RESEARCH ARTICLE

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2017, 8, 2238Identification of novel *N*-acetylcysteine derivatives
for the treatment of hepatocellular injuryShourong Liu,^{†a} Yanmei Zhao,^{†a} Ruoyu He,^a Limin Kong,^b Jianjun Xi,^a
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New anti-hepatocellular injury drugs with better curative effects and fewer side effects are urgently needed at present. In this study, a series of novel *N*-acetylcysteine (NAC) derivatives were designed, synthesized and biologically evaluated for their anti-hepatocellular injury activities against two different cell models. In the biological evaluation against hydrogen peroxide (H₂O₂)-induced LO2 hepatocytes, half of the target compounds exhibited moderate to potent activities in improving the model cell viability, and two compounds (**6a** and **6b**) displayed more potent activities in decreasing malondialdehyde (MDA) levels than the positive control NAC. In further 4-acetamidophenol (APAP)-induced LO2 cell experiment, compounds **6a** and **6b** could not only improve the cell viability but also significantly reduce the secretion of MDA. Additionally, compound **6a** displayed excellent Caco-2 permeability and oral bioavailability in rats. All these experimental results suggested that compounds **6a** and **6b** could serve as potential lead molecules for further development of anti-hepatocellular injury drugs.

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1. Introduction

Liver diseases are highly prevalent all over the world. Regardless of different etiologies, inflammation and oxidative stress (OS) are the most important pathogenetic events in hepatic diseases.^{1–3} Chemical drugs, re-oxidation after hypoxia and all kinds of acute or chronic inflammations can cause oxidative damage to hepatocytes, accompanied by the changes of the cellular structure and function, which allows the liver cells to produce excessive reactive oxygen species (ROS).^{4–6} Several studies on hepatic diseases indicated that cells have intrinsic antioxidant mechanisms, such as peroxidases, catalases and superoxide dismutases to scavenge ROS. Among these antioxidant systems, the most abundant and outstanding cellular thiol antioxidant glutathione (GSH) exhibits numerous and versatile functions and therefore protects cells against oxidative stress.⁷

As an antioxidant, *N*-acetylcysteine (NAC) could directly increase intracellular GSH, especially on hepatic tissue, and it has been in clinical practice for several decades with relatively low toxicity and good efficacy. As a small molecule, NAC has been elucidated to interact with numerous meta-

bolic pathways including, but not limited to, regulation of the cell cycle and apoptosis, carcinogenesis and tumor progression, mutagenesis, gene expression and signal transduction, immune modulation, cytoskeleton organization and trafficking and mitochondrial functions.^{8–10} The molecular mechanisms of NAC are complex which makes it have many metabolic pathways. The insignificant route under physiological conditions of NAC is serving as a precursor of cysteine for GSH synthesis which can help in the detoxification of reactive metabolites and scavenging of free radicals.¹¹ The second important mechanism of NAC is attributed to the anti-oxidative activity of its sulfhydryl group, which has a fast binding rate to 'OH, 'NO₂, CO₃[–] and thiyl radicals as well as restoration of impaired targets in vital cellular components.¹² In the meantime, the uniqueness of NAC is most probably due to efficient reduction of disulfide bonds in proteins thus altering their structures and disrupting their ligand bonding and competition with larger reducible molecules in sterically less accessible spaces. Possible chemical and biochemical routes involving NAC are summarized in Fig. 1.¹³

At present, administered NAC has been mostly used as a mucolytic agent. IV NAC is used for various types of liver injury and the early stage of liver failure on the basis of comprehensive treatment in order to reduce bilirubin and increase prothrombin activity.¹⁴ According to the literature, the human plasma terminal half-life of NAC after single intravenous administration is 5.6 h where 30% of the drug is cleared by renal excretion. NAC's oral bioavailability is less than 5%, which is mainly thought to be associated with its

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Molecular mechanisms of NAC

Metabolic pathways affected by NAC

Fig. 1 Metabolic pathways affected by NAC and its molecular mechanisms.

N-deacetylation in the intestinal mucosa and first pass metabolism in the liver.¹⁵ Studies indicated that NAC is negatively charged under physiological conditions and its neutral, membrane permeating form constitutes as little as 0.001% of the total NAC.¹⁶ Therefore, in order to achieve a certain good clinical efficacy, higher doses and longer treatment cycles should be performed, which also bring more drug toxicity risks. The side effects that accompany the use of high doses of IV NAC include rash, pruritus, angioedema and bronchospasm, while oral administration of NAC may be associated with vomiting and diarrhea along with unpleasant odor.

Therefore, more effective and safer drugs are urgently required, which would be of great value for the treatment of various diseases, especially for liver injury. To improve the stability and activity of NAC, structure optimization was performed at the carboxyl, sulfhydryl and acetyl groups in this research (Fig. 2). Firstly, different carboxylic esters of NAC were designed and synthesized to increase the molecular permeation rate through the biological membranes by adjusting the molecule pK_a . Additionally, methyl-substituted thiol could not only prevent the self-oxidation and improve the stability of the compound, but also exhibit an antioxidant effect after demethylation by demethylase *in vivo*.¹⁷ Finally,

in order to prevent NAC from *N*-deacetylation in the intestinal mucosa and first pass metabolism in the liver, acetyl was replaced with other more stable acyl, which was expected to prolong the compound retention time *in vivo* and improve its bioavailability, thus overcoming the adverse side effects of this drug.

2. Synthesis

The synthetic route for all the target compounds is outlined in Scheme 1. Firstly, Fmoc-Cys(Me)-OH treated with corresponding carboxylic acid or ester in the presence of *N,N*-diisopropylethylamine (DIEA) and *N*-methylmorpholine (NMM) to give the target compounds 2a–e by using standard Fmoc-chemistry. The target compounds 2a–e were also important intermediates for the next step, which were then treated with HCl and MeOH to afford methyl ester analogues 3a–e. As illustrated in Scheme 1, the treatment of 2a–e with 2-*tert*-butyl-1,3-diisopropyl-isourea provided *tert*-butyl ester compounds 4a–e, while those reacted with methylamine hydrochloride, cyclohexylamine and aniline afforded target compounds 5a–c and 6a–c, respectively, with suitable reagents and under appropriate conditions. The treatment of 2a–e with aniline and HATU afforded target compounds 7a and 7b with the structure of phenyl amide. Commercially available *N*-(*tert*-butoxycarbonyl)-*S*-methyl-L-cysteine 8 was converted to phenyl amide 9 in the presence of NMM and IBCF at 15 °C which was then deprotected with TFA to afford amine 10. Subsequently, the condensation reaction of compounds 10 and benzoyl chloride provided target compound 7c.

3. Pharmacology

3.1 Cell viabilities and MDA measurement on the H₂O₂-induced LO2 cell injury model

Human hepatocyte cell line LO2 was used to build a hepatocyte injury model by H₂O₂ treatment. Cell Counting Kit-8 (CCK-8) was utilized to detect cell proliferation and determine the optimal damage conditions. All the target compounds were screened for their cell viabilities on this model

Fig. 2 Design of novel NAC derivatives.

