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# Diacylfuroxans Are Masked Nitrile Oxides That Inhibit GPX4 Covalently

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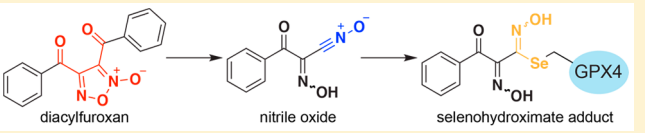
Supporting Information

**ABSTRACT:** GPX4 represents a promising yet difficult-to-drug therapeutic target for the treatment of, among others, drug-resistant cancers. Although most GPX4 inhibitors rely on a chloroacetamide moiety to modify covalently the protein's catalytic selenocysteine residue, the discovery and mechanistic elucidation of structurally diverse GPX4-inhibiting molecules have uncovered novel electrophilic warheads that bind and inhibit GPX4. Here, we report our discovery that diacylfuroxans can act as masked nitrile oxide prodrugs that inhibit GPX4 covalently with unique cellular and biochemical reactivity compared to existing classes of GPX4 inhibitors. These observations illuminate a novel molecular mechanism of action for biologically active furoxans and also expand the collection of reactive groups capable of targeting GPX4.






## INTRODUCTION

Glutathione peroxidase 4 (GPX4) plays a critical role in the dissipation of cellular lipid oxidative stress.[1,2] Among antioxidant proteins, GPX4 has the unique ability to reduce complex lipid hydroperoxides,[3,4] the accumulation of which leads to ferroptotic cell death.[1,5] The direct inhibition of GPX4 enzymatic function is an effective means to induce ferroptosis in certain cellular contexts,[1,6,7] most notably to kill cancer cells that are in a pan-therapy-resistant state.[6−8]

GPX4 is challenging to inhibit with small molecules because it lacks a well-defined active site or known allosteric regulatory sites.[9,10] To date, the only cell-active GPX4 inhibitors that have been reported rely on reactive warheads that covalently engage the catalytic selenocysteine (Sec, U) residue of GPX4.[1,10,11] Notably, nearly all validated GPX4 inhibitors, such as RSL3, ML162, and DPI17 (Figure 1A), rely on a chloroacetamide warhead or related chloromethyltriazine group.[1,12] Despite the high nucleophilicity of the selenocysteine selenol,[13,14] it has not been possible to replace the chloroacetamide functionality of GPX4 inhibitors with less reactive alternatives.[1,10,15] Furthermore, reported cellular[1,16] and biochemical[9] screens have not identified other reactive groups capable of inhibiting GPX4. The limitations of chloroacetamide-containing inhibitors, including low target selectivity and poor pharmacokinetic properties,[10,17] have motivated us to identify GPX4-targeting small molecules with alternative electrophilic chemotypes.

We have recently reported that an unusual class of masked nitrile oxide electrophiles, exemplified by ML210, JKE-1674, and JKE-1716 (Figure 1A,B) can target GPX4 selectively in cells.[10] On the basis of these findings, we hypothesized that GPX4 represents a promising model system for identifying additional novel masked electrophilic warheads that rely on unexpected cellular chemical transformations. Such warheads may be useful not only for targeting GPX4 but more broadly for the development of covalent chemical biology tools for other proteins.

In our continuing pursuit to catalog chemotypes that are able to bind and inhibit GPX4, we investigated a small subset of compounds with NCI-60 cell line sensitivity profiles that correlate with known ferroptosis inducers.[18] One of these compounds is NSC144988 (**1**), which contains an unusual furoxan heterocycle (Figure 2A). Here, we report our discovery that **1** and related diacylfuroxans represent a novel class of masked nitrile oxide electrophiles that covalently target GPX4 and other (seleno)cysteine-containing proteins.

## RESULTS

**Diacylfuroxans Are Inducers of Ferroptotic Cell Death.** To determine if diacylfuroxans, exemplified by **1** and related analogs **2** and **3** (Figure 2A), induce ferroptosis, we assessed their effects on the viability of cells in the presence and absence of ferrostatin-1 (fer-1), a lipophilic radical-trapping antioxidant that inhibits ferroptosis.[5,19,20] The cell-killing activity of **1**−**3** was suppressed by cotreatment with fer-1 (Figures 2B,C and S1A) and other ferroptosis inhibitors[5,21] (Figure S1B). Treatment of cells with **1**−**3** led to the accumulation of cellular lipid hydroperoxides that could be prevented by fer-1 cotreatment (Figures 2D and S1C), indicating that diacylfuroxans are indeed capable of inducing ferroptotic cell death.

