A hydrogen peroxide responsive prodrug of Keap1-Nrf2 inhibitor for improving oral absorption and selective activation in inflammatory conditions

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\textbf{ARTICLE INFO}

\textbf{Keywords:}
Keap1-Nrf2 pathway
Protein-protein interaction
H\textsubscript{2}O\textsubscript{2}
Prodrug
Inflammation
Oral administration

\textbf{ABSTRACT}

Transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2) and its negative regulator, the E3 ligase adaptor Kelch-like ECH-associated protein 1 (Keap1), control the redox and metabolic homeostasis and oxidative stress. Inhibitors of Keap1-Nrf2 interaction are promising in oxidative stress related inflammatory diseases but now hit hurdles. By utilizing thiazolidinone moiety to shield the key carboxyl pharmacophore in Keap1-Nrf2 inhibitor, a hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-responsive prodrug pro2 was developed. The prodrug modification improved the physicochemical properties and cell membrane permeability of the parent drug. Pro2 was stable and stayed inactive under various physiological conditions, while become active by stimulation of H\textsubscript{2}O\textsubscript{2} or inflammation derived reactive oxygen species. Moreover, pro2 exhibited proper pharmacokinetic profile suitable for oral administration and enhanced anti-inflammatory efficiency \textit{in vivo}. Thus, this novel prodrug approach may not only provide an important advance in the therapy of chronic inflammatory diseases with high level of H\textsubscript{2}O\textsubscript{2}, but also offer a fresh solution to improve the drug-like and selectivity issues of Keap1-Nrf2 inhibitors.

\textbf{1. Introduction}

Transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), as the master regulator of multiple cytoprotective responses, is pivotal in redox and metabolic homeostasis, as well as the regulation of oxidative stress [1,2]. Nrf2 activation enhances the anti-oxidant capacity and provides cryoprotection against oxidative stress and inflammatory insults [3,4]. Recently, therapeutic targeting of protein-protein interaction (PPI) of Nrf2 and its principal negative regulator, the E3 ligase adaptor Kelch-like ECH-associated protein 1 (Keap1) is emerging as a new strategy for drug development of chronic diseases [5–10]. Several types of PPI inhibitors with high potency in disrupting Keap1-Nrf2 interaction have been reported by both pharmaceutical companies and academic institutes [11–17]. However, concerns regarding pharmacokinetic properties and safety still remain. Most of the currently reported inhibitors are molecules with fairly high molecule weight and several polar functional groups [18–20], requirements for blocking the large and high polar Keap1-Nrf2 interface, and thus these compounds with high potency \textit{in vitro} at present exhibit poor absorption, distribution, metabolism and excretion properties and relative low efficacy \textit{in vivo}. Safety issues also challenge the further development of Keap1-Nrf2 inhibitors, since Nrf2 activation in normal cells can excessively strengthen the antioxidant system, resulting in the clearance of reactive oxygen species (ROS), which may disturb the pathological function of ROS [21]. Moreover, a concern about the increased cancer risk of Nrf2 activators also appears [22–25]. Somatic mutations in KEAP1 and NFE2L2, resulting in high and unrestrained Nrf2 activity, have been regarded as driving factors of several tumors [26–28]. Therefore, the selective inhibition of Keap1-Nrf2 PPI in oxidative-stressed tissue is a key challenge, as it would dramatically benefit the therapeutic application. Designing a prodrug of Keap1-Nrf2 inhibitor that can reveal carboxyl acid group upon activation by ROS could kill two birds with one stone. It can not only improve the poor pharmacokinetic properties caused by the polar and ionizable characters of

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https://doi.org/10.1016/j.redox.2020.101565
Received 26 March 2020; Received in revised form 22 April 2020; Accepted 29 April 2020
Available online 11 May 2020
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carboxyl acid group, but also shield the key group of Keap1-binding until reaching the target cells, specifically rebalancing the redox state in pathological cells while not affecting normal cells.