Scheme 1 Synthesis of target compounds **2a–e**, **3a–e**, **4a–e**, **5a–c**, **6a–c** and **7a–c**. Reagents and conditions: (I) DIEA, Fmoc-Cys(Me)-OH, NMM, DMF, DCM, rt; (II) HCl, MeOH, 15 °C; (III) 2-*tert*-butyl-1,3-diisopropyl-isourea, DCM, 80 °C to rt; (IV) methylamine hydrochloride, EDCl, HOBt, DMF, DIEA, 15 °C; (V) NMM, IBCF, THF, 0 °C; (VI) aniline, HATU, DMF, 15 °C or aniline, EDCl, pyridine, 15 °C; (VIII) TFA, DCM, 15 °C; (IX) benzoyl chloride, NaHCO₃, THF, H₂O, 0 °C.

using NAC as the positive control, and the results are illustrated in Fig. 3.

As the results displayed in Fig. 3, compared with the model group, some of the target compounds (**2a**, **2b**, **2c**, **2d**, **5c**, **6a**, **6b** and **7a**) exhibited moderate to potent cell protective and repair effects against H₂O₂-treated LO2 cells. In Fig. 3(a), compounds **2a–e** had a slight improvement in cell viability when the carboxyl groups were retained and different substituents were introduced into the R_1 position, while compounds **3a–e** with carboxylic methyl ester had slight inhibitory effects on cell proliferation. Additionally, compound **2a** could slightly improve the cell viability and reduce the MDA level, which indicated that the increase of the antioxidant damage ability at the cell level was limited after the introduction of methyl to the R_2 position compared with NAC. In Fig. 3(b),

the inhibition of cell viability increased from compound **4a** to **4e** with *tert*-butyl ester being substituted at the R_3 position. In contrast, the inhibitory activity decreased from compound **5a** to **5c** with formamide at the R_3 position, which indicated that the structure–activity relationship of these two series of compounds is not clear and compounds **4a–e** have a certain degree of cytotoxicity. Among the formamide derivatives (**5a–c**), compound **5c** with phenyl at the R_1 position has a certain recovery effect on the cell viability, which displayed more potent cell protective activity than compounds **5a** and **5b**. Compounds **6a–c** and **7a–c** with different substituents at the R_1 position, displayed the same regularity, in which methyl-replaced analogues (**6a** and **7a**) exhibited more potent cell protective activities than ethyl-substituted compounds (**6b** and **7b**), and compounds with benzene at this position (**6c**

Fig. 3 Effect of NAC and target compounds on cell proliferation in H₂O₂-treated LO2 cells. (a) Viabilities of the H₂O₂-treated LO2 cell line cultured with compounds 2a–e and 3a–e. (b) Viabilities of the H₂O₂ treated LO2 cell line cultured with compounds 4a–e and 5a–c. (c) Viabilities of H₂O₂-treated LO2 cell line cultured with compounds 6a–c and 7a–c. **, significantly different from the model group with $p < 0.01$; ***, significantly different from the model group with $p < 0.001$.

and 7c) displayed the worst potent activities. These data also suggested that minor changes at the R₁ position could greatly influence the cell viabilities of the target compounds (4a–b, 4d, 6a–c and 7a–c) when R₃ was substituted with *tert*-butyl, phenyl or cyclohexyl. Among all these derivatives (except 2a and 5a), the ones substituted with methyl at the R₁ position displayed more potent activities than the other ones. The most potent compound 6a, whose cell protective and repair activity was superior to the model group, deserved further study with regard to its application potential in the treatment of anti-hepatic injury.

Based on the results of cell viabilities of all the target compounds, selected analogues (2a–e, 5b–c, 6a and b and 7a) were screened for their effects on malondialdehyde (MDA) secretion in H₂O₂-treated LO2 cells using a lipid oxidation (MDA) assay kit using NAC as the positive control. The results are shown in Fig. 4. Except for compound 5c, all the selected compounds could decrease the MDA level in the LO2 cells injured by H₂O₂ compared to the model group. In particular, compounds 6a, 6b and 7a exhibited more potent MDA secretion inhibitory activities than the positive control, which indicated that these active compounds could protect injured liver cells through inhibition of MDA secretion.

3.2 Cell viabilities and MDA measurement on the APAP-induced LO2 cell injury model

To further confirm the cell protective activities of compounds 6a, 6b and 7a on oxidatively injured liver cells, an additional experiment was performed to determine the effects of the three selected compounds on APAP-induced LO2 injured cells. As displayed in Fig. 5, compared with the model group, compounds 6a and 6b exhibited moderate improvement in cell viability while 7a had almost no effect. At the same time, the effects of compounds 6a, 6b and 7a on MDA secretion in APAP-treated LO2 cells were studied by using the lipid oxidation (MDA) assay kit. As shown in Fig. 6, compared with the model group, compounds 6a, 6b and 7a could significantly reduce the level of MDA in the APAP-treated LO2 cells. However, the recovery ability of compounds 6a and 6b was no better than that of the positive control NAC.

3.3 ADME properties

The preliminary ADME profiles of compound 6a were evaluated by determining its Caco-2 permeability (Table 1) and pharmacokinetic properties (Table 2). As displayed in Table 1, compound 6a exhibited a better Caco-2 permeability compared to the positive control NAC, which also validated the initial structural optimization ideas. In addition,

MDA secretion in H₂O₂ induced hepatocellular injury model

Fig. 4 Effect of NAC and selected compounds on MDA secretion on H₂O₂-treated LO2 cells.

Fig. 5 Effect of NAC and selected compounds on cell viability in APAP-induced LO2 cells. *, significantly different from the model group with $p < 0.05$.

Fig. 6 Effect of NAC and selected compounds on MDA secretion in APAP-treated LO2 cells. ***, significantly different from the model group with $p < 0.001$.

as the pharmacokinetic data depicted in Table 2, the terminal half-life of compound 6a after single oral administration was 5.62 h and its oral bioavailability could reach 52.8% in SD rats.

4. Conclusion

A novel series of NAC derivatives were designed, synthesized and biologically evaluated for their cell protective activities on H_2O_2 -treated LO2 hepatocytes, and half of the derivatives exhibited moderate to potent activities. Additionally, selected ten compounds were further screened for their effect on MDA secretion. What encouraged us was that three com-

pounds (6a, 6b and 7a) exhibiting more potent activities than the positive control NAC were successively obtained. In further APAP-treated LO2 cell study, compounds 6a and 6b could not only improve the cell viability but also significantly reduce the level of MDA. However, the effect of different substituents on the selectivity and pharmacological activity of target compounds was not very clear in this study, and the main reason for this unclear structure–activity relationship was that the target compounds were pharmacologically screened on the cell model. The cell viabilities of various target compounds were affected by both the electronegativity and size of different substituents and the physicochemical properties of these compounds. If the tests were carried out at a molecular level, the structure–activity relationship would be more explicit, and we will focus on this issue in subsequent research.

In the ADME studies, compound 6a showed excellent Caco-2 permeability and good oral bioavailability in rats. Overall, although we did not obtain a compound significantly superior to the precursor NAC in pharmacodynamics, the membrane permeability and pharmacokinetic profiles of compound 6a were significantly better than those of NAC. All these results suggested that compound 6a could be a potential lead molecule for further structural optimization and investigation.