Ferroptotic cell death can be induced not only by direct inhibition of GPX4 but also through the inhibition of upstream targets in the glutathione biosynthesis pathway.[22,23] To rule

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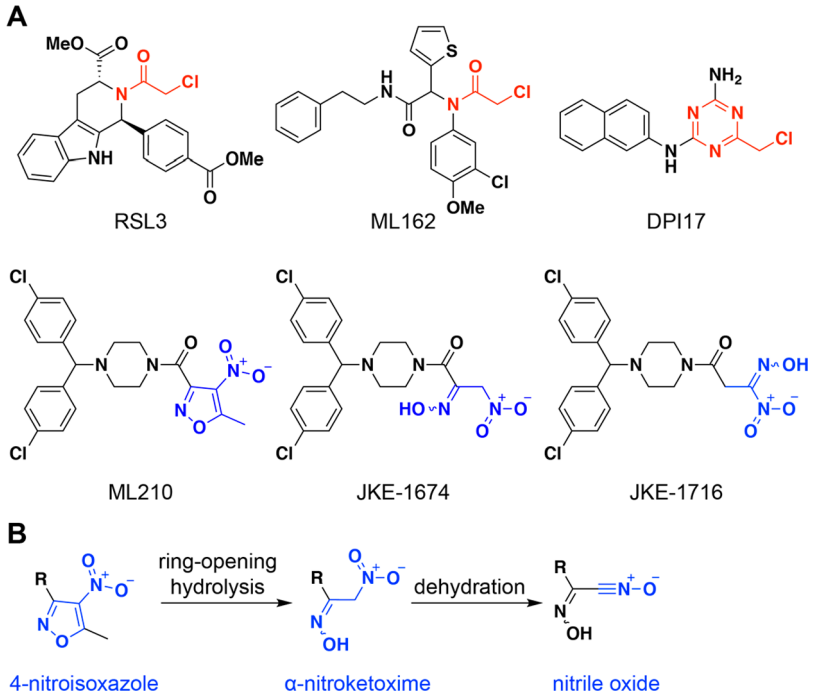
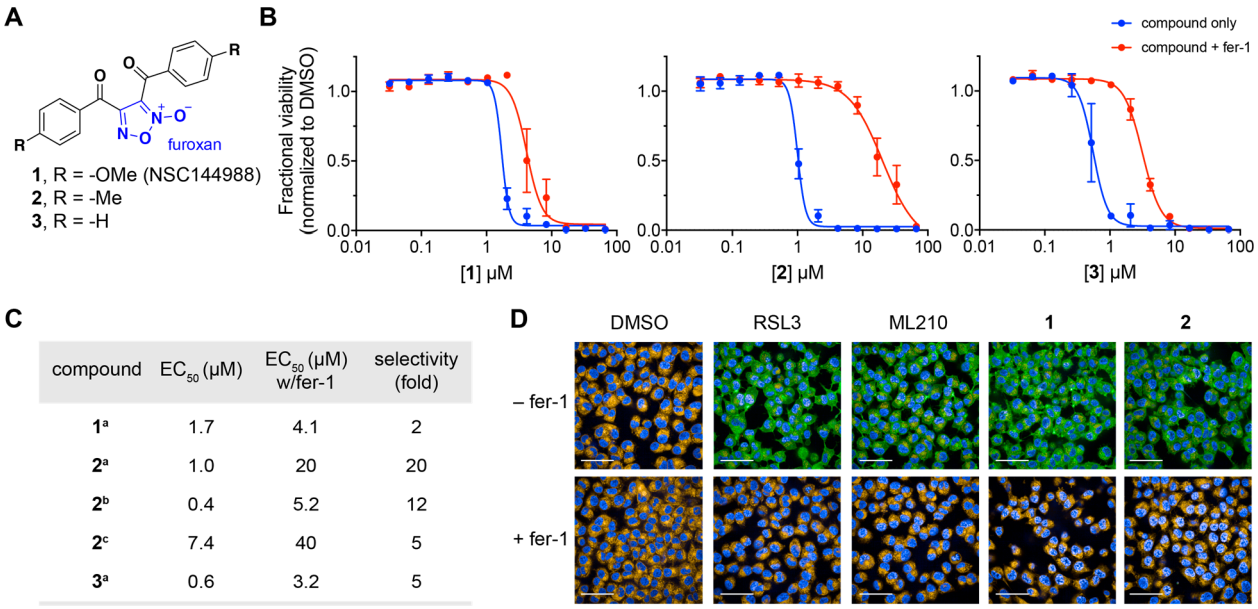



**Figure 1.** Summary of small-molecule GPX4 inhibitors. (A) Structures of selected small-molecule GPX4 inhibitors. Chloroacetamide and chloromethyltriazine groups of RSL3, ML162, and DPI17 are shown in red. Masked nitrile oxide groups of ML210, JKE-1674, and JKE-1716 are shown in blue. (B) Proposed pathway for the unmasking of nitrile oxide electrophiles in cells.




| compound | $EC_{50}$ (μM) | $EC_{50}$ (μM) w/fer-1 | selectivity (fold) |
|---|---|---|---|
| **1**[a] | 1.7 | 4.1 | 2 |
| **2**[a] | 1.0 | 20 | 20 |
| **2**[b] | 0.4 | 5.2 | 12 |
| **2**[c] | 7.4 | 40 | 5 |
| **3**[a] | 0.6 | 3.2 | 5 |

