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7,8-Dihydroxyflavone alleviates ulcerative colitis by inhibiting ferroptosis

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ABSTRACT: As a naturally occurring flavonoid, 7,8-dihydroxyflavone (7,8-DHF) exhibits protective effects in preventing neurodegeneration by activating the tropomyosin receptor kinase B (TrkB). However, whether 7,8-DHF influences the progression of chronic diseases through TrkB-independent mechanisms remains largely unclear. Here, we discovered that 7,8-DHF could protect cells from oxidative stress-induced cell death by acting as a previously unrecognized inhibitor of ferroptosis. Ferroptosis is a form of programmed cell death caused by uncontrolled peroxidized phospholipids. Although 7,8-DHF functions as a neuron protector by acting as a TrkB agonist, our data demonstrate that 7,8-DHF suppresses ferroptosis independently of activating TrkB signaling pathways. Indeed, as natural radical-trapping antioxidants, 7,8-DHF directly neutralizes peroxidized phospholipids to inhibit ferroptosis in a GSH/GPX4-independent manner. Since the low bioavailability of 7,8-DHF *in vivo* limits its clinical uses, we developed engineered 7,8-DHF nanoparticles (7,8-NPs) to enhance its absorption and utilization in a pharmacokinetic model. Consistent with previous studies, we confirmed that 7,8-NPs effectively reduce Alzheimer's disease pathology. Moreover, we found that 7,8-NPs could alleviate ulcerative colitis by reducing inflammation and ferroptotic stresses, suggesting the critical role of 7,8-DHF against ferroptosis *in vivo*. These observed effects on ferroptosis inhibition demonstrated that 7,8-DHF exhibits a molecular signature associated with improved colon health.

Keywords: 7,8-dihydroxyflavone; lipid peroxidation; ferroptosis; radical trapping antioxidants; ulcerative colitis.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by relapsing and remitting inflammation of the colon and rectum ¹. The interplay between inflammation and cell death is the critical process in UC pathology. Dysregulated cell death contributes to epithelial barrier disruption by directly killing cells. In addition, cell death-induced damage-associated molecular patterns (DAMPs) release enhances the inflammatory microenvironment and aberrant immune response ². Apoptosis, necroptosis, and

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pyroptosis have been identified as the main types of cell death in a caspase-dependent manner, however, the intervention by applying the pan-caspase inhibitor (such as emricasan) of these cell deaths demonstrates unfavorable results, suggesting that an additional type of cell death may contribute to the chronic pathology^{3, 4}. Recently, ferroptosis has been considered as the potential target in UC therapy, while the efficient anti-ferroptosis drugs to ameliorate UC require further investigation⁵.

The progression from IBD to colitis-associated colorectal cancer (CAC) underscores a direct link between chronic inflammation and carcinogenesis⁶. CAC demonstrates distinct characteristics to sporadic colorectal cancer (CRC), such as diffuse pattern with flatter dysplastic lesions, a higher malignant grade, and unique genetic alterations (higher frequency of *TP53* and *KRAS*, but lower frequency of *APC* mutations)⁷. Vitexin, a natural flavonoid, postpones the transition from inflammatory colitis to CRC by acting as a Vitamin D Receptor agonist and modulating macrophage polarization⁸. Whether other plant-derived compounds can mitigate the transition of CAC requires further investigation.

Bidirectional communication across the gut microbiota-brain axis is involved in multiple biological processes. The nervous system rapidly transmits information between the gut and the brain to coordinate intestinal function. Conversely, gut microbiota can regulate the nervous system to contribute to mood, cognition, and behavior^{9, 10}. Alzheimer's disease (AD), one of the most common neurodegenerative diseases, is characterized by some neuropathological hallmarks, such as β -amyloid ($A\beta$) aggregation. Patients with AD are at high risk of developing UC¹¹. AD patients have shown a decrease in microbial richness and diversity¹². Especially, the increase of pro-inflammatory microbiome was discovered in elderly AD patients, whereas the anti-inflammatory microbiome was reduced¹³, suggesting the regulatory role of the gut-brain axis in inflammation and AD pathology. Aging, chronic stress, or other conditions lead to perturbations in intestinal permeability and the mucosal defense system, affecting gut microbiota populations and, consequently, in turn mediating the $A\beta$ deposition through microglia during AD in mice^{14, 15}. UC patients induced microglial and astrocyte activation, $A\beta$ accumulation, and cognitive impairment compared with age-matched individuals without IBD¹⁶⁻¹⁸. Overall incidence of AD in patients with IBD was significantly increased, and the risk of developing AD appeared to elevate with IBD chronicity¹⁹. Thus, the gut-brain axis is critical for host intestinal and neuropsychiatric health.

Brain-derived neurotrophic factor (BDNF) supports motor neuron differentiation and survival by activating its receptor, tropomyosin receptor kinase B (TrkB)^{20, 21}. TrkB is a potent therapeutic target in several neurodegenerative disorders such as Parkinson's disease and AD; however, the low efficiency of drug delivery to the central nervous system restrains the application of TrkB agonist²². Previous studies identified 7,8-DHF as the natural compound that efficiently crosses the blood-brain barrier (BBB), binds TrkB, and activates TrkB signaling, providing a surprising solution of TrkB agonist against neurodegenerative diseases²³⁻²⁵. 7,8-DHF is a naturally occurring flavonoid found in the tree *Godmania aesculifolia* and the weed *Tridax procumbens*. In addition, the protective function of 7,8-DHF was discovered in chronic human diseases, such as depression, osteoporosis, and obesity²⁶⁻²⁸. Although 7,8-DHF manifests powerful effects in chronic

diseases, the undetermined working mechanism and low absorption rate *in vivo* hinder its application. For example, some studies indicate that 7,8-DHF fails to activate the TrkB receptor or downstream signaling pathways under certain conditions^{29, 30}, indicating that 7,8-DHF-mediated protective effects could have undescribed mechanisms. Moreover, 7,8-DHF has a short half-time and is rapidly degraded in the body, which declines its bioavailability (4.6%)²⁵. Therefore, overcoming these issues will promote the application of 7,8-DHF.

Ferroptosis is a newly defined type of programmed cell death triggered by excessive lipid peroxidation in the presence of iron³¹. Since the definition of ferroptosis, the regulatory mechanisms of ferroptosis have shown robust relationships with the pathology of chronic diseases³². The ways to eliminate ferroptotic cell death demonstrate promising potential in mitigating chronic organ injuries. Glutathione peroxidase 4 (GPX4) is the cornerstone in directly detoxifying peroxidized lipids into their alcohol isoforms by utilizing glutathione (GSH)^{33, 34}. The uptake of cystine by the cystine/glutamate transporter system SLC7A11 and subsequent GSH synthesis pathways are essential for GPX4-mediated ferroptosis defense³⁵⁻³⁷. Repression of the SLC7A11/GSH/GPX4 axis promotes vascular calcification phenotype in chronic kidney disease by triggering ferroptosis³⁸. The progression of nonalcoholic steatohepatitis induced by GPX4 inactivation can also be alleviated by inhibition of ferroptosis³⁹.

Recent studies reveal that GSH/GPX4 independent anti-ferroptosis mechanisms can effectively eliminate lipid peroxides and inhibit ferroptosis⁴⁰. Ferroptosis suppressor protein 1 (FSP1, encoded by *AIFM2*) or dihydroorotate dehydrogenase converts CoQ₁₀ to CoQ₁₀H₂ to protect cells from ferroptosis⁴¹⁻⁴³. Mechanistically, CoQ₁₀H₂ acts as a type of lipophilic antioxidant, called radical-trapping antioxidants (RTAs), to clear out the peroxidized lipids directly. Beyond CoQ₁₀H₂, the reduced form of vitamin K, VKH₂, is the other type of RTAs, which can also inhibit ferroptosis. Vitamin K reductase, FSP1, or vitamin K epoxide reductase complex subunit 1-like 1 mediates vitamin K reduction, which is critical for lipid peroxidation elimination and ferroptosis defense^{44, 45}. These studies emphasize that the identification of novel RTAs is critical in understanding the mechanism of ferroptosis regulation and discovering potent metabolites against ferroptosis. Nevertheless, the relationship between RTAs and chronic injuries remains unclear.

In this study, we identified 7,8-DHF as a potent and universal ferroptosis inhibitor. We found that 7,8-DHF directly counteracts lipid peroxidation in neither GSH/GPX4-independent nor TrkB-independent manner, indicating a previously undescribed anti-oxidative function beyond its role as a mimic of BDNF. The application of low concentrations of 7,8-DHF efficiently protects mice's colon from the dextran sulfate sodium (DSS)-induced acute colitis by inhibiting ferroptosis. Thus, our results demonstrate 7,8-DHF as a dual-alleviator of ulcerative colitis and neurodegenerative diseases through distinct mechanisms, thus providing intriguing and novel insights into applying 7,8-DHF in treating chronic diseases.

2. Materials and methods

2.1. Cell culturing.

Human fibrosarcoma HT1080 cells, human melanoma A375 cells, human neuroblastoma SK-N-BE(2)-C cells, human neuroblastoma LAN-1 cells, mouse hippocampal HT-22 cells, human colon epithelial NCM460 cells, human colorectal adenocarcinoma Caco-2 cells, and human colorectal adenocarcinoma HT-29 cells were previously obtained from American Type Culture Collection (ATCC). HT1080 and A375 cells were well-accepted model cells to investigate the universal role of 7,8-DHF against ferroptosis; HT-22, SK-N-BE(2)-C, and LAN-1 cells were used to investigate the specific role of 7,8-DHF in nerve cell lines; NCM460, Caco-2, and HT-29 cells were used to investigate the specific role of 7,8-DHF in intestinal epithelial cell lines. GPX4 knockout and GPX4/ACSL4 double knockout cells were previously described^{45,46}. Cells were cultured with DMEM containing 10% (v/v) fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin, and 100 µg/ml streptomycin (Gibco) at 37 °C with a humidified atmosphere of 5% CO₂. All cells were cultured in 10 cm plates and seeded into 6-well plates, 12-well plates, or 96-well plates for cell viability and lipid peroxidation analysis. All cell lines have been regularly tested to be negative for mycoplasma contamination every month. No cell lines used in this work were listed in the ICLAC database.

2.2. siRNA-mediated *TrkB* ablation.

TrkB knocking down was performed by transfection of SK-N-BE(2)-C, LAN-1, NCM460, or Caco-2 cells with *TrkB* targeting siRNA oligo for 24 h using Lipofectamine 3000 (Invitrogen) and then transfected again according to the manufacturer's protocol. The siRNA sequences are listed below:

TrkB #1: GAGGAAGAACAUCAAGGGCAU;

TrkB #2: CCAGAUUGAAAGGGAGUGAUU.

2.3. Chemicals.

Reagents and compounds were used as follows. IKE was synthesized by MedChemExpress. 7,8-Dihydroxyflavone (7,8-DHF, 16946) and 1-Stearoyl-2-15(S)-HpETE-sn-glycero-3-PE (15(S)-HpETE-SAPE, 25856) were purchased from Cayman. Other compounds were all purchased from Sigma-Aldrich: tert-butyl hydroperoxide (TBH, 458139) and RSL-3 (SML2234), N-acetyl-L-cysteine (NAC, A7250), Ferrostatin-1 (ferr-1, SML0583), Liproxstatin-1 (lipro-1, SML1414), Deferoxamine (DFO, D9533), α-Tocopherol (Vitamin E, T3251), α-Ascorbic acid (Vitamin C, A92902).