Hydrogen peroxide (H2O2), an uncharged molecule and a stable ROS, is endogenously produced and ubiquitous existed in living organisms [29,30]. Physiologically, H2O2 plays an active role in redox signaling through reversible redox post-translational modifications, and its level is fine-tuned by anti-oxidant system [31–33]. Nevertheless, high level of H2O2 has been closely associated with several pathological conditions, including inflammation [34], neurodegenerative disorders [35] and cancer [36]. On one hand, H2O2 overabundance, together with the aberrant oxidative stress, contributes to the pathology of these conditions. On the other hand, excessive level of H2O2 in pathological microenvironments could be an ideal trigger for targeting activation of therapeutic agents [37]. Chang’s group reported the pioneer work of H2O2-responsive aryl boronate trigger [38,39], inspiring the rapid development of aryl boronate-based probes of H2O2 [40–45]. Using arylboronates or boronic acids as the trigger units, Peng’s group developed the first H2O2-activated DNA cross-linking agents [46], and various H2O2-activated anti-cancer agents have been identified [47–53].

More recently, increasing evidence from different studies supports the relation between oxidative stress and the pathogenesis of inflammation, and it inspired the research around the H2O2-responsive cytoprotective and anti-inflammation agents, including prodrugs of methotrexate and aminopterin for the treatment of rheumatoid arthritis [54,55], prodrugs of H2S donors [56,57] and CO donors [58], and prodrug of angioeniin for neuroprotective activity [59]. The presence of high level of H2O2 in inflammatory conditions also provides an ideal trigger for the selective activation of Nfr2. To the best of our knowledge, an oral H2O2-responsive prodrug for Nfr2 activation is still unavailable, which limits therapeutic usage of Nfr2 activation agents in chronic inflammatory diseases. It is therefore envisioned that improvements could be made to develop new orally administered H2O2-responsive prodrug of Keap1-Nfr2 PPI inhibitors.

Here, we report a H2O2-responsive prodrug of Keap1-Nfr2 inhibitor by shielding the key carboxyl pharmacophore with the H2O2-sensitive thiazolidinone moiety. Our study showed that prodrug modification of the parent drug can improve the drug-like properties and enable the molecule suitable for oral administration. We proved that the caged inhibitor lost its activity in disrupting Keap1-Nfr2 interaction and inflammation derived ROS allowed for the release of the active entity to antagonize the inflammatory conditions in both cellular and in vivo inflammatory models. To the best of our knowledge, it is the first example of H2O2-responsive prodrug suitable for oral administration, and this study highly stresses the in vivo anti-inflammation efficacy of small molecule Keap1-Nfr2 inhibitory agents.

2. Materials and methods

2.1. Chemistry

The synthesis of prodrugs is highlighted in Scheme 1. All chemicals purchased from commercial suppliers were used as received unless otherwise stated. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. Reactions were monitored by thin-layer chromatography on silica gel plates (GF-254) visualized under UV light. Melting points were determined on a Mel-Temp II melting point apparatus without correction. 1H NMR and 13CNMR spectra were recorded in DClO or DMSO-d6 on a Bruker Avance-300 instrument. Chemical shifts (δ) are reported in parts per million (ppm) from tetramethylsilane (TMS) using the residual solvent resonance (CDCl3: 7.26 ppm for 1H NMR, 77.16 ppm for 13C NMR; DMSO: 2.5 ppm for 1H NMR, 39.5 ppm for 13C NMR). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet). HR-MS spectra were recorded on a Water P200 micro mass spectrometer. Flash column chromatography was performed with 100–200 mesh silica gel and yields refer to chromatographically and spectroscopically pure compounds. The purity (≥95%) of the compounds was verified by the HPLC study performed on an Agilent C18 (4.6 mm × 150 mm, 3.5 μm) column using a mixture of solvent methanol/water at a flow rate of 0.5 mL/min and monitored by UV absorption at 254 nm. The petroleum ether used in the study was the grade of analytical pure and boiled over the range 60–90 °C.

2.2. Biology

2.2.1. HPLC assay for drug release study

Compounds were dissolved in DMSO as solutions (10 mM) and stored at −20 °C. The incubation was initiated by the addition of compound (10 mM) to phosphate buffer solution (PBS) (10 mM, pH 7.4) to obtain a final concentration of 50 μM and then added H2O2 followed by vortex mixing. The solution was incubated at 37 °C and conducted in triplicate. Samples were taken at appropriate time intervals and directly analyzed by HPLC analysis. Peak areas were recorded to calculate the percentage of compounds. Agilent 1260 HPLC and DAD detector with conditions: Agilent C18 column (4.6 × 150 mm, 3.5 μm); Mobile phase: methanol 70%; Flow rate: 0.5 mL/min. A standard curve for compounds was made to fit the measured concentrations.

