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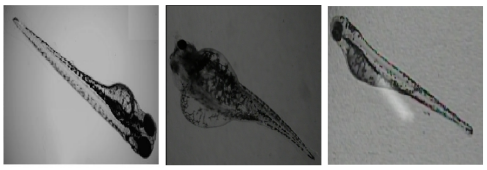
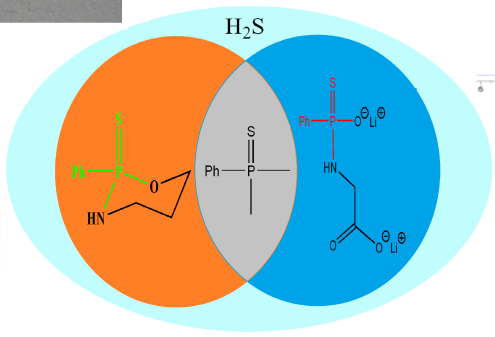
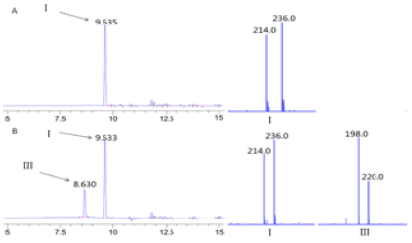
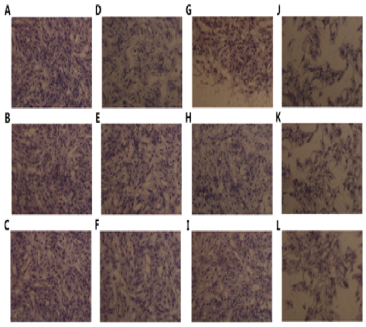
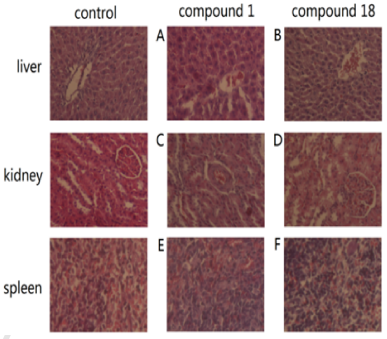










Toxicity, bioactivity, release of H_2S *in vivo* and pharmaco-kinetics of H_2S-donors with thiophosphamide structure

Jinlong Zhang[1], Qiuping Zhang[1], Yanni Wang[1], Jili Li[1], Zhongjie Bai[1], Quanyi Zhao *[1], Dian He[1], Zhen Wang[1], Jingke Zhang[2], Yonglin Chen[3]

(1 Institute of Medicinal Chemistry, School of Pharmacy of Lanzhou University; 2 GLP Lab Centre, School of Basic Medicine, Lanzhou University; 3 The First Affiliated Hospital of Lanzhou University, Lanzhou, 730000, China)

Abstract: H_2S donors are substitutes of H_2S with various biological activities like inhibiting the inflammatory response and protecting myocardial cells from injury. In order to confirm whether the H_2S donors have drug-like properties, two series thiophosphamide H_2S donors were evaluated including toxicity, bioactivity and pharmacokinetic properties *in vivo* and *in vitro*. The following results were obtained. Firstly, all the compounds released H_2S under measuring condition; with the increase of pH value, the H_2S release rate of all the compounds decreased and the amount reduced, but pH value had little effect on the maximum release of H_2S. Secondly, in the organs and tissues of rats, the compounds released H_2S in the same way as in PBS. In plasma, compound **1** reached the C_{max} after administration 55 min, and no compound **1** was detected after 12 h; for compound **18**, the C_{max} reached only after administration 100 min, and after 6 h, compound **18** was not detected; in organs and tissues, the H_2S-release rates were different from those in PBS, but the mechanism of H_2S release was the same. Thirdly, in the test of toxicity, all the compounds displayed low toxicities to 5 cancer cells and W138 cell lines; compounds **1** and **18** had slight effect on the physiological tissue and function of rat liver at low concentration; the compounds had almost no effect on the hatching rate, survival rate of zebrafish embryos, and the spontaneous movement of zebrafish embryos at below 0.5 μM, but when they were over 1 μM, the compounds displayed inhibitory effect in the manner of concentration dependence. Fourthly, in the course of anti-inflammatory test, all the tested compounds significantly reduced the level of TNF-α and increased the level of IL-10; when they were 100 μM, the levels of IL-10 were three times as high as those in the control group. Among them, compounds **10** and **18** displayed stronger activities than the others. In addition, the compounds protected H9c2 cells from injure and improved myocardial injury through anti-oxidation pathway. In summary, the compounds have druglike properties due to low toxicity, better activity and good pharmacokinetic property. Therefore, they have potential to be as candidates to investigate further.

Corresponding author, Email: zhaoqy@lzu.edu.cn

Keywords: H_2S donors; thiophosphamide; toxicities; anti-inflammation; myocardial protection; pharmacokinetic

Introduction

H_2S is considered as the third gas signal molecule after CO and NO.[1-6] Many tests confirmed H_2S displayed various biological activity, for example, inhibiting the inflammatory response.[7,8] Endogenous H_2S was produced by enzymatic reaction using cysteine and homocysteine as substrates.[9-15] In the past decade, a lot of testing results show H_2S plays an important role in physiology and pathology process, especially in the cardiovascular system and nervous system.[16-22] Besides that, H_2S also induced cancer cells apoptosis by activating map kinase and cysteinase-3. [23] All indicates H_2S has a big potential to be candidate drugs.[24,25]

However, H_2S is a rotten-egg gas, and inconvenient to use in clinic due to uncontrollable dose. Consequently, using H_2S donors taking the place of H_2S gas to use in clinic is a practical way. H_2S donors have inorganic sulfide salts and organic H_2S donors. The salts like NaSH, Na_2S and CaS, release H_2S almost immediately, [26] and possibly lead to the toxic injury of tissue cells because the concentration of H_2S is too high. [27] The organic H_2S donors may overcome these faults to get controllable dose and targeting tissues and organs. At present, they involve mainly thioamides, disulfide and compounds containing polysulfide atoms. [28-34]

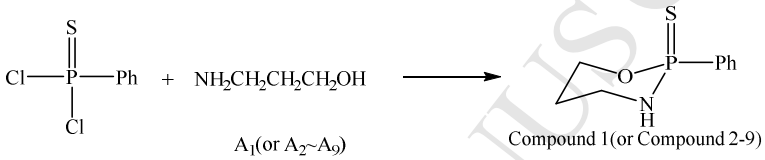
Among the organic H_2S donors, thiophosphamide derivative is a kind of H_2S donors with low cytotoxicity. The structures of thiophosphoramide donors include cyclic phosphoramide structure and chain phosphoramide structure.[35-36] They all released H_2S in the process of test, and exhibited various biological activities, such as anti-inflammation, myocardial protection. [35] However, although there are many reports about H_2S donors, there is still no any report on the kinetic properties of H_2S donors *in vivo*. Whether the H_2S donors can be used in clinic as medicines, there are many determinants. Among them, the toxicity and the pharmacokinetic properties of compound *in vivo* are crucial. In addition, H_2S donor is only a carrier of H_2S, they do not take effect until H_2S released from it arrives at the effective concentration. *In vivo*, the microenvironments (such as pH) of organs and tissues maybe affect the H_2S releasing rates. In order to understand deeply and comprehensively the properties of H_2S donor compounds *in vivo*, it is very necessary to investigate the H_2S-release in organs and tissues.

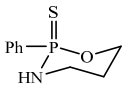
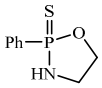
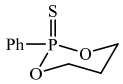
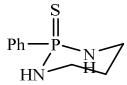
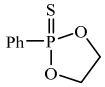
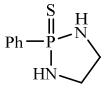
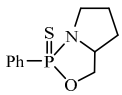
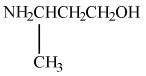
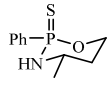
Therefore, in this paper, based on the synthesis of two series of thiophosphamide H_2S donors, we evaluated the toxicity and bioactivity using several models, and investigated their pharmacokinetic properties *in vivo*. By which, we hope to obtain some information about whether the H_2S donors can be as candidates to be further study, and also provide theoretical basis for the clinical application.

Results and discussion

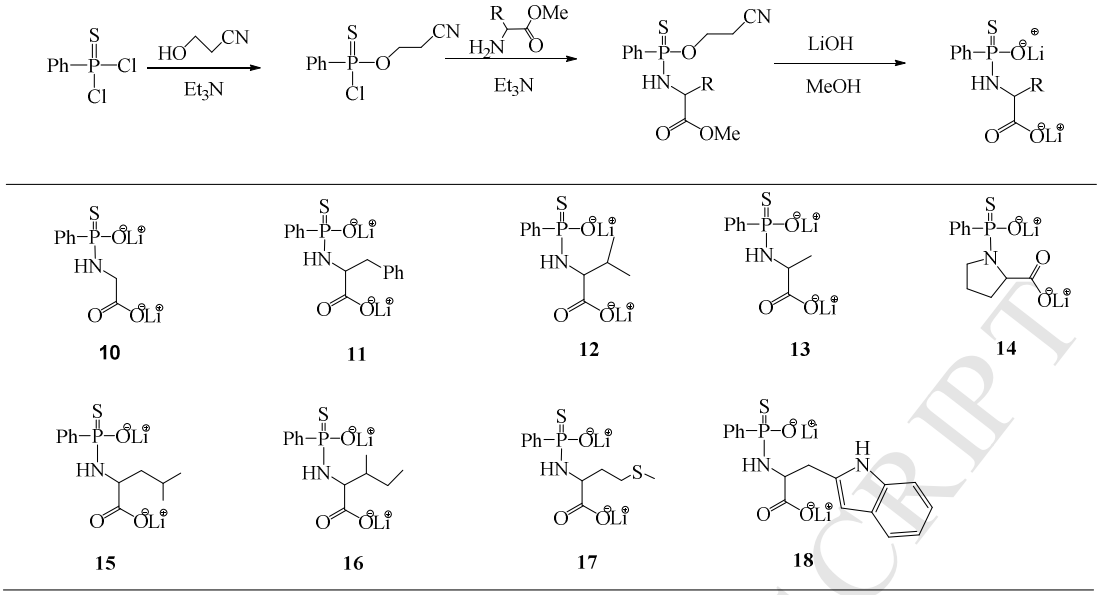
1. Synthesis and characterization of compounds:

Compounds **1-9** were synthesized by the reaction of phenylthiophosphoryl dichloride with different amino alcohols, diols or diamines in the presence of triethylamine. The product was purified by column chromatography to obtain white granular solid. [36]Compounds **10-18** also took phenyl thiophosphonyl dichloride as the starting material, reacting with 3-hydroxypropionitrile and amino acid methyl ester to obtain ester intermediates; then the esters hydrolyzed with LiOH to form lithium salt, following freeze-dried to form white granular products.[35] Nine amino acid methyl esters were involved (glycine, phenylalanine, valine, alanine, proline, leucine, isoleucine, tryptophan and methionine). All the compounds were characterized by ^{1}H NMR, ^{13}C NMR, ESI-MS and IR.



A		Compounds	
A_1	$NH_2CH_2CH_2CH_2OH$	1	
A_2	$NH_2CH_2CH_2OH$	2	
A_3	$HOCH_2CH_2CH_2OH$	3	
A_4	$NH_2CH_2CH_2CH_2NH_2$	4	
A_5	$HOCH_2CH_2OH$	5	
A_6	$NH_2CH_2CH_2NH_2$	6	
A_7		7	
A_8		8	
A_9	$NH_2CH_2CH_2CH_2CH_2OH$	9	

Scheme 1 The synthetic route and structures of compounds **1-9**



Scheme 2 The synthetic route and structures of compounds **10**-**18**

The spectra of all the compounds are consistent with the expected ones. IR data and other main characteristics are shown in **Table 1**. In the ^{1}H NMR spectra of compounds **1**-**9**, the chemical shifts of hydrogen atoms in benzene ring appeared in the range of 7.88-7.28 ppm. For all the compounds containing O atom (all but compounds **4** and **6**), the signals of protons on OCH_2 appeared in the range of 3.40-4.49 ppm. For compounds containing NH group (compounds **4**, **6** and **9**), the chemical shifts of hydrogen in NH are in the range of 3.15-3.44 ppm. In the hydrogen spectra of compounds **10**-**18**, the chemical shifts of hydrogen in benzene ring were in the range of 7.81-7.07 ppm; meanwhile, the chemical shifts of hydrogen in NH appeared in the range of 3.42-3.86 ppm and those in NCH_2 in the range of 3.52-3.82 ppm.

Table 1 The main characteristics (solubility, IR absorption) of the compounds

Compds	solubility	IR characteristic absorption (cm^{-1})	stability	trait
1	insoluble in water; easily soluble in organic solvents such as DMSO, dichloromethane,acetone, and so on	3274m (NH),1438s (P=S), 1120s (C-O)	stable in dry air at r.t ; do not decay for long periods of time	white granular solid
2		3265m (NH), 1436s (P=S), 1121s (C-O)		light yellow oil
3		1440s (P=S), 1125s (C-O)		white oil solid
4		3252m (NH), 1436s (P=S)		white oil solid
5		1439s (P=S), 1118s (C-O)		white oil solid
6		3317m (NH), 1437s (P=S)		yellow granular solid
7		3448m (NH), 1437s (P=S), 1115s (C-O)		white oil solid
8		3418m (NH), 1437s (P=S), 1120s (C-O)		white oil solid
9		3437m (NH), 1439s (P=S), 1161s (C-O)		white granular solid
10	easily soluble in water, methanol and DMSO; insoluble in dichloromethane, ethanol, acetone	3370m (NH), 1593s (COO), 1436.9 (s, P=S)	easily deliquescent and tend to deteriorate in humid air	white granular solid
11		3476s (NH), 1636s (COO), 1411s (P=S)		
12		3472m (NH), 1634s (COO), 1418s (P=S)		
13		3428m (NH), 1641s (COO), 1416 (s, P=S)		
14		3434m (NH), 1641s (COO), 1410 (s, P=S)		
15		3294m (NH), 1638s (COO), 1437s (P=S)		
16		3420m (NH), 1638s (COO), 1422s (P=S)		
17		3404m (NH), 1636s (COO), 1420s (P=S)		
18		3431m (NH), 1638s (COO), 1418s (P=S)		

2. H_2S release ability of the compound and its influencing factors

Among the methods used to detect H_2S, methylene blue method was used generally, but it needs to be carried out under strong acid condition, and the promotors such as cystine often lead to a large deviation in the measurement results.[37] In contrast, the fluorescence probe method is obviously superior to methylene blue method. The fluorescence probe is an important tool for selective and sensitive detection of H_2S. The fluorescence probe based on sulfonyl azide can react with H_2S in a few minutes,

showing a higher fluorescence change and excellent linear correlation. So the H_2S released from the donor can be measured quantitatively by the fluorescence value of the probe. [38]

In the testing of process, we chose fluorescence probe based on sulfonyl azide to detect the H_2S released from the compounds. The results show all the compounds released H_2S at room temperature, and most of the H_2S release curves are in accordance with the first order kinetic equation. All the data is shown in **SI-Table 1**.

