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Discovery of selective non-small cell lung cancer toxins that target mitochondrial Complex I

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Abstract

Selective toxicity among cancer cells of the same lineage is a hallmark of targeted therapies. As such, identifying compounds that impair proliferation to a subset of non-small lung cancer (NSCLC) cell lines represents one strategy to discover new drugs for lung cancer. Previously, phenotypic screens of 202,103 compounds led to the identification of 208 selective NSCLC toxins¹. The mechanism of action for the majority of these compounds remains unknown. Here, we discovered the target for a series of quinazoline diones (QDC) that demonstrate selective toxicity among 96 NSCLC lines. Using photoreactive probes, we found that the QDC binds to both mitochondrial Complex I of the electron transport chain and HADHA, which catalyzes long-chain fatty acid oxidation. Inhibition of Complex I is the on-target activity for QDC, while binding to HADHA is off-target. The sensitivity profile of the QDC across NSCLC lines correlated with the sensitivity profiles of six additional structurally distinct compounds. The anti-proliferative activity of these compounds is also the consequence of binding to mitochondrial Complex I, reflecting significant structural diversity among Complex I inhibitors. Small molecules targeting Complex I are currently in clinical development for the treatment of cancer. Our results highlight Complex I as a target in NSCLC and report seven structurally diverse scaffolds that inhibit Complex I.

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Author contributions

N.M. designed and performed target identification and compound selectivity studies; B.H. synthesized all of the quinazoline dione analogs and probes used in this study; P.M. provided guidance on seahorse bioanalyzer experiments; J.F.C.M. screened quinazoline dione analogs to identify SW217808 (**13**), a p75-specific competitor; K.P. and R.B. performed LC-MS/MS analysis of hydroxyacylcarnitines; J.M.R. designed and supervised chemical synthetic strategies and target identification studies; D.N. designed and supervised target identification and compound selectivity studies. N.M., D.N. and J.M.R. wrote the manuscript.

Introduction

Large scale sequencing studies demonstrate that human cancers, even those originating from the same tissue, have disparate genotypes. As a result, most successful cancer therapies not only require specific and potent on-target activity, but also knowledge of which genetic subtypes are more likely to respond. One strategy to exploit genetic heterogeneity in cancer is to identify small molecules that exhibit selective toxicity across cancer cell lines of the same tissue lineage.

A viability screen of 202,103 small molecules has recently been used to identify 208 compounds that demonstrate selective toxicity amongst 12 non-small cell lung cancer (NSCLC) cell lines¹. These compounds were subsequently evaluated for toxicity against 96 different NSCLC cell lines and 4 non-transformed bronchial epithelial cell lines in dose response experiments. The NSCLC lines have been characterized for gene expression, protein expression, and exome sequencing. Genomic and expression profiling were used to identify genetic interactions for 171 of these compounds¹. Notwithstanding, the development of these compounds depends on understanding their mechanism of action. To date, the mechanism of action for the majority of these selective toxins remains unknown. We have previously used photochemical probes to identify Stearoyl CoA desaturase (SCD) as the direct target of an oxalamide and benzothiazole, both of which are NSCLC selective toxins. These inhibitors are pro-drugs, which are activated by CYP4F11 into covalent SCD inhibitors. The selectivity of these SCD inhibitors depends on CYP4F11 expression².

Here, we have used photochemical probes to determine the mechanism of action for another NSCLC selective toxin, a quinazoline dione compound (QDC). QDC inhibits cancer cell proliferation by directly binding to the ubiquinone binding pocket of mitochondrial Complex I. Two other NSCLC selective toxins, a nitroarene and carboxysulfonamide, have a similar toxicity profile to the QDC. We found that both the nitroarene and carboxysulfonamide also inhibit proliferation by targeting mitochondrial Complex I.

