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ORIGINAL ARTICLE

Deuterium modification of tyrosine kinase inhibitors contributes to reversing ferroptosis resistance through upregulation of aldehyde oxidase 1 in hepatocellular carcinoma



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Abstract The susceptibility to ferroptosis partially determines the efficacy of tyrosine kinase inhibitors (TKIs) in hepatocellular carcinoma (HCC), exposing a mechanistic vulnerability that can be therapeutically exploited. The development of deuterated compounds is a promising strategy for the improvement of anti-tumor efficacy. Here, we identified HCC with higher level of ferroptosis-resistance exhibited insensitive to TKIs, which could be reversed by deuterated TKIs. Aldehyde oxidase 1 (AOX1) was screened as a critical gene mediating the responsiveness to deuterated TKIs-induced ferroptosis in HCC. The presence of a pyridyl tri-deuterated methanamide contributed to the upregulation of AOX1 in a structure-dependent manner, thereby promoting ferroptosis. Mechanistically, AOX1 inhibited sirtuin 6-mediated deacetylation of H3K9 and H3K56, leading to transcriptional activation of acyl-CoA synthetase long chain family member 5, which resulted in poly-unsaturated fatty acids hyperaccumulation-induced ferroptosis. Additionally, HCC with lower AOX1 expression conferred better efficacy to

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deuterated TKIs. In patient cohorts with HCC, those with lower AOX1 expression exhibited a more pronounced therapeutic response to deuterated sorafenib. Overall, the present study elucidates the mechanism by which deuterated TKIs reverse TKI resistance by promoting ferroptosis and suggests that AOX1 could serve as a biomarker to guide clinical decision-making for deuterated TKI treatment in HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer death worldwide¹. More than 50% of patients with HCC are diagnosed at an advanced stage, and systemic therapy based on tyrosine kinase inhibitors (TKIs) is an important therapeutic strategy for patients with unresectable HCC (uHCC)². However, TKIs including sorafenib, lenvatinib, and regorafenib have demonstrated limited efficacy³, leaving HCC as a challenging disease to manage. Ferroptosis is an iron-dependent form of regulated cell death driven by the overloaded lipid peroxides on cellular membranes^{4,5}, and has been proved to be an effective anti-tumor mechanism⁶. Several studies have reported that multiple TKIs can act as ferroptosis inducers in HCC^{7,8}. However, the high degree of heterogeneity of HCC facilitates primary resistance to ferroptosis, leading to the insufficient efficacy of TKIs⁹. Therefore, seeking alternative strategies is urgently needed to improve clinical outcomes for patients with HCC.

Drug deuteration is a new strategy in which hydrogen atoms at specific sites of a molecule are replaced with deuterium¹⁰. The drug stability is enhanced since deuterium-carbon is ten times stronger than hydrogen-carbon bond, resulting in improved efficacy and reduced toxic side effect¹¹. The substitution site of deuterium plays a major role in influencing the efficacy and safety of deuterated derivatives¹². Pyridine is an important scaffold that is widely present in pharmaceutical compounds¹³. Pyridine-containing compounds have demonstrated excellent anti-tumor activities, and researchers are increasingly focusing on the development of new pyridine-based derivatives for cancer treatment¹⁴. A series of pyridine derivatives have shown higher therapeutic potential by modulating the expression of VEGF, p53, and cyclin D compared to their respective standard drugs¹⁵. Newly developed pyridine-based deuterated derivatives of EGFR inhibitors overcome the drug resistance¹². Notably, the approved TKIs for HCC, including sorafenib and regorafenib, are all pyridine-containing compounds, and their deuterated derivatives differ from their prototype by deuterium substitution on the pyridine methanamide moiety¹⁶. Although studies have demonstrated the improved efficacy of deuterated sorafenib¹⁷, the specific mechanism by which deuterium modification affects sorafenib and regorafenib require further exploration.

In this study, ferroptosis resistance led to insensitivity to multiple TKIs in HCC, whereas deuterium modification re-sensitized HCC cells to sorafenib and regorafenib by promoting ferroptosis. Pyridyl tri-deuterated methanamide, the distinct structure of deuterated-TKIs, conferred vulnerability to ferroptosis by upregulating aldehyde oxidase 1 (AOX1). AOX1 facilitated the transcription of acyl-CoA synthetase long chain family member 5 (ACSL5) through a chromatin-dependent mechanism, thereby

promoting poly-unsaturated fatty acids (PUFAs) hyperaccumulation-induced ferroptosis. Finally, we proposed that HCC with lower AOX1 expression may potentially benefit more from deuterated TKIs treatment.

2. Materials and methods

2.1. Ethical approval and clinical specimens

Two cohorts including 50 patients with uHCC receiving deuterated sorafenib neoadjuvant therapy were enrolled to evaluate the response from Huashan Hospital ($n = 30$) (Supporting Information Table S1) and Tongji Hospital ($n = 20$) (Supporting Information Table S2). Two cohorts including 50 patients with primary HCC underwent resection for primary tumors and received adjuvant therapy of deuterated sorafenib, which was enrolled to evaluate the short-term recurrence from Huashan Hospital ($n = 30$) (Supporting Information Table S3) and Tongji Hospital ($n = 20$) (Supporting Information Table S4). Patients were diagnosed as HCC according to the clinical practice guidelines of the European Association for the Study of the Liver¹⁸. Patients enrolled were diagnosed at Barcelona Clinic Liver Cancer B or C stage and specimens were collected by biopsy or surgery prior to the treatment of deuterated sorafenib. Treatment response was evaluated every 3 months according to modified Response Evaluation Criteria in Solid Tumors (mRECIST)¹⁹. Informed consent was obtained from each patient for their information and specimens to be used for research purposes. This study was performed under the principle of the Declaration of Helsinki and Istanbul and approved by the Ethics Committee of Huashan Hospital Affiliated to Fudan University (No. KY2025-1406).

2.2. Mice

Wild-type C57BL/6 and BALB/c nude mice were purchased from GemPharmatech (Nanjing, China). All experiments were performed on 4–6-week-old male mice. Hepa1-6 ($1 \times 10^6/30 \mu\text{L}$) cells were injected into subcapsular region of median liver lobe to construct the orthotopic HCC model. HCC-LM3 ($6 \times 10^6/100 \mu\text{L}$) cells were injected into subcutaneous of right inguinal fold regions to construct a subcutaneous HCC model. Tumors reached a measurable size (about 100 mm^3) after 10 days and then allocated randomly according to mouse number ($n = 6$ each group, 3 groups). For drugs intervention, sorafenib, regorafenib or deuterated derivatives were reconstituted in 0.5% sodium carboxymethyl cellulose (CMC-Na) and intragastric administrated at 60 mg/kg every 2 days to treat mice. Mouse weight and subcutaneous tumor volume were measured every 2 days. Tumor volume was calculated using Eq. (1):

$$\text{Volume} = (\text{Length} \times \text{Width}^2)/2 \quad (1)$$

Mice completing 14 days of therapy were sacrificed and sampled for subsequent analysis. All animals were humane cared according to the criteria outlined in the NIH "Guide for the Care and use of Laboratory Animals". All mouse experiments were performed following the protocols approved by The Committee on Animal Welfare and Ethics of the Laboratory Animal Center of Fudan University (No. A20250009).

2.3. Patient derived xenograft (PDX)

Human HCC tissues were acquired immediately after hepatectomy. Tissues were cut into 2 mm × 2 mm pieces and transplanted into subcutaneous of 4–6-week-old male severely immunodeficient mice ($n = 12$) (NSG) (Biomodel Organism, Shanghai, China). When the tumors reached a volume of approximately 100 mm³, they were passed into NSG mice to establish the second generation, and the third-generation tumors were used for subsequent experiments. Tumor-bearing mice were allocated randomly according to mouse number and treated as indicated ($n = 6$ each group, 6 groups). The tumor tissues were photographed after fixation in formalin. Informed consent was obtained from each patient for their information and specimens to be used for research purposes. Patients' information was provided in Supporting Information Table S5. This study was approved by the Ethics Committee of Huashan Hospital Affiliated to Fudan University (No. KY2025-1406). All animals were humane cared according to the criteria outlined in the NIH "Guide for the Care and use of Laboratory Animals". All mouse experiments were performed following the protocols approved by The Committee on Animal Welfare and Ethics of the Laboratory Animal Center of Fudan University (No. A20250009).

2.4. Human and mouse cell lines and cell lines transfection

Cell lines used in this article are as follows: Huh7, Hep3B, PLC, HCC-LM3, MHCC-97H, HepG2, HEK293T, Hepa1-6. All of these cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 mg/mL streptomycin and 100 U/mL penicillin at 37 °C in an atmosphere of 5% CO₂. Cells were transfected with plasmids, shRNA or sgRNA, and selected with puromycin (2–5 µg/mL) for 1 week. The stable cell lines were verified by Western blotting.

2.5. Plasmids and RNA interference

The cDNA encoding *AOX1* and *ACSL5* were amplified by PCR and cloned into pCDH-CMV-MCS-EF1-Puro according to the standard protocols. For RNA interference, shRNA sequences against *AOX1* and *ACSL5* were obtained from Sigma–Aldrich and cloned into pLKO.1 TRC (#10879; Addgene, Cambridge, MA, USA). The shRNA sequence with the best effect of knockdown to target gene can be found in Supporting Information Table S6.

2.6. CRISPR/Cas9-mediated gene knockout

The sequence of CRISPR single-guide RNA (sgRNA) against human *AOX1* was designed on <http://crispr-era.stanford.edu/index.jsp> (Lei Stanley Qi Lab). The two complementary sequences were incubated at 95 °C for 4 min and then cooled to

room temperature slowly to allow for annealing. The annealing products were cloned to lentiCRISPR v2 (#52961; Addgene, Cambridge, MA, USA). The sgRNA sequences with the best effect of knockout to target gene are as follows:

Forward, 5'-CACCGACAGGAAGTATGGCTG-3';
Reverse, 5'-AAACCAGCCATACTTAGTTCCTGTC-3'.

2.7. Transcriptional sequencing (RNA-Seq)

Samples were isolated and RNA was extracted with TRIzol Reagent. Library was prepared with NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs) and sequenced on a HiSeq 4000 platform (Novogene, Beijing, China). Gene expression was analyzed with DESeq2. Selected genes were compared with heatmap, and the gene expression values were row z-scored in the heatmap.

Published HCC transcriptional profiles were downloaded from TCGA and ICGC data portal (<https://tcga-data.nci.nih.gov/tcga/>, <https://dcc.icgc.org/>) and re-analyzed.

2.8. Non-targeted metabolomics

Non-targeted metabolomic profiling was performed at Shanghai Biotree Biotech Co., Ltd. according to the standard procedures. Briefly, metabolites were extracted by extract solution (methanol:water = 3:1, with isotopically labelled internal standard mixture). LC–MS/MS analyses were run with UHPLC system with a UPLC HSS T3 column. The QE HFX mass spectrometer acquired MS/MS spectra in the control of the acquisition software.