2.4. Preparation of 7,8-DHF nanoparticles (7,8-NPs).

Oleanolic acid-mediated nanoparticles were prepared by the ultrasonic emulsification method. 20 mg oleanolic acid was accurately weighed and dissolved in 2 mL methanol, 2 mg 7,8-DHF was added into 1 mL solution, which was dissolved by ultrasound (SCIENTZ). 2.5 mL of 2.5% PVA solution was added and emulsified under vortex conditions for 90 seconds, then quickly transferred to a beaker containing 35 mL of 0.3% PVA solution. Place it on a magnetic stirrer (LICHEN, LC-MSB-S) and stir continuously for 12 h to evaporate the solvent. The solution was collected and centrifuged for 30 min (1200 r/min, 4°C), and the precipitate was gathered to obtain nanoparticles loaded with 7,8-DHF.

2.5. Pharmacokinetics detection of 7,8-NPs.

A total of twenty-five C57BL/6 male mice (5-6-week-old) were randomly divided into 5 groups ($n = 5$). Mice were intraperitoneally injected with 7,8-NPs at a dose of 5 mg/kg. Tail vein blood was collected at 0 h, 0.5 h, 2 h, 6 h, and 24 h, and the content of 7,8-DHF in the blood was detected by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford). Then the content curve was drawn to observe pharmacokinetic characteristics of 7,8-NPs. This part of the HPLC-MS/MS detections was performed by Beijing Junfei Technology (Beijing, China).

2.6. DPPH assay.

2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma D9132) was prepared in a 50 μ M solution with methanol. And 1 μ l indicated compound in DMSO was added to 1 ml prepared DPPH buffer and rotated for 5 min at room temperature. Every group was set for triplicate. 1 ml reactions were measured at an absorbance of 517 nm with Ultrospec 2100 Pro UV/Spectrometer (Amersham Biosciences). Methanol was used as the blank.

2.7. Cystine starvation and glutamate treatment.

For cystine starvation, cells were trypsinized and washed with PBS three times. Then, cells were seeded into cystine-deficient DMEM (21013024, Invitrogen) supplemented with 4 mM glutamine (Sigma-Aldrich, G8540) and 200 μ M methionine (Sigma-Aldrich, M5308) containing 10% FBS in the presence of the indicated concentration of cystine (Sigma-Aldrich, C7602). For glutamate treatment, HT22 cells were sub-cultured into regular DMEM medium with or without 4 mM glutamate (Sigma-Aldrich, G1251) for the indicated time.

2.8. Cell viability assay.

Without specific mention, 5×10^3 per well indicated cells were seeded into a 96-well microplate (PerkinElmer) overnight. Cystine starvation, glutamate treatment, or Ferrostatin-1 withdrawal were applied to cells during seeding on 96-well plates with the indicated chemicals for the indicated time. For overnight adhered cells, cells were treated with ferroptosis inducers for the indicated time, accompanied by adding the indicated chemicals.

There were three biological replicates per experiment condition. Before testing cell viability, the 96-well plate stood still at room temperature for 30 min. And cellular ATP levels were measured with CellTiter-Glo 2.0 Cell Viability Assay reagent (G9243, Promega) following the manufacturer's instructions. Then, the plates were immediately transferred to the GloMax Discover Microplate Reader (Promega). Cell viability was calculated by the ATP level of the treated group compared to the untreated group. 100 percent of cell viability was regarded as 0 percent of cell death. Nonlinear regression analysis was used to measure the fit curves of cell viability using GraphPad Prism 8.0.

2.9. Cell death staining.

4×10^4 cells were seeded in a 12-well plate per well one day prior to the experiment. After indicated treatment, the medium was replaced with fresh medium containing 30 nM SYTOX green dead cell stain

(Invitrogen, S34860) and incubated for 1 h at 37°C. Three images per experiment condition were captured with both the bright channel and the green channel by microscopy (Olympus, IX51).

2.10. GSH/GSSG detection.

Cells were treated under the indicated conditions for the indicated time by adding the indicated chemicals. GSH/GSSG ratio was measured by following the manufacturer's instructions of GSH/GSSG-Glo Assay (V6611, Promega).

2.11. Liposome preparation.

Chloroform-solved egg PC (Sigma-Aldrich, P3556) was mixed in a dry vial and then evaporated under vacuum to yield a thin film on the vial wall. The lipid film was then re-suspended in PBS, yielding a 20 mM lipid suspension. The lipid suspension was subjected to 3 times of 2 min dry ice freezing, 5 min room temperature thawing, and 1 min sonication cycles. The lipid suspension was then extruded 25 times using a mini extruder (Avanti Polar Lipids, 610000) equipped with a 100 nm polycarbonate membrane to form homogeneous liposomes.

2.12. *In vitro* STY-Bodipy autoxidation assay.

1mM Egg PC liposomes, 1μM STY-BODIPY probes (Cayman, 27089), and indicated RTAs were pre-mixed together for 5min at 37°C in a Black 96-well Microplate (PerkinElmer 6005320) in PBS (pH = 7.4). The autoxidation was initiated by adding 1mM 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH, Sigma-Aldrich, 440914) followed by vigorously mixing for 30s. The data was acquired by the fluorescence signals of excitation 488 nm and emission 518 nm every minute for 180 to 200 min using GloMax Discover Microplate Reader.

2.13. Real-time quantitative PCR (RT-qPCR).

Total RNA of cells or tissues was harvested using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription of template RNA into cDNA was conducted by SuperScript IV VILO Master Mix (Invitrogen). qPCR was performed with SYBR Green Master Mix (Invitrogen) to detect the target genes' mRNA expression levels. All samples were run in triplicate. Human *GAPDH* or mouse *Actin* expression level was used for normalization. The sequence of primers is shown in Supplementary Table S1.

2.14. Western blot and antibodies.

Indicated cells or tissues were lysed in Flag lysis buffer [50 mM Tris-HCl (pH 7.9), 137 mM NaCl, 1% Triton X-100, 0.2% Sarkosyl, 1 mM NaF, 1 mM Na₃VO₄, and 10% glycerol] containing protease inhibitor cocktail and 1 mM dithiothreitol, and 1 mM phenylmethyl sulfonyl fluoride. Lysis was centrifuged, and the supernatant was collected. Protein samples were quantified with Bio-Rad Protein Assay Dye Reagent (Bio-Rad, 5000006). The same amount of protein from different experiment groups was examined with Western blot analysis. Western blot was conducted for protein analysis according to standard methods with 4%-20% pre-cast SDS-PAGE gel (Invitrogen, XP0420). Commercial antibodies were shown as below: Vinculin (Sigma-Aldrich, V9131, 1:10000 dilution), ZO-1 (Proteintech, 21773-1-AP, 1:500 dilution)

Occludin (Proteintech, 27260-1-AP, 1:1000 dilution), ACSL4 (Santa Cruz, sc-271800, 1:1000 dilution), GPX4 (Abcam, ab125066, 1:500 dilution), Phospho-ERK1/2 (Cell Signaling Technology, 4370S, 1:1000 dilution), ERK1/2 (Cell Signaling Technology, 4695S, 1:1000 dilution), Phospho-S6 (Cell Signaling Technology, 4858S, 1:1000 dilution), S6 (Cell Signaling Technology, 2217S, 1:1000 dilution). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibody (Jackson ImmunoResearch) were visualized by ECL (ThermoFisher Scientific, 32106) or SuperSignal (Thermo Fisher Scientific, 34076).

2.15. Lipid peroxidation analysis using C11-BODIPY.

For the cystine starvation or glutamate treatment, 12×10^4 cells were seeded in a 6-well plate per well (Corning) the day before the experiment with cystine starvation or 4 mM glutamate, followed by adding the indicated reagents. For other ferroptosis inducer treatment, 12×10^4 cells were seeded in a 6-well plate per well the day before the experiment overnight. The next day, cells were treated with RSL3 in the absence or presence of indicated reagents for the indicated time. Next, Cells were labelled in PBS containing 2 μ M BODIPY 581/591 C11 dye (ThermoFisher Scientific, D3861) for 30 min at 37°C. Subsequently, cells were trypsinized and rinsed twice with PBS, re-suspended in 500 μ L PBS, and strained through a 35 μ m cell strainer (Falcon, 352235), followed by flow cytometry analysis (Canto II, BD Bioscience).

2.16. ELISA assay.

The expression levels of IL-6, IL-1 β , and TNF- α were detected from colonic epithelial tissues using the Mouse IL-6 Uncoated ELISA Kit (ThermoFisher Scientific, 88-7064-88), Mouse IL-1 β Uncoated ELISA Kit (ThermoFisher Scientific, 88-7013A-88), and Mouse TNF alpha Uncoated ELISA Kit (ThermoFisher Scientific, 88-7324-88), respectively, according to the manufacturer's protocols. The absorbance was recorded using GloMax Discover Microplate Reader.

2.17. Lipid peroxidation analysis by MDA or 4-HNE.

MDA or 4-HNE levels were detected from colonic epithelial tissues using the Lipid Peroxidation (MDA) Assay Kit (Abcam, ab118970) or Lipid Peroxidation (4-HNE) Assay Kit (Abcam, 238538) according to the manufacturer's protocol, respectively.

2.18. Phospholipidomics LC-MS/MS analysis.

Quantitative phospholipidomics were performed on an UPLC-MS/MS system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA) (Metabo-Profile, Shanghai, China). Lipid was extracted by 5mM ammonium acetate in methanol containing internal standards. Supernatant was transferred to a 96-well plate for further LC-MS analysis. All analyses were according to standard lipid peaks.

2.19. Transmission electron microscope.

NCM460 cells or 7,8-NPs were collected, fixed, dehydrated, and then embedded into resin blocks. The resin blocks were cut to 60 – 80 nm thin on the ultra microtome (UC7, LEICA). After 2% uranium acetate saturated alcohol solution staining for 8 min and 2.6% lead citrate staining for 8 min, the cuprum grids were

put into the grids board and dried overnight. The next day, the cuprum grids are observed under transmission electron microscope (HT7800, HITACHI).

2.20. Animal husbandry.

All the mice were housed in a temperature-controlled room (65–75 °F) with 40–60% humidity, with a light/dark cycle of 12 h/12 h. All animal experiments in this study were approved by the Zhongyan Zichuang Animal Ethics Committee of Beijing Changping District Life Science Park (Experimental Ethics Approval No. ZYZC202211008S).

2.21. MEF cells generation and genotyping.

MEFs were generated from day 13.5 embryos according to standard procedures. FBS used for MEFs was heat-inactivated and supplemented with 1% non-essential amino acids. The *Slc7a11*^{-/-} or *Slc7a11*^{+/+} cells were PCR genotyped using forward primer: 5'-TGTGGCAAGAACCTGCCCTCATA-3', and reverse primer: 5'-CCTGAACAGTATCTTCTGATACAC-3'.

2.22. APP/PS1 transgenic mice model.

Ten male APP/PS1 transgenic mice (3-month-old) or corresponding control mice were randomly divided into the control group or the 7,8-NPs-treated group (referred to as 7,8-NPs) (n = 5). Mice were administered 7,8-NPs by intraperitoneal injection at 10 mg/kg 7,8-NPs daily for a total of 15 days, and the corresponding mice were injected with normal saline. Mice were sacrificed 4 weeks after the last dose, after which the brains were collected for further analysis.