Results

An anti-cancer toxin, SW069087 (**1**), hereafter referred to as the quinazoline dione compound (QDC), was identified from a small molecule screen aimed at discovering selective toxins against non-small cell lung cancer (NSCLC) cell lines (Figure 1a)¹. The proliferative impact across a range of concentrations of QDC on 96 different NSCLC lines has previously been reported¹. The dose that results in a 50% reduction in viability (IC₅₀) was less than 1 μ M for 37% (n=37), while 41% (n=41) cell lines had no loss in viability at 50 μ M, the highest concentration tested (Figure 1b and Table S1). Importantly, four non-transformed cell lines (HBEC34KT, HBEC3KT, HBEC13KT, and HBEC30KT) were insensitive to QDC with IC₅₀ greater than 8 μ M, suggesting that QDC has cancer-specific activity. We sought to characterize the QDC mechanism of action by identifying proteins that bind to QDC.

To identify proteins that bind to QDC, we synthesized a chemical probe SW218360 (**2**), hereafter referred to as QDC-probe, with a benzophenone and terminal alkyne group (Figure

2a). The benzophenone group is photo-reactive, and in the presence of ultraviolet (UV) light is expected to generate a reactive di-radical that will covalently modify a nearby amino acid. The alkyne group permits conjugation of QDC-probe to the azide moiety via a copper-catalyzed [3+2] cycloaddition, commonly referred to as a “click” reaction. The QDC-probe inhibited the proliferation of K-562 cells in a dose-dependent manner (Figure 2b). We treated K-562 cells with several concentrations of QDC-probe with and without UV (310 nm) light. After UV treatment, we labeled QDC-probe bound proteins by performing a click reaction between protein lysates and a fluorescent azide. QDC-probe bound protein was visualized by SDS-PAGE and in-gel fluorescence scanning (Figure 2c). Without UV light, no protein bands were identified. By contrast, several protein bands were observed in the cells treated with UV light, representing potential non-covalent binding partners for the QDC-probe. These QDC-probe bound proteins are candidate targets.

We next sought to determine if any of the QDC-probe UV-crosslinked proteins are responsible for QDC’s anti-proliferative activity. We reasoned that only active QDC analogs would compete for the QDC target. To this end, we synthesized derivatives of compound **1** featuring modifications at various positions (Table 1). For example, extending the N-alkyl chain (**3**) increased potency, and replacing the linear alkyl chain with a benzyl group (**10**) maintained cytotoxicity. Interestingly, an N-hexyl analog (**9**) proved less active than either the N-pentyl (**1**) or the N-heptyl (**3**) congeners. Introducing polar functionality such as an N(H)Boc group abrogated activity completely (**8**). Replacing one of the quinazoline dione carbonyl with an amine (**11**) or removing it altogether (**7**) was associated with a slight decrease in potency. However, removal of both the N-alkyl and carbonyl group, as in quinazoline **4**, resulted in an inactive analog. A piperidine linker (**10**) improved activity relative to the piperazine **1**. This result proved critical to our target identification efforts because the piperazine analog of photocrosslinker **2**, while cytotoxic, failed to give any detectable crosslinked bands. Finally, substitution on the nitroarene ring resulted in a modest loss in potency (**11**).

We treated K-562 cells with 0.3 μ M of QDC-probe (**2**) along with increasing concentrations of SW218336 (**3**) (IC_{50} = 0.23 μ M in K-562). Compound **3** competed two UV-dependent bands at 25kD and 75kD, which we refer to as p25 and p75 respectively (Figure 2d). Importantly, the inactive QDC analog SW218973 (**4**) (IC_{50} > 50 μ M) does not compete p25 or p75 (Figure 2e). These experiments implicate p25 or p75 as the target but do not distinguish between them. To distinguish between p25 and p75, we correlated the anti-proliferative activity of seven additional QDC derivatives with their ability to bind either p25 or p75 (Figure S1). From this analysis, we found a higher correlation between anti-proliferative toxicity and p25 binding ($R^2=0.91$) (Figure 2f) versus p75 binding ($R^2=0.63$) (Figure 2g). The reduced correlation for p75 is primarily the result of two outliers, compound **3** and **5**, which demonstrated EC_{50} for p75 competition that is approximately 10-fold higher than their cellular IC_{50} . Since these two analogs are toxic to cells at concentrations lower than those at which they compete p75, we reasoned that p75 is likely an off-target of the QDC scaffold. By contrast the EC_{50} for p25 competition closely matches the IC_{50} for cellular toxicity across several QDC analogs. Based on these observations, we hypothesized that binding to p25 is likely responsible for QDC’s anti-cancer activity.