Seen from the data, the first series compounds released H_2S far more slowly than the second series, and accordingly the H_2S release half lives were longer than those of the second; moreover, the maximum amounts of H_2S-release of the first series compounds were also lower than those of the second series under the same condition. For compounds **1**-**9**, their maximum releases of H_2S were all in range from 25 to 30 μM, which indicates that the size of cycle-type structure and the function groups like ester and amide had little effect on the release of H_2S. Compounds **1**-**9** were H_2S releasers with medium release rates, and their half-lives were about 30 min. For compounds **10**-**18**, except for **14**, the maximum release of H_2S of each compound was more than 100 μM and in the range of 100-120 μM. Compound **14** released H_2S a small amount and only 4.03 μM at 22 □. This is possibly because the influence of the rigid structure of tetrahydropyrrole gave rise to the carboxyl anion far away the N-P double bond not to take part in the intramolecular substitution reaction. The half-life of compound **14** was about 10 min while the others were about 3.5 min, which indicates the second series of compounds were fast H_2S releasers.

In the body, the pH value of each organ and tissue varies, for example, the pH of gastric juice is about 1.8, and the pH of small intestine is about 8.0 when the body temperature is 37 □. When the H_2S donor enters the body, is the H_2S-release affected by the pH value and temperature? In order to simulate as much as possible the real situation of the release of H_2S *in vivo*, the half-life and maximum release of compound were measured under different pH and temperature conditions. All data is also shown in **Table 2** and **SI-Table 1**.

Table 2 H_2S C_{max} released from the compounds at 150 μM

H_2S C_{max}(μM)								
Com-pds	22℃				37℃			
	pH 1.8	pH 5.0	pH 7.4	pH 8.0	pH 1.8	pH 5.0	pH 7.4	pH 8.0
1	25.0	24.9	25.1	25.6	34.8	35.8	26.5	35.1
2	30.0	29.9	29.9	29.7	35.6	37.0	31.1	35.4
3	24.8	25.7	24.4	25.7	38.4	37.4	27.2	35.7
4	27.0	27.2	27.2	27.1	35.7	35.5	27.7	35.7

5	28.4	27.9	27.7	27.5	35.7	35.7	29.1	35.7
6	28.5	28.4	28.4	28.3	35.7	35.4	29.9	34.8
7	27.1	27.1	26.9	27.1	36.2	36.8	28.2	33.7
8	25.5	25.5	25.4	28.1	36.7	36.6	26.4	35.4
9	28.3	28.5	27.8	29.2	35.4	34.5	30.0	34.7
10	101.4	99.4	99.5	99.4	102.6	103.0	102.2	101.4
11	121.5	121.0	121.5	120.3	128.8	128.6	128.3	127.0
12	121.4	121.5	121.5	121.5	125.2	125.9	125.3	124.2
13	104.2	102.9	102.4	102.7	109.7	106.5	107.7	105.8
14	4.0	4.2	4.0	4.1	4.2	4.2	4.2	4.3
15	113.1	111.8	111.5	111.4	118.6	118.3	118.2	115.5
16	118.6	117.0	117.3	117.0	122.9	123. 0	122.7	122.4
17	114.3	112.6	112.3	112.6	120.0	119.8	119.9	118.1
18	118.4	117.2	117.3	118.4	124.4	124.4	124.3	123.5

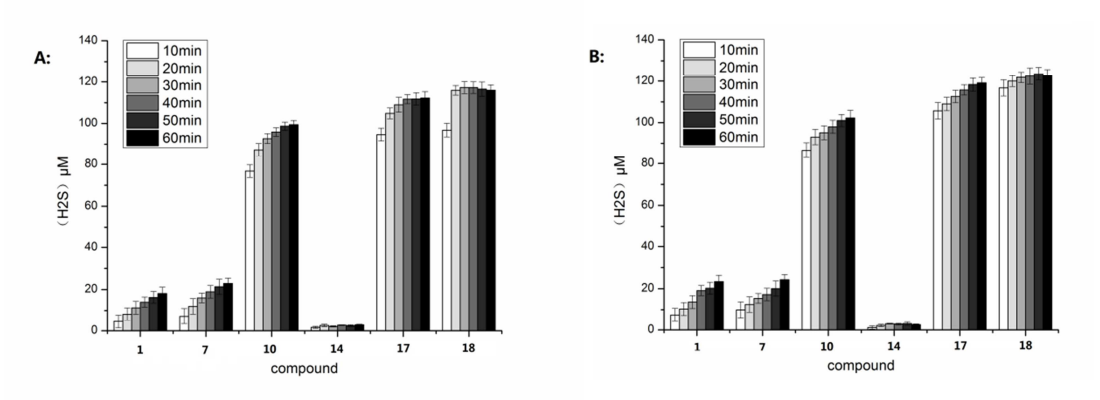


Fig. 1 H_2S concentration change with time when compound 1,7,10,14,17 and 18 at 150 μM, and probe 200 μM: A for 22°C; B for 37°C.

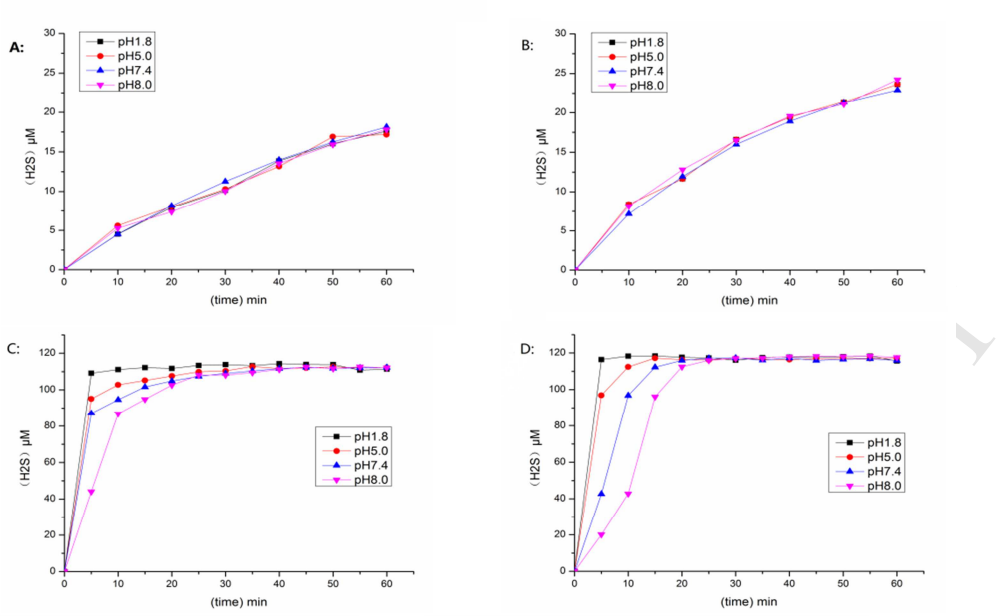


Fig.2 The effect of pH on H_2S release of the compounds at 150 μM, probe 200 μM. (A) for compound **1**; (B) for compound **7**; (C) for compound **17**; (D) for compound **18.**

Seen from **Fig.1**, compared with room temperature, when the temperature was at 37 □, the maximum release of H_2S of compounds **1** and **7** increased slightly, but their half-lives did not change significantly. For the second series, the maximum releases of H_2S of compounds **10**, **17** and **18** were also slightly increased, and their half lives of release of H_2S were also shortened, while compound **14** did not change. In general, at 37 □, the release rates of all the compounds increased and the release amount increased. Meanwhile, the pH value of the solution also affected the H_2S release. As shown by **Fig.2**, the release half lives and maximum releases of compounds **1** and **7** changed little, but compounds **17** and **18**, the smaller pH value, the faster the H_2S releasing rate. With the increase of pH value, the release of H_2S became slow and the amount became less, but pH had no effect on the maximum release of H_2S. Of course, except pH value and temperature, the substances with nucleophilic group also promote H_2S release, such as cysteine [39-41] and glutathione.

3. H_2S release ability of the compounds in organs and tissues

3. 1. The mechanism of the compound releasing H_2S

The H_2S release ability of the compound is closely related to its structure. The first series compounds (such as compound **1**) are more stable, and the H_2S released slowly in PBS; the mechanism of H_2S-release was proposed as showing in **Fig.3** (**a**). The second series compounds (e.g compound **18**) released H_2S more quickly, and the process of H_2S release in PBS was considered as showing in **Fig.3** (**b**). [35] Whether the H_2S-release process as we expected? Is there any difference between *in vivo* and *in*

vitro? *In vivo*, whether the products formed by the compound after releasing H_2S are the same as those done in PBS?

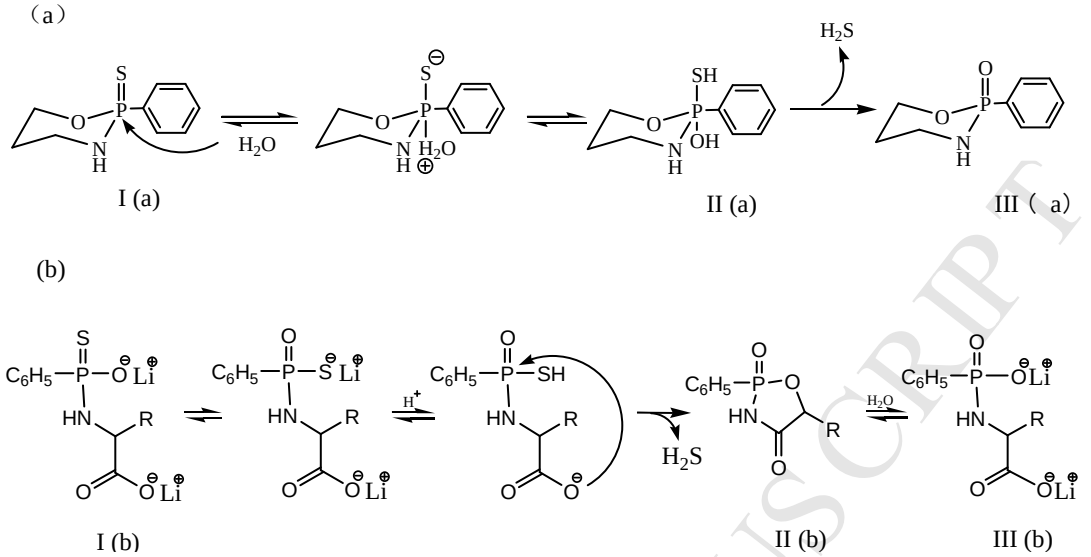


Fig. 3 Mechanism of H_2S- release of the compounds as H_2S donors: (a) for compound 1, (b) for compound 18.

To illustrate this problem, we used LC-MS to detect the products after the compound releasing H_2S in PBS and serum. Seen from **Fig.4** and **SI-Fig. 2**(**a**), in PBS, the retention time (Rt) of compound **1** was 9.649 min, and the characteristic peaks in MS appeared at m/z 214.0 ($[M+H]^+$) and m/z 236.0 ($[M+Na]^+$). The Rt of the product was about 8.652 min, and the characteristic peaks appeared at m/z 198.00 ($[M+H]^+$) and m/z 220.00 ($[M+Na]^+$). In serum (**Fig.4** and **SI-Fig.2**(**b**)), the Rt of compound **1** was 9.647 min, and the characteristic peaks also appeared at m/z 214.0 ($[M+H]^+$) and m/z 236.0 ($[M+Na]^+$); the Rt of the product was 8.851 min, and the characteristic peaks also appeared at m/z 198.00 ($[M+H]^+$ and m/z 220.00 ($[M+Na]^+$), which indicated the product was the same as that in PBS. The product was identified as compound **III-(a)** by comparison of known substance. Therefore, we infer that compound **1** released H_2S in serum and in PBS in the same manner, and is consistent with our expectation.

There are many various components in serum, whether they have an effect on the release rate of H_2S. To answer this question, the concentrations of compound in both PBS and serum were detected every 12 h using LC-MS in the process of test. Seen from **Fig.4**, in PBS, the concentration of compound **1** decreased gradually and the product **III-(a)** increased gradually with the prolongation of time in 36 h; in contrast, in serum, it was much lower than that in PBS buffer of pH 7.4 at the same time point. This is because compound **1** had a certain protein binding rate. At the same time, in serum, the concentration of the product **III-(a)** was higher than that in PBS. This indicates compound **1** released H_2S faster in serum than in PBS.

