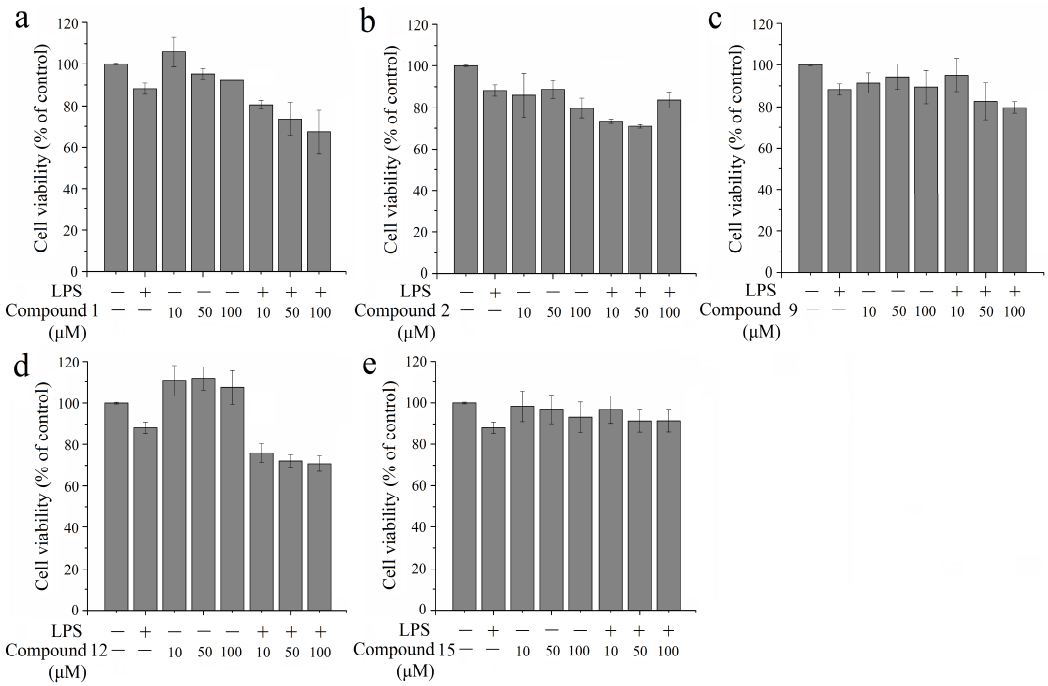



**Fig. 8** Effects of the compounds on cell viability. Measurement in the media of murine RAW264.7 macrophages exposed for 24 h to 10, 50 and 100 μM of each compound in the presence or absence of 1 μg $mL^{-1}$ LPS. a for compound **1**, b for **2**, c for **9**,d for **12**,e for **15**. Each bar represents the mean ± SD of four independent experiments.

### 2.6.2 Effect of the compounds on LPS-mediated nitrite production

NO is one of the inflammatory mediators. Excessive NO up-regulates inducible NO synthase (iNOS), which is a critical step in the initiation and propagation of the inflammatory response [40, 41]. NO is oxidated easily to nitrite by superoxide ions *in vivo*. $H_2S$ not only can quench peroxynitrite but also interacts chemically with NO to yield a novel nitrosothiol [42, 43]. The anti-inflammatory activity of $H_2S$ donors can be assessed by measurement of the amount of nitrite.

LPS can activate iNOS to increase NO level. In order to make the effect more pronounced, LPS-activated macrophages were used to evaluate the anti-inflammatory activities of the compounds. The testing results show tested compounds all decreased nitrite levels in the presence of LPS. As shown in Fig. 9, they attenuated LPS-induced nitrite production in a concentration-dependent manner. Among five compounds, compound **9** displayed the strongest activity to inhibit iNOS; compared with the control, it inhibited approximately 60% nitrite produce at 50μM. From the $H_2S$

releasing curves, we can see that compound **9** released $H_2S$ most among five compounds. Maybe, this is one of the reasons why compound **9** showed better anti-inflammatory activity than the others.

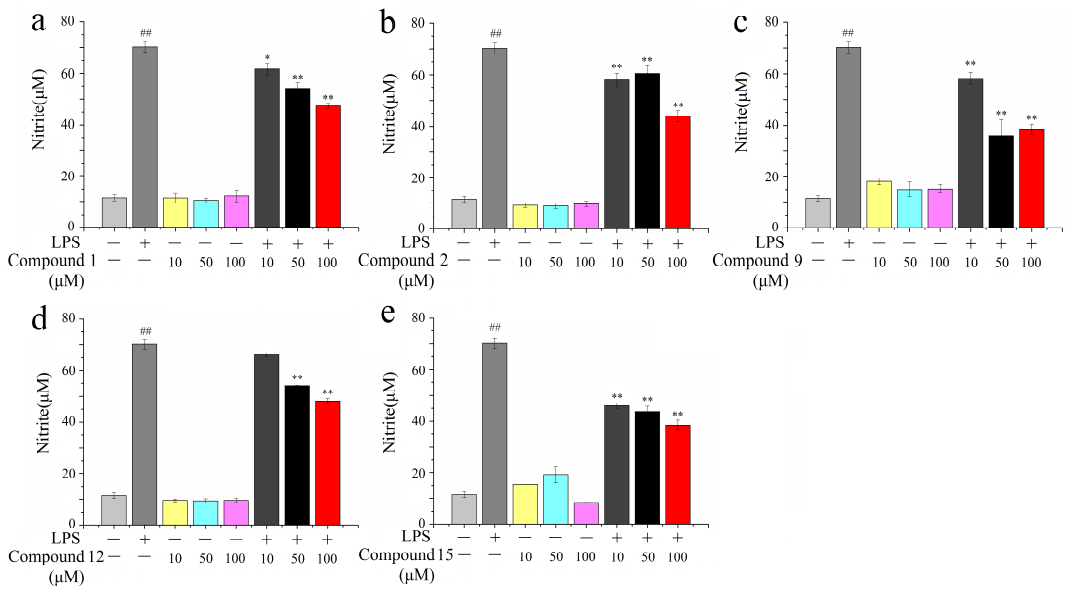



**Fig.9** Effect of the compounds on nitrite. Measurement in the media of murine RAW 264.7 macrophages exposed to 10, 50 and 100 μM of each compound for 24 h. a for compound**1**, b for **2**, c for **9**, d for **12**, e for **15**. Each bar represents the mean ± SD of four independent experiments. # $p < 0.05$, ##$p < 0.01$ vs. untreated cells. *$p < 0.05$, **$p < 0.01$, vs. LPS-treated cells

### 2.6.3 Effect of the compounds on LPS-mediated TNF-α and IL-10

TNF-α is a tumor necrosis factor, and it promotes T cells to produce a variety of inflammatory factors, thereby promoting the occurrence of inflammatory response. Studies have found $H_2S$ significantly can reduce LPS-induced neutrophils in the tissues and organs (liver and lung), decrease the level of TNF-α and increase IL-10 levels in plasma, thereby inhibiting inflammatory mediators [8]. Therefore, the level of TNF-α in cell was used as an important index to assess anti-inflammatory activity of $H_2S$ donors.