2.2.2. Physicochemical property and cell membrane permeability determination

The pKa and partition coefficient (log D, pH 7.4) were determined according to the methods of Avdeef and Tsinman on a Gemini Profiler instrument (pION) by the “gold standard” Avdeef–Bucher
Scheme 1. Reagents and conditions: (a) NH₂OH·HCl, 95% ethanol, MeOH, 60 °C, 2 h, yield 57%; (b) Pd/C, H₂, THF, rt, 4 h; (c) 4-Methoxybenzenesulfonyl chloride, Na₂CO₃, THF, 0 °C, 2 h, yield 83%; (d) 4-Methoxybenzenesulfonyl chloride, toluene, pyridine, 100 °C, 2 h, yield 64%; (e) DMF, K₂CO₃, ethyl bromoacetate, rt, 3 h, yield 84%; (f) LiOH, MeOH, H₂O, 2 h, yield 94%; (g) thiazolidin-2-one, DMAP, DCC, DMF, rt, 6 h, yield 76%.

Fig. 1. Proposed scheme for activation of the thiazolidinone prodrugs.
potentiometric titration method. The pH-metric method was used to determine the intrinsic solubility. The potentiometric solubility data were obtained with the pSOL model 3 instrument (pION INC., Cambridge, MA, USA). Permeability coefficients were determined via double sink PAMPA on a PAMPA Explorer instrument (pION).

2.2.3. Cell culture and ARE-luciferase activity assay

The mouse RAW 264.7 cell line was obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. HepG2 cells stably transfected with a luciferase reporter (HepG2-ARE-C8) were kindly provided by Professor Dr. A. N. Tony Kong (Rutgers University, Piscataway, NJ) and Prof. Rong Hu (China Pharmaceutical University, Nanjing). The cells were maintained by regular passage in modified RPMI-1640 medium (Gibco, Invitrogen Corp., USA) supplied with 10% FBS, 100 units per mL penicillin and 100 μg/mL streptomycin, cultured at 37 °C in a water vapour saturated atmosphere with 5% CO₂. The experimental procedures were carried out as reported previously [60].

2.2.4. RNA extraction and qRT-PCR analysis

The experimental procedure of quantitative real-time RT-PCR was previously reported. Total RNA of RAW264.7 cells was extracted from the treated cells using TRizol reagent (Invitrogen). Then the RNA was converted to cDNA by reverse transcriptase (PrimeScript RT reagent kit) according to the manufacturer’s instructions. Quantitative real-time RT-PCR analysis of Nrf2, HO-1, NQO1 and GCLM were performed by using the StepOne System Fast Real Time PCR system (Applied Biosystems). The values are expressed as the fold of the control. All genes’ mRNA expression was normalized against β-Actin expression.

2.2.5. Western blot analysis

Anti-Nrf2 (ab62352), anti-IL-1β (ab45692) antibodies were purchased from Abcam Technology (Abcam Technology, England). Anti-HO-1 (SC-136960) and anti-NQO1 (SC-271116) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin (60,008-1-lg), anti-GCLM (14241-1-AP) antibodies were purchased from Proteintech Group (Proteintech Group, USA). Briefly, the extracts were separated by SDS-PAGE and then electro-transferred to PVDF membranes (PerkinElmer, Northwalk, CT, USA). Membranes were blocked with 1% BSA for 1 h followed by incubation with a primary antibody at 4 °C overnight. Then they were washed and treated with a DyLight 800 labeled secondary antibody at 37 °C for 2 h. The membranes were screened through the odyssey infrared imaging System (LI-COR, Lincoln, Nebraska, USA).
2.2.6. IL-1β, IL-18, IL-6, TNF-α and NO production

Levels of IL-1β (IL-1β (m) ELISA kit, EK0394, Boster), IL-18 (IL-18 (m) ELISA kit, #EMC011, NeoBioscience), IL-6 (IL-6 (m) ELISA kit, EK0411, Boster), TNF-α (TNF-α (m) ELISA kit, EK0527, Boster) and NO production (Nitrate/Nitrite Assay Kit, S0023, Beyotime, China) were evaluated using commercially available kits according to the manufacturer’s instructions.