**Figure 2.** Diacylfuroxans induce ferroptosis. (A) Structures of diacylfuroxans **1** (NSC144988), **2**, and **3**. The furoxan group (1,2,5-oxadiazole 2-oxide) is highlighted in blue. (B) Cotreatment with fer-1 (1.5 μM) rescues the cell-killing effects of **1**, **2**, and **3** in LOX-IMVI cells. Data are plotted as the mean ± s.e.m., with $n = 4$ technical replicates. (C) Summary of diacylfuroxan activity and selectivity in cancer cell lines. [a]LOX-IMVI, [b]KP4, and [c]PANC02. See also Figure S1A. (D) Treating cells with GPX4 inhibitors (RSL3 and ML210; 10 μM, 1 h) or diacylfuroxans **1** and **2** (20 μM, 1 h) leads to the accumulation of lipid hydroperoxides as assessed by C11-BODIPY 581/591 fluorescence in LOX-IMVI cells. The C11-BODIPY dye emission shifts from orange to green upon oxidation. Fer-1 cotreatment (1.5 μM) prevents lipid hydroperoxide accumulation. Scale bars, 50 μm. See also Figure S1C.

out that diacylfuroxans may be inhibiting glutathione biosynthesis, we assessed the effects of these compounds on cellular glutathione. We found that treating cells with **1**–**3** did not perturb glutathione levels to the extent expected for glutathione biosynthesis inhibitors such as erastin[1,24] (Figure S1D). We also ruled out the prospect that nitric oxide (NO) donation, a common mechanism of action of furoxan-containing compounds,[25−27] underlies the ferroptosis-inducing activity of **1**–**3** (Figure S2). These observations left open the possibility that diacylfuroxans induce ferroptosis by targeting GPX4 directly.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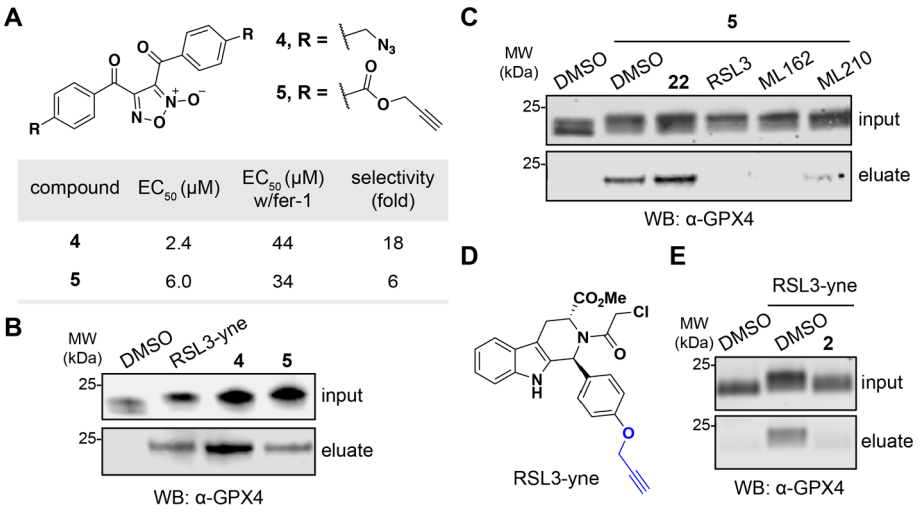


**Figure 3.** Diacylfuroxans target GPX4 covalently. (A) Structures of diacylfuroxan affinity probes **4** and **5** and cellular activity in LOX-IMVI cells. See also Figure S3A,B. (B) GPX4 pulldown by diacylfuroxan affinity probes **4** and **5** (20 $\mu$M, 1 h) after cell treatment. (C) GPX4 pulldown by **5** (20 $\mu$M, 30 min) can be blocked by pretreatment with GPX4 inhibitors (10 $\mu$M, 30 min). (D) Structure of the RSL3-yne affinity probe with the alkyne group shown in blue. (E) Pulldown of GPX4 by RSL3-yne (10 $\mu$M, 30 min) can be blocked by the pretreatment of cells with **2** (20 $\mu$M, 30 min). See also Figure S3C.