For this reason, we measured the levels of TNF-α in LPS-induced macrophages RAW264.7. As shown in Fig.10, all tested compounds significantly reduced levels of TNF-α. Among them, compounds **12** and **15** displayed stronger activities than compound **9**, the levels of TNF-α decreased to 5-20% of the control when the cells treated with both compounds at 100μM; in contrast, compound **9** only reached approximately 50% under the same condition. We know compound **9** released more $H_2S$ than compounds **12** and **15** at 40μM, why its activity is lower than the two compounds? In fact, there are many factors to impair the effects of action for the compounds. Compound **9** has longer $H_2S$-releasing half-life than compounds **12** and

**15**, which means compound **9** takes effects more slowly than both of them; possibly, the concentrations were up to 100µM, the effects were more distinct. In addition, TNF-α possibly has a different sensibility to all kinds $H_2S$ donors. As a whole, the compounds reduce TNF-α level in dose-dependent manner. This further shows $H_2S$ plays an important role in anti-inflammatory process.

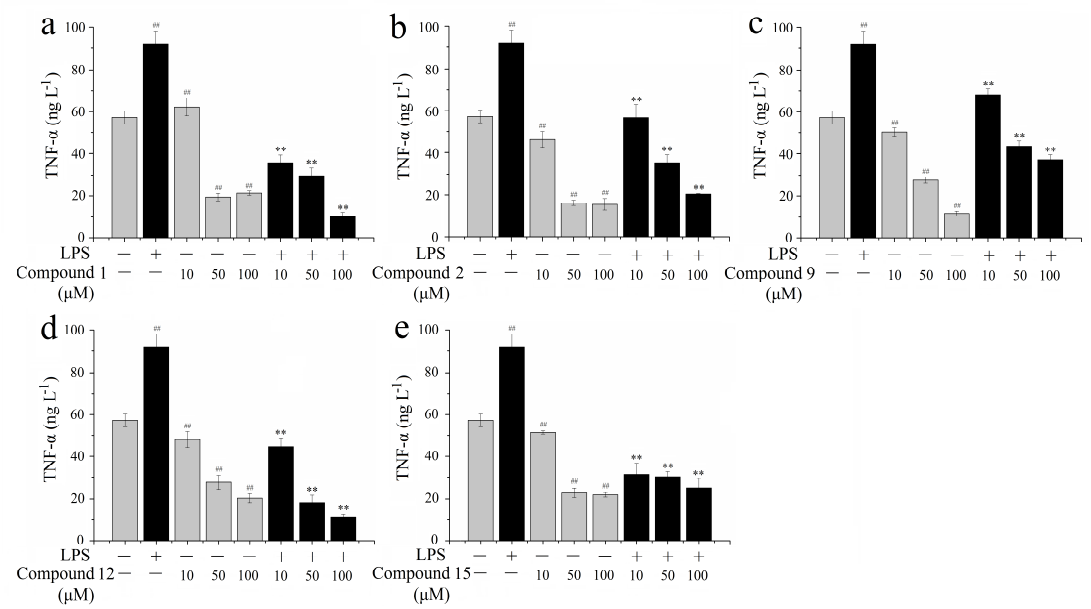



**Fig.10** Effect of the compounds on TNF-α. Measurement in the media of murine RAW 264.7 macrophages exposed to 10, 50 and 100 µM of each compound for 24 h. a for compound **1**, b for **2**, c for **9**, d for **12**, e for **15**. Each bar represents the mean ± SD of four independent experiments. # $p < 0.05$, ##$p < 0.01$ vs. untreated cells; *$p < 0.05$, **$p < 0.01$, vs. LPS-treated cells

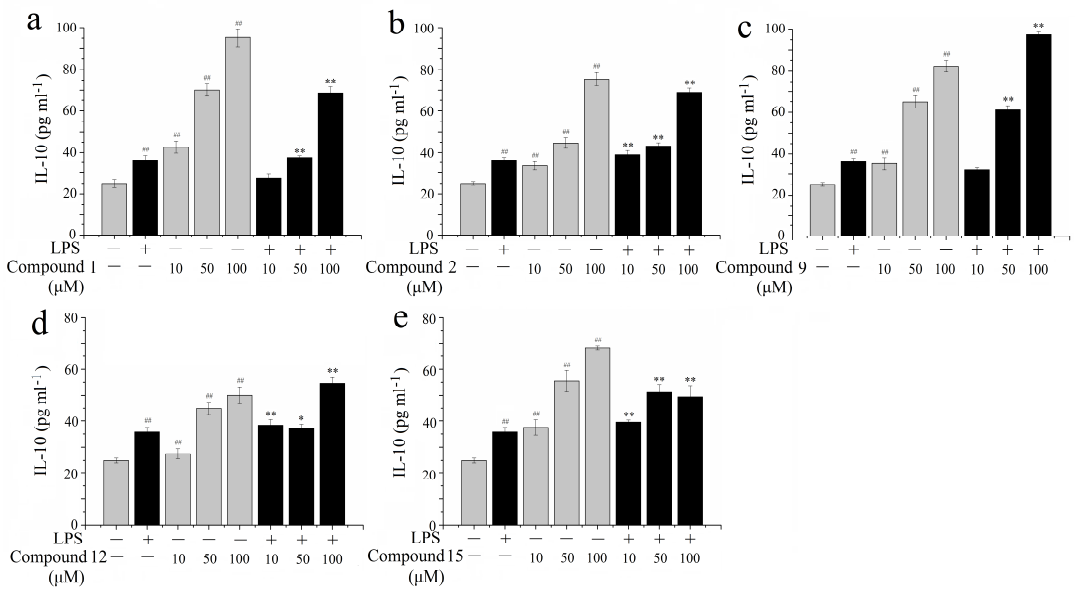



**Fig.11** Effect of the compounds on interleukin-10 (IL-10). Measurement in the media of murine RAW 264.7 macrophages exposed to 10, 50 and 100 µM of each compound for 24 h. a for

compound **1**, b for **2**, c for **9**, d for **12**,e for **15**. Each bar represents the mean ± SD of four independent experiments. # $p < 0.05$, ## $p < 0.01$ vs. untreated cells. *$p < 0.05$, **$p < 0.01$, vs. LPS-treated cells

Like TNF-α, IL-10 also regulates the inflammatory response; however, it is an anti-inflammatory factor. The level of IL-10 in cell can reflect the anti-inflammatory activity of compound. We found the tested compounds all significantly increased the level of IL-10 in both the presence and absence of LPS, as shown in Fig. 11. Among the compounds, compound **9** displayed stronger activity to increase IL-10 level than the others. The levels of IL-10 in cells treated with compound **9** at 100μM are as 3 times as the controls of blank and with LPS. We infer that more $H_2S$ released from compound **9** maybe is one of the reasons why it showed better activity to increase the level of IL-10.