Table 1
| Compd | pKa  | LogD at pH 7.4 | Aqueous solubility at pH 7.4 (μg/mL) | Pe at pH 7.4 (10⁻⁶ cm/s) |
|-------|------|----------------|-------------------------------------|--------------------------|
| Pro2  | 4.53 | 2.34           | 879.6                               | 6.35                     |
| 2     | 4.31 | 1.88           | 426.2                               | 0.80                     |

Fig. 4. Comparison of activities of pro2, 2 and 3. (A) Relative ARE induction activity in HepG2-ARE-C8 cells under normal conditions. (B) Relative ARE induction activity under various concentrations of LPS in HepG2-ARE-C8 cells. Cells were pretreated by various concentrations of LPS for 8 h, and then treated with selected compounds (200 nM) or DMSO (for use as the control) for another 12 h. The values are expressed as the fold of the control. (C) The EC₅₀ curves of the relative ARE induction activity. Cells were pretreated by LPS (20 ng/mL) for 8 h, and then treated with various concentrations (0.001–50 μM) of selected compounds or DMSO (for use as the control) for another 12 h. The values are expressed as the fold of the control.
Fig. 5. Prodrug activated the Nrf2-ARE regulated antioxidant system in the RAW264.7 cells. RAW264.7 cells were pretreated with 20 ng/mL LPS for 8 h and then treated with compounds for another 16 h. PCR analysis of HO-1 (A), NQO1 (B) and GCLM (C) in the RAW264.7 cells. (D) Western blot analysis of the Nrf2-targeted proteins in the RAW264.7 cells. (E-H) Measurement of SOD, GSH-PX, GSH/GSSG and MPO level in the RAW264.7 cells. The values shown are the means ± SEM (n = 3 independent observations). *p < 0.05, **p < 0.01, ***p < 0.001, which were calculated with one-way ANOVA.

Fig. 6. Prodrug reduced inflammatory factors production induced by LPS in the RAW264.7 cells. RAW264.7 cells were pretreated with 20 ng/mL LPS for 8 h and then treated with compounds for another 16 h. PCR analysis of IL-1β (A) and IL-6 (B) in the RAW264.7 cells. (C) Western blot analysis of IL-1β and IL-6 protein levels in the RAW264.7 cells. Concentrations of IL-1β (D), IL-6 (E), TNF-α (F) and NO (G) in the RAW264.7 cell culture supernatants. The values shown are the means ± SEM (n = 3 independent observations). *p < 0.05, **p < 0.01, ***p < 0.001, which were calculated with one-way ANOVA.
The prodrug reduced the LPS-induced production of the pro-inflammatory factors in vivo. Levels of serum IL-1β (A), IL-6 (B), TNF-α (C) and IFN-γ (D) were measured by Elisa kits. *p < 0.05, **p < 0.01, ***p < 0.001, which were calculated with one-way ANOVA.
agents. With these considerations in mind, we designed and synthesized the thiazolidinone-based prodrugs of potent Keap1-Nrf2 inhibitors (pro1 and pro2, Fig. 1 and Scheme 1). These two prodrugs demonstrated no inhibition against Keap-Nrf2 interaction in the FP assay, indicating that the thiazolidinone pro moiety did abolish the activity of parent drugs.

The synthesis of the prodrugs is shown in Scheme 1. Amination of commercially available 1-nitronaphthalene afforded 4-Nitronaphthalen-1-amine (4). The nitro group of 4 was reduced by hydrogen and Pd/C, and the subsequent condensation with 4-Methoxybenzenesulfonyl chloride gave compound 5. Compound 6 were obtained by nucleophile substitution of NH by ethyl bromoacetate in the presence of K₂CO₃ in DMF, and the subsequent condensation with 4-Methoxybenzenesulfonyl chloride gave compound 7. Hydrolysis of the ester group of 7 resulted in the parent compound 2. The thiazolidinone moiety was then introduced through a DCC/DMAP-mediated coupling with 2 to give the desired prodrug pro2. The synthesis for CPUY192002 (1) had been previously reported [61]. Amide bond formation between the carboxylic acid compound 1 and the thiazolidinone moiety was also performed via addition of DCC and DMAP in DMF, which finally gave the prodrug pro1.