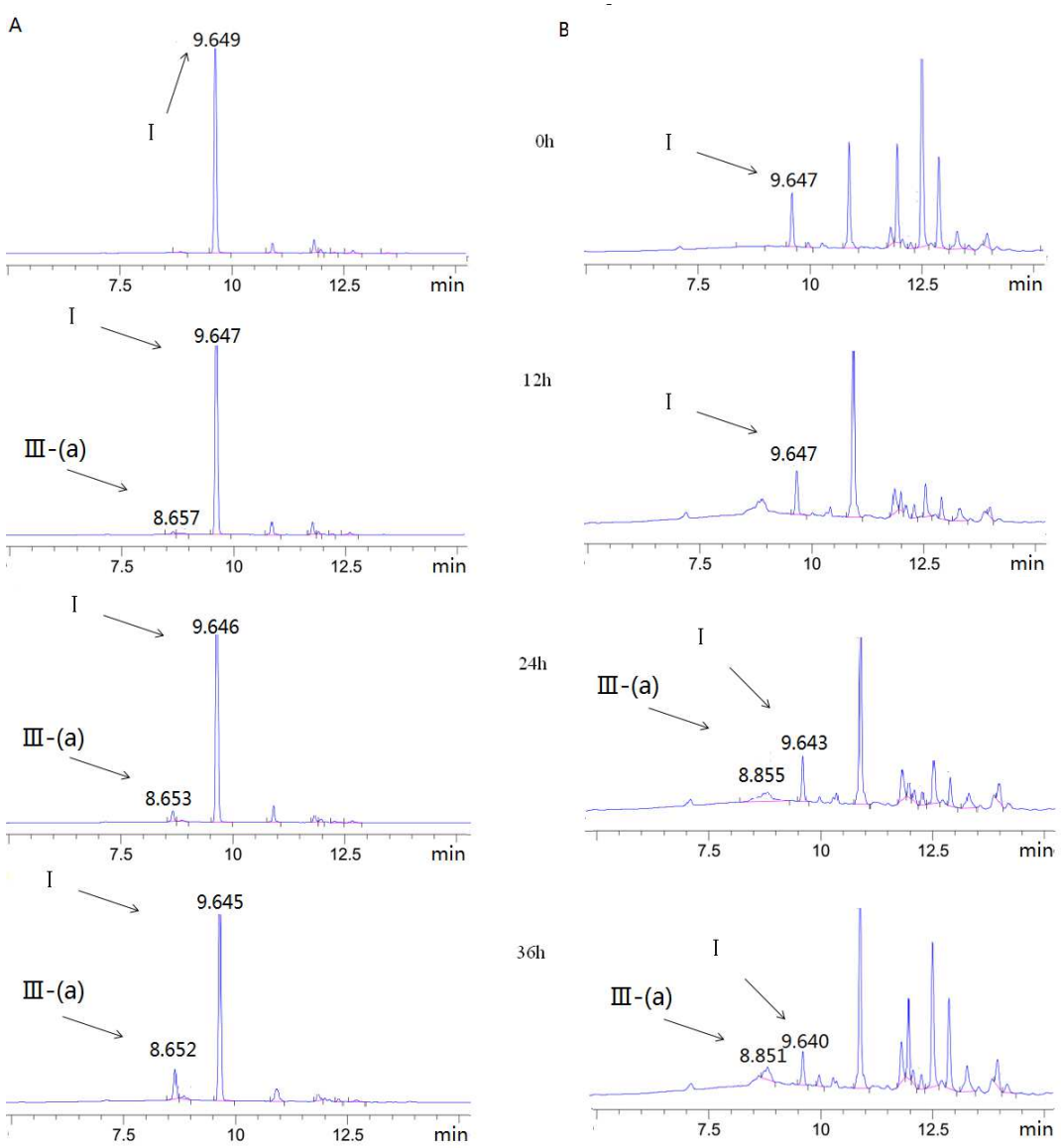


Fig. 4 LC diagrams of compound **1** over time in PBS (A) and serum (B)

Compound **18** was detected every 30 min because of the short half-life of H_2S release. Seen in **SI-Fig.4**, the Rt of compound **18** was about 8.08 min, and the characteristic peak appeared at m/z 318.10 ($[M+H]^+$). Compound **18** released H_2S easily in serum, and **III-(b)** was detected at the beginning of test. The content of compound **III-(b)** increased quickly with the prolongation of time (**SI-Fig.5b**). Compared with PBS buffer (**SI-Fig.5a**), the content of compound **18** was much lower, which shows it had a higher protein binding rate. After H_2S release, **III-(b)** was the main product which Rt was about 7.27 min, and characteristic peak at m/z 302.10 ($[M+H]^+$). It was reported that compound **18** released H_2S by forming cyclic compound **II-(b)**, [35] but we did not detect compound **II-(b)** in the process of test. This suggests the formation of cyclic compound **II-(b)** is not the driving force of H_2S release from compound **18**. However, we found compound **18** also released H_2S in methanol solution (containing little water) during the treatment of serum samples, and cyclic compound **II-(b)** was the main product except H_2S.

3.2. Process of H_2S release of the compounds in liver, heart and kidney

The compounds displayed the pharmacological activity of H_2S in the organs and tissues like myocardial tissue, which demonstrates them having been distributed to target organs and tissues and released H_2S. When the compound enters the body, it may be disposed by the enzymes in various tissue cells. In the liver, the compounds possibly do not release H_2S due to being oxidized. In cardiomyocyte, is there any toxical substance produced after H_2S release from the compound? In kidney, does the compound also release H_2S in the same manner as in other organs? In order to figure out this problem, we used the liver, heart and kidney homogenate of WISTAR rats to simulate the process of H_2S release *in vivo*. During the process of test, the method of H_2S measure was the same as above. The results showed all the tested compounds released H_2S in various tissue homogenates.

In liver homogenate, compound **1** decreased with time. As shown in **Fig.5(B)**, after incubation for 36 h, most of compound **1** still remained intact; the primary metabolite increased gradually, which the retention time was 8.63 min, and the characteristic peaks appeared at m/z 198.0 ($[M+H]^+$) and 220.0 ($[M+Na]^+$). It is obvious that the primary metabolism is compound **III-(b)**.

In myocardial homogenate, compound **1** released H_2S was also slow. As shown in **Fig.6**, except for the metabolite **III-(b)**, two new products appeared, and their retention times were 11.9 min and 12.4 min, respectively. The characteristic peaks in appeared at m/z 449.0 and m/z 662.1. This indicates that compound **1** transformed into new products in myocardial tissue after releasing H_2S; however, these two new compounds also appeared in kidney tissue homogenate (**SI-Fig.2(c)** and **3**).

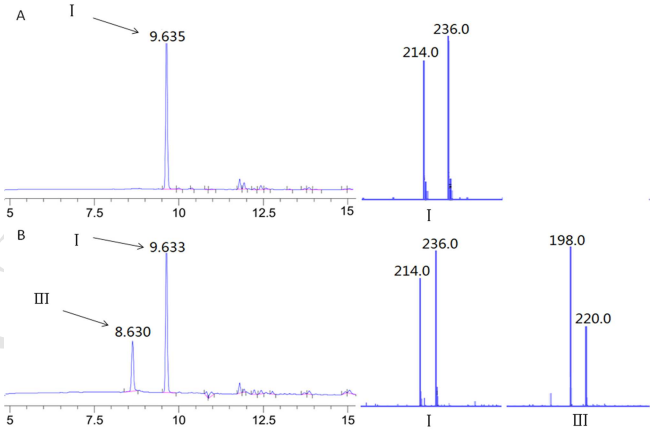
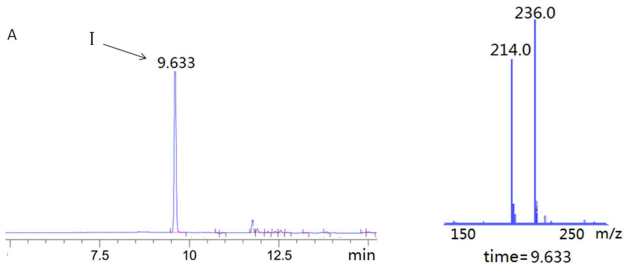


Fig. 5 LC-MS diagram of compound **1** in liver homogenate. **A** for 0 h and **B** for 36 h



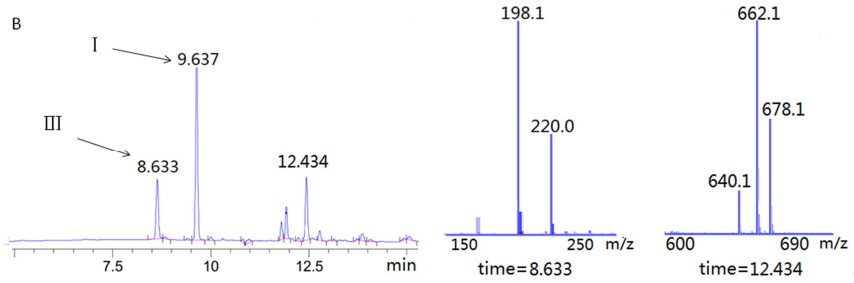


Fig. 6 LC-MS diagram of compound **1** in heart homogenate. A for 0 h; B for 36 h

For compound **18**, the release rate of H_2S in liver homogenate was still fast. In the LC diagram, with exception of the original compound **18** (Rt 8.3 min, m/z 318.1 $[M+H]^+$) and III-(b) (Rt 7.9 min, m/z 302.1$[M+H]^+$), there were two products, which their retention times were 10.2 min and 10.6 min; moreover, their contents changed obviously with time. These two substances were also found in serum, heart, and kidney tissues (**SI-Fig.5(c, d, e)**), and their concentrations also decreased with time. Their m/q values are higher, so we thought they maybe were small peptide conjugates of compound **18**. These conjugates prolonged the action time of H_2S. Possibly, this is the reason why the half life of H_2S release was short in PBS, but it had obvious myocardial protection *in vivo*. Therefore, it was in liver, heart and kidney tissues that compound **18** released H_2S in the same way as in PBS, but the release rates and the amount of metabolites are slightly different.

Seen from the above results, these two series compounds released H_2S in both PBS and various organs and tissues in the same way. The protein binding rate of compound **1** in serum, while compound **18** had high protein binding rate, which making H_2S release slowly. In different tissues, the rates of release H_2S of these two compounds *in vitro* were different due to the effect of plasma protein, but the mechanism of H_2S release was the same.

4. Pharmacokinetics of the compounds as H_2S donors

Male WISTAR rats were chosen as animal model, and injected intraperitoneally with compound **1** or compound **18** of 200 mg/kg. The blank group was injected with the solution without containing compound **1** or **18**. The blood was taken from canthus eye of the rats every set time point. After treatment, the compound was analyzed by LC-MS.

As shown in **Fig.7**, compound **1** was absorbed quickly into blood. After 50 min post administration, the plasma concentration of compound **1** reached maximum, and then it decreased sharply over time, which indicates the distribution rate of compound **1** into organs and tissues was also fast. After 6 h, the concentration of compound **1** was very low and tended to the detection limit. After 12 h, compound **1** was not detected in the plasma of rats, which suggests compound **1** was metabolized into other substances or excreted completely. For compound **18**, the plasma concentration reached maximum post 100 min administration. After 6 h, compound **18** was not detected in the plasma, indicating that it was metabolized into other substances or

excreted out of the body. In comparison, compound **1** was absorbed faster than compound **18**, while compound **1** was eliminated slower than compound **18**, possibly because the polarity of compound **1** is so small that it easily passes through the cell membranes to be absorbed. Compound **18** was easily eliminated because it has a big polarity.

For the same compound, there is a big difference of property between *in vivo* and *in vitro*. This is because the compound was distributed, metabolized and eliminated gradually *in vivo*. This explains why compound **1** still can be detected *in vitro* after 36 h while cannot be detected *in vivo* after 12 h.

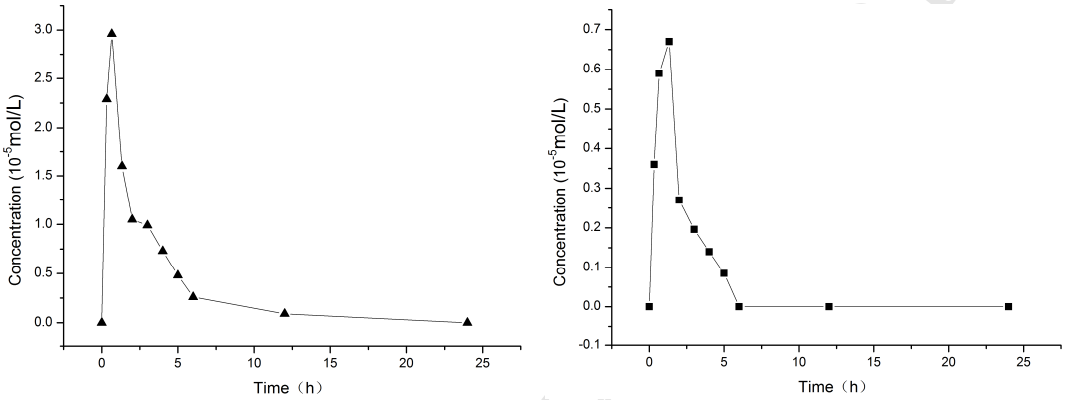


Fig.7 Blood concentration curves of compounds vs time, **1** (left) and **18** (right). Each data was measured three times and its average value was taken.

5. Toxicity of the compounds as H_2S donors

5.1 Cytotoxicity of the compounds as H_2S donors

In order to evaluate the cytotoxicity of the compounds to cells, six cell lines (HeLa, A549, HePG2, MCF-7, HT-29 and W138 cell line) were chosen to evaluate the inhibitory effect of the compounds on the growth of the cells, 5-FU as positive control. The results showed all the compounds had little inhibitory effect on the growth of six cell lines compared with 5-FU; some of the IC_{50} values were above 800 μM (**Table 3**). Among all the first series compounds, compound **2** displayed a slight effect against HT-29 cells, with IC_{50} 539.1 μM; compound **5** had a better inhibitory effect on HeLa cells than the others.

The second series of compounds also displayed low toxicities to the cells, possibly because their structures contain amino acid fragments. Among the second series, compound **16** displayed the strongest inhibitory effect on the growth of A549 cells with an IC_{50} of 236.7 μM. The anti-proliferative activity of compound **17** to HePG2 cells was higher than the others, and its IC_{50} was 285.9 μM. That indicates the toxicity of compound **17** was much lower than that of 5-FU. Compound **12** displayed higher anti-proliferation against MCF-7 cells than the others, but its IC_{50} was still very large. All in all, all 18 compounds had a low inhibitory activity on the growth of HeLa,

A549, HePG2, MCF-7, HT-29 and W138 cell lines; Moreover, the compounds displayed no selectivity to cancer cells and normal cells.