### 2.6.4 Effects of the compounds on Heme-oxygenase-1(HO-1)

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the heme degradation processes, and it catalyzes heme to transform into biliverdin, iron ion and CO. CO and biliverdin were shown to mediate many beneficial effects, including modulation of inflammation. And several studies have demonstrated HO-1 has anti-inflammatory property in itself *in vivo* [44, 45]. The level of HO-1 expression reflects anti-inflammatory ability of the cell in some degree. Recently, the scientists found that $H_2S$ donor, like NaHS, enhanced hypoxia-induced HO-1 expression in smooth muscle cells of small pulmonary arteries [46].

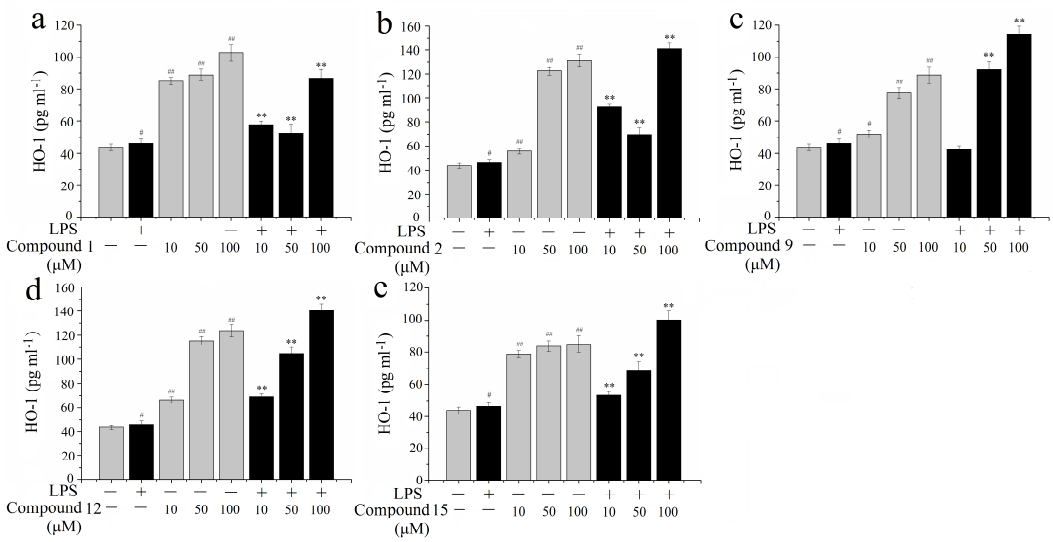


**Fig.12** Effect of the compounds on Heme-oxygenase-1(HO-1). Measurement in the media of murine RAW 264.7 macrophages exposed for 24 h to 10, 50 and 100 μM of each compound after 1 μg $mL^{-1}$ LPS administration for 4h. a for compound **1**, b for **2**, c for **9**, d for **12**, e for **15**. Each bar represents the mean ± SD of four independent experiments. # $p < 0.05$, ##$p < 0.01$, vs. untreated cells; *$p < 0.05$, **$p < 0.01$, vs. LPS-treated cells.

In order to evaluate the effects of the compounds on HO-1, we treated RAW264.7 macrophages with the compounds at set concentrations, and examined HO-1 expression level. As shown in Fig.12, the tested compounds all increased HO-1 levels in a concentration dependent manner. When the tested compounds arrived at 100μM, the level of HO-1 in cells rose up to 2 times as high as the controls; for compounds **2** and **12**, the expression levels were beyond 3 times as much as the controls. This suggests the more $H_2S$ released from the compound, the more HO-1 expression.

### 2.6.5 Effects of the compounds on COX-2

The inducible cyclooxygenase enzymes COX-2 is key enzyme which catalyzing the biotransformation of arachidonic acid to the prostaglandin E, the latter is one of inflammatory mediators. Traditional NSAIDs produce anti-inflammatory effect by inhibiting the activity of COX-2. Because the compounds contain anti-inflammatory agent structural fragments, and link them with ester bond, these compounds must be had anti-COX-2 activities. In order to test these compounds whether have better activities against COX-2, we examined the effects of the compounds on COX-2 expression in RAW264.7 macrophages. As shown in Fig.13, all tested compounds displayed strong activities to COX-2. Among the tested compounds, compound **12** displayed stronger activity to decrease COX-2 level than the others; when it was 100 μM, COX-2 level decreased to approximately 40% of the controls.

## 2.7 Conclusion

To avoid the unnecessary loss, early failing and early eliminating is the best way to seek a new drug. In this paper, to establish the drug-like properties of all the $H_2S$ donors, we evaluated all compounds from several aspects including $H_2S$-releasing ability, toxicity and anti-inflammatory activities.

As a result, all the compounds were fast $H_2S$ releasing agents, their half-lives were in range of 0–20 min. Among them, compounds **1** and **2** had higher toxicity to HepG2 cells ($IC_{50}$ 89.3 μM for compound **2**), the others displayed much lower toxicities to both LO2 cells and HepG2 cells, their $IC_{50}$ values were above 800μM and 300μM respectively. After zebrafish embryos were exposed to the tested compounds, the survival rate, hatchability and spontaneous movement all decreased with the concentration increasing; the survival rate and hatchability decreased in dose

dependent manner. Moreover, tested compounds caused a dose-dependent malformation of zebrafish. On the other hand, the tested compounds had strong anti-inflammatory activities, compound **9** displayed better anti-inflammatory activity than the others. Under the same condition, compound **9** released more $H_2S$ than compounds **2** and **15**, which is corresponding with their activities.