The raw data were transformed into mzXML format by ProteoWizard and processed with XCMS package for peak detection, extraction, alignment and integration. Then BiotreeDB (v2.1) database was applied for metabolite annotation, and the cutoff for annotation was set at 0.3.

2.9. Quantitative lipidomics

Quantitative lipidomic profiling was performed at Shanghai Biotree Biotech Co., Ltd. according to the standard procedures. Briefly, samples were preprocessed in 250 µL ice-water and 50 µL homogenate was obtained for BCA quantification. Then metabolites were extracted by extract solution (MTBE:MeOH = 5:1, containing internal standard and transferred to a sample vial for LC–MS/MS analysis. The UHPLC separation was performed with a SCIEX ExionLC series UHPLC System, and the AB Sciex QTrap 6500+ mass spectrometer was applied for assay development.

The quantification of the target compounds was conducted with Biobud-v2.1.4.1 Software. The absolute content of individual lipid corresponding to the internal standard was calculated on the basis of peak area and actual concentration of the identical lipid class internal standard.

2.10. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurements

Blood was sampled by extracting eyeballs and centrifuged for serum collection. ALT and AST were measured by ALT or AST assay kit according to the manufacture's instruction.

2.11. Cellular nicotinamide adenine dinucleotide (NAD⁺) level measurement

Level of cellular NAD⁺ was detected using the NAD⁺/NADH Assay Kit with WST-8 (Beyotime, Shanghai, China) according to the manufacturer's instruction. In detail, cells were washed with pre-cooled PBS buffer and lysed with NAD⁺/NADH extraction buffer. Cell lysis was centrifuged at 13,300×g, 4 °C for 10 min and the supernatant were collected and split into two parts for separate measurement: 1) the total NAD (NAD⁺ & NADH) and 2) NADH only. To measure total NAD, 20 µL cell lysis and 90 µL alcohol dehydrogenase working solution were added into 96-well plates and incubated at 37 °C for 10 min protected from light. Then added 10 µL chromogenic solution and incubated at 37 °C for 30–60 min, aiming to convert NAD⁺ into NADH. The absorbance was measured at 450 nm. To measure the level of NADH, NAD⁺ was cleared at 60 °C for 30 min and collected the supernatant after centrifuging at 13,300×g for 10 min. The level of NADH was measured as total NAD. NADH and total NAD were calculated using a standard curve. The level of NAD⁺ was calculated as Eq. (2):

$$\text{NAD}^+ = \text{NAD}_{\text{total}} - \text{NADH} \quad (2)$$

The value of NAD⁺ was normalized to the total protein concentration of each sample. Total proteins were quantified with BCA protein assay kit (Beyotime, Shanghai, China) according to the manufacture's instruction (Supporting Information Table S7).

2.12. Measurement of cellular Fe²⁺

Cellular Fe²⁺ was measured using Ferrous Ion Content Assay Kit (Solarbio, Beijing, China) according to the manufacture's induction. Briefly, cells were lysed with lysis buffer then fragmented by sonication. Cell lysates were centrifuged and the supernatant was harvested. 200 µL cell lysates and 100 µL chromogen were mixed and then incubated in 37 °C for 10 min. Following adding chloroform, cell lysates were centrifuged at 12,000×g for 10 min then the inorganic phase was used for absorbance measurement at 593 nm. The level of Fe²⁺ was normalized to the total protein concentration of each sample. Total proteins were quantified with BCA protein assay kit according to the manufacture's instruction (Table S7).

2.13. Determination of reactive oxygen species (ROS) and lipid peroxidation

The intracellular ROS level was detected by DCFH-DA as manufacture's instruction (Table S7). Cells were collected and washed with DMEM free of FBS. Then cells were incubated with 10 µmol/L DCFH-DA in DMEM at 37 °C incubator for 20 min protected from light, followed by washing with DMEM. Then cells were examined using FACS Calibur flow cytometer (488 nm) (Supporting Information Table S8). For determination of lipid peroxidation (Table S7), cells were seeded into 24-well plates with glass coverslips in each well. After adherence overnight, cells were treated with indicated compounds. Cells were washed with PBS then incubated with 5 µmol/L BODIPY 581/591 C11 at 37 °C incubator for 30 min protected from light. Nuclei were counterstained with 1 µg/mL Hoechst 33,342 for 10 min before imaging. Images were captured with Leica confocal microscope.

The images of both non-oxidized (C11^{Non-ox}, 581 nm) and oxidized (C11^{Ox}, 500 nm) C11 were monitored for each sample. The gates were set up based on untreated cells.

For malondialdehyde (MDA) (Table S7), cells were lysed with PBS and centrifuged at 12,000×g for 10 min. The MDA detection working solution for each sample included 50 µL TBA stock (0.37%), 150 µL TBA diluent and 3 µL antioxidant. The working solution was added to the supernatant and incubated at 100 °C for 15 min and cooled to temperature, followed by centrifuging at 12,000×g for 10 min. Then transferred 200 µL supernatant to 96-well plates, and the absorbance was detected at 532 nm.

2.14. Cell viability

Cell viability was determined by using CCK8 assay following the protocol.

2.15. Transmission electron microscopy (TEM) analysis

Cells were harvested and fixed with 2% v/v glutaraldehyde for 30 min in room temperature and then transferred to 4 °C. Samples were washed, dehydrated and embedded in Eponate 12 resin according to the standard procedures. The embedded samples were then cut into 60 nm thick sections and stained with uranyl acetate and lead citrate. Finally, representative images were acquired using a Philips CM 100 Transmission EM (Table S8).

2.16. Half maximal inhibitory concentration (IC₅₀) determination

The value of IC₅₀ was calculated by cell counting kit-8 (CCK-8). Cells were seeded into 96-well plates. After adherent, cells were treated with the increasing concentration of drugs as indicated and incubated for 48 h at 37 °C containing 5% CO₂ incubator. Then discarded the medium and then incubated with 100 µL DMEM containing 10% CCK-8 solution for 90 min and read at 450 nm. The value of IC₅₀ was calculated based on the absorbance of untreated cells.

2.17. Long-term colony formation assay

Cells were seeded into 12-well plates at a density of 2000–3000 cells per well and treated with indicated drugs for 10 days (medium was changed once every three days). Cells were fixed with 4% (w/v) formaldehyde for 20 min and stained with 0.1% (w/v) crystal violet for 30 min. Cell confluence was quantified by ImageJ software.

2.18. Oil red O (ORO) staining

Cells were seeded and pretreated as before. Cells were fixed with 4% (w/v) paraformaldehyde for 20 min and stained with 3 mg/mL Red Oil O solution in 60% (v/v) isopropanol for 30 min protected from light. Nuclei were counterstained with hematoxylin for 3 min, then acid alcohol differentiation solution was used to differentiate for 15 s followed by washing with tap water to return to blue.

2.19. Quantitative real-time quantitative PCR (qRT-PCR)

Cells were washed with pre-cooled PBS three times. Total RNA was extracted with TRIzol Reagent. Reverse-transcriptional PCR

was carried out with 5×PrimeScript Buffer according to the manufacturer's instruction. Quantitative PCR analysis of ACSL5 and β -actin was performed with TB Green Premix Ex Taq with Applied Biosystems (Table S8). The results were analyzed with the relative quantification ($2^{-\Delta\Delta C_t}$) method. Primers can be found in Table S9.

2.20. Western blot (WB)

Cells were lysed with RIPA (P0013C, Beyotime) supplemented with 1/100 protease inhibitor cocktail and proteins were quantified with BCA protein assay kit according to the manufacturer's instruction followed by denaturation at 100 °C for 10 min. Proteins were fractionated by SDS-PAGE gels and transferred to nitrocellulose membrane. The nonspecific binding sites of nitrocellulose membrane were blocked with 5% (w/v) Bovine Serum Albumin (BSA) in TBST for 1 h at RT. The membranes were incubated with primary antibodies overnight at 4 °C. Then the membranes were washed with TBST to remove unbound antibodies and incubated with HRP-conjugated secondary antibody. Finally, chemiluminescence substrate was added to the membranes and visualized using Amersham Imager600 (Table S8). Antibodies used in WB are listed in Table S10.

2.21. Immunohistochemistry

Immunohistochemistry (IHC) was performed according to the standard procedure. Briefly, tissues were fixed with 4% formaldehyde and paraffin-embedded sections were obtained at 5 μ m/slide. Slides were incubated with anti-AOX1, anti-ACSL5 and anti-4-HNE antibodies. Then slides were stained with corresponding peroxidase-conjugated secondary antibody. The 3-amino-9-ethylcarbazole chromogen was used to visualize the target protein. Finally, the sections were scored independently by two pathologists. The number of cells with positive staining was scored using a 4-point scale (1 = 0–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100%). Intensity was scored as 0 (negative), 1 (weak), 2 (medium) and 3 (strong). The two scores were multiplied to yield a final score per pathologist per slide. Antibodies used in IHC are listed in Table S10.

2.22. Chromatin immunoprecipitation (ChIP)

The chromatin immunoprecipitation assay was performed with SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling Technology, MA, USA) (Table S7) as the manufacturer's instructions. Briefly, cells were crosslinked with 37% formaldehyde and collected. Chromatin fragments were prepared by Micrococcal Nuclease and incubated with anti-H3K9ac, anti-H3K56ac, anti-H3. Then DNA was purified and analyzed by qRT-PCR. Amplification condition was as follow: 95 °C for 2 min, 40 cycles consisting of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s, and 72 °C for 5 min. The primers were used as follows: Forward 5'-CCGCTTAAGCCCATTGCATA-3'; Reverse 5'-GACACACCCGAAGCTACTGT-3'.

2.23. Molecular docking

In order to explore the binding activity between drugs and protein molecules, MOE 2019 software was used for molecular docking. The 2D structure was obtained from the PubChem database (<http://pubchem.ncbi.nlm.nih.gov/>) and was imported into Chem

Office software to synthesize 3D structure and saved as mol2 file. The Molecular Operating Environment was used to search for active pockets of protein molecules. Finally, MOE was applied for molecular docking. The binding activity was evaluated based on binding energy (BE). The docking results were visualized by PyMOL and Discovery studio.

2.24. Surface plasmon resonance (SPR) analysis

Prior to immobilization, the activator solution is freshly prepared by combining 400 mmol/L EDC with 100 mmol/L NHS. The CM5 sensor chip surface is then activated using this mixture at a flow rate of 10 μ L/min for 420 s. AOX1 protein is diluted to a concentration of 20 μ g/mL using the immobilization buffer and injected into sample channel Fc2 at flow rate of 10 μ L/min, typically achieving an immobilization level of approximately 12,600 response units. Channel Fc1 is used as a reference and does not undergo ligand immobilization. Following protein coupling, the chip surface is blocked with 1 mol/L ethanolamine hydrochloride at 10 μ L/min for 420 s.

The compounds are diluted in analyte buffer to generate eight serial concentrations ranging from 0.78 to 50 μ mol/L. Each compound is injected over both Fc1 and Fc2 at a flow rate of 20 μ L/min, with an association phase lasting 100 s, followed by a 180-s dissociation phase. All binding interactions occur in the same analyte buffer. Each compound is tested in ascending concentration order across eight cycles. Following each binding cycle, the chip surface is regenerated to remove residual analyte and restore binding capacity for the next injection.

