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# Five-Membered Ring Peroxide Selectively Initiates Ferroptosis in Cancer Cells

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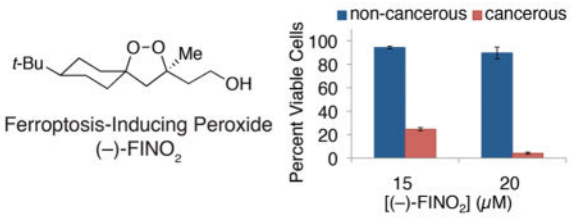
## Abstract

A 1,2-dioxolane ($FINO_2$) was identified as a lead compound from a screen of organic peroxides. $FINO_2$ does not induce apoptosis, but instead initiates ferroptosis, an iron-dependent, oxidative cell death pathway. Few compounds are known to induce primarily ferroptosis. In contrast to the perceived instability of peroxides, $FINO_2$ was found to be thermally stable to at least 150 °C. $FINO_2$ was more potent in cancer cells than nonmalignant cells of the same type. One of the enantiomers was found to be more responsible for the observed activity.

## Graphical Abstract




In the United States, cancer is the primary cause of death in ages 40–79 and the second leading cause of death overall, making the need for the development of new drugs a necessity.[1,2] Conventional agents used in cancer therapy operate by a variety of mechanisms, but the vast majority induce cell death by initiating the mitochondrial pathway of apoptosis.[3,4] This common pathway of cell death, while effective for many tumors, is associated with resistance mechanisms, such as modulation of the expression levels of the BCL-2 family of proteins or mutation of the p53 protein, that limit therapeutic efficacy.[3–6]

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Supporting Information

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Methods, synthetic procedures, and spectra (PDF)

X-ray crystallographic data (CIF)

Screening libraries of compounds can identify drugs with new mechanisms for the treatment of cancer. A potential problem with this method is that restrictions on the types of compounds included in libraries may limit the possible agents identified. To reduce the risk of mistaking generally reactive and unselective compounds as leads for further development, functional groups that are deemed undesirable are often excluded from these screens.[7,8] A class of compounds largely excluded in screening platforms due to perceived instability are the organic peroxides.[7–9] Peroxides continue to be excluded even though the World Health Organization recommends derivatives of the peroxide artemisinin (Figure 1a), such as artesunate, in combination therapies for the treatment of uncomplicated malaria.[10] Artemisinin, as well as several derivatives including artesunate, are under current investigation as potential anticancer agents.[11] A clinical trial in which colorectal cancer patients were treated with artesunate prior to curative resection showed that artesunate was well tolerated and had antiproliferative effects.[12] An active phase I clinical trial has the goal of establishing safety and maximum tolerated dose of artesunate for treatment of patients with solid tumors (NCT02353026).

To determine if organic peroxides with structures dissimilar from the artemisinin family (Figure 1a) could be anticancer agents,[13,14] we investigated peroxides containing the 1,2-dioxolane scaffold. This scaffold is found in only a few compounds in nature, such as the plakinic acids[14] (Figure 1c). The plakinic acids exhibit cytotoxicity toward a few cancer cell lines, but their mechanism of action is unknown.[14] The minimal availability of the plakinic acids from the source from which they were isolated, a sponge of the genus *Plakortis*, limits the quantity of these compounds. Consequently, chemical synthesis was necessary to study the anticancer activity of compounds containing the 1,2-dioxolane scaffold. In this article, we report the first 1,2-dioxolane-containing compound subjected to extensive mechanism of action studies in cancer. This 1,2-dioxolane targets cancer cells by a mechanism distinct from standard chemotherapy drugs. Using a B-lymphoblastic cell leukemia line, RS4;11,[15] as a model system, we provide evidence indicating that the 1,2-dioxolane under investigation induces ferroptosis, a nonapoptotic, iron- and reactive oxygen species-dependent form of cell death.[16] Furthermore, *in vitro* this compound is capable of bypassing common chemoresistance pathways such as mutation of p53 and modulation of the expression levels of the BCL-2 proteins.