Table 3 IC_{50} values of all compounds against cells proliferation

Compds	IC_{50}(μM)					
	HeLa	A549	HePG2	MCF-7	HT-29	W138
1	826.3±13.4	886.1± 11.8	628.7±9.6	752.0±16.2	1054.9±10.8	720.5±12.8
2	929.9±8.7	621.7±16.9	775.0±11.7	797.5±14.8	539.1±16.0	758.8±16.4
3	1278.8±16.0	569.3±11.6	612.0±15.4	852.9±13.1	991.1±16.7	652.8±10.7
4	793.3±9.0	875.6±10.9	800.6±17.4	850.1±13.0	600.4±14.0	380.6±17.4
5	678.7±14.6	678.6±15.3	804.3±15.7	748.5±9.8	882.1±17.0	381.0±9.5
6	967.5±8.5	641.4±11.2	656.9±10.1	712.8±13.4	780.5±12.3	957.8±14.8
7	984.3±10.0	543.8±11.3	531.0±10.2	920.5±17.7	635.5±13.5	502.9±16.5
8	1429.8±15.9	886.4±12.7	596.7±8.8	979.9±9.3	1005.4±8.8	481.1±8.3
9	945.3±13.8	675.4±16.0	798.1±17.0	890.5±8.0	653.9±9.5	990.2±10.5
10	913.1±12.9	377.7±14.0	770.7±16.9	875.7±8.2	912.4±13.3	508.5±13.1
11	711.9±13.5	236.7±15.9	671.1±12.5	662.8±9.8	542.1±15.2	689.5±11.3
12	809.4±10.8	294.5±8.9	689.6±17.0	542.4±13.2	602.3±13.6	545.7±9.1
13	888.4±17.3	345.6±10.4	858.2±11.2	832.0±15.5	689.3±13.9	351.4±12.2
14	838.7±13.4	518.7±10.7	815.5±12.1	874.4±11.6	932.2±18.0	871.6±14.7
15	835.2±11.0	273.3±14.4	414.7±11.1	643.3±9.9	756.4±11.5	438.5±16.3
16	972.1±15.3	236.3±18.0	603.9±16.6	601.3±15.3	782.4±17.7	658.2±15.8
17	919.1±17.8	308.8±10.8	285.9±8.9	671.4±12.2	826.2±14.9	546.2±17.5
18	712.3±13.1	433.3±12.7	315.1±13.0	837.6±12.1	741.6±9.0	723.6±13.5
5-FU	120.4±12.6	104.5±11.9	112.1±13.4	136.5±14.1	146.2±16.2	201.9±8.7

Note: Values represent mean ± SD from at least three independent experiments.

5.2 Toxicity of the compound after successive administration

In the set dose range, the single dose of mice did not show obvious toxicity, so we selected the rats to study the toxicity of the compounds by multiple doses. The liposolubility of the first series of compounds is large, and their lipids and water partition coefficients are between 2.1 and 3.1, indicating they are easy to permeate the membrane and are easily absorbed; the second series of molecules are the lithium salt structure, which is not easy to absorb and can only be administered by intraperitoneal injection. The tested compounds were injected intraperitoneally with 0.01 $mmol•kg^{-1}$ per dose each day. After 14 days, the rats were killed; then the blood, liver, kidney and other main organs were collected, and examined at the same time.

The results of routine blood test (**SI-Table 2**) showed, after the rats were administrated the compound successively for 14 days, the number of white blood cells decreased compared with the control (5.82×10^9/L); and the numbers were 3.23 and

4.01×10^9/L for compound **1** and **18**, respectively. The platelet numbers were 628 and 530×10^9/L, which was also lower than that of the control group (857×10^9/L). However, the hemoglobin content and erythrocyte number were increased. This indicates that the negative impacts of the compound on the blood system are in controllable range.

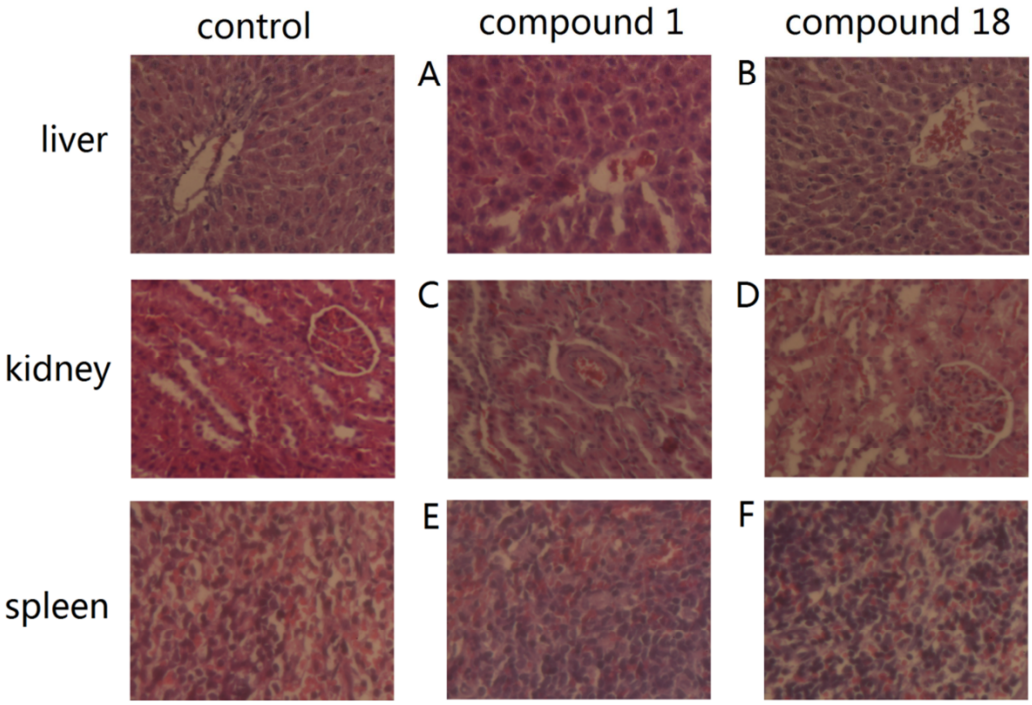


Fig.8 Morphology of liver (A and B), kidney (C and D) and spleen (E and F) of the rats after successive administration for 14 days. A, C and E for compound **1**; B, D and F for compound **18**

In order to understand the degree of damage caused by the compound to the physiological structure of organs of the rats, we observed the liver, kidney and spleen tissues under the microscopic after H-E staining. The results (**Fig.8**) showed, in the control group, the hepatic lobules were well structured, the hepatocytes were arranged neatly, and there was no obvious gap between the cells. In test group, the hepatic lobules were still intact after 14 days of administration (**Fig.8** (**A** and **B**)), but center venous and the hepatic sinuses were slight congestion. The hepatocytes were swollen and marked gaps were observed. In the kidney section, the glomerular was intact in the control group, the space of proximal convoluted tubules was normal; in the test group, the glomeruli were damaged slightly after administration (**Fig.8** (**C** and **D**)): the cells of proximal convoluted tubules were swollen and marked gaps were observed. For splenic sections, compared with the control, some nucleus became larger, and there was slight inflammation (**Fig.8** (**E** and **F**)). These results suggest the compounds will damage to the liver, kidney and spleen of rats after long-term administration. Therefore, the compounds are suitable for short-term successive administration.

6. Effect of the compound on the development of zebrafish

6.1 Effect of the compound on hatching rate and survival rate of zebrafish embryos

In order to assess the development toxicity of the compounds, we selected zebrafish embryos as model animal. The hatching rate and survival rate of zebrafish embryos were measured at 24, 48, 72, 96 and 120 hpf. The results show: when the compound was below 0.5 μM, the compound had almost no effect on survival rate of zebrafish embryos after 120 hpf compared with the control (**Fig.9** (**A** and **B**)), and also had less effect on hatching rate of zebrafish (**Fig.9**(**C**)). For each tested compound, the embryo survival rate decreased with the increase of the compound concentration. When compound **1** or **18** was up to 20 μM, at 120 hpf, both the embryo survival rate and hatching rate decreased to 50% or so; moreover, the longer the incubating with the compound under the same condition, the lower both of them.

The incubation period of normal zebrafish embryos ranges from 48 to 72 hpf. In the incubation process, the compounds inhibited the key enzymes to promote the embryonic development, and then led to the embryonic of zebrafish stopping the growth.[42] Seen from **Fig.9**(**C**), for compounds **1** and **18**, the hatching rate of zebrafish embryos was almost the same as the survival rate of embryos: at low concentrations (0.5 and 1.0 μM), the hatching rate of zebrafish embryos was not significantly affected by the compounds compared with the control; however, it decreased sharply when the compounds were over 5.0 μM. All these results indicate it is the compounds with low concentration that will be safe to the development of zebrafish embryos.

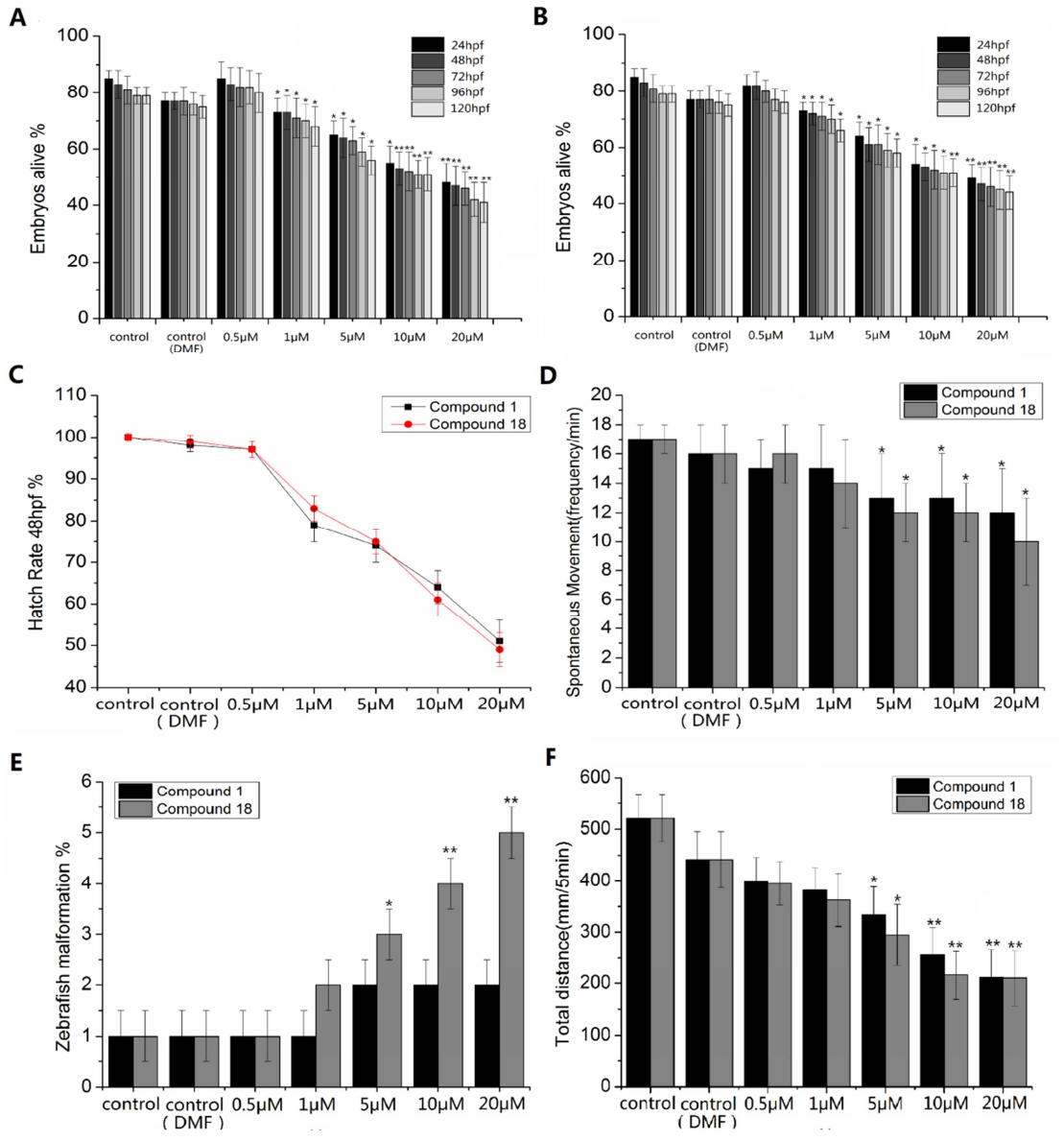


Fig.9 A(for compound 1) and **B** (for compound 18) is the effect of the concentration of compound on the embryo survival of zebrafish; **C** is the effect of the concentration of compound on the hatching rate of zebrafish embryosat 48 hpf; **D** is the effect of the concentration of compound on the spontaneous movement of zebrafish at 24hpf; **E** is the effect of the concentration of compound on the deformity rate of zebrafish embryos; **F** is the effect of the concentration of compound on the total swimming distance of juvenile zebrafish; n = 100. * compared with the control (DMF), * $p < 0.05$, * * $p < 0.01$. The data shown represent mean value ±SD for three independent experiments. DMF as solvent control, and compared with blank control, the diluted DMF (1: 1000) had no effect on zebrafish experiment.

6.2 The effect of compound on the spontaneous movement of zebrafish embryos

In order to evaluate the neurotoxicity of compound, the spontaneous motion frequency of zebrafish embryos was measured in 1min at 24 hpf. The results are shown in **Fig.9** (**D**). As a whole, the spontaneous motion frequency of zebrafish

embryos decreased gradually with the increase of compound concentration. When the concentration was below 0.5 μM, compound **18** showed no effect on the spontaneous motion frequency of zebrafish embryos compared with the control (17 times / min). However, at higher concentration, this effect was significant. Compound **1** of 5.0 μM decreased the motion frequency to 12 times /min while compound **18** decreased it to 13 times /min. This suggests the both compounds had slight neurotoxicity to zebrafish embryos at higher concentration, and this effect was concentration dependent.

6.3 Teratogenicity of the compound on zebrafish

At 24 hpf, zebrafish embryos were treated with the compound solution of 0.5 μM, and then observed under microscope at 96 hpf. Seen from **Fig.9** (**E**), the compounds had no effect on the development of zebrafish embryos at below 0.5 μM. When compound **1** was in the range of 1-20 μM, the embryo malformation rate was about 2%; but as for compound **18**, the embryo malformation rate increased with the increase of concentration under the same condition, and when it reached 20 μM, the embryo malformation rate was about 5%. This suggests the teratogenicity was concentration-dependent. The embryos incubated with compound **1** (or **18**) appeared pericardial edema, spinal curvature and yolk cysts (**Fig.10**). Therefore, we conclude that the compounds caused embryo malformation at over 1 μM, and they are safe at below 0.5 μM.

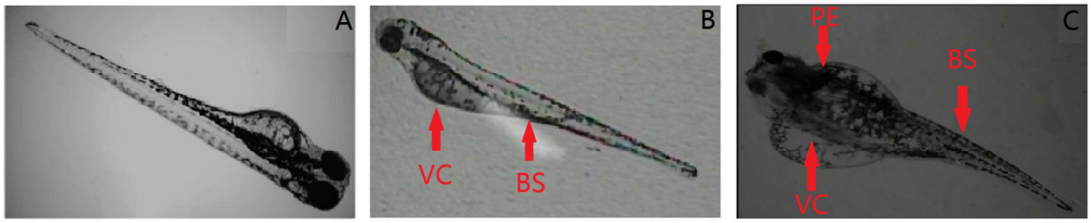


Fig.10 The morphology of zebrafish embryos after incubated with the compound; A for normal zebrafish; B for compound **1**; C for compound **18**. Red arrows indicate malformation. PE stands for pericardial edema; VC for yolk sac; BS for spinal curvature.