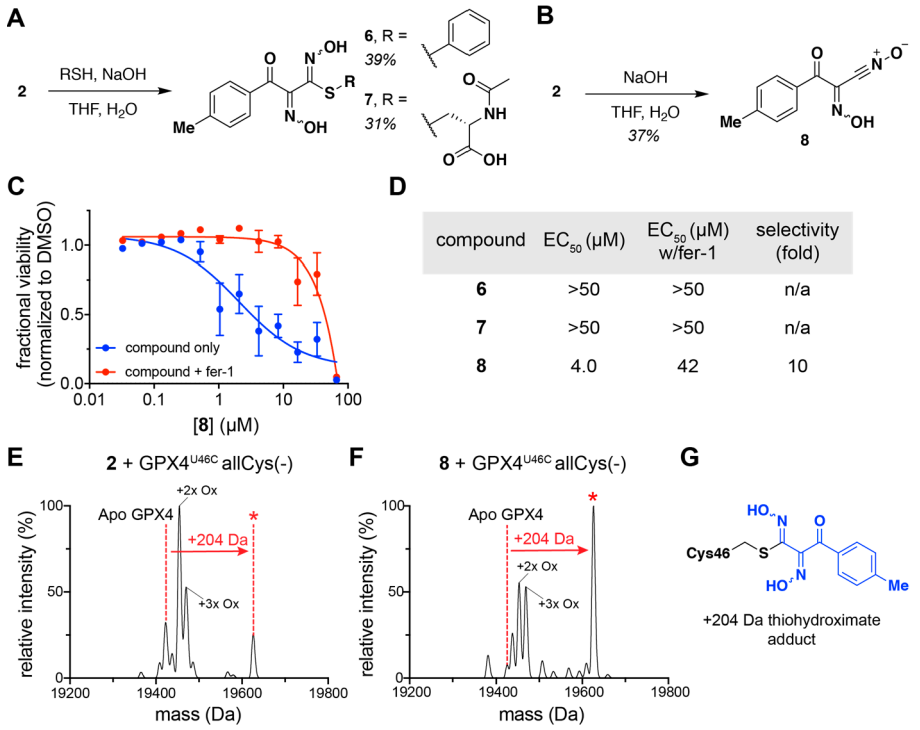


**Figure 4.** Diacylfuroxans bind GPX4 via a nitrile oxide intermediate. (A) Diacylfuroxan **2** reacts with thiols to yield adducts containing a thiohydroximate structure. (B) The hydrolysis of **2** yields nitrile oxide **8** with the loss of 4-methylbenzoic acid. (C) Cotreatment with fer-1 (1.5 $\mu$M) rescues the cell-killing effects of **8** in LOX-IMVI melanoma cells. Data are plotted as the mean ± s.e.m., with $n = 4$ technical replicates. (D and E) Incubation of GPX4$^{U46C}$ allCys(−) (5 $\mu$M) with diacylfuroxan **2** (50 $\mu$M, 1 h) produces a +204 Da adduct based on intact protein mass spectrometry. (F) Incubation of GPX4$^{U46C}$ allCys(−) (5 $\mu$M) with nitrile oxide **8** (50 $\mu$M, 1 h) produces the same +204 Da adduct as **2**. (G) Structure of proposed thiohydroximate adduct derived from **2** and **8**.

**Diacylfuroxans Bind GPX4 Covalently.** To determine if diacylfuroxans induce ferroptosis by binding and inhibiting GPX4 directly, we developed two affinity probe derivatives of **1**−**3** containing azide (**4**) or alkyne (**5**) groups (Figure 3A). These analogs have cellular potency similar to that of **1**−**3** and retain the ability to induce ferroptotic cell death (Figures 3A and S3A,B). Both **4** and **5**, like established GPX4 inhibitor affinity probes,[10] enable the direct pulldown of GPX4 from cells after conjugation to the appropriate alkyne- or azide-biotin reagent via copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC) chemistry,[28] affinity enrichment with solid-supported streptavidin, and visualization by SDS-PAGE and western blotting (Figure 3B). The ability of these probes to pull down GPX4 in this assay under denaturing conditions and to induce a GPX4 gel shift, in a manner similar to that of established irreversible GPX4 inhibitors,[10] is consistent with


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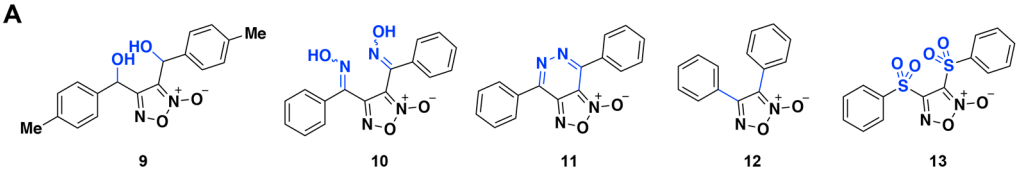
A




B

| compound | $EC_{50}$ (μM) | $EC_{50}$ (μM) w/fer-1 | Selectivity (fold) |
|---|---|---|---|
| **9** | 38 | 38 | 1 |
| **10** | >66 | >66 | n/a |
| **11** | 15 | 15 | 1 |
| **12** | >66 | >66 | n/a |
| **13** | 0.8 | 1.8 | 2 |

C

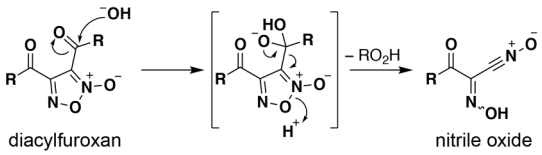



**Figure 5.** Diacylfuroxan structure–activity relationship (SAR) studies. (A) Analogs of diacylfuroxans **2** and **3**. Modifications to the acyl groups are highlighted in blue. (B) Summary of diacylfuroxan and 4-nitroisoxazole activity and selectivity in LOX-IMVI cells. See also Table S1 and Figures S7 and S8. (C) Proposed mechanism for diacylfuroxan hydrolysis to generate a nitrile oxide and carboxylic acid.

diacylfuroxans acting as covalent GPX4 inhibitors. Compound **5** is unable to engage GPX4 in cells pretreated with selenocysteine-binding GPX4 inhibitors[10,12,29] (Figure 3C), suggesting that diacylfuroxans also interact covalently with the selenocysteine residue.

