Phenylpyrrole fungicides act on triosephosphate isomerase to induce methylglyoxal stress and alter hybrid histidine kinase activity

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Fludioxonil, a natural product of pyrrolnitrin, is a potent fungicide used on crops worldwide. Drug action requires the presence of a group III hybrid histidine kinase (HHK) and the high osmolarity glycerol (HOG) pathway. We have reported that the drug does not act directly on HHK, but triggers the conversion of the kinase to a phosphatase, which dephosphorylates Ypd1 to constitutively activate HOG signaling. Still, the direct drug target remains unknown and mode of action ill defined. Here, we heterologously expressed a group III HHK, dimorphism-regulating kinase 1 (Drk1) in Saccharomyces cerevisiae to delineate fludioxonil's target and action. We show that the drug interferes with triosephosphate isomerase (TPi) causing release of methylglyoxal (MG). MG activates the group III HHK and thus the HOG pathway. Drug action involved Drk1 cysteine 392, as a C392S substitution increased drug resistance in vivo. Drug sensitivity was reversed by dimedone treatment, indicating Drk1 responds in vivo to an aldehydic stress. Fludioxonil treatment triggered elevated cytosolic methylglyoxal. Likewise, methylglyoxal treatment of Drk1-expressing yeast phenocopied treatment with fludioxonil. Fludioxonil directly inhibited TPi and also caused it to release methylglyoxal in vitro. Thus, TPi is a drug target of the phenylpyrrole class of fungicides, inducing elevated MG which alters HHK activity, likely converting the kinase to a phosphatase that acts on Ypd1 to trigger HOG pathway activation and fungal cell death.

Fungicides are used worldwide to combat fungal infection in agriculture and in human and animal disease. Fludioxonil, an agricultural fungicide of the phenylpyrrole class, is widely used on crops both pre- and post-harvest, originally finding applications as a preservative for seed storage. Fludioxonil is a chemical derivative of the natural product pyrrolnitrin, initially isolated from Pseudomonas. Despite extensive study and the discovery that the drug's action requires the presence of group III hybrid histidine kinases (HHKs), its molecular target is unknown and mode of action incompletely understood.

Group III HHKs have been widely studied for their role in pathogenesis, morphogenesis, and fungicide sensitivity. HHKs are sensor kinases that regulate environmental stress response pathways, such as the high osmolarity glycerol (HOG) pathway. Under non-stress conditions, the prototypical HHK from Saccharomyces cerevisiae, a group VI HHK called Sln1, is active and negatively regulates the HOG pathway. Exposure to hyperosmotic or extra-cellular aldehydic stress, however, abrogates Sln1 activity, leading to phosphorylation of the Hog1 transcription factor and activation of the cell's stress response elements. When cells expressing group III HHKs are exposed to fludioxonil, Hog1 becomes constitutively activated leading to cell-cycle arrest, glycerol accumulation, cell swelling, and rupture.

Group III HHKs like Nik1 of Candida albicans or Drk1 of B. dermatitidis are required for the fungicidal action of fludioxonil. When elements of the HOG pathway are deleted, fungal cells become resistant to fludioxonil. Conversely, heterologous expression of a group III HHK in S. cerevisiae, which has no group III kinase, renders it sensitive to fludioxonil. Group III HHKs structurally diverge from the other classes of fungal HHKs due to

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the HAMP domain repeats at the N-terminus of the protein. Importantly, when the HAMP repeats are deleted or modified so the HHK becomes a constitutive kinase, it no longer engenders fludioxonil sensitivity.

We recently clarified the action of fludioxonil by showing that the drug induced the group III HHK Drk1 to act as a phosphatase and dephosphorylate its downstream phosphotransfer protein, Ypd1, in vivo. Ypd1 dephosphorylation leads to Hog1 phosphorylation. This finding revealed how group III HHKs act to inappropriately activate the HOG pathway in response to fludioxonil. We also showed that fludioxonil does not induce purified Drk1 protein to produce the same effect in vitro, suggesting fludioxonil may not act directly on group III HHKs. This discrepancy between fludioxonil induced Drk1 behavior in vitro and in vivo led us to investigate whether group III sensor kinases alter their activity in response to a stress condition elicited by exposure to fludioxonil rather than by the direct action of the fungicide itself upon the HHK.

Herein, we heterologously expressed Drk1 in S. cerevisiae to (i) induce sensitivity to fludioxonil, (ii) investigate the drug target and mode of action, and (iii) decipher how Drk1 senses changes in intracellular homeostasis. Sensor kinases may rely on the reaction of signaling molecules with sentinel cysteine thiols rendered reactive by their chemical environment. We hypothesize that Drk1 behaves as such a sensor, responding to drug-induced stress as one or more of its cysteine residues become modified. We furnish evidence to support this hypothesis through a mutational analysis of Drk1 cysteines, which enhances drug resistance. We tested intracellular stresses (e.g. nitrosation, oxidation, and glycation) that can modify cysteine thiols, and defined the intracellular target(s) that lead to their generation.

We report that the Drk1 HHK responds to aldehydic stress induced by elevated cytosolic methylglyoxal (MG) treatment upon fludioxonil treatment. Elevated MG can result from blocked clearance by the glyoxalase system, build-up of a precursor dihydroxyacetone phosphate (DHAP, which is normally converted to glyceraldehyde 3-phosphate [G3P] by triosephosphate isomerase [TPI]), or allosteric interference with the active site of TPI thus promoting decomposition of the phospho-enediol intermediate to MG. We show that fludioxonil treatment both inhibits TPI and causes it to convert triosephosphate into MG, likely by the latter mechanism. We offer a new model for the target and action of phenylpyrrole class drugs: upon modification of TPI function, group III HHKs sense the engaged MG stress, which modifies the sensor at one or more of its cysteine residues converting it from a kinase to a phosphatase, inducing constitutive activation of HOG signaling and cell death.

Results

A cysteine mutation of Drk1 diminishes fludioxonil sensitivity. We hypothesized that Drk1 cysteine thiols act as reactive "sentinels," responding to stress induced by fludioxonil treatment as one or more of these cysteines undergo modification. To test this premise, we mutated each of the Drk1 cysteines to serines, individually or in pairs, and determined the effect of these mutations upon fludioxonil sensitivity. Drk1 has nine cysteines, (Fig. 1A,B). We first screened the Drk1 cysteine mutants for resistance to a concentration of 1 µg/ml fludioxonil. Mutation of cysteines 392 and 856 increased fludioxonil resistance (Fig. 1C). We then tested these two single mutants, a C392S/C856S double mutant, and a control C75S mutant that did not exhibit increased fludioxonil resistance against a range of fludioxonil concentrations. The mutants gave different sensitivity profiles (Fig. 1D). The C856S mutant had the same EC50 as the wild-type Drk1 and control C75S mutant, although the growth profile may suggest delayed pathway activation at the highest concentrations of fludioxonil. The C392S mutant, on the other hand, had close to a one-log increase in EC50 compared to wild-type Drk1 and the control C75S mutant. At the highest concentrations of fludioxonil, however, this mutant showed the same (poor) growth as the wild-type Drk1, i.e., sensitive to the fungicide. A double C392S/C856S Drk1 mutant had the same resistance profile as the single C392S mutant (data not shown). These data suggest that fludioxonil sensitivity is mediated, at least in part, in a manner dependent upon Drk1 C392.