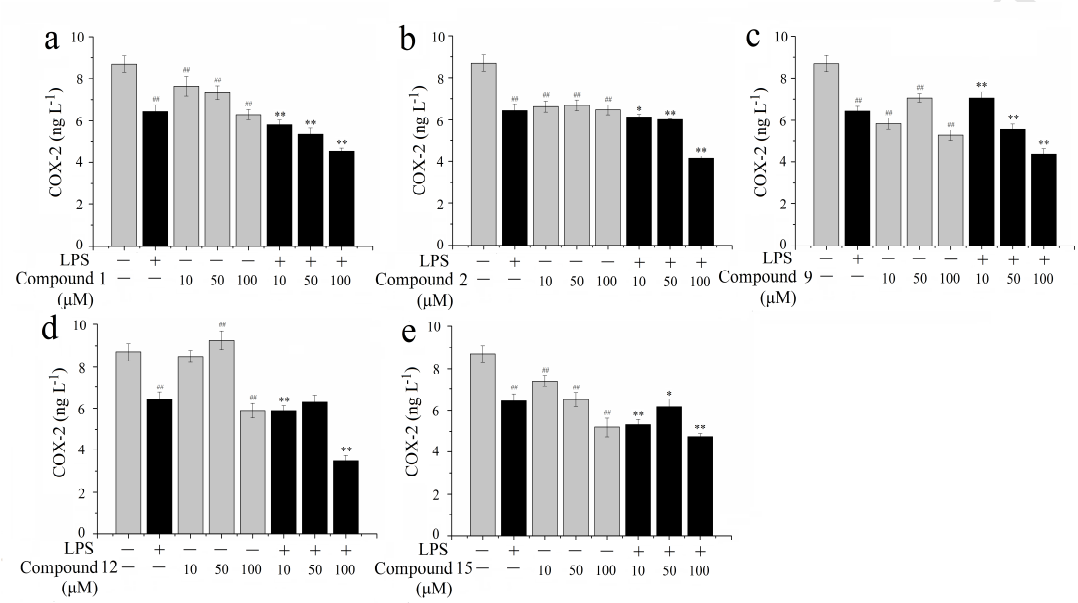



**Fig.13** Effect of the compounds on cyclooxygenase-2 (COX-2). Measurement in the media of murine RAW 264.7 macrophages exposed for 24 h to 10, 50 and 100 μM of each compound after 1 μg $mL^{-1}$ LPS administration for 4h. a for compound **1**, b for **2**, c for **9**, d for **12**, e for **15**. Each bar represents the mean ± SD of four independent experiments. # $p < 0.05$, ## $p < 0.01$, vs. untreated cells. * $p < 0.05$, **$p < 0.01$, vs. LPS-treated cells.

The compounds not only decreased PGE level (inflammatory mediator) by inhibiting COX-2 activity, but decreased NO level (another inflammatory mediator) through inhibiting iNOS. Meanwhile compound **9** also took effects by TNF-α and IL-10 pathway which regulated by $H_2S$. This demonstrates that the anti-inflammatory action of compound is a result of synergistic effect involving in several pathways.

Furthermore, the toxicities of these compounds are very low, and $H_2S$ released from the compounds played a role during the anti-inflammation, such as inhibiting iNOS, decreasing TNF-α levels and increasing HO-1 and IL-10 express levels, and so on. All suggest the compounds maybe are potential anti-inflammatory drugs for substitution for classical NSAIDs.

## 3. Experimental

### 3.1 Reagents and instruments

All reactions were carried under nitrogen atmosphere. Solvents for reactions were degassed and distilled from the proper drying agents. Column chromatography was carried out using 200–300 mesh silica gel; RAW264.7macrophages lines, HepG2 cell lines and LO2 cell lines from cell resources Center for Shanghai Life Science Institute of Chinese Academy of Sciences (China), zebrafish from Center for Shanghai Life Science Institute of Chinese Academy of Sciences (China).

IR spectra were recorded on a Nicolet NEXUS 360 spectrophotometer, and NMR spectra on a BrukerAM-400 MHz spectrometer. A Lambda 25 UV–Visible spectrophotometer and a Maxis-4G TOF Mass spectrometer (ESI) were used.

### 3.2 Experimental protocol

#### 3.2.1 $H_2S$ Measurement

A 5mM solution of $Na_2S$ in sodium phosphate buffer (20 mM, pH 7.4) was prepared ($Na_2S{\cdot}9H_2O$, 120.20 mg in 100 mL volumetric flask) and used as the stock solution. Aliquots of 50, 100, 200, 400, 600, 800, 1000, 1500 µL of the $Na_2S$ stock solution were added into a 50 mL volumetric flask and dissolved in sodium phosphate buffer to obtain the standard solutions in 5, 10, 20, 40, 60, 80, 100, 150 µM, respectively. 1 ml aliquot of the respective solution was reacted with the methylene blue ($MB^+$) cocktail: 30mM $FeCl_3$ (200 µL) in 1.2 M HCl, 20 mM of N, N-dimethyl-1, 4-phenylenediamine sulfate (200 µL) in 7.2 M HCl, 1%w/v of $Zn(OAc)_2$ (100 µL) in $H_2O$ at room temperature for at least 15 min (each reaction was performed in triplicate). The absorbance of methylene blue was measured at $\lambda_{max}$=670 nm in UV-Vis spectrophotometer (Lambda 25). The $Na_2S$ calibration curve was obtained.

The reaction was initiated by adding 75 µL of stock solution of the donor (40 mM, in THF) into pH 7.4 phosphate buffer (30 mL) containing accelerator for TECP (1.0 mM) or L-Cysteine (1.0 mM). Then 2.0 mL of reaction aliquots were periodically taken and transferred to colorimetric cuvette containing zinc acetate (1% w/v, 200 µL) and N,N-dimethyl-1,4-phenylenediamine sulfate (20 mM, 400 µL) in 7.2 M HCl and ferric chloride (30 mM, 400 µL) in 1.2 M HCl. The absorbance (670 nm) of the resulted solution was determined 15 min thereafter using an UV−Vis spectrometer (Lambda 25). The $H_2S$ concentration of each sample was calculated against a calibration curve of $Na_2S$. The $H_2S$ releasing curve was obtained by plotting $H_2S$ concentration versus time.

#### 3.2.2 Cytotoxic assay

The LO2 cells and HepG2 cells were cultivated at 37 □, 10% $CO_2$, 100% humidity in RPMI1640 medium, enriched with glucose and supplemented with 10% fetal bovine serum, non-essential amino acids, antibiotics (penicillin/streptomycin) and antifungals. Typically, 0.08mmol of the compound was dissolved in 0.5 mL of DMSO, which after homogenization was injected over 10mL of culture medium with vigorous stirring at 55 °C. Growth inhibitory effect toward LO2 (or HepG2) cells line was determined by means of MTT colorimetric assay. The cells were seeded into 96-well plates at $6\times10^3$ cells/well and a certain volume of the above solution was added. Culture medium was added until completion of 0.2 mL and the final concentration of the compound in each well was 12.5–800 μM. Triplicate cultures were established for all compounds and for the control. The mixture was incubated at 37 °C in a humid atmosphere containing 10% $CO_2$ for 24h. Cell viability was quantified after 24h by the MTT method. Descriptive data were expressed as mean± standard deviation and values $IC_{50}$ were carried out using SPSS 20.0 software.