To identify both p25 and p75, we repeated our prior photo-crosslinking experiments on a larger scale and used a diazo-biotin-azide in place of the fluorophore-azide. We included the following two controls, which are conditions that are not expected to crosslink p25 or p75: 1) no UV treatment and 2) excess active derivatives as competitors (Figure 3a). We used click chemistry to label p25 and p75 with diazo-biotin-azide and then enriched biotinylated proteins with streptavidin-bound resin. The azide moiety participates in the click conjugation, while the diazo-linker provides a cleavage site for elution from streptavidin. Streptavidin-bound proteins were eluted with either sodium dithionite, which reductively cleaves the diazo-biotin, or extended denaturation by boiling in SDS. Following SDS-PAGE, gels were subject to silver (Figure 3b) or Coomassie staining. Gel slices of p25 and p75 as well as a gel section containing the full eluates from each condition were treated with trypsin, and the resulting peptides were fractionated by liquid chromatography and characterized by mass spectrometry. Peptides arising from MT-ND1 and NDUFS7 were identified in the crosslinked condition but were not detected in the two control samples (Figure 3c). MT-ND1 and NDUFS7 encode core subunits of mitochondrial Complex I. Human MT-ND1 was previously shown to migrate on denaturing gels at 24kD, consistent with the molecular weight of p25³. This led us to hypothesize that p25 is the Complex I subunit MT-ND1. HADHA (hydroxyacyl CoA dehydrogenase subunit alpha) peptides were identified in gel slices of p75 at levels 10-fold higher than in the negative control samples (Figure 3d). HADHA has a predicted molecular weight of 83 kD and encodes a subunit of the mitochondrial trifunctional protein, which catalyzes the three terminal steps of β -oxidation to liberate acetyl-CoA from long chain fatty acids.

After identifying p25 and p75 as MT-ND1 and HADHA respectively, we sought to determine the biochemical consequence of QDC binding to either protein. HADHA encodes one subunit of the mitochondrial trifunctional protein (TFP), which catalyzes three steps in long-chain fatty acid oxidation. TFP is peripherally associated with the mitochondrial inner membrane. It is comprised of two subunits α and β , which are encoded for by the genes HADHA and HADHB, respectively. We used CRISPR/Cas9 to silence HADHA and then isolated three independent clones that lack HADHA expression (Figure 4a). HADHA knockout resulted in the inability of the QDC-probes SW218360 (**2**) and SW218443 (**12**) (Figure 4b) to crosslink p75, providing independent genetic evidence that p75 is indeed HADHA (Figure 4c and Figure 4d). Compound **12** is a diazirine QDC-probe that crosslinks p75 (HADHA) but not p25 (Complex I). We subsequently found that HADHA loss does not alter sensitivity to the QDC (Figure 4e), which indicates that binding HADHA does not account for the anti-proliferative effect of QDC.

Even though HADHA binding is off-target for QDC, it is important to know whether QDC inhibits HADHA, as this could lead to potential adverse events in pre-clinical systems. Since HADHA activity could be influenced by QDC binding to mitochondrial Complex I, we repeated the competition experiments described in Figure 2 with the objective of identifying a QDC analog that binds to HADHA but not mitochondrial Complex I. SW217808 (**13**) (Figure 5a), is a QDC analog that binds specifically to p75 (HADHA) and not to p25 (Complex I) at 2 μ M (Figure 5c). We also identified a structurally similar compound that binds neither HADHA nor Complex I, SW213110 (**14**) (Figure 5b), to serve as a negative

control. To measure HADHA activity, we analyzed hydroxyacylcarnitine levels in parental H157 cells treated with compounds **13** and **14** as well as HADHA KO H157 cells (Figure 5e). As expected, genetic loss of HADHA leads to an increase in hydroxyacylcarnitines. By contrast, compound **13** has no effect on HADHA activity, thereby mitigating concerns that HADHA is a relevant off-target.