To ensure that a mutation of cysteine 392 did not alter the structure and function of Drk1 generally, we investigated its kinase activity. We transformed C392S Drk1 into a strain of S. cerevisiae harboring a temperature sensitive version of Snl1 (TM229). At a permissive (26 °C) temperature, Snl1 is functional, HOG1 signaling is inhibited, and yeast are viable. At a non-permissive (37 °C) temperature, Snl1 is inactive and yeast are inviable due to constitutive activation of HOG1 signaling. A functional wild-type Drk1 kinase, conditionally expressed upon growth in galactose, rescues yeast lacking functional Snl1 (Fig. 1E lower right-hand plate). Likewise, conditional expression of either the C392S and C856S mutants of Drk1 rescued yeast grown at a non-permissive temperature of 37 °C. Thus, these Drk1 mutants retained sufficient kinase activity to inhibit constitutive HOG1 activation.

A model for Drk1 sensing and conversion from kinase to phosphatase. Sensitivity to modification of a chemically reactive thiol has been reported for many other sensor kinases. Based on the effect that pyrrolnitrin has on mitochondrial electron transport chains, we postulated that Drk1 thiols might be sensitive to reactive oxygen species (ROS) within the cell. However, thiol reactive stress intermediates besides ROS could also act in this capacity. We thus tested several stress intermediates that could react with cysteine thiols, including ROS, nitrosating species and glycation (Fig. 2).

Dismissal of elevated nitrosative stress, induction of glutathionylation and/or dimerization of thiols as modes of fludioxonil action. Despite fludioxonil’s behavior bearing some similarity to nitrosogluthionine reductase inhibitors (Supplementary Fig. S1), and possessing some inhibitory capacity of this target, we dismissed a nitrosative stress mechanism as the mode of fludioxonil action after generating substantial negative data (Supplementary Fig. S2). Based on an in-depth MS analysis of Drk1 isolated from fludioxonil-treated yeast, we also dismissed glutathionylation of sensitive cysteine residues and catalysis of disulfide formation as modes of fludioxonil action (Supplementary Fig. S3).
Fludioxonil causes oxidative stress in vivo.  It has been conjectured that pyrrolnitrin causes oxidative stress in fungi due to its established effect upon the mitochondrial electron transport chain. As fludioxonil is derived from pyrrolnitrin, we hypothesized that the drug might likewise induce oxidative stress. To test this, we employed the redox-responsive FRET reporter yeast, RedoxFluor. This reporter responds to oxidative stress with decreased FRET signal. We used RedoxFluor yeast to measure redox environment in S. cerevisiae after exposure to DMSO, fludioxonil or H2O2 (positive control). We saw a decreased FRET signal in response to fludioxonil and H2O2, indicating oxidative stress in the yeast (Fig. 3A). An assay based on dihydroethidium oxidation (DHE)
Fludioxonil evolves ROS. Using MitoTracker Green dye, we tested wild-type yeast directly by treating yeast with dimedone. Dimedone reacts with aldehydes and partially oxidized cysteine thiols, and we sought to “capture” intermediate forms of oxidized thiol that might be too labile to observe. The presence of oxidative stress molecules like hydrogen peroxide or superoxide. During our pursuit of evidence for oxidative stress induced by fludioxonil and the positive control, H$_2$O$_2$ (Fig. 3C). After fludioxonil exposure, 44% of the cells showed a similar increase in oxidation of DHE in yeast that were incubated in fludioxonil compared to DMSO vehicle (Fig. 3B).

We next used a complementary approach to monitor oxidative stress: histone H2A.x phosphorylation. Histone H2A.x is phosphorylated during DNA damage and serves as a marker of oxidative stress. This method is used in the Environmental Protection Agency’s (EPA) ToxCast “toxicity forecaster” screen, which uses high-throughput bioassays to evaluate potentially toxic side effects of environmentally relevant small molecules. Fludioxonil has been tested in the ToxCast system using HepG2 cells, a liver hepatocellular carcinoma cell line, and was found to increase H2A.x phosphorylation, indicating oxidative stress, at concentrations equal to or greater than 12.5 μM (~3.1 μg/ml) after 24 hours. To see if fludioxonil induces this same response in fungi, we used wild-type S. cerevisiae to measure H2A.x phosphorylation following fludioxonil exposure. We observed evidence of oxidative stress induced by fludioxonil and the positive control, H$_2$O$_2$ (Fig. 3C). After fludioxonil exposure, 44% of the cells stained positive for H2A.x phosphorylation, compared to 17% with the vehicle control, DMSO.

Oxidative stress does not substitute for fludioxonil treatment. We reasoned that if fludioxonil acted by inducing oxidative stress, we should expect to observe toxicity in Drk1-expressing yeast after the direct application of reagents that induce oxidative stress. This was not the case. Growth of yeast expressing Drk1 was not inhibited more than yeast carrying the empty vector upon exposure to H$_2$O$_2$ (Fig. 3E). Extensive MS analysis of Drk1 thiols found no evidence of thiol oxidation in fludioxonil-treated cells beyond that observed in control yeast treated with 1% DMSO alone (data not shown). Whereas oxidative modification events were observable via MS due to background levels of these modified thiols, no increase in these events was noted following fludioxonil exposure.

Though our work demonstrates that a substantial oxidative stress is induced by fludioxonil treatment, in a manner that is independent of the presence of the Drk1 kinase, fludioxonil toxicity does not hinge on the presence of oxidative stress molecules like hydrogen peroxide or superoxide. During our pursuit of evidence for oxidized thiols, we sought to “capture” intermediate forms of oxidized thiol that might be too labile to observe directly by treating yeast with dimedone. Dimedone reacts with aldehydes and partially oxidized cysteine thiols like sulfenic acid. MS analysis demonstrated that there was no reaction of dimedone with oxidized thiols in Drk1. No increase in dimedone adducts was observable between fludioxonil-treated cells and controls treated with DMSO control (data not shown).

Interestingly, and unexpectedly, dimedone treatment was seen to attenuate the toxic effects of fludioxonil in Drk1-expressing yeast (Fig. 4A). This protective effect was significant and concentration dependent. This observed protective effect of dimedone was revealing, since this reagent is a standard treatment for the protection of cells against aldehydic stress (below). This dimedone effect could be replicated in a native, fludioxonil-sensitive strain of Candida albicans (K1). Thus, dimedone protected C. albicans against fludioxonil toxicity just as it did Drk1 expressing S. cerevisiae (Supplementary Fig. S4).