6.4 Effects of the compounds on juvenile movement of zebrafish

In order to make out whether the compounds have effect on the development of nervous system of zebrafish, we assessed the effect of compound on the motor ability of juvenile zebrafish at 144 hpf. The results show, under visible light irradiation, the total swimming distance of juvenile decreased with the increase of compound concentration. Seen from **Fig.9** (**F**), the compounds with higher concentration significantly inhibited juvenile movement, but when they were below at 1.0 μM, there was less difference in total swimming distance compared with the control. When compound **1** was 10 μM, the total swimming distance of juvenile zebrafish in 5 min was 257 mm; but for compound **18**, it was 216 mm under the same condition. Therefore, we conclude the tested compounds have a concentration dependent toxicity to the development of the nervous system of juvenile zebrafish. Compared with

compound **1**, compound **18** displayed more serious toxicity to the development of nervous system at the same concentration.

7. The anti-inflammatory activity of the compounds

We chose to use macrophage RAW264.7 as model to test and verify the anti-inflammatory activities of the compounds. In order to obtain more accurate results and exclude the effects of cell death on the experimental results, the survival rate of cells was measured by MTT method after treated with tested compounds at different concentrations (20 μM and 100 μM) for 24 h in the presence or absence of LPS (1 μg/ml). The results showed that the tested compounds had little toxicity to macrophages (**Fig.11**), and the survival rates of macrophages reached 70-110%. This indicates the anti-inflammatory results are authentic when the tested compounds are used in the range of 20-100 μM.

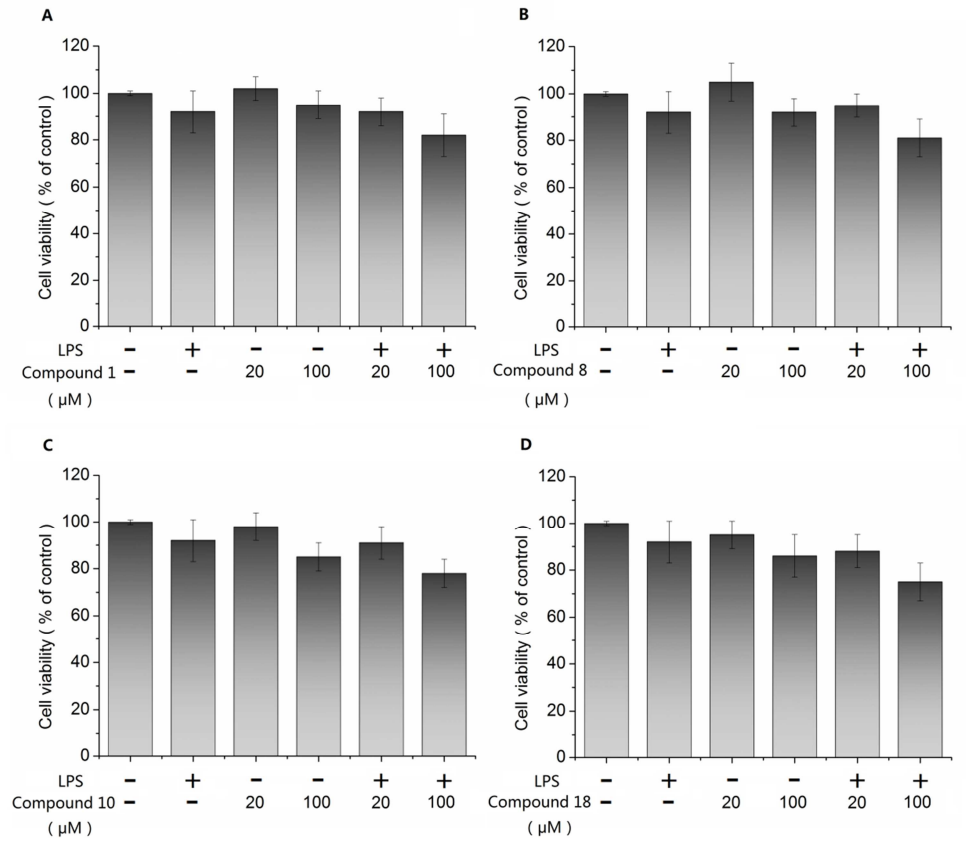


Fig.11 The effect of the compound on macrophage RAW 264.7 survival rate after treated for 24 h. In each experiment, four groups of parallel experiments were set, and the data were represented by Mean ±SD.

NO is one of the inflammatory mediators, and excessive NO up-regulates the level of inducible nitric oxide synthase (iNOS), which is a key step in the development of inflammation.[43-44] NO can be easily oxidized to nitrite by superoxide ions in the body. H_2S can not only quench peroxynitrite, but also react with NO to form nitromercaptan.[45-47] Therefore, the anti-inflammatory activities of the compounds as H_2S donors were evaluated by measuring the amount of nitrite.

LPS can activate iNOS to generate more NO. To make the effect more obvious, the macrophages activated by LPS were used to evaluate the anti-inflammatory activities of the compounds. The test results showed all the tested compounds reduced the nitrite levels in RAW 264.7 cells in a concentration-dependent manner, as shown in **Fig.12**. Among the tested compounds, compound **18** exhibited the strongest activity and decreased by about 50% nitrite level at 100 μM compared with the control. This is possibly because compound **18** has the largest H_2S release amount among four compounds.

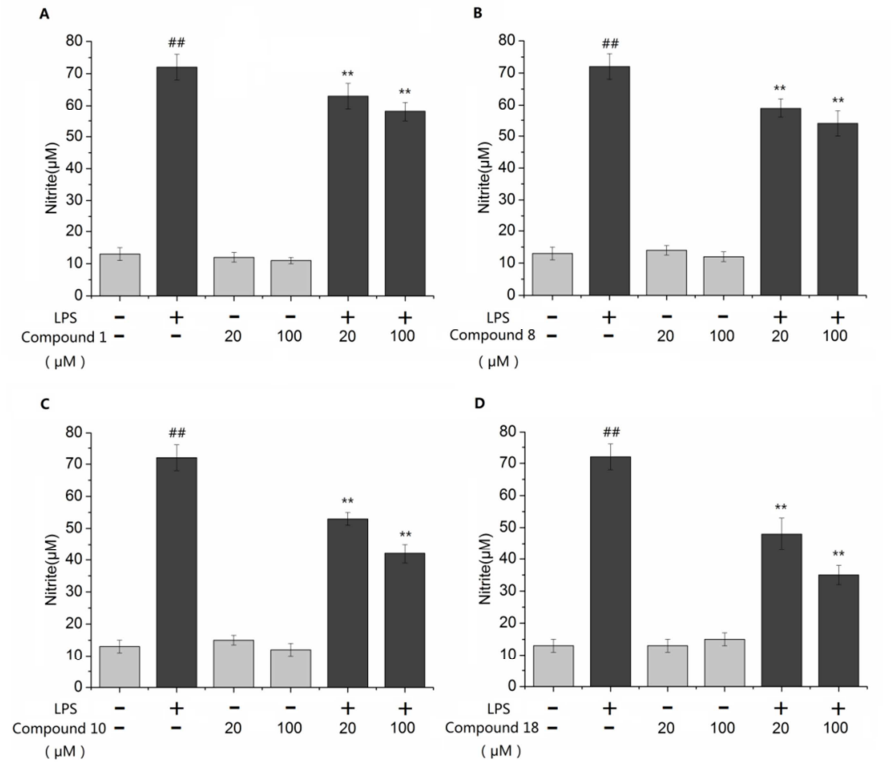


Fig.12 The effect of the concentration of compound on nitrite in macrophages RAW264.7 after treated for 24 h, A for compound **1**, B for **8**, C for **10** and D for **18**. Compared to untreated cells, $^{\#}p < 0.05$, $^{\#\#}$ $p < 0.01$, compared to LPS treated cells, * $p < 0.05$, ** $p < 0.01$.In each experiment, four groups of parallel experiments were set, and the data were represented by mean ±SD.

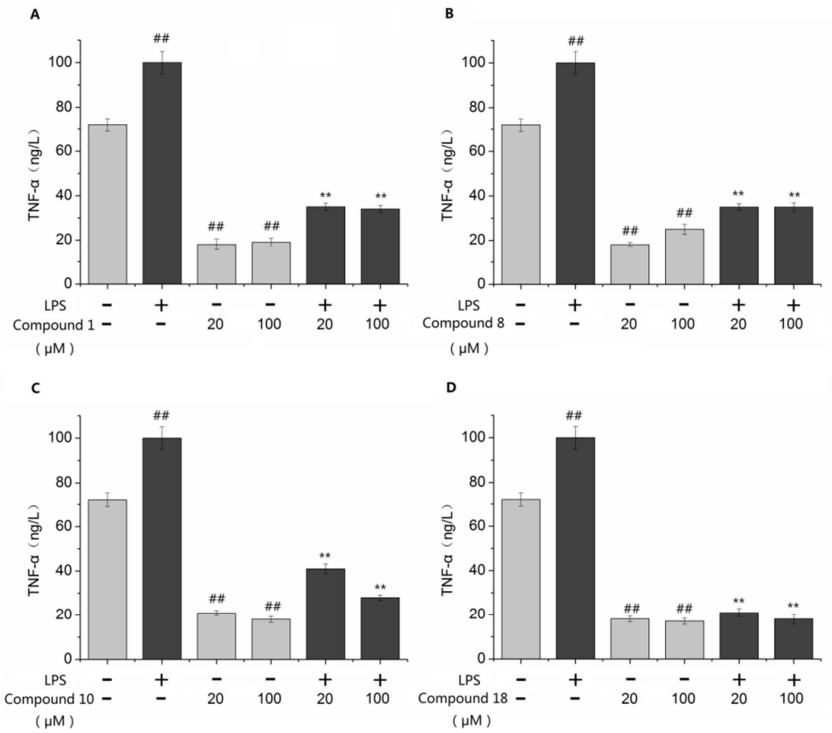


Fig.13 The effect of the concentration of compound on the level of TNF-α in macrophages after treated for 24 h; A for compound **1**, B for **8**, C for **10** and D for **18**. Compared to untreated cells, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to LPS treated cells, $^{*}p < 0.05$, $^{**}p < 0.01$.In each experiment, four groups of parallel experiments were set, and the data were represented by mean ±SD.

TNF-α is a tumor necrosis factor, which promotes T cells to produce various inflammatory factors, further promoting the occurrence of inflammatory reaction. As shown in **Fig.13**, all the tested compounds significantly reduced the level of TNF-α; among them, compounds **10** and **18** displayed stronger activities than compounds **1** and **8** when their concentrations were 20 μM. The level of TNF-α decreased to 20-30% of the control group. In contrast, compounds **1** and **8** reduced TNF-α level to 30-40% of the control group under the same conditions. IL-10 is an anti-inflammatory factor. The level of IL-10 in cell also is one of indexes of inflammation. Seen in **Fig.14**, all the tested compounds increased the level of IL-10 in the cells. Among four compounds, compounds **10** and **18** exhibited better activities than compounds **1** and **8**. When compounds **10** and **18** were 20 μM, the levels of IL-10 were two times as high as those in the control group. Therefore, we conclude compounds **10** and **18** have better anti-inflammatory activity than compounds **1** and **8**.

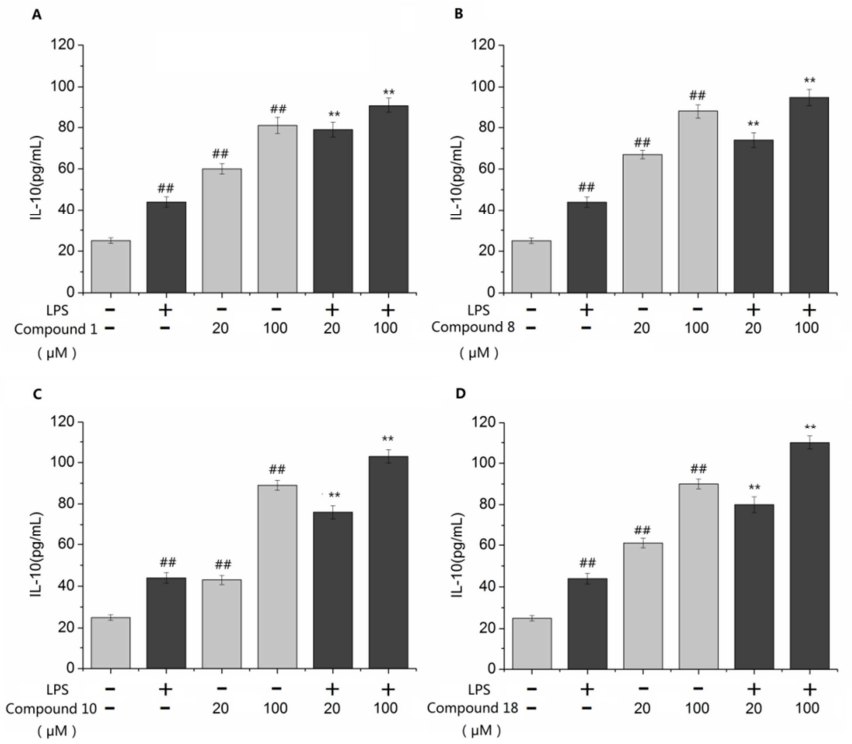
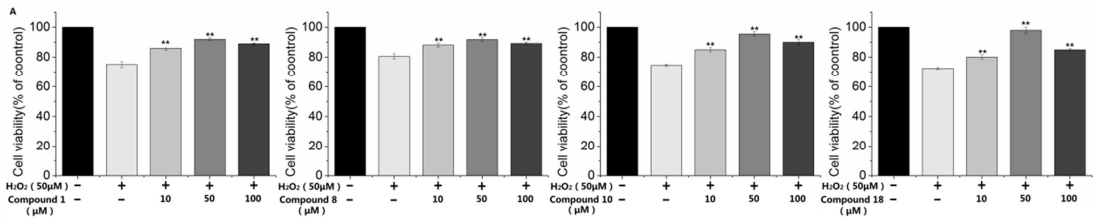


Fig.14 The effect of the concentration of compound on IL-10 expression in macrophages after treated for 24 h, A for compound **1**, B for **8**, C for **10** and D for **18**. Compared to untreated cells, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to LPS treated cells, $^{*}p < 0.05$, $^{**}p < 0.01$. In each experiment, four groups of parallel experiments were set, and the data were represented by mean ±SD.