2.23. Dextran sulfate sodium (DSS)-induced acute ulcerative colitis (UC) mice model.

For wild-type C57BL/6 mice, thirty male mice (5-6-week-old) were randomly divided into six groups. For survival analysis, two groups of mice (DSS group and DSS+7,8-NPs group, n = 5) drank water containing 2.5% DSS (MP Biomedicals) for 7 days, and a 10 mg/kg 7,8-NPs intervention was performed daily in the DSS+7,8-NPs group. Then, the DSS or DSS+7,8-NPs mice drank water containing 1% DSS for an additional 18 days, and a 10 mg/kg 7,8-NPs intervention was performed once every three days in the DSS+7,8-NPs group. The experiment was stopped once all the mice had died. For other analysis, 4 groups (CTRL group, DSS group, 7,8-NPs group, and DSS+7,8-NPs group, n = 5) of mice drank water with or without 2.5% DSS in the presence or absence of 10 mg/kg 7,8-NPs intervention for 7 days. Mice were administered 7,8-NPs by intraperitoneal injection. During the experiment, body weight, water intake, fecal consistency, and occult blood were recorded and scored daily. The disease activity index (DAI) scores were calculated according to a previous study^{8, 47}. Briefly, weight loss score, stool consistency score, and rectal bleeding score were determined on a scale of 0 to 4, and DAI was calculated as the total score of weight loss, stool consistency, and rectal bleeding. On the seventh day post DSS-administration, mice were fasted for 6 h prior to gavage 20 mg/kg FITC-dextran (Sigma-Aldrich, FD4). 3 h later, mice were euthanized, and blood was drawn. Blood FITC-dextran concentration was determined by using GloMax Discover Microplate Reader with excitation at 485 nm and emission at 535 nm.

For APP/PS1 transgenic C57BL/6 mice, fifteen male mice (3-month-old) were randomly divided into three groups ($n = 5$). APP/PS1 mice represent a pre-plaque or initial-plaque stage and memory impairments by 3 months of age, which were used for investigating the role of gut inflammation and AD pathology. Mice drank normal water or water containing 2.5% DSS in the presence or absence of 10 mg/kg 7,8-NPs intervention for 7 days. Mice were administered 7,8-NPs by intraperitoneal injection at 10 mg/kg 7,8-NPs. During the experiment, body weight, fecal consistency, and occult blood were recorded and scored daily. The DAI scores were calculated.

2.24. H&E staining.

Mouse colon tissues were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin using tissue embedding machine (EG1550, LEICA), then cut into pieces using rotary microtome (RM2235, LEICA). The slices were stained with hematoxylin-eosin buffers and sealed with neutral gum. The pathological changes of the colon tissues were observed with an microscopy (Olympus, IX51).

2.25. Nissl body staining.

The brain tissue slices were treated with conventional dewaxing in water and flushed in distilled water for 5 min, after which they were stained with 1.2% toluidine blue for 35 min. Later, the slices were washed with distilled water to remove the dye and dehydrated in ethanol. Finally, the slices were rendered transparent using xylene and sealed with neutral gum. The numbers of Nissl bodies in different groups were calculated with an microscopy (Olympus, IX51).

2.26. Quantification, statistics, and reproducibility.

All experiments were independently repeated at least twice. A two-tailed unpaired Student's *t*-test by GraphPad Prism 8.0 was done for the statistical analyses without specific statements. Data represented in the figures were shown with error of mean ($\text{mean} \pm \text{SD}$) without specific statements. $P < 0.05$ was considered statistically significant between groups. n.s. indicated not significant; * indicated $P < 0.05$; ** indicated $P < 0.01$; *** indicated $P < 0.001$. Western blot densitometry qualification was performed by ImageJ. Flow cytometry data were analyzed by FlowJo. Data were graphed using GraphPad Prism 8.0.

3. Results

3.1. 7,8-DHF demonstrates protective effects against oxidative stresses.

It is well known that 7,8-DHF supports neurogenesis and exhibits potent anti-depressant effects by acting as the agonist of TrkB. Given that high oxidative stresses are accompanied during the progression of neurodegeneration⁴⁸, it is convincing that 7,8-DHF may possess anti-oxidative abilities through activating TrkB. To this end, we first assessed the anti-oxidative effects of 7,8-DHF in mouse hippocampal neuronal HT22 cells under high concentrations of glutamate treatment. Glutamate treatment is widely used in evaluating glutamate-induced oxidative stress that is caused by inhibiting cystine import⁴⁹. We observed that an overdose of glutamate restrained the uptake of cystine to promote cell death, which could be abrogated by canonical ferroptosis inhibitors, ferrostatin-1 (ferr-1), liproxstatin-1 (lipro-1), or deferoxamine (DFO). As

shown in **Figure 1A, B**, HT22 cells died severely upon glutamate treatment, while ferr-1, lipro-1, and DFO co-treatment completely inhibited cell death. Surprisingly, we found that the treatment of 7,8-DHF could equally abolish cell death compared to the ferroptosis inhibitors (**Figure 1A, B**), suggesting that 7,8-DHF effectively protects neuron cells from oxidative stress-induced cell death. Meanwhile, we tested the lipid ROS levels of HT22 cells under the same conditions. 7,8-DHF demonstrated the same abilities as ferr-1, lipro-1, or DFO in eliminating the upregulation of lipid ROS induced by glutamate treatment (**Figure 1C, D**), while high concentrations of 7,8-DHF had no apparent effects on the lipid ROS levels (**Figure S1A, B**), suggesting that 7,8-DHF possesses protective effects upon metabolic stresses.

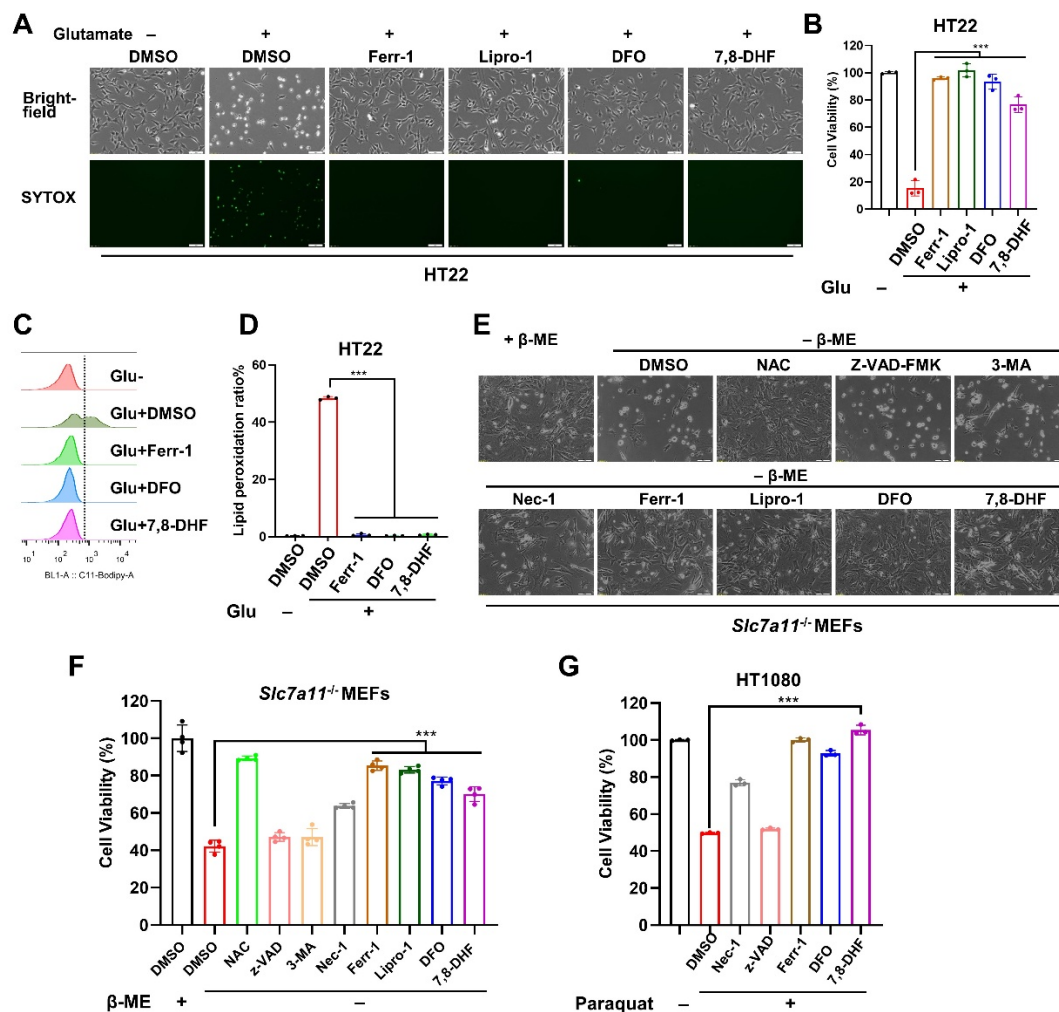


Figure 1 7,8-DHF demonstrates protective effects against oxidative stresses

(A and B) Representative phase-contrast SYTOX staining images (A) or cell viability (B) of HT22 cells incubated with glutamate in the absence or presence of ferr-1 (5 μ M), lipro-1 (5 μ M), DFO (50 μ M), or 7,8-DHF (5 μ M) for 24 h. Scale bars, 100 μ m. (C and D) Lipid peroxidation measurement (C) and ratio (D) of HT22 cells incubated with glutamate in the absence or presence of ferr-1 (5 μ M), DFO (50 μ M), or 7,8-DHF (5 μ M) for 16 h. (E and F) Representative phase-contrast images (E) or cell viability (F) of *Slc7a11*^{-/-} MEF cells with 2-Mercaptoethanol (β -ME, 50 μ M) or without β -ME treatment. *Slc7a11*^{-/-} MEF cells without β -ME treatment were incubated with NAC (500 μ M), Z-VAD-FMK (10 μ M), 3-Methyladenine (3-MA, 1 mM), necrostatin-1 (nec-1, 10 μ M), ferr-1 (5 μ M), lipro-1 (5 μ M), DFO (50 μ M), or 7,8-DHF (5 μ M) for 24 h. Scale bars, 100 μ m. (G) Cell viability of HT1080 cells treated with paraquat in the absence or presence of nec-1 (10 μ M), Z-VAD-FMK (10 μ M), ferr-1 (5 μ M), DFO (50 μ M), or 7,8-DHF (5 μ M) for 24 h. Data are the mean $\pm$ SD of n = 3 biological repeats. *p* values were calculated using unpaired, two-tailed Student's *t* test. *** indicated *P* < 0.001.

Next, we applied the spontaneous ferroptosis model using *Slc7a11* knockout (*Slc7a11*^{-/-}) mouse embryonic fibroblasts (MEFs) (**Figure S1C**). Chintala et al. reported that the sulfhydryl-reducing agent,

2-mercaptoethanol (β -ME), maintains the viability of *Slc7a11*^{-/-} MEFs⁵⁰. Consistent with previous studies, *Slc7a11*^{-/-} MEFs died without the protection of β -ME (**Figure 1E, F**). As expected, the subjection of N-acetyl-L-cysteine (NAC), ferr-1, lipro-1, or DFO largely inhibited cell death of *Slc7a11*^{-/-} MEFs in the absence of β -ME. Moreover, these cell deaths were specifically blocked by 7,8-DHF but not by inhibitors of other cell death pathways, such as apoptosis (Z-VAD-FMK), autophagy (3-MA), or necroptosis (nec-1) (**Figure 1E, F**).

To further prove our point that 7,8-DHF possesses the capacity against oxidative stress, we assessed the other reagent, paraquat. Paraquat exerts its oxidative effects through the production of superoxide anion. As shown in **Figure 1G**, in comparison to nec-1, ferr-1, or DFO, 7,8-DHF demonstrated the best efficiency in supporting cell growth upon paraquat treatment, further proving that 7,8-DHF acts as a natural anti-oxidative reagent. Taken together, these results indicate the potency of 7,8-DHF in defense against oxidative stress.