5. Experimental procedures

5.1 Chemistry and general methods

Mass spectra (MS) were taken in the ESI mode on an Agilent 1100 LC-MS (Agilent, Palo Alto, CA, U.S.A.). 1H NMR and ^{13}C NMR spectra were recorded on Bruker 400 MHz spectrometers (Bruker Bioscience, Billerica, MA, USA) with TMS as an internal standard and $CDCl_3$ or $DMSO-d_6$ as solvents. Coupling constants (J) were reported in Hertz (Hz). Splitting patterns were designated as singlet (s), broad singlet (brs), doublet (d), double doublet (dd), triplet (t), quartet (q), and multiplet (m). All materials were obtained from commercial suppliers and were used without further purification. The reaction time and purity of the products were monitored using TLC on FLUKA silica gel aluminum cards (0.2 mm thickness) with a fluorescent indicator of 254 nm. Column chromatography was run on silica gel (200–300 mesh) from Qingdao Ocean Chemicals (Qingdao, Shandong, China). All yields were unoptimized and generally represent the result of a single experiment.

Table 1 Caco-2 permeability of compound 6a

Compound	A–B permeability ^a (Caco-2, $cm\ s^{-1}$) (10^{-6})	B–A permeability ^a (Caco-2, $cm\ s^{-1}$) (10^{-6})	Papp (B–A)/Papp (A–B)
6a	19.73	21.81	1.11
NAC	<1.09 ^b	<0.61 ^b	N/A

^a Each value is an average of $n = 3$, measured at $c = 10\ \mu M$. ^b Papp values were expressed as “<” the values that were calculated using the minimum concentration of the standards for receiver sides due to the fact that the real concentrations in the receivers were below quantification limit (BQL). N/A: Not acquired.

Table 2 Pharmacokinetic data of compound **6a**

Compound	Species	$C_{\max}^a$ (ng mL ⁻¹)	Terminal $t_{1/2}^a$ (h)	AUC ^a (ng h mL ⁻¹)	F^a (%)
6a	SD rat ^b	2138 ± 233	5.62 ± 0.501	3082 ± 446	52.8 ± 4.64

^a Data represent means ($n = 5$). ^b Male.

5.2 Synthetic procedures

5.2.1 Method for the preparation of compound *N*-acetyl-*S*-methyl-*L*-cysteine (2a**).** Compound **2a** was synthesized using standard Fmoc chemistry. The mixture of chlorotrityl chloride (CTC) resin (18.2 mmol), Fmoc-Cys(Me)-OH (5.2 g, 41.6 mmol, 0.8 eq.) and DCM (200.0 mL) was stirred at 25 °C in a vessel for 12 h under a N₂ atmosphere, then DIEA (3.2 eq.) was added dropwise and the mixture was stirred for another 2 h. Then, MeOH (20.0 mL) was added, and the mixture was stirred for 30 min and washed with DMF (10 × 5.0 mL). Subsequently, 20% piperidine/DMF was drained and reacted for 30 min, and the mixture was then washed with DMF again (10 × 5.0 mL). A solution of 85% DMF/10% acetyl acetate/5% NMM (200 mL) was added and reacted under a N₂ atmosphere for 0.5 h. Finally, 20% piperidine in DMF was used for Fmoc deprotection, and the reaction lasted for 30 min. The coupling reaction was monitored by a ninhydrin test, and the resin was washed with DMF (10 × 5.0 mL). In the peptide cleavage and purification phase, cleavage buffer (80% DCM/20% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)) was added to the flask at room temperature for 0.5 h. Then the reaction mixture was filtered and the HFIP-mixture was concentrated under reduced pressure to remove the solvent. The crude peptide was purified by pre-HPLC (A: 0.1% TFA in H₂O, B: acetonitrile) to give the compound **2a**.

White solid; yield: 91%; purity: 95%; ¹H NMR (400 MHz, *d*₆-DMSO): δ = 8.23 (d, 1H, J = 8.1 Hz, NH), 4.40 (m, 1H, J = 8.3, 5.0 Hz, CH), 2.77 (ddd, 2H, J = 22.0, 13.6, 6.7 Hz, CH₂), 2.07 (s, 3H, CH₃), 1.86 (s, 3H, CH₃); ¹³C NMR (101 MHz, DMSO): δ = 172.78, 169.77, 52.01, 35.54, 22.82, 15.68; HRMS (ES⁺) m/z 178.0452 (178.0460 calcd for C₆H₁₁NO₃S M + H).

5.2.2 Preparation of compounds **2b–e.** Peptides **2b–e** were also synthesized using standard Fmoc chemistry. The mixture of CTC resin (18.2 mmol), Fmoc-Cys(Me)-OH (5.2 g, 41.6 mmol, 0.8 eq.) and DCM (200.0 mL) was stirred at 25 °C in a vessel for 12 h under a N₂ atmosphere, and then DIEA (3.2 eq.) was added dropwise and the resulting mixture was stirred for 2 h. MeOH (20.0 mL) was added, and the mixture was mixed for 30 min and washed with DMF (10 × 5.0 mL). After that, 20% piperidine/DMF was drained and reacted for 30 min, and the mixture was washed with DMF again (10 × 5.0 mL), and then the corresponding acid was added to the mixture which was mixed for 30 s. The activation buffer was added under a N₂ atmosphere for 0.5 h. Finally, 20% piperidine in DMF was used for Fmoc deprotection for 30 min. The coupling reaction was monitored by a ninhydrin test, and the resin was washed with DMF (10 × 5.0 mL). In the peptide cleavage and purification phase, cleavage buffer (80% DCM/

20% HFIP) was added to the flask at room temperature for 0.5 h. Then the reaction mixture was filtered and the HFIP-mixture was concentrated under reduced pressure to remove the solvent. The crude peptides were purified by pre-HPLC (A: 0.1% TFA in H₂O, B: acetonitrile) to give the compounds **2b–e**.

***S*-Methyl-*N*-propionyl-*L*-cysteine (**2b**).** White solid; yield: 80%; purity: 96%; ¹H NMR (400 MHz, *d*₆-DMSO): δ = 8.13 (d, 1H, J = 8.1 Hz, NH), 4.40 (td, 1H, J = 8.3, 5.0 Hz, CH), 2.77 (ddd, 2H, J = 22.2, 13.7, 6.8 Hz, CH₂), 2.14 (q, 2H, J = 7.6 Hz, CH₂), 2.07 (s, 3H, CH₃), 0.99 (t, 3H, J = 7.6 Hz, CH₃); ¹³C NMR (101 MHz, DMSO): δ = 173.47, 172.84, 51.94, 35.56, 28.64, 15.69, 10.24; HRMS (ES⁺) m/z 192.0608 (192.0616 calcd for C₇H₁₃NO₃S M + H).