Competition experiments identified a correlation between binding to mitochondrial Complex I and anti-proliferative activity providing evidence that it is on-target (Figure 2f). Complex I is a large L-shaped protein complex of approximately 45 subunits, with a hydrophobic arm embedded in the inner mitochondrial membrane and a hydrophilic arm extending into the mitochondrial matrix. Complex I oxidizes NADH and shuttles electrons to ubiquinone, which generates ubiquinol. Ubiquinol donates electrons to downstream components of the mitochondrial electron transport chain. Complex I couples the reduction of ubiquinone by NADH to the pumping of protons from the mitochondrial matrix into the intermembrane space. This provides the electro-chemical potential required for ATP synthesis⁴. Cryo-EM structural studies of Complex I indicate that NDUFS7 and MT-ND1, the proteins identified as candidate QDC binding partners, line the ubiquinone binding pocket^{5–6}. Rotenone and piericidin are chemically distinct from QDC and are known to inhibit Complex I by binding to MT-ND1⁷, and therefore, we hypothesized that these molecules would compete for QDC binding to MT-ND1. Both piericidin and rotenone competed for QDC-probe binding to p25 (Figure 6a). In contrast, antimycin and oligomycin, which inhibit downstream components of the electron transport chain, Complex III and Complex V respectively, do not compete p25 (Figure 6b). These data confirm that QDC binds to MT-ND1 in the Complex I ubiquinone pocket.

We performed two experiments to test the hypothesis that the QDC inhibits mitochondrial respiratory activity and Complex I activity specifically. First, we measured oxygen consumption in H157 cells upon treatment with the active QDC analog SW218336 (**3**) (IC₅₀ 0.15 μ M in H157 cells) using a Seahorse bioanalyzer. We observed a dose-dependent decrease in oxygen consumption in H157 cells upon treatment with compound **3**, which is consistent with inhibition of Complex I activity (Figure 6c). We then measured oxygen consumption in permeabilized cells in the presence of either Complex I substrates (pyruvate and malate (PM)), or Complex II substrates (succinate and rotenone (SR)). Under PM conditions, permeabilized cells respire primarily through Complex I, while SR conditions promote Complex II-mediated respiration⁸. The QDC demonstrated a dose-dependent inhibition of respiration under PM conditions (Figure 6d), but not under SR conditions (Figure 6e). These experiments demonstrate that the QDC specifically inhibits Complex I activity.

Cultured cells that are unable to engage in oxidative phosphorylation (OxPhos) become pyruvate auxotrophs⁹. To confirm that the QDC impairs cell proliferation by inhibiting OxPhos we evaluated how pyruvate supplementation influences QDC activity. Pyruvate supplementation rescues the toxicity of piericidin (Figure 7a) as well as an active QDC analog, SW218336 (**3**) (Figure 7b). Taken together, these experiments provide evidence that QDC impairs cell proliferation by blocking OxPhos via inhibition of Complex I.

We subsequently tested the activity of the QDC and piericidin in six NSCLC lines from the initial screen, three of which were predicted to be sensitive and the other three insensitive. Concordant with the original screening data, we found that H157, DFCI-024 and H1581 cells were sensitive to both QDC (IC₅₀ range of 0.2-0.5 μ M) and piericidin (IC₅₀ range of 3-10 nM) while H1437, H1693 and H2882 cells were insensitive to both compounds even at concentrations as high as 50 μ M (Figure 7c). One explanation for resistance is that the compounds fail to inhibit Complex I in insensitive cell lines, either because of metabolism of the compound or differences in the target protein. To address this possibility, we measured oxygen consumption in three insensitive cell lines. QDC and piericidin inhibit oxygen consumption in the three insensitive cell lines, thereby ruling out lack of target engagement as an explanation for resistance (Figure S2). These data support the notion that the QDC and piericidin have the same mechanism of action and that Complex I inhibition is indeed selectively toxic across NSCLC lines. This observation is consistent with previous studies demonstrating that OxPhos inhibition is selectively toxic in cultured cancer cells¹⁰⁻¹¹.