### 3.2.3 Zebrafish toxicity test

Adult zebrafish of the wild-type strain (AB) were raised and maintained at 28± 1 °C with a 14h light/10h dark photoperiod (lights on at 8:00) in a recirculation system. The fish water supplied to the system was filtered by reverse osmosis (pH6.5–7.5), and instant ocean salt was added to the water, to raise the conductivity to 450–500 μs/cm. The zebrafish were fed twice daily with decapsulated, freshly hatched brine shrimps (Brine Shrimp Direct, USA) according to the description of Zhou [47]. The zebrafish embryos were obtained from spawning adults in tanks overnight with a sex ratio of 1:1. The embryos were collected within 1h after the light was switched on and washed using standard zebrafish E3culture medium. The zebrafish use and handling protocol conformed to the Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Use and Care Committee (IAUCC) of the Lanzhou University. At 4 hour post-fertilization (hpf), the embryos were examined under a dissecting light microscope, and the specimens that had developed normally were selected for the further experiments according to the description of Kimmel [48].

The zebrafish embryos were exposed to compound for 24, 48, 72, 96, 120, 144 and 168 hpf and then assessed for toxicity. The toxicological end points were determined based on previous reports in the literature [49]. Each group consisted of 100 embryos randomly divided into three replicate groups. The embryos were kept in sterile 96-well plates with one embryo per well containing 200 μL of the solution. The

plates were covered with sealing film to prevent evaporation and the mortality of the zebrafish was recorded at 24, 48, 72, 96, 120, 144 and 168 hpf.

Normal embryos were exposed to control vehicle and complex from 4 to 24 hpf. Ten embryos were selected randomly from control and experimental groups. The zebrafish embryonic spontaneous movement (5min) was recorded using a stereoscopic dissecting microscope and Media Cruiser recording software (Canopus Corporation, Kobe, Japan). Data were analyzed using the Etho Vision XT10.0 software (Noldus Information Technology, Wageningen, Netherlands). When normal embryos were exposed to the control vehicle and compound from 4 to 72 hpf, the hatch rate was measured at 48 hpf. The normal embryos (4 hpf) were exposed to the control vehicle compound for 96 hpf, and then the malformation of the zebrafish were observed using a stereoscopic dissecting microscope.

### 3.2.4 Anti‑inflammatory actions assays

Murine RAW264.7 monocyte macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) with high-glucose from HyClone which is supplemented with 2mM glutamine, antibiotics (100 U/ml penicillin A and 100 U/ml streptomycin), and 10% heat-inactivated fetal bovine serum and maintained in a 37 °C humidified incubator containing 5% $CO_2$.

Macrophages were exposed for 24 h to LPS (1μg $mL^{-1}$) in the presence or absence of compound 1, 2, 9, 12 and 15 (10, 50 and 100 μM), cell viability, nitrite levels and TNF-α, HO-1, IL-10, and COX-2 protein expression were determined at the end of the incubation. In each experiment, compound **1**, **2**, **9**, **12** and **15** (10, 50 and 100 μM) were added to cells 24 h after incubation with LPS for 4 h.

### 3.2.5 Cell viability

Cell viability was determined using MTT method and it was previously reported [50].The assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of living, not dead cells, it has become a very popular technique for quantification of viable cells in culture. Briefly, after the cells were exposed for 24h in 96-well plate, MTT was added to each well (20μL per well) for 4h, after that discard the culture, and then DMSO was added to each well (100μL per well), the plate was shaken for 10 min to make sure all of blue formazan were dissolved in DMSO, which is calculated as the absorbance in 570nm on a microplate reader and expressed as a percentage of control.

### 3.2.6 Assay for nitrite levels

Nitrite levels were determined using the Griess method as previously described [51]. The measurement of this parameter is widely accepted as indicative of NO production. Briefly, the medium from treated cells was removed and placed into a 96-well plate (50μL per well). The Griess reagent Ⅰ and Ⅱ were added to each well to begin the reaction, the plate was shaken for 10 min and the absorbance read at 570 nm on a microplate reader. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite (0–100 μM in cell culture medium).

### 3.2.7 Bicinchoninic acid (BCA) Protein Assay

In order to exclude the influence of the total amount of protein on the determination of the following cytokine levels, protein quantification of each protein was carried out using BCA protein Assay. Briefly, samples were removed and placed into a 96-well plate (20 μL per well). BCA reagent A and reagent B were formulated as working solutions, which was added to each well (200 μL per well) and left to react for 0.5 h in 37 ℃. And then the plate was shaken for 10 min and the absorbance read at 562 nm on a microplate reader. The concentration of protein in each sample was calculated from a standard curve generated with standard solution.

### 3.2.8 Determination of four cytokines (TNF-*α*, HO-1, IL-10, and COX-2) levels

The level of four cytokines present in each sample was determined using a commercially available kit from Shanghai MLBIO Biotechnology Co.Ltd. The assay was performed according to the manufacturers' instructions. Briefly, cell were collected from 6-well plate immediately after lysed by RIPA Lysis Buffer and spun at 12,000×g for 10 min to remove any particulates. Each protein solution was measured by BCA Protein Assay Kit. The protein solution for concentration of 0.1mg $ml^{-1}$ was added to a 96-well plate precoated with affinity-purified polyclonal antibodies specific for the mouse of our cytokines. An enzyme-linked polyclonal antibody specific for the mouse of our cytokines was added to the wells and left to react for 0.5 h followed by a final wash to remove any unbound antibody-enzyme reagent. The intensity of the color detected at 450 nm was measured after addition of chromogen solution A, B and stop solution and was proportional to the amount of four cytokines produced. The four cytokines levels which in each sample was calculated from a standard curve generated with standard solution.