***N*-(Cyclohexanecarbonyl)-*S*-methyl-*L*-cysteine (**2c**).** White solid; yield: 86%; purity: 95%; ¹H NMR (400 MHz, *d*₆-DMSO): δ = 7.15 (d, 1H, J = 6.4 Hz, NH), 3.87 (dd, 1H, J = 10.9, 5.4 Hz, CH), 2.83 (ddd, 2H, J = 18.5, 13.1, 5.0 Hz, CH₂), 2.11 (t, 1H, J = 11.1 Hz, CH), 1.97 (s, 3H, CH₃), 1.77–1.53 (m, 5H, cyclohexane-H), 1.35–1.10 (m, 5H, cyclohexane-H); ¹³C NMR (101 MHz, DMSO): δ = 174.38, 173.14, 53.95, 44.66, 37.50, 30.06, 29.64, 26.03, 25.77, 25.71, 16.16; HRMS (ES⁺) m/z 246.1090 (246.1086 calcd for C₁₁H₁₉NO₃S M + H).

***N*-Benzoyl-*S*-methyl-*L*-cysteine (**2d**).** White solid; yield: 81%; purity: 95%; ¹H NMR (400 MHz, *d*₆-DMSO): δ = 7.95 (d, 1H, J = 6.4 Hz, NH), 7.84–7.72 (m, 2H, Ar-H), 7.58–7.38 (m, 3H, Ar-H), 4.10 (dd, 1H, J = 10.3, 6.1 Hz, CH), 2.98 (ddd, 2H, J = 19.2, 13.2, 5.1 Hz, CH₂), 2.01 (s, 3H, CH₃); ¹³C NMR (101 MHz, DMSO): δ = 173.11, 165.60, 135.50, 131.43, 128.83, 127.35, 54.57, 37.34, 15.99; HRMS (ES⁺) m/z 240.0604 (240.0616 calcd for C₁₁H₁₃NO₃S M + H).

***N*-(4-Fluorobenzoyl)-*S*-methyl-*L*-cysteine (**2e**).** White solid; yield: 81%; purity: 95%; ¹H NMR (400 MHz, *d*₆-DMSO): δ = 7.98 (d, 1H, J = 6.6 Hz, NH), 7.88 (dd, 2H, J = 8.7, 5.6 Hz, Ar-H), 7.29 (t, 2H, J = 8.9 Hz, Ar-H), 4.12 (dd, 1H, J = 8.5, 4.5 Hz, CH), 2.96 (ddd, 2H, J = 19.8, 13.2, 5.4 Hz, CH₂), 2.01 (s, 3H, CH₃); ¹³C NMR (101 MHz, DMSO): δ = 172.96, 164.61, 130.04, 129.96, 115.79, 115.57, 54.64, 37.37, 15.91; HRMS (ES⁺) m/z 258.0528 (258.0522 calcd for C₁₁H₁₂NO₃S M + H).

5.2.3 General methods for preparation of the target compounds **3a–e.** Appropriate intermediates **2a–e** (1.1 mmol) in HCl/MeOH (4 M, 5.0 mL) were stirred at 15 °C for 3 h. The solvent was removed under reduced pressure and the residue was purified by pre-HPLC (acid condition, TFA) to give desired compounds **3a–e**.

***Methyl N*-acetyl-*S*-methyl-*L*-cysteinate (**3a**).** White solid; yield: 78%; purity: 97%; ¹H NMR (400 MHz, MeOD): δ = 4.65 (dd, 1H, J = 8.2, 5.2 Hz, CH), 3.75 (s, 3H, CH₃), 2.89 (ddd, 2H,

$J = 22.2, 13.9, 6.8$ Hz, CH_2), 2.13 (s, 3H, CH_3), 2.01 (s, 3H, CH_3); ^{13}C NMR (101 MHz, DMSO): $\delta = 171.87, 169.85, 52.48, 51.98, 35.24, 22.73, 15.58$; HRMS (ES^+) m/z 192.0610 (192.0616 calcd for $\text{C}_7\text{H}_{13}\text{NO}_3\text{S M} + \text{H}$).

Methyl *S*-methyl-*N*-propionyl-*L*-cysteinate (3b). White solid; yield: 84%; purity: 99%; ^1H NMR (400 MHz, MeOD): $\delta = 4.65$ (dd, 1H, $J = 8.4, 5.2$ Hz, CH), 3.75 (s, 3H, CH_3), 2.89 (ddd, 2H, $J = 22.3, 13.9, 6.8$ Hz, CH_2), 2.29 (q, 2H, $J = 7.6$ Hz, CH_2), 2.13 (s, 3H, CH_3), 1.15 (t, $J = 7.6$ Hz, 3H, CH_3); ^{13}C NMR (101 MHz, DMSO): $\delta = 173.56, 171.93, 52.46, 51.94, 35.25, 28.56, 15.59, 10.17$; HRMS (ES^+) m/z 206.0787 (206.0773 calcd for $\text{C}_8\text{H}_{15}\text{NO}_3\text{S M} + \text{H}$).

Methyl *N*-(cyclohexanecarbonyl)-*S*-methyl-*L*-cysteinate (3c). White solid; yield: 86%; purity: 99%; ^1H NMR (400 MHz, MeOD): $\delta = 4.62$ (dd, 1H, $J = 8.6, 5.2$ Hz, CH), 3.75 (s, 3H, CH_3), 2.89 (ddd, 2H, $J = 22.5, 13.9, 6.9$ Hz, CH_2), 2.29 (tt, 1H, $J = 11.7, 3.3$ Hz, CH), 1.88–1.67 (m, 5H, cyclohexane-H), 1.53–1.21 (m, 5H, cyclohexane-H); ^{13}C NMR (101 MHz, DMSO): $\delta = 175.83, 171.96, 52.44, 51.78, 44.00, 35.19, 29.62, 29.46, 25.90, 25.65, 25.16, 15.56$; HRMS (ES^+) m/z 260.1228 (260.1242 calcd for $\text{C}_{12}\text{H}_{21}\text{NO}_3\text{S M} + \text{H}$).

Methyl *N*-benzoyl-*S*-methyl-*L*-cysteinate (3d). White solid; yield: 81%; purity: 98%; ^1H NMR (400 MHz, MeOD): $\delta = 8.75$ (d, 1H, $J = 7.0$ Hz, NH), 7.87 (dd, 2H, $J = 5.3, 3.3$ Hz, Ar-H), 7.62–7.54 (m, 1H, Ar-H), 7.54–7.43 (m, 2H, Ar-H), 4.87–4.82 (m, 1H, CH), 3.79 (s, 3H, CH_3), 3.05 (ddd, 2H, $J = 23.0, 13.9, 7.1$ Hz, CH_2), 2.17 (s, 3H, CH_3); ^{13}C NMR (101 MHz, DMSO): $\delta = 171.85, 166.91, 134.04, 132.07, 128.82, 127.91, 52.59, 52.57, 34.85, 15.45$; HRMS (ES^+) m/z 254.0763 (254.0773 calcd for $\text{C}_{12}\text{H}_{15}\text{NO}_3\text{S M} + \text{H}$).