After confirming that Complex I inhibition leads to selective toxicity in NSCLC cell lines, we considered the possibility that other compounds identified in the same phenotypic screen might also inhibit Complex I. Each of 221 (208 selective toxins plus 13 known toxins) compounds was evaluated in dose response for anti-proliferative activity to 96 different NSCLC cell lines¹. We correlated the area under the dose response curve (AUC) for each of the 220 NSCLC selective toxins to that of the QDC (Figure 8a and Table S4). The correlation coefficients (r) ranged from -0.22 to 0.73, and six compounds (compounds **15-20**) had a correlation coefficient greater than 0.60 (Figure 8a-g). The correlation between the selectivity profiles of these six compounds and QDC raised the hypothesis that **15-20** also target Complex I. Compounds **15-20** compete with the QDC-probe (**2**) binding to Complex I (Figure 8h). Pyruvate supplementation rescues the anti-proliferative effects of these compounds confirming that their activity is indeed due to Complex I inhibition (Figure 9). These experiments, taken together, support the hypothesis that Complex I is the target of compounds **15-20**.

Discussion

We have identified mitochondrial Complex I as a target in NSCLC by discovering the mechanism of action for three compounds that demonstrate selective toxicity across a well-annotated panel of 96 NSCLC lines. First, we used photochemical probes for a quinazoline dione to identify mitochondrial Complex I and HADHA as a binding partners. To distinguish between selective and non-selective binding events, we performed dose-response competition experiments with multiple analogs. These analogs featured modifications at multiple locations on the chemical scaffold and varied in potency by over 100-fold. Competition experiments indicated that the correlation between binding and toxicity was incomplete for p75 (HADHA), suggesting that HADHA is an off-target. By contrast, binding p25 (Complex I) correlated closely with toxicity, suggesting that Complex I is the functional target of the QDC.

We also identified six additional compounds (representing five unique scaffolds), compounds **15-20**, from the same phenotypic screen, as Complex I inhibitors. These

compounds have similar selectivity profiles to the quinazoline dione across NSCLC lines. Even though these five scaffolds have unique chemical structures, they bind to the same pocket of Complex I. The QDC and carboxysulfonamides both feature an aliphatic tail that impacts potency. This group is linked to an amide by way of an aromatic ring. Nonetheless, the specific functionality is rather different between the two scaffolds. Likewise, the nitroarene scaffold does not show clear structural overlap with either the QDC or the carboxysulfonamide scaffold. Major substituents are displayed meta on the central aromatic ring in the former scaffold and para in the latter two scaffolds. Thus, in addition to variation in the specific functional groups, the overall shapes of these inhibitors are distinct. Taken together, these results suggest that Complex I is able to bind a wide diversity of chemical matter, reminiscent of the hERG channel or the aromatic hydrocarbon receptor.

Mitochondrial Complex I is a known cancer metabolism target. A recently reported Complex I inhibitor, IACS-010759, demonstrated potent anti-tumor activity in pre-clinical animal models and is distinct from the three aforementioned scaffolds¹². IACS-010759 is currently in clinical trials for solid tumors and leukemia. In preclinical models, IACS-010759 has a narrow therapeutic index: 10 mg/kg is efficacious, but 25 mg/kg is toxic to mice harboring tumors derived from human cancer cells (xenografts). It is unknown whether the observed toxicity is on or off-target. In this regard, it will be important to optimize at least one of the three novel, independent Complex I inhibitors identified herein for animal use. If novel scaffolds also have a narrow therapeutic index, it will support the hypothesis that toxicity is mechanism related. By contrast, if the toxicity of IACS-010759 is off-target, one of these scaffolds might afford an opportunity to widen the therapeutic index.