In conclusion, all the tested compounds showed good anti-inflammatory activity. They all reduced nitrite content in macrophage RAW264.7, and compound **18** displayed the highest activity among them, which closely related to the maximum release of H_2S. The compounds reduced the level of TNF-α and increased the level of IL-10 in a dose-dependent manner, thus inhibiting inflammatory mediators and exerting anti-inflammatory activity. This further indicates that H_2S play an important role in the anti-inflammatory process.

8. Myocardial protection of compounds

In order to avoid the test error due to H9c2 cells death caused by the compound, we first use MTT method to measure the survival rate of H9c2 cells after treated with the compound. Seen from **SI-Fig.6**, the four compounds have no significant effect on the proliferation of H9c2 cells. When the concentration reached 100 μM, the survival rate still exceeded 90%. This indicates that the concentration below 100 μM is reliable and effective during the process of testing. Consequently, the tested compounds all were used below 100 μM in the process of following test.



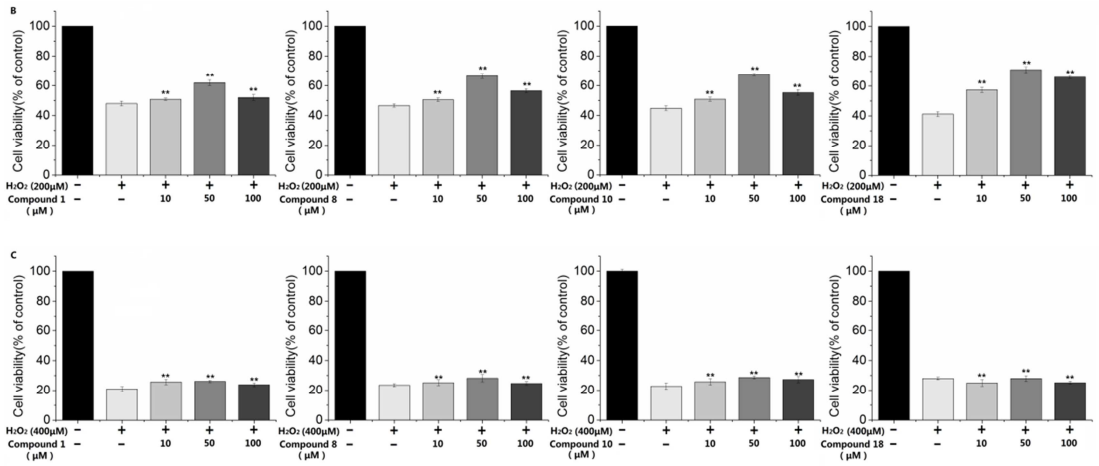


Fig.15 The effect of the concentration of compound on the H9c2 cell survival rate after treatment with H_2O_2 for 8h: A for 50μM; B: for 200 μM H_2O_2; C for 400 μM H_2O_2. Each bar represents the average of six independent experiments ±sd, compared with model group, * $p < 0.05$; ** $p < 0.01$.

In order to get the protective effect of the compound on myocardial injury more remarkable, the H9c2 cells were treated with different concentration H_2O_2 for 8 h.[48] Seen from **Fig.15**, after treatment with H_2O_2 of 50 μM, 200 μM and 400 μM for 8 h, the survival rates of H9c2 cells were 75.6%, 45.6% and 20.3%, respectively; however, after the H9c2 cells were incubated with tested compounds, the survival rates increased. When H_2O_2 was 50 μM, compound **1** of 50 μM made the cell survival rate increase by 17.1%; however, when H_2O_2 was 200 and 400 μM, the cell survival rates were only increased by 13.9% and 5.1%, respectively. The more serious the cell damage, the lower the protective effect of the compound. When H_2O_2 was 400 μM, the damage of the cells is too serious to repair. In the range of 10-100μM, the activity of compound **1** was not concentration-dependent. When H_2O_2 was 50 μM and 200 μM, the optimal dose of the compound was 50 μM, and when the concentration was increased to 100 μM, the protective effect of the cells began to decrease.

As for compound **18**, when H_2O_2 was 50 μM and 200 μM, compound **18** of 50 μM increased the survival rates of the cells by 25.5% and 30.1%, respectively. However, after the cells treated with 400 μM H_2O_2 for 8 h, the cell survival rate still decreased even though compound **18** arrived at up to 100 μM, even was lower than that of H_2O_2 control group. It is clear that H_2O_2 of 400 μM is beyond the safe concentration range of the cells, which is consistent with the results of toxicity test. Compared with compound **1**, compound **18** displayed higher activity than compound **1**. This is because compound **18** released more H_2S than compound **1**.

After the H9C2 cells treated with H_2O_2 and compound were stained with Gimsa dye for 30 min, microscope was used to observe morphology of H9c2 cells.

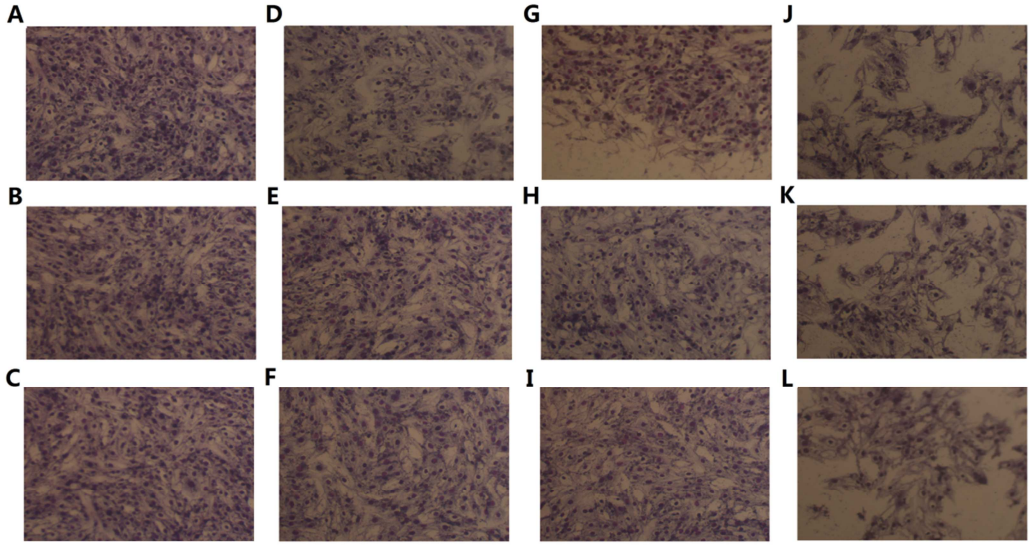


Fig.16 Morphology of H9c2 cells after treatment with H_2O_2-compound (50 μM), blank group (A, B and c); H_2O_2 group (D, G and J); H_2O_2-compound **1** group (E, H and K); H_2O_2-compound **18** group (F, I and L). Cells were cultured in a 24-well plate and treated for 24 h. From left to right, 50 μM, 200 μM and 400 μM H_2O_2 were used for 8h, respectively.

As shown in **Fig. 16**, in the control group, H9c2 cells were long fusiform, firmly adhered and not abnormal (**Fig. 16** (**A**, **B** and **C**)), but in H_2O_2 group, the cells were irregular, not closely arranged, and some cells became round (**Fig. 16** (**D**, **G** and **J**)). With the increase of oxidative damage of H_2O_2, more and more deformable cells were damaged and the damage was more and more serious. In the presence of compounds **1** or **18**, some of the injured cells caused by H_2O_2 returned to their original state. However, when H_2O_2 was 400 μM, most injured cells did not restore to their original state, which indicates that the damage of the cells was too serious and the compound did not display the protective role. The results correspond to the above experiments.

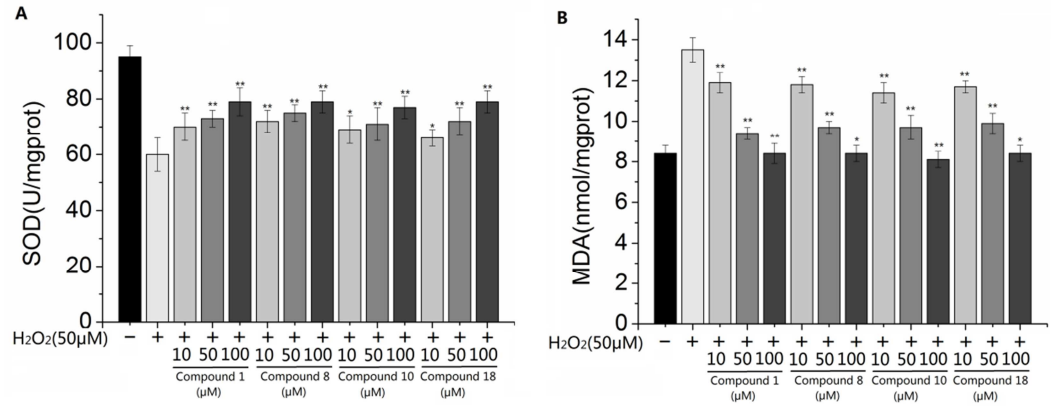


Fig.17 The effect of the concentration of compound on SOD and MDA levels in H9c2 cells: A: SOD; b: MDA. The data showed an average ±SD, n = 6. * $p < 0.05$ * $p < 0.01$.

Generally, the oxidative stress reaction happened in cells leads to cell damage and even cell death. MDA and SOD levels in cells are two indexes in oxidative stress reaction. [49-50] In order to seek the reason why the compound protected myocardial cells from oxidative injury, we measured the levels of SOD and MDA levels in H9c2 cells after treatment with H_2O_2-compound. The results show cell damage caused by H_2O_2 resulted in a decrease in the level of SOD in the cells, but in the presence of the tested compound, the SOD level increased distinctly (**Fig.17** (A)). Meanwhile, treatment of H9c2 cells with H_2O_2 resulted in an increase in MDA, but MDA levels significantly decreased after incubating with the compound (**Fig.17** (**B**)). Moreover, the antioxidant capacity was concentration dependent. Among the tested compounds, compound **18** showed better antioxidant activity than the others. All suggest the compounds protected myocardial cells from injure possibly by through increasing SOD level and decreasing MDA level.

Conclusion

H_2S is a gas signal molecule and has various biological activities like inhibiting the inflammatory response and inducing cancer cells apoptosis. H_2S plays an important role in the cardiovascular system and nervous system. H_2S donors are substitutes of H_2S. Many tests indicate H_2S donors have a big potential to be used in clinic.

In order to confirm whether the H_2S donors have drug-like properties, two series thiophosphamide H_2S donors were synthesized here. The drug-like properties involve several aspects, including bioactivity, toxicity, pharmacokinetic properties and so on. Because H_2S donors reported focused on synthesis and bioactivity, there is no any study about pharmacokinetic properties *in vivo*. Consequently, the pharmacokinetic property of the compounds is our main issue to be solved. We found pH value was different, the H_2S-release rates and amounts of the compounds were different, which suggests the compounds release H_2S differently in different organs and tissues *in vivo*. Importantly, each compound released H_2S in both PBS and organ and tissues in the same way. In rat models, the pharmacokinetic properties of the compounds vary with their structures: compound **1** had a certain protein binding rate in plasma; the plasma concentration reached maximum after administration 55 min and no compound **1** was detected in the plasma after 12 h; whereas compound **18**, the protein binding rate was high; the maximum plasma concentration reached after administration 100 min, and it was metabolized or eliminated completely 6 h later. In addition, in organs and tissues *in vivo*, the rates of release H_2S of the compounds were different from those *in vitro*, but we found the mechanism of H_2S release was the same.

In toxicity aspect, all the compounds displayed lower cytotoxicity, and displayed almost no toxicity to the mice in the process of single dose administration. But after successive administration for 14d, the number of white blood cells in the blood of rats reduced, and the liver and kidney of rats slightly damaged. In addition, when the compounds were below 0.5 μM, they had almost no effect on the hatching rate and survival rate of zebrafish embryos, the spontaneous movement of zebrafish embryos, and it did not caused embryo malformation. But when the concentration was over 1

μM, the compounds displayed significant inhibitory effect in the manner of concentration dependence. This suggests the compounds are safe for short-term period of application at low dose. Finally, we testified the compounds not only displayed better anti-inflammation activity, but also protected H9c2 cells from injure through increasing SOD level and decreasing MDA level, such as **18**.

In summary, based on the above, we draw a conclusion the compounds have druglike properties, and they have potential to be as candidates to investigate further. However, we have to see that the H_2S donor as a drug will be used in clinic, there's a long way to go. Since H_2S as an endogenous gas signaling molecule, has a variety of biological activities, such as anti-inflammatory, myocardial protection, anti-tumor and so on, what patients are fit to H_2S donor to deal with? This may be a question we will discuss in the future.

Experimental

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; RAW264.7 macrophages lines, HeLa, A549, HePG2, MCF-7, HT-29 and WI38 cell lines were 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 Bruker AM-400 MHz spectrometer. A Lambda 25 UV–Visible spectrophotometer and a Maxis-4G TOF Mass spectrometer (ESI) were used. Fluorescence intensity was recorded using an RF-5301PC fluorescence spectrophotometer. Pharmacokinetics of the compounds was studied using the Agilent 1260 ininfinity II LC and Agilent infinity Lab LC/MSD.

Preparation of compounds 1~9

Compound **1**:

A mixture solution of 3-aminopropanol (0.52 mL, 6.77 mmol) and triethylamine (2.0 mL, 9.0×10^{-5} mol) in CH_2Cl_2 was added dropwise to a solution of phenylthiono phosphonic dichloride (1mL, 6.77 mmol) in CH_2Cl_2 with stirring. The mixture was stirred for 12 h in room temperature, the precipitate was filtered off, the filtrate was washed with cold water (3x20 mL), dried with Na_2SO_4, and the solvent was removed in vacuum. The crude product was chromatographed on a silica gel (PE/EA 3:1), and a white granular solid was obtained. Yield: 37.4%. IR (KBr, cm^{-1}): 3274 (NH),1438 (P=S), 1120 (C-O); ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.79 (m, 2H, Ar-H), 7.42

(m, 3H, Ar-H), 4.42 (m, 1H, OCH_2), 4.02 (m, 1H, OCH_2), 3.38(br, 1H, NH), 3.11 (m, 2H, NCH_2), 2.00 (m, 1H, CH_2), 1.58 (m, 1H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 27.4, 45.6, 59.3, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2. ESI-HRMS (m/z): calcd. for $C_9H_{12}NOPSNa$ $[M+Na]^+$: 236.0275; found 236.0270.