Methyl *N*-(4-fluorobenzoyl)-*S*-methyl-*L*-cysteinate (3e). White solid; yield: 79%; purity: 98%; ^1H NMR (400 MHz, MeOD): $\delta = 8.78$ (d, 1H, $J = 7.2$ Hz, NH), 8.02–7.80 (m, 2H, Ar-H), 7.23 (dd, 2H, $J = 9.7, 7.8$ Hz, Ar-H), 4.86–4.80 (m, 1H, CH), 3.78 (s, 3H, CH_3), 3.04 (ddd, 2H, $J = 23.1, 13.9, 7.1$ Hz, CH_2), 2.17 (s, 3H, CH_3); ^{13}C NMR (101 MHz, DMSO): $\delta = 171.62, 165.97, 130.66, 130.57, 115.91, 115.69, 52.61, 52.60, 34.82, 15.43$; HRMS (ES^+) m/z 272.0660 (272.0678 calcd for $\text{C}_{12}\text{H}_{14}\text{NO}_3\text{S M} + \text{H}$).

5.2.4 General procedure for the preparation of 4a–e. A solution of appropriate intermediates 2a–e, 2-*tert*-butyl-1,3-diisopropyl-isourea (507.8 mg, 2.5 mmol) in DCM (5.0 mL) was stirred at 80 °C for 12 h. Then 2-*tert*-butyl-1,3-diisopropyl-isourea (507.0 mg, 2.5 mmol) was added and the resulting mixture was stirred at 80 °C for another 12 h. The mixture was cooled to room temperature and filtered, and the filtrate was concentrated in a vacuum. The residue was purified by pre-HPLC (acid condition, TFA) to obtain the desired compounds 4a–e.

***tert*-Butyl *N*-acetyl-*S*-methyl-*L*-cysteinate (4a).** Colorless oil; yield: 65%; purity: 99%; ^1H NMR (400 MHz, MeOD): $\delta = 4.51$ (dd, 1H, $J = 8.2, 5.3$ Hz, CH), 2.85 (ddd, 2H, $J = 21.9, 13.8, 6.8$ Hz, CH_2), 2.14 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 1.50 (s, 9H, C_4H_9); ^{13}C NMR (101 MHz, DMSO): $\delta = 170.49, 169.78, 81.45, 52.73, 35.49, 28.08, 22.77, 15.68$; HRMS (ES^+) m/z 178.0456 (178.0460 calcd for $\text{C}_6\text{H}_{11}\text{NO}_3\text{S M} + \text{H}-\text{C}_4\text{H}_9$).

***tert*-Butyl *S*-methyl-*N*-propionyl-*L*-cysteinate (4b).** Yellow oil; yield: 72%; purity: 98%; ^1H NMR (400 MHz, MeOD): $\delta = 4.57$ (s, 1H, CH), 3.08–2.76 (m, 2H, CH_2), 2.43–2.28 (m, 2H, CH_2), 2.21 (d, $J = 2.0$ Hz, 3H, CH_3), 1.56 (s, 9H, C_4H_9), 1.30–1.15 (m, 3H, CH_3); ^{13}C NMR (101 MHz, DMSO): $\delta = 173.50, 170.50, 81.38, 52.69, 35.46, 28.64, 28.08, 15.69, 10.28$; HRMS (ES^+) m/z 192.0602 (192.0616 calcd for $\text{C}_7\text{H}_{13}\text{NO}_3\text{S M} + \text{H}-\text{C}_4\text{H}_9$).

***tert*-Butyl *N*-(cyclohexanecarbonyl)-*S*-methyl-*L*-cysteinate (4c).** Yellow oil; yield: 71%; purity: 95%; ^1H NMR (400 MHz, MeOD): $\delta = 8.13$ (d, 1H, $J = 6.8$ Hz, NH), 4.47 (td, 1H, $J = 8.2, 5.2$ Hz, CH), 2.86 (ddd, 2H, $J = 22.2, 13.7, 6.8$ Hz, CH_2), 2.29 (tt, 1H, $J = 11.8, 3.3$ Hz, CH), 2.13 (s, 1H, CH_3), 1.77 (dd, 5H, $J = 40.8, 9.4$ Hz, cyclohexane-H), 1.57–1.16 (m, 14H, cyclohexane-H + C_4H_9); ^{13}C NMR (101 MHz, DMSO): $\delta = 175.68, 170.51, 81.25, 52.59, 44.01, 35.31, 29.61, 29.52, 28.08, 25.91, 25.66, 25.63, 15.65$; HRMS (ES^+) m/z 246.1094 (246.1086 calcd for $\text{C}_{11}\text{H}_{19}\text{NO}_3\text{S M} + \text{H}-\text{C}_4\text{H}_9$).

***tert*-Butyl *N*-benzoyl-*S*-methyl-*L*-cysteinate (4d).** Yellow oil; yield: 61%; purity: 99%; ^1H NMR (400 MHz, MeOD): $\delta = 8.64$ (d, 1H, $J = 7.0$ Hz, NH), 7.92–7.79 (m, 2H, Ar-H), 7.65–7.43 (m, 3H, Ar-H), 4.76–4.64 (m, 1H, CH), 3.02 (ddd, 2H, $J = 22.8, 13.8, 7.1$ Hz, CH_2), 2.17 (s, 3H, CH_3), 1.51 (s, 9H, C_4H_9); ^{13}C NMR (101 MHz, DMSO): $\delta = 170.44, 166.95, 134.33, 131.95, 128.79, 127.86, 81.52, 53.33, 35.01, 28.11, 15.50$; HRMS (ES^+) m/z 240.060 (240.0616 calcd for $\text{C}_{11}\text{H}_{13}\text{NO}_3\text{S M} + \text{H}-\text{C}_4\text{H}_9$).

***tert*-Butyl *N*-(4-fluorobenzoyl)-*S*-methyl-*L*-cysteinate (4e).** Yellow oil; yield: 75%; purity: 99%; ^1H NMR (400 MHz, MeOD): $\delta = 8.67$ (d, 1H, $J = 7.4$ Hz, NH), 8.00–7.81 (m, 2H, Ar-H), 7.33–7.12 (m, 2H, Ar-H), 4.69 (ddd, 1H, $J = 9.0, 8.0, 5.1$ Hz, CH), 3.02 (tt, 2H, $J = 22.9, 6.8$ Hz, CH_2), 2.17 (s, 3H, CH_3), 1.51 (s, 9H, C_4H_9); ^{13}C NMR (101 MHz, DMSO): $\delta = 170.39, 165.89, 130.60, 130.51, 115.86, 115.64, 81.57, 53.37, 35.01, 28.10, 15.49$; HRMS (ES^+) m/z 258.0536 (258.0522 calcd for $\text{C}_{11}\text{H}_{12}\text{NO}_3\text{S M} + \text{H}-\text{C}_4\text{H}_9$).

5.2.5 Preparation of compounds 5a–5c. To a mixture of an appropriate intermediate (2b, 2c or 2e, 1.2 mmol), EDCI (189.2 mg, 1.5 mmol), DIEA (315.4 mg, 2.4 mmol), and HOBT (197.8 mg, 1.5 mmol) in DMF (5.0 mL) was added methanamine hydrochloride (90.6 mg, 1.3 mmol) at 15 °C. Then the mixture was stirred at 15 °C for 16 h, diluted with DCM (100.0 mL), washed with 1 M HCl (1 × 20.0 mL), H_2O (1 × 20.0 mL), and brine (1 × 20.0 mL), dried over anhydrous Na_2SO_4 , and concentrated under reduced pressure. The residue was purified by pre-HPLC (acid condition, TFA) to obtain desired compounds 5a–5c as white solids.