The promiscuity of the ubiquinone binding pocket for small molecules could be problematic to the development of other drugs¹³. Along these lines, an earlier phenotypic screen for anti-cancer molecules identified a photoreactive probe and chemical scaffold that bind Complex I¹⁴. More recent work has demonstrated that Complex I inhibition is the on-target effect of a purported ERBB2 inhibitor, Mubrutinib, in acute myeloid leukemia¹⁵. Given the structural diversity of compounds that bind Complex I, it is plausible that Complex I might be a frequent off-target in drug development. In this regard, the QDC-probe (**2**), along with a previously reported Complex I probe¹⁴, constitute valuable tools to test whether a compound binds to the ubiquinone binding pocket of mitochondrial Complex I. Off-target profiling is a routine aspect of drug discovery, and the tools provided herein could add a new dimension to that analysis.

Complex I activity is not essential for all cancer cells to grow in culture or *in vivo*. The Complex I inhibitors identified in this study were evaluated for toxicity in 96 different NSCLC cell lines including four non-cancerous immortalized bronchial epithelial cells. Approximately 40% of cell lines, including the immortalized epithelial cells, were resistant to Complex I inhibition. On the other hand, around a third of cell lines were sensitive to Complex I inhibition. The clinical development of Complex I inhibitors for the treatment of NSCLC depends on identifying tumor properties that dictate reliance on Complex I.

Pyruvate supplementation or expression of NADH oxidases regenerates NAD⁺ and also rescues proliferative defects caused by Complex I inhibition^{9–10, 16}. Complementation of

redox imbalance maintains proliferation in the presence of OxPhos inhibition by driving aspartate synthesis^{17–18}. Aspartate is a substrate for nucleotide synthesis and is therefore essential for proliferation. Exogenous aspartate can restore proliferation in cells treated with mitochondrial inhibitors, supporting the notion that maintaining aspartate synthesis is an important function of the electron transport chain^{17–18}. In regards to factors that predict sensitivity to Complex I inhibition, prior work shows that high basal levels of aspartate correlate with resistance to OxPhos inhibitors¹¹. Specifically, cell lines that are able to scavenge aspartate, are more resistant to mitochondrial inhibition. Thus, it is possible that intracellular aspartate availability, as dictated by uptake or endogenous synthesis, determines sensitivity of tumors to Complex I inhibition.

Methods

Cell culture, dose response curves and cell viability measurements

All NSCLC lines were obtained from the Minna and Gazdar laboratories at UT Southwestern. Cell lines were screened for mycoplasma and authenticated by short-tandem repeat analysis through the McDermott Core at UT Southwestern. The K-562 cell line used in this study was a gift from the laboratory of Jonathan Weissman (UCSF) and expresses dCAS9-KRAB-BFP¹⁹. NSCLC lines and K-562 cells were cultured in RPMI 1640 (Sigma) supplemented with 5% FBS (Sigma) and 2 mM glutamine (Sigma).

For dose-dependent compound viability assays involving K-562 cells, cells were plated at 1000-1500 cells per well in 96-well plates in 100 μ L of media. Immediately after plating, cells were treated with compounds in dose response. DMSO concentration was kept constant at 0.5%, and each dose of compound was tested in triplicate. Cells were incubated at 37°C and 5% CO₂ for 72h with compound at which point cell viability was assayed using CellTiter-Glo® (Promega G7570) per manufacturer instructions.

For viability assays involving adherent NSCLC cells, cells were plated at 1000-4000 cells per well (depending on cell line) in 96-well plates in 100 μ L of media. Cells were allowed to adhere overnight and were treated with compounds in dose response the following day. Compounds were added from DMSO stocks using a Tecan D300e digital dispenser. Each dose of compound was tested in triplicate and DMSO concentration was kept constant at 0.1%-0.5% depending on cell line. Cells were incubated for at least 3 doublings (which ranged from 3-5 days) with compound at which point cell viability was assayed using CellTiter-Glo® (Promega G7570) per manufacturer instructions. In the case of adherent cells, cells were allowed to grow until they reached 80% confluency in the vehicle treated condition for the dose response. Data were plotted and IC₅₀ values were determined by using GraphPad Prism to generate a four-parameter dose response curve.