To characterize the interaction between GPX4 and diacylfuroxans further, we performed competitive GPX4 pulldown experiments with RSL3-yne (Figure 3D),[10] an alkyne probe derived from selenocysteine-binding GPX4 inhibitor RSL3.[12,29] We found that the pretreatment of cells with diacylfuroxans blocks GPX4 pulldown by RSL3-yne, which is otherwise able to enrich GPX4 in the absence of competitors (Figures 3E and S3C). These observations confirm the mutually exclusive nature of GPX4 binding by diacylfuroxans and other GPX4 inhibitors and support the fact that the catalytic selenocysteine residue is the binding site for diacylfuroxans.

**Diacylfuroxans Bind GPX4 via a Nitrile Oxide Electrophile.** To investigate the mechanism by which diacylfuroxans covalently bind GPX4, we initially assessed the reactivity of **2** toward nucleophiles. We found that thiols react directly with **2** to form covalent adducts with the concomitant loss of a single 4-toluoyl group, exemplified by thiohydroximate adducts **6** and **7** (Figure 4A). This transformation is strongly suggestive that a nitrile oxide intermediate is the active species derived from **2**, and the structure of these adducts is similar to those formed by the active nitrile oxide electrophile derived from ML210 and related nitrile oxide precursors (e.g., JKE-1674).[10]

We found that the putative nitrile oxide species derived from **2**, compound **8**, could be isolated along with byproduct *p*-toluic acid following the treatment of **2** with aqueous base (Figure 4B). This hydrolysis of **2** occurs in aqueous solutions including PBS buffer (pH 7.4) (Figure S4A) and can be controlled by adjusting the solution pH (Figure S4B). The rate of diacylfuroxan hydrolysis increases with increasing solution basicity but can be suppressed completely in acidic buffers (Figure S4B). We confirmed that other ferroptosis-inducing diacylfuroxans are similarly hydrolyzed (Figure S4C). The isolation of compound **8** is noteworthy because while this type of conversion of diacylfuroxans to nitrile oxides has been inferred from their previously reported reactivity with dipolarophiles[30−32] or nucleophiles,[33] the nitrile oxide intermediates were not observed directly in these cases.

Several observations support that diacylfuroxan **2** reacts via nitrile oxide **8** to inhibit GPX4 and induce ferroptotic cell death. First, **8** exhibits the ability to induce ferroptosis in LOX-IMVI cells with a potency and degree of fer-1 rescue similar to those of **2** (Figure 4C). Second, **8** forms thiol adducts which are identical to those formed by **2** (Figure S5A−C). These adducts are not able to induce ferroptosis in cells (Figures 4D and S5D,E) and therefore do not explain the cellular effects of **2**. Finally, **2**, nitrile oxide **8**, and other diacylfuroxans react with purified $GPX4^{U46C}$ allCys(−) protein (Figure S6A; see Materials and Methods) to produce adducts with masses consistent with thiohydroximate structures (Figures 4E,F and S6). These results strongly suggest that diacylfuroxan-derived nitrile oxides bind the catalytic (seleno)cysteine 46 residue of GPX4 because $GPX4^{U46C}$ allCys(−) contains only a single cysteine residue at this position. The observation that GPX4 binding by diacylfuroxans and established (seleno)cysteine-binding inhibitors is mutually exclusive (Figure 3C,E) further supports that the active-site (seleno)cysteine residue interacts with diacylfuroxan-derived nitrile oxides covalently.