3.2. Validation of H₂O₂-responsive parent drug release and evaluation of stability

To verify that H₂O₂ was able to deprotect the thiazolidinone group, H₂O₂-induced prodrug transformation experiments were performed and monitored by HPLC. However, in the presence of H₂O₂, the prodrug pro1, which contains two thiazolidinone pro-moieties, was hydrolyzed to generate the compound with only one free carboxylic acid, identified by UPLC/HRMS, and the production of compound 1 cannot be observed even after 12 h (Fig. S2). In the case of the prodrug pro2, as expected, after incubating 24 h, a H₂O₂ concentration-dependent release of the parent compound 2 was observed, and approximately 60% prodrug can be released upon treatment with 1 mM H₂O₂ (Fig. 2A). Upon treatment with 10 mM H₂O₂, pro2 was activated with increasing incubating time and completely released to the parent compound 2 within 3 h (> 98% conversion determined by HPLC, Fig. 2B and C). Then, the H₂O₂-responsive activation of pro2 was further examined in live cells. Murine macrophage cells (RAW264.7) were stimulated with H₂O₂ for 24 h to induce the intracellular ROS and then culture medium was replaced with fresh medium. Pro2 (1 mM) was added to stimulated or non-stimulated cells for incubation and the cell lysates were analyzed by LC-MS. No peak of 2 was observed in non-stimulated cells, while obvious signal of 2 appeared in stimulated cell lysate (Fig. S3). The results demonstrated that pro2 can be taken up by cells and transformed to 2 by intracellular H₂O₂.

Then, the stability of the synthesized prodrug was evaluated in different relevant physiological conditions. It was observed that pro2 was relatively stable to hydrolysis in PBS of different pH values from 4 to 10 after incubating 24 h (Fig. 3A). In bio-relevant media, including simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), more than 50% of pro2 remained after 8 h (Fig. 3B). Moreover, pro2 is quite stable in rat plasma (Fig. 3C) and in co-incubation with rat liver microsomes (Fig. 3D), indicating the high metabolic stability.

3.3. Physicochemical properties and cell membrane permeability

Then, we determined the physicochemical properties of pro2 as well as the parent compound 2. As shown in Table 1, the pKa and logD values of 2 are 4.31 and 1.88, while the pKa and logD of pro2 are 4.53 and 2.34, respectively. These results confirmed that pro2 is a less ionizable and less polar molecule than 2, which can enhance the passive cell membrane permeability of pro2. We then examined the cell membrane permeability by using a standard parallel artificial membrane permeability assay (PAMPA). The parent compound 2 showed a permeability coefficients (Pe) value of 0.80 × 10⁻⁶ cm/s, while pro2 gave a Pe value of 6.35 × 10⁻⁶ cm/s, which proved that pro2 can permeate the cell membrane more easily.

3.4. Selective activation of prodrug by inflammation-derived ROS

Next, the cellular biological activity of pro2 was investigated. Initial studies aimed at demonstrating selective Nrf2 activation effects of pro2 upon ROS. The Nrf2/ARE luciferase reporter assay was applied to evaluate the cellular Nrf2 activity, and the methyl ester prodrug 3, which is not responsive to cellular ROS, was used as an unsel ective control to demonstrate the ROS-responsive selectivity of pro2. As shown in Fig. 4A, both of 2 and 3 concentration-dependently induced the ARE activity. The methyl ester prodrug 3 showed higher ARE-induction activity compared to the active compound 2. However, pro2 kept nearly inactive even at the highest concentration, which indicated that pro2 is stable and does not affect the Nrf2-ARE system under physiological conditions. In order to simulate the inflammation related high ROS microenvironment, cells were exposed to lipopolysaccharide (LPS), a widely used inflammation inducer which can enhance the production of intracellular ROS. Treatment with LPS can elevate ROS level with the increase of LPS concentration (Fig. S4). Then, Nrf2-ARE induction activities of these compounds were examined under the gradient concentrations of LPS. In order to exclude the LPS-induced Nrf2 activation effects, cells pretreated with gradient concentration of LPS alone were used as the control. As shown in Fig. 4B, LPS-stimulation before drug exposure (200 nM) activated pro2 and resulted in the LPS concentration-dependent enhancement of Nrf2-ARE inducing activity, while the potency of 2 and 3 did not show obvious changes. Under LPS stimulation, pro2 showed the lowest EC₅₀ value (0.32 μM) among them in the ARE induction activity (Fig. 4C). Together, the thiazolidinone-based prodrug pro2 can be selectively activated by LPS-induced intracellular ROS, enhancing the Nrf2-ARE system at a much lower concentration than the parent drug.