2.25. Detection of AOX1 enzymatic activity

Cells were dissected into the ice-cold saline buffer pH 7.4. Place the tube in an ice bath and sonicate using an ultrasonic homogenizer. Typical settings are 20%–30% amplitude, with 5-s pulses followed by 10-s intervals, repeated 3 times. Supernatants of cell homogenates were collected after centrifugation (15 min at 12,000×g) and stored at –80 °C.

A fluorescence assay was used to assess AOX1 activity by monitoring the oxidation of 4-dimethylaminocinnamaldehyde (DMAC) to 4-dimethylaminocinnamic acid (DMACA) in black 384-well plates. Cell lysates (16 μ g protein/well) were incubated in 100 μ L of 0.1 mol/L Tris-HCl buffer (pH 7.4) at 37 °C for 5 min to measure background fluorescence²⁰. The reaction was initiated by adding 100 μ mol/L DMAC (final DMSO concentration: 0.1%). Fluorescence was recorded every 25 s for 10 min (Ex, 340 nm; Em, 440 nm) using a SpectraMax Gemini EM reader. A standard curve of DMACA (0.045–33 μ mol/L) was used to calculate specific activity. Blank values were subtracted to correct for background, and assay linearity was confirmed in pilot experiments.

2.26. Immunoprecipitation (IP)

Cells were collected and lysed on ice for 30 min using an immunoprecipitation buffer supplemented with protease and phosphatase inhibitors (types A and B). The lysates were then clarified by centrifugation at 12,000×g for 10 min. For each immunoprecipitation reaction, 600 μ g of cellular protein or 1000 μ g of tissue lysate was incubated with 2–5 μ g of specific antibody overnight at 4 °C on a shaker. Subsequently, 20 μ L of protein A/G agarose beads were added and the mixture was further

incubated for 4 h at 4 °C with gentle agitation. Beads were washed thoroughly with IP buffer, followed by the addition of 20 µL of SDS loading buffer. The samples were then subjected to WB analysis.

2.27. Weighted gene co-expression network analysis (WGCNA)

WGCNA was performed using R package “WGCNA”. Calculate the correlation between genes and build a weighted network based on the correlation matrix using Spearman correlation coefficient. The correlation matrix is converted into a similarity matrix using a power function to convert the correlation value into a similarity measure to highlight highly related gene pairs. Construct gene co-expression network based on similarity measurement using Topological Overlap Matrix, where each gene represents a node in the network, and edge represents the similarity relationship between genes. The application of clustering algorithms (such as hierarchical clustering) to identify gene modules in the network, that is, clusters of highly related genes. Each module typically represents a group of genes with similar expression patterns. Gene modules are correlated with external phenotypic data to discover gene modules that are closely related to the phenotype of interest.

2.28. Kyoto encyclopedia of genes and genomes (KEGG) analysis

KEGG was performed by “KEGG” R package. Gene IDs were mapped to pathway information in the KEGG database to determine the function and role of genes in different pathways. By performing pathway enrichment analysis on the gene set of interest, the number of genes in the KEGG pathway is determined to significantly exceed random expectations. The functional enrichment analysis of genes in the pathway was visualized with “ggplot” package.

2.29. Gene set enrichment analysis (GSEA)

GSEA was performed by GSEA (v4.1.0) software. Rank all genes based on their expression changes between the two conditions using a metric like *t*-statistic. Define gene sets representing biological pathways and functional annotations. Calculate an enrichment score for each gene set to determine if the genes in the set are randomly distributed or clustered towards the top or bottom of the ranked list. Assess the statistical significance of the enrichment scores by permutation testing to determine if the observed enrichment is significant. Visualize the results using enrichment plots showing the running enrichment score and the positions of genes in the ranked list.

2.30. Single sample GSEA (ssGSEA)

ssGSEA, computed by GSVA Bioconductor package from R, was applied to assign an enrichment score for each sample²¹. The enrichment score characterizes joint up- or down-regulation of a set of genes in relation to remaining reference genes measured in the sample.

2.31. Statistical analysis

Difference between two groups was tested by Student's *t*-test. Difference between multiple groups was tested by one-way ANOVA with Tukey's multiple comparisons test. Frequency

comparisons were analyzed by Fisher exact probability test. The differences of survival were analyzed with log-rank (Mantel–Cox) test. The statistical significance was set at a value of $P < 0.05$ (ns no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

3. Results

3.1. Deuterium modification of TKIs reverse resistance to TKIs through promoting ferroptosis in HCC

To verify the role of ferroptosis in determining responsiveness to TKIs, we first analyzed the correlation between ferroptosis resistance scores and the corresponding IC₅₀ values of TKIs across multiple HCC cell lines. Cell lines with high degree of ferroptosis resistance tended to be more tolerant to multiple TKIs (Fig. 1A). Furthermore, ferroptosis was identified as the major factor conferring responsiveness to sorafenib, as patients with enhanced ferroptosis defense mechanisms were less likely to respond to sorafenib (Supporting Information Fig. S1A). To validate the correlation between ferroptosis resistance and TKI sensitivity, we first measured IC₅₀ values of four representative TKIs across six HCC cell lines. Cells demonstrated distinct sensitivity to TKIs (Fig. S1B), indicating a possible association between ferroptosis sensitivity and TKI responsiveness in HCC cells. Then, the six HCC cell lines were subsequently treated with sorafenib, regorafenib, and their deuterated derivatives. Different HCC cell lines showed distinct vulnerability to TKIs through long-term clonogenic assays (Fig. 1B; Fig. S1C). Notably, HCC-LM3, MHCC-97H, and HepG2, which were relatively resistant to TKIs, could be re-sensitized by deuterated sorafenib and regorafenib derivatives (Fig. 1B; Fig. S1C). The accumulation of Fe²⁺ and MDA (a degradation product of lipid peroxidation) increased in TKIs-resistant cells following treatment with deuterated TKIs compared to their prototypes (Fig. 1C and D). Then, cells were subject to WB analysis to detect key markers of ferroptosis²². TKI-tolerant cells exhibited higher level of SLC7A11 and NRF2 upon treatment with TKIs, whereas deuterated TKIs suppressed the expression of these ferroptotic defense markers, potentially explaining the enhanced sensitivity (Fig. 1E). Collectively, these results suggest that deuterium modification of TKIs might be an effective therapeutic option for patients with ferroptosis-resistant HCC.

Deuterated sorafenib and regorafenib shares the same molecular targets as their prototype but are structurally distinguished by a pyridyl tri-deuterated methanamide modification. To further verify their therapeutic effects, we treated HCC cells with deuterated TKIs and their corresponding prototypes. Compared with the prototype, deuterated TKIs significantly promoted ferroptosis in HCC cells (Supporting Information Figs. S2A–S2C, S3A–S3C) and exhibited comparable inhibitory effects on angiogenesis-related signaling pathways (Fig. S2D). In addition, deuterated TKIs demonstrated enhanced anti-tumor efficacy (Figs. S2E–S2G, S3D–S3F) and similar anti-angiogenesis (Fig. S2I) *in vivo* relative to their prototype counterparts. Importantly, these treatments had minimal impact on mouse body weight, liver function and renal function (Figs. S2H and S3G). Subsequently, high-throughput RNA sequencing was conducted on samples treated with either sorafenib or deuterated sorafenib. GSEA revealed enhanced execution of ferroptosis in the deuterated sorafenib-treated group (Fig. 1F), whereas other form of cell death, including apoptosis, autophagy, and necrosis, showed

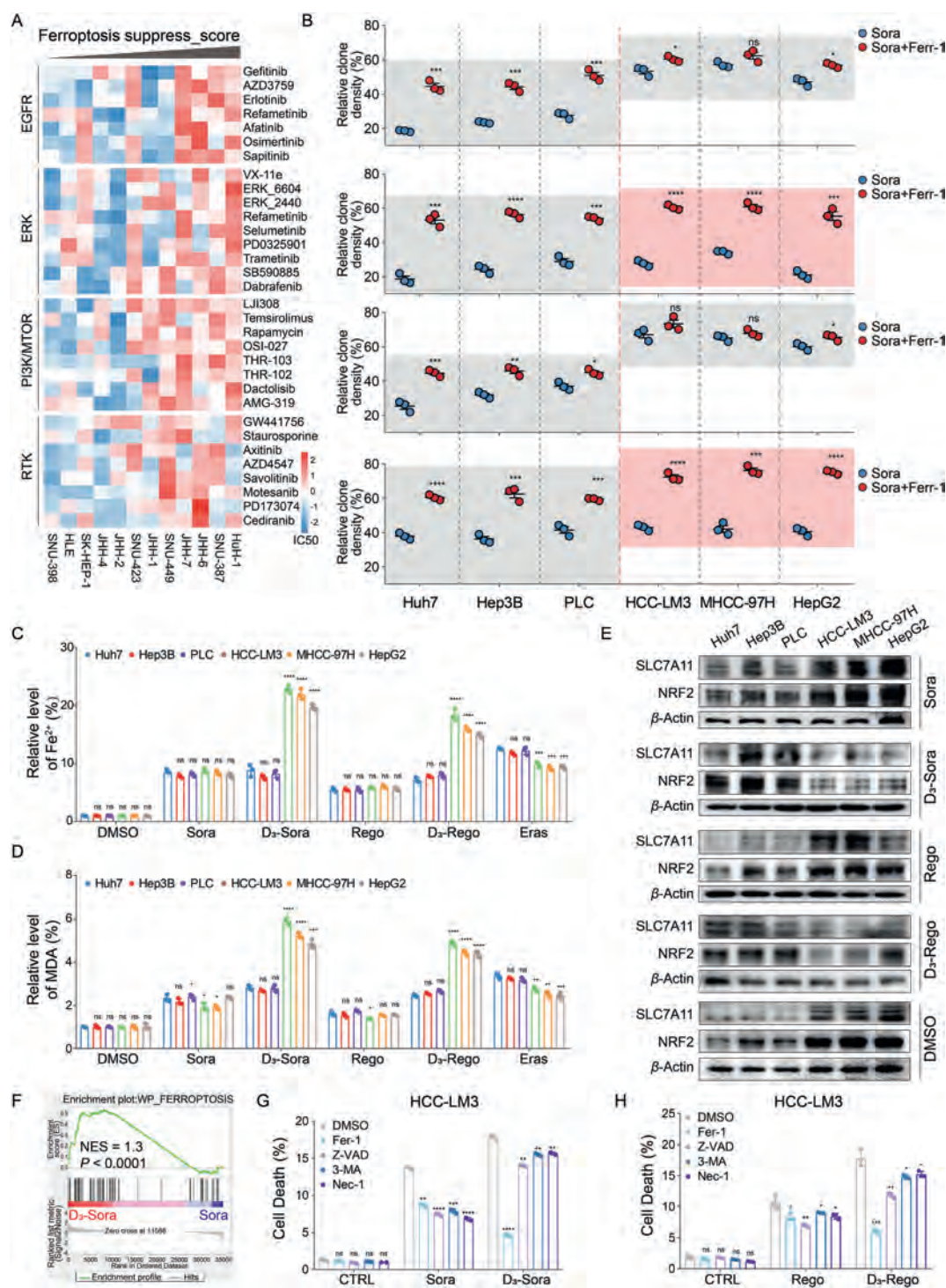


Figure 1 Deuterium modification of TKIs reverse resistance to TKIs through promoting ferroptosis in HCC. (A) Heatmap of IC_{50} values for various TKIs in HCC cells (GDSC database). (B) Responses of six HCC cell lines to TKIs and deuterated TKIs analyzed by long-term colony-formation assay ($n = 3$). (C–E) Response of 6 cell lines to TKIs-induced ferroptosis analyzed by Fe^{2+} measurement (C) ($n = 3$), MDA measurement (D) ($n = 3$) and WB analysis (E). (F) GSEA of the ferroptosis between sorafenib and deuterated sorafenib group. (G, H) Cells were treated with TKIs or deuterated TKIs (6 μ mol/L) in the presence of Ferrostatin-1 (2 μ mol/L), Z-VAD-FMK (10 μ mol/L), 3-methyladenine (50 μ mol/L) or necrostatin-1 (1 μ mol/L) for 48 h as indicated. Cell viability was analyzed by CCK8 ($n = 3$). Data are shown as mean $\pm$ SEM, ns, no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