(*R*)-*N*-Methyl-3-(methylthio)-2-propionamidopropanamide (5a). White solid; yield: 82%; purity: 97%; ^1H NMR (400 MHz, MeOD): $\delta = 8.08$ (s, 1H, NH), 4.50 (ddd, 1H, $J = 8.1, 7.0, 4.3$ Hz, CH), 2.96–2.65 (m, 5H, $\text{CH}_2 + \text{CH}_3$), 2.30 (q, $J = 7.6$ Hz, 2H, CH_2), 2.12 (s, 3H, CH_3), 1.15 (t, $J = 7.6$ Hz, 3H, CH_3); ^{13}C NMR (101 MHz, DMSO): $\delta = 173.55, 171.22, 52.24, 36.30, 28.70, 26.07, 15.52, 10.20$; HRMS (ES^+) m/z 205.0516 (205.0932 calcd for $\text{C}_8\text{H}_{16}\text{N}_2\text{O}_2\text{S M} + \text{H}$).

(*R*)-*N*-(1-(Methylamino)-3-(methylthio)-1-oxopropan-2-yl)cyclohexanecarboxamide (5b). White solid; yield: 79%; purity: 98%; ^1H NMR (400 MHz, MeOD): $\delta = 7.96$ (d, 1H, $J =$

7.8 Hz, NH), 4.55–4.40 (m, 1H, CH), 2.96–2.65 (m, 5H, CH₂ + CH₃), 2.29 (tt, 1H, *J* = 11.7, 3.4 Hz, CH), 2.12 (s, 3H, CH₃), 1.91–1.65 (m, 5H, cyclohexane-H), 1.53–1.18 (m, 5H, cyclohexane-H); ¹³C NMR (101 MHz, DMSO): δ = 175.67, 171.28, 52.01, 44.15, 36.34, 29.94, 29.32, 26.08, 25.94, 25.77, 25.63, 15.48; HRMS (ES⁺) *m/z* 259.1419 (259.1402 calcd for C₁₂H₂₂N₂O₂S M + H).

(*R*)-*N*-(1-(Methylamino)-3-(methylthio)-1-oxopropan-2-yl)benzamide (**5c**). White solid; yield: 89%; purity: 99%; ¹H NMR (400 MHz, MeOD): δ = 7.89 (dd, 2H, *J* = 5.3, 3.3 Hz, Ar-H), 7.63–7.43 (m, 3H, Ar-H), 4.74 (dd, 1H, *J* = 8.6, 5.8 Hz, CH), 2.97 (ddd, 2H, *J* = 22.5, 13.9, 7.2 Hz, CH₂), 2.79 (s, 3H, CH₃), 2.17 (s, 3H, CH₃); ¹³C NMR (101 MHz, DMSO): δ = 171.21, 166.82, 134.47, 131.83, 128.65, 128.03, 53.07, 36.02, 26.18, 15.42; HRMS (ES⁺) *m/z* 253.0912 (253.0932 calcd for C₁₂H₁₆N₂O₂S M + H).

5.2.6 General procedure for the synthesis of 6a–6c. To a solution of an appropriate intermediate (**2a**, **2b** or **2e**, 1.1 mmol) and NMM (137.2 mg, 1.4 mmol) in THF (4.0 mL) was added isobutyl chloroformate (IBCF) (169.8 mg, 1.2 mmol) dropwise at 0 °C. After addition, the mixture was stirred at 0 °C for 1 h and cyclohexanamine (123.3 mg, 1.2 mmol) was added at the same temperature. The resulting mixture was stirred at 0 °C for another 1 h, which was then diluted with DCM (50.0 mL), washed with 1 M HCl (1 × 20.0 mL) and brine (1 × 20.0 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was purified by pre-HPLC (acid condition, TFA) to obtain the desired compounds **6a–6c** as white solids.

(*R*)-2-Acetamido-*N*-cyclohexyl-3-(methylthio)propanamide (**6a**). White solid; yield: 68%; purity: 92%; ¹H NMR (400 MHz, MeOD): δ = 8.12 (dd, 1H, *J* = 55.0, 7.6 Hz, NH), 4.50 (td, 1H, *J* = 7.9, 4.7 Hz, CH), 3.65 (tdt, 1H, *J* = 11.6, 7.9, 3.9 Hz, CH), 2.78 (ddd, 2H, *J* = 21.6, 13.7, 7.1 Hz, CH₂), 2.14 (s, 3H, CH₃), 2.01 (s, 3H, CH₃), 1.92–1.59 (m, 5H, cyclohexane-H), 1.44–1.14 (m, 5H, cyclohexane-H); ¹³C NMR (101 MHz, DMSO): δ = 169.86, 169.52, 52.28, 48.08, 36.62, 32.77, 32.62, 25.66, 24.99, 24.93, 22.97, 15.60; HRMS (ES⁺) *m/z* 259.1416 (259.1402 calcd for C₁₂H₂₂N₂O₂S M + H).

(*R*)-*N*-Cyclohexyl-3-(methylthio)-2-propionamidopropanamide (**6b**). White solid; yield: 63%; purity: 99%; ¹H NMR (400 MHz, MeOD): δ = 4.50 (dd, 1H, *J* = 7.7, 6.6 Hz, CH), 3.66 (td, 1H, *J* = 10.7, 5.6 Hz, CH), 2.79 (ddd, 2H, *J* = 21.6, 13.7, 7.2 Hz, CH₂), 2.28 (q, *J* = 7.6 Hz, 2H, CH₂), 2.14 (s, 3H, CH₃), 1.93–1.60 (m, 5H, cyclohexane-H), 1.45–1.19 (m, 5H, cyclohexane-H), 1.15 (t, 3H, *J* = 7.6 Hz, CH₃); ¹³C NMR (101 MHz, DMSO): δ = 173.27, 169.74, 52.23, 48.06, 36.62, 32.77, 32.66, 28.71, 25.66, 24.96, 24.90, 15.62, 10.30; HRMS (ES⁺) *m/z* 273.1546 (273.1558 calcd for C₁₃H₂₄N₂O₂S M + H).

(*R*)-*N*-(1-(Cyclohexylamino)-3-(methylthio)-1-oxopropan-2-yl)benzamide (**6c**). White solid; yield: 73%; purity: 99%; ¹H NMR (400 MHz, MeOD): δ = 8.42 (d, 1H, *J* = 7.8 Hz, NH), 8.15 (d, 1H, *J* = 8.2 Hz, NH), 7.93–7.76 (m, 2H, Ar-H), 7.53 (dt, 3H, *J* = 14.9, 7.3 Hz, Ar-H), 4.80–4.65 (m, 1H, CH), 3.77–3.60 (m, 1H, CH), 2.94 (ddd, 2H, *J* = 22.1, 13.8, 7.2 Hz, CH₂), 2.18 (s, 1H, CH₃), 1.95–1.62 (m, 5H, CH₅), 1.48–1.12 (m, 5H, CH₅);

¹³C NMR (101 MHz, DMSO): δ = 169.77, 166.69, 134.54, 131.81, 128.69, 127.95, 53.08, 48.19, 36.29, 32.78, 32.69, 25.66, 24.99, 24.95, 15.51; HRMS (ES⁺) *m/z* 321.1552 (321.1558 calcd for C₁₇H₂₄N₂O₂S M + H).