3.2. Ferroptotic cell death is repressed by 7,8-DHF.

Given that 7,8-DHF plays an important role against oxidative stress-induced ferroptotic phenotype, we then investigated whether 7,8-DHF can directly inhibit ferroptosis (**Figure 2A**). As shown in **Figure S2A**, A375 cells were treated with GPX4 inhibitor, RSL-3, in the presence of different concentrations of 7,8-DHF. RSL-3-induced cell death was significantly abrogated in the presence of 7,8-DHF (EC₅₀ = 0.45 μ M in A375 cells). The lipid peroxidation levels of A375 cells were examined through flow cytometry analysis. We observed that the lipid peroxidation induced by GPX4 inhibition was totally abrogated by 7,8-DHF or other canonical ferroptosis inhibitors (**Figure S2B, C**). We obtained consistent results by using HT1080 cells: 7,8-DHF inhibited the ferroptotic cell death at EC₅₀ around 0.6 μ M and disrupted the upregulation of lipid ROS levels triggered by RSL-3 (**Figure S2D-F**). Furthermore, we applied human colon mucosal epithelial NCM460 cells to verify whether the 7,8-DHF could inhibit ferroptosis in normal cells. As expected, 7,8-DHF efficiently inhibited the cell death and lipid peroxidation triggered by RSL-3 (**Figure 2B, C**). In addition, we performed transmission electron microscopy (TEM) experiments to visualize the 7,8-DHF-mediated anti-ferroptosis effects at a deeper resolution (**Figure 2D**). We treated NCM460 cells with RSL-3 and observed that the mitochondrial membrane density increased and the mitochondrial cristae were lost significantly, while 7,8-DHF treatment completely rescued the morphological alterations of mitochondria (**Figure 2D**). To further prove our point, the protective effects of 7,8-DHF were reproduced using Caco-2 and HT-29 cells. Consistently, the cell death induced by RSL-3 was completely rescued in the presence of a high dose of 7,8-DHF (**Figure 2E, F**).

Next, we applied another ferroptosis inducer, tert-Butyl hydroperoxide (TBH), which is involved in p53-dependent ferroptosis by mimicking elevated levels of ROS^{46, 51, 52}. As expected, high levels of ROS-induced ferroptosis can be eliminated by 7,8-DHF in a series of intestinal epithelial cell lines, such as NCM460, Caco-2, and HT-29 cells (**Figure 2G-I**). We obtained consistent results by using A375 and HT1080 cells (**Figure S2G, H**). We also tested the role of 7,8-DHF in ferroptosis under other ferroptosis induction conditions. 7,8-DHF suppressed the ferroptotic cell death caused by the second-generation SLC7A11

inhibitor, imidazole ketone erastin (IKE) (**Figure S2I, J**). Consistently, the protective effects of 7,8-DHF were achieved upon cystine-deprivation-induced ferroptosis at EC₅₀ around 2.82 μM (**Figure S2K**). Indeed, by accessing the flow cytometry results, we found that 7,8-DHF demonstrated the same ability as ferr-1 in inhibiting cystine-deprivation-induced ferroptosis (**Figure S2L, M**). Taken together, our data prove that 7,8-DHF is a *bona fide* ferroptosis suppressor.

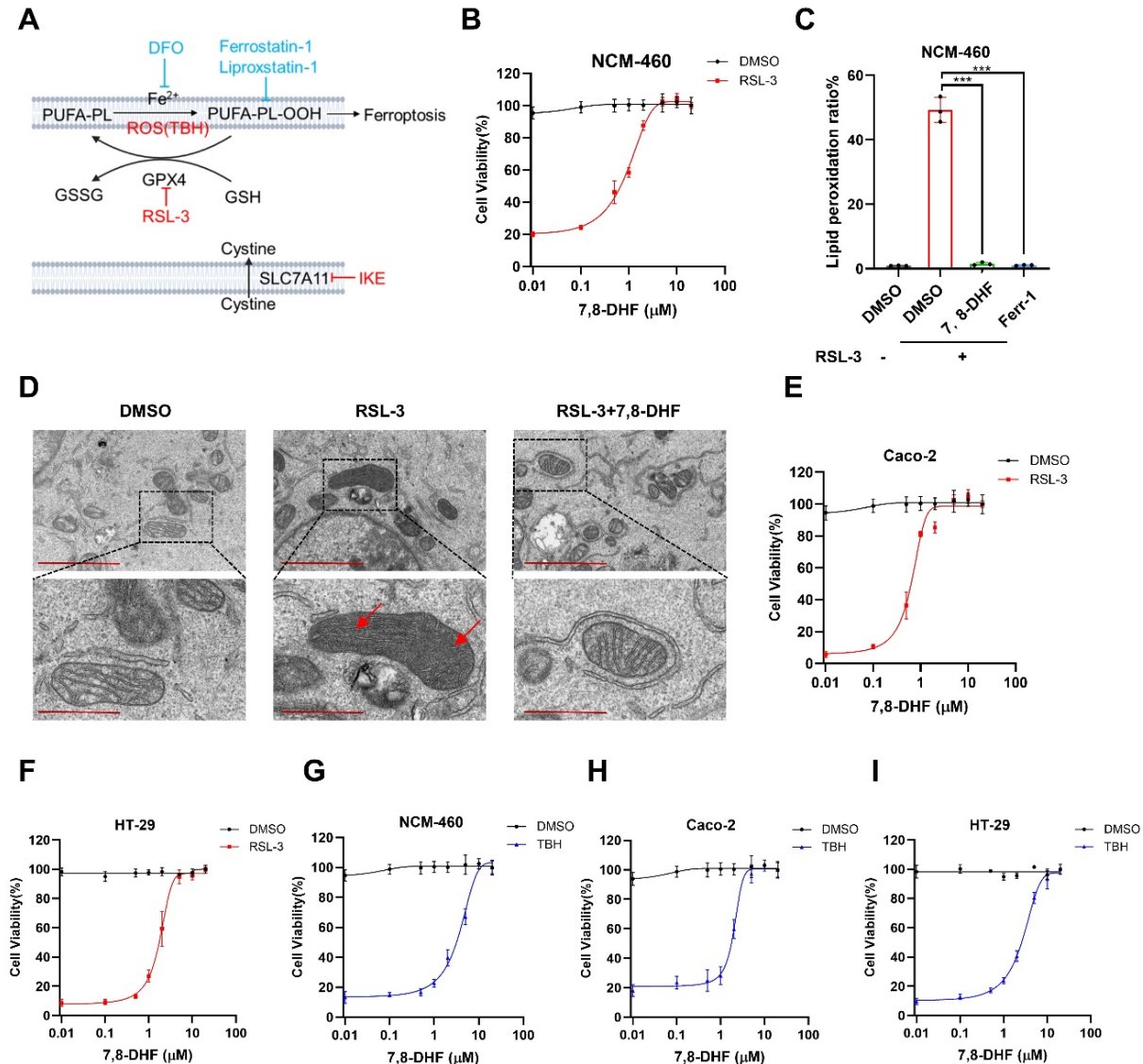


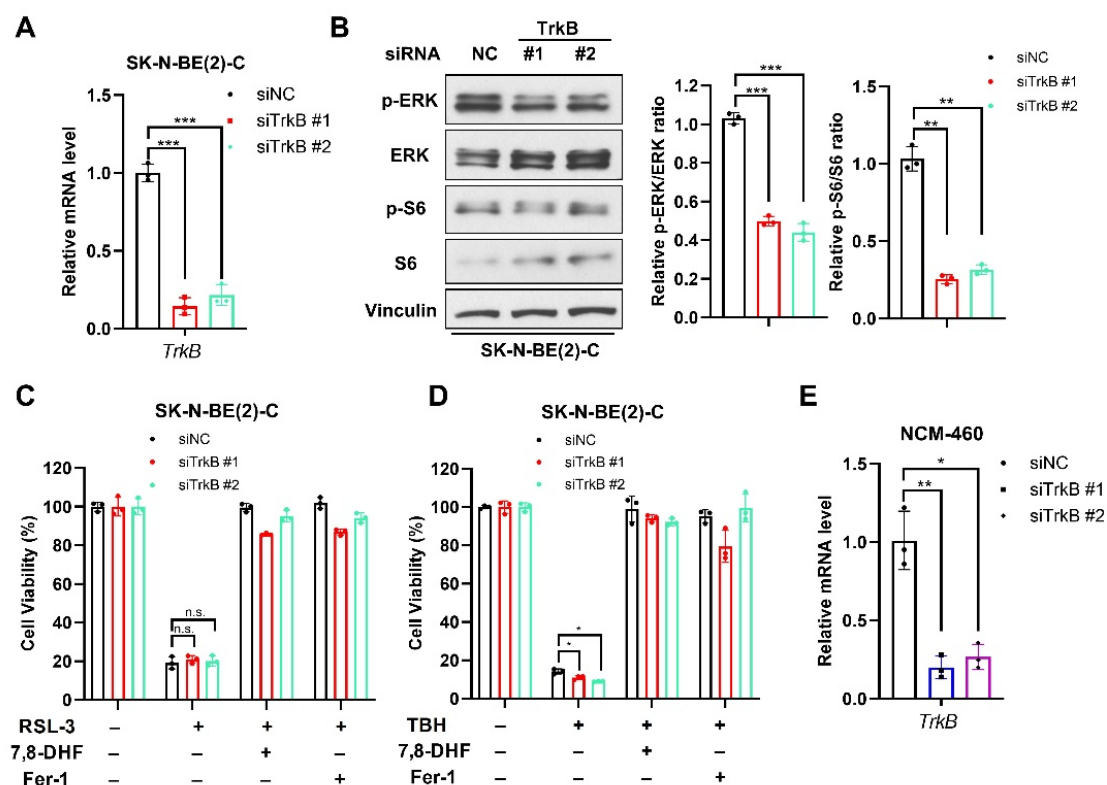
Figure 2 Ferroptotic cell death is repressed by 7,8-DHF

(A) Model of core ferroptosis regulation and the inducers/inhibitors of ferroptosis. Ferroptosis inhibitors are labeled in blue; ferroptosis inducers are labeled in red. Ferr-1 and lipro-1 directly react with peroxidized lipid and inhibit lipid peroxidation. DFO is an iron chelator. TBH promotes ferroptosis by acting as a ROS generator; RSL-3 promotes ferroptosis by inhibiting GPX4 activity; IKE promotes ferroptosis by inhibiting SLC7A11 activity. (B) Cell viability assay for evaluating the protective effects of 7,8-DHF against RSL-3 (10 μM)-induced ferroptosis in NCM460 cells for 8 h. (C) Lipid peroxidation ratio of NCM460 cells treated with RSL-3 in the absence or presence of ferr-1 (5 μM) or 7,8-DHF (10 μM) for 6 h. (D) Representative transmission electron microscopy images of mitochondria of NCM460 cells with indicated treatment. Scale bars, 2 μm . Scale bars of enlarged images, 500 nm. (E) Cell viability assay for evaluating the protective effects of 7,8-DHF on RSL-3 (5 μM)-induced ferroptosis in Caco-2 cells for 8 h. (F) Cell viability assay for evaluating the protective effects of 7,8-DHF on RSL-3 (10 μM)-induced ferroptosis in HT-29 cells for 8 h. (G) Cell viability assay for evaluating the protective effects of 7,8-DHF on TBH (100 μM)-induced ferroptosis in NCM460 cells for 8 h. (H and I) Cell viability assay for evaluating the protective effects of 7,8-DHF on TBH (200 μM)-induced ferroptosis in Caco-2 (H) or HT-29 (I) cells for 8 h. Data are the mean $\pm$ SD of n = 3 biological repeats. p values were calculated using unpaired, two-tailed Student's t test. *** indicated $P < 0.001$.