comparable activity between the two treatments (Fig. S2J). Besides, a stronger activation of ferroptotic signaling was observed in the deuterated sorafenib group (Fig. S2K). In HCC cells,

treatment with the ferroptosis inhibitor ferrostatin-1 (Ferr-1) effectively protected against deuterated TKIs-induced cell death. In contrast, inhibitors of apoptosis (Z-VAD-FMK), autophagy (3-

methyladenine, 3-MA), or necrosis (necrostatin-1, Nec-1) exerted only marginal effects on cell viability (Fig. 1G and H). Taken together, these findings indicate that HCC exhibits variable susceptibility to TKIs-induced ferroptosis. Deuterated TKIs display enhanced anti-tumor efficacy in ferroptosis-resistant HCC, primarily through the robust activation of ferroptotic cell death.

3.2. *AOX1 confers a distinct susceptibility to ferroptosis on HCC upon the treatment of deuterated TKIs*

To identify the co-expression pattern genes associated with the distinct susceptibility to deuterated TKIs-induced ferroptosis in HCC, we performed WGCNA in patients with differential responses to sorafenib. Ten co-expression modules were identified (Fig. 2A, left), among which genes in module “MEDdarkgreen” module showed a strong correlation with responsiveness to sorafenib ($P < 0.0001$). In total, 31 overlapping genes were identified among the hub genes of the “MEDdarkgreen” module, the differentially expression genes (DEGs) between responder and non-responder patients, and the DEGs between tumors treated with deuterated sorafenib and sorafenib (Fig. 2A, right). Then, aldehyde oxidase 1 (AOX1) was identified as the most strongly associated gene with the differential response to ferroptosis induced by deuterated TKIs, based on both Pearson’s correlation analysis and survival analysis (Fig. 2B; Supporting Information Fig. S4A and S4B).

Deuterated sorafenib and regorafenib are distinguished from their prototypes by a pyridyl tri-deuterated methanamide structure (D₃-pyridine ring) (Fig. 2C and D). Notably, aldehyde oxidases plays crucial roles in the oxidation of pyridine²³. Deuterated TKIs share the same targets as their prototypes, suggesting that their effect to induce ferroptosis may depend on structural modification rather than conventional target-dependent mechanisms. We conducted molecular docking to evaluate binding affinities of H₃-pyridine ring and D₃-pyridine ring to AOX1. The D₃-pyridine ring showed a lower binding energy (BE) of -5.2597 kcal/mol compared to the H₃-pyridine ring (BE = -5.0790 kcal/mol), indicating a stronger interaction (Fig. 2E). SPR analysis further verified the enhanced binding affinity of deuterated compounds to AOX1. The D₃-pyridine ring exhibited a lower dissociation constant (K_d) (59.1 μ mol/L) compared to the H₃-pyridine ring (74.3 μ mol/L) (Fig. 2F). Immunoblot analysis revealed that AOX1 expression was upregulated following D₃-pyridine ring treatment in a dose- and time-dependent manner (Supporting Information Fig. S5A). Functionally, the D₃-pyridine ring enhanced cellular sensitivity to ferroptosis, whereas the H₃-pyridine ring and Urea have minimal effects in the presence of Erastin (a ferroptosis inducer) (Fig. 2G and H). Similarly, molecular docking showed that deuterated sorafenib (BE = -7.6149 kcal/mol) and deuterated regorafenib (BE = -7.660 kcal/mol) exhibited stronger binding to AOX1 than their respective prototypes (sorafenib, BE = -7.1668 kcal/mol; regorafenib, BE = -6.8261 kcal/mol) (Fig. 2I; Fig. S5B). SPR analysis proved that the K_d values of deuterated sorafenib and deuterated regorafenib to AOX1 were 16.5 and 21.9 μ mol/L, respectively, while the non-deuterated counterparts showed weaker affinities (sorafenib, 60.9 μ mol/L; regorafenib, 42.7 μ mol/L) (Fig. 2J; Fig. S5C). Correspondingly, AOX1 expression was upregulated by deuterated TKIs in a similar manner (Fig. S5D and S5E). In addition, the enzymatic activity of AOX1 was not affected after deuteration (Fig. S5F), indicating that the deuterium modification affects expression of AOX1 but does not interfere with its enzymatic function. We tentatively investigated how deuterated sorafenib

influenced AOX1 expression level. The expression of AOX1 was stabilized when treated with deuterated sorafenib in the presence of protein biosynthesis inhibitor cycloheximide (CHX), and the proteasome inhibitor MG132 rather than lysosome inhibitor chloroquine (CQ) could block the degradation of AOX1 when treated with sorafenib (Supporting Information Fig. S6A). Furthermore, deuterated sorafenib could slow down the degradation of AOX1 and inhibit the ubiquitin-bound AOX1 (Fig. S6B and S6C). Collectively, these findings demonstrate that deuterated TKIs promote ferroptosis through structural modification-dependent upregulation of AOX1.

Given the different binding activity of TKIs with AOX1 and their varied responsiveness to ferroptosis, we first investigated the role of AOX1 in HCC. AOX1 expression was positively correlated with ferroptosis scores in both the TCGA and ICGC databases (Supporting Information Fig. S7A), and AOX1 enhanced the sensitivity to ferroptosis in the presence of Erastin (Fig. S7B–S7H). To further explore the role of AOX1 in deuterated TKIs-induced ferroptosis, we generated AOX1-knockout (AOX1-KO) HCC cells using the CRISPR/Cas9 system (Fig. 3A). We found that AOX1 deficiency abrogated ferroptosis induced by deuterated TKIs, but not by their prototypes, as evidenced by long-term clonogenic assays, cell viability measurement, MDA measurement, and Fe²⁺ measurement (Fig. 3B–E, Supporting Information Fig. S8A–S8D). These results suggest that deuterated TKIs induce ferroptosis *via* a mechanism distinct from that of their prototypes, relying on D₃-pyridine ring-mediated upregulation of AOX1 in HCC.

3.3. *AOX1 promotes ferroptosis through ACSL5-mediated accumulation of PUFAs*

Since ferroptosis is a by-product of cellular metabolism⁵, we employed LC-MS/MS-based untargeted metabolomics to characterize metabolic profile. Compared with sorafenib, deuterated sorafenib group was enriched in poly-unsaturated fatty acids (PUFAs), which have been reported to be central to the execution of ferroptosis⁵ (Fig. 4A). We also verified that deuterated sorafenib increased the accumulation of lipid droplets, a storage form of excess PUFAs during lipid peroxidation²⁴ (Fig. 4B). Subsequent reanalysis of gene expression profile revealed significant activation of PUFAs synthesis in the deuterated sorafenib group (Fig. 4C). Integrating gene expression data with qPCR validation, we identified that acyl-CoA synthetase long chain family member 5 (ACSL5) was significantly upregulated upon deuterated sorafenib treatment (Fig. 4D). Pearson correlation analysis between AOX1 and PUFAs synthesis-related signatures further suggested that ACSL5 might be the most likely downstream target of AOX1 (Supporting Information Fig. S9A). We then confirmed that AOX1 regulates ACSL5 expression in HCC cell lines (Fig. 4E).

Based on the above evidence, we hypothesized that the AOX1 upregulation induced by deuterated sorafenib might facilitate ACSL5-mediated PUFAs accumulation, ultimately resulting in ferroptotic death. Indeed, the lipidomic profiling showed that AOX1 increased the abundance of multiple PUFAs species through ACSL5 (Fig. 4F). To verify the role of ACSL5 in AOX1-induced ferroptosis, we first examined the effect of ACSL5 on ferroptosis (Fig. S9B). ACSL5 promoted the accumulation of lipid droplets, lipid peroxidation, MDA and Fe²⁺ (Fig. S9C–S9J), indicating that ACSL5 functions as a pro-ferroptosis factor. Moreover, we knocked down ACSL5 in AOX1-OE-cell and upregulated ACSL5 in shAOX1-cell in order to prove the