5.2.7 General procedure for the preparation of 7a and b

(*R*)-2-Acetamido-3-(methylthio)-*N*-phenylpropanamide (**7a**). A solution of compound **2a** (200.0 mg, 1.1 mmol), aniline (160.8 mg, 1.7 mmol), and HATU (515.6 mg, 1.4 mmol) in DMF (3.0 mL) was stirred at 15 °C for 16 h. Then DMF was removed under reduced pressure. The residue was diluted with DCM (100.0 mL), washed with 1 M HCl (1 × 30.0 mL), H₂O (1 × 30.0 mL), and brine (1 × 30.0 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure to obtain compound **7a** as a white solid.

White solid; yield: 61%; purity: 99%; ¹H NMR (400 MHz, MeOD): δ = 10.02 (s, 1H, NH), 7.66–7.51 (m, 2H, Ar-H), 7.33 (dd, 2H, *J* = 10.8, 5.2 Hz, Ar-H), 7.13 (t, 1H, *J* = 7.4 Hz, Ar-H), 4.76–4.59 (m, 1H, CH), 2.90 (ddd, 2H, *J* = 21.7, 13.8, 7.2 Hz, CH₂), 2.17 (s, 3H, CH₃), 2.04 (s, 3H, CH₃); ¹³C NMR (101 MHz, DMSO): δ = 169.85, 169.77, 139.25, 129.18, 123.96, 119.82, 53.36, 36.14, 22.91, 15.67; HRMS (ES⁺) *m/z* 253.0922 (253.0932 calcd for C₁₂H₁₆N₂O₂S M + H).

(*R*)-3-(Methylthio)-*N*-phenyl-2-propionamidopropanamide (**7b**). A solution of compound **2b** (300.0 mg, 1.6 mmol), aniline (160.8 mg, 1.7 mmol), and EDCI (451.5 mg, 2.4 mmol) in pyridine (5.0 mL) was stirred at 15 °C for 16 h. The solvent was removed under reduced pressure. The residue was diluted with DCM (100.0 mL), washed with 1 M HCl (1 × 30.0 mL), H₂O (1 × 30.0 mL), and brine (1 × 30.0 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was purified by pre-HPLC (acid condition, TFA) to obtain compound **7b** as a white solid.

White solid; yield: 55%; purity: 97%; ¹H NMR (400 MHz, MeOD): δ = 10.02 (s, 1H, NH), 7.64–7.52 (m, 2H, Ar-H), 7.33 (dd, *J* = 10.8, 5.1 Hz, 2H, Ar-H), 7.13 (t, *J* = 7.4 Hz, 1H, Ar-H), 4.77–4.62 (m, 1H, CH), 2.90 (ddd, *J* = 21.8, 13.8, 7.2 Hz, 2H, CH₂), 2.32 (q, *J* = 7.6 Hz, 2H, CH₂), 2.17 (s, 3H, CH₃), 1.17 (t, *J* = 7.6 Hz, 3H, CH₃); ¹³C NMR (101 MHz, DMSO): δ = 173.49, 169.90, 139.27, 129.18, 123.95, 119.81, 53.31, 36.18, 28.65, 15.68, 10.26; HRMS (ES⁺) *m/z* 267.1095 (267.1089 calcd for C₁₃H₁₈N₂O₂S M + H).

5.2.8 Preparation of the target compound 7c

tert-Butyl (*R*)-3-(methylthio)-1-oxo-1-(phenylamino)propan-2-ylcarbamate (**9**). To a solution of compound **8** (700 mg, 2.97 mmol) and NMM (360.50 mg, 3.56 mmol) in THF (20 mL) was added IBCF (446.21 mg, 3.27 mmol) dropwise at 0 °C. The mixture was stirred at 15 °C for 1 h. Then the mixture was added to aniline (331.92 mg, 3.56 mmol) at 0 °C. The mixture was stirred at 15 °C for 1 h, diluted with EA (50 mL), washed with 1 M HCl (20 mL), H₂O (20 mL) and brine (20 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure to obtain compound **9** as a white solid.

White solid; yield: 79%; purity: 95%; ESI-MS: *m/z* = 254.8[M + H–C₄H₉]⁺.

(*R*)-2-Amino-3-(methylthio)-*N*-phenylpropanamide (**10**). To a solution of compound **9** (700.00 mg, 2.26 mmol) in DCM

(10.00 mL) was added TFA (67.53 mmol, 5.00 mL), which was stirred at 15 °C for 1 h. The solvent was removed under reduced pressure to obtain the crude product as brown oil. The product was put into the next step directly without further purification. Yield: 91%.

Preparation of (R)-N-(3-(Methylthio)-1-oxo-1-(phenylamino)propan-2-yl)benzamide (7c). To a mixture of compound **10** (700.00 mg, 2.16 mmol), NaHCO₃ (725.30 mg, 8.64 mmol) in THF (10 mL) and H₂O (5 mL) was added benzoyl chloride (425.08 mg, 3.02 mmol) at 0 °C. Then the mixture was stirred at 0 °C for 2 h. The solvent was removed under reduced pressure. The residue was diluted with DCM (50 mL), washed with 1 M HCl (20 mL), H₂O (20 mL), and brine (20 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was purified using a silicagel column (PE:EA = 5:1) to obtain compound **7c** as a white solid.

White solid; yield: 61%; purity: 96%; ¹H NMR (400 MHz, MeOD): δ = 10.13 (s, 1H, NH), 8.63 (d, *J* = 7.5 Hz, 1H, NH), 8.00–7.82 (m, 2H, Ar–H), 7.56 (ddd, 5H, *J* = 31.8, 11.9, 4.2 Hz, Ar–H), 7.34 (t, 2H, *J* = 8.0 Hz, Ar–H), 7.13 (t, 1H, *J* = 7.4 Hz, Ar–H), 3.06 (ddd, 2H, *J* = 22.1, 13.8, 7.3 Hz, CH₂), 2.22 (s, 1H, CH₃); ¹³C NMR (101 MHz, DMSO): δ = 169.41, 166.46, 138.82, 133.84, 131.43, 128.69, 128.22, 127.54, 123.45, 119.35, 53.73, 35.29, 15.12; HRMS (ES⁺) *m/z* 315.1064 (315.1089 calcd for C₁₇H₁₈N₂O₂S M + H).