Toxicity of fludioxonil on Drk1-expressing yeast can be duplicated by aldehydic stress. Treatment of yeast with allyl alcohol induces intracellular aldehydic stress in the form of acrolein. Acrolein is an aldehydic toxin with well-established fungicidal properties. Yeast induced to express Drk1 exhibited more...
Figure 3. Fludioxonil induces oxidative stress in vivo. (A) YRF-1A yeast exposed to either 25 µg/ml fludioxonil, 1% DMSO (solvent control), or 1 mM H2O2 for 20 minutes. Left: FRET ratio image of FRET signal/CFP signal. Shift from green to blue indicates a decreased FRET ratio (oxidative stress). Right: The FRET ratio of 30 cells for each condition was measured every five min. for 20 min. Data are mean ± SEM. (B) DMSO was added to 1%, and fludioxonil in DMSO at 25 µg/ml and 1%, respectively. Dihydroethidium (DHE) was added to 20 µM. Incubation was for 1 hr at 30 °C with shaking. DHE fluorescence was recorded on a Filtermax F5 multi-mode microplate reader. (C) H2A.x phosphorylation of wild-type BY4741 yeast exposed to 1 µg/ml fludioxonil in 1% DMSO or 0.9 mM H2O2 for 60 min. The negative control for fludioxonil was 1% DMSO, the negative control for H2O2 was dH2O. Data are shown as percent of H2A.x positive cells, indicating oxidative stress. Gates were set using fluorophore minus one (FMO) gatings. (D) Upper panel: Relative fluorescence of total cellular mitochondria in BY4741 cells exposed to fludioxonil in 1% DMSO. Representative single channel flow plots showing fluorescence intensity of cell population. Lower panel: Mean fluorescence intensity of total
sensitivity to allyl alcohol compared to uninduced controls (Fig. 4B). This increased sensitivity could be reversed almost entirely through the addition of dimedone, in a concentration dependent manner. These results argue that the stress condition triggering the Drk1 kinase in vivo is likely an aldehydic stress.

Aldehydic stress can derive from various stimuli. The most common are aldehydes derived from the oxidative breakdown of cellular lipids. With this in mind, we initially searched for adducts that form between lipid-derived aldehydes and cysteine thiols, using MS of PAGE-isolated Drk1 protein. The evidence for these known adducts forming in fluodioxinol treated yeast, compared to controls, was entirely negative (data not shown).

Another, more elusive form of cellular aldehydic stress are aldehydes derived from a product of glycolysis, DHAP (dihydroxyacetone phosphate). DHAP can spontaneously convert into the damaging aldehyde MG, but while MG reacts rapidly with sensitive cysteine residues, this reaction may be quickly reversed. Searching for MG modifications directly was not fruitful, so we assessed reactivity of the Drk1 cysteine thiols through a surrogate stressor. We examined allyl alcohol treated yeast, employing nanoLC-MS/MS to seek adducts formed between the aldehyde acrolein and the protein thiols of Drk1 (mass of +56 initially, converting to a more stable +38 form over time) (Fig. 4C). A tryptic digest of Drk1 provided sequence coverage of all but two cysteines (Supplementary Fig. S3). Four of the cysteine residues examined bore modifications with these masses in treated vs. untreated yeast (Supplementary Fig. S5). Modifications of two cysteines (C392 and C1112) were detectable with a relative abundance of ~10–20% (Fig. 4C); two (C1039 and C1209) were modified at trace levels, and three (C75, C-1062 and C-1184) were unmodified. Drk1 thus becomes modified at specific, sensitive cysteine thiols during the response to this surrogate aldehydic stress. This is not inconsistent with MS experiments in which yeast treated with fluodioxinol showed a depletion of reduced and DTT-reducible cysteines in the Drk1 HHK compared to DMSO-treated controls. Three cysteines (C75, C392 and C1112) showed significant (50–60%) reductions in the reduced thiol form, while one (C1209) showed no change (Supplementary Fig. S3). The specific mass/derivation of the DTT-resistant modifications that accumulated in response to fluodioxinol was not resolvable.

Despite the fact that C392S mutations to Drk1 rendered S. cerevisiae significantly less sensitive to the effect of fluodioxinol, this result was not observed in experiments in which the yeast were exposed to allyl alcohol (Supplementary Fig. S6) or MG (data not shown). These results suggest that, while cysteine 392 of the Drk1 HHK appears to be involved in the molecular response to fluodioxinol, its reactivity is not solely sufficient to drive the mechanism(s) of response to aldehydic stress.

Wild-type S. cerevisiae bears Sln1, a group VI HHH, on its surface, which responds to osmotic stress, but also to extracellular aldehydic stress. This makes direct application of exogenous MG problematic for distinguishing the response of Sln1 versus Drk1. To circumvent this issue, we engineered the TM229 strain, in which Sln1 is inactive at restrictive temperatures, to express Drk1 (Fig. 1E). Growth of this strain at the permissive temperature (26°C) allowed us to probe and validate the native, Sln1-dependent sensitivity of the yeast to MG. By growing the yeast in galactose at 26°C, Drk1 and Sln1 were expressed together, and by growing the yeast at the non-permissive temperature (37°C) in galactose, we probed the capacity of Drk1 to respond to MG in the absence of native Sln1 (Fig. 1E). Modification of about 10% of the native Sln1 cysteine thiols was detectable in yeast (Supplementary Fig. S5). Modifications of two cysteines (C392 and C1112) were detectable with a relative abundance of ~10–20% (Fig. 4C); two (C1039 and C1209) were modified at trace levels, and three (C75, C-1062 and C-1184) were unmodified. Drk1 thus becomes modified at specific, sensitive cysteine thiols during the response to this surrogate aldehydic stress. This is not inconsistent with MS experiments in which yeast treated with fluodioxinol showed a depletion of reduced and DTT-reducible cysteines in the Drk1 HHK compared to DMSO-treated controls. Three cysteines (C75, C392 and C1112) showed significant (50–60%) reductions in the reduced thiol form, while one (C1209) showed no change (Supplementary Fig. S3). The specific mass/derivation of the DTT-resistant modifications that accumulated in response to fluodioxinol was not resolvable.

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Fludioxonil inhibits TPI, the enzyme that controls DHAP. For MG to accumulate in cells, one of two things must occur. Either one (or more) of the pathways that degrade MG (e.g. Glo1) must be inhibited, or, alternatively, TPI must be prevented from converting the MG precursor, DHAP, to G3-P45 (Fig. 5A). We investigated the effect of fluodioxinol on TPI activity and found dose-dependent inhibition with an IC50 of ~100 μg/ml (Fig. 5B). In contrast, fluodioxinol had no effect on Glo1 activity (Fig. 5C). Thus, drug inhibition of TPI could potentially contribute to the elevation of MG levels seen in our yeast model of fluodioxinol action.

Yeast exposed to fluodioxinol exhibit elevated levels of MG. Following the action of MG can be difficult - thiold modifications at sensitive “sentinel” thiols form rapidly, but appear to be transient, either reverting to reduced thiols just as quickly as MG concentrations drop or maturing into more complex adducts (of unknown mass). Glycation at cysteine thiols can also serve as an intermediate step in either transferring the modification to nearby amino groups or catalyzing the formation of disulfide bonds, thus a cysteine thiol may be critical to the function of an MG sensor kinase yet display no evidence of modification in MS analysis. Fortunately,
elevated cellular MG can be trapped as a 2-methylquinoxaline adduct via reaction with 2,4-diaminobenzene 49. Increases in this adduct can then be measured by HPLC.