## RESULTS AND DISCUSSION

### Identification of a 1,2-Dioxolane As a Lead Compound

Several 1,2-dioxolanes with structures resembling the plakinic acids were prepared[17,18] and submitted for testing at the Developmental Therapeutics Program of the National Cancer Institute (NCI) through the In vitro Cell Line Screening Project. Examination of the cytotoxicity profiles of the 1,2-dioxolanes identified a lead compound for further investigation. Keeping with the nomenclature for ferroptosis-inducing compounds as “FIN” compounds,[19] the 1,2-dioxolane in our studies will be referred to as $FINO_2$, for ferroptosis-inducing peroxide. $FINO_2$’s cytotoxicity is not limited to one tissue of origin (see Supporting Information Figure 1 for sensitivity across the NCI cancer cell line panel). The lowest concentration of $FINO_2$ to cause 50% growth inhibition ($GI_{50}$) was observed in an

ovarian cancer line, IGROV-1 ($GI_{50}$ 435 nM), and the highest in a lung cancer line, NCI-H322 M ($GI_{50}$ 42 $\mu$M). Eighteen out of the 59 cell lines tested showed a $GI_{50}$ below 2.5 $\mu$M, and the average $GI_{50}$ across all cell lines was 5.8 $\mu$M. By comparison, many successful chemotherapeutic agents display average activity across the NCI60 in the micromolar range.[20,21] For example, out of 27 Federal Drug Administration (FDA)-approved DNA-damaging agents, 24 possess mean $GI_{50}$ potency in the micromolar range.[20,21]

Metrics other than $GI_{50}$ potency are important when determining the suitability of a lead compound, such as the concentration required to achieve complete cell death and the steepness of the dose–response curve.[22] Because many FDA-approved chemotherapy agents have been tested in the NCI60 and the data are publicly available,[21,23] the average cytotoxicity of these compounds can be compared to potential lead compounds.[24,25] We performed this analysis with respect to $FINO_2$ (Figure 2). When comparing the average concentration needed to induce 50% lethality ($LC_{50}$) instead of $GI_{50}$ (Figure 2a), $FINO_2$ has similar potency (average $LC_{50}$ 46 $\mu$M) to conventional chemotherapeutic agents such as paclitaxel (75 $\mu$M), doxorubicin (13 $\mu$M), and vorinostat (70 $\mu$M).[21] The small difference in concentrations of $FINO_2$ required to cause growth inhibition versus that required to kill the cells suggests $FINO_2$ has a steep dose–response curve. Even though the traditional chemotherapy agents shown have more potent growth inhibition numbers, the distribution of growth inhibition activity across all cell lines is comparable to $FINO_2$.

The CellMiner program[23,26] was used to compare $FINO_2$ to other agents tested in the NCI60. Using the $GI_{50}$ data of several FDA-approved drugs, the averages, standard deviations ($\sigma$), and coefficient of variation ($C_V$, standard deviation divided by the mean) were calculated (Figure 2b). The coefficient of variation, a representation of the relative variation within a data set, shows that, out of the compounds analyzed, doxorubicin displays the most variability in its effect on different cell lines and sorafenib displays the least variability, with $FINO_2$ falling in the middle of this list (Figure 2b). Additional analysis using the CellMiner program indicated no significant correlations between $FINO_2$ and the activity of other compounds available through the database or between the activity of $FINO_2$ and the available gene transcript levels.

Having identified $FINO_2$ as a candidate for further study, it was necessary to establish its safety and selectivity. The previously reported synthesis[17] was optimized to obtain gram quantities of $FINO_2$ (Supporting Information Figure 2a). To address potential stability concerns on the use of organic peroxides as drugs, $FINO_2$ was subjected to thermal stress and demonstrated stability to at least 150 °C (Supporting Information Figure 2b). One system to assess the selectivity index of drug candidates as anticancer agents compares the effect of compounds in cancerous vs noncancerous cells of the same type.[27,28] This approach uses a defined model of oncogenic transformation to allow for direct comparison of a noncancerous, immortalized fibroblast cell line (BJ-hTERT) with the same cell line transformed to possess a cancerous phenotype (BJ-ELR).[29] This transformation was accomplished by the introduction of Simian Virus 40 large and small T oncoproteins and an oncogenic allele of *HRAS*.[29] Despite the observation that the potency of $FINO_2$ against tumor line BJ-ELR was higher than the average $GI_{50}$ for the NCI60, treatment with $FINO_2$ had a larger effect on cell viability of the tumor vs the nontumor cell lines (Figure 3a). The

steep dose–response curve suggested by the NCI data is clearly indicated by the dramatic decrease in cancer cell viability over only a few micromolar differences. Viability continues to decrease over time in the cancerous cells, but not in the noncancerous cells (Supporting Information Figure 3). This and all experiments were performed in triplicate, and error bars reflect the standard deviation of one experiment. A representative graph of at least three independent experiments is shown.