**Structure–Activity Relationship (SAR) Studies of Diacylfuroxans.** Although compounds containing furoxan heterocycles are known to be reactive in biological contexts,[25,26] the ability of diacylfuroxans to act as masked nitrile oxides has not been described previously. Structure–activity relationship (SAR) studies of diacylfuroxans (Figure 5A,B and Table S1) revealed the critical role of the acyl groups during the transformation of diacylfuroxans into nitrile oxide electrophiles. We found that a variety of diacyl-substituted furoxans (**15**, **17**−**20**) exhibit the ability to induce ferroptosis (Table S1) by targeting GPX4 (Figure S6G). In contrast, furoxan analogs lacking acyl groups (**9**−**12**) (Figure 5A) show no ability to target GPX4 as determined by cellular lipid peroxidation and ferroptotic cell death assays (Figures 5B and S7). One exception to this trend is structurally similar disulfonylfuroxan **13** (Figure 5A), which we found binds GPX4 covalently (Figure S8A,B) and rapidly depletes cellular glutathione levels (Figure S8C). Despite these observations, **13** reacts in a manner chemically distinct from that of diacylfuroxans and does not involve a nitrile oxide intermediate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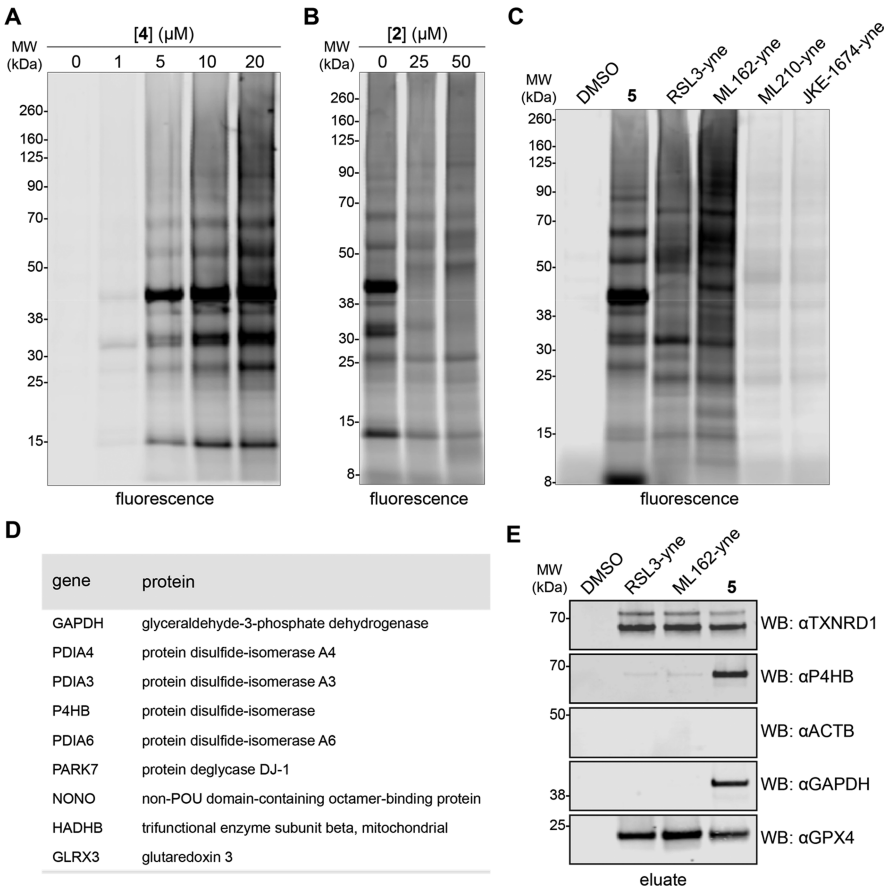


**Figure 6.** Chemoproteomic characterization of diacylfuroxans. (A) Assessment of the proteome-wide reactivity of **4** in LOX-IMVI cells by ABPP (1 h treatment). See Figure S10B for an assessment of probe **5**. (B) Pretreatment of LOX-IMVI cells with **2** (30 min) blocks, in a concentration-dependent manner, the labeling of proteins by **4** (5 $\mu$M, 30 min). See Figure S10D for an assessment of probe **5**. (C) Comparison of cellular reactivity profiles of GPX4-inhibitor probes (10 $\mu$M, 1 h) in LOX-IMVI cells. See also Figure S11A–C. (D) Proteins enriched specifically by diacylfuroxan **5**. See also Table S2. Listed proteins were enriched ≥5-fold vs the vehicle (DMSO) and ≥5-fold vs both RSL3-yne and ML162-yne. (E) Validation of selected proteins targets of **5** by western blotting. See also Figure S12.

(Figure S8D).[34,35] The ability of **13** to promote lipid peroxidation and kill cells cannot be suppressed efficiently by fer-1 cotreatment (Figure S8E,F), consistent with its extensive and nonselective proteome-wide reactivity (Figure S8G). These findings highlight the diverse reactivity that is possible for furoxans and reinforce the unique ability of diacylfuroxans among this class of heterocycles to act as masked nitrile oxides (Figure 5C).

Another finding from our SAR studies is that 4-nitroisoxazoles **21**–**23** (Figure S9A), which are isomeric with diacylfuroxans **1**–**3**, respectively, are unable to target GPX4 or induce ferroptosis as assessed by competitive binding experiments (Figure S3C), cell viability assessments with fer-1 cotreatment (Figure S9B), and lipid peroxidation measurements (Figure S9C). These 4-nitroisoxazoles arise as byproducts from common procedures used to synthesize diacylfuroxans (Figure S9D),[36,37] and their inability to induce ferroptosis is notable given the structural similarity with nitroisoxazole-containing ML210. The inactivity of **21**–**23** may reflect the poorly understood SAR trends underlying the cellular transformation of 4-nitroisoxazoles into GPX4-targeting nitrile oxides. These observations also underscore the different chemical mechanisms involved in the unmasking of 4-nitroisoxazole and diacylfuroxan heterocycles to reveal nitrile oxide electrophiles in cells.