3.3. 7,8-DHF suppresses ferroptosis in a TrkB-independent manner.

As 7,8-DHF is a well-known BDNF mimicker that can activate TrkB downstream signaling, we explored whether TrkB is critical for the ferroptosis inhibitory role of 7,8-DHF. To this end, we first used nerve cell lines and knocked down TrkB in SK-N-BE(2)C neuroblastoma cells by applying the small-interference RNA (siRNA) technique (**Figure 3A**). As shown in **Figure 3B**, the knocking down of TrkB decreased the phosphorylation of S6 and ERK. Under this condition, we treated control or TrkB siRNA-expressing cells with RSL-3 or TBH in the absence or presence of ferr-1 or 7,8-DHF and performed the cell viability analysis. Although TrkB knockdown had different effects on the cell sensitivity to GPX4-inactivation-induced or high levels of ROS-induced ferroptosis, either ferr-1 or 7,8-DHF can ultimately rescue the cell death, indicating the 7,8-DHF-mediated ferroptosis defense is independent of TrkB activities (**Figure 3C, D**). We also repeated these assays using the other neuroblastoma LAN-1 cells; we knocked down TrkB under the same conditions (**Figure S3A**). We found that the decreased TrkB expression could sensitize LAN-1 cells to ferroptosis; however, TrkB did not affect the anti-ferroptosis capability of 7,8-DHF (**Figure S3B, C**).

To further prove our point, we applied intestinal epithelial NCM460 and Caco-2 cell lines to examined whether 7,8-DHF-mediated ferroptosis defense is independent on TrkB. Consistent with the data from nerve cells, although TrkB knocking down caused higher NCM460 cells' sensitivity to ferroptosis inducer, 7,8-DHF possessed the same ability as ferrostatin-1 to inhibit ferroptosis (**Figure 3E, F**). Same results were also obtained in Caco-2 cells, suggesting that TrkB is dispensable for 7,8-DHF-mediated ferroptosis defense (**Figure 3G, H**). Collectively, these data demonstrate that 7,8-DHF-mediated anti-ferroptosis effects are independent of its TrkB agonist function.



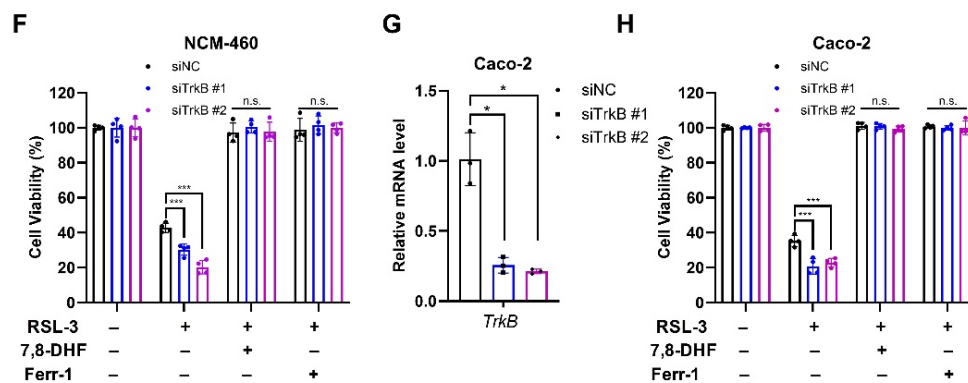


Figure 3 7,8-DHF suppresses ferroptosis in a TrkB-independent manner (A) RT-qPCR of SK-N-BE(2)-C cells expressing negative control (NC) siRNA or TrkB siRNA. (B) Western blot analysis of SK-N-BE(2)-C cells expressing NC siRNA or TrkB siRNA. ERK and S6 are TrkB downstream targets. The relative p-ERK/ERK ratio or p-S6/S6 ratio was analyzed. (C) Cell viability of SK-N-BE(2)-C cells expressing NC siRNA or TrkB siRNA treated with RSL-3 in the absence or presence of 7,8-DHF (5 μ M) or ferr-1 (5 μ M) for 8 h. (D) Cell viability of SK-N-BE(2)-C cells expressing NC siRNA or TrkB siRNA treated with TBH in the absence or presence of 7,8-DHF (5 μ M) or ferr-1 (5 μ M) for 8 h. (E) RT-qPCR of NCM460 cells expressing NC siRNA or TrkB siRNA. (F) Cell viability of NCM460 cells expressing NC siRNA or TrkB siRNA treated with RSL-3 in the absence or presence of 7,8-DHF (5 μ M) or ferr-1 (5 μ M) for 8 h. (G) RT-qPCR of Caco-2 cells expressing NC siRNA or TrkB siRNA. (H) Cell viability of Caco-2 cells expressing NC siRNA or TrkB siRNA treated with RSL-3 in the absence or presence of 7,8-DHF (5 μ M) or ferr-1 (5 μ M) for 8 h. Two TrkB siRNA sequences were applied in the indicated experiments. Data are the mean $\pm$ SD of $n = 3$ biological repeats. p values were calculated using unpaired, two-tailed Student's t test. n.s. indicates $P > 0.05$; * indicated $P < 0.05$; ** indicated $P < 0.01$; *** indicated $P < 0.001$.

3.4. 7,8-DHF protects cells from ferroptosis in a GSH/GPX4-independent manner.

To dissect the mechanism of 7,8-DHF in modulating cell sensitivities to ferroptosis, we performed phospholipidomics analysis to investigate the potential regulatory role of 7,8-DHF in lipid metabolism (**Figure S4A**). Cells were treated without or with 7,8-DHF for 24 h and analyzed by phospholipidomics. It is well-established that PUFA-PE is the main substrate in inducing ferroptosis⁵³; thus, we checked whether 7,8-DHF affected the contents of PUFA-PE. We found that all detected species of PUFA-PE had negligible changes (**Figure S4B**). We also found that MUFA-containing PE was not affected. Considering the recent progress revealing PC, PS, PA, or PI could initiate ferroptosis^{46, 54, 55}, we then examined the effects of 7,8-DHF on the content of these types of phospholipids. However, neither PUFA-containing nor MUFA-containing PC, PS, PA, or PI was affected upon treatment with 7,8-DHF, suggesting that 7,8-DHF had no obvious effects on phospholipid metabolism (**Figure S4C-E**).

As 7,8-DHF had no obvious effects on the contents of phospholipids, we then examined whether 7,8-DHF was the modulator of GPX4. We knocked out GPX4 in A375 cells and tested the effects on the ferroptosis that occurred in GPX4-null cells (**Figure 4A**). Consistent with our previous data, loss of GPX4 causes spontaneous cell death without the protection of ferrostatin-1^{45, 46}. Remarkably, 7,8-DHF rescued the cell lethality induced by the loss of GPX4 (**Figure 4B, C**), suggesting GPX4 is dispensable for 7,8-DHF-mediated ferroptosis defense.

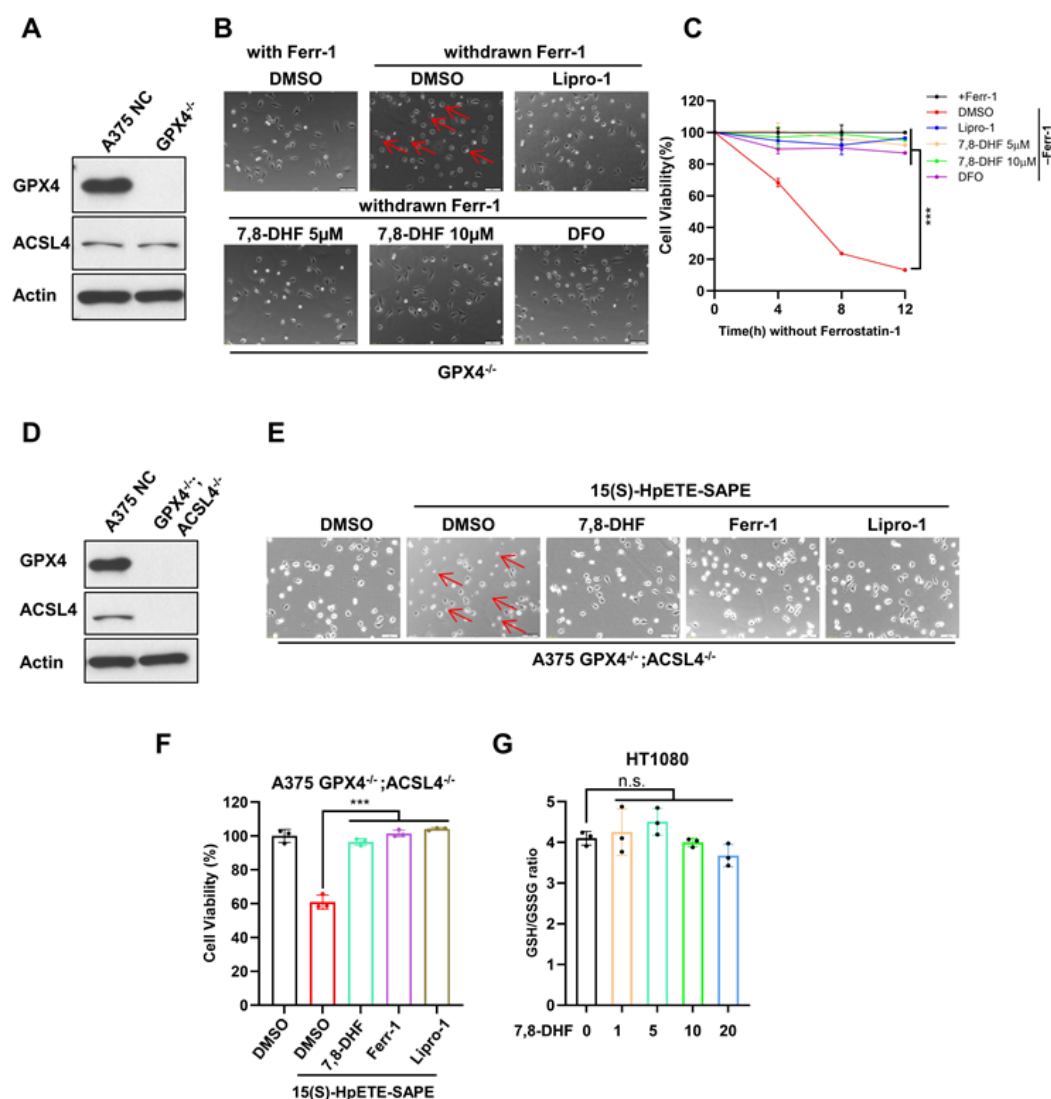


Figure 4 7,8-DHF protects cells from ferroptosis in a GSH/GPX4-independent manner (A) Western blot analysis of A375 NC and GPX4^{-/-} cells. (B) Representative phase-contrast images of A375 GPX4^{-/-} cells withdrawn ferr-1 in the absence or presence of lipro-1 (5 μM), 7,8-DHF (5 μM or 10 μM), or DFO (50 μM) for 8 h. Scale bars, 100 μm. (C) Cell viability assay for evaluating the protective effects of lipro-1 (5 μM), 7,8-DHF (5 μM or 10 μM), or DFO (50 μM) in A375 GPX4^{-/-} cells withdrawn from ferr-1 for periods as indicated. (D) Western blot analysis of A375 NC, GPX4^{-/-};ACSL4^{-/-} cells. (E) Representative phase-contrast images of A375 GPX4^{-/-};ACSL4^{-/-} cells treated with 15(S)-HpETE-SAPE in the absence or presence of lipro-1 (5 μM), ferr-1 (5 μM), or 7,8-DHF (5 μM) for 12 h. Scale bars, 100 μm. (F) Cell viability of A375 GPX4^{-/-};ACSL4^{-/-} cells treated with 15(S)-HpETE-SAPE in the absence or presence of lipro-1 (5 μM), ferr-1 (5 μM), or 7,8-DHF (5 μM) for 12 h. (G) GSH/GSSG ratio of HT1080 cells treated with titrated concentration of 7,8-DHF as indicated for 24 h. Data are the mean ± SD of n = 3 biological repeats. *p* values were calculated using unpaired, two-tailed Student's *t* test. n.s. indicated not significant; *** indicated *P* < 0.001.