3.5. Activation of the Nrf2-ARE regulated antioxidant system in the RAW264.7 cells

To ascertain the effects of pro2 on the transcription of Nrf2-ARE-driven genes, the mRNA levels of Nrf2 downstream genes, Heme oxygenase 1 (HO-1), NAD(P)H: Quinone Oxidoreductase 1 (NQO1) and glutamate-cysteine ligase, modifier subunit (GCLM), were examined (Fig. 5A–C). The quantitative real-time PCR (qRT-PCR) analysis showed that exposure of RAW264.7 cells to 20 ng/mL LPS for 8 h slightly increased the transcription of Nrf2 targeted genes. Addition of pro2 (500 nM) highly enhanced the transcription, superior to the effects of 2 and its methyl ester prodrug at the same concentration. The protein levels of these genes were measured by immunostaining, and the methyl ester prodrug pro2 (Fig. 5D).

Subsequently, to explore the effects of pro2 on antioxidant capacity under inflammatory conditions, the activities of representative enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), were determined. Mouse RAW 264.7 cells treated with LPS (20 ng/mL) alone showed the obvious decrease in activities of SOD and GSH-Px (Fig. 5E and F). Treatment with pro2 significantly restored the activities of SOD and GSH-Px, while the parent compound and its methyl ester analog were less efficient. We also measured the GSH/GSSG ratio and myeloperoxidase (MPO) activity (Fig. 5G and H), two important markers for indication of oxidative stress. LPS exposure caused huge decline in the GSH/GSSG ratio and sharp rise in the MPO activity, confirming that LPS could cause the oxidative stress. The addition of pro2 restored the levels nearly back to normal, the parent drug and its methyl analog showed similar trend but much lower activity.
3.6. Anti-inflammatory effects of pro2 in the LPS-induced RAW264.7 cells

Nrf2 activation has been proven to be an effective way to relieve the inflammatory conditions. We first examined the mRNA levels of IL-1β and IL-6, which can be transcriptionally repressed by Nrf2 [66]. After LPS (20 ng/ml) stimulation for 8 h, the mRNA levels of IL-1β and IL-6 were remarkably induced in the RAW264.7 cells. Treatment with 2 or the methyl ester prodrug 3 slightly inhibited the transcription of IL-1β and IL-6. Of note, treatment with pro2 resulted in significant suppression effects (Fig. 6A and B). Consistent with the results of the PCR analyses, 2 and 3 showed moderate effects on the inhibition of LPS-induced elevation of IL-1β and IL-6 protein, but pro2, at the same concentration, almost completely suppressed the elevation (Fig. 6C). Then, we quantitatively evaluated several inflammatory mediators that are closely related with ROS, including IL-1β, IL-6, TNF-α and NO. As expected, all these inflammatory factors increased markedly in the LPS-treated groups, and pro2 showed obvious superiority in the suppression of IL-1β, IL-6 and TNF-α production (Fig. 6D–F). The LPS triggered NO production was also remarkably diminished by pro2 with an IC50 of 0.12 μM, while compound 2 and 3 showed higher EC50 values (Fig. 6G).

3.7. In vivo efficacy of pro2 against the LPS-induced inflammatory conditions

After confirming the ROS-responsive Nrf2 activation effects of pro2 in live cells, we finally investigated the therapeutic potential of pro2 in vivo. To explore the suitability of thiazolidinone-based prodrug for oral administration, we evaluated the pharmacokinetics of pro2 for both IV and PO, and the oral pharmacokinetics with bioavailability of 68.1% and half-life of 2.09 ± 0.93 h (Table 2) indicated pro2 could be an effective oral medication.

Then, the murine LPS challenged acute inflammation model was used to evaluate anti-inflammation effects in vivo. C57BL/6 mice were challenged with LPS (300 μg/kg, IP) and then orally administrated with compound 4 h after the LPS challenge for 3 days. The blank group only received saline during the experiment. Dexamethasone (DXM), the widely used steroid anti-inflammatory drug, was used as the positive control. Animals were sacrificed 24 h after the last dose of compound and sera were collected. LPS challenge markedly elevated the pro-inflammatory cytokines in mouse sera, including IL-1β, IL-6, TNF-α and IFN-γ. DXM treatment (10 mg/kg) diminished inflammatory response (Fig. 7A–D). Oral administration of 2 (40 mg/kg) showed moderate anti-inflammation activity, much less potent than DXM. Pro2 showed comparable therapeutic effects with DXM at the same dose (10 mg/kg), and high dose of pro2 showed more potent effects, indicating the dose-dependent behavior. Taken together, these results suggested that the thiazolidinone-based ROS-responsive prodrug of the Keap1-Nrf2 PPI inhibitor is an effective oral medication for oxidative stress related inflammatory conditions.