The synthesis of $FINO_2$ produces a racemic mixture,[17] so we devised a route to separate the enantiomers so they could be tested individually in the selectivity assay. Oxidation of the pendant hydroxyl group on $FINO_2$ to the carboxylate allowed for coupling to a chiral auxiliary.[30] The resulting diastereomers could then be separated. Because one of the diastereomers was crystalline, X-ray crystallography was employed to establish the absolute stereochemistry of the two isomers. Reductive removal of the auxiliary provided the two enantiomerically pure forms of $FINO_2$ (Figure 3b). The (*S*) enantiomer, (−)-$FINO_2$, was found to be more responsible for the observed activity and selectivity of $FINO_2$ for cancerous fibroblasts (Figure 3c). The steep dose–response curve was again observed with both enantiomers of $FINO_2$. The increased activity of one enantiomer suggests that $FINO_2$ is not simply degrading in an unregulated fashion to form hydrogen peroxide or some other reactive oxygen species (ROS) but instead might have a specific protein interaction necessary for its activity.[31] This observation is in contrast to the report that enantiomers of compounds possessing the same core as artemisnin (a 1,2,4-trioxane) display equivalent *in vitro* activity against malaria parasites.[32]

## $FINO_2$ Induces a Nonapoptotic Form of Cell Death with Features Characteristic of Ferroptosis

Several experiments were performed to discount the mitochondrial apoptotic pathway as a mechanism of cell death induced by $FINO_2$. No increase in Annexin V staining was observed prior to 7-aminoactinomycin D (7AAD) incorporation (Figure 4a), indicating that phosphatidylserine is not exposed to the outer leaflet of the cell membrane upon treatment with $FINO_2$, as would be expected if apoptosis occurred (Supporting Information Figure 4).[33] The BioVision MitoCapture Mitochondrial Apoptosis Detection Kit was used to measure the occurrence of mitochondrial outer membrane permeabilization (MOMP). While a shift in fluorescence indicated that MOMP had occurred, this shift occurred only after the majority of cells had died, not before (Supporting Information Figure 5), suggesting MOMP is not necessary for $FINO_2$ to induce cell death. This result was confirmed by overexpression of the Bcl-2 protein to prevent MOMP. Unlike many conventional chemotherapy drugs,[5] such as etoposide (Figure 4b), overexpression of the Bcl-2 protein had no effect on $FINO_2$'s ability to induce cell death (Figure 4b and Supporting Information Figure 6). Further experiments showed that caspase activation is not involved in the cell death pathway induced by $FINO_2$, as would be expected for apoptosis (Supporting Information Figure 7).[34] The steep dose–response curve observed in the fibroblast cells was even more pronounced in these mechanistic studies (performed in a leukemia cell line), which were quantified by cell death, as opposed to cell viability.

The nomenclature committee on cell death suggests that apoptosis remain a morphological description, as it was first defined.[35–37] Therefore, electron microscopy was performed to elucidate the cell death pathway caused by $FINO_2$. Electron microscopy images showed no morphological features characteristic of apoptosis (chromatin condensation, fragmentation of the nucleus, and plasma membrane blebbing), necrosis (plasma membrane rupture), or autophagy (formation of double membrane vacuoles in the cytoplasm, Figure 5a and Supporting Information Figure 8).[37] The absence of these features indicates $FINO_2$ is initiating an alternative form of cell death. Having ruled out the apoptotic pathway, it was then possible to consider alternative forms of cell death.