Wild-type yeast exposed to 0.1% DMSO vehicle alone showed a small elevation of MG (as 2-methylquinoxaline) over time, but when they were exposed to fludioxonil, cellular MG rose to much higher levels (Fig. 5D). The presence of Drk1 was not required for MG to accumulate. The peaks in Drk1-expressing yeast were actually smaller than in empty vector control-yeast at later time points (Fig. 5E), possibly because of the rapid rate at which fludioxonil renders yeast non-viable. Within 3 hours of fludioxonil exposure, viability in Drk1-expressing yeast is halved 26. These results are consistent with a mechanism in which fludioxonil disrupts cellular homeostasis by inhibiting TPI, thus causing DHAP to accumulate.

Fludioxonil alters the activity of TPI, causing the enzyme to convert triosephosphate directly into MG in vitro. To see if a model involving TPI inhibition was plausible, we looked at changes in the concentration of DHAP in response to fludioxonil vs. DMSO vehicle control. While small but significant changes in DHAP were noted (Fig. 5F), they seemed insufficient to explain MG levels that rose up to 10-fold (Fig. 5D,E). On this basis we concluded that TPI inhibition by itself was insufficient to explain the increases in cellular MG observed following treatment with fludioxonil.

Interference with the function of the loop 6 component of the TPI active site catalyzes the degradation of the triosephosphate intermediate into MG and free phosphate with increased exposure to solvent 24. Since there is precedent...
Figure 5. Fludioxonil inhibits the activity of triosephosphate isomerase (TPI) leading to elevation of methylglyoxal (MG). (A) MG concentration may be increased by inhibition of TPI, which acts upon a product of glycolysis, DHAP, or by inhibition of Glo1, which degrades MG with the assistance of a glutathione cofactor. (B) TPI was inhibited by incubation with fludioxonil for two hours at 37 °C. TPI activity was measured by NADH consumed by glycerocephosphate dehydrogenase as it was processed from DHAP, which was generated by TPI from an excess of glyceraldehyde 3-phosphate. Loss of OD340 as NADH was consumed was monitored every 0.5 min. Data averaged from 4 replicates. (C) Glo1 was not inhibited by incubation with fludioxonil (10 mM in 1% DMSO) for two hours at 37 °C compared to a known glo1 inhibitor, S-hexylglutathione (10 mM in 1% DMSO). Components were incubated for 10 min. in 50 mM sodium phosphate buffer (pH 6.6) at 37 °C and OD240 monitored for comparison to starting value. (D, E) Cellular MG was measured by lysing cells at various time points after they were exposed to fludioxonil and measuring the 2-methylquinoxaline (MQ) adduct formed by the reaction of MG with 1,2-diaminobenzene. Quinoxalines were separated by RP HPLC.
and detected by absorbance at 220 nm. In panel D, pure MQ (red trace) is included as a positive control for time of elution from the HPLC column. (F) DHAP was quantified by fluorometric kit, which follows conversion of DHAP to G3P by linking the latter molecule to the activation of a fluorescent probe. (G) Release of MG by TPI was quantified by running the reaction in the presence of 1,2-diaminobenzene and measuring formation of MQ adduct (by increase in OD at 320). *p < 0.05, **p < 0.01 vs. background or the respective control. One-way ANOVA analysis with Bonferroni’s or Tukey’s multiple comparison test.

for small hydrophobic inhibitors of TPI to act at the dimerization interface in a fashion that constrains this same loop39, we tested whether fludioxonil might induce TPI to release MG in this same way. We found that purified TPI treated with fludioxonil did convert a significant amount of triosephosphate substrate into MG, compared to background levels (Fig. 5G). This increase in vitro was more in keeping with the increases in MG we observed in vivo.

Database docking study of fludioxonil at the dimerization interface of TPI. If fludioxonil acts upon TPI by binding within the hydrophobic tunnel region at the TPI dimer interface, as has been reported for TPI inhibitors like the benzoazole derivative bt102, docking studies for fludioxonil would be expected to show clustering within this same cleft. Our docking study of the bt10 molecule was consistent with the previous study39, with the molecule docking in the cleft and interacting with hydrophobic residues including Tyr103 in 1tcn (3D TPI structure from Trypanosoma cruzi) (Fig. 6A). Cluster analysis of 30 dockings gave the best cluster of 17 dockings having a top/average binding energy −9.04/−8.88 kcal/mol. For fludioxonil, 5 out of 30 were in the top cluster with a best/mean binding energy of −5.89/−5.81 Kcal/mol (In 1yp, TPI from S. cerevisiae). Fludioxonil binds in the same cleft as bt10, interacting with homologous residues (Phe102) (Fig. 6B) but in a larger variety of orientations. The drug would be expected to yield a less favorable binding energy than bt10 because it is smaller, does not have the sulfate group driving specificity in bt10, and has a less favorable directionality. The binding energy of fludioxonil is, however, comparable to many smaller, benzothiazole TPI inhibitors31.

We postulate that these molecules act as steric effectors, inducing an inflexibility in loop 6 that permits the release of MG into the cytosol of our model yeast. This signal, in turn, converts the group III HHK of sensitive yeast into a phosphatase by modifying or activating one or more cysteine thiols, likely including C392. De-phosphorylation of Ypd1 leads to activation of Hog1 signaling, resulting in death of the yeast when this activation proceeds unabated.

Discussion

Despite the worldwide application of fludioxonil to myriad agricultural products (>900), the mode of action of this fungicide is incompletely understood and its target is unknown. Due to the necessity of group III HHKs for sensitivity to fludioxonil, it was initially posited that these kinases fulfilled the role of direct target; this model went unchallenged for decades. Little data supported such a model and recent findings argue against direct drug sensitivity to fludioxonil, it was initially posited that these kinases fulfilled the role of direct target; this model went unchallenged for decades. Little data supported such a model and recent findings argue against direct drug action on HHKs37. Producers of the fungicide have since advanced a mode of action suggesting inhibition of transport-associated glucose phosphorylation34, a model that is not consonant with prior assumptions on direct action on HHKs. Here, we propose that the mechanism of fludioxonil is best explained by direct action upon TPI, which induces an unreported cytosolic stress – MG – that, in turn, triggers the activation of HHKs.

We show that fludioxonil modifies the action of TPI, inducing elevation in MG, an aldehydeic stress. This drug target and mechanism is consistent with prior observations that fludioxonil treatment (i) impairs mitochondrial electron transport chains, (ii) reduces transport-associated phosphorylation of glucose38 and (iii) leads to accumulation of glycerol32. The first two effects can be a consequence of elevated MG7–57, whereas glycerol overproduction is promoted by an elevation in DHAP45. Thus, our data support a novel drug target and model in which the group III HHK Drk1 functions as a sensor of aldehydeic stress intermediates.

The sensitivity of sensor kinases often hinges on modification of highly reactive “sentinel” cysteine thiols38. To test our hypothesis that fludioxonil affects Drk1 function indirectly via interaction of a stress-derived chemical signal with one or more cysteine residues, we tested fludioxonil sensitivity in S. cerevisiae bearing mutations in heterologously expressed Drk1. Mutants were created in which each of the 9 cysteine residues of Drk1 was replaced with a serine residue. One mutant, C392S, resulted in a log-fold increase in fludioxonil resistance. This partial effect suggests that C392 plays a role in drug sensitivity, but is insufficient by itself to mediate sensitivity, as this mutant remained sensitive to high concentrations of the fungicide and was as sensitive as the yeast with unmutated Drk1 to allyl alcohol-derived acrolein. This is not unexpected, as there has long been evidence that the mechanism of fludioxonil toxicity is multi-factorial and unlikely to be simplified to a single signaling event. For example, in the fungus Botrytis cinerea, the Hog1 pathway is surprisingly uninolved in the organism’s sensitivity to fludioxonil30 and other investigators have reported that stress response pathways are critical to fludioxonil resistance, potentially complementing osmoregulation mechanisms40.