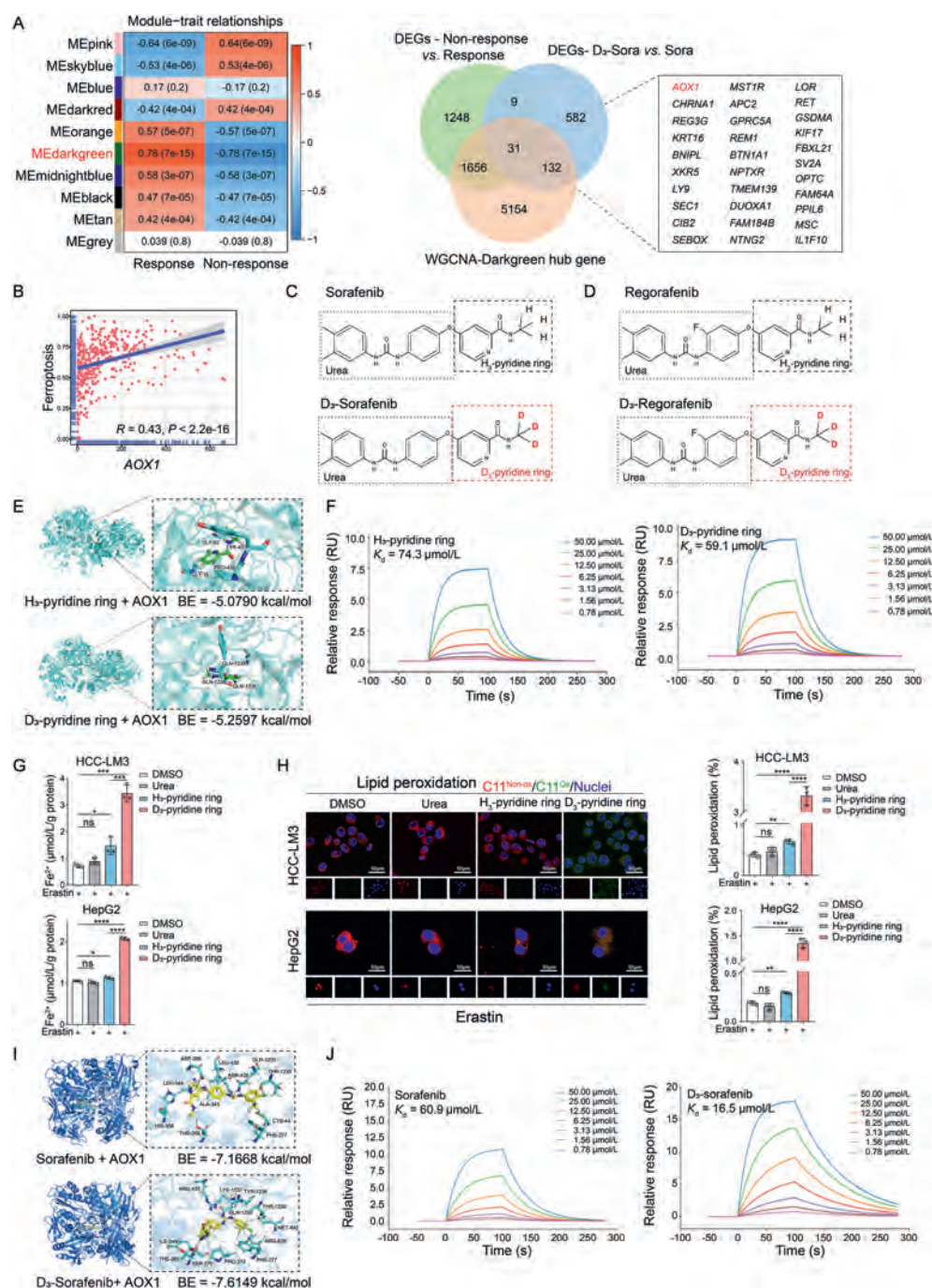


Figure 2 AOX1 confers distinct ferroptosis susceptibility to HCC cells upon deuterated TKI treatment. (A) WGCNA between patients with differential response to sorafenib (left); Venn diagram of hub genes in “MEdarkgreen” module, DEGs between non-response and response patients and DEGs between deuterated sorafenib and sorafenib-treated tumors (right). (B) Correlation between the expression of *AOX1* and ferroptosis in TCGA database. (C) Molecular structures of sorafenib and deuterated sorafenib. (D) Molecular structures of regorafenib and deuterated regorafenib. (E) Molecular docking of H₃-pyridine ring and D₃-pyridine ring to AOX1. (F) SPR analysis of the binding affinity of H₃-pyridine ring and D₃-pyridine ring to AOX1. (G, H) Cells were treated with substructures of sorafenib and deuterated sorafenib as indicated (8 $\mu\text{mol/L}$) in the presence of erastin (5 $\mu\text{mol/L}$) for 48 h. Level of Fe²⁺ was measured (G) ($n = 3$). Lipid peroxidation was analyzed, and quantitative results were presented (scale bar = 50 μm) ($n = 3$) (H). (I) Molecular docking of sorafenib and deuterated sorafenib to AOX1. (J) SPR analysis of the binding affinity of sorafenib and deuterated sorafenib to AOX1. Data are shown as mean $\pm$ SEM, ns, no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

regulation of ferroptosis by AOX1 through ACSL5 (Supporting Information Fig. S10A). Knockdown of *ACSL5* in AOX1-overexpressing cells abolished the excessive accumulation of

lipid droplets, lipid peroxidation, Fe²⁺, and restored mitochondrial morphology in the presence of erastin (Fig. S10B, S10D–S10F). Conversely, overexpression of *ACSL5* in AOX1-deficient HCC

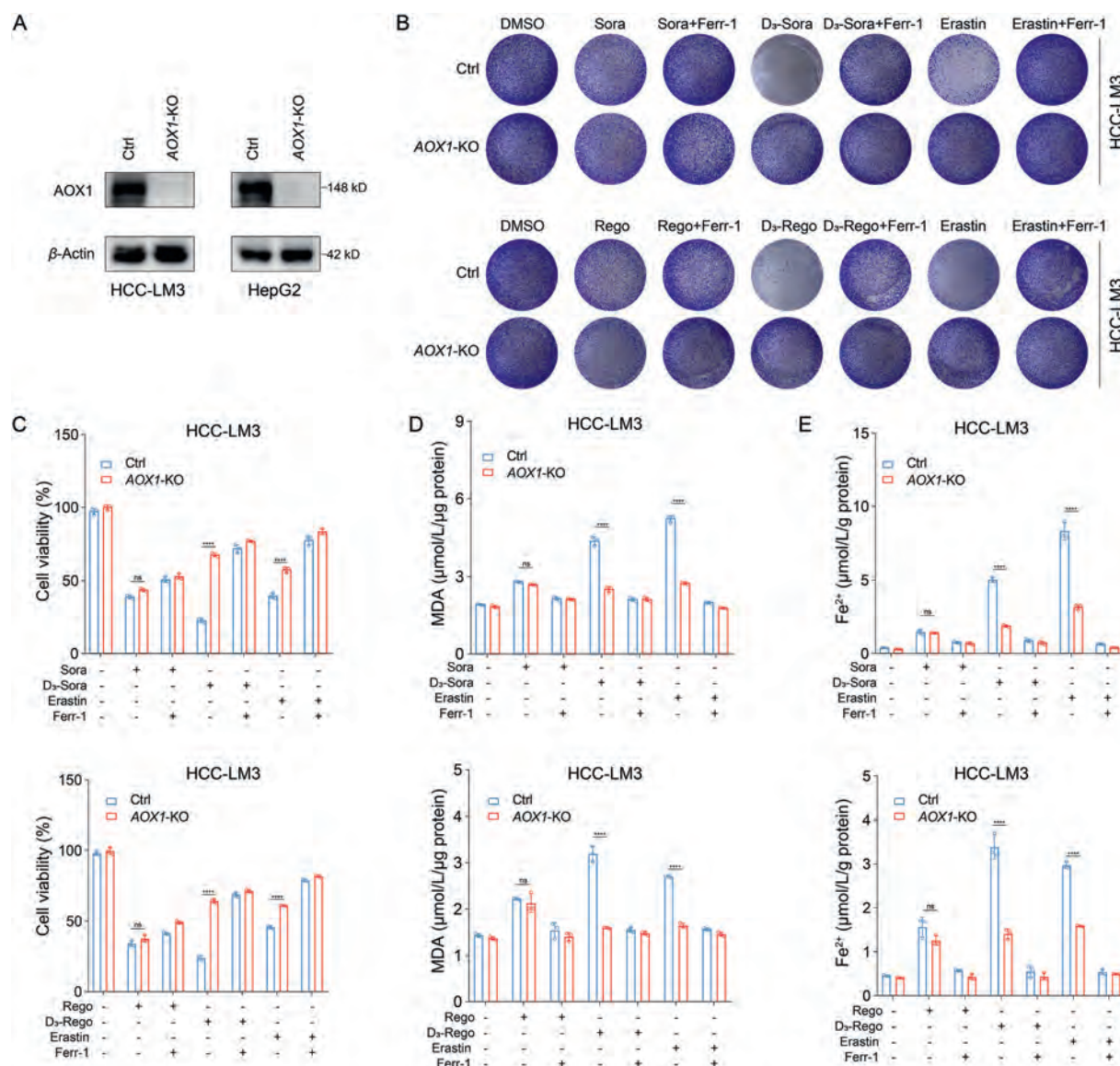


Figure 3 Deuterated TKIs promotes ferroptosis through upregulation of AOX1. (A) Verification of AOX1-KO cells. β -Actin was a loading control. (B–E) Cells (Ctrl, AOX1-KO) were treated with TKIs (6 $\mu\text{mol/L}$), deuterated TKIs (6 $\mu\text{mol/L}$) or erastin (5 $\mu\text{mol/L}$) with or without ferrostatin-1 (2 $\mu\text{mol/L}$) for 48 h. Cell viability was determined by long-term clonogenic assay and CCK8 (B, C); levels of MDA and Fe^{2+} were measured by biochemical tests (D, E) ($n = 3$). Data are shown as mean $\pm$ SEM, ns, no significance, **** $P < 0.0001$.

cells eliminated the protective effect conferred by AOX1 knock-down, leading to enhanced ferroptosis (Fig. S10C, S10G–S10I). We further investigated whether ACSL5 is involved in the regulation of deuterated TKIs-induced ferroptosis. Loss of AOX1 partially suppressed ferroptosis induced by deuterated TKIs, whereas exogenous restoration of ACSL5 significantly enhanced ferroptotic cell death (Fig. 4G–I, Supporting Information Fig. S11A–S11C). Together, these results suggest that AOX1-mediated upregulation of ACSL5 upregulation is sufficient to reprogram the lipidome in HCC, thereby sensitizing cells to deuterated TKIs-induced ferroptosis.

3.4. AOX1 blocks SIRT6-mediated deacetylation of H3K9 and H3K56 to promote transcription of ACSL5

AOX1 is one of the key enzymes involved in the clearance of NAD^+ , an essential cofactor in numerous enzymatic redox

reactions²⁵ (Fig. 5A). Indeed, AOX1 reduced the abundance of NAD^+ in HCC cell lines (Fig. 5B). NAD^+ is primarily consumed by three classes of enzymes: sirtuins (SIRT6), CD38, and poly (ADP-ribose) polymerase (PARPs) (Fig. 5A). Therefore, the availability of NAD^+ influences the biological processes mediated by these consumer²⁶. WB analysis showed that SIRT6 expression was suppressed by AOX1 overexpression but restored by NAD^+ supplementation in HCC cells, while the other NAD^+ consumers showed no significant changes (Fig. 5C). TCGA and ICGC databases also demonstrated a significant negative correlation between AOX1 and SIRT6 (Supporting Information Fig. S12A–S12C). SIRT6 is known to deacetylate histone H3 at lysine 9 and 56 (H3K9 and H3K56) and is critical for regulating genes involved in diverse biological processes, including lipid metabolism^{27,28}. High levels of H3K9ac and H3K56ac are typically associated with transcriptional activation²⁹. We speculated that AOX1 may regulate ACSL5 transcription via SIRT6-mediated