Further, we established a peroxidized lipids-induced ferroptosis model by using 1-Stearoyl-2-15(S)-HpETE-sn-glycero-3-PE (15(S)-HpETE-SAPE) and examined the potential role of 7,8-DHF in eliminating peroxidized lipids. 15(S)-HpETE-SAPE is considered the signal in ferroptosis initiation and propagation⁵³, and direct incubation of 15(S)-HpETE-SAPE promotes ferroptosis in the absence of GPX4, providing us a useful tool in investigating the anti-ferroptosis effects of the reagent. To this end, we applied ACSL4/GPX4 double knockout A375 cells (**Figure 4D**). Loss of ACSL4 abrogates the spontaneous cell death caused by loss of GPX4⁵⁶. We incubated ACSL4/GPX4 double knockout cells with 15(S)-HpETE-SAPE and observed an obvious cell death rate. Under the same conditions, 7,8-DHF

maintained the cell survival upon 15(S)-HpETE-SAPE treatment (**Figure 4E, F**), further proving that the anti-ferroptosis activities of 7,8-DHF are GPX4-independent.

Last, we examined whether 7,8-DHF can directly regulate the contents of GSH. As shown in **Figure 4G**, the intracellular GSH/GSSG ratio was not affected by titrated concentration of 7,8-DHF treatment. Taken together, these results indicate that 7,8-DHF counteracts ferroptosis in a GSH/GPX4-independent manner.

3.5. 7,8-DHF acts as RTAs to directly eliminate lipid peroxides.

Given that 7,8-DHF inhibits ferroptosis neither through TrkB signaling nor the GSH/GPX4 axis, we next explored the potential mechanism of 7,8-DHF-mediated ferroptosis defense. We first assessed 7,8-DHF's radical scavenging capacity by applying the DPPH assay⁵⁷. We observed that 7,8-DHF displayed significant radical scavenging capacities as ferr-1/lipiro-1 (**Figure 5A**). And 7,8-DHF reacted with DPPH in a dose-dependent manner, suggesting 7,8-DHF could react with free radicals directly (**Figure 5B**).

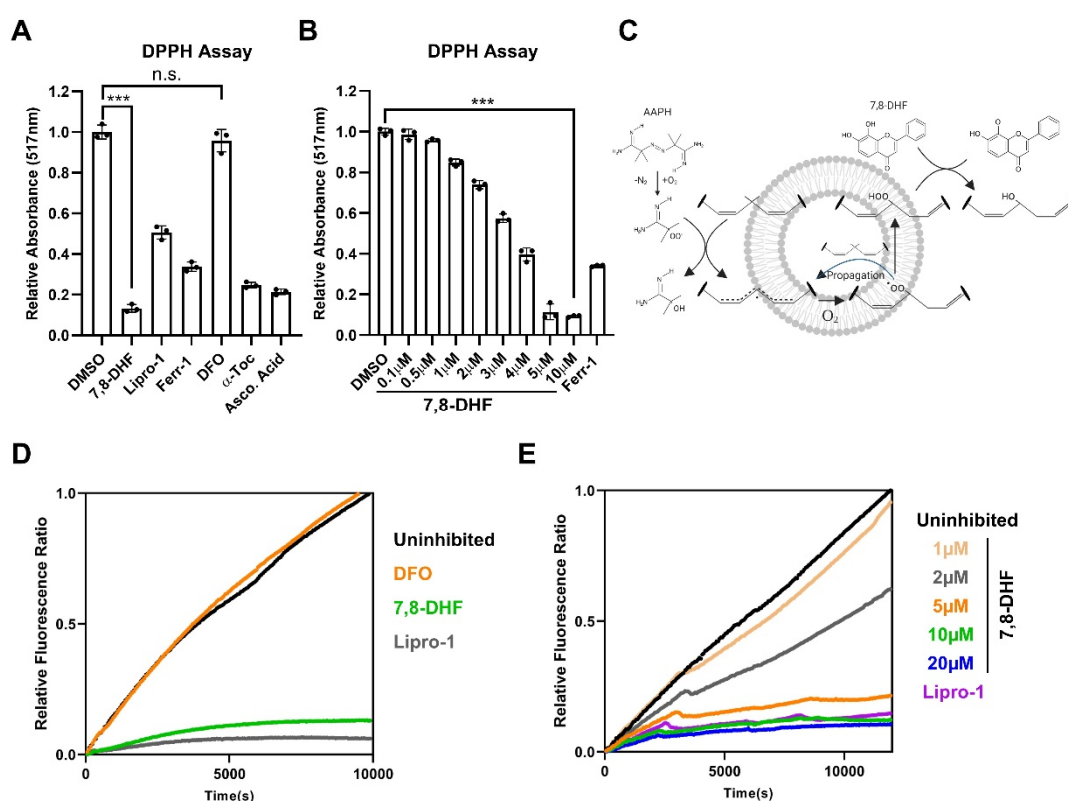


Figure 5 7,8-DHF acts as RTAs to directly eliminate lipid peroxides. (A) *In vitro* DPPH assay. Lipiro-1 (5 μ M), ferr-1 (5 μ M), α -tocopherol (5 μ M), and ascorbic acid (5 μ M) were used as positive controls. DFO (50 μ M) was the negative control. 5 μ M 7,8-DHF was used. (B) *In vitro* DPPH assay. Ferr-1 (5 μ M) was used as the positive control. Titrated concentration of 7,8-DHF was used as indicated. (C) Model of STY-BODIPY autoxidation assay to evaluate RTAs activities. AAPH was used as a radical initiator to initiate the lipid peroxidation in egg phosphatidylcholine liposomes. Relative STY-BODIPY autoxidation rate was monitored by measuring the change of fluorescence at excitation 488nm/emission 518nm. 7,8-DHF reduced polyunsaturated fatty acids to polyunsaturated fatty alcohols and became oxidized isoforms. At the endpoint, the relative ratio of changed fluorescence of the uninhibited group was defined as 1. (D) Relative STY-BODIPY autoxidation rate by 7,8-DHF (5 μ M). DFO (50 μ M) or lipiro-1 (5 μ M) was used as the negative or positive control, respectively. (E) Relative STY-BODIPY autoxidation rate by titrated 7,8-DHF as indicated. Lipiro-1 (5 μ M) was used as a positive control. Data are the mean $\pm$ SD of $n = 3$ biological repeats. p values were calculated using unpaired, two-tailed Student's t test. n.s. indicated not significant; *** indicated $P < 0.001$.

Based on the results of the DPPH assay, we speculated that 7,8-DHF could have the same abilities as canonical ferroptosis inhibitors (ferr-1, lipiro-1, etc.) to directly react with peroxidized phospholipids⁵⁸. We

applied the STY-Bodipy autooxidation assay to examine whether 7,8-DHF could reduce lipid peroxidized phospholipids. Lipophilic STY-Bodipy autooxidation has been well-established in evaluating the potential RTAs (**Figure 5C**)^{58, 59}. As shown in **Figure 5D**, DFO showed negligible effects in inhibiting STY-Bodipy autooxidation (orange line vs. black line), while significant inhibition was observed by 7,8-DHF or lipro-1 (gray line / green line vs. black line), indicating that 7,8-DHF can eliminate lipid peroxidation *in vitro* (**Figure 5D**). Additionally, the inhibiting effects of 7,8-DHF were in a dose-dependent manner; 5 μ M 7,8-DHF can completely prohibit the accumulation of lipid peroxidation (**Figure 5E**). Thus, our results provide the intriguing mechanism of 7,8-DHF-mediated ferroptosis inhibition through acting as RTAs.

3.6. Engineered 7,8-DHF nanoparticles (7,8-NPs) facilitate effective bioavailability and neurodegenerative protection.

Since the low bioavailability of 7,8-DHF impeded its clinical application, we assessed the nanoparticle drug delivery systems to enhance the absorption and prolong the release time. To this end, we synthesized oleanolic acid nanoparticles based on self-assembled oleanolic acid to carry 7,8-DHF (referred to as 7,8-NPs). Nano encapsulated particles appear circular or elliptical in shape, with a diameter of approximately 100-200 nanometers, and are evenly dispersed. Scanning electron microscope (SEM) and high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) were employed to analyze the formation of 7,8-NPs (**Figure 6A, S5A, B**), indicating that 7,8-DHF could be packaged and released successfully. As assessed by pharmacokinetics, 2 h after intraperitoneal (IP) injection, the 7,8-DHF concentration of the nanoparticles-packaged group increased over 25-fold in comparison to the unpackaged group (11331 ng/L versus 439 ng/L) (**Figure 6B**). At the prolonged time post IP, the release rate of 7,8-DHF of the nanoparticles-packaged group was consistently higher than the unpackaged group (**Figure 6B**), suggesting the engineered 7,8-NPs sustainably release 7,8-DHF in the circulation system. We next examined whether 7,8-NPs and 7,8-DHF had the same effects on ferroptosis. As expected, in NCM460 cells, 7,8-NPs can inhibit ferroptosis and lipid peroxidation, suggesting nanoparticle packaged 7,8-DHF had the same ability as natural 7,8-DHF in inhibiting ferroptosis (**Figure S5C-E**).

We tested the efficacy of 7,8-NPs in ameliorating neurodegenerative AD model. Wild-type or APP/PS1 mice that develop amyloid pathologies were administered OA nanoparticles alone (control) or 7,8-NPs. We monitored the signature of amyloid pathologies in the mouse hippocampus by immunohistochemistry (IHC) analysis with A β antibody. The A β positive count in the hippocampus was lower in the 7,8-NPs-treated group than in the control group (**Figure 6C**). Next, we stained the Nissl bodies to assess the neuronal loss during the pathological process. As shown in **Figure 6D, E**, Nissl bodies of APP/PS1 mice were decreased compared to normal mice, and 7,8-NPs efficiently prevented the Nissl bodies decrement, suggesting 7,8-NPs can delay the neuronal damage. Microglia and astrocytes are brain macrophages and inflammatory modulators that display critical interactions for understanding the pathogenesis of AD. Immunofluorescence (IF) analysis was performed to examine the efficacy of 7,8-NPs in retarding AD progression. We found that glial fibrillary acidic protein (GFAP)-stained astrocytes were increased in APP/PS1 mice in comparison to normal mice;

7,8-NPs treatment, but not control treatment, could significantly alleviate the increment of astrocytes (**Figure 6F**). Similar results were obtained in ionized calcium binding adaptor molecule 1 (Iba1)-stained microglia (**Figure 6G-H**). Taken together, our data indicate that the engineered 7,8-NPs partially solve the low availability of 7,8-DHF *in vivo* and demonstrate robust effects in alleviating AD progression.