Drk1 could possess two or more sensitive cysteine residues that respond to different concentrations of fludioxonil, possibly dependent upon the reductive environment created by the amino acids surrounding them. By this model, C392 might respond to stress signal molecules engendered by a low concentration of fludioxonil, but would be superseded by the response of one or more of the other cysteine residues at higher concentrations. For example, the Yap1 transcription factor in S. cerevisiae responds to MG via cysteine modification37. Yap1 can translocate to the nucleus so long as it possesses one of its 6 cysteines. It is not until all 6 cysteines are mutated that Yap1 loses its capacity for nuclear localization in response to MG.

While mutations such as C392S could potentially lead to mis-folding of enzymes that compromise their function, we verified that wild-type Drk1 and the C392S mutant were capable of rescuing a lethal, temperature-sensitive mutation of Sln1 that renders yeast non-viable at elevated temperature35. Thus, kinase activity of Drk1 is active in Saccharomyces indicating that our cysteine substitutions did not abrogate that function.
The fact that C392 resides in a HAMP domain reinforces the idea that C392 is involved in sensing or signaling. The means by which group III HHKs sense and respond to fludioxonil involves HAMP domains. Since group III HHKs are required for fludioxonil sensitivity, others have hypothesized that HAMP domain repeats unique to group III HHKs are involved in sensing fludioxonil. Of fludioxonil resistant mutants isolated in the field or laboratory, nearly all contain mutations in HAMP domains. The HAMP domain region is also involved in regulating kinase activity of other group III HHKs. Thus, it is not surprising that a cysteine in the HAMP domain region is involved in sensitivity to fludioxonil.

After fludioxonil exposure in vivo, the group III HHK Drk1 converts from a kinase, which phosphorylates its downstream target Ypd1, into a phosphatase. In vitro, however, fludioxonil does not induce this change in Drk1 function, suggesting that the drug may not directly act on Drk1. These data, together with the observation that lead compounds identified in our drug screen share structural similarities with certain GSNOR inhibitors, led us to initially ask if fludioxonil acts as a GSNOR inhibitor, increasing intracellular GSNO. Our data showed that while fludioxonil weakly inhibited GSNOR this inhibition does not appear to be a significant element of fludioxonil's mode of action. Additionally, neither SNO levels nor protein nitrosation increased intracellularly during fludioxonil exposure. Thus, nitrosation of Drk1 thiols is unlikely to be relevant in fludioxonil's action.

Cysteine modifications are key elements in detection and response to oxidative stress. Based on pyrrolnitrin's propensity to block mitochondrial respiration, a capacity to induce oxidative stress is plausible. We used multiple techniques to establish that fludioxonil induces oxidative stress in vivo. We also sought evidence for modifications catalyzed by this stress. For instance, S-glutathionylation of cellular proteins is higher under conditions of oxidative stress. We thus investigated whether glutathionylation of sensitive cysteine residues proceeds from fludioxonil treatment; however, evidence of glutathionylation of Drk1 in response to fludioxonil in vivo was lacking, either at cysteine residue 392 or any other residue.

Exposure of fludioxonil-sensitive yeast to different oxidative stress inducing molecules, such as H$_2$O$_2$, failed to phenocopy the effects of fludioxonil. Examination of Drk1 protein from fludioxonil-treated and untreated yeast using nanoLC-MS/MS also failed to detect oxidized forms of its cysteine thiols. Even though fludioxonil triggers oxidative stress, this stress is not sufficient, by itself, to mediate the toxicity of the drug.

During our search for oxidized cysteine residues, we treated yeast with dimedone to react with, and mass-label, any labile sulfenic acid groups that formed. No such groups were detected by MS, but we did find that dimedone protected yeast against fludioxonil toxicity. Dimedone is a known protectant against aldehydic reactants, and this suggested that the action of fludioxonil on yeast could involve aldehydic stress. We found that allyl alcohol,

![Docking Bt-10 into T. cruzi TPI](image)

![Docking Fludioxonil into S. cerevisiae TPI](image)

**Figure 6.** TPI inhibitors occupy the hydrophobic dimer interface region of TPI. Results of the top hits in the Autodock4 docking of (A) bt-10 in *T. cruzi* TPI and (B) fludioxonil in *S. cerevisiae* TPI (displayed in Pymol). Solvent accessible surface viewed along the interface between the dimerized TPI molecules (cyan) and (green) emphasizing the hydrophobic tunnel. The mean binding energy of $-8.88$ Kcal/mol for bt-10 in cluster 1 is lower than the mean binding energy of $-5.98$ Kcal/mol for fludioxonil because the ligand bt-10 contains a sulfate group H-bonded to a lysine residue in the protein, and has more atoms to contact the protein.
which induces intracellular aldehydic stress, phenocopied the toxicity of fludioxonil, and this effect was blocked by dimedone just as it countered the effect of fludioxonil. This data establishes that the toxic effect fludioxonil has upon sensitive fungi may be reversed by a reagent that blocks aldehydes.

In wild-type yeast, Sln1 on the surface is sensitive to extracellular aldehydes. Activation of Sln1 with exogenous MG over-stimulates the HOG pathway, just as fludioxonil kills fungi through activation of the group III HHKs and constitutive HOG signaling. We found that a temperature sensitive Sln1 mutant remained highly sensitive to MG at Sln1 non-permissive temperature, when rescued by Drk1. This established that Drk1 also responds to this aldehydic stress.

Since oxidative stress accompanies fludioxonil exposure, and oxidative intermediates can convert cellular lipids into aldehydic species, we sought evidence of these in the form of known adducts derived from their reaction with cysteine thiols. NanoLC-MS/MS analysis of Drk1 failed to find adducts formed from a reaction with acrolein or any other lipid-derived aldehydes, making it unlikely that these were the source of aldehydic stress. We theorized that fludioxonil might instead evolve aldehydic stress in the form of MG by disrupting one of the enzymes responsible for its normal metabolism. Under normal circumstances, TPI isomerizes the MG precursor, DHAP, into G3-P, minimizing MG creation, while Glo1 is the enzyme responsible for the breakdown of MG into lactic acid. We thus investigated the capacity of fludioxonil to inhibit TPI and Glo1, discovering that it did inhibit the former, but not the latter.

TPI inhibition engenders a known toxic effect in actively metabolizing cells. This toxicity is sufficiently strong that TPI inhibitors exercise a chemotherapeutic effect and new TPI inhibitors are sought to treat cancer and parasitic infections. In a small molecule screen for TPI inhibitors, a frequently seen structural motif was the linkage of a benzyl group to a substituted pyrrole by a single carbon-carbon bond such that the aromatic linkage of a benzyl group to a substituted pyrrole by a single carbon-carbon bond such that the aromatic structures may achieve a co-planar configuration. This configuration allows the molecules to fit into the narrow, hydrophobic tunnel at TPI’s dimerization interface. Fludioxonil’s structure is comprised of these same features. Of potential interest is the fact that beta-carbolines that inhibit TPI also display a fungicidal effect.