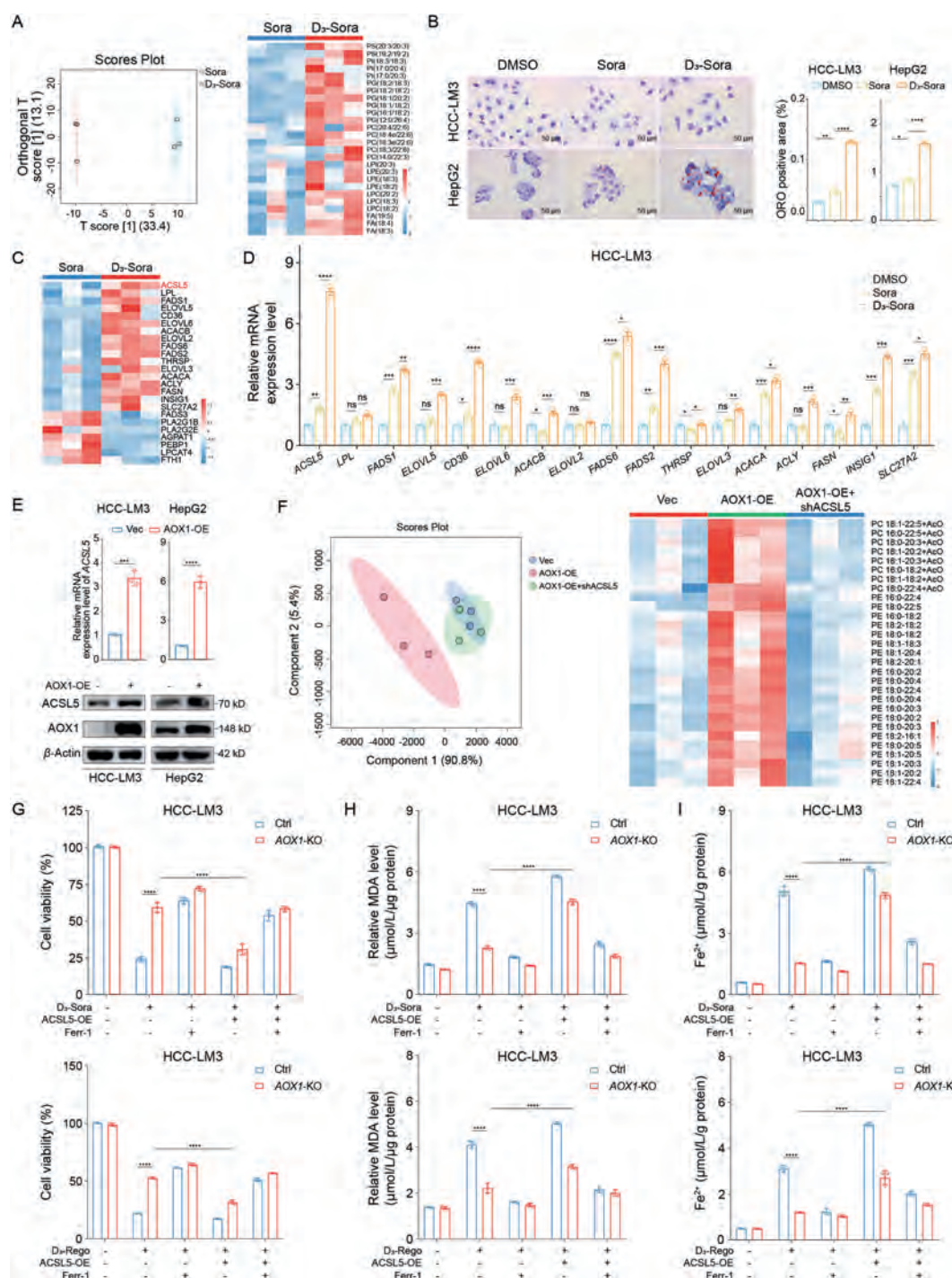


Figure 4 AOX1 promotes ferroptosis through ACSL5-mediated accumulation of PUFAs. (A) Scatter plot of OPLS-DA model and heatmap of Z-score transformed abundance of PUFAs for tumors treated with sorafenib and deuterated sorafenib ($n = 3$). (B) Representative image of lipid droplet in cells treated as indicated and quantification results were presented (scale bar = 50 μm) ($n = 3$). (C) Heatmap of Z-score transformed expression of genes involved in biosynthesis of PUFAs ($n = 3$). (D) Cells were treated with sorafenib (6 μmol/L) and deuterated sorafenib (6 μmol/L) for 48 h and level of ACSL5 was detected by qRT-PCR ($n = 3$). (E) Cells were treated with control and AOX1-OE followed by qRT-PCR ($n = 3$) and WB analysis of ACSL5. β-Actin was a loading control. (F) Scatter plot of OPLS-DA model and heatmap of Z-score transformed abundance of PUFAs for cells treated with control, AOX1-OE and AOX1-OE + shACSL5 lentivirus as indicated ($n = 3$). (G–I) Cells (Ctrl, AOX1-KO) were treated with deuterated TKIs and ACSL5-OE lentivirus with or without ferrostatin-1 (2 μmol/L) as indicated. Cell viability was determined by CCK8 (G); level of MDA and Fe²⁺ were measured by biochemical tests (H, I) ($n = 3$). Data are shown as mean ± SEM. ns, no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

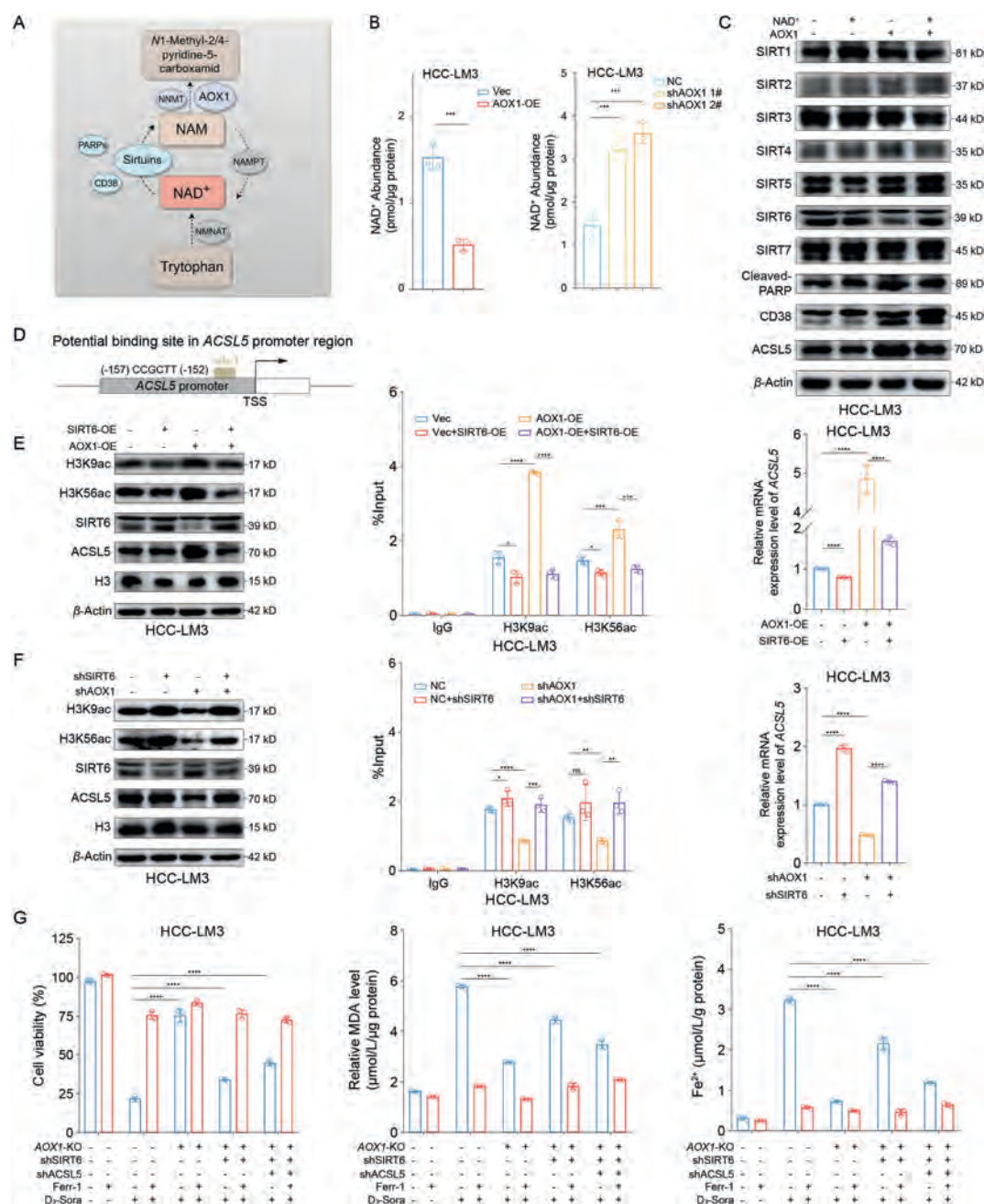


Figure 5 AOX1 blocks SIRT6-mediated deacetylation of H3K9 and H3K56 to promote transcription of ACSL5. (A) Enzymatic reactions involved in the NAD⁺ metabolism including NAD⁺ synthesis (NMNAT), consuming (Sirtuins, PARPs, CD38) and clearance pathway (NNMT, AOX1). (B) Level of NAD⁺ was measured in cells treated with control and AOX1-OE lentivirus or control and shAOX1 lentivirus ($n = 3$). (C) Cells were treated with NAD⁺ (200 μmol/L, 24 h) and AOX1-OE lentivirus and protein level of NAD⁺ consumers were subject to WB analysis. β-Actin was a loading control. (D) Potential binding sites of H3K9ac and H3K56ac in the promoter region of ACSL5. (E) Cells were treated with AOX1-OE and SIRT6-OE lentivirus as indicated. Protein levels were assessed by WB and H3 and β-Actin were loading control (left). The enrichment of H3K9ac and H3K56ac at promoter of ACSL5 was determined by ChIP assay. IgG served as a negative control (median) ($n = 3$). The mRNA level of ACSL5 was determined by qRT-PCR analysis (right) ($n = 3$). (F) Cells were transfected with shAOX1 and shSIRT6. Protein levels were subject to WB analysis for quantitation and H3 and β-actin were loading control (left). The enrichment of H3K9ac and H3K56ac at promoter of ACSL5 was determined by ChIP assay. IgG served as a negative control (median) ($n = 3$). The mRNA level of ACSL5 was determined by qRT-PCR analysis (right) ($n = 3$). (G) Cells (Ctrl, AOX1-KO) were treated with deuterated sorafenib (6 μmol/L), shSIRT6 lentivirus and shACSL5 lentivirus with or without ferrostatin-1 (2 μmol/L) as indicated. Cell viability was determined by CCK8 (left); level of MDA and Fe²⁺ were measured by biochemical tests (median, right) ($n = 3$). Data are shown as mean ± SEM. ns, no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

deacetylation of H3K9 & H3K56. We first predicted potential SIRT6 binding sites in the promoter region of *ACSL5* (Fig. 5D). WB results showed that H3K9 and H3K56 were acetylated upon AOX1 overexpression and deacetylated by SIRT6 (Fig. 5E, left). ChIP-qPCR further revealed that H3K9ac and H3K56ac were significantly enriched at the promoter region of *ACSL5* in AOX1-overexpressing cells, but this enrichment was diminished in the presence of SIRT6 (Fig. 5E, median). In addition, the mRNA level of *ACSL5* was re-suppressed following SIRT6 overexpression (Fig. 5E, right). Conversely, SIRT6 deficiency protected H3K9 and H3K56 from deacetylation caused by AOX1 inhibition, thereby enhancing their enrichment at the *ACSL5* promoter and upregulating *ACSL5* mRNA expression (Fig. 5F). We next investigated the effect of the AOX1–SIRT6–*ACSL5* axis on deuterated TKIs-induced ferroptosis by measuring cell viability, MDA levels, and Fe^{2+} accumulation. As expected, AOX1 deletion partially abolished ferroptosis induced by deuterated TKIs while SIRT6 silencing restored ferroptotic vulnerability, however, *ACSL5* inhibition counteracted this effect by reducing MDA and Fe^{2+} levels and attenuating ferroptotic cells in HCC (Fig. 5G, Fig. S12C). Together, these results indicate that AOX1 impairs SIRT6-mediated deacetylation of H3K9 and H3K56, thereby promoting *ACSL5* transcription and increasing susceptibility to ferroptosis.

3.5. AOX1 acts as a biomarker to distinguish the efficacy of deuterated TKIs in preclinical model with HCC

The present study further evaluated the potential of AOX1 as a biomarker for predicting the efficacy of deuterated TKIs. HCC cells with different AOX1 expression levels were treated with either deuterated TKIs or their prototypes for short-term viability assay and long-term clonogenic assay. In the presence of deuterated sorafenib, the IC_{50} of cells with shAOX1 was 7.476 $\mu\text{mol/L}$ while the IC_{50} of the overexpression of AOX1 (AOX1-OE) cells was 11.32 $\mu\text{mol/L}$, indicating that the expression of AOX1 was associated with sensitivity to deuterated sorafenib (Fig. 6A). In the long-term clonogenic assay, deuterated sorafenib markedly inhibited cell proliferation in shAOX1 cells (Fig. 6B, Supporting Information Fig. S13A). In contrast, the efficacy of sorafenib was not affected by AOX1 expression status (Fig. S13C and S13D). We next examined changes in AOX1 expression following treatment with sorafenib or deuterated sorafenib in cells with varying baseline AOX1 levels. Notably, deuterated sorafenib induced the most pronounced upregulation of AOX1 in cells with initially low AOX1 expression (Fig. 6C, Fig. S13B), which may account for the enhanced efficacy of deuterated sorafenib in low AOX1 expression cells. Similarly, low AOX1 expression also conferred better efficacy of deuterated regorafenib in both short-term and long-term assay (Fig. S13E–S13H). Next, we established subcutaneous HCC models with differential AOX1 expressions to evaluate the relevance of AOX1 to the efficacy of deuterated sorafenib. Treatments were administered as indicated (Fig. 6D). The efficacy of deuterated sorafenib in tumors with AOX1-OE was comparable to that of sorafenib, whereas tumors with low AOX1 expression were more sensitive to deuterated sorafenib (Fig. 6E and F). Neither sorafenib nor deuterated sorafenib caused significant liver or kidney injury in HCC model with varying AOX1 levels (Fig. S13I and S13J). In addition, IHC analysis showed the expression levels of AOX1, *ACSL5* and 4-HNE were significantly upregulated in shAOX1 tumor-bearing mice following treatment with deuterated sorafenib (Fig. 6G; Fig. S13K). These results

suggest that deuterated sorafenib enhances the therapeutic efficacy in HCC with low AOX1 expression by promoting AOX1-*ACSL5*-mediated ferroptosis. Collectively, these preclinical data suggest that AOX1 expression level serves as a biomarker to predict the efficacy of deuterated TKIs. Deuterated TKIs exhibit enhanced efficacy in HCC with low AOX1 expression by promoting ferroptosis.

3.6. HCC with lower AOX1 expression confers better clinical efficacy to deuterated sorafenib

We next investigated the association between AOX1 expression levels and the clinical response to deuterated sorafenib in HCC. Patient-derived xenografts (PDX) tumors from HCC patients were established and treated with either sorafenib or deuterated sorafenib (Fig. 7A). Compared to tumors with high AOX1 expression (AOX1^{high}), those with low AOX1 expression (AOX1^{low}) benefited more from deuterated sorafenib treatment than from sorafenib (Fig. 7B and D). Tumors were further analyzed by IHC to assess ferroptotic responses after treatment. Consistent with the above findings, AOX1^{low} PDX tumors exhibited more pronounced upregulation of AOX1, *ACSL5* and 4-HNE following deuterated sorafenib treatment, while such histological changes were less evident in the sorafenib-treated group (Fig. 7C and E). Deuterated sorafenib has currently been officially approved as a first-line treatment option for advanced HCC¹⁶, thus we collected primary HCC specimens *via* biopsy from 50 patients who received deuterated sorafenib in Huashan Hospital and Tongji Hospital. In Huashan cohort, 5 (16.7%) uHCC patients responded to deuterated sorafenib, of these, the majority (4/5) had low AOX1 expression, whereas most non-responders (7/9) exhibited high AOX1 expression (Fig. 7F). In the Tongji cohort, patients with low AOX1 expression were more sensitive to deuterated sorafenib (3/4), while those with high AOX1 (1/4) were less likely to benefit (Supporting Information Fig. S14A–S14D). According to the Chinese Expert Consensus on Postoperative Adjuvant Therapy for Hepatocellular Carcinoma (2023 edition), deuterated sorafenib is recommended as one of the adjuvant therapies for patients at high risk of recurrent following curative resection. Primary HCC specimens were collected *via* hepatectomy from 50 high-risk patients before receiving deuterated sorafenib as adjuvant therapy at Huashan Hospital and Tongji Hospital. In the Huashan cohort, 8 (53.3%) patients with high AOX1 expression experienced recurrence within two years (median recurrence-free survival, mRFS = 17.0 months), whereas only 4 (26.7%) patients with low AOX1 expression recurred (mRFS, not reached) (Fig. 7G). Similarly, in the Tongji cohort, patients with low AOX1 levels exhibited significantly higher RFS rate ($P = 0.0365$) (Fig. S14E–S14G). In summary, data from the PDX models and two patient cohorts suggest the clinical benefit of deuterated sorafenib is more pronounced in HCC patients with AOX1^{low} than those with high AOX1 expression.

4. Discussion

For more than a decade, targeted therapy has remained the mainstay treatment for advanced HCC. However, intrinsic resistance to TKIs remains a major hurdle to achieve favorable clinical responses in HCC treatment^{30–32}. This study reveals ferroptosis resistance as a pivotal mechanism contributing to the limited

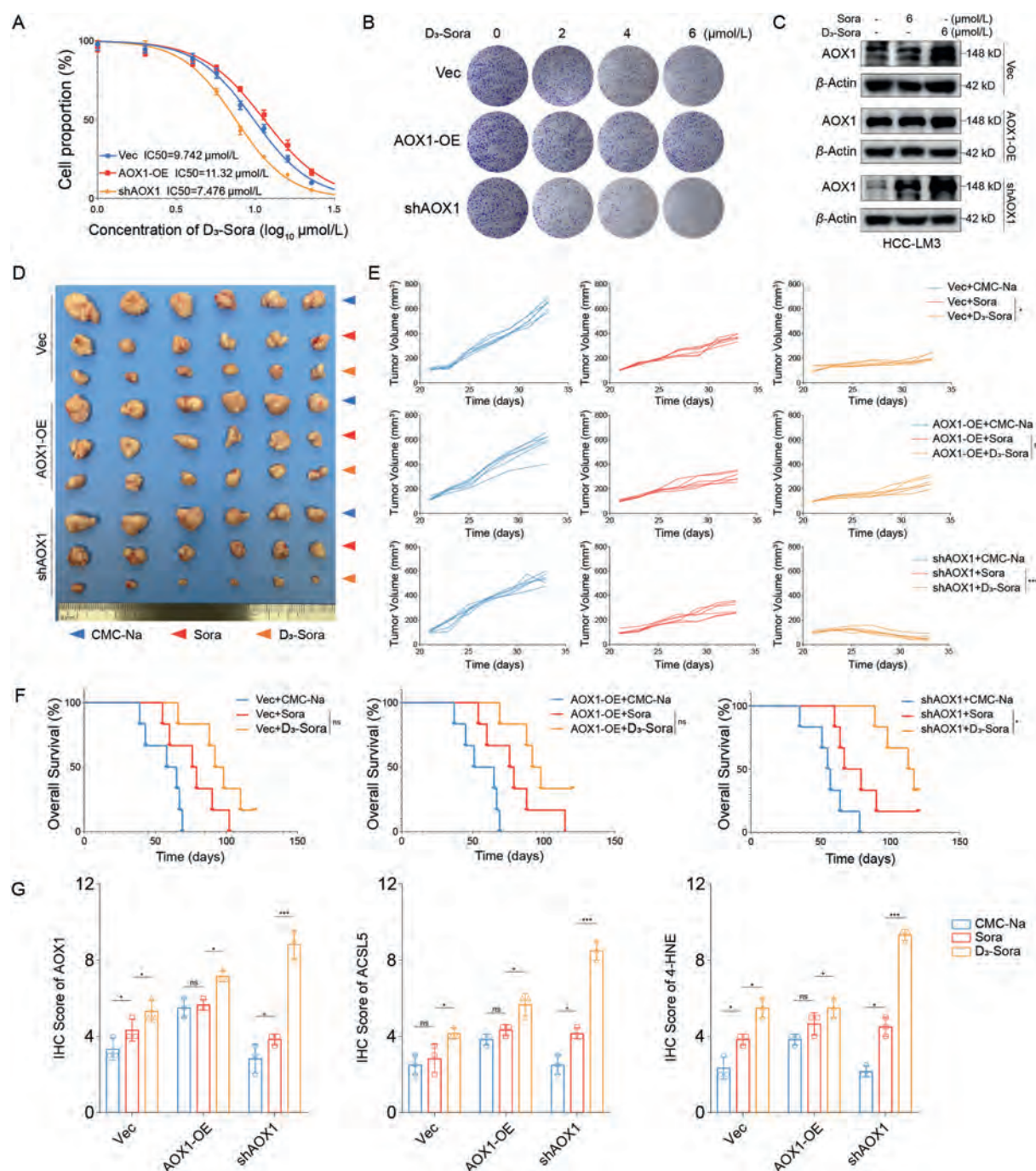


Figure 6 AOX1 acts as a biomarker to distinguish the efficacy of deuterated TKIs in preclinical model with HCC. (A) Cells with different levels of AOX1 were treated with increasing concentration of deuterated sorafenib for 48 h, and the IC₅₀ values were calculated ($n = 3$). (B) Clonogenic image of cells with different levels of AOX1 treated with increasing dose of deuterated sorafenib for 10 days. (C) Cells with different levels of AOX1 were treated as indicated for 48 h and then immunoblotted. (D) Representative image of subcutaneous HCC model of nude mice with the treatment as indicated ($n = 6$). (E) Anti-tumor effect of sorafenib or deuterated sorafenib on tumors with different AOX1 expression in nude mice ($n = 6$). (F) Overall survival time in each group of nude mice ($n = 6$). (G) IHC score of AOX1, ACSL5 and 4-HNE (production of lipid peroxidation) in each group were presented as histogram ($n = 3$). Data are shown as mean $\pm$ SEM. ns, no significance, $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$.