Compound treatment, cell lysis and click chemistry

For crosslinking experiments involving the quinazoline dione probe SW218360 (**2**), 10⁶ K-562 cells were first plated in 12-well plates at cells per well in 1mL of media. Following plating, cells were treated with probe and/or quinazoline dione competitors and incubated at 37°C/5% CO₂ for 2 hours. All compounds were dissolved in DMSO. After incubation, the

lids were taken off the cell plates. The plates were placed in a glass dish filled with ice and then subjected to 15 minutes of UVB irradiation in a Stratalinker® UV Crosslinker. After crosslinking, cells were collected into 1.5 mL tubes, centrifuged at 1200 rpm for 5 minutes at room temperature, washed once with PBS, centrifuged once again at 1200 rpm for 5 minutes and then lysed in 1% SDS Buffer A (50 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl₂) supplemented with benzonase nuclease (Sigma E1014) at a 1:10000 dilution. Protein concentration among different cell lysates were normalized using A₂₈₀ measurements. Equal amounts of protein were then subject to a click reaction with 100 μM Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) dissolved in 4:1 DMSO: t-butanol solvent, 1 mM sodium ascorbate, 2 mM CuSO₄, and 25 μM AlexaFluor 532 azide for 1 hour at room temperature. Synthesis of AlexaFluor 532 azide was previously described². The click reaction was quenched using 4x Laemmli sample buffer supplemented with 50 mM 2-mercaptoethanol. Lysates were subjected to SDS-PAGE and imaged with a Typhoon Scanner (excitation: 532 nm, emission filter: 555 nm) to detect fluorescently labeled proteins. Following in-gel fluorescence scanning, gels were fixed and stained with Coomassie to ensure equal protein loading. The densitometry feature of ImageJ was used to quantify intensity of probe-crosslinked bands and competition EC₅₀ values.

For crosslinking experiments involving adherent cell lines such as NCI-H157, cells were plated in 12-well plates at a density of 250,000-300,000 cells per well in 1 mL of media and allowed to adhere overnight. Compound treatment and UV-crosslinking was performed the following day as described in previous paragraph. After crosslinking, cells were washed once with PBS and lysed directly in the 12-well plate using the 1% SDS Buffer A supplemented with benzonase (described in previous paragraph). Lysates were collected into 1.5 mL microcentrifuge tubes and then subjected to a click reaction with AlexaFluor-532 azide (described in previous paragraph).

Enrichment of compound bound proteins

To enrich proteins that are crosslinked by the quinazoline dione probe, three 1L cultures of K-562 cells were grown to maximum density (1 million cells/mL). Two flasks of cells were treated with 300 nM SW218360 (**2**), while the third flask was treated with 300 nM SW218360 (**2**) + 6 μM SW218336 (**3**) (competitor) for 2 hours. After treatment, cells were spun down at 1200 rpm for 10 minutes at room temperature. Approximately 800-900 mL of media was removed from each of the three 1L K-562 cultures and cells were resuspended in the remaining media. Cells from each of the three cultures were evenly distributed into four 15 cm tissue culture plates for UV-crosslinking. One of the K-562 cultures treated with just 300 nM SW218360 (**2**) was subject to 15 minutes of UV-Crosslinking on ice, while the other was simply kept on ice (no UV treatment). The cells treated with both probe and competitor were also subject to 15 minutes of UV-crosslinking. This resulted in three different experimental conditions: probe – UV, probe + UV, probe + competitor + UV. Following crosslinking, cells were spun down at 1200 rpm for 10 minutes, washed once in PBS and then lysed in 30-40 mL of 1% SDS Buffer A (50 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl₂). After lysis, cells were homogenized using a 22-gauge needle 5-10 times and then supplemented with 5 μL of undiluted benzonase nuclease. Cell lysates were rocked at room temperature for 25 minutes to ensure complete digestion of nucleic acids by benzonase.