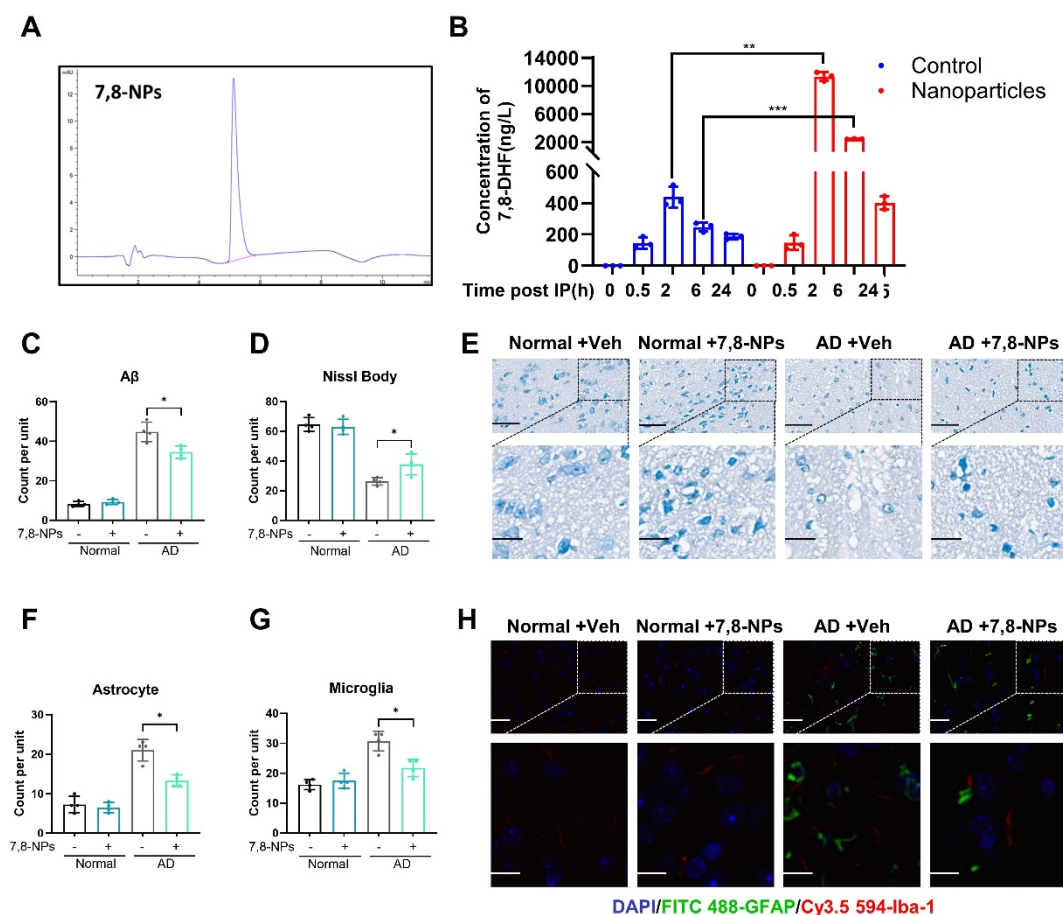


Figure 6 Engineered 7,8-DHF nanoparticles (7,8-NPs) facilitate effective bioavailability and neurodegenerative protection. (A) Extracted ion chromatograms of 7,8-NPs analyzed by HPLC-MS/MS. The retention time of 7,8-NPs is 5.125 min. (B) Pharmacokinetics analysis of 7,8-NPs. Unpackaged 7,8-DHF or nanoparticle-packaged 7,8-DHF was intraperitoneally injected into C57BL/6J mice. The blood concentration of 7,8-DHF was analyzed post-intraperitoneal injection at the indicated time. (C) Quantification of immunostaining of Aβ in the hippocampus region. Four tissue slides were collected per group. (D) Quantification of toluidine blue staining of Nissl bodies in the hippocampus region. Four tissue slides were collected per group. (E) Representative images of toluidine blue staining of Nissl bodies in the hippocampus region from indicated groups. Scale bars, 50 μm. Scale bars of enlarged images, 20 μm. (F and G) Quantification of immunostaining of astrocytes (F) and microglia (G) in the hippocampus region. Four tissue slides were collected per group. (H) Representative images of immunostaining of astrocytes and microglia in the hippocampus region. Scale bars, 20 μm. Scale bars of enlarged images, 10 μm. For (B), data are the mean ± SD of n = 3 biological repeats. For (C), (D), (F), and (G), Data are the mean ± SD of n = 4 biological repeats. *p* values were calculated using unpaired, two-tailed Student's *t* test. * indicated *P* < 0.05; ** indicated *P* < 0.01; *** indicated *P* < 0.001.

3.7. 7,8-NPs alleviate ulcerative colitis in normal mice.

Accumulating studies have revealed that IBD is associated with the risk of developing AD, cognitive impairment, and depression⁶⁰. Since 7,8-NPs had positive effects in preventing AD pathology, we hypothesized that 7,8-NPs possesses dual-protective effects against IBD. To verify our hypothesis, we generated an acute colitis mouse model by administering dextran sulfate sodium salt (DSS) using wild-type C57BL/6 mice (**Figure 7A**). The water uptake of the 2.5% DSS administration groups has a negligible

difference from the normal groups (**Figure S6A**). We observed that the DSS group had obvious body weight loss compared with the normal group, while 7,8-NPs can mitigate the body weight loss (**Figure 7B**). We obtained a consistent trend of the disease activity index (DAI) in the absence or presence of 7,8-NPs: DSS group showed rapidly increased DAI scores after two days of DSS treatment, while 7,8-NPs treatment obviously delayed the process (**Figure 7C**). It is worth mentioning that 7,8-NPs intervention delayed the rectal bleeding by around two days (DSS group started at day 5 vs. DSS+7,8-NPs group started at day 7). Post-dissection observations revealed that, in comparison to the control group, the colons of mice with DSS administration were significantly shortened, but the shortness was mitigated after intervention with 7,8-NPs (**Figure 7D, E**). We next evaluated the epithelial barrier function of colon tissues by FITC-dextran and Western blot analysis. We found that FITC-dextran concentration was significantly increased in the DSS mice, and 7,8-NPs treatment showed a decreased concentration of FITC-dextran (**Figure 7F**). Consistent results were obtained by detecting the expression levels of ZO-1 and Occludin, suggesting that the severe damage of colonic barriers was ameliorated by 7,8-NPs (**Figure 7G**). Furthermore, pathology observation by assessing H&E staining revealed that DSS treatment induced barrier disruption and extensive inflammatory infiltration, while 7,8-NPs alleviated inflammatory responses and enhanced intestinal barrier integrity (**Figure 7H**).

Next, we examined the inflammation levels of colonic tissues using the enzyme-linked immunosorbent assay (ELISA) method. We observed that the IL-6 levels of DSS mice significantly increased compared with the control group; 7,8-NPs treatment decently mitigated the increment (**Figure 7I**). The other types of cytokines, such as IL-1 β and TNF- α , displayed a consistent trend with IL-6, indicating that 7,8-NPs ameliorated the progression of colitis by decreasing the inflammation (**Figure 7J, K**).

Furthermore, to dissect the anti-ferroptosis effects of 7,8-NPs, which play critical roles in mitigating colitis pathology, we first detected the levels of MDA and 4-HNE, which are both markers of lipid peroxidation and ferroptosis *in vivo*. Indeed, the levels of MDA and 4-HNE of colonic tissues were significantly increased under DSS treatment, while 7,8-NPs administration partially decreased the levels of MDA and 4-HNE (**Figure 7L, M**). We observed that the upregulation of *Ptgs2* and *Chac1* expression of the DSS group was also restored by 7,8-NPs treatment (**Figure 7N, S6B**), suggesting that ferroptotic stresses are largely reduced by 7,8-NPs. However, the mortality of long-term DSS-treated mice was not significantly prolonged upon 7,8-NPs intervention ($p=0.183$), suggesting that although 7,8-NPs partially ameliorated the phenotype of colitis, 7,8-NPs cannot completely reverse the progression of disease (**Figure S6C**).

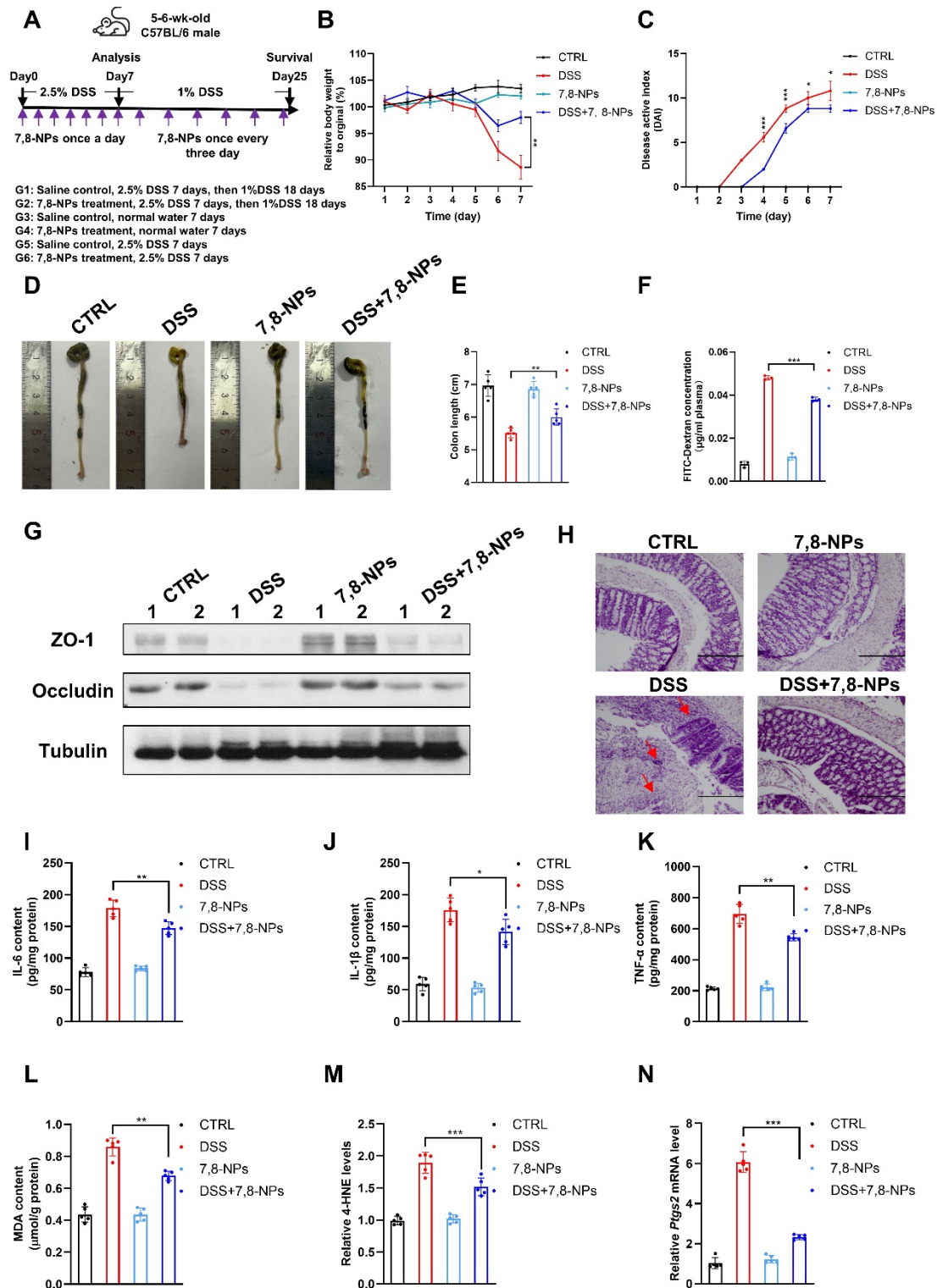
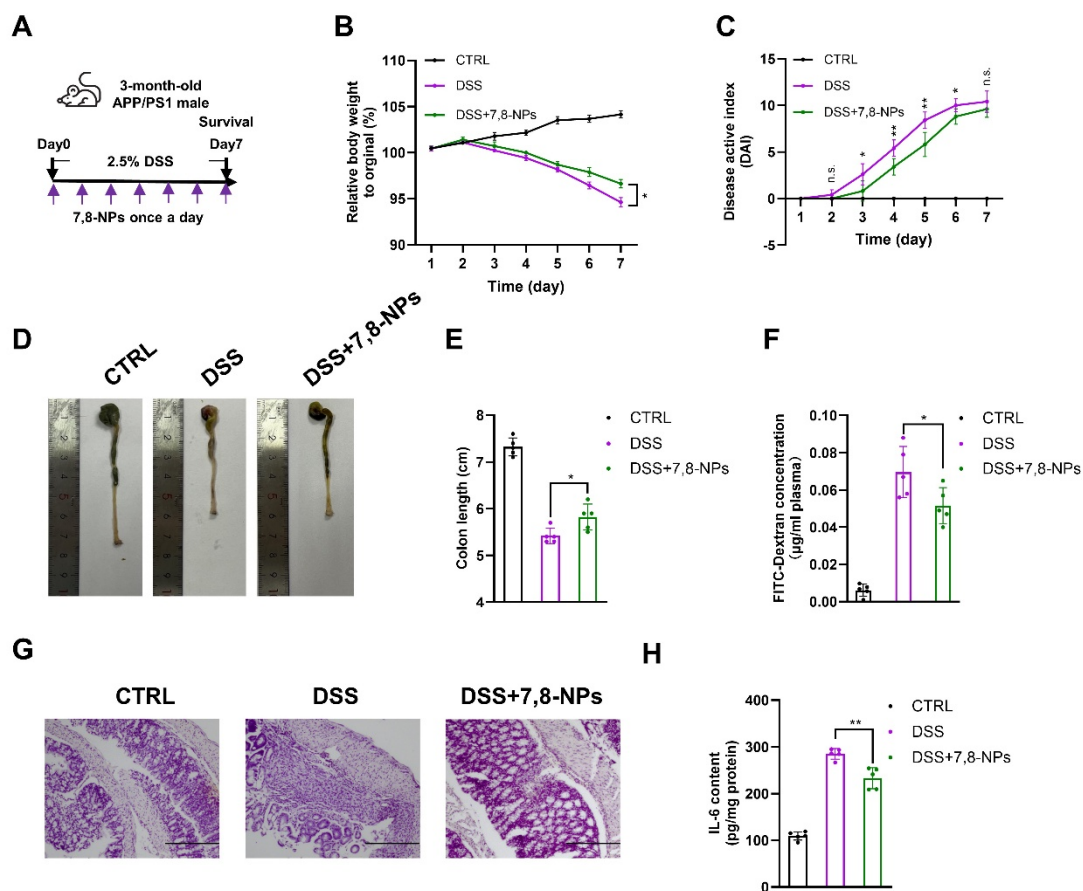