Additional experiments suggest that cell death is due to ferroptosis. Ferroptosis is a nonapoptotic form of cell death that is induced by the accumulation of lipid peroxides.[16] This form of cell death is known to occur in response to a limited number of compounds that inactivate the essential selenium-dependent antioxidant enzyme, glutathione peroxidase IV (GPX4).[19,38–41] General oxidative stress upon treatment with $FINO_2$ was indicated by use of a fluorogenic probe, CellROX Green Reagent, that emits at 520 nm upon oxidation and binding to DNA (Figure 5b). This effect was observed about 6 h after the addition of $FINO_2$ but before significant cell death had occurred. To detect for the formation of lipid peroxides in response to $FINO_2$, the membrane-targeted lipid peroxidation detection reagent, C11-BODIPY, was utilized.[42] An increase in lipid peroxidation upon treatment with $FINO_2$ (Figure 5c) indicated that oxidative stress in the lipid membrane occurred before death. A time-course experiment with $FINO_2$ using both the CellROX and C11-BODIPY probes ensured that $FINO_2$ did not interact directly with the probes, but that $FINO_2$ did initiate an increase in cellular oxidative stress (Supporting Information Figure 9). In contrast, no oxidative stress occurred upon treatment with the control compound, etoposide. As observed for other ferroptosis-inducing compounds, iron is necessary for $FINO_2$'s mechanism of action. After pretreatment with an iron chelator, deferoxamine (DFO), $FINO_2$ did not cause cell death. By contrast, the addition of exogenous iron, as ferric ammonium citrate (FAC), accelerated cell death by $FINO_2$ beyond what was observed in control experiments (Figure 5d).

Potent inhibition of cell death was observed upon pretreatment with lipophilic antioxidants that inhibit ferroptosis, ferrostatin[16,43] and liproxstatin[41] (Figure 5e and f). While water-soluble antioxidants do not inhibit all forms of ferroptosis,[19] these compounds prevent cell death by eliminating lipid peroxides.[43] Because the lipid peroxides implicated specifically in ferroptotic cell death are produced by the oxidation of arachidonic acid,[40] the involvement of arachidonic acid metabolism in response to $FINO_2$ treatment was examined. The addition of arachidonic acid increased the potency of $FINO_2$ (Supporting Information Figure 10). The broad-spectrum lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), completely inhibited $FINO_2$-induced cell death (Figure 5g), just as it inhibits the induction of ferroptosis by a knockout of glutathione peroxidase IV (GPX4).[40] Partial inhibition of cell death induced by $FINO_2$ was observed by treatment with indomethacin, a broad-spectrum cyclooxygenase inhibitor (Figure 5h). By contrast, a protective effect by indomethacin treatment was not observed with previously reported inducers of ferroptosis.[19,40]

Because the mechanism of cell death induced by $FINO_2$,ferroptosis, bypasses Bcl-2 expression levels, and the mitochondrial pathway as a whole, this mechanism could be used as a platform for identifying efficacious agents when patients are refractory to other treatments due to modulation of the mitochondrial pathway of apoptosis.[4,5] The *BCL2* gene was identified as one of six genes that can be used to predict overall survival in patients with B-cell lymphoma.[44] Additionally, Bcl-2 protein expression was found to be an independent prognostic marker in triple negative breast cancer.[45] Several compounds that aim to inhibit Bcl-2 are in clinical trials,[46,47] but other BCL-2 proteins such as Mcl-2 can compensate for Bcl-2 inhibition, limiting the effectiveness of these therapies.[48]

Resistance to chemotherapeutic agents can also occur because many agents depend on the utilization of p53 in the apoptotic process.[49] Wild type p53 cells respond more effectively than mutant p53 cells to many chemotherapies and radiation therapy.[50] About 50% of cancers, however, harbor a mutation in the *TP53* gene,[51] leading to the transcription of a nonfunctional, mutant p53 protein. The absence of functional p53 makes establishing a successful treatment regimen for these patients more difficult.[6] Upon further examination of the screening data from the NCI, it was noted that $FINO_2$ was cytotoxic toward several cell lines with altered p53 status.[52] For example, the $GI_{50}$'s for mutant p53-expressing nonsmall cell lung cancer cells, NCI-H522 and HOP-92, and the p53-null leukemia cells,[52] HL-60, are 1.2 $\mu$M, 1.4 $\mu$M, and 2.2 $\mu$M, respectively. These observations are consistent with the fact that treatment with $FINO_2$ did not increase p53 protein expression even in cells expressing wild type p53 (Supporting Information Figure 11).