## 3.3 Syntheses

### 3.3.1 Preparation of compounds 1-8

Compound **1:** To the solution of (naproxen, 230mg 1.0mmol) in 8mL of acetonitrile, DMAP (18mg, 0.15mmol) and EDCI (190mg, 1.0mmol) were added with stirring at room temperature for 0.5h. To the reaction mixture 4-hydroxythiobenzamide (153 mg, 1.0 mmol) was added and stirred for 4 h at room temperature. After filtration, the filtrate was evaporated under reduced pressure to remove the solvent. The oily residue thus obtained was dissolved in trichloromethane; the organic layer was washed with brine, with NaCl 5%, and then dried on anhydrous $Na_2SO_4$, filtered and the solvent evaporated. The crude product was chromatographed on a silica gel ($CHCl_3$/$CH_3OH$ 30:1), and 148.8 mg pale yellow solid was obtained. Yield: 42.0%. IR (KBr, $cm^{-1}$): 3398 (w, $NH_2$), 3298 (w, $NH_2$), 1755 (v, COO), 1214 (s, COC), 1170 (v, C=S). $^1$H NMR (DMSO-$d_6$, TMS，ppm): δ 1.59 (d, J=7.2Hz, 3H, Ar-C-$CH_3$), 3.88 (s, 3H, O-$CH_3$), 4.25 (q, J=7.2Hz, 1H, Ar-CH), 7.09 (d, J=8.8Hz, 2H, Ar-H), 7.18 (dd, J=8.8Hz, 2.4Hz, 1H, Ar-H), 7.33 (s, 1H, Ar-H), 7.52 (d, J=8.4Hz, 1H, Ar-H), 7.85 (d, J=4.4Hz, 2H, Ar-H), 7.87 (s, 1H, Ar-H), 7.90 (d, J=8.4Hz, 2H, Ar-H), 9.53 (s, 1H, NH), 9.91 (s, 1H, NH). $^{13}$C NMR (DMSO-$d_6$, TMS, ppm): δ18.9, 45.0, 55.7, 106.3, 119.4, 121.5, 126.4, 126.7, 127.8, 129.0, 129.3, 129.7, 133.9, 135.6, 153.2, 157.8, 173.1. ESI-HRMS (m/z): calcd. for $C_{21}H_{19}NO_3SNa$ $[M+Na]^+$: 388.0983; found 388.0979.

The preparation and workup of compounds **2-8** were similar to compound **1**.

Compound **2:** a pale yellow solid 145.8 mg. Yield: 43.0%. IR (KBr, $cm^{-1}$): 3374 (w, $NH_2$), 3281 (w, $NH_2$), 1741 (v, COO), 1152 (v, C=S). $^1$H NMR (DMSO-$d_6$, TMS, ppm): δ 0.86 (d, J=6.4Hz, 6H, $C(CH_3)_2$), 1.50 (d, J=6.8Hz, 3H, Ar-C-$CH_3$), 1.83 (m, 1H, Bn-CH), 2.44 (d, J=7.2Hz, 2H, Ar-$CH_2$), 4.07 (q, J=7.2Hz, 1H, Ar-CH), 7.07 (d, J=8.4Hz, 2H, Ar-H), 7.17 (d, J=7.6Hz, 2H, Ar-H), 7.21 (d, J=8.0Hz, 2H, Ar-H), 7.91 (d, J=8.8Hz, 2H, Ar-H), 9.54 (s, 1H, NH), 9.92 (s, 1H, NH). $^{13}$C NMR (DMSO-$d_6$, TMS, ppm): δ19.2, 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. ESI-HRMS (m/z): calcd. for $C_{20}H_{23}NO_2SNa$ $[M+Na]^+$: 364.1347; found 364.1337.

Compound **3:** pale yellow solid 307.1 mg. Yield: 62.5%. IR (KBr, $cm^{-1}$): 3371 (w, $NH_2$), 3267 (w, $NH_2$), 1740 (v, COO), 1328 (m, S=O), 1168 (v, C=S). $^1$H NMR (DMSO-d6, TMS, ppm): δ 2.22 (d, J=24.0Hz, 3H, Cp-$CH_3$), 2.82 (d, J=12.0Hz, 3H, O=S-$CH_3$), 4.03 (s, 2H, COO-$CH_2$), 6.76 (m, 1H, C=C-H), 7.21 (m, 4H, Ar-H), 7.45 (s, 1H, Ar-H), 7.79 (m, 4H, Ar-H), 7.93 (m, 2H, Ar-H), 9.52 (s, 1H, NH), 9.89 (s, 1H, NH). $^{13}$C NMR (DMSO-$d_6$, TMS, ppm): δ10.9, 31.2, 43.6, 106.6, 106.8, 111.0, 121.6, 123.7, 123.8, 124.4, 129.3, 129.8, 130.4, 130.8, 131.6, 137.7, 138.9, 140.6, 146.9,

153.1, 169.2, 199.5. ESI-HRMS (m/z): calcd. for $C_{27}H_{23}FNO_3S_2$ $[M+H]^+$: 492.1103; found 492.1365.

Compound **4:** pale yellow solid 130.9 mg. Yield: 34.5%. IR (KBr, $cm^{-1}$): 3379 (w, $NH_2$), 3281 (w, $NH_2$), 1748 (v, COO), 1276 (s, C-F), 1163 (v, C=S). $^1H$ NMR ($CDCl_3$, TMS, ppm): δ 1.58 (d, J=8.0Hz, 3H, CH-$CH_3$), 4.23 (q, J=8.0Hz, 1H, Ar-CH), 7.15 (d, J=8.0Hz, 2H, Ar-H), 7.37 (m, 3H, Ar-H), 7.48 (m, 2H, Ar-H), 7.55 (m, 3H, Ar-H), 7.93 (d, 2H, J=8.0Hz, Ar-H), 9.53 (s, 1H, NH), 9.90 (s, 1H, NH). $^{13}C$ NMR (DMSO-$d_6$, TMS, ppm): δ19.1, 44.7, 115.9, 116.2, 121.7, 124.8, 128.6, 129.3, 129.4, 129.5, 131.7, 135.8, 137.7, 142.6, 153.7, 158.3, 163.5, 173.1, 199.6. ESI-HRMS (m/z): calcd. for $C_{23}H_{20}NO_3S$ $[M+H]^+$: 380.1042; found 380.1131.

Compound **5:** pale yellow solid 51.5 mg. Yield: 18.2%. IR (KBr, $cm^{-1}$): 3359 (w, $NH_2$), 3279 (w, $NH_2$), 1729 (v, COO), 1630 (v, C=C), 1139 (v, C=S). $^1H$ NMR (DMSO-d6, TMS, ppm): δ 6.91 (d, J=16Hz, 1H, C=O-CH), 7.27 (d, J=8.8Hz, 2H, Ar-H), 7.48 (d, J=4.0Hz, 3H, Ar-H), 7.82 (d, J=4,0Hz, 2H, Ar-H), 7.89 (d, J=7.2Hz, 1H, Ar-CH), 7.96 (d, J=8.4Hz, 2H, Ar-H), 9.54 (s, 1H, NH), 9.90 (s, 1H, NH). $^{13}C$ NMR (DMSO-$d_6$, TMS, ppm): δ117.4, 121.8, 129.2, 129.3, 129.5, 131.5, 134.3, 137.5, 147.3, 153.2, 165.2, 199.5. ESI-HRMS (m/z): calcd. for $C_{16}H_{13}NO_2SNa$ $[M+Na]^+$: 306.0565; found 306.0556.