Were fludioxonil to inhibit TPI, exposed cells would accumulate the MG precursor DHAP, which is normally maintained at low concentrations by the action of TPI. Since DHAP converts to MG spontaneously, TPI inhibition would be predicted to drive up the concentration of MG in affected cells. We found that MG levels increased in S. cerevisiae in response to fludioxonil. In cells expressing Drk1, this increase waned at approximately the rate that yeast lost viability. The increase in DHAP levels, however, was not commensurate with the increase in MG. TPI inhibition alone therefore did not adequately explain the acute spike in MG observed in our yeast model of fludioxonil activity.

Mutations in loop 6 of the TPI active site greatly increase the rate at which the triosephosphate substrate is released prematurely, degrading into MG and free phosphate. Certain small, hydrophobic inhibitors can also bind to the dimerization interface of TPI in a way that modifies the mobility of loop 6. We reasoned that fludioxonil, a small, hydrophobic molecule, might work the same way, not only inhibiting TPI, but accelerating the generation of MG by a previously unreported mechanism. Indeed, our in vitro MG assay showed that approximately 2% of the triose processed by TPI was released from the enzyme as MG. This amount is greatly inflated compared to the normal rate, which is estimated to be 0.3% in yeast systems. Our docking analysis showed that binding of fludioxonil to the hydrophobic tunnel at the dimeric interface of TPI is thermodynamically favorable, and comparable to the binding by known inhibitors of T. cruzii TPI to the same region in that enzyme.

MG inhibits the electron transport pathway of mitochondria at complex 1, causing oxidative stress to build-up. It also suppresses the transport-associated phosphorylation of glucose, just as the phenylpyrroles do, and excess DHAP leads to the substrate-driven overproduction of glycerol. These effects are also hallmarks of fludioxonil toxicity. Our results suggest they are side effects of the primary mechanism by which fludioxonil exerts its fungicidal effect e.g. a spike in cytosolic MG.

In summary, we propose that fludioxonil exerts its drug effect as follows. Fludioxonil modifies the activity of TPI, causing release of MG into the cytosol. MG modifies one or more cysteine thiols of group III HHKs, converting it to a phosphatase, which dephosphorylates Ypd1, setting off a cascade that activates the HOG1 pathway constitutively, resulting in cell death.

Though our primary focus in this work has been to ascertain the mechanism by which fludioxonil causes the Drk1 HHK to turn into a phosphatase, it is also important to note that the fungicide fludioxonil has been deemed safe for application to food post-harvest by the EPA, and at fairly high concentrations, because it was believed to act directly upon group III HHKs which human beings lack. If its actual target is TPI, fludioxonil could exert an insidious and difficult to detect toxic effect upon human beings. In fact, elevated MG is co-morbid with countless human disease states and its effect upon cells is difficult to distinguish from the natural effects of aging. As several other research groups have concluded, and as summarized in our own review, it is critical that the effects of fludioxonil upon human beings be re-evaluated at this time.

**Methods**

**Strains, vectors, and growth conditions.** The strains and vectors used in this study are listed in Tables S1 and S2, respectively. S. cerevisiae was grown in either yeast peptone dextrose (YPD) or synthetic complete (SC) media lacking uracil with either 2% glucose, 2% galactose/1% raffinose (induction media) or 2% raffinose (overnight growth) as the carbon source. S. cerevisiae cells were grown at 30 °C either stationary (96 well plate assays) or shaking at 200 rpm in Erlenmeyer flasks. To make the YRF-1A RedoxFluor strain, we first digested the plasmid with MfeI and transformed 0.5 µg of linear plasmid into S. cerevisiae BY4741 and plated on SC-ura + glucose. Candida albicans strain K1 was grown on YPD medium.
**E. coli and S. cerevisiae transformation.** *E. coli* was transformed by electroporation\(^79\). *S. cerevisiae* was transformed using the LiAc/SS carrier DNA/PEG method with the addition of 10mM DTT\(^80\).

**Mutagenesis of Drk1 cysteine residues.** Eight of the nine Drk1 cysteines were mutated using the QuickChange Multi Site-Directed mutagenesis kit (Agilent) with primers SML166-168, 170–174 (Table S3) and pYES-DEST52 Drk1 as template. Following mutagenesis, all sequences were confirmed with BigDye Terminator v3.1 mix (Applied Biosystems). Samples were cycled (96 °C for 2’, then 35 cycles of 96 °C for 10’, 52 °C for 15”, 60 °C for 3’, followed by 1 min at 72 °C) and heated up using Cleaver clean up kit (Agencourt, Bioscience). The samples were sequenced at the University of Wisconsin Biotechnology Center (UWBC).

For C934, when we employed the above method, we only recovered C934S mutants that had extra, unwanted mutations (insertions, deletions, or rearrangements). Considering the possibility of a toxic mutation, we mutagenized initially with a non-functional Drk1 (Drk1 D1140N\(^8\)). The Drk1 C934S mutant was created by performing SRM as above with pYES-DEST52 Drk1 and SML169. The D1140N mutation was reverted by PCR with primers IRK099 and IRLK100 using Phusion polymerase (NEB) and sequence-confirmed mutants pYES-DEST52 Drk1 C934S D1140N. Linear DNA was phosphorylated with PNK enzyme (NEB) and self-ligated with T4 DNA ligase (NEB) in the same reaction. Ligation products were transformed into *E. coli* DH10B by electroporation. Mutants were confirmed by Sanger sequencing. The C392S/C856S double mutant was made using pYES-DEST52 Drk1 C932S as the template and primer SML168 to mutate the C856 to serine.

**GSNO synthesis.** To synthesize GSNO, 620 µl 12 M HCl (Fisher) was added to 5.9 ml of water in a fume hood in the dark on ice with stirring. To this solution, 1.54 g GSH was added and allowed to dissolve, and then 1 ml of 0.5 M NaNO₂, was slowly added and stirred for five minutes. The reaction was neutralized with 710 µl of freshly made 10 M NaOH and brought to 10 ml with 18.2 MΩ water. The concentration of GSNO was determined by measuring the absorbance at 335 nm and calculated using a millimolar extinction coefficient of 0.92. GSNO stocks were stored at −80 °C. All experiments involving GSNO, including production, were performed in the dark.

**NIH Small Molecule Screen.** We undertook a screen with the National Institutes of Health (NIH) designed to discover small molecules that worked in a similar way to fludioxonil, i.e. compounds that killed fungi in a Drk1- and Hog1-dependent manner. We noticed that some of the hits that emerged from this screen were structurally similar to S-nitrosoglutathione reductase (GSNOR) inhibitors (Fig. S1). This led us to initially question whether fludioxonil might also act as a GSNOR inhibitor.