Compounds that induce ferroptosis are proposed to be selective because many cancer cells have an increased concentration of iron relative to healthy cells.[16,53,54] Ferroptosis was identified by screening compounds selective for engineered tumor cells but not their nonmalignant counterparts.[28] The tumor cells were created using serial introduction of genetic elements necessary to induce a cancerous phenotype.[29] Compounds that were selective due to an increased rate of proliferation exhibited an incremental increase in cytotoxicity with the introduction of each genetic mutation. The compound that induced ferroptosis, however, only caused an increase in cytotoxicity upon introduction of all genetic elements necessary for malignant transformation.[28] Therefore, it was hypothesized that a factor other than the increased proliferation rate of the BJ-ELR cells was causing the observed selectivity of ferroptosis-inducing agents. Upon examination of the iron levels of each cell type, it was discovered that the cancerous BJ-ELR cells have an increased iron content relative to not only the noncancerous BJ-hTERT cells but also the noncancerous cells lacking only the final mutation necessary to induce a cancerous phenotype (cells possessing Simian Virus 40 large and small T oncoproteins but not an oncogenic allele of *HRAS*).[54] These observations suggest the relative levels of iron in different cell types could be the cause of the observed selectivity of $FINO_2$ and other ferroptosis-inducing compounds.

Ferroptosis is dependent on the oxidation of arachidonic acid by the iron-dependent lipoxygenase enzymes.[41,55] The products of the lipoxygenase reactions can be further processed to produce the leukotrienes,[55,56] while excess hydroperoxide accumulation is controlled by glutathione peroxidase IV (GPX4). GPX4 is responsible for reducing these peroxides to keep their concentration at an appropriate level for cell homeostasis.[57] Direct

inhibition of GPX4 by agents such as RSL3[19,54] leads to a toxic accumulation of lipid peroxides, resulting in ferroptosis. Another ferroptosis-inducing compound, erastin,[19] is proposed to cause depletion in glutathione levels by inhibiting cystine uptake, a pathway that can be sensitized by both wild type and mutant p53.[58] Because glutathione is necessary for GPX4's antioxidant activity, erastin indirectly causes GPX4 inhibition, resulting in the enzyme's inability to reduce lipid peroxides effectively.[59] As would be expected for a compound that induced ferroptosis, cell death induced by $FINO_2$ was inhibited by an iron chelator and a broad-spectrum lipoxygenase inhibitor, nordihydroguaiaretic acid. These results suggest the lipoxgenase enzymes play a key role in cell death caused by $FINO_2$.

None of the ferroptosis-inducing compounds identified from a high-throughput screen contained the peroxide functionality;[28,54] however, in two recent reports, the authors demonstrate that, in some contexts, artemisinin compounds are capable of inducing ferroptosis.[60,61] $FINO_2$ and artemisinin share many common characteristics: they both have a requirement of iron for cell death to occur, involve the generation of ROS, and are inhibited by lipophilic antioxidants such as ferrostatin.[60–63] There is evidence, however, that artemisinin can be dependent on MOMP and caspase activation.[62,64–66] Additionally, the artemisinin family of compounds induce G1 cell cycle arrest.[67,68] Cell cycle analysis revealed no change in distribution upon treatment with $FINO_2$, however (Supporting Information Figure 12). Using the CellMiner program to analyze the NCI60 data, it was determined that there was no correlation between the activity of $FINO_2$ and artesunate (−0.478) or dihydroartemisinin (−0.455). In contrast, there is a high level of correlation between artesunate and dihydroartemisinin (0.802). Another noteworthy difference in biological activity of $FINO_2$ and artemisinin compounds is that while $FINO_2$ is more potent in cancer cell lines (AVG $GI_{50}$ of 5.8 $\mu$M) than artemisinin (AVG $GI_{50}$ of 93 $\mu$M) or artesunate (AVG $GI_{50}$ of 12 $\mu$M), artemisinin is more potent (~4 nM) against the malaria parasite than a closely related derivative of $FINO_2$ (~200 nM).[69]

In conclusion, we have identified a small molecule endoperoxide that warrants further investigation for the potential treatment of cancer. Our results indicate that the peroxide functional group may have broader applications to the treatment of disease than previously suggested. We show that peroxide scaffolds outside that of the artemisinin family may exhibit similar stability profiles, suggesting this class of compounds should not be immediately excluded from investigation due to suspected, but not experimentally verifiable, stability issues.[7–9] $FINO_2$ shows selectivity for cancer cells, suggesting that compounds of this kind may be a clinically relevant treatment strategy to pursue. The nonapoptotic mechanism of cell death induced by $FINO_2$ is a desirable feature for the treatment of tumors that have limited response to other chemotherapeutic agents due to either the presence of nonfunctional p53 or inhibition of the mitochondrial pathway of apoptosis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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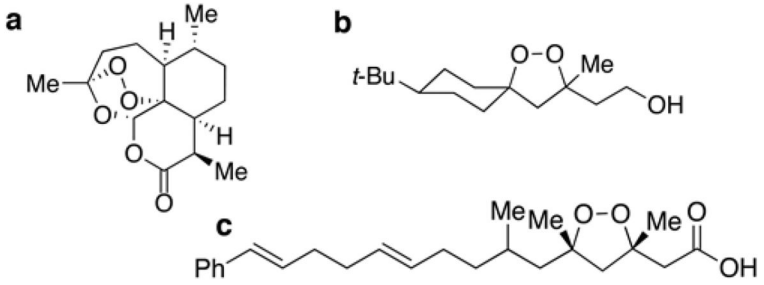
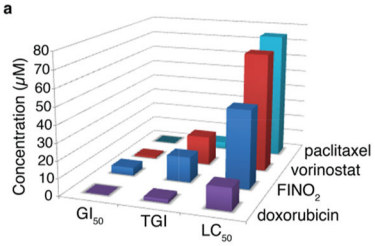