**Diacylfuroxans Exhibit Distinct Proteome-Wide Reactivity in Cells Compared to Other GPX4 Inhibitors.** Our findings reveal that diacylfuroxans are readily converted into electrophilic nitrile oxides with high reactivity toward thiol and selenol nucleophiles. Because of this reactivity, we expected that diacylfuroxans would interact with many proteins in cells beyond GPX4. To explore the cellular proteome-wide reactivity of this compound class, we used diacylfuroxan affinity probes **4** and **5** to perform activity-based protein profiling (ABPP)[38] experiments. These experiments involve the treatment of intact cells or cell lysates with affinity probes, subsequent CuAAC conjugation with azide- or alkyne-bearing fluorescent dyes, and the visualization of probe-labeled proteins by fluorescence imaging of SDS-PAGE gels. We observed that both **4** (Figures 6A and S10A) and **5** (Figure S10B) label cellular proteins in a dose-dependent manner and that their overall patterns of labeling are similar. The labeling of several prominent proteins (e.g., at ∼42 kDa) could be blocked by the pretreatment of cells with **2**, suggesting specific binding interactions,[39] while other proteins were insensitive to pretreatment with **2**, indicating nonspecific protein labeling (Figures 6B and S7C,D).

Diacylfuroxan **5** exhibits a distinct pattern of proteome reactivity in cells (Figures 6C and S11B,C) compared to chloroacetamide (RSL3-yne, ML162-yne) and ML210-family


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probes (ML210-yne, JKE-1674-yne) (Figure S11A). Competitive labeling experiments further corroborate that the major protein targets of **5** are not shared with other GPX4 inhibitors (Figure S11D). To identify the protein targets of **5**, RSL3-yne, and ML162-yne in cells, we performed mass spectrometry (MS)-based chemoproteomics experiments (Figures 6D and S12A and Table S2). Although we were unable to detect GPX4 in these mass spectrometry- and gel-based ABPP studies, likely because of the relatively low expression of this protein, we confirmed the enrichment of GPX4 in the same samples by western blotting (Figures 6E and S12B).

Notably, nearly all proteins enriched by **5** (Figure 6D, Table S2) are known to contain ligandable (seleno)cysteine residues,[29,40,41] and we validated a subset of these proteins by western blotting (Figures 6E and S12B,C). The top protein enriched specifically by **5**, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ∼42 kDa band in Figure 6A−C), likely reacts via its catalytic cysteine residue, which has been previously identified as ligandable[40] and is known to bind the covalent inhibitor koningic acid.[42] Other highly enriched proteins identified as unique targets of **5** are members of the protein disulfide-isomerase (PDI) family, including PDIA4, PDIA3, P4HB (PDI), and PDIA6 (Figure 6D and Table S2), which contain multiple ligandable cysteine residues.[40] Although PDI is a known target of both RSL3[1] and structurally similar compounds,[43] our findings indicate that **4** and **5** interact with PDI to a greater extent compared to other GPX4 inhibitors. The prevalence of (seleno)cysteine-containing proteins enriched by **5** suggests that proteins are bound covalently by a **5**-derived nitrile oxide to form seleno- or thiohydroximate adducts in a manner consistent with our diacylfuroxan reactivity studies with GPX4. It is possible that some proteome-wide reactivity is due to different chemical reactions of diacylfuroxans (e.g., protein benzoylation) or the labeling of non(seleno)cysteine residues, although we found no examples of such reactivity.

Despite the unique cellular reactivity profile of diacylfuroxans, there are common targets with chloroacetamide-based probes that further reinforce the expected (seleno)cysteine reactivity of diacylfuroxans. For example, probes **4**, **5**, RSL3-yne, and ML162-yne enrich selenoprotein thioredoxin reductase 1 (TXNRD1) (Figure S12B,C). A recent report identified the TXNRD1 selenocysteine residue as the binding site of RSL3,[29] an observation that indicates that **4**, **5**, and ML162-yne may also bind at this site. Similarly, diacylfuroxan probes enrich glutathione *S*-transferase omega 1 (GSTO1) (Figure S12C), which binds ML162 and other chloroacetamides via its active-site cysteine residue.[44,45] This overlap between targets of diacylfuroxans and chloroacetamide-based GPX4 inhibitors further supports that the cellular reactivity of diacylfuroxans is due to the binding of the (seleno)cysteine residue via their active nitrile oxide forms.