The preparation and workup of compounds **2**-**9** were similar to that of compound **1.** The difference was using the corresponding amine instead of ethanolamine.

Compound **2**:

Compound **2** was obtained as light yellow viscous oil. Yield: 42.4%. IR (KBr, cm^{-1}): 3265 (NH), 1436 (P=S), 1121 (C-O); ^1H NMR (400 MHz, $CDCl_3$) δ7.72 (s, 1H, Ar-H), 7.45-7.28 (m, 4H, Ar-H), 3.92 (s, 1H, NH), 3.40 (q, J = 7.0 Hz, 1H, O-CH_2), 3.16-3.05 (m, 1H, O-CH_2), 2.08 (s, 1H, CH_2), 1.14 (dt, J = 13.9, 7.9 Hz, 1H, CH_2).^{13}C NMR (400 MHz, Chloroform-*d*) δ46.9, 67.6, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2. ESI-HRMS (m/z): calcd. for $C_8H_{10}NOPSNa$ $[M+Na]^+$: 222.0118; found 222.0121.

Compound **3**:

The preparation and workup of compound **3** were similar to compound **1** except 1,3-propanediol (0.4 ml, 6.63×10^{-6} mol) were used instead of 3-aminopropanol. Chromatography on silica gel with (PE/EA 5:1) as eluent gave the product. White oil was obtained. Yield: 35.4%. IR (KBr, cm^{-1}): 1440 (s,P=S), 1125 (s,C-O); ^1H NMR (400 MHz, Chloroform-*d*) δ7.88 (ddd, J = 14.6, 5.4, 3.4 Hz, 2H, Ar-H), 7.55 – 7.36 (m, 3H, Ar-H), 4.71 (dddd, J = 11.6, 9.9, 8.5, 3.0 Hz, 2H, O-CH_2), 4.19 (ddt, J = 18.3, 11.5, 4.4 Hz, 2H, O-CH_2), 2.18 (dtt, J = 14.3, 9.4, 4.3 Hz, 1H, CH_2), 1.87 (dtq, J = 14.5, 4.9, 2.7 Hz, 1H, CH_2); ^{13}C NMR (400 MHz, Chloroform-*d*) δ26.2, 58.2, 58.2, 128.7, 128.7, 129.0, 129.0, 129.0, 131.2. ESI-HRMS (m/z): calcd. for $C_9H_{11}O_2PSNa$ $[M+Na]^+$: 237.0115 ; found 237.0110.

Compound **4**:

The preparation and workup of compound **4** were similar to compound **1** except 1,3-diaminopropane(0.4 ml, 6.63×10^{-6} mol) were used instead of 3-aminopropanol. Chromatography on silica gel with (PE/EA 3:1) as eluent gave the product. A white granular solid was obtained. Yield: 42.0%. IR (KBr,cm^{-1}): 3252 (m, C-NH), 1436 (s,P=S); ^1H NMR (400 MHz, Chloroform-*d*) δ7.80 (dtd, J = 13.5, 4.9, 2.7 Hz,2H, Ar-H), 7.40 (dt, J = 5.3, 2.7 Hz, 3H, Ar-H), 3.31-3.15 (m, 2H, N-CH_2), 3.05-2.91 (m, 2H, N-CH_2), 2.86 (s, 2H, NH), 1.82-1.66 (m, 1H, CH_2), 1.46-1.35 (m, 1H, CH_2); ^{13}C NMR (400 MHz, Chloroform-*d*) δ25.8, 58.2, 58.2, 128.7, 128.7, 129.0, 129.0, 129.0, 131.2. ESI-HRMS (m/z): calcd. for $C_9H_{14}N_2PS$ $[M+H]^+$: 212.0537 ; found 213.0567.

Compound **5**:

The preparation and workup of compound 5 were similar to compound 1 except ethylene glycol (0.4 ml, 6.63×10^{-6} mol) were used instead of 3-aminopropanol. Chromatography on silica gel with (PE/EA 3:1) as eluent gave the product. A white

granular solid was obtained. Yield: 56.4%. IR (KBr,cm^{-1}): 1438.8 (s, P=S), 1118.2 (s, C-O); ^{1}H NMR (400 MHz, Chloroform-*d*) δ7.92-7.84 (m, 2H, Ar-H), 7.61-7.45 (m, 3H, Ar-H), 4.65-4.57 (m, 2H, O-CH_2), 4.47-4.36 (m, 2H, O-CH_2); ^{13}C NMR (400 MHz, Chloroform-*d*) δ63.4, 63,4, 128.7, 128.7, 129.0, 129.0, 129.0, 131.2. ESI-HRMS (m/z): calcd. for $C_8H_9O_2PSNa$ $[M+Na]^+$:222.9959; found 222.9906.

Compound **6**:

The preparation and workup of compound **6** were similar to compound **1** except ethylenediamine (0.4 ml, 6.63×10^{-6} mol) were used instead of 3-aminopropanol. Chromatography on silica gel with (PE/EA 3:1) as eluent gave the product. A yellow granular solid was obtained. Yield: 35.0%. IR (KBr, cm^{-1}): 3316.6 (m, C-NH), 1436.9 (s, P=S). ^{1}H NMR (400 MHz, Chloroform-*d*) δ7.92 (ddd, J = 14.8, 8.2, 1.6 Hz, 2H, Ar-H), 7.42-7.29 (m, 3H, Ar-H), 3.57-3.46 (m, 2H, CH_2), 3.35-3.26 (m, 2H, CH_2), 2.65 (d, J = 9.6 Hz, 2H, NH); ^{13}C NMR (400 MHz, Chloroform-*d*) δ45.7, 45.7, 128.1, 128.1, 131.2, 131.2, 131.2, 132.0. ESI-HRMS (m/z): calcd. for C $_8H_{11}N_2PSNa$ $[M+Na]^+$: 221.0278; found 221.0266.

Compound **7**:

The preparation and workup of compound **7** were similar to compound **1** except 2-(hydroxymethyl)pyrrolidine (0.6 ml, 6.63×10^{-6} mol) were used instead of 3-aminopropanol. Chromatography on silica gel with (PE/EA 4:1) as eluent gave the product. A white solid was obtained. Yield: 42.4%. IR (KBr, cm^{-1}): 3447.8 (m, C-NH), 1436.9 (s, P=S), 1114.5 (s, C-O). ^{1}H NMR (400 MHz, Chloroform-*d*) δ7.80 (dd, J = 15.0, 7.5 Hz, 2H, Ar-H), 7.41-7.25 (m, 3H, Ar-H), 4.23 (dddd, J = 22.3, 8.6, 6.2, 2.1 Hz, 1H, O-CH_2), 4.00 (qd, J = 7.1, 3.9 Hz, 1H, O-CH_2), 3.80-3.63 (m, 2H, N-CH_2), 2.88-2.71 (m, 1H, N-CH), 1.98-1.87 (m, 1H, CH_2), 1.83 (td, J = 8.2, 4.7 Hz, 2H, CH_2), 1.62 (dt, J = 11.8, 5.3 Hz, 1H, CH_2). ^{13}C NMR (400 MHz, Chloroform-*d*) δ24.3, 28.8, 48.1, 64.0, 70.7, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2.ESI-HRMS (m/z): calcd. for $C_{11}H_{14}NOPSNa$ $[M+Na]^+$: 262.0431; found 262.0369.

Compound **8**:

The preparation and workup of compound 8 were similar to compound 1 except 4-amino-1-butanol (0.4 ml, 6.63×10^{-6} mol) were used instead of 3-aminopropanol. Chromatography on silica gel with (PE/EA 3:1) as eluent gave the product. A white granular solid was obtained. Yield: 56.8%. IR (KBr, cm^{-1}): 3418.0 (m, C-NH), 1436.9.8 (s, P=S), 1120.1 (s, C-O); ^{1}H NMR (400 MHz, Chloroform-*d*) δ7.82 (ddt, J = 14.0, 6.7, 1.6 Hz, 2H, Ar-H), 7.38 (dddt, J = 12.6, 8.5, 6.1, 1.9 Hz, 3H, Ar-H), 4.58-4.46 (m, 1H, O-CH_2), 4.21-4.05 (m, 1H, O-CH_2), 3.13-2.96 (m, 1H, O-CH_2), 2.80-2.66 (m, 1H, O-CH_2), 1.91-1.82 (m, 1H, NH), 1.78 (dp, J = 13.2, 3.5 Hz, 2H, CH_2), 1.74-1.67 (m, 1H, CH_2), 1.58-1.45 (m, 1H, CH_2); ^{13}C NMR (400 MHz, Chloroform-*d*) δ27.8, 30.2, 37.5, 70.5, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2. ESI-HRMS (m/z): calcd. for $C_{10}H_{14}NOPSNa$ $[M+Na]^+$: 250.0431; found 250.0429.

Compound **9**:

The preparation and workup of compound 9 were similar to compound 1 except 3-aminobutan-1-ol (0.6 ml, 6.63×10^{-6} mol) were used instead of 3-aminopropanol. Chromatography on silica gel with (PE/EA 5:1) as eluent gave the product. White oil was obtained. Yield: 47.4%. IR (KBr, cm^{-1}): 3436.6 (m, C-NH), 1438.8 (s, P=S), 1161.1 (s, C-O). ^{1}H NMR (400 MHz, Chloroform-*d*) δ8.14-8.05 (m, 2H, Ar-H), 7.58-7.43 (m, 3H, Ar-H), 4.73-4.61 (m, 1H, O-CH_2), 4.31-4.18 (m, 1H, O-CH_2), 3.92-3.79 (m, 1H, CH), 2.39 (s, 1H, NH), 1.90-1.74 (m, 2H, CH_2), 1.21 (dd, J = 6.3, 2.1 Hz, 3H, CH_3); ^{13}C NMR (400 MHz, Chloroform-*d*) δ23.2, 42.6, 56.8, 57.8, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2. ESI-HRMS (m/z): calcd. for $C_{10}H_{14}NOPSNa$ $[M+Na]^+$: 227.0534.; found 227.0528.

Preparation of compounds 10~18

Compound **10**:

The phenylthionophosphonic dichloride (0.45 mL, 3 mmol) was dissolved in 3.5 mL anhydrous CH_2Cl_2. To the solution, 3-hydroxypropionitrile (0.2 mL, 3 mmol) and triethylamine (0.45 ml, 3 mmol) were added at 0□ under an atmosphere of Ar (g). The reaction was stirred at 0□ for 15 min and at room temperature for 3 h. A solution of glycine methyl ester (0.414 g, 3.3 mmol), triethylamine (1.25 ml, 9 mmol), and 3.5 mL anhydrous CH_2Cl_2 was added and the reaction was stirred for an additional 3 h. The mixture was diluted with 10 ml CH_2Cl_2, washed with 10 mL of 2M H_2SO_4, dried (Na_2SO_4), and concentrated under reduced pressure. The crude material was purified by flash chromatography to afford yellow oil. Yield: 35%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.77 (ddt, *J* = 14.2, 6.9, 1.5 Hz, 2H, Ar-H), 7.45-7.33 (m, 3H, Ar-H), 4.21 (dtd, *J* = 9.5, 6.2, 3.4 Hz, 2H, O-CH_2), 3.86 (dt, *J* = 9.5, 6.6 Hz, 1H, NH), 3.65 (dd, *J* = 12.7, 6.3 Hz, 2H, N-CH_2), 3.59 (d, *J* = 2.8 Hz, 3H, CH_3), 2.69 (t, *J* = 6.1 Hz, 2H, CH_2); ^{13}C NMR (400 MHz, Chloroform-*d*) δ20.8, 41.1, 51.6, 56.7, 117.7, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2, 168.5. ESI-HRMS (m/z): calcd. for $C_{12}H_{16}N_2O_3PS$ $[M+H]^+$: 299.0613.; found 299.0623.

2mL of freshly prepared 1 M aqueous LiOH solution was added dropwise to 2 ml of methanol containing 150 mg (0.5 mmol) yellow oil compound, and the resulting solution was stirred at room temperature for 12 hours and then concentrated to dryness. The obtained solid was suspended in anhydrous methanol, filtered (0.2 μm Teflon membrane) and concentrated. After lyophilization, the final product 10 was obtained as a white solid. Yield: 72%. IR (KBr, cm^{-1}): 3369.5 (m, C-NH), 1593.4 (s, COO), 1436.9 (s, P=S). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.76 (dt, *J* = 12.7, 5.7 Hz, 2H, Ar-H), 7.25 (d, *J* = 6.3 Hz, 3H, Ar-H), 3.17 (s, 1H, NH), 2.98 (q, *J* = 12.1, 7.4 Hz, 2H, N-CH_2); ^{13}C NMR (400 MHz, DMSO-d_6) δ43.3, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2, 173.1. ESI-HRMS (m/z): calcd. for $C_8H_7NO_3PS^{2-}$ $[M-H]^-$: 227.9895; found 228.0542.

The procedure and workup of compounds **11**-**18** were similar to compound **10**.

Compound **11**: Yield: 64%. IR (KBr, cm^{-1}): 3475.7 (s, NH), 1636.3 (s, COO), 1410.8 (s, P=S); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.72 (ddd, *J* = 25.6, 12.6, 6.7 Hz, 1H, Ar-H), 7.39 – 6.94 (m, 9H, Ar-H), 3.17 (s, 1H, NH), 3.05 – 2.92 (m, 1H, N-CH), 1.85 (s, 2H, CH_2); ^{13}C NMR (400 MHz, DMSO-d_6) δ38.6, 64.0, 125.9, 127.7, 127.7, 128.5, 128.6, 128.6, 128.7, 128.7, 131.2, 131.2, 131.2, 136.6, 178.4. ESI-HRMS (m/z): calcd. for $C_{15}H_{13}NO_3PS^{2-}$ $[M-H]^-$: 318.0365; found 318.0879.

Compound **12**: Yield: 74%. IR (KBr, cm^{-1}): 3472 (m, C-NH), 1634 (s, COO), 1418 (s, P=S); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.85 – 7.67 (m, 2H, Ar-H), 7.38 – 7.15 (m, 3H, Ar-H), 3.32 (s, 1H, NH), 2.45 (d, 1H, N-CH), 1.84 (m, 1H, CH), 0.98 – 0.54 (m, 6H, CH_3); ^{13}C NMR (400 MHz, DMSO-d_6) δ18.9, 18.9, 31.6, 62.7, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2, 174.7. ESI-HRMS (m/z): calcd. for $C_{11}H_{13}NO_3PS^{2-}$ $[M-H]^-$: 270.0365; found 270.0861.