Lysates were then supplemented with an additional 3% SDS for a final concentration of 4% SDS in the lysis buffer. Lysates were clarified by centrifugation at 30,000 RPM at room temperature for 1h in a swinging bucket rotor ultracentrifuge and then filtered through a 0.22 micron filter using a syringe. Protein concentration was determined using a Pierce BCA assay and protein concentrations were normalized to 5mg/mL or less. Equal amounts of protein (100-130mg) from each experimental condition were taken and subject to a click reaction in 100 μ M Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) dissolved in 4:1 DMSO:t-butanol solvent, 1 mM tris(2-carboxyethyl)phosphine (TCEP), 2 mM CuSO₄, and 100 μ M diazo biotin-azide (Click chemistry tools 1041-25) for 1 hour at room temperature. The diazo biotin-azide powder was dissolved in DMSO. Protein was precipitated in four volumes of cold acetone (pre-chilled at -80°C for 1h) for 30 minutes at -80°C . Insoluble protein was spun down at 4000rpm for 10 minutes at 4°C . Acetone was removed and protein pellet was washed twice more with cold acetone to remove all free diazo biotin-azide dye. After the final acetone wash, cell pellet was dried for 10 minutes at room temperature and resuspended in 30-40 mL of 4% SDS in Dulbecco's Phosphate Buffered Saline (DPBS). Cell pellets were rocked at room temperature overnight to resolubilize protein. Protein estimation was performed using the Pierce BCA assay, and protein concentration was normalized between samples. An equal amount of protein (100-130 mg) was subject to treatment with 150 μ L of streptavidin agarose ultra-performance beads (TriLink Biotechnologies N-1000). Beads were vortexed hard for 30 seconds prior to being added to cell lysates. Lysates were incubated with streptavidin beads for 30 minutes at room temperature with gentle rocking. Lysates were then centrifuged at 1200 rpm for 5 minutes at room temperature to pellet streptavidin beads. Supernatant was removed, and beads were washed three times (10 minutes per wash) in 15 mL volumes of 4% SDS/DBPS. For dithionite elution, beads were washed a final time in 1 mL of 4% SDS Buffer B (200 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl₂), after which biotinylated species were chemically eluted with 100 μ L 4% SDS Buffer B supplemented with 50 mM sodium dithionite (Sigma Aldrich 157953). Buffer B was supplemented with fresh sodium dithionite powder right before elution. For elution, beads were incubated with 4% SDS Buffer B + 50 mM sodium dithionite with agitation on an Eppendorf ThermoMixer© at room temperature for 15 minutes. Samples were then spun for 3 minutes at 1200rpm at room temperature and the eluate was moved to a clean 1.5 mL tube. Beads were then subject to another 15-minute round of sodium dithionite elution (sodium dithionite powder was once again dissolved fresh in 4% SDS Buffer B). Samples were then spun for 3 minutes at 1200rpm at room temperature and the eluate was pooled with sample from the previous dithionite elution. After two rounds of dithionite elution, beads were boiled at 95°C for 15 minutes in 100 μ L of 4% SDS Buffer A (50 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl₂) with vortexing every 5 minutes. Samples were then spun at 1200 rpm for 3 minutes at room temperature and the supernatant was taken to a clean 1.5 mL tube. Sodium dithionite and boiled eluates were supplemented with 4x Laemmli Sample buffer (with 50 mM 2-mercaptoethanol). 35 μ L of each sample was run on 12% NuPage® Bis-Tris gels (ThermoFisher) in MOPS SDS running buffer at 120V and the gels were silver stained. Protein bands of interest were excised with a clean blade, destained, and sent for proteomics analysis. For shotgun proteomics experiments involving analysis of all proteins in the eluate (unfractionated), samples were run until they just penetrated the gel, at which time the gel

was immediately fixed and stained with Coomassie. The Coomassie stained gel section containing the unfractionated proteins were excised with a clean blade and sent for proteomics analysis.

Methods for Protein Sequence Analysis by LC-MS/MS.

Excised gel bands were cut into approximately 1 mm³ pieces. Gel pieces were then subjected to a modified in-gel trypsin digestion procedure²⁰. Gel pieces were washed and dehydrated with acetonitrile for 10 min. followed by removal of acetonitrile. Pieces were then completely dried in a speed-vac. Rehydration of the gel pieces was with 50 mM ammonium bicarbonