

# A multicomponent reaction-enabled discovery platform reveals a TXNRD1-targeted ferroptosis inducer

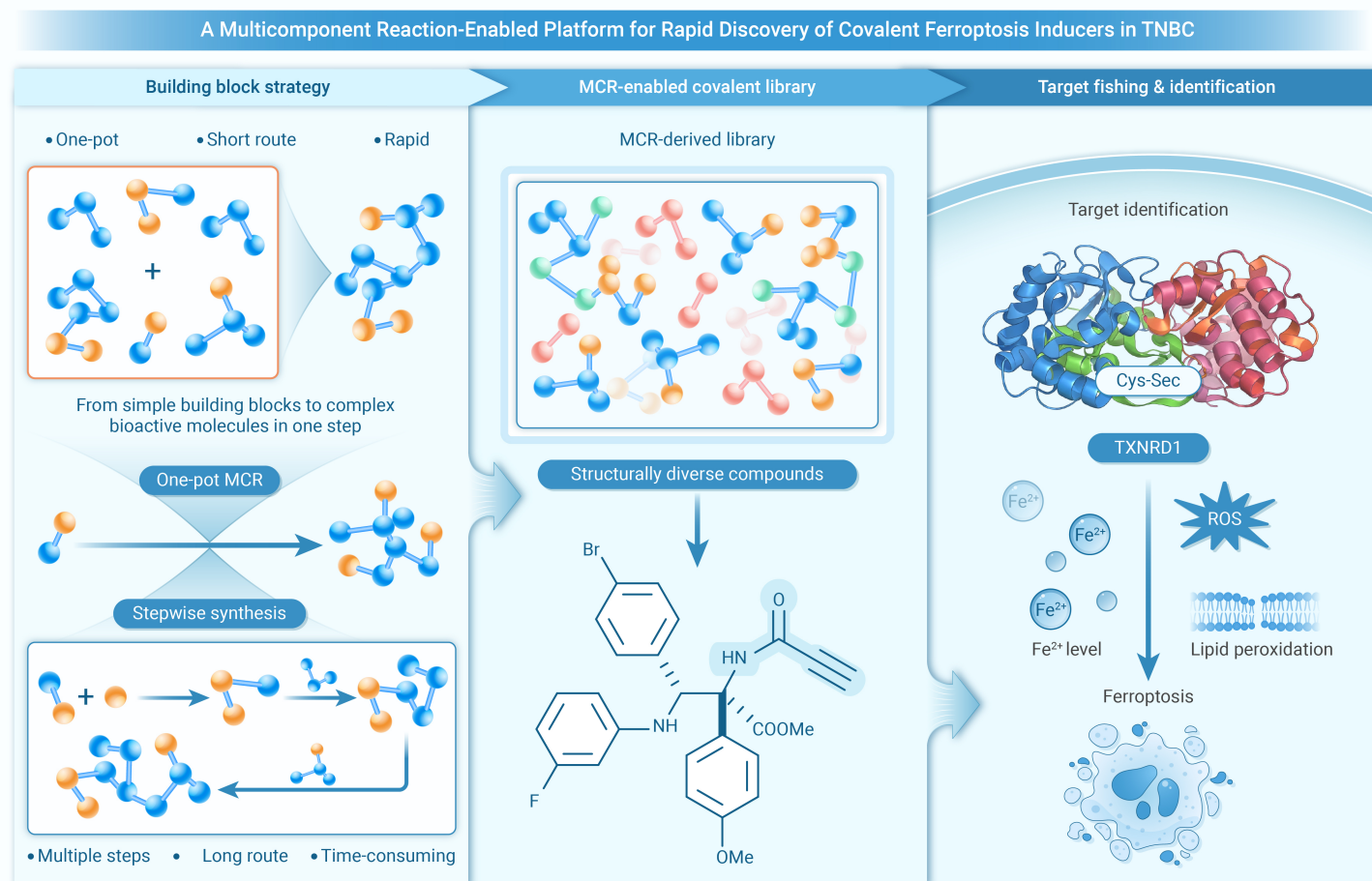
Xiang Fu,<sup>1,3,4</sup> Qiong Wu,<sup>2,4</sup> Siyu Wu,<sup>1,4</sup> Gejun Niu,<sup>1,4</sup> Zhonghui Zhang,<sup>1</sup> Yue Lin,<sup>2</sup> Yu Cao,<sup>2</sup> Yaxin Li,<sup>1</sup> Feng Xiong,<sup>1</sup> Yu Qian,<sup>1</sup> Taoda Shi,<sup>1,\*</sup> Albert Sun Chi Chan,<sup>1</sup> Jinsai Shang,<sup>2,\*</sup> and Wenhao Hu<sup>1,\*</sup>

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## GRAPHICAL ABSTRACT



## PUBLIC SUMMARY

- A multicomponent reaction-based platform enabled efficient discovery of covalent inhibitors.
- HF-4aa was identified as a covalent probe targeting antioxidant enzyme TXNRD1 with nanomolar potency.
- HF-4aa inhibits TXNRD1, triggers redox imbalance and ferroptosis, with preclinical antitumor activity.

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Covalent inhibitors offer durable target engagement, but their discovery is often serendipitous and constrained by limited structural diversity. We introduce a multicomponent reaction (MCR)-based platform that combines modular synthesis, phenotypic screening, and proteome-level target deconvolution to identify HF-4aa, a covalent inhibitor of thioredoxin reductase 1 (TXNRD1). Stereodivergent synthesis enabled access to all four HF-4aa stereoisomers using Rh(II)/chiral phosphoric acid cocatalysis. HF-4aa displays nanomolar potency in cancer cells and modifies TXNRD1 via selenocysteine/cysteine targeting, supported by chemoproteomics and intact-protein mass spectrometry. TXNRD1 inhibition is functionally linked to redox collapse characterized by ROS accumulation, Fe<sup>2+</sup> elevation, and lipid peroxidation, thereby inducing ferroptotic cell death. *In vivo*, HF-4aa suppresses tumor growth, and its efficacy is reversed by Ferrostatin-1, confirming ferroptosis dependence. Liposomal encapsulation overcomes the short metabolic half-life of free HF-4aa, and the resulting formulation demonstrates potent antitumor activity including in paclitaxel-resistant tumor models. HF-4aa serves as a proof-of-concept covalent chemical probe for TXNRD1-directed ferroptosis induction, and this study establishes a scalable MCR-based framework for systematic covalent inhibitor discovery.

## INTRODUCTION

Covalent inhibitors have redefined therapeutic strategies by enabling precise and sustained interaction with recalcitrant targets via irreversible or reversible covalent bond formation.<sup>1–4</sup> Notable examples (Scheme 1A) include Osimertinib (AstraZeneca) an epidermal growth factor receptor (EGFR) inhibitor,<sup>5</sup> and Ibrutinib, a Bruton's tyrosine kinase (BTK) inhibitor,<sup>6</sup> which have generated approximately \$22 billion and \$15 billion in sales over the past three years, respectively. Additionally, Sotorasib (Amgen), an inhibitor of the KRAS<sup>G12C</sup> mutant, marked a breakthrough by securing FDA approval for non-small cell lung cancer (NSCLC) and colorectal cancer (CRC), effectively overturning the historical classification of KRAS as an undruggable target.<sup>7–9</sup> Beyond therapeutic applications, these inhibitors serve as indispensable chemical biology tools to map functional protein domains, develop activity-based probes, and dissect dynamic molecular mechanisms.<sup>10,11</sup> However, the discovery of novel covalent inhibitors has historically relied on serendipitous identification from natural products,<sup>12–15</sup> which are limited in structural diversity and scalability. This bottleneck highlights the urgent need for innovative synthetic methodologies to systematically generate covalent compound libraries with tunable reactivity and spatial precision.

Multicomponent reactions (MCRs) represent a powerful synthetic paradigm for drug discovery, enabling the efficient construction of structurally diverse compound libraries from simple building blocks in a single operation.<sup>16–20</sup> By integrating three or more reactants into complex three-dimensional architectures, MCRs achieve superior atom economy and synthetic efficiency compared to traditional stepwise approaches (Scheme 1B). Our group has pioneered novel MCR methodologies through trapping of transient intermediates, including ylides and zwitterions generated from diazo compounds and nucleophiles, with electrophiles as shown in Scheme 1.C<sup>21,22</sup>

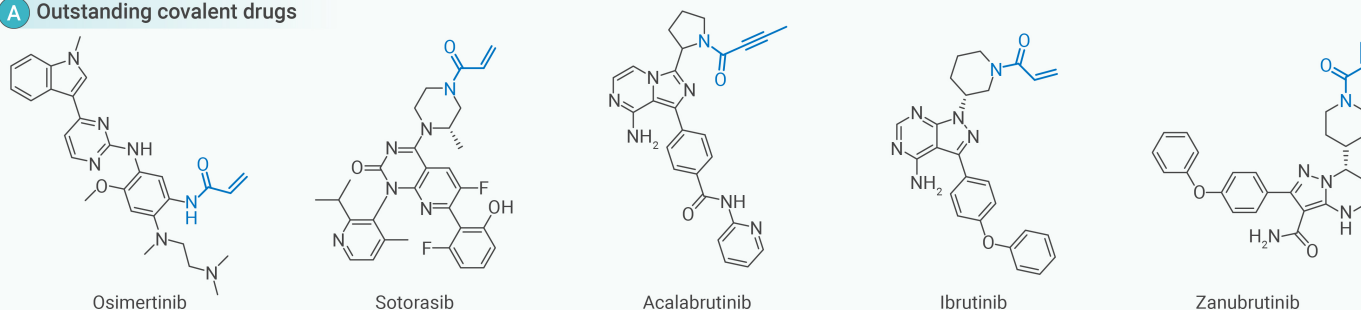
This strategy leverages abundant chemical building blocks (diazo compounds, nucleophiles, electrophiles) to rapidly generate structurally enriched libraries (Scheme 1D), providing a robust platform for phenotypic screening. These MCR-driven libraries have yielded validated hits across therapeutic areas including antitumor agents,<sup>23</sup> PTP1B inhibitors,<sup>24</sup> and Nav channel blockers<sup>25</sup> (Scheme 1E). Critically, the inherent modularity of MCRs enables precise control over chemical selectivity and spatial configurations, permitting strategic placement of covalent warheads within pharmacophores. This spatial precision ensures optimal alignment of electrophilic moieties with target nucleophilic residues like cysteine or selenocysteine, thereby enhancing binding specificity for challenging biological targets and positioning MCRs as indispensable tools for accelerating covalent inhibitor discovery. Recent studies have established MCRs as a powerful strategy for exploring covalent inhibitor space through modular assembly of electrophilic scaffolds.<sup>26</sup> Building upon this conceptual foundation, we sought to harness the stereochemical programmability of MCR chemistry to achieve precise spatial positioning of covalent warheads and to integrate it within a phenotypic-to-mechanistic discovery pipeline.

Ferroptosis, a unique iron-dependent form of regulated cell death mechanistically distinct from apoptosis and necrosis, is driven by the lethal accrual of lipid peroxides concurrent with dysregulated ferrous iron (Fe<sup>2+</sup>) metabolism.<sup>27</sup> Hallmark morphological alterations include mitochondrial shrinkage and cristae fragmentation, while molecular dysregulation involves glutathione (GSH) depletion,<sup>28</sup> inactivation of glutathione peroxidase 4 (GPX4),<sup>29</sup> and impaired detoxification of lipid peroxidation products, which is reported to be buffered by the transcription factor Nrf2<sup>30,31</sup> or by malic enzyme 1.<sup>32</sup> Iron-mediated Fenton reactions catalyze the oxidation of polyunsaturated fatty acids, generating reactive oxygen species (ROS) that propagate membrane damage and culminate in cellular collapse. Pathophysiologically implicated in oncogenesis, neurodegeneration, and ischemia-reperfusion injury, ferroptosis represents a promising therapeutic avenue for recalcitrant malignancies such as triple-negative breast cancer (TNBC).<sup>33</sup> While GPX4 remains a central ferroptosis regulator,<sup>34–36</sup> recent studies reveal thioredoxin reductase 1 (TXNRD1), which is a selenocysteine-dependent oxidoreductase, as a critical redox node whose inhibition amplifies ferroptosis vulnerability.<sup>37</sup> Existing TXNRD1 inhibitors have laid a foundation for redox-targeted therapy,<sup>38–40</sup> yet many interact broadly with antioxidant pathways or suffer limited *in vivo* efficacy. Selective covalent targeting of the selenocysteine active site therefore offers an opportunity to achieve clearer mechanistic outcomes together with more precise ferroptotic modulation.

In this study, we report the discovery and mechanistic elucidation of HF-4aa, a potent covalent inhibitor of TXNRD1 derived from MCR chemistry, which induces ferroptosis in TNBC. Initial phenotypic screening identified this  $\alpha,\beta$ -diamino acid derivative as exhibiting exceptional anti-proliferative activity against TNBC cells. Through integrated proteomic approaches, we definitively mapped TXNRD1, a selenocysteine-dependent master regulator of cellular redox homeostasis, as the molecular target of HF-4aa. Mechanistic validation confirmed covalent inhibition of TXNRD1 by HF-4aa, resulting in profound disruption of redox equilibrium. This manifested as elevated ROS,

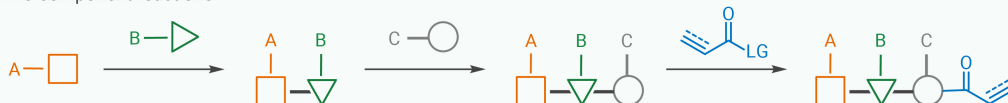


## A Outstanding covalent drugs



## B Strategies for covalent drug synthesis

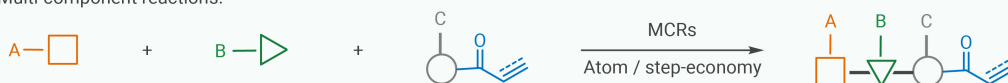
Two-component reactions:



• Multi-step synthesis

• Single-step bond formation

Multi-component reactions:

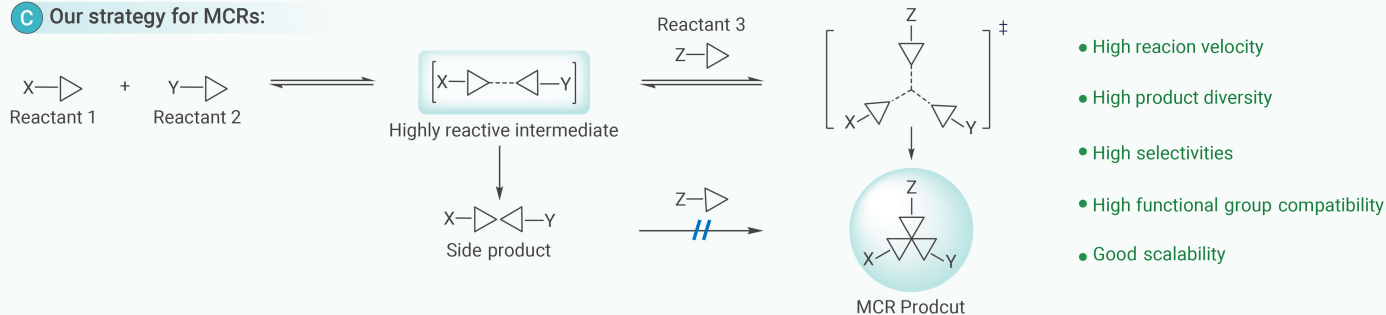


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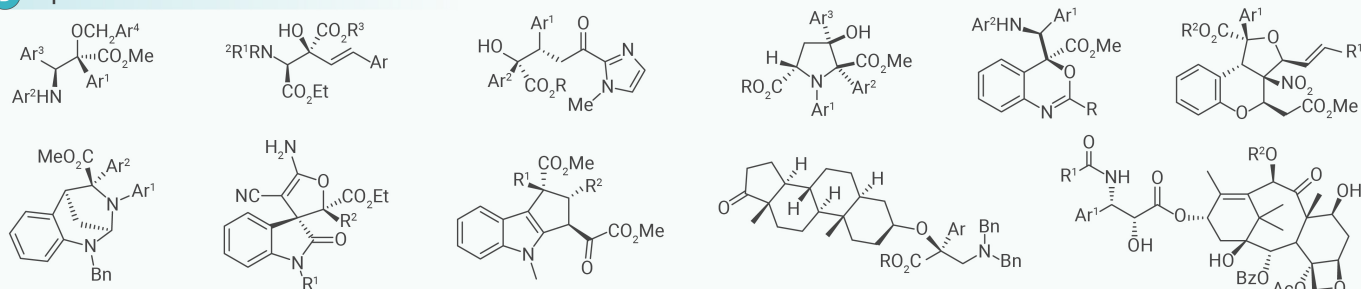
• One step synthesis

• Single-step multiple bonds formation

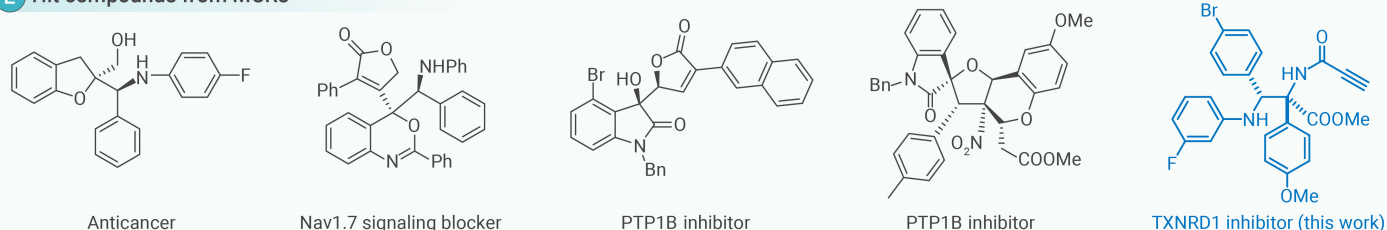
## C Our strategy for MCRs:



## D Representative scaffolds from our MCRs



## E Hit compounds from MCRs



Scheme 1. MCR-driven Compound Synthesis and Hit Compound Discovery

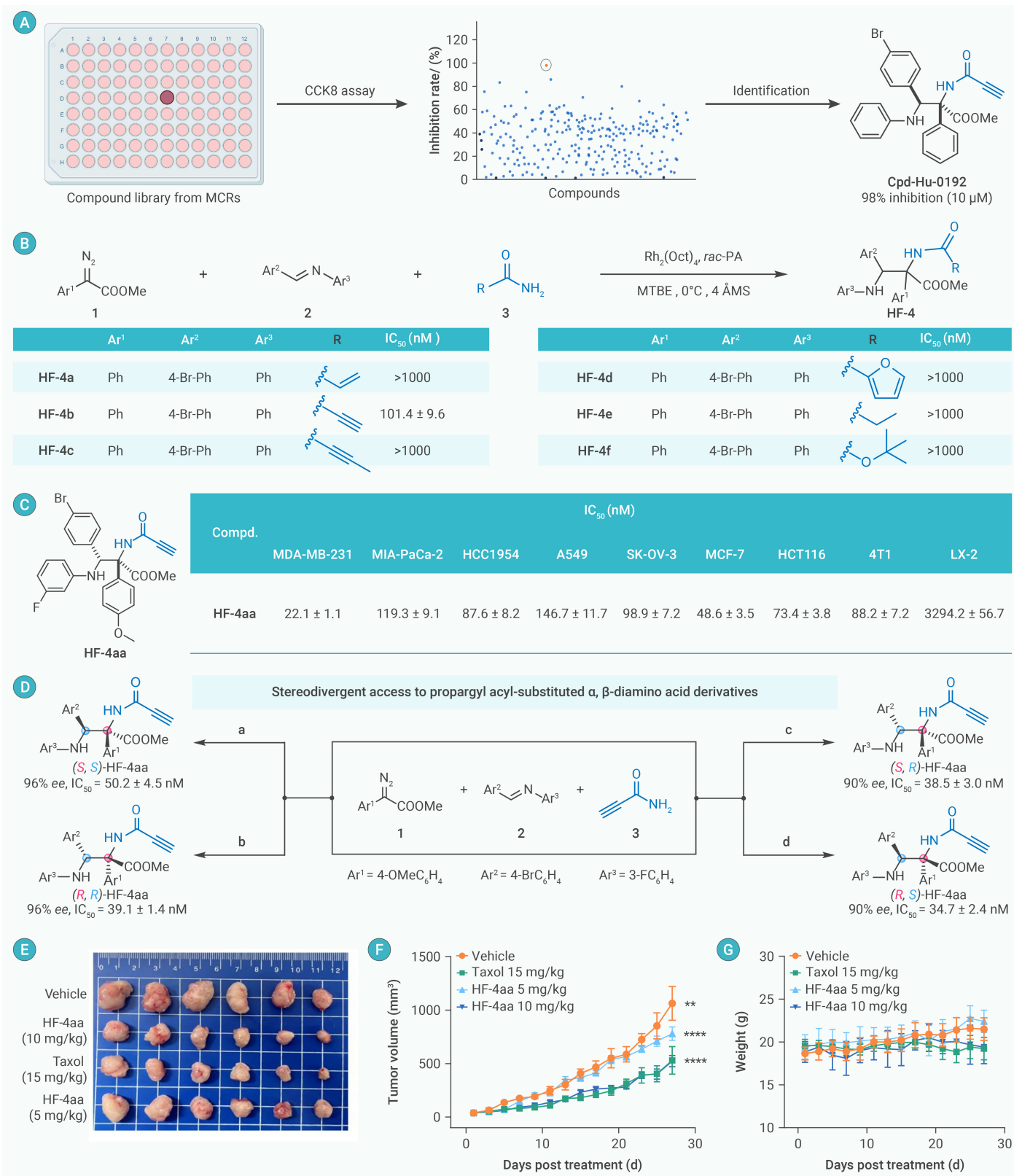
accumulation of lipid peroxidation products, and dysregulated labile  $\text{Fe}^{2+}$  levels, collectively triggering ferroptotic cell death. Crucially, HF-4aa demonstrated significant tumor growth suppression *in vivo*. These findings establish HF-4aa as a covalent chemical probe targeting TXNRD1 that induces ferroptosis in TNBC, demonstrating the utility of this MCR-based discovery

platform.

## RESULTS

## Discovery of HF-4aa by anticancer phenotypic screening

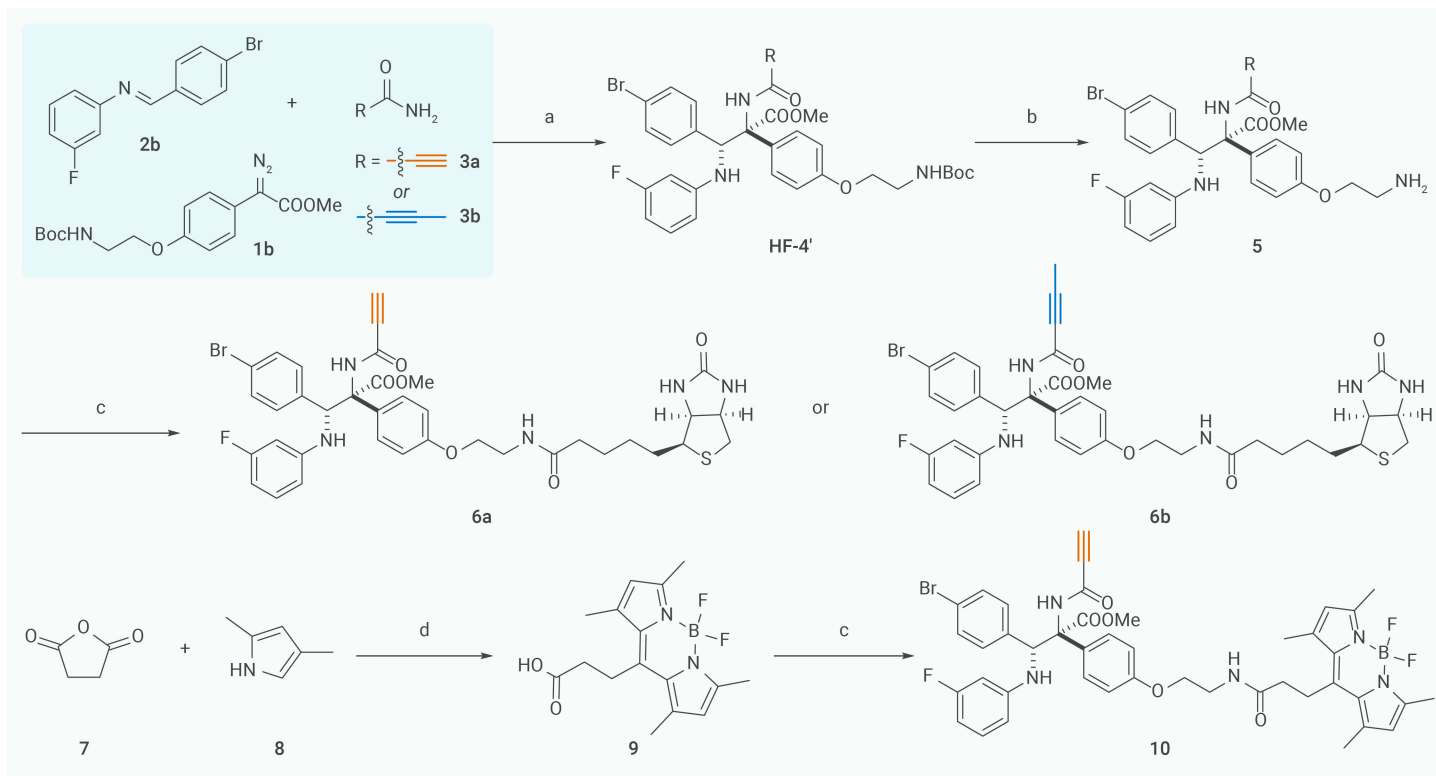
To identify small-molecule modulators with anticancer potential, we



**Figure 1. Discovery of the anti-TNBC hit HF-4aa and possible mechanisms** (A) Screening scheme for identifying anti-TNBC hits. Test compounds (10 μM) were added to the cultured MDA-MB-231 cells. After 48 h treatment, CCK-8 assay was used to detect cell viability. (B) Synthesis of α,β-diamino acid derivatives by multicomponent reaction and analysis of the structure-activity relationship for the covalent warhead part. (C) Chemical structure of HF-4aa and its in vitro anti-proliferative activity against various cell lines. (D) Synthesis of all stereoisomers of HF-4aa. Reaction conditions: a) Rh<sub>2</sub>(Oct)<sub>4</sub>, SPINOL-derived CPA-a, tBuOMe, 4 ÅMS, rt, 2 h. b) Rh<sub>2</sub>(Oct)<sub>4</sub>, SPINOL-derived CPA-b, tBuOMe, 4 ÅMS, rt, 2 h. c) Rh<sub>2</sub>(OAc)<sub>4</sub>, BINOL-derived CPA-c, DCM, 4 ÅMS, rt, 2 h. d) Rh<sub>2</sub>(OAc)<sub>4</sub>, BINOL-derived CPA-d, DCM, 4 ÅMS, rt, 2 h. (E-G) Data are represented as mean ± SEM. Female Balb/c mice bearing the 4T1 murine breast cancer (n = 6 mice per group) received the vehicle, Paclitaxel (*i.p.*, 10 mg/kg, once daily), HF-4aa (*i.p.*, 5, 10 mg/kg, once daily), as indicated. Tumor images (E). Tumor volume (F). Mouse body weight (G).

screened an in-house library of ~1400 MCR-derived compounds using a CCK-8-based phenotypic assay. Through this unbiased screening approach, the

α,β-diamino acid derivative Cpd-Hu-0192 emerged as a promising hit, showing 98% growth inhibition against MDA-MB-231 TNBC cells at 10 μM (Figure



**Scheme 2. Synthesis of Probes 6a and 6b** <sup>a</sup>Reagents and reaction conditions. (a)  $\text{Rh}_2(\text{OAc})_4$ , *rac*-PA, DCM, 4 ÅMS, rt, 2 h. (b) TFA, HFIP, 0 °C, 1 h. (c)  $\text{EDCl}$ , DCM, rt, 4 h. (d) Step 1.  $\text{BF}_3 \cdot \text{Et}_2\text{O}$ , toluene, 80 °C, reflux, 4 h; Step 2.  $\text{BF}_3 \cdot \text{Et}_2\text{O}$ ,  $\text{Et}_3\text{N}$ , rt, 18 h.

1A). To explore structure-activity relationships (SAR) and improve potency, we modified three key structural elements of the  $\alpha,\beta$ -diamino acid scaffold—diazo precursors, imines, and the Michael acceptor warhead—and synthesized >40 derivatives through one-pot, three-component reactions. SAR analysis revealed that altering the propiolamide warhead to acrylamide or methyl-propiolamide markedly reduced activity (Figure 1B), whereas introducing aromatic substituents on either the diazo or imine component enhanced potency (Figure S3). Among these derivatives, HF-4aa displayed exceptional activity, with an  $\text{IC}_{50}$  of 22.1 nM in MDA-MB-231 cells. To evaluate its broader antiproliferative profile, HF-4aa was further tested across a panel of cancer cell lines (MIA-PaCa-2, HCC1954, A549, SK-OV-3, MCF-7, HCT116, and 4T1), exhibiting  $\text{IC}_{50}$  values ranging from 48.6 to 146.7 nM (Figure 1C). Notably, its cytotoxicity toward a normal hepatic stellate cell line (LX-2) was significantly lower ( $\text{IC}_{50}$  = 3.3  $\mu\text{M}$ ), indicating preferential activity in cancer cells (Figure S4).

Given that HF-4aa contains two stereocenters and thus exists as four stereoisomers, we next sought to investigate whether configurational differences influence its anti-TNBC activity. To access all isomers in enantiopure form, we developed an asymmetric three-component reaction enabled by synergistic catalysis. Screening of various chiral phosphoric acid (CPA) catalysts revealed that SPINOL-derived CPAs preferentially afforded the *syn*-diastereomer with high enantioselectivity, whereas BINOL-based CPAs favored formation of the *anti*-diastereomer (Table S1). Ultimately, by modulating catalyst structure and reaction conditions, we achieved stereodivergent synthesis of all four HF-4aa stereoisomers (Figure 1D and S1). Surprisingly, evaluation of their anti-proliferative activities against MDA-MB-231 cells revealed no significant differences in potency among the isomers, suggesting that cytotoxicity is largely stereochemistry-independent under these conditions.

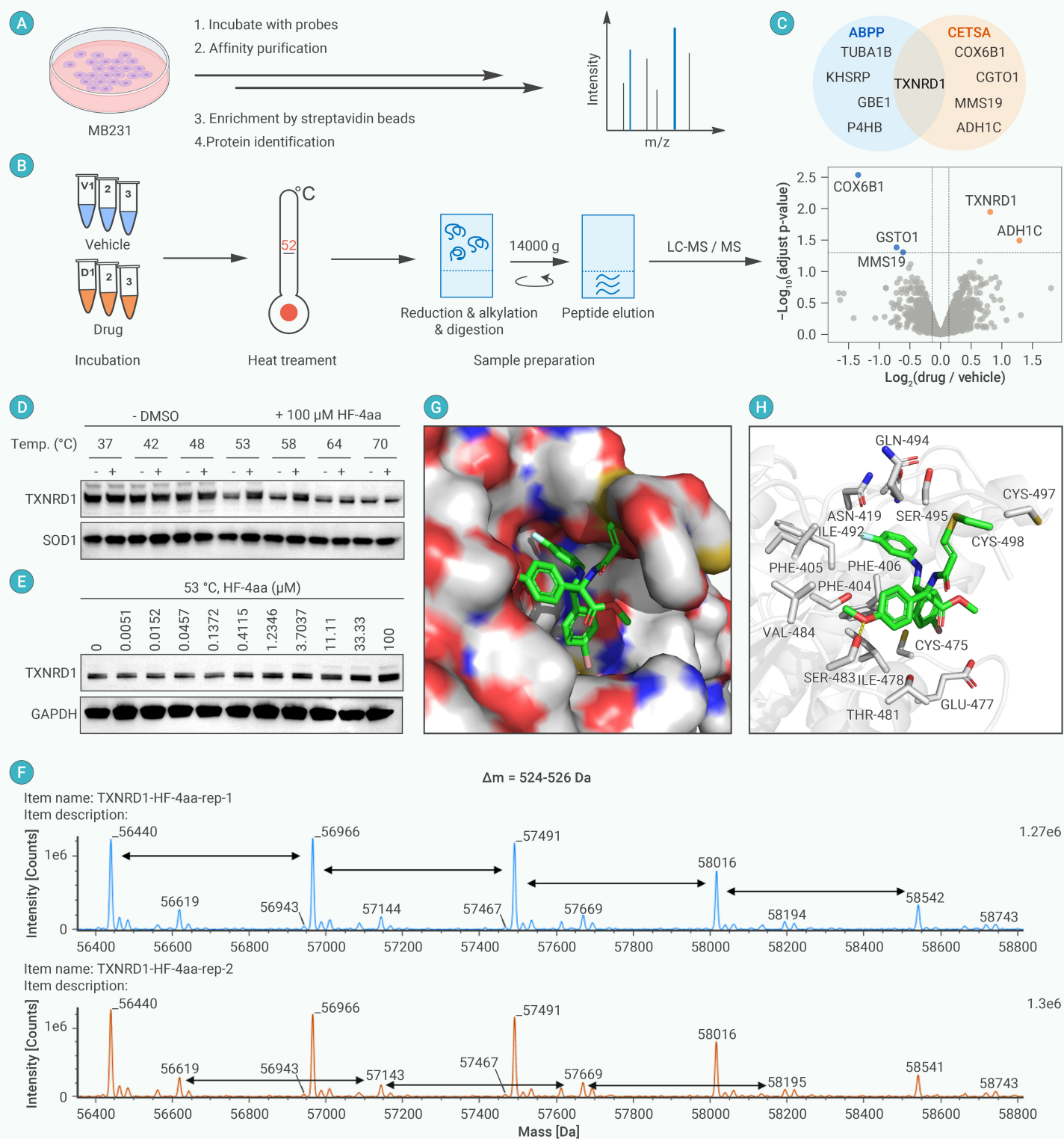
We also conducted a preliminary assessment of the *in vivo* antitumor efficacy and biological safety of HF-4aa using a 4T1 murine breast cancer model in BALB/c mice. After 4 weeks of treatment, HF-4aa significantly reduced tumor weights with dose-dependent inhibition, and the tumor growth inhibition (TGI) values were 27.7% and 66.3% at 5 and 10 mg/kg, respectively. Notably, 10 mg/kg treatment of HF-4aa exhibited comparable tumor growth

inhibition to paclitaxel administered at 15 mg/kg (Figure 1E-G). Furthermore, histological examination by H&E staining of major organs (including the heart, liver, spleen, lung, and kidney) revealed no significant morphological abnormalities, indicating a preliminary favorable safety profile for HF-4aa at the tested doses (Figure S5).

#### Identification of TXNRD1 as the primary biological target of HF-4aa

HF-4aa contains electrophilic warheads designed to react with nucleophilic residues such as cysteine or selenocysteine, which are commonly found in proteins that regulate redox homeostasis. To identify potential molecular targets, we selected MDA-MB-231 TNBC cells, which are highly sensitive to HF-4aa and strongly reliant on redox balance for survival.<sup>41,42</sup> Two biotinylated probes (6a and 6b) bearing distinct covalent warheads were synthesized *via* MCRs for chemoproteomic profiling (Scheme 2). Probe 6a retained inhibitory activity similar to HF-4aa (78% inhibition at 1  $\mu\text{M}$ ), confirming that the modifications did not compromise bioactivity. Cells were incubated with the probes with or without HF-4aa competition, followed by streptavidin pull-down, trypsin digestion, and LC-MS/MS analysis (Figure 2A). After filtering for nonspecific interactions, several candidate targets were identified, including TXNRD1 (Supplemental Data S1). Target engagement was further validated by cellular thermal shift assay coupled with MS (CETSA-MS) (Figure 2B), which showed pronounced stabilization of TXNRD1 and alcohol dehydrogenase 1C (ADH1C) upon HF-4aa treatment. Both proteomic approaches consistently implicated TXNRD1 as a primary molecular target, in line with the probe design aimed at redox-active cysteine/selenocysteine residues (Figure 2C and Supplemental Data S2).

We subsequently conducted two distinct WB experiments based on CETSA (CETSA-WB) to validate TXNRD1 as a putative target of HF-4aa, including (i) the melt curve CETSA approach, in which protein thermal stability is monitored at different distinct temperatures in the presence or absence of drug; (ii) the isothermal dose-response (ITDR), in which protein thermal stability is monitored at multiple drug concentrations at a single temperature. As the temperatures increased, TXNRD1 gradually underwent denaturation and precipitation. TXNRD1 was more stabilized in the presence of HF-4aa than that in the absence of HF-4aa, especially at 53 °C and 58 °C (Figure 2D).



**Figure 2. HF-4aa covalently targets TXNRD1 via propiolamide warhead engagement** (A) Competitive chemoproteomic workflow for target identification of HF-4aa in MDA-MB-231 cells using biotinylated probes: Cells were incubated with probe 6a (1  $\mu$ M)  $\pm$  competitor HF-4aa (1  $\mu$ M) for 6 h. Negative controls included D-biotin (1  $\mu$ M) and probe 6b (structural analog). Three competitive sets (6a vs D-biotin, 6a vs 6b, and 6a vs HF-4aa + 6a) enabled streptavidin-affinity purification and mass spectrometry identification of candidate targets including TXNRD1. (B) CETSA-MS workflow for target engagement analysis of HF-4aa in MDA-MB-231 cell lysates: Lysates were treated with HF-4aa (20  $\mu$ M) or DMSO control, heated at 52°C (3 min), and soluble proteins underwent tryptic digestion followed by TMT10-plex labeling and quantitative LC-MS/MS. (C) Identification of TXNRD1 as a target of HF-4aa. D.E CETSA-WB validation. Thermal melt curve analysis showed HF-4aa stabilized TXNRD1 against denaturation, particularly at 53°C and 58°C (D), while ITDR-CETSA demonstrated dose-dependent stabilization (E), confirming TXNRD1 as a target. (F) Covalent binding assay of TXNRD1 and intact protein mass analysis. (G-H) Preferential binding mode between HF-4aa and TXNRD1 (PDB: 1H6V) according to docking simulation using Autodock4 software.

Besides, we observed a dose-dependent stabilization of TXNRD1 by HF-4aa at elevated concentrations (Figure 2E). These results demonstrated TXNRD1 as a definite target for HF-4aa.

HF-4aa contains an  $\alpha,\beta$ -unsaturated carbonyl functionality, which may

attack thiol or selenolthiol in proteins through 1,4-Michael addition in a covalent manner. To determine the covalent modification of TXNRD1 by HF-4aa, we expressed and purified truncated TXNRD1 (M151-G649), then incubated TXNRD1 with HF-4aa and subjected to LC-MS for intact protein mass spec-



trometry analysis. TXNRD1 is generally regarded as homodimeric/heterodimeric enzyme containing two forms, selenolthiol-free (-GC) with a detectable molecular weight (MW) of 56440 Da and selenolthiol-containing (-GCUG) with a detectable MW of 56619 Da in the intact LC-MS analysis. In addition, the truncated TXNRD1 has two active sites, C-terminal and N-terminal, which contains four redox-capable selenol/thiol groups. LC-MS result indicated that 3-4 adduct peaks of HF-4aa occurred for both forms (Figure 2F), with an additional molecular weight of 524–526 Da, which is exactly the molecular weight of HF-4aa (525 Da). The above results demonstrated HF-4aa covalently bound with TXNRD1.

To further predict the binding sites and modes of HF-4aa with TXNRD1, we performed covalent docking using the TXNRD1 crystal structure (PDB ID: 1H6V). Prioritizing the four specific thiols in the FAD-binding domain (C59, C64, C497, C498), we calculated binding for four configurations of HF-4aa against these sites. After preprocessing the ligand to define rotatable bonds, covalent docking calculations (Autodock suite) generated 160 conformations across the four sites and four configurations (see Table S4–7). We found that the lowest-energy conformations showed a clear preference for C498 or C497 across all isomers, forming a stable covalent bond within the TXNRD1 pocket. HF-4aa also formed a hydrogen bond with Ser483 and engaged in van der Waals interactions with surrounding residues, including Ser404, Phe405, Phe406, Asn419, Cys475, Glu477, Ile478, Thr481, Val484, Ile492, Gln494, Ser495, and Cys497 (Figure 2G–H). These findings indicate that HF-4aa may irreversibly inhibit the redox function of TXNRD1 by preferentially covalently binding to C498 or C497.

An *in vitro* assay employing the 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) method was conducted to evaluate the direct inhibitory effect of HF-4aa on TXNRD1 activity at enzymatic levels (Figure 3A–B). Results demonstrated that HF-4aa exhibited potent inhibition with an  $IC_{50}$  of 15.1 nM, slightly outperforming the positive control Tri-1 ( $IC_{50}$  = 32.7 nM). Crucially, structural integrity of the propiolamide warhead was essential for maintaining inhibitory potency, as modifications to this covalent motif significantly diminished activity. Notably, the structurally truncated derivative HF-11, despite retaining the propiolamide warhead, showed reduced activity in enzymatic assays ( $IC_{50}$  = 92.9 nM), underscoring that non-warhead regions of the molecule are critical for optimal target engagement. Furthermore, the diastereomer HF-4aa-*syn* ( $IC_{50}$  = 47.7 nM) and its corresponding enantiomers ( $IC_{50}$  = 41.3 nM and 56.9 nM) were marginally less active than HF-4aa and its enantiomers ( $IC_{50}$  = 13.1 nM and 25.7 nM), though without statistically significant differences. To validate intracellular target specificity, MDA-MB-231 cells were co-incubated with a red-fluorescent probe derivative (10) of HF-4aa, followed by immunostaining using a TXNRD1-specific primary antibody and a green-fluorescent secondary antibody. Confocal microscopy revealed pronounced co-localization of TXNRD1 (green signal) and probe 10 (red signal) within cytoplasmic compartments, with merged fluorescence intensity confirming spatial overlap (Figure 3C). These integrated findings robustly establish HF-4aa as a specific TXNRD1 inhibitor within the cellular milieu.

### Ferroptosis induction in MDA-MB-231 cells by HF-4aa

We evaluated the engagement of HF-4aa in regulating ferroptosis *via* TXNRD1 inhibition by assessing hallmark biomarkers, including intracellular ROS accumulation,  $Fe^{2+}$  elevation, and lipid peroxidation. As a key indicator of ferroptosis, intracellular ROS levels in HF-4aa-treated MDA-MB-231 cells were quantified using high-content imaging. Treatment with HF-4aa (5–20 nM) induced a dose-dependent increase in ROS, as visualized by enhanced green fluorescence signals using the ROS-sensitive probe DCFH-DA (Figure 3F), indicating progressive oxidative stress. Notably, at 20 nM, HF-4aa demonstrated significantly stronger ROS induction compared to both the TXNRD1 inhibitor positive control (Tri-1) and the canonical ferroptosis inducer RSL-3. These observations were quantitatively confirmed by high-content imaging analysis (Figure 3D). To validate ferroptosis-associated iron dysregulation, intracellular labile  $Fe^{2+}$  levels were measured using the  $Fe^{2+}$ -specific fluorescent probe FerroOrange. Quantitative high-content imaging analysis revealed that HF-4aa significantly elevated cytosolic  $Fe^{2+}$  accumulation in a dose-dependent manner (Figure 3E). This spatial distribution pattern was further confirmed by imaging analysis (Figure 3G). Notably, HF-4aa (20 nM) treatment induced  $Fe^{2+}$  levels slightly higher than those observed with

the canonical ferroptosis inducer RSL-3 at an equivalent concentration, consistent with its potent ferroptosis-inducing activity.

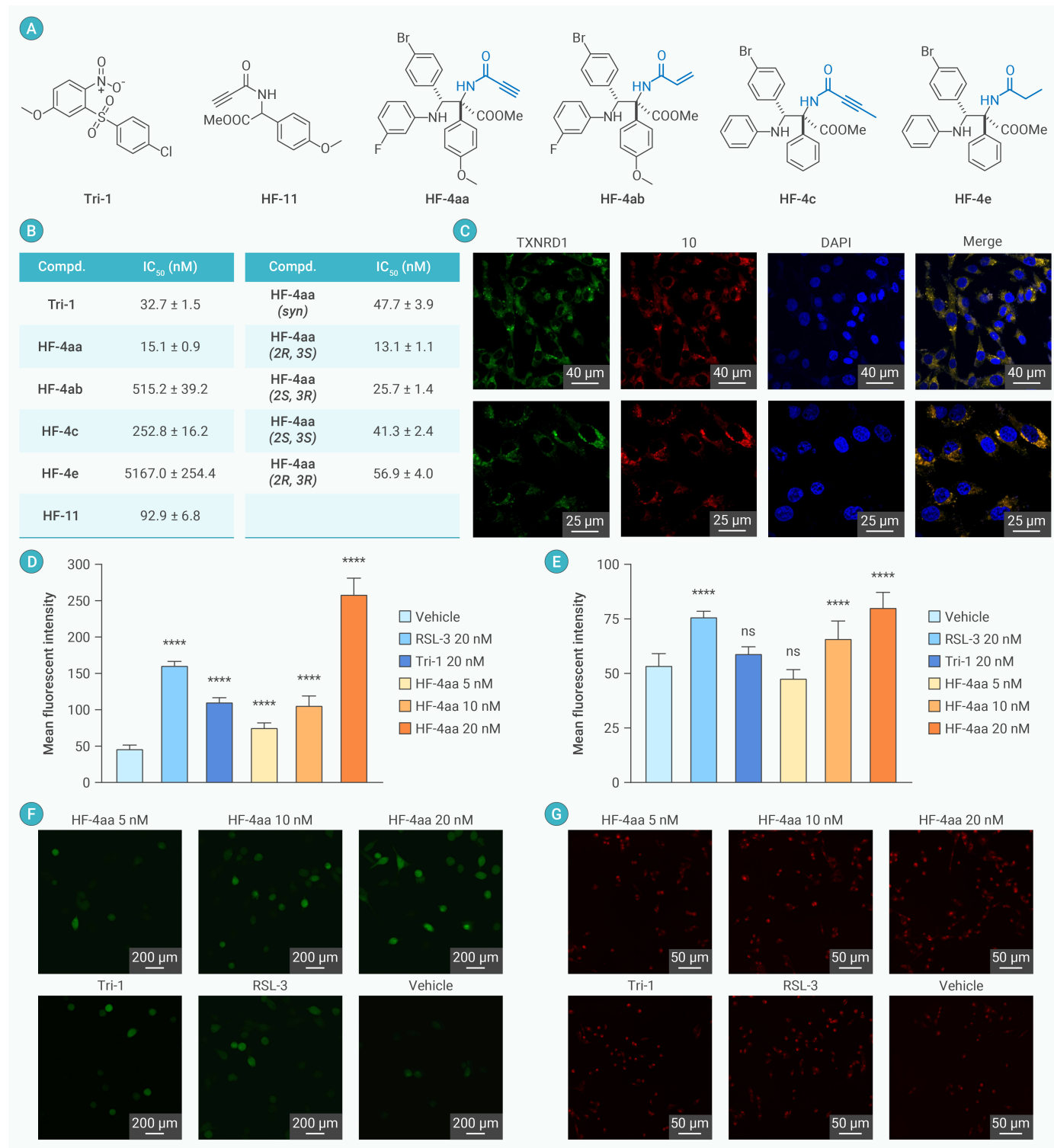
Protection studies demonstrated that ferroptosis inhibitors (DFO, Fer-1) partially rescued HF-4aa-induced cytotoxicity, while inhibitors of apoptosis (Z-VAD-FMK), necroptosis (Nec-1), and pyroptosis (VX765) had no significant effects (Figure 4A). To further quantify ferroptosis-specific protection, MDA-MB-231 cells were treated with increasing concentrations of HF-4aa in the presence or absence of Fer-1, and cell survival was measured. Fer-1 significantly increased cell viability in a concentration-dependent manner, confirming that HF-4aa-induced cell death is primarily ferroptotic (Figure 4B). To investigate HF-4aa's impact on ferroptosis-associated lipid peroxidation, MDA-MB-231 cells were treated with increasing concentrations (5–20 nM) of HF-4aa. High-content imaging using the oxidation-sensitive probe C11-BODIPY (581/591) revealed a dose-dependent enhancement of oxidized probe signal (green fluorescence) in treated cells (Figure 4C). Quantitative analysis confirmed a concentration-dependent elevation in lipid peroxidation levels, consistent with ferroptosis induction (Figure 4D). Notably, at 20 nM, HF-4aa exhibited slightly stronger activity than RSL-3. Transmission electron microscopy further revealed ultrastructural changes in mitochondria of HF-4aa-treated cells consistent with ferroptosis, including reduced mitochondrial volume, loss of cristae integrity, and increased membrane density (Figure 4E). These findings collectively support the notion that HF-4aa exerts its antitumor effect primarily by inducing ferroptosis in cancer cells.

### *In vivo* anti-tumor effect of HF-4aa

To further validate the ferroptosis-inducing effect of HF-4aa *in vivo*, we established a BALB/c mouse model bearing subcutaneous 4T1 tumors. Mice were administered four treatment regimens: HF-4aa monotherapy, Fer-1 monotherapy, HF-4aa/Fer-1 co-administration, and vehicle control. HF-4aa effectively suppressed breast tumor growth (TGI = 57.3%), and this antitumor effect was partially reversed by co-treatment with the ferroptosis inhibitor Ferrostatin-1 (10 mg/kg), which reduced TGI to 27.9% (Figure 5A–D). Moreover, immunohistochemical analysis of tumor tissues revealed markedly reduced Ki67 proliferation index in HF-4aa-treated groups, further supporting its antitumor efficacy (Figure 5E). Quantification confirmed that HF-4aa significantly decreased Ki67 positivity, and peripheral blood analysis showed no evident hematological toxicity in the Fer-1 reversal experiment (Figure S8 and Table S8). Biochemical assays demonstrated that HF-4aa elevated intratumoral ROS levels and enhanced lipid peroxidation, consistent with ferroptosis induction (Figure 5F–G).

Hepatic microsomal stability assays revealed rapid degradation of free HF-4aa, with an extremely short half-life ( $t_{1/2}$  < 5 min). To address this metabolic liability and enhance plasma pharmacokinetics, ginsenoside Rg3-functionalized biomimetic liposomes encapsulating HF-4aa (mean size:  $89.4 \pm 1.2$  nm; encapsulation efficiency: 91.2%) were engineered (Figure S2). This delivery platform significantly improved compound stability while enabling preferential tumor accumulation. In orthotopic 4T1 breast cancer models, blank liposomes exhibited moderate antitumor activity (18.7% inhibition). HF-4aa-liposomes administered at 5 mg/kg and 10 mg/kg demonstrated efficacy comparable to paclitaxel-liposomes (10 mg/kg), with no significant difference observed between the two HF-4aa-liposome doses, both outperformed the 2.5 mg/kg group. Notably, HF-4aa-liposomes (5 mg/kg) showed superior antitumor activity relative to equivalent doses of free HF-4aa and standard ferroptosis inducers RSL3 (10 mg/kg) and Tri-1 (10 mg/kg) (Figure 5H–I). A modest decrease in body weight was associated with the administration of free HF-4aa (5 mg/kg), paclitaxel-liposomes (10 mg/kg), and HF-4aa-liposomes (10 mg/kg), suggesting low-grade toxicity that may be related to the intravenous dosing regimen (Figure 5J).

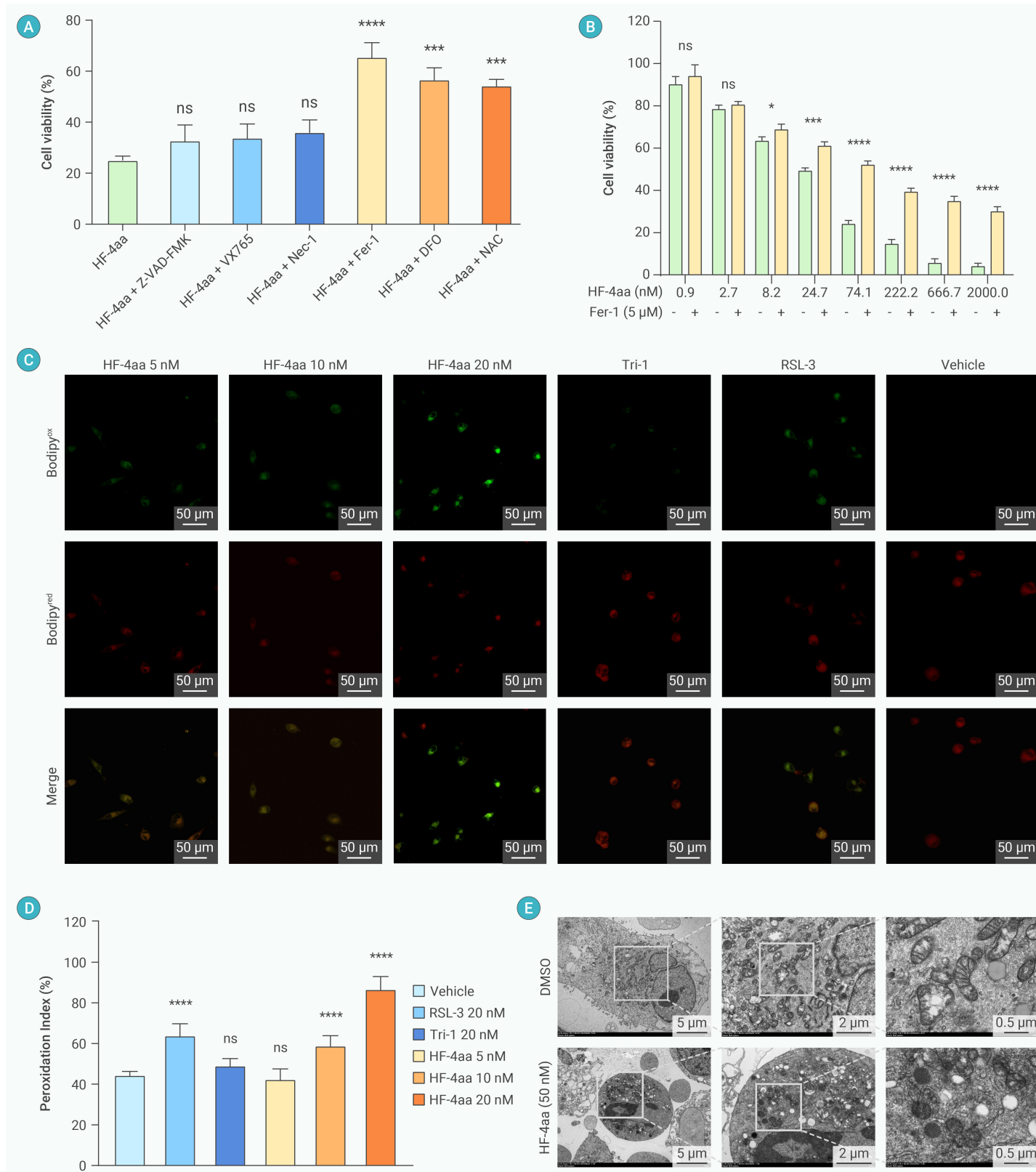
Previous studies have suggested that ferroptosis activation may help address paclitaxel resistance.<sup>43</sup> To further assess the potential of covalent ferroptosis induction in this context, we evaluated HF-4aa in a paclitaxel-resistant tumor model (Figure 6). Paclitaxel-liposomes (10 mg/kg) alone produced only modest tumor growth inhibition (TGI = 29%), reflecting the resistant phenotype. In contrast, HF-4aa-liposomes (5 mg/kg) demonstrated a strong single-agent effect (TGI = 71%), indicating that ferroptosis induction can effectively overcome paclitaxel resistance. The combination of HF-4aa with paclitaxel yielded an enhanced TGI of 80%, suggesting a degree of func-



**Figure 3. HF-4aa promotes ferroptosis hallmarks in MDA-MB-231 cells** (A) Structures of the  $\alpha,\beta$ -diamino acid derivatives evaluated at the enzymatic level. (B) Inhibitory activity of  $\alpha,\beta$ -diamino acid derivatives and HF-4aa stereoisomers. (C) Colocalization of probe 10 with TXNRD1 in MDA-MB-231 cells. Fixed and permeabilized MDA-MB-231 cells were treated with the BODIPY-conjugated probe 10 (red) and immunolabeled for TXNRD1 (green). Nuclei were visualized with DAPI (blue). Scale bars = 40  $\mu$ m. (D) Data are represented as mean  $\pm$  SEM. Quantitative high-content imaging analysis of intracellular ROS levels in MDA-MB-231 cells treated with HF-4aa (5, 10, 20 nM), Tri-1 (20 nM), or RSL-3 (20 nM), stained with DCFH-DA (10  $\mu$ M). (E) Data are represented as mean  $\pm$  SEM. HF-4aa dose-dependently increases cytosolic Fe<sup>2+</sup> accumulation. Quantitative analysis of intracellular labile Fe<sup>2+</sup> levels in MDA-MB-231 cells treated with HF-4aa (5, 10, 20 nM), Tri-1 (20 nM), RSL-3 (20 nM), or vehicle (0.01% DMSO) for 4 hours. Fe<sup>2+</sup> was detected using FerroOrange probe (1  $\mu$ M, 40 min) and quantified by high-content imaging (Ex/Em: 543/580 nm). (F) Representative high-content images of DCFH-DA-stained MDA-MB-231 cells. Scale bar: 200  $\mu$ m. (G) Subcellular localization of HF-4aa-induced Fe<sup>2+</sup> accumulation. Scale bar: 50  $\mu$ m. Images were acquired using a 40 $\times$  oil objective.

tional cooperativity despite their distinct mechanisms of action. Peripheral blood analysis in the paclitaxel-resistant model showed no evident hematological toxicity associated with HF-4aa (Table S9). These results support the concept that covalent ferroptosis inducers such as HF-4aa represent a promising approach for targeting paclitaxel-resistant tumors and may

complement existing chemotherapies. While further optimization of pharmacokinetic and toxicity profiles will be required for translational application, this study provides important proof-of-concept for covalent ferroptosis induction as a strategy to address drug-resistant cancers, warranting further pharmacological optimization.



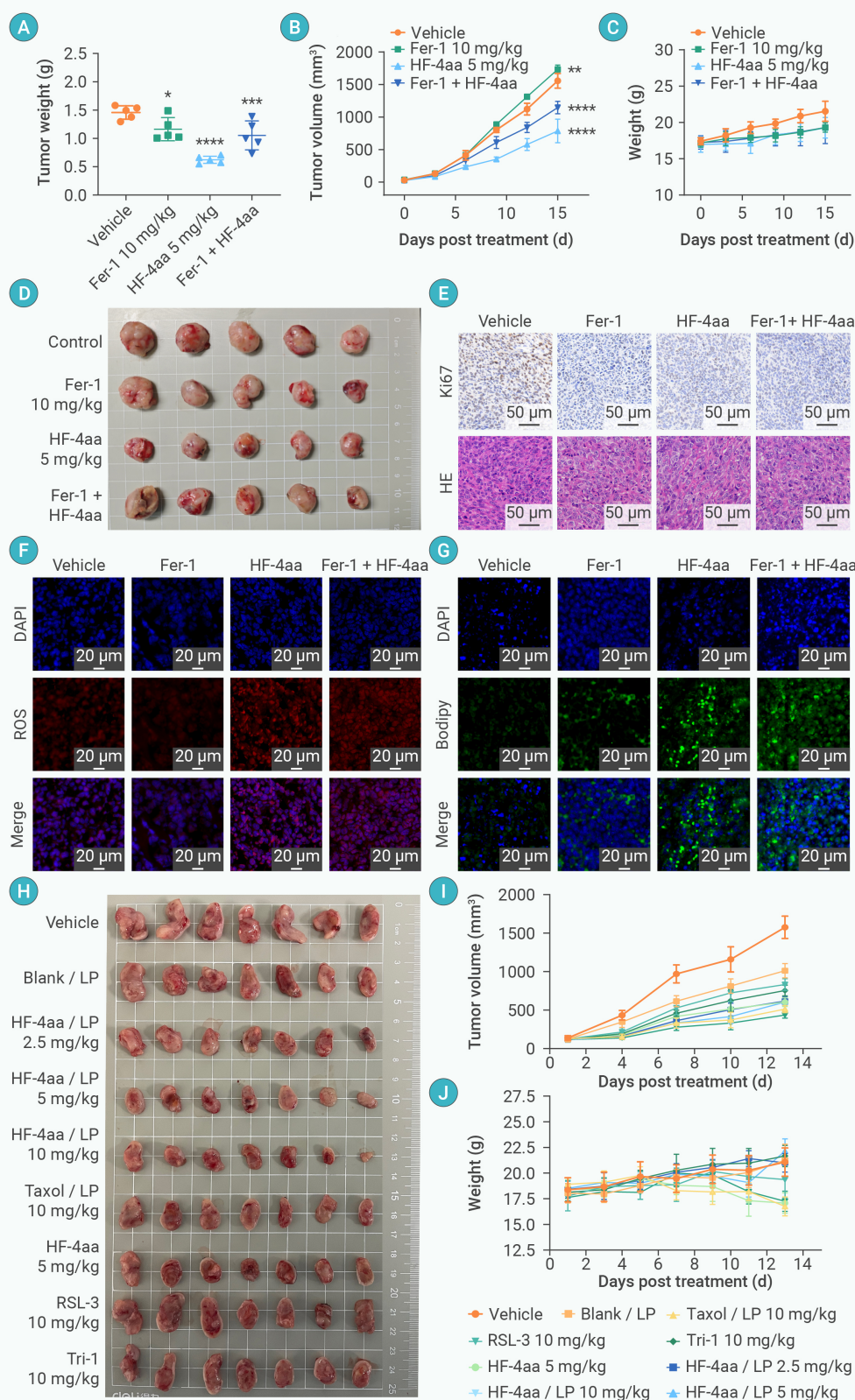
**Figure 4. HF-4aa induces lipid peroxidation and ferroptotic phenotypes** (A) Data are represented as mean  $\pm$  SEM. MDA-MB-231 cells were treated with HF-4aa (50 nM) in the absence or presence of Apoptosis inhibitor Z-VAD-FMK (10  $\mu$ M), Pyroptosis inhibitor VX765 (2  $\mu$ M), Necroptosis inhibitor Nec-1 (2  $\mu$ M), Ferroptosis inhibitor Fer-1 (5  $\mu$ M), Ferroptosis inhibitor DFO (10  $\mu$ M), Antioxidant NAC (10  $\mu$ M). (B) Data are represented as mean  $\pm$  SEM. MDA-MB-231 cells were treated with increasing concentrations of HF-4aa (0.9, 2.7, 8.2, 24.7, 74.1, 222.2, 666.7, 2000.0 nM) in the presence or absence of Fer-1 (5  $\mu$ M), and cell viability was determined. (C) HF-4aa induces dose-dependent lipid peroxidation in MDA-MB-231 cells. Representative high-content images of intracellular lipid peroxidation detected by C11-BODIPY 581/591 probe (green: oxidized signal) in MDA-MB-231 cells treated for 4 hours with: Vehicle (0.01% DMSO), Tri-1 (20 nM), RSL-3 (20 nM), HF-4aa (5, 10, 20 nM). Scale bars = 50  $\mu$ m. (D) Data are represented as mean  $\pm$  SEM. Quantitative analysis of HF-4aa-driven lipid peroxidation. (E) Electron micrographs in MDA-MB-231 cells treated with vehicle (DMSO) or HF-4aa (50 nM) for 12 h.

## DISCUSSION

Covalent inhibitors have re-emerged as powerful chemical probes and

therapeutic modalities because they can irreversibly engage reactive amino acids to modulate traditionally undruggable proteins. However, their discov-





**Figure 5. HF-4aa induces ferroptosis and suppresses breast cancer growth *in vivo*** (A-D) *In vivo* antitumor activity and ferroptosis dependency of HF-4aa. Data are represented as mean ± SEM. Analysis of 4T1 tumor-bearing BALB/c mice that were treated with vehicle, HF-4aa (5 mg/kg), Fer-1 (10 mg/kg), or a combination of HF-4aa and Fer-1. All treatments were administered every 2 days for 15 days. (A) Quantification of tumor weight at endpoint. (B) Tumor volume over time. (C) Mouse body weight changes during treatment. (D) Representative photographs of excised tumors. (E) H&E and IHC images of the indicated proteins in the randomly selected 4T1 tumor section. Scale bars = 50 μm. (F-G) HF-4aa induces ferroptosis biomarkers in tumor tissues: (F) Elevated intratumoral ROS and (G) enhanced lipid peroxidation. Scale bars = 20 μm. (H-J) Superior efficacy of Rg3-functionalized HF-4aa-liposomes in orthotopic 4T1 models. Data are represented as mean ± SEM. Analysis of 4T1 tumor-bearing BALB/c nude mice treated with: Vehicle (5% glucose), Blank liposomes, Free HF-4aa (5 mg/kg, *i.v.*), RSL-3 (20 mg/kg, *i.v.*), Tri-1 (10 mg/kg, *i.v.*), Paclitaxel-liposomes (10 mg/kg, *i.v.*), or HF-4aa-liposomes (2.5, 5, 10 mg/kg, *i.v.*) administered every 2 days for 13 days. (H) Representative photographs of excised tumors at study endpoint. (I) Tumor volume over time. (J) Mouse body weight changes during treatment.

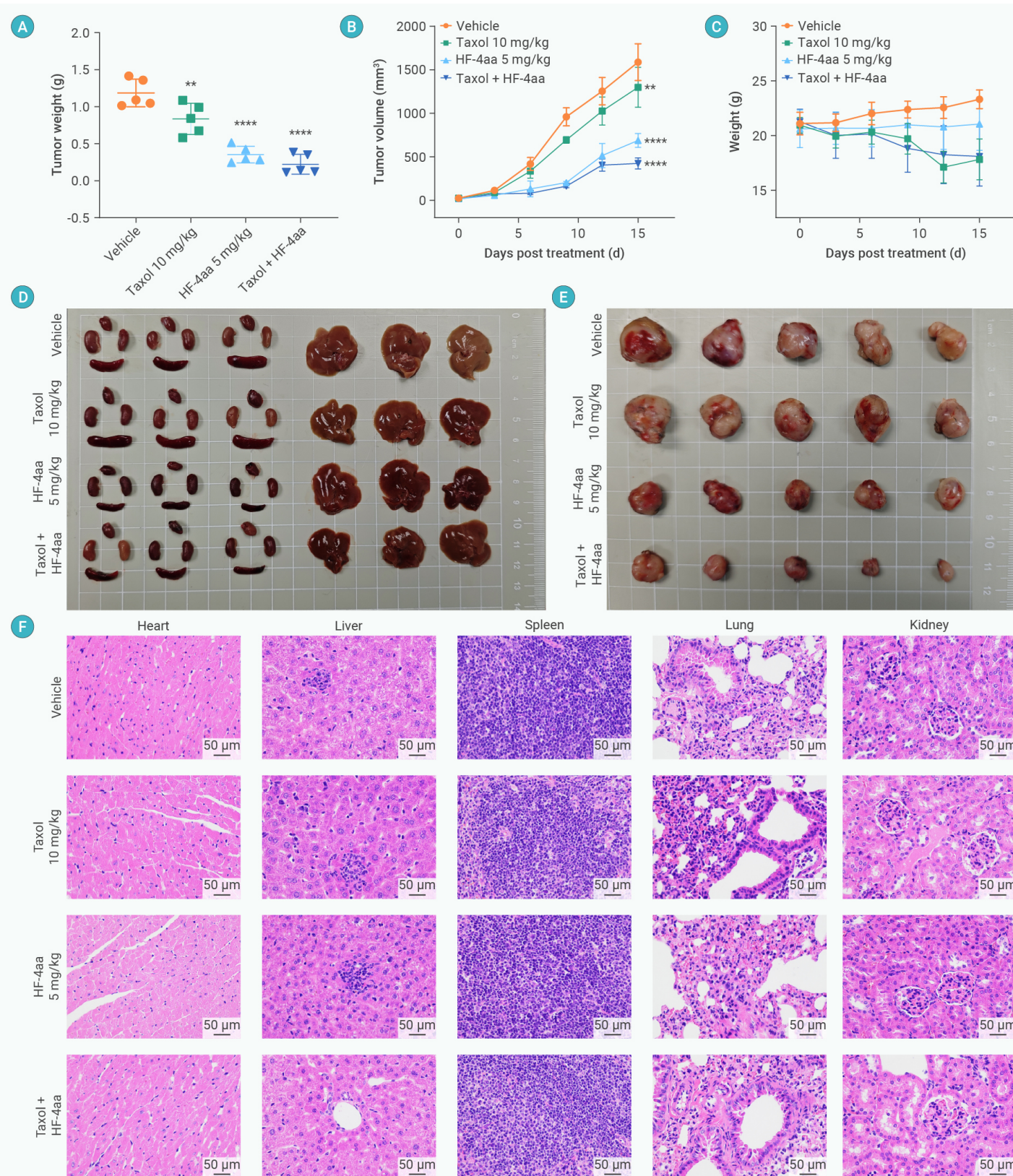
A key advantage of the platform is that the same MCR logic used for hit discovery also enables fast generation of covalent probes with conserved electrophilicity and steric architecture. This allowed direct translation of SAR insights into competitive chemoproteomic profiling, followed by unbiased CETSA-MS validation, rapidly converging on TXNRD1 as the primary functional target. Such continuity, from hit to probe and to proteome annotation, addresses a long-standing bottleneck in phenotype-initiated drug discovery, where target deconvolution typically lags behind compound identification due to the need for bespoke probe synthesis. By eliminating this synthetic barrier, MCR chemistry transforms covalent proteomics from an auxiliary step into an integrated driver of mechanistic progression.

Mechanistically, HF-4aa covalently modifies the selenocysteine/cysteine redox couple of TXNRD1, collapsing antioxidant buffering in a manner functionally linked to ferroptotic death, as evidenced by ROS accumulation, labile Fe<sup>2+</sup> elevation, and lipid peroxidation. Ferrostatin-1 rescue confirms ferroptosis as a causal death pathway, while comparison with the benchmark inhibitor Tri-1 highlights MCR-enabled scaffolds as a viable alternative to SNAr-substitution based electrophiles. *In vivo*, HF-4aa suppressed tumor growth through TXNRD1-dependent ferroptosis, and its efficacy extended to paclitaxel-resistant tumors, suggesting that covalent targeting of redox metabolism may circumvent apoptosis-based drug tolerance.

ery is still constrained by stochastic hit identification and limited access to electrophilic chemotypes that balance reactivity, selectivity, and structural diversity. Here, we establish an MCR-driven chemical biology framework that unifies hit generation, probe development, target elucidation, and mechanistic application. The identification of HF-4aa from an MCR-derived library exemplifies how modular synthesis can accelerate phenotypic exploration: a single convergent step installs both the molecular scaffold and covalent warhead, enabling rapid diversification of electrophiles without sacrificing pharmacophoric or stereochemical features.

Together, these findings position the catalytic asymmetric MCR chemistry as both a scalable engine for electrophilic diversity and an enabling technology for rapid mechanistic annotation. Beyond TXNRD1, such covalent MCR platforms offer a broadly applicable strategy to interrogate nucleophilic nodes across redox signaling, post-translational redox regulation, metal-dependent enzymology, and ferroptosis biology. By tightly coupling synthesis with proteomics, this approach provides a blueprint for systematic discovery, optimization, and biological decoding of next-generation covalent modulators.





**Figure 6. LLC Paclitaxel-Resistant Mouse Model.** A-C Data are represented as mean  $\pm$  SEM (A-C) Data are represented as mean  $\pm$  SEM. Quantification of tumor weight at endpoint (A). Tumor volume over time (B). Mouse body weight changes during treatment (C). (D) Morphological images of heart, liver, spleen, kidney and liver. (E) Representative photographs of excised tumors. (F) H&E stain of heart, liver, spleen, lung, and kidney.

## MATERIALS AND METHODS

General procedures for three-component reactions, synthesis of all stereoisomers, synthetic procedure for the probes, and characterization of products. Details are provided in Supplemental Information.

## Cell Cultures

MDA-MB-231, MIA-PaCa-2, MCF-7, 4T1 and KYSE-520 cell lines were purchased from Procell Life Science & Technology Co., Ltd. The LX-2, SK-OV-3, HCC1954, A549, HCT116 cell lines was received as a gift from the New

Drug R&D Technology Center, School of Pharmaceutical Sciences, Sun Yat-sen University. MCF-7, LX-2, MDA-MB-231 and MIA-PaCa-2 were maintained in DMEM, and the A549 cell line was grown in Ham's F-12K. HCC1954, 4T1 and KYSE-520 were cultured in RPMI-1640 medium. SK-OV-3 and HCT116 were cultured in McCoy's 5A. All media were supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). All cells were grown at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Compound Screening and IC<sub>50</sub> Determination

For single-concentration screening, MDA-MB-231 cells in logarithmic growth were plated at 5,000 cells per well in 96-well plates. After medium removal, cells were treated with 10 µM test compounds (from 10 mM DMSO stocks; final DMSO ≤ 0.1%) for 48 h. For IC<sub>50</sub> assays, cells were seeded similarly, exposed to compound gradients (0.0001–20 µM) after 24 h, and incubated for 48 h. Cell viability was determined using the Cell Counting Kit-8 (CCK-8) assay. Briefly, 10 µL of CCK-8 reagent was added to each well, and after incubation at 37 °C for 1–3 h, the absorbance at 450 nm was measured. IC<sub>50</sub> values were calculated by nonlinear regression of dose-response curves (GraphPad Prism 8.01). Data were normalized to DMSO-treated controls and represent mean ± SD from at least three independent experiments.

### Multimodal Death Inhibitor Reversal Assay *In Vitro*

Cells in logarithmic phase were seeded in 96-well plates at 5,000 cells per well. After 24 h, the following inhibitors were added: Z-VAD-FMK (apoptosis, 10 µM), Necrostatin-1 (necroptosis, 2 µM), VX765 (pyroptosis, 2 µM), DFO (ferroptosis, iron chelation, 100 µM), Ferrostatin-1 (ferroptosis, lipid peroxidation suppression, 50 µM), and NAC (antioxidant, ROS scavenger, 100 µM). Inhibitor-free wells served as negative controls. Following 1 h pre-incubation, cells were treated with HF-4aa (5 nM) and inhibitors for 48 h. Cell viability was assessed using CCK-8 reagent, with absorbance measured at 450 nm after 1–3 h incubation. All inhibitors were prepared in DMSO and diluted in complete medium, with final DMSO concentration maintained below 0.1% (v/v).

### Ultrastructural Analysis of Subcellular Morphology *via* TEM

MDA-MB-231 cells were seeded in 6-well plates and allowed to adhere overnight. Following attachment, cells were treated with 50 nM compound in serum-free medium for 12 h. Cells were then harvested, fixed primarily with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 4 h, and post-fixed with 1% osmium tetroxide. After dehydration through a graded ethanol series, samples were embedded in EPON 812 resin, polymerized, and sectioned at 70 nm. Ultrathin sections were stained with uranyl acetate and lead citrate, and imaged using a transmission electron microscope operated at 110 kV.

### Pull-Down and Target Validation

Log-phase MDA-MB-231 cells were treated with biotinylated probe 6a (1 µM) alone or in combination with competitor HF-4aa (1 µM), D-biotin, or probe 6b for 6 h. Cells were lysed in RIPA buffer with phosphatase inhibitors, and clarified lysates (800 µg protein) were incubated overnight with streptavidin-agarose beads at 4 °C. The beads were washed three times with 1 mL of lysis buffer containing 0.1% SDS, followed by two washes with 1 mL of PBS, bound proteins were eluted in loading buffer at 100 °C. Eluates were separated by SDS-PAGE and silver-stained. Target bands were excised, subjected to in-gel reduction, alkylation, and tryptic digestion. Extracted peptides were analyzed by LC-MS/MS on a Q Exactive Plus mass spectrometer using a 30-min acetonitrile gradient. Data were processed with Proteome Discoverer 2.4 against the human proteome database, allowing for methionine oxidation and N-terminal acetylation.

### CETSA-MS and Target Validation

MDA-MB-231 cell lysates were treated with 20 µM HF-4aa or DMSO, heated at 52 °C, and cooled. After supernatant collection and protein quantification, equal protein amounts were reduced, alkylated, and digested into peptides, which were then TMT10plex-labeled, fractionated, and analyzed by LC-MS/MS using an Easy-nLC 1200 coupled to an Orbitrap Eclipse mass spectrometer with a 120-min gradient and FAIMS voltages of –45 V and –65

V. Raw data were processed using Proteome Discoverer against a human UniProt database, and results were visualized in volcano plots with ProSAP, applying Benjamini-Hochberg correction for statistical significance. Detailed methods are provided in the Supplemental Information.

### CETSA-WB

In MDA-MB-231 cell lysates, CETSA-WB was performed in two formats: melt curve and ITDR. For melt curve assays, lysates treated with 100 µM HF-4aa or DMSO were heated at seven temperature points, cooled, and centrifuged. For ITDR assays, lysates treated with a range of HF-4aa concentrations were heated at 53 °C, cooled, and centrifuged. Supernatants from both assays were subjected to protein quantification, followed by SDS-PAGE, transfer to PVDF membranes, and immunoblotting with antibodies against TXNRD1, SOD1, and GAPDH. Protein bands were visualized using enhanced chemiluminescence.

### Protein Expression and Purification

The human TXNRD1 plasmid, together with the pACYC184-selABC plasmid, was co-transformed into *E. coli* BL21 (DE3) cells for protein expression. Cells were cultured in LB medium at 37 °C, and protein expression was induced with 0.5 mM IPTG in the presence of 5 mM Na<sub>2</sub>SeO<sub>3</sub> and 100 µg/mL cysteine at 18 °C. The harvested cells were lysed by ultrasonication, and the recombinant TXNRD1 protein was purified from the supernatant using Ni-NTA affinity chromatography followed by gel filtration chromatography<sup>44</sup>.

### Covalent Binding Assay and Intact Protein MS Analysis

Purified TXNRD1 (1 µM) was pre-incubated with 50 µM NADPH for 10 min at room temperature, followed by treatment with 200 µM HF-4aa in the dark for 2 h. The reaction was quenched with 1% formic acid (final concentration). The samples were analyzed by LC-MS on a BioAccord system (Waters) equipped with a C4 reversed-phase column using a 10-min gradient. Acquired mass spectra were deconvoluted to obtain intact protein masses using UNIFI software (Waters).

### TXNRD1 Activity Assay

Recombinant TXNRD1 was diluted 60-fold in phosphate buffer. A mixture of 1 µL of serially diluted compound and 59 µL of the diluted enzyme solution was incubated in a 96-well plate at 37 °C for 20 min. Then, 20 µL of 1 mM NADPH and 20 µL of 15 mM DTNB were added, and kinetic measurements were immediately initiated. Absorbance at 412 nm was recorded every 30 s for 10 min at 37 °C. Enzyme activity was calculated from the slope between 0 s and 90 s, and inhibition (%) was determined relative to inhibitor-free controls. IC<sub>50</sub> values were derived by nonlinear regression analysis using GraphPad Prism 8.01.

### Immunofluorescence Staining and Lipid Peroxidation Imaging

MDA-MB-231 cells were seeded in glass-bottom confocal dishes. At ~90% confluency, cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.3% Triton X-100 for 15 min. After blocking with 10% goat serum for 1 h, cells were incubated with an anti-TXNRD1 primary antibody overnight at 4 °C, followed by a species-matched fluorescent secondary antibody for 1 h. Lipid peroxidation was detected by co-incubating the cells with the BODIPY 581/591 C11 probe (5 µM) in serum-free medium for 1 h. Nuclei were counterstained with DAPI. All steps were followed by three washes with PBST. Imaging was performed using a laser scanning confocal microscope under consistent exposure settings.

### Intracellular ROS, Fe<sup>2+</sup>, and Lipid Peroxidation Levels *In Vitro*

MDA-MB-231 cells were seeded in 12-well glass-bottom plates (2×10<sup>4</sup> cells/well) and cultured to ~90% confluency. Cells were treated with test compounds (5–20 nM), Tri-1 (2 µM), RSL-3, or vehicle (0.01% DMSO) for 4 h at 37 °C and 5% CO<sub>2</sub>. For ROS detection, cells were incubated with 10 µM DCFH-DA for 40 min, and fluorescence was quantified (Ex/Em: 488/525 nm). For Fe<sup>2+</sup> measurement, cells were stained with 1 µM FerroOrange for 40 min, followed by imaging (Ex/Em: 543/580 nm) and subcellular localization analysis using a 40× oil objective. Lipid peroxidation was assessed using 5 µM C11-BODIPY 581/591 in serum-free medium for 1 h, and fluorescence was



measured at 488 nm (oxidized) and 565 nm (reduced). The lipid peroxidation index was calculated as the ratio of mean fluorescence intensities (488 nm/565 nm). All samples were imaged using high-content analysis with  $\geq 9$  fields per well.

### Liver Microsomal Stability

The metabolic stability of the test compound was assessed in mouse liver microsomes. The incubation mixture, containing liver microsomes (0.33 mg/mL) in 0.1 M Tris buffer (pH 7.4), 5 mM  $MgCl_2$ , 0.005% BSA, and the test compound, was pre-incubated at 37 °C for 10 min. The reaction was initiated by adding 1 mM NADPH. Aliquots were collected at 0, 5, 15, 30, and 60 min. The reaction was terminated immediately by adding methanol pre-cooled to 4 °C. After centrifugation at 4000 rpm for 5 minutes, analyze the resulting supernatants using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

### Mouse Models and Treatments

Female Balb/c or C57BL/6 mice (3–5 weeks old) were housed under specific pathogen-free (SPF) conditions. For therapeutic efficacy studies, four distinct models were established: (1) 4T1 subcutaneous xenografts ( $1 \times 10^7$  cells/injection) treated with HF-4aa (5–10 mg/kg, *i.p.*, daily) or paclitaxel (10 mg/kg) for 28 d; (2) 4T1 orthotopic implants ( $5 \times 10^5$  cells/mammary fat pad) receiving HF-4aa liposomes (2.5–10 mg/kg, *i.v.*), TXNRD1 inhibitor (10 mg/kg), RSL-3 (20 mg/kg), or paclitaxel liposomes (10 mg/kg, *i.v.*) every 2 d for 13 d; (3) 4T1 tumor fragment implantation models administered HF-4aa (5 mg/kg, *i.p.*) with/without ferrostatin-1 (10 mg/kg) every other day for 15 d; and (4) LLC paclitaxel-resistant tumor models ( $IC_{50} \approx 500$  nM) treated with HF-4aa liposomes (5 mg/kg, *i.v.*) and/or paclitaxel liposomes (10 mg/kg, *i.v.*) every other day for 15 d. All test articles were formulated in castor oil ethoxylate:Solutol HS 15:5% glucose (1:1.5:8, v/v). Tumor volumes were measured every 2 d using the formula ( $\text{length} \times \text{width}^2$ )/2. Mice were euthanized when tumors exceeded 1500 mm<sup>3</sup> or reached 15 mm diameter; tumors and major organs were collected within 12 h post-final dose. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University (SYSU-IACUC-2023-000016, SYSU-IACUC-2023-000091, SYSU-IACUC-2025-000115, SYSU-IACUC-2025-001250) and performed in accordance with national guidelines.

### Protocol for BODIPY, Ki67, and H&E Staining in Tumor Tissue

Tissue samples were fixed, paraffin-embedded, and sectioned for H&E and Ki67 staining, or cryosectioned for BODIPY staining. For H&E, sections were dewaxed, rehydrated, stained with hematoxylin and eosin, dehydrated, and mounted. For Ki67 immunohistochemistry, antigen retrieval was performed in EDTA buffer (pH 8.0), followed by peroxidase blocking with 3%  $H_2O_2$ , incubation with anti-Ki67 antibody (1:1000) overnight at 4 °C, HRP-conjugated secondary antibody (1:200), and DAB development. Ki67 positivity was calculated as Ki67-positive nuclei / total nuclei  $\times 100\%$ . Six randomly selected high-power fields per sample were quantified, with  $n = 6$  per group. Statistical analysis was performed using one-way ANOVA followed by Dunnett's or Tukey's multiple comparisons test. For BODIPY staining, cryosections were incubated with 1–5  $\mu M$  BODIPY 493/503 (37 °C, 20 min), counterstained with DAPI, and mounted. All stained sections were imaged using appropriate filters: BODIPY (Ex/Em: 488/505–550 nm), Cy3 (561/570–620 nm), and DAPI (405/420–480 nm).

### REFERENCES

- Gehring, M., Laufer, S.A. (2019). Emerging and re-emerging warheads for targeted covalent inhibitors: Applications in medicinal chemistry and chemical biology. *J. Med. Chem.* **62**:5673–5724. DOI:10.1021/acs.jmedchem.8b01153
- Baillie, T.A. (2016). Targeted covalent inhibitors for drug design. *Angew. Chem. Int. Ed.* **55**:13408–13421. DOI:10.1002/anie.201601091
- Bauer, R.A. (2015). Covalent inhibitors in drug discovery: From accidental discoveries to avoided liabilities and designed therapies. *Drug Discov. Today* **20**:1061–1073. DOI:10.1016/j.drudis.2015.05.005
- Boike, L., Henning, N.J., Nomura, D.K. (2022). Advances in covalent drug discovery. *Nat. Rev. Drug Discov.* **21**:881–898. DOI:10.1038/s41573-022-00542-z
- Soria, J.-C., Ohe, Y., Vansteenkiste, J., et al. (2018). Osimertinib in Untreated EGFR - Mutated Advanced Non-Small-Cell Lung Cancer. *N. Engl. J. Med.* **378**:113–125. DOI:10.1056/NEJMoa1713137
- Byrd, J.C., Furman, R.R., Coutre, S.E., et al. (2013). Targeting BTK with Ibrutinib in Relapsed Chronic Lymphocytic Leukemia. *N. Engl. J. Med.* **369**:32–42. DOI:10.1056/NEJMoa1215637
- Skoulidis, F., Li, B.T., de Langen, A.J., et al. (2025). Molecular determinants of sotorasib clinical efficacy in KRASG12C-mutated non-small-cell lung cancer. *Nat. Med.* **31**:2755–2767. DOI:10.1038/s41591-025-03732-5
- Passiglia, F., Pepe, F., Russo, G., et al. (2025). Circulating tumor DNA dynamic variation predicts sotorasib efficacy in KRASG12C-mutated advanced non-small cell lung cancer. *Cancer* **131**:e35917. DOI:10.1002/cncr.35917
- Lanman, B.A., Parsons, A.T., Zech, S.G. (2022). Addressing atropisomerism in the development of sotorasib, a covalent inhibitor of KRAS G12C: Structural, analytical, and synthetic considerations. *Acc. Chem. Res.* **55**:2892–2903. DOI:10.1021/acs.accounts.2c00479
- London, N., Miller, R.M., Krishnan, S., et al. (2014). Covalent docking of large libraries for the discovery of chemical probes. *Nat. Chem. Biol.* **10**:1066–1072. DOI:10.1038/nchembio.1666
- Hartung, I.V., Rudolph, J., Mader, M.M., et al. (2023). Expanding chemical probe space: Quality criteria for covalent and degrader probes. *J. Med. Chem.* **66**:9297–9312. DOI:10.1021/acs.jmedchem.3c00550
- Kornienko, A., La Clair, J.J. (2017). Covalent modification of biological targets with natural products through Paal-Knorr pyrrole formation. *Nat. Prod. Rep.* **34**:1051–1060. DOI:10.1039/C7NP00024C
- Byun, D.P., Ritchie, J., Jung, Y., et al. (2023). Covalent inhibition by a natural product-inspired latent electrophile. *J. Am. Chem. Soc.* **145**:11097–11109. DOI:10.1021/jacs.3c00598
- Takemoto, M., Delghandi, S., Abo, M., et al. (2025). Covalent plant natural product that potentiates antitumor immunity. *J. Am. Chem. Soc.* **147**:2902–2912. DOI:10.1021/jacs.4c17837
- Li, L., Wang, Y., Wang, Y., et al. (2025). Dimeric natural product panepocyclinol A inhibits STAT3 via di-covalent modification. *Acta Pharm. Sin. B* **15**:409–423. DOI:10.1016/j.japsb.2024.10.001
- Neochoritis, C.G., Zhao, T., Dömling, A. (2019). Tetrazoles via multicomponent reactions. *Chem. Rev.* **119**:1970–2042. DOI:10.1021/acs.chemrev.8b00564
- Zhou, X., Lai, Z., Li, J., et al. (2025). Novel Mannich-type multicomponent reactions: Discovery, mechanism, and application. *Acc. Chem. Res.* **58**:2317–2331. DOI:10.1021/acs.accounts.5c00338
- De Graaff, C., Ruijter, E., Orru, R.V.A. (2012). Recent developments in asymmetric multicomponent reactions. *Chem. Soc. Rev.* **41**:3969–3989. DOI:10.1039/C2CS15361K
- Dömling, A., Wang, W., Wang, K. (2012). Chemistry and biology of multicomponent reactions. *Chem. Rev.* **112**:3083–3135. DOI:10.1021/cr100233r
- Cioc, R.C., Ruijter, E., Orru, R.V.A. (2014). Multicomponent reactions: advanced tools for sustainable organic synthesis. *Green Chem.* **16**:2958–2975. DOI:10.1039/C4GC00013G
- Zhang, D., Hu, W. (2017). Asymmetric multicomponent reactions based on trapping of active intermediates. *Chem. Rev.* **17**:739–753. DOI:10.1002/tcr.201600124
- Guo, X., Hu, W. (2013). Novel multicomponent reactions via trapping of protic onium ylides with electrophiles. *Acc. Chem. Res.* **46**:2427–2440. DOI:10.1021/ar300340k
- Fu, X., Yang, H., Li, Y., et al. (2025). Multicomponent reaction-enabled semisynthesis of taxanes yields an analogue with reduced chemotherapy-induced neuropathy. *JACS Au* **5**:4299–4308. DOI:10.1021/jacsau.5c00675
- Shi, T., Li, Y., Yang, J., et al. (2025). Multicatalysis-enabled multicomponent reactions generate a PTP1B inhibitor. *ACS Cent. Sci.* **11**:938–949. DOI:10.1021/acscentsci.5c00041
- Shu, J., Wang, Y., Guo, W., et al. (2024). Carbenoid-involved reactions integrated with scaffold-based screening generate a Nav1.7 inhibitor. *Commun. Chem.* **7**:135. DOI:10.1038/s42004-024-01213-3
- Sutanto, F., Shaabani, S., Neochoritis, C.G., et al. (2021). Multicomponent reaction-derived covalent inhibitor space. *Sci. Adv.* **7**:eabd9307. DOI:10.1126/sciadv.abd9307
- Dixon, S.J., Lemberg, K.M., Lamprecht, M.R., et al. (2012). Ferroptosis: An iron-dependent form of nonapoptotic cell death. *Cell* **149**:1060–1072. DOI:10.1016/j.cell.2012.03.042
- Niu, B., Liao, K., Zhou, Y., et al. (2021). Application of glutathione depletion in cancer therapy: Enhanced ROS-based therapy, ferroptosis, and chemotherapy. *Biomaterials* **277**:121110. DOI:10.1016/j.biomaterials.2021.121110
- Doll, S., Proneth, B., Tyurina, Y.Y., et al. (2017). ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. *Nat. Chem. Biol.* **13**:91–98. DOI:10.1038/nchembio.2239
- Shakya, A., McKee, N.W., Dodson, M., et al. (2023). Anti-ferroptotic effects of Nrf2: Beyond the antioxidant response. *Mol. Cells* **46**:165–175. DOI:10.14348/molcells.2023.0005
- Anandhan, A., Dodson, M., Shakya, A., et al. (2023). NRF2 controls iron homeostasis and ferroptosis through HERC2 and VAMP8. *Sci. Adv.* **9**:eade9585. DOI:10.1126/sciadv.ade9585
- Fang, X., Zhang, J., Li, Y., et al. (2023). Malic enzyme 1 as a novel anti-ferroptotic regulator in hepatic ischemia/reperfusion injury. *Adv. Sci.* **10**:2205436. DOI:10.1002/adv.202205436

33. Ding, Y., Chen, X., Liu, C., et al. (2021). Identification of a small molecule as inducer of ferroptosis and apoptosis through ubiquitination of GPX4 in triple-negative breast cancer cells. *J. Hematol. Oncol.* **14**:19. DOI:10.1186/s13045-020-01016-8
34. Huang, B., Wang, H., Liu, S., et al. (2025). Palmitoylation-dependent regulation of GPX4 suppresses ferroptosis. *Nat. Commun.* **16**:867. DOI:10.1038/s41467-025-56344-5
35. Ma, T., Du, J., Zhang, Y., et al. (2022). GPX4-independent ferroptosis—A new strategy in disease therapy. *Cell Death Discov.* **8**:434. DOI:10.1038/s41420-022-01212-0
36. Lancaster, G.I., Murphy, A.J. (2025). GPX4 methylation puts a brake on ferroptosis. *Nat. Cell Biol.* **27**:556–557. DOI:10.1038/s41556-025-01640-x
37. Stafford, W.C., Peng, X., Olofsson, M.H., et al. (2018). Irreversible inhibition of cytosolic thioredoxin reductase 1 as a mechanistic basis for anticancer therapy. *Sci. Transl. Med.* **10**:eaaf7444. DOI:10.1126/scitranslmed.aaf7444
38. Zhang, J., Xu, Q., Ma, D. (2022). Inhibition of thioredoxin reductase by natural anticancer candidate  $\beta$ -lapachone accounts for triggering redox activation-mediated HL-60 cell apoptosis. *Free Radic. Biol. Med.* **180**:244–252. DOI:10.1016/j.freeradbiomed.2022.01.019
39. Yang, R., Sun, S., Zhang, Q., et al. (2025). Pharmacological inhibition of TXNRD1 by a small molecule flavonoid butein overcomes cisplatin resistance in lung cancer cells. *Biol. Trace Elem. Res.* **203**:1949–1960. DOI:10.1007/s12011-024-04331-0
40. Torrente, L., Prieto-Farigua, N., Falzone, A., et al. (2020). Inhibition of TXNRD or SOD1 overcomes NRF2-mediated resistance to  $\beta$ -lapachone. *Redox Biol.* **30**:101440. DOI:10.1016/j.redox.2020.101440
41. Rodrigues, J.A., Pires, B.R.B., de Amorim, I.S.S., et al. (2024). STAT3 regulates the redox profile in MDA-MB-231 breast cancer cells. *Cell Biochem. Biophys.* **82**:3507–3516. DOI:10.1007/s12013-024-01439-x
42. Zhu, Y., Yue, P., Dickinson, C.F., et al. (2022). Natural product preferentially targets redox and metabolic adaptations and aberrantly active STAT3 to inhibit breast tumor growth in vivo. *Cell Death Dis.* **13**:1022. DOI: 10.1038/s41419-022-05477-2. DOI:10.1038/s41419-022-05477-2
43. Lin, C., Sun, J., Yang, Y., et al. (2025). Peptide-based nanoassembly enhances ferroptosis in cancer to overcome paclitaxel resistance. *J. Controlled Release* **384**:113895. DOI:10.1016/j.jconrel.2025.113895
44. Ni, Y., Chen, H., Li, R., et al. (2025). Thimerosal, a competitive thioredoxin reductase 1 (TrxR1) inhibitor discovered via high-throughput screening. *Biochem. Biophys. Res. Commun.* **650**:117–122. DOI:10.1016/j.bbrc.2023.02.014

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## AUTHOR CONTRIBUTIONS

W.H. and X.F. conceptualized and designed the study. X.F., Q.W., S.W., G.N., Z.Z., Y.L. and Y.C. performed the experiments. X.F., Q.W., G.N., Y.L. and F.X. analyzed the results. W.H., J.S., A.C. T.S. and Y.Q. provided technical and material support. W.H., X.F., T.S. and J.S. wrote the paper. W.H., J.S. and T.S. were responsible for research supervision. All authors contributed to the manuscript and approved the final version.

## DECLARATION OF INTERESTS

Albert Sun Chi Chan is the Editor-in-Chief of The Innovation Drug Discovery. Wenhao Hu is an Editorial Board member of The Innovation Drug Discovery. Both Albert Sun Chi Chan and Wenhao Hu were blinded from reviewing or making final decisions on the manuscript. Peer review was handled independently of this member and their research group. The other authors declare no conflicts of interest.

## ETHICAL STATEMENT AND PATIENT CONSENT

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University (SYSU-IACUC-2023-000016, SYSU-IACUC-2023-000091, SYSU-IACUC-2025-000115, SYSU-IACUC-2025-001250) and performed in accordance with national guidelines.

## DATA AND CODE AVAILABILITY

The data supporting the findings of this study are available within the article and its Supplementary Information. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre (CCDC) under deposition numbers 1972426 and 2526472. Additional supporting data are available upon reasonable request from the corresponding author. Source data are provided with this paper.

## SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-drugdisc.2026.100011>.