Compound **6**: pale yellow solid 73.4 mg. Yield: 25.2%. IR (KBr, $cm^{-1}$): 3321 (w, $NH_2$), 3275 (w, $NH_2$), 1767 (v, COO), 1171 (v, C=S). $^1H$ NMR (DMSO-$d_6$, TMS, ppm): δ 7.36 (d, J=8.0Hz, 2H, Ar-H), 7.70 (d, J=8.0Hz, 2H, Ar-H), 7.99 (d, J=8.0Hz, 2H, Ar-H), 8.15 (d, J=8Hz, 2H, Ar-H), 9.57 (s, 1H, NH), 9.94 (s, 1H, NH). $^{13}C$ NMR (DMSO-d6, TMS, ppm): δ121.0, 126.7, 127.2, 129.9, 131.7, 132.0, 133.8, 135.1, 147.1, 153.5, 167.2, 199.6. ESI-HRMS (m/z): calcd. for $C_{14}H_{10}ClNO_2SNa$ $[M+Na]^+$: 314.0018; found 314.0044.

Compound **7:** pale yellow solid 103.4 mg. Yield: 35.4%. IR (KBr, $cm^{-1}$): 3387 (w, $NH_2$), 3281 (w, $NH_2$), 1740 (v, COO), 1168 (v, C=S). $^1H$ NMR (DMSO-$d_6$, TMS, ppm): δ 7.38 (d, J=8.0Hz, 2H, Ar-H), 7.57 (m, 1H, Ar-H), 7.70 (d, J=8.0Hz, 2H, Ar-H), 8.00 (d, J=8Hz, 2H, Ar-H), 8.12 (d, J=8Hz, 1H, Ar-H), 9.58 (s, 1H, NH), 9.95 (s, 1H, NH). $^{13}C$ NMR (DMSO-d6, TMS, ppm): δ120.8, 127.0, 128.3, 128.5, 132.1, 139.3, 146.9, 153.4, 165.7, 199.5. ESI-HRMS (m/z): calcd. for $C_{14}H_{10}ClNO_2SNa$ $[M+Na]^+$: 314.0018; found 314.0046.

Compound **8:** pale yellow oil 137.2 mg. Yield: 36.2%. IR (KBr, $cm^{-1}$): 3388 (w, $NH_2$), 3284 (w, $NH_2$), 1751 (v, COO), 1660 (s, CO), 1181 (v, C=S). $^1H$ NMR ($CDCl_3$, TMS, ppm): δ 1.57 (d, J=8.0Hz, 3H, CH-$CH_3$), 4.30 (q, J=8.0Hz, 1H, Ar-CH), 7.12 (d, J=8.0Hz, 2H, Ar-H), 7.57 (m, 3H, Ar-H), 7.69 (m, 2H, Ar-H), 7.77 (m, 4H, Ar-H), 7.94 (d, 2H, J=12.0Hz, Ar-H), 9.54 (s, 1H, NH), 9.92 (s, 1H, NH). $^{13}C$ NMR (DMSO-$d_6$, TMS, ppm): δ19.2, 44.9, 121.6, 129.3, 129.5, 129.8, 130.3, 132.6, 133.5, 137.6, 137.9, 138.1, 141.2, 153.3, 154.5, 156.1, 163.8, 172.9, 199.6. ESI-HRMS (m/z): calcd. for $C_{23}H_{19}NO_3SNa$ $[M+Na]^+$: 412.0983; found 412.1045.

### 3.3.2 Preparation of compounds 9-10

Compound **9**: A solution of α-thioctic acid (1mmol), DCC (1.1mmol) and paracetamol (150 mg, 1.0mmol) (1mmol) in dichloromethane (10 ml) with catalytic amount of DMAP was stirred mechanically at room temperature until esterification was complete. The N, N-dicyclohexylurea was filtered off and the filtrate was washed with water (3×50 ml), 5% acetic acid (3×50 ml) again with water (3×50 ml) and then dried over anhydrous sodium sulphate. The solvent was removed under reduced pressure to give the ester which was chromatographed over a column of silica gel using (trichloromethane /methyl alcohol 30:1) as eluent gave 103.0 mg pale yellow solid. Yield: 30.4%. IR (KBr, $cm^{-1}$): 1751 (v, COO), 1663 (v, C=O), 604 (w, C-S). $^1H$ NMR (DMSO-$d_6$, TMS, ppm): δ 1.55 (m, 2H, Dithiolane-C-$CH_2$), 1.76 (m, 4H, Dithiolane-$CH_2$, O=C-C-$CH_2$), 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=8.8Hz, 2H, Ar-H), 7.47 (d, J=8.8Hz, 2H, Ar-H), 7.53 (s, 1H, NH). $^{13}C$ NMR (DMSO-$d_6$, TMS, ppm): δ24.4, 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. ESI-HRMS (m/z): calcd. for $C_{16}H_{21}NO_3S_2Na$ $[M+Na]^+$: 362.0861; found 362.0857.

Compound **10:** pale yellow solid 127.4 mg. Yield: 36.4%. IR (KBr, $cm^{-1}$): 1748 (v, COO), 1705 (v, C=O), 625 (w, C-S). $^1H$ NMR ($CDCl_3$, TMS, ppm): δ 1.53 (m, 2H, Dithiolane-C-$CH_2$), 1.78 (m, 4H, Dithiolane-$CH_2$, O=C-C-$CH_2$), 1.92 (m, 1H, 5H-Dithiolane), 2.47 (m, 1H, 3H-Dithiolane), 2.63 (t, J=7.6Hz, 2H, 4H-Dithiolane), 3.12 (m, 2H, O=C-$CH_2$), 3.60 (m, 1H, 5H-Dithiolane), 6.40 (d, J=9.6Hz, 1H, 3H-Coumarin), 7.05 (dd, J=8.8Hz 2.0Hz, 1H, Ar-H), 7.11 (s, 1H, Ar-H), 7.50 (d, J=8.4Hz, 1H, Ar-H), 7.70 (d, J=9.6Hz, 1H, 4H-Coumarin). ESI-HRMS (m/z): calcd. for $C_{17}H_{18}O_4S_2Na$ $[M+Na]^+$: 373.0544; found 373.0587.