**Diacylfuroxans Exhibit Distinct Proteome-Wide Reactivity in Cell Lysates Compared to Other GPX4 Inhibitors.** ABPP experiments in cell lysates highlight another distinct reactivity of diacylfuroxans compared to other classes of GPX4 inhibitors. Although diacylfuroxan **5** exhibits comparable proteome labeling in both cells and cell lysates, chloroacetamide- and ML210-derived probes display reactivity in cell lysates that is diminished compared to their reactivity in intact cells (Figure S13A). The discrepancy between compound reactivity in intact cells versus lysates is especially notable for RSL3-yne which, like prodrugs ML210-yne and JKE-1674-yne, does not interact readily with GPX4 in the cell lysate under the assay conditions (Figure S13B) despite the greater cellular potency of RSL3-yne (>100-fold) compared to that of **5** (Figure S13C). We found previously that RSL3 exhibits biochemical activity that is weaker than expected given its cellular potency, including reactivity toward GSH and the ability to bind wild-type GPX4 protein.[10] Consistent with our findings, a recent study reported that RSL3 does not efficiently bind purified GPX4 without the assistance of an adaptor protein.[46] These observations highlight the limitations of established small-molecule GPX4 inhibitors as biochemical probes and suggest that **5** and other diacylfuroxans may have unique advantages due to their greater ability to react with GPX4 outside of the cellular context.

## DISCUSSION

We have discovered that diacylfuroxans can act as masked nitrile oxide electrophiles to target GPX4 covalently and induce ferroptotic cell death. Our discovery illuminates diacylfuroxans as a novel class of masked electrophilic warheads with unique reactivity properties, both biochemically and in intact cells, making them useful tool compounds for studying GPX4. The ability to study the effects that small-molecule inhibitors have on GPX4 has been hindered by both the difficulty of obtaining selenocysteine-containing wild-type GPX4 protein[3,10,47] and the lack of structurally diverse tool compounds with activity in biochemical assays. Because diacylfuroxans interact more readily with GPX4 outside of the cellular context compared to established inhibitors (e.g., RSL3 and ML210), they may enable the determination of cocrystal structures with GPX4 to better understand the structural basis of GPX4-inhibitor binding. Such information may help to elucidate the discrepancy we have noted between the potency of GPX4 inhibitors in cellular versus biochemical contexts and may provide useful information for the rational design of improved GPX4 inhibitors.

On the basis of our investigation of diacylfuroxans as well as our previous studies of ML210,[10] it appears that the masking elements of prodrug nitrile oxides do not necessarily contribute directly to their selectivity in cells. Rather, these features confer nitrile oxides, which are inherently unstable due to multiple decomposition pathways,[10,48,49] with the stability necessary for routine use in biochemical and cellular experiments. This is similar conceptually to the masked covalent inhibitor omeprazole and related compounds, which are prodrug proton-pump inhibitors requiring conversion to their active sulfenamide and sulfenic acid forms under acidic conditions.[50−52] Like nitrile oxides, the decomposition of these sulfenamides and sulfenic acids yields a mixture of inactive products,[50,53] and it would not be possible to deliver these compounds if not for their more stable masked prodrug forms.

A variety of structurally diverse masked electrophiles have been reported previously,[54−59] many of which rely on nonenzymatic activation, such as diacylfuroxans and omeprazole. However, the enzymatic formation of nitrile oxides has been reported in the context of drug metabolism[60] and natural product biosynthesis,[61,62] suggesting that there may be additional strategies for developing prodrugs that act via nitrile oxides in cells. More broadly, diverse methods for masking the reactivity of nitrile oxides may enable the development of improved covalent inhibitors of GPX4 and other proteins.

Taken together, our observations of diacylfuroxans reinforce our recent discovery that masked nitrile oxides may be well


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suited for targeting GPX4 and broaden the scope of specific chemotypes available for delivering highly reactive nitrile oxide electrophiles to cells. The unique *in situ* reactivity and structural diversity of masked nitrile oxides including 4-nitroisoxazoles, $\alpha$-nitroketoximes, nitrolic acids, and now diacylfuroxans, warrant additional study and exploration as electrophilic probes of cellular circuitry.

## CONCLUSIONS

Diacylfuroxans represent a new chemotype capable of targeting GPX4 in cells to induce ferroptosis. Mechanistically, diacylfuroxans act as masked nitrile oxides that are activated under aqueous conditions. Compared to established small-molecule GPX4 inhibitors, diacylfuroxans possess unique reactivity properties in biochemical and cellular contexts. We anticipate that diacylfuroxans and other masked nitrile oxides will serve as promising chemical tools for the study of GPX4 and other protein targets.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.9b10769.

Materials and methods, figures and tables, and NMR spectra (PDF)

Label-free quantification of proteins enriched from LOX-IMVI cells and summary of proteins enriched selectively by diacylfuroxan **5** (XLSX)

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### Notes

The authors declare the following competing financial interest(s): S.L.S. is a member of the Board of Directors of the Genomics Institute of the Novartis Research Foundation (GNF); a shareholder and member of the Board of Directors of Jnana Therapeutics; a shareholder of Forma Therapeutics; a shareholder of and adviser to Decibel Therapeutics and Eikonizo Therapeutics; an adviser to Eisai, Inc., the Ono Pharma Foundation, and F-Prime Capital Partners; and a Novartis Faculty Scholar.

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