**GSNOR activity assay.** To determine the effects of fludioxonil on GSNOR, we added 50 µl of 2 mM NADH (Sigma) (0.2 mM final concentration) to 425 µl GSNOR reaction buffer (20 mM Tris pH 7.5, 0.5% Deoxycholic Acid (Sigma), 1.6 µg/ml purified GSNOR [Proteintech] or 10% murine liver lysate, 1% DMSO) to the indicated concentration of fludioxonil) and incubated for seven minutes. The liver lysate was prepared by homogenizing fresh murine liver from C57BL/6 mice using a rotor-stator homogenizer, pelleting insoluble matter, and passing supernatant through a 0.22 µm filter. Deoxycholic acid was used to solubilize fludioxonil. The initial absorbance at 340 nm (0 minute time point) was measured using a Cary 60 Spectrophotometer (Agilent), after which 25 µl GSNO was added to a final concentration of 0.4 mM and mixed by pipetting to start the reaction. Reactions were performed in triplicate. The absorbance at 340 nm was measured every 60 s for 3 minutes. A decrease in ΔA\(^{340}\) of the control.

**Microtiter dilution assays.** Overnight cultures of yeast grown in SC-ura + raffinose were passaged to an OD600 of 0.025 in SC-Ura + glucose or SC-ura + galactose. Standard MIC dilution broth testing procedures were used with the above media. The final volume in each well was 200 µl. Plates were incubated at 30 °C for 48 hours. Yeast were suspended by shaking prior to OD600 measurement. The growth was calculated as a percentage of the growth relative to DMSO alone.

**Nitrile oxide analysis.** A wild-type yeast culture grown overnight in YPD was diluted to OD600 of 0.4 and grown for another 2 hours at 30 °C. 5 mM GSNO and either 1% DMSO, 25 µg/ml fludioxonil, or 25 µg/ml N6022 was added to 25 ml of culture and the culture was grown another 2hrs at 30 °C, while shaking. Cells were centrifuged and the cell pellets were stored at −80 °C. To lyse cells, pellets were resuspended in 500 µl Yeast Lysis Buffer (50 mM Tris-HCl, pH 8.0, 1 mM DTPA, 5 mM NEM, 0.1% Triton X-100) and transferred to a 2 ml tube containing 0.5 g of 0.5 mm acid-washed glass beads. Tubes were vortexed for 30 seconds followed by 30 seconds on ice for a total of 10 minutes (5 minutes of total vortex time). Crude lysates were centrifuged for 10 minutes at 10,000 rpm and 4 °C to pellet unbroken cells and insoluble material.

The clarified lysates were transferred to a new tube and stored at −80 °C. Protein concentration was determined with a BCA Protein Assay Kit (Pierce). S-nitrosothiol (SNO) levels were measured using the tri-iodide-dependent ozone-based chemiluminescence method, as previously described\(^81\). Briefly, 0.1 volume of...
Green fluorescence was measured with a BD LSRFortessa flow cytometer and the data was analyzed with FlowJo.

**Dfk1 predicted structure modeling and domain identification.** The 3D structure of Dfk1 was predicted using the RaptorX Structure Alignment server (http://raptorx.uchicago.edu/)82. The images were constructed in MacPyMOL (Schrödinger, LLC) using the output from RaptorX. Dfk1 domain designations were from the results of the NCBI Conserved Domain Database83.

**FRET assay.** The YRF-1A RedoxFluor strain was grown overnight in SC-ura−glucose, backpassed into the same medium, and grown to around OD600 of ~0.9. 0.5 ml of yeast was then placed onto Concanavalin A (ConA)-coated 35 mm glass bottom dishes (MatTek, #P35G-1.5-14-C). Yeast were allowed to adhere for 2–3 minutes and unadhered yeast were removed. The dishes were carefully washed twice with media and fresh media added to each dish. A 0 minute reading was taken on an Olympus IX83 microscope with a Semrock CFP/ YFP/HeRed-3 × 3 M-B-000 filter, Hamamatsu ORCA Flash 4.0 camera, and X-Cite 120PC light source, all controlled by the MetaMorph Software platform. The CFP signal was read first (427 nm excitation/472 nm emission) followed by the FRET signal (427 nm excitation/542 nm emission). After taking the 0 minute image, water (no treatment), 1 mM H2O2, 1% DMSO, or 25 μg/ml fludioxonil (in 1% DMSO) was immediately added to the dish. Images were taken every five minutes for 20 minutes total. To obtain the composite image, both sets of images were background subtracted and then the FRET image was divided by the CFP image84 (Fiji, ImageJ). For quantification, a 5 × 5 square was drawn around 30 cells in each image and the intensity in that square was measured84 (Fiji, ImageJ). The FRET signal (Ic, Intensity of the acceptor) was divided by the CFP signal (Ia, Intensity of the donor). Ic/Ia ratios were normalized to the starting 0 minute value. ConA-coated dishes were prepared by adding 0.5 ml of 1 mg/ml ConA (in sterile dH2O) to the center of the dish and incubating the dish for 5 minutes at room temperature. The unadhered ConA was removed, and the dishes were washed twice with sterile dH2O and allowed to dry. Coated dishes were stored at 4 °C for a maximum of two weeks before use.

**H2A.x phosphorylation assay.** Wild-type BY4741 *S. cerevisiae* yeast were grown overnight with YPD and backpassed into fresh YPD to an OD600 of 0.3–0.5. Cultures were grown another 4–5 hours and were split into treatment groups. dH2O, 1% DMSO, or the indicated concentrations of either H2O2 (in dH2O) or fludioxonil (in 1% DMSO) were added and cells were grown for 1 hour. Cells were collected by centrifugation and suspended in 1 ml of KS buffer (0.1 M Potassium phosphate buffer pH 7.4, 1 M sorbitol) with 3% formaldehyde and incubated for 15 minutes at 30 °C, with shaking. 3% ethanolamine was added and incubated with shaking for 15 min at 30 °C. Cells were then transferred to a deep well microtiter plate where they were washed twice with KS buffer. Cells were collected by centrifugation and suspended in 1 ml KS buffer after which 2.5U/ml Zymolase (Zymo Research) (in KS buffer) was added to cells and incubated for at least one hour at 37 °C to digest the cell wall, followed by washing with KS buffer. Cell permeabilization was performed by suspension and incubation in 500 μl of ice-cold methanol for 5 min at −20 °C. Cells were then incubated in 500 μl of ice-cold acetone and incubated for 30 seconds at −20 °C. After permeabilization, cells were washed twice with KS buffer. Blocking was performed for one hour at 4 °C in PBS containing 10% normal Donkey serum. Cells were incubated with 1:1000 anti-γH2A.x antibody (EMB-Millipore Rabbit anti-γ H2A.x [Ser129], #07-745-I) in PBS with 1% normal donkey serum at 4 °C overnight. Cells were washed twice with PBS and incubated with 1:500 anti-rabbit secondary antibody (donkey-anti-rabbit AF488, Jackson Labs) in PBS with 10% normal donkey serum and washed twice in PBS with 1% BSA. Cells were strained through 40 μm mesh immediately prior to cytometric analysis. The cells were analyzed for H2A.x phosphorylation with a BD LSR II flow cytometer and the data was processed with FlowJo.

**Enzymatic “In gel” digestion for mass spectrometry and NanoLC-MS/MS.** All work was done by the Mass Spectrometry Facility (UWBC) in accordance with their standard protocols for these techniques. https://www.biotech.wisc.edu/services/massspec/protocols/ingelprotocol.