Compound **13**: Yield: 45%. IR (KBr, cm^{-1}): 3428 (m, C-NH), 1641 (s, COO), 1416 (s, P=S). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.80 (dq, *J* = 17.8, 6.5 Hz, 2H, Ar-H), 7.34 – 7.16 (m, 3H, Ar-H), 3.17 (s,1H, NH), 2.59 (dd, *J* = 11.7, 5.6 Hz, 1H, N-CH), 1.20 – 0.83 (m, 3H, CH_3).^{13}C NMR (400 MHz, DMSO-d_6) δ19.1, 59.0, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2, 178.4. ESI-HRMS (m/z): calcd. for $C_9H_9NO_3PS^{2-}$ $[M-H]^-$: 242.0052; found 242.1047.

Compound **14**: Yield: 52%. IR (KBr, cm^{-1}): 3434 (m, C-NH), 1641 (s, COO), 1410 (s, P=S). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.93 (ddd, *J* = 12.5, 6.8, 3.1 Hz, 1H, Ar-H), 7.81 (ddd, *J* = 11.7, 6.7, 2.9 Hz, 1H, Ar-H), 7.26 (dt, *J* = 5.0, 2.5 Hz, 3H, Ar-H), 3.35 (t, *J* = 8.5 Hz, 2H, N-CH_2), 3.16 (s, 1H, N-CH), 2.48 (s, 2H, CH_2), 1.70 – 1.55 (m, 2H, CH_2).^{13}C NMR (400 MHz, DMSO-d_6) δ23.7, 30.7, 54.0, 69.0, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2, 178.4. ESI-HRMS (m/z): calcd. for $C_{11}H_{13}NO_3PS^{2-}$ $[M+H]^+$: 270.0365; found 270.0397.

Compound **15**: Yield: 67%. IR (KBr, cm^{-1}): 3293.7(m, C-NH), 1638.2 (s, COO), 1436.9 (s, P=S). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.05 – 7.45 (m, 2H, Ar-H), 7.36 – 6.83 (m, 3H, Ar-H), 3.04 – 2.84 (m, 1H, N-CH), 2.54-2.23(m,1H, CH),1.57 (s, 1H, NH), 1.33 – 0.92 (m, 2H, CH_2), 0.69 – 0.30 (m, 3H, CH_3), 0.27 – -0.10 (m, 3H, CH_3).^{13}C NMR (400 MHz, DMSO-d_6) δ22.9, 22.9, 24.8, 42.4, 60.0, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2, 178.4. ESI-HRMS (m/z): calcd. for $C_{12}H_{15}NO_3PS^{2-}$ $[M-H]^-$: 284.0521; found 284.1028.

Compound **16**: Yield: 48%. IR (KBr, cm^{-1}): 3419.8 (m, C-NH), 1638.2 (s, COO), 1422.0 (s, P=S). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.70 (s, 1H, NH), 7.92 – 7.85 (m, 1H, Ar-H), 7.41 (dt, *J* = 7.5, 4.5 Hz, 3H, Ar-H), 7.00 (d, *J* = 13.8 Hz, 3H, Ar-H), 6.90 – 6.80 (m, 2H, Ar-H), 3.18 (s, 1H, NH), 1.85 (s, 2H, CH_2), 1.26 (d, *J* = 16.6 Hz, 1H, CH).^{13}C NMR (400 MHz, DMSO-d_6) δ32.3, 66.0, 109.7, 111.1, 118.8, 119.8, 121.7,

123.0, 127.4, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2, 136.5, 178.4. ESI-HRMS (m/z): calcd. for $C_{17}H_{14}N_2O_3PS^{2-}$ $[M-H]^-$: 357.0474; found 357.0900.

Compound **17**: Yield: 52%. IR (KBr, cm^{-1}): 3404.2 (m, C-NH), 1636.3 (s, COO), 1420.1 (s, P=S). ^1H NMR (400 MHz, DMSO-d_6) δ 7.85 (ddd, *J* = 12.6, 6.7, 2.8 Hz, 2H, Ar-H), 7.25 (tq, *J* = 5.5, 2.5 Hz, 3H, Ar-H), 3.21 – 3.09 (m, 1H, N-CH), 2.78 (td, *J* = 10.8, 7.1 Hz, 1H, CH), 1.72 (s, 2H, CH_2), 1.29 (q, *J* = 9.9, 7.7 Hz, 1H, CH), 0.92 – 0.72 (m, 3H, CH_3), 0.61 – 0.47 (m, 3H, CH_3); ^{13}C NMR (400 MHz, DMSO-d_6) δ11.3, 15.0, 25.1, 38.1, 67.0, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2, 178.4. ESI-HRMS (m/z): calcd. for $C_{12}H_{15}NO_3PS^{2-}$ $[M-H]^-$: 284.0521; found 284.0960.

Compound **18**: Yield: 67%. IR (KBr, cm^{-1}): 3431.0 (m, C-NH), 1638.2 (s, COO), 1418.3 (s, P=S). ^1H NMR (400 MHz, DMSO-d_6) δ 7.88 – 7.75 (m, 2H, Ar-H), 7.24 (s, 3H, Ar-H), 3.18 (dd, *J* = 12.4, 6.0 Hz, 1H, N-CH), 2.09 (q, *J* = 9.8, 7.5 Hz, 2H, CH_2), 1.93 (s, 1H, NH), 1.78 (s, 3H, CH_3), 1.70 (s, 2H, CH_2).^{13}C NMR (400 MHz, DMSO-d_6) δ15.4, 29.7, 32.8, 61.0, 128.5, 128.7, 128.7, 131.2, 131.2, 131.2, 178.4. ESI-HRMS (m/z): calcd. for $C_{11}H_{13}NO_3PS_2^{2-}$ $[M-H]^-$: 302.0085; found 302.0467.

H_2S measurement

1.5 mL of the probe solution dissolved in a buffer solution concentration of 400 μM was added to the quartz cuvette, and then the Na_2S solution dissolved in a buffer solution concentration of 400 μM was also added to the quartz cuvette, and finally the buffer solution was used to make the total volume reached 3 mL. The final probe concentration was 200 μM and the final Na_2S concentrations were 0, 20, 40, 80, 160 and 200 μM, respectively, depending on the amount of Na_2S added. Fluorescence readings for each Na_2S concentration were measured by a fluorescence spectrophotometer, three measurements per concentration, and three readings per measurement.

First, prepare a buffer solution of different pH buffer and acetonitrile (1: 9 by volume), then add 1.5 mL of the probe solution dissolved in a buffer solution of 400 μM to the quartz cuvette, and then the quartz ratio 30 μL of a 15 mM hydrogen sulfide donor solution dissolved in a buffer solution was added to the dish, and finally a buffer solution was added to make a total volume of 3 mL, a probe concentration of 200 μM, and a hydrogen sulfide donor compound concentration of 150 μM. During the experiment, the quartz cuvette was placed in a water bath at room temperature of 22 °C or 37 °C, and then the fluorescence readings of the samples were recorded three times at intervals by a fluorescence spectrophotometer. The amount of H_2S released was calculated by a standard curve (SI-2).

Determination of the distribution coefficient of lipid water

The *n*-octanol and the twice distilled water were shaken at room temperature for 24 hours with a constant temperature (37±1) °C shaker to saturate each other. After standing overnight for stratification, the two phases were separated and stored for

later use. Accurately weigh the appropriate amount of the test compound in a 10 mL volumetric flask, dissolve it with water-saturated *n*-octanol, shake it for 30 min, and dilute to give a solution with a concentration of 1 mM.

Separate a certain amount into a 10 mL volumetric flask with a pipette, dilute with water-saturated *n*-octanol and dilute to a concentration of 6-90 μM. The concentration gradient is 6 μM. Scanning in the wavelength range of 400 nm, taking the absorbance at the maximum absorption peak to obtain the standard regression equation of the compound: A=a+bC, where A is the absorbance, C is the sample concentration (M), and a and b are the fitting coefficients.

750 μL and 1500 μL of the total solution were accurately weighed in a 250 mL volumetric flask with a pipette, dissolved in water-saturated *n*-octanol, sonicated for 30 min, and made up to a concentration of 30 μM and 60 μM solution, respectively. Then take 5ml 3 parts of the solution and 5 mL of *n*-octanol-saturated water in a constant temperature shaker, shake at room temperature for 24 h, and centrifuge for 20 min to separate the two phases. The ultraviolet absorption spectra of the compounds in the organic and aqueous phases were determined at room temperature using an ultraviolet-visible spectrophotometer. The concentration of the compound in the oil phase and the aqueous phase can be calculated according to the standard regression equation, and then the lipid-water partition coefficient of the compound is calculated according to the formula logP=log (c organic phase /c aqueous phase).

Toxicity test

(1) **Cytotoxic assay**

The HeLa, A549, HePG2, MCF-7, HT-29 and WI38 cells were cultivated at 37°C, 10% CO_2, 100% humidity in RPMI 1640 medium, enriched with glucose and supplemented with 10% fetal bovine serum, non-essential amino acids, antibiotics (penicillin/streptomycin) and antifungals. Typically, 0.08 mmol of the compound was dissolved in 0.5 mL of DMSO, which after homogenization was injected over 10 mL of culture medium with vigorous stirring at 55°C. Growth inhibitory effect toward HeLa, A549, HePG2, MCF-7, HT-29 and WI38 cells line was determined by means of MTT colorimetric assay. The cells were seeded into 96-well plates at 6×10^3 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 24 h. Cell viability was quantified after 24 h by the MTT method. Descriptive data were expressed as mean± standard deviation and values IC_{50} were carried out using SPSS 20.0 software.

(2) **Multiple dose toxicity**:

We selected Wistar rats to study the toxicity of target molecules through multiple doses. The compound **1** and **18** was dissolved in 10% DMSO to prepare a standard solution of the compound, and then normal Wistar rats were administered by intraperitoneal injection according to the dose of 0.01 mol•kg^{-1}, and the control group was administered to the rats by intraperitoneal injection. An equal amount of 10% DMSO blank solution (6 rats per group) was administered continuously for 14 days. After the end of the administration, blood was collected through the eyeball, and the liver, kidney and other tissues of the rat were collected, and then the tissue was immediately fixed using a 4% glutaraldehyde solution. After sectioning, the physiological condition of the liver and kidney tissues of the rats was observed by HE staining. It was judged whether the target compound had an effect on the structure and function of the liver and kidney of the rats. Moreover, the blood of the rat was analyzed by a biochemical instrument to further evaluate the effect of the target compound on the physiological condition of the rat.

(3) **Zebrafish toxicity test**

Adult zebrafish of the wild-type strain (AB) were raised and maintained at 28±1°C with a 14 h light/10 h dark photoperiod (lights on at 8:00) in a recirculation system. The fish water supplied to the system was filtered by reverse osmosis (pH 6.5–7.5), 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.[51] The zebrafish embryos were obtained from spawning adults in tanks overnight with a sex ratio of 1:1. The embryos were collected within 1 h after the light was switched on and washed using standard zebrafish E3 culture 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.[52]

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. [53] 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 (5 min) 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 malformations of the zebrafish were observed using a stereoscopic dissecting microscope.

Anti-inflammatory test

(1) Anti-inflammatory activity 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 2 mM 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 (1 μg mL^{-1}) in the presence or absence of compound **1**, **8**, **10** or **18** (20 and 100 μM), cell viability, nitrite levels and TNF-α and IL-10 protein expression were determined at the end of the incubation. In each experiment, compound **1**, or **8**, or **10** or **18** (20 and 100 μM) was added to cells 24 h after incubation with LPS for 4 h.

(2) Cell viability

Cell viability was determined using MTT method which previously reported. [54]The assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes; 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 24 h in 96-well plate, MTT was added to each well (20 μL per well) for 4 h, after that discard the culture, and then DMSO was added to each well (100 μL 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 570 nm on a microplate reader and expressed as a percentage of control.

(3) Assay for nitrite levels

Nitrite levels were determined using the Griess method. [55] 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 (50 μL 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).

(4) 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°C. 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.

(5) Determination of four cytokines (TNF-α and IL-10) levels

The level of two 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 0.1 mg 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 two cytokines produced. The two cytokines levels which in each sample was calculated from a standard curve generated with standard solution.

Pharmacokinetics of the compounds

Nine male WISTAR rats were randomly divided into three groups, blank group, compound-**1**-group and compound-**18**-group; each group has 3 rats. Rats were injected intraperitoneally with compound **1** or **18** of 200 mg/kg, and those in the blank group were injected with the solution without containing the compound **1** or **18**. At 20 min, 40 min, 80 min, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h and 24 h after administration, about 0.3 mL blood was taken from canthus eye, respectively; then anticoagulant was added to the blood. After centrifugation, the plasma of 0.1 mL was taken and 400 μL dichloromethane was added. Centrifugation for 10 min after vortex mixing, the rotational speed was 10000 rpm. After the lower layer solution separated, it was dried with nitrogen. The residue was dissolved with 500 μL methanol. After centrifugation, the supernatant of 10 μL was taken and analyzed by LC-MS. The peak areas of the compound and external standard in the LC-MS results were used to calculate the blood compound concentration. Finally, the curve of blood concentration of the compound vs time was obtained.

Myocardial protection test

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 μg•mL^{-1} of streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 .

Cardiomyocyte survival rate: The H9c2 cells were cultured in 96 well plates at a density of 5 × 10^4 cells /mL and cultured for 8 h at 37°C and 5 % CO_2 incubator for 8 h. The tests were divided into blank group, H_2O_2 group, compound (10 μM, 50 μM and 100 μM)-H_2O_2 group. H_2O_2 was 50 μM, 200 μM and 400 μM, and the control group only added cells without the compounds. The cells survival rate was detected after 24 h. After cells were treated with H_2O_2 for 8 h, the culture medium was discarded and the H_2O_2 group was supplemented with fresh medium. Then the cell viability was measured after 24 h of incubation.

Assays for enzyme activities: H9c2 cells were cultured in 6-well plates overnight and then treated with H_2O_2 (50 μM) for another 8 h. Then compound **1**, or **8**, or **10** or **18** (10, 50, and 100 μM) was added and the cells were cultured for additional 24 h. The activities of SOD and the MDA content were measured using commercial kits following the manufacturer's instruction (Nanjing JianCheng Bioengineering Institute, Nanjing, China).

Conflict of Interest

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

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Highlights

- ►First evaluated the toxicity of two series of thiophosphoramide H_2S donors
- ►Investigate the release mechanism of two series of thiophosphoramide H_2S donors
- ►Exploring the pharmacokinetics of two series of thiophosphoramide H_2S donors