**Figure 1.**
Chemical structures of peroxides. (a) Artemisinin. (b) Lead 1,2-dioxolane ($FINO_2$). (c) Plakinic acid D.

a




b

| | AVG $GI_{50}$ ($\mu$M) | $\sigma$ ($\mu$M) | $C_V$ |
|---|---|---|---|
| sorafenib | 2.4 | 0.6 | 0.3 |
| vorinostat | 1.3 | 0.9 | 0.7 |
| cisplatin | 3.9 | 3.3 | 0.9 |
| $FINO_2$ | 5.8 | 6.2 | 1.1 |
| etoposide | 19.5 | 30.8 | 1.6 |
| paclitaxel | 0.1 | 0.4 | 3.1 |
| doxorubicin | 0.5 | 2.4 | 4.9 |

**Figure 2.**

Comparison of $FINO_2$ to FDA-approved anticancer agents. (a) $GI_{50}$, TGI (total growth inhibition), and $LC_{50}$ values for $FINO_2$ compared to three other anticancer agents. (b) Average (AVG), standard deviation ($\sigma$), and coefficient of variation ($C_V$) of FDA-approved chemotherapeutic agents compared to $FINO_2$ to demonstrate similarity in the amount of variation between cell lines.

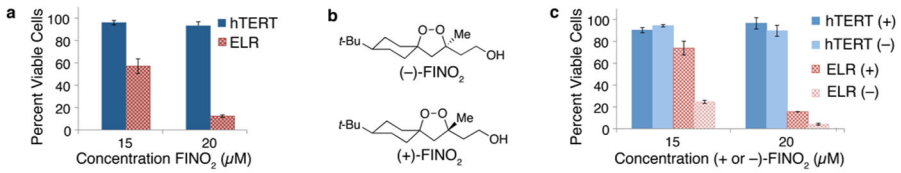



**Figure 3.**

Selectivity of $FINO_2$ measured by Promega CellTiter–Glo Luminescence Assay. (a) Comparison of noncancerous (BJ-hTERT; blue) and oncogenically transformed (BJ-ELR; red) fibroblast cells treated with the racemic form of $FINO_2$. (b) Structures of enantiomers of $FINO_2$. (c) Comparison of noncancerous (BJ-hTERT; blue) and oncogenically transformed (BJ-ELR; red) cells treated with individual enantiomers of $FINO_2$. All experiments were performed in triplicate, and error bars reflect the standard deviation of one experiment. A representative graph of at least three independent experiments is shown.