Peptides were analyzed by nanoLC-MS/MS using the Agilent 1100 nanoflow system connected to a hybrid linear orbitrap mass spectrometer (LTQ-Orbitrap Elite™, Thermo Fisher Scientific) equipped with an EASY-Spray™ electrospray source. Chromatography of peptides prior to mass spectral analysis was accomplished using capillary emitter column (PepMap® C18, 3 μM, 100 Å, 150 × 0.075 mm, Thermo Fisher Scientific) onto which 3 μl of extracted peptides was automatically loaded. NanoHPLC system delivered solvents A: 0.1% (v/v) formic acid, and B: 99.9% (v/v) acetonitrile, 0.1% (v/v) formic acid at 0.5 L/min to load the peptides (over a 30 minute period) and 0.3 μl/min to elute peptides directly into the nano-electrospray with gradual gradient from 3% (v/v) B to 20% (v/v) B over 18 minutes and concluded with 5 minute fast gradient from 20% (v/v) B to 50% (v/v) B at which time a 4 minute flash-out from 50–95% (v/v) B took place.

**MitoTracker Green staining of mitochondria.** Cultures of wild-type BY4741 *S. cerevisiae* grown in YPD overnight were passaged to fresh YPD to OD600 0.3–0.5 and grown another 4–5 hours. The culture was divided and dH2O (no treatment), 1% DMSO or the indicated concentrations of fludioxonil (in 1% DMSO) was added to each aliquot. Cells were grown for 30 minutes, after which 100 nM MitoTracker Green (ThermoFisher) was added. Cultures were grown for a further 30 minutes. Cells were collected by centrifugation and suspended in KS buffer with 3% formaldehyde. Cells were washed twice with PBS, collected by centrifugation and suspended in PBS with 1% BSA. Cells were strained through 40 μm mesh immediately prior to cytometric analysis. MitoTracker Green fluorescence was measured with a BD LSRFortessa flow cytometer and the data was analyzed with FlowJo.
Triosephosphate Isomerase inhibition assay. Triosephosphate isomerase activity was measured by the technique of Ensafi29, linking the isomerization reaction to an NAD dependent enzymic reaction. Briefly, we treated 5 μg of commercial Triosephosphate isomerase (from S. cerevisiae) in 20 μl triethanolamine-HCl buffer (25 mM, pH 7.9) with 0, 25, 50, 100, and 200 μg/ml fludioxonil and 1% DMSO or with DMSO alone for 30 minutes prior to beginning assay. To a cuvette with a 1-cm light path, we added and mixed 1.4 ml of triethanolamine-HCl buffer, 50 μl of glyceraldehyde phosphate (10 mM), 25 μl of NADH (disodium salt, 7 mM), and 5 μl of α-glycerophosphate dehydrogenase (10 mg/ml). The reaction was started by adding the triosephosphate isomerase/inhibitor combination. The decrease in absorbance of the solution at 340 nm was monitored at 25 °C for 10 minutes on a Genesys 20 spectrophotometer (Thermo Scientific). Inhibition is inversely correlated with the ratio of (final OD340-background)/(starting OD340-background).

Glo1 inhibition assay. Glo1 inhibition by Fludioxonil and the positive control inhibitor S-hexylglutathione was measured by a glo1 assay kit (MAK114, Sigma). Samples were exposed to either fludioxonil in 1% DMSO (10 mM) or S-hexylglutathione in 1% DMSO (10 mM) for 2 hours before the activity of glo1 was measured by production of S-lactoylglutathione from methylglyoxal and glutathione (followed by an increase in absorbance at 240 nm). Samples were read on a Filtermax F5 multi-mode microplate reader (Molecular devices).

HPLC analysis of methylglyoxal in yeast by measurement of diaminobenzene adduct. BY4741 S. cerevisiae, bearing the empty vector plasmid or the Drk1 gene were cultured overnight and back-passed as above. After 3 hours of induction with 2% galactose, either DMSO was added to a final concentration of 0.1% or fludioxonil in DMSO was added to 10 μg/ml and 0.1% respectively (DMSO vehicle was minimized in this assay due to its effect on background MG stress). After 15 min, 1 hour and 3 hours of fludioxonil exposure, yeast were collected, washed once in H2O and suspended in 0.5 M perchloric acid containing 10 mM 1,2-diaminobenzene (DAB). Yeast were lysed by vortexing with glass beads as described above. Lyed yeast and denatured proteins were pelleted and supernates were retained. Supernates were incubated at RT for 30 min to allow methylglyoxal (MG) to form the 2-methylquinoxaline (MQ) adduct with DAB29. Quinoxalines were extracted using 500 μg (DAB). Yeast were lysed by vortexing with glass beads as described above. Lyed yeast and denatured proteins were pelleted and supernates were retained. Supernates were incubated at RT for 30 min to allow methylglyoxal (MG) to form the 2-methylquinoxaline (MQ) adduct with DAB29. Quinoxalines were extracted using 500 μg DAB. Y25 °C for 10 minutes on a Genesys 20 spectrophotometer (Thermo Scientific). Inhibition is inversely correlated with the ratio of (final OD340-background)/(starting OD340-background).

DHAP determination in vivo and MG in vitro. DHAP in vivo was determined using a DHAP assay kit (Abcam), as per the accompanying protocols. Production of MG by TPI reaction in vitro used this kit with the addition of 1 mM DAB. Methylglyoxal adduct was then detected by OD220 on a Genesys 20 spectrophotometer (Thermo Scientific).

Fludioxonil Docking in Triosephosphate Isomerase. Small molecules were built in Sybyl (CertaCorp) and saved in mol2 format. The two TPI protein receptors from Trypanosoma cruzi (PDB code 1tbj) and S. cerevisiae (1ypi) were prepared in Sybyl using the protein preparation tools to add hydrogens, properly type atoms, and remove any clashes. AutoDock Tools were used to prepare the ligand and receptor pdbqt files and to select the docking box size for Autodock4. The docking box size was large enough to include the dimer interface described by Kurkcuoglu et al. Autodock4.2.6 was run with Lamarckian Genetic Algorithm, with 25 M energy evaluations for 27,000 generations. This method includes a Solis & Wets local search of the ligand in the receptor after docking. Autodock 4.2.6 calculates a binding energy by summation of the molecular energy components (desolvation + HBonding + desolvation + electrostatics + ligand torsional free energy) minus the unbound system energy. The 30 best-docked ligands were examined. Optimal docking results were displayed in Pymol (PyMOL Mol. Graphics System, Ver. 1.8.2, Schrodinger, LLC).

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Author Contributions

I.K. constructed the Drk1 cysteine mutant strains, performed the H2A.x and MitoTracker Red oxidative stress assays, and assisted with the GSNOR activity assay. T.B. wrote the final draft of the manuscript, helped with the GSNOR and TPI activity assays, and performed HPLC, TPI, DHAP and MS analyses. S.L. performed the remainder of the experiments and wrote an early draft of this manuscript. D.W. designed, performed and analyzed flow cytometry studies of yeast. B.K. conceived the study and helped design and analyze experiments and write the manuscript.

Additional Information

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