5.3 Pharmacology

5.3.1 Cell proliferation assay against the hepatocellular injury model induced by H₂O₂. Human hepatocyte cell line LO2 (provided by Shanghai Institutes for Biological Sciences, SIBS) was used to build a hepatocyte injury model induced by H₂O₂ or APAP. The liver cell protective activities of the target compounds were evaluated against the hepatocellular injury model induced by H₂O₂, and the Cell Counting Kit-8 (CCK-8, Beyotime, C0038) kit was utilized to detect the cell proliferation rates. Approximately 2 × 10⁶ LO2 cells were suspended in 10 mL fresh medium and cultured in a relative saturation humidity incubator in 5% CO₂ at 37 °C for 24 h. Then the culture liquid was removed and 300 μL H₂O₂ fresh culture liquid with no fetal bovine serum (FBS) was added under the same conditions for 6 h. The culture fluid was removed again, washed with phosphate buffered saline (PBS) three times, and underwent trypsin digestion and suspension. Subsequently, the culture liquid was centrifuged with the supernatant being discarded, and then 1 mL PBS was added and the cells were suspended and counted. Finally, the cells were transferred to a 1.5 mL EP tube and centrifuged (4000 r × 10 min). The supernatant was discarded and 150 μL PBS was added for cell suspension, and then the cells were kept at –20 °C. All of the following procedures were carried out strictly in accordance with the instruction of the CCK-8 kit. The absorbance was measured at 450 nm using a microplate reader. All the target compounds were screened against this cell model and the processing of the model group was similar

to that of the compound test group, except that the tested compounds were not included in the hepatocellular injury cells induced by H₂O₂ when the CCK-8 kit was used. The results were calculated by using the GraphPad Prism 5.

5.3.2 Cell proliferation assay against hepatocellular injury model induced by APAP. The liver cell protective activities of selected compounds were evaluated against hepatocellular injury model induced by APAP, and CCK-8 kit was used to detect the cell proliferation rates. Similar to the injury model induced by H₂O₂, approximate 2 × 10⁶ cells were suspended in fresh medium and cultured in 5% CO₂ at 37 °C for 24 h, then the culture liquid was removed and 1% DMSO plus 40 mmol APAP with no FBS was added for incubation with 5% CO₂ at 37 °C for 3 h. The following operation referred to the experimental procedures illustrated in paragraph 5.3.1.

5.3.3 In vitro MDA secretion assay. In this experiment, human normal LO2 cells were cracked directly and measured to determine the MDA level of normal group. The –20 °C stored cells (hepatocellular injury cells induced by H₂O₂ or APAP) were cracked with repeated freezing and thawing method, and the following assay was determined according to the instruction of the MDA kit (Beyotime, S0131). 0.37% thiobarbituric acid (TBA) storage liquid was prepared and the MDA detection work fluid was diluted according to the number of samples. 100 μL (100 mmol) of various target compounds was added and treated with 200 μM of MDA test solution. The processing of the model group was similar to that of the compound test group, except that the tested compounds were not included in the hepatocellular injury cells induced by H₂O₂ or APAP in the above experimental step. The solution was mixed at 100 °C for 15 min, cooled to room temperature and centrifuged (1000g) for 10 min. Finally, the supernatant (200 μL) was preloaded in a 96-well plate and the absorbance was determined using an enzyme-meter 532.

5.4 ADME assay

5.4.1 Caco-2 permeability assay. Caco-2 cells (human colorectal adenocarcinoma line) were cultivated in MEM medium supplemented with 10% FBS, 1% non-essential amino acid solution and 0.1% penicillin streptomycin in a humidified atmosphere at 37 °C in 5% CO₂ and then were seeded at 4.8 × 10⁴ cells per well on 24-well semipermeable insert plates (Millicell # PSHT 010 R5). The medium was changed every two days. After 21 days of cell growth, the integrity of differentiated Caco-2 monolayers was verified by trans-epithelial electrical resistance (TEER) measurements using a Millicell-ERS VoltOhmmeter (Millipore # MERS 000 01). Caco-2 cell monolayers were considered acceptable for transport studies if the final values of TEER were greater than 250 Ω cm^{–2}. For the permeability studies, a 24-well insert plate was removed from its feeder plate and placed in a new sterile 24-well receiver plate. The cell layer was washed twice with HBSS (# 14025-092). In the A–B direction, aliquots (400 μL) of the test compound solution (in duplicates, at 10 μM, in HBSS with 5.56 mM glucose buffered with 25 mM HEPES, pH 7.4) were

added into the apical compartments of the trans-well insert and 1000 μL of the same buffer was added to the basolateral compartments; in the B–A direction, aliquots (1000 μL) of the test compound solution (in duplicates, at 10 μM , in HBSS with 5.56 mM glucose buffered with 25 mM HEPES, pH 7.4) were added into the basolateral compartments of the trans-well insert and 400 μL of the same buffer was added to the apical compartments. The plates were then incubated for 1.5 h at 37 °C. High, low and Pgp substrate permeability controls (atenolol, metoprolol, erythromycin) were run with every experimental batch to verify assay validity. The concentrations of the compounds tested in the permeability assay were determined using the LC-MS-MS method. The formula for calculating Papp (expressed in $10^{-6} \text{ cm s}^{-1}$) was as follows:

$$\text{Papp} = (\text{VA}/(\text{Area} \times \text{time})) \times ([\text{drug}]_{\text{acceptor}}/([\text{drug}]_{\text{initial, donor}} \times \text{Dilution Factor}))$$

VA – the volume in the acceptor well, area – the surface area of the membrane, time – the total transport time in seconds, [drug]_{acceptor} – the concentration of the test compound in the acceptor well, [drug]_{initial, donor} – the initial concentration of the test compound in the donor well.

5.4.2 Pharmacokinetic studies. The protocol of the rat pharmacokinetics study was reviewed and approved by the Institutional Animal Care and Use Committee, Shanghai ChemPartner (protocol number: A998HL0002) prior to being instituted, and the experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Shanghai ChemPartner Co., Ltd. The compounds were dissolved in 5% DMSO + 5% Solutol HS 15 + 90% (20% HP- β -CD in water) as vehicles and were administered intravenously (i.v.) or orally (p.o.) to SD rats at 10 or 20 mg kg⁻¹. Ten male animals (bodyweight: 220–240 g) were used for the i.v. or p.o. group, and blood was collected *via* a tail vein at 5, 15, and 30 min, 1, 2, 4, 8 and 24 h or 15 and 30 min, 1, 2, 4, 8 and 24 h for i.v. or p.o. administration, respectively. All animals had fasted overnight and were fed 4 h post dosing with free access to water. EDTA-K2 was used as an anticoagulant, and blood samples were centrifuged at 4 °C (2000g, 5 min) to obtain plasma within 15 min after sample collection. Plasma samples were analyzed after protein precipitation with ACN and centrifuged at 5800 rpm for 10 min by liquid chromatography coupled to mass spectrometry (LC-MS-MS) using appropriate calibration curves, an internal standard (Diclofenac) and bioanalytical quality controls. Pharmacokinetic parameters were calculated using the Phoenix WinNonlin 6.4 software package (Pharsight) using non-compartmental analysis. The area under the plasma concentration *versus* time curve (AUC) was calculated using the linear trapezoidal linear interpolation rule and bioavailability (BA, %) was calculated using $(\text{AUC}_{\text{INF-PO}} \times \text{DOSE}_{\text{IV}})/(\text{AUC}_{\text{INF-IV}} \times \text{DOSE}_{\text{PO}}) \times 100\%$.

Conflicts of interest

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

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