### 3.3.3 Preparation of compounds 11-15

Compound **11-15** were prepared using a procedure similar to that for compound **1** except ADTOH (226mg, 1.0mmol) were used instead of 4-hydroxythiobenzamide. Chromatography on silica gel with ($CHCl_3$/$CH_3OH$ 50:1) as eluent gave the product.

Compound **11**, brownish red solid 98.4 mg. Yield: 22.5%. IR (KBr, $cm^{-1}$): 1744 (s, COO), 1217 (s, Ar-O-C), 1165 (v, C=S). $^1H$ NMR (DMSO-$d_6$, TMS, ppm): δ 1.73 (d, J=8.0Hz, 3H, Ar-C-$CH_3$), 3.95 (s, 3H, Ar-O-$CH_3$), 4.15 (d, J=8.0Hz, 1H, Ar-CH), 7.17 (m, 4H), 7.39 (s, 1H, 4H-1,2-dithiole-3-thione), 7.51 (d, J=8.0Hz, 1H, Ar-H), 7.64 (d, J=8.0Hz, 2H, Ar-H), 7.80 (m, 3H, Ar-H). $^{13}C$ NMR (DMSO-$d_6$, TMS, ppm): δ 18.8, 31.2, 45.0, 55.7, 106.3, 119.4, 123.3, 126.4, 126.7, 127.8, 129.0, 129.2, 129.3, 129.7, 134.0, 135.5, 136.3, 153.9, 157.8, 172.9, 173.0, 216.0. ESI-HRMS (m/z): calcd. for $C_{23}H_{18}NO_3S_3$ $[M–H]^-$: 437.0418; found 437.1935.

Compound **12:** brownish red oil180.0 mg. Yield: 44.1%. IR (KBr, $cm^{-1}$): 1753 (s, COO), 1136 (v, C=S). $^1H$ NMR (DMSO-$d_6$, TMS, ppm): δ 0.94 (d, J=6.0Hz, 6H, $C(CH_3)_2$), 1.65 (d, J=8.0Hz, 3H, Ar-C-$CH_3$), 1.90 (m, 1H, Ar-C-CH), 2.50 (d, 2H, Ar-$CH_2$), 3.98 (q, J=8.0Hz ,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=8.0Hz, 2H, Ar-H). $^{13}C$ NMR (DMSO-$d_6$, TMS, 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. ESI-HRMS (m/z): calcd. for $C_{22}H_{22}O_2S_3Na$ $[M+Na]^+$: 437.0680; found 437.0701.

Compound **13:** brownish red oil124.7 mg. Yield: 27.0%. IR (KBr, $cm^{-1}$): 1751 (s, COO), 1660 (s, CO), 1146 (v, C=S). $^1H$ NMR (DMSO-$d_6$, TMS, ppm): δ 1.59 (d, J=8.0Hz, 3H, $CH_3$), 4.00 (q, J=8.0Hz, 1H, Ar-CH), 7.07 (m, 2H, Ar-H), 7.28 (s, 1H, 4H-1,2-dithiole-3-thione), 7.41 (m, 3H, Ar-H), 7.53 (m, 4H, Ar-H), 7.64 (m, 1H, Ar-H), 7.73 (m, 2H, Ar-H), 7.78 (s, 1H, Ar-H). $^{13}C$ NMR (DMSO-$d_6$, TMS, ppm): δ17.9, 42.7, 121.5, 123.3, 128.5, 128.8, 129.1, 130.3, 131.2, 132.4, 135.1, 136.2, 137.6, 140.6, 172.9, 173.1, 215.9. ESI-HRMS (m/z): calcd. for $C_{25}H_{18}NO_3S_3$ $[M+H]^+$: 463.0496; found 463.0487.

Compound **14:** brownish red solid 109.0 mg. Yield: 30.5%. IR (KBr, $cm^{-1}$): 1739 (s, COO), 1630 (s, C=C), 1130 (v, C=S). $^1H$ NMR (DMSO-$d_6$, TMS, ppm): δ 6.66 (d, J=16.0Hz, 1H, -CHCO), 7.36 (m, 2H, Ar-H), 7.45 (s, 1H, 4H-1,2-dithiole-3-thione), 7.47 (m, 3H, Ar-H), 7.63 (m, 2H, Ar-H), 7.74 (m, 2H, Ar-H), 7.93 (d, J=16.0Hz, 1H, Ar-CH). $^{13}C$ NMR (DMSO-$d_6$, TMS, ppm): δ117.3, 123.7, 129.2, 129.3, 129.5, 131.5, 134.3, 136.3, 147.6, 154.0, 165.0, 173.2, 216.0. ESI-HRMS (m/z): calcd. for $C_{18}H_{13}O_2S_3$ $[M+H]^+$: 357.0078; found 357.0134.

Compound **15:** brownish red solid 197.0 mg. Yield: 34.9%. IR (KBr, $cm^{-1}$): 1757(m, COO), 1681 (s, CO), 1320 (s, Ar-O-C), 1121 (v, C=S). $^1$H NMR (DMSO-$d_6$, TMS, ppm): δ 2.41 (s, 3H, Cp-$CH_3$), 3.77 (s, 3H, Ar-$OCH_3$), 3.87 (s, 2H, COO-$CH_2$), 6.64 (dd, J=4.0Hz, J=8.0Hz, 1H), 6.81 (d, J=8.0Hz, 1H, Ar-H), 6.97 (d, J=2.0Hz, 1H, Ar-H), 7.15 (m, 3H, Ar-H), 7.32 (s, 1H, 4H-1,2-dithiole-3-thione), 7.42 (m, 2H, Ar-H), 7.60 (m, 4H, Ar-H). $^{13}$C NMR (DMSO-d6, TMS, ppm): δ13.8, 29.8, 56.0, 102.2, 112.0, 112.5, 115.2, 123.5, 129.2, 129.4, 129.6, 130.9, 131.7, 134.5, 136.3, 136.4, 138.2, 154.0, 156.2, 169.6, 173.1, 216.0. ESI-HRMS (m/z): calcd. for $C_{28}H_{21}ClNO_4S_3$ $[M+H]^+$: 566.0321; found 566.0407.

**Acknowledgements**

This work was financially supported by the National Natural Science Foundations of China (no. 21171079) and Science Development Project of Lanzhou (No. 2014-2-34).

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Highlights

- ► Firstly evaluate the toxicity of $H_2S$-donors based on NSAIDs
- ► Investigate the effects of $H_2S$-donors on levels of inflammatroy factors.
- ► Explore the synergistic effect of $H_2S$ and NSAIDs.