efficacy of TKIs in HCC. Importantly, deuterated TKIs overcome this barrier by reactivating ferroptotic cell death in resistant HCC cells, highlighting a novel therapeutic avenue for enhancing the responsiveness of HCC to targeted therapy. Further investigation identified AOX1 as a key determinant of the differential

susceptibility of HCC cells to ferroptosis. Deuterated TKIs stabilized AOX1 in a structure-dependent manner, resulting in ACSL5-mediated polyunsaturated fatty acid accumulation and subsequent induction of ferroptotic cell death. Clinically, our findings establish AOX1 as a predictive biomarker that delineates

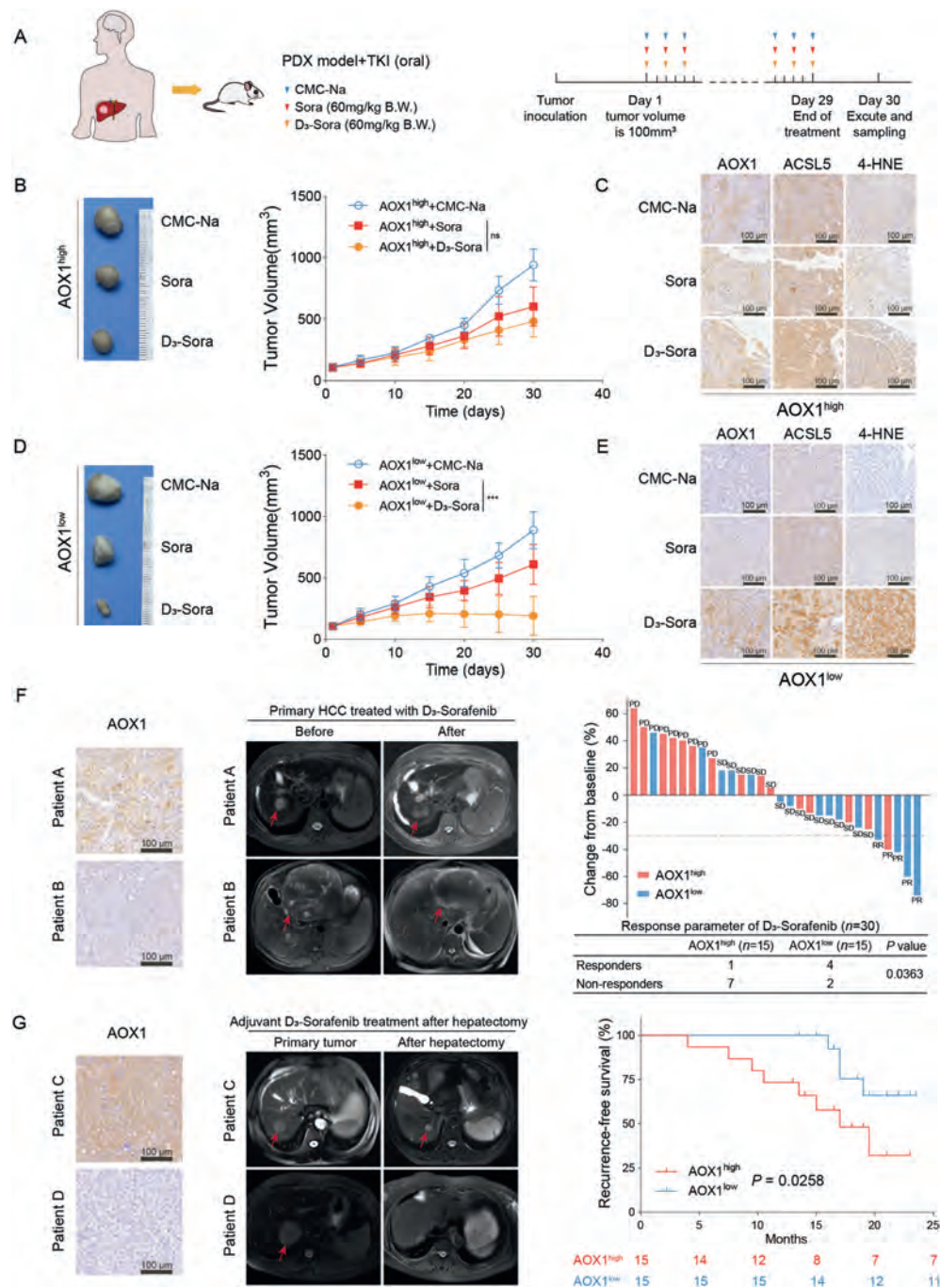


Figure 7 HCC with lower AOX1 expression confers better clinical efficacy to deuterated sorafenib. (A) Scheme for construction of PDX model and therapeutic administration. (B, D) Representative image and growth curve of PDX tumors treated with sorafenib and deuterated sorafenib ($n = 6$). (C, E) Representative IHC staining of AOX1, ACSL5 and 4-HNE in PDX tumors at the end of treatment period (scale bar = 100 μ m). (F) Analysis of AOX1 expression in primary HCC before the treatment of deuterated sorafenib (scale bar = 100 μ m) (left). Images for lesions before and after treatment of deuterated sorafenib (median). Percentage changes of target lesion from baseline and response parameter of deuterated sorafenib treatment in primary HCC ($n = 30$) (right). P value by Fisher's exact test. (G) Analysis of AOX1 expression in primary HCC after hepatectomy (scale bar = 100 μ m) (left). Images for lesions before and after hepatectomy (median). RFS rate of patients receiving deuterated sorafenib adjuvant therapy after hepatectomy ($n = 30$) (right). P value by Log-rank test. Data are shown as mean $\pm$ SEM. ns, no significance, *** $P < 0.001$.

patient responsiveness to deuterated TKIs. The observation that patients with low AOX1 expression derived substantial benefit from deuterated sorafenib underscores the translational potential of AOX1-guided precision therapy in HCC.

Ferroptosis has been recognized as a novel tumor-suppressive mechanism³³. Previous studies have demonstrated that resistance to ferroptosis is a significant contributor to TKIs tolerance³⁴. The intricate interplay among various signaling pathways regulates the

ferroptotic response in multiple cancers, providing potential therapeutic targets to enhance ferroptosis and improve anti-tumor efficacy³⁵. In this study, resistance to ferroptosis contributing to non-responsiveness to TKIs in HCC was supported by both cancer genomics data from GEO database and experiments in several HCC cell lines. In contrast, deuterated TKIs overcame ferroptosis resistance and demonstrated superior efficacy in HCC. Tumor genetic heterogeneity is well known to influence TKIs resistance^{36,37}. Our findings further revealed that AOX1 status confers differential susceptibility to ferroptosis. Deuterated sorafenib and regorafenib differ only by the presence of a pyridyl tri-deuterated methanamide moiety. To explain the observed difference in susceptibility to ferroptosis, we provided a structural rationale and molecular basis. Molecular docking simulations suggested that deuterated TKIs exhibit a higher binding affinity to AOX1 than their prototype counterparts. The unique “D₃-pyridine ring” of deuterated TKIs upregulated AOX1 expression in a dose- and time-dependent manner. Importantly, deuterated TKIs depended on AOX1 to increase susceptibility to ferroptosis in HCC. Pyridine, as a scaffold, is widely found in anti-tumor agents¹³. Pyridine-based derivatives have demonstrated substantial anti-cancer potential by effectively suppressing tumor oncogenes^{15,38}. Notably, AOX1-mediated oxidation of pyridine has attracted increased attention among pharmacologists²³. Pyridine-based modifications provide opportunities for drug design and facilitate pharmacokinetic optimization^{13,39}. In this study, deuterated modifications of sorafenib and regorafenib conferred enhanced sensitivity to ferroptosis through a mechanism distinct from that of the prototype drugs. These findings may provide new insights into the development of deuterate anti-tumor agents.

Ferroptosis is triggered by unrestrained lipid peroxidation⁴⁰. Membrane lipid composition is dynamically regulated⁴⁰. Saturated fatty acids (SFA) render cell membrane rigidity, while polyunsaturated fatty acids (PUFA) facilitate cells intrinsic susceptibility to ferroptosis⁴⁰. In this study, metabolomic and RNA-Seq analyses revealed that excessive PUFAs accumulation was the predominant alteration following deuterated sorafenib treatment compared with sorafenib. Further investigation demonstrated that deuterated sorafenib induced AOX1 expression, which in turn enhanced ACSL5-mediated PUFA accumulation, thereby rendering HCC cells more vulnerable to ferroptosis. ACSL5 is a key enzyme in fatty acid metabolism and plays a role in the regulation of intracellular lipid accumulation⁴¹. ACSL5 has been identified as a tumor suppressor that promotes both apoptosis and ferroptosis⁴²⁻⁴⁴. We proved that AOX1-mediated reduction of NAD⁺ suppressed SIRT6 expression. Inhibition of SIRT6 led to increased levels of H3K9ac and H3K56ac at the promoter region of ACSL5, thereby promoting its transcriptional upregulation.

Previous studies have reported that AOX1 expression is associated with favorable prognosis in bladder cancer, prostate cancer, and clear cell renal cell carcinoma⁴⁵⁻⁴⁷. Consistently, higher AOX1 expression has also been associated with improved survival in hepatocellular carcinoma⁴⁸. Given the differential regulation of AOX1 by deuterated TKIs, we explored its implications in guiding the selection of deuterated TKIs. Preclinical and PDX models consistently demonstrated enhanced tumor suppression by deuterated sorafenib in HCC with low AOX1 expression, while the efficacy of sorafenib remained unaffected by AOX1 levels. This differential dependency provides a mechanistic basis for the superior activity of deuterated sorafenib. Clinical cohort analyses further confirmed that low AOX1 expression correlated with higher response rates and longer recurrence-free survival

following deuterated sorafenib treatment. Collectively, these findings establish AOX1 as a promising biomarker for optimizing patient selection and clinical decision-making in deuterated TKI therapy, also warranting further validation in larger, diverse populations.

5. Conclusions

In conclusion, this study identifies a novel mechanism by which deuterated TKIs reverse resistance in hepatocellular carcinoma through AOX1-dependent induction of ferroptosis. The findings highlight AOX1 as a clinically potential biomarker for guiding patient selection and optimizing the therapeutic use of deuterated TKIs.

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Author contributions

Yue Ma: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Visualization, Writing-Original Draft. Chenhe Yi: Conceptualization, Methodology, Investigation, Resources, Writing-Review & Editing. Ning Cai: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Funding acquisition. Baorui Tao: Methodology, Software, Visualization. Yan Geng: Formal analysis, Resources. Weiqing Shao: Resources. Rongquan Sun: Formal analysis. Zhenmei Chen: Resources. Yitong Li: Resources. Bo Zhang: Formal analysis, Resources. Xiangyu Wang: Formal analysis, Resources. Jing Lin: Formal analysis, Resources. Wenwei Zhu: Formal analysis, Resources. Lu Lu: Formal analysis, Resources. Wanguang Zhang: Conceptualization, Resources, Supervision, Funding acquisition. Jinhong Chen: Conceptualization, Writing-Review & Editing, Supervision, Project Administration, Funding acquisition.

Conflicts of interest

The authors declare no potential conflicts of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2025.12.007>.

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