Figure 7 7,8-NPs alleviate ulcerative colitis in normal mice (A) The schematic diagram displayed the timeline of the generation of the DSS-induced UC model in the absence or presence of 7,8-NPs by using C57BL/6 mice. (B and C) Body weight change (B) and Disease activity index (DAI) scores (C) were recorded from the indicated groups. (D and E) Colon images (D) and length measurement (E) of mice in each group was determined. (F) Quantification of FITC-Dextran concentration in blood. (G) The protein levels of ZO-1 and Occludin of colon tissues were determined by Western Blot in the indicated groups. (H) Representative images of H&E staining of the colon from the indicated groups. Scale bar, 100 μm. (I-K) ELISA analysis of IL-6 (I), IL-1β (J), and TNF-α (K) levels in colonic tissues. (L) MDA levels of colonic tissues dissected from indicated groups according to the MDA assay kit. (M) Relative 4-HNE levels of colonic epithelial tissues dissected from indicated groups according to the Lipid Peroxidation (4-HNE) assay kit. (N) RT-qPCR of *Ptgs2* expression level in colonic epithelial tissues dissected from the indicated groups. For (B), (C), (E), and (I-N) Data are the mean ± SD of n = 5 biological repeats. For (F), Data are the mean ± SD of n = 3 biological repeats. *p* values were calculated using unpaired, two-tailed Student's *t* test. * indicated *P* < 0.05; ** indicated *P* < 0.01; *** indicated *P* < 0.001.

3.8. 7,8-NPs alleviate ulcerative colitis in APP/PS1 transgenic mice.

Since 7,8-NPs demonstrated anti-inflammatory and protective effects in the progression of acute colitis, we then examined whether the alleviation of intestinal inflammation ameliorates the pathology of AD. To this end, the same conditions were applied to generate the DSS-induced acute colitis mouse model using APP/PS1 mice (**Figure 8A**). As expected, APP/PS1 mice resulted in rapid body weight loss under DSS treatment, along with higher DAI scores and shorter colon length, while 7,8-NPs partially mitigated the phenotypes (**Figure 8B-E**). Consistent results were obtained by analyzing colonic barrier function, pathology, and inflammation (**Figure 8F-I**). Next, we applied Nissl body staining to test whether gut inflammation accelerates the progression of AD and whether 7,8-NPs could alleviate the progression. As shown in Figure 8J, the staining of Nissl bodies of the DSS group was significantly decreased compared to the control group, indicating that the pathology of AD was increased upon UC progression. Importantly, the decrement of Nissl bodies staining of the DSS group was abrogated by treatment with 7,8-NPs, indicating that the elimination of intestinal inflammation can also alleviate the progression of AD (**Figure 8J**). Together, these data demonstrate that 7,8-NPs-mediated intestinal inflammation elimination, in turn, ameliorates the pathological progression of Alzheimer's disease.



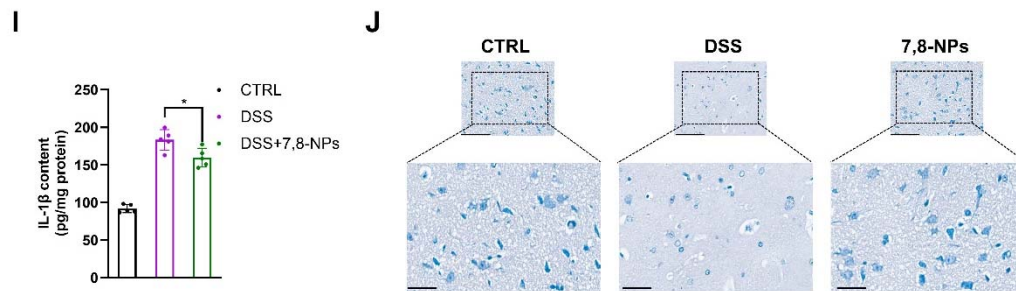


Figure 8 7,8-NPs alleviate ulcerative colitis in APP/PS1 transgenic mice (A) Schematic diagram shows the timeline of acute colitis mouse model by using APP/PS1 mice. (B and C) Body weight change (B) and Disease activity index (DAI) scores (C) were shown. (D and E) Colon images (D) and length measurement (E) of mice in each group was determined. (F) Quantification of FITC-Dextran concentration in blood from the indicated groups. (G) Representative images of H&E staining of the colon from the indicated groups. Scale bar, 100 μ m. (H and I) ELISA analysis of IL-6 (H) and IL-1 β (I) levels in colon tissues. (J) Representative images of toluidine blue staining of Nissl bodies in the hippocampus region from indicated groups. Scale bars, 100 μ m. Scale bars of enlarged images, 20 μ m. For (B), (C), (E), (F), (H), and (I) Data are the mean $\pm$ SD of $n = 5$ biological repeats. p values were calculated using unpaired, two-tailed Student's t test. n.s. indicates not significant; * indicated $P < 0.05$; ** indicated $P < 0.01$.

4. Discussion

Since the discovery of 7,8-DHF as a potent TrkB agonist in 2010, the pre-clinical application of 7,8-DHF has been continuously investigated. 7,8-DHF possesses protective effects in multiple diseases, such as Parkinson's disease, Alzheimer's disease, depression, osteoporosis, and obesity²³⁻²⁷. However, whether acting as the TrkB agonist is the only mechanism by which 7,8-DHF facilitates treating chronic diseases is undetermined. Indeed, the capacities of 7,8-DHF in activating TrkB signaling were challenged recently^{29,30}. 7,8-DHF fails to activate the TrkB signaling pathways under certain conditions, raising the possibility that the protective effects of 7,8-DHF may go through another path. Here, we identified 7,8-DHF as a universal inhibitor of ferroptosis, which is induced by GPX4 inactivation (RSL-3), metabolic stresses (glutamate treatment and cystine starvation), or direct ROS treatment (TBH). We prove that 7,8-DHF directly counteracts lipid peroxidation by acting as radical trapping antioxidants. Notably, the anti-ferroptotic cell death abilities of 7,8-DHF are independent of TrkB signaling, explaining the fact that 7,8-DHF mitigates chronic disease without activating TrkB signaling.

The other issue restraining the application of 7,8-DHF is that the drug has low bioavailability *in vivo* due to unfavorable absorption or rapid drug catabolism. In this study, to solve this problem, we prepared 7,8-NPs and proved that 7,8-NPs could sufficiently increase the absorption and prolong the release, delaying the progression of Alzheimer's disease and ulcerative colitis, and ameliorating pro-inflammation and oxidative stress.

Ferroptotic cell death is observed in numerous diseases, such as Parkinson's disease, Alzheimer's disease, nonalcoholic steatohepatitis, and kidney failure, emphasizing the importance of ferroptosis inhibitor application to ameliorate the progression of these diseases. During tissue culture, the canonical ferroptosis inhibitors are well accepted since the ferroptotic cell death induced could be completely inhibited. On the contrary, the performance of canonical ferroptosis inhibitors is doubtful due to the high toxicity and low bioavailability. In comparison to current ferroptosis inhibitors, 7,8-DHF has two advantages. Firstly, low

toxicity. DFO is a widely used iron chelator that decreases the ability of free iron and inhibits ferroptosis. However, long-term subjection of DFO may cause ocular symptoms, growth retardation, local reactions, and allergy⁶¹. Although additional experiments are required, we found that 7,8-NPs have negligible effects on the weight of mice and burden to the liver, suggesting 7,8-DHF may be safe to use. Second, 7,8-DHF can cross the BBB. BBB is a major obstacle in hindering the utilization of ferroptosis inhibitors in curing neurodegenerative diseases. We found that 7,8-NPs decreased the pathology of Alzheimer's disease, building up a new solution between ferroptosis and neurodegenerative diseases. Therefore, our study revealed that the 7,8-DHF-mediated ferroptosis defense mitigated ulcerative colitis, indicating that the naturally occurring plant derivative, 7,8-DHF, could be a promising drug in treating chronic human diseases. The clinical implication of the current work is evident.

5. Conclusion

This study identified 7,8-DHF as a potent ferroptosis inhibitor through directly counteracting lipid peroxidation. The anti-ferroptotic cell death abilities of 7,8-DHF are independent of TrkB signaling, explaining the fact that 7,8-DHF mitigates chronic disease without activating TrkB signaling. The engineered 7,8-DHF nanoparticles overcome the issue of low bioavailability of 7,8-DHF and improve the pathogenesis of ulcerative colitis and Alzheimer's disease.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated during the current study are available from the corresponding authors upon reasonable request.

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