4. Conclusions

In this study, a new ROS-activated prodrug pro2 was developed by utilizing H2O2-responsive thiazolidinone moiety to shield the key carboxyl pharmacophore in Keap1-Nrf2 inhibitor. Inflammation derived intracellular H2O2 can deprotect carboxyl group, producing the potent Keap1-Nrf2 inhibitor to selectively activate Nrf2-regulated antioxidant system in target cells. Moreover, the thiazolidinone modification of the carboxyl group improve the physicochemical properties and cell membrane permeability of the parent drug. Further cellular studies showed that pro2 can stay inactive at physiological conditions and be much more potent than the parent drug at inflammatory conditions. In addition, thiazolidinone-based drug displayed good stabilities at various physiological conditions and appropriate PK profile for oral administration. Finally, in vivo therapeutic activity was demonstrated by oral use of pro2 in LPS challenged acute inflammation model.

An increasing body of literature has revealed that over and unselective activation of Nrf2 may cause unexpected risks and particularly contribute to the initiation and progression of cancer [67–70], inspiring the discovery of precision Nrf2 activators. Direct Keap1-Nrf2 PPI inhibitors are assumed to have higher target selectivity than electrophilic Nrf2 activators. However, one critical challenge for the Keap1-Nrf2 inhibitors has also emerged. Direct Keap1-Nrf2 inhibitors are designed to bind to Keap1 with a similar pattern of Nrf2 ETGE motif, which may also affect functions of other Keap1 substrates with a similar recognition motif [71,72]. Developing Keap1-Nrf2 inhibitors selectively activating Nrf2 in specific pathologic conditions is a new avenue. The carbonyl group, the key pharmacophore in the direct Keap1-Nrf2 inhibitors, can be utilized to design a targeted prodrug that not only improves the ADMET properties but also selectively activates Nrf2. The high concentrations of H2O2 in the inflammatory environment can serve as the stimulus for prodrug activation. This pathologic site-selective drug delivery system therefore restricts the effects of Keap1-Nrf2 inhibitors on Keap1-involved interacome of normal tissues.

Taken together, our study confirmed that this H2O2-activated prodrug can achieve selective Nrf2 activation and enhanced in vivo efficacy simultaneously, providing an attractive approach for the further development of Keap1-Nrf2 inhibitors.

Declaration of competing interest

All authors have given approval to the final version of the manuscript. The authors have no conflicts of interest to declare.

Acknowledgments

This study was supported by Projects 81773639, 81773581, 81803363 and 81930100 of the National Natural Science Foundation of China; National Science & Technology Major Project ‘Key New Drug Creation and Manufacturing Program’, China (No: 2018ZX09711002 and 2017ZX09302003); the Priority Academic Program Development of Jiangsu Higher Education Institutions; China Postdoctoral Science Foundation-funded Project (2017M620231 and 2018T110576); CPU2018GY02 of Double First Class Innovation Team of China Pharmaceutical University; Program for Outstanding Scientific and Technological Innovation Team of Jiangsu Higher Education, Jiangsu Qing Lan Project and the Young Elite Scientists Sponsorship Program by CAST.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101565.

Abbreviations

| Acronym | Description |
|---------|-------------|
| Nrf2    | Nuclear factor erythroid 2-related factor 2 |
| Keap1   | Kelch-like ECH-associated protein 1 |
| H2O2    | Hydrogen peroxide |
| ROS     | Reactive oxygen species |
| ARE     | Antioxidant response element |
| PPI     | Protein-protein interaction |
| ITC     | Isothermal Titration Calorimetry |
| PAMPA   | Parallel artificial membrane permeability assay |
| Pe      | Permeability coefficients |
| LPS     | Lipopolysaccharide |
| HO-1    | Heme oxygenase 1 |
| NQO-1   | NAD(P)H dehydrogenase (quinone) 1 |
| GCLM    | Glutamate-cysteine ligase regulatory subunit |
| qRT-PCR | Quantitative real-time PCR |
| SOD     | Superoxide dismutase |
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