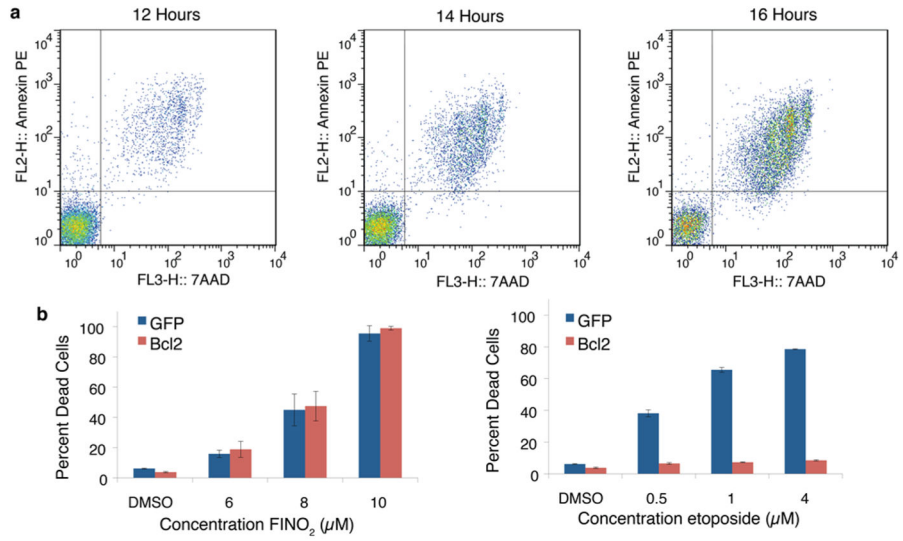



**Figure 4.**

A nonapoptotic form of cell death induced by $FINO_2$. (a) Annexin V staining is not observed prior to 7AAD incorporation with 6 $\mu$M $FINO_2$ treatment at indicated time points. (b) RS4;11 cells overexpressing either GFP or Bcl-2 treated with and $FINO_2$ (left) and etoposide (right). Percent dead cells indicate the Annexin V and 7AAD positive cell population. The nature of the steep dose–response curve induced by $FINO_2$ causes a slight difference in concentrations necessary to induce cell death depending on the preparation of the stock solutions of $FINO_2$ and the passage number of the cells. All experiments were performed at a large concentration range, and the appropriate data are reported.

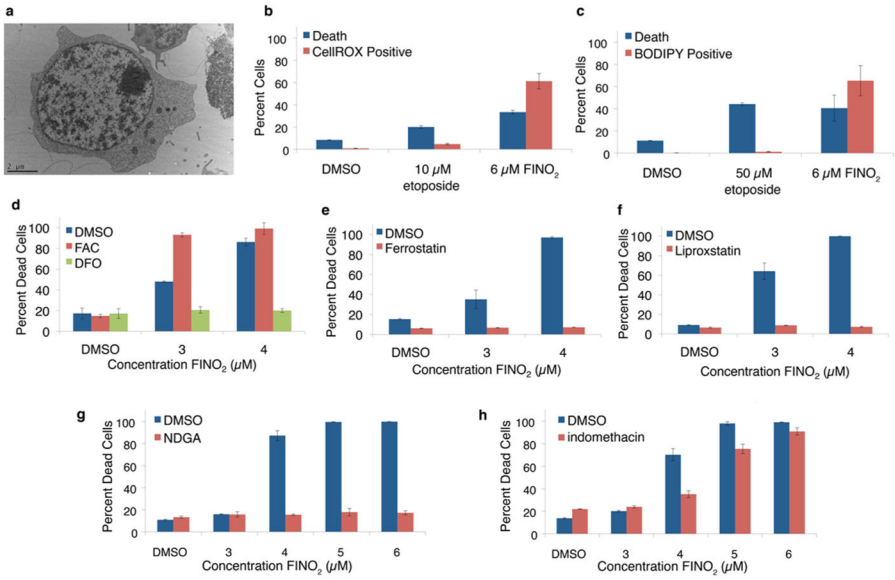



**Figure 5.**

Many features of ferroptosis exhibited by $FINO_2$-induced cell death. (a) Electron microscopy image of typical RS4;11 cell treated with $FINO_2$ showing a lack of hallmarks of apoptosis, necrosis, and autophagy (performed by the Microscopy Core at New York University Langone Medical Center). Detection of oxidative stress 6 h after indicated treatment by (b) CellROX Green Reagent, Molecular Probes, and (c) BODIPY 581/591 C11. (d) Dependence on iron is shown by iron chelation with 20 $\mu$M deferoxamine (DFO) or iron addition with 38 $\mu$M ferric ammonium citrate (FAC). (e) Pretreatment with lipophilic antioxidants 500 nM ferrostatin and (f) 200 nM liproxstatin prevent cell death. (g) Pretreatment with 5 $\mu$M nordihydroguaiaretic acid (NDGA) prevents cell death. (h) Pretreatment with 100 $\mu$M indomethacin partially inhibits cell death. Percent dead cells indicate the Annexin V and 7AAD positive cell population. The nature of the steep dose–response curve induced by $FINO_2$ causes a slight difference in concentrations necessary to induce cell death depending on the preparation of the stock solutions of $FINO_2$ and the passage number of the cells. All experiments were performed at a large concentration range, and the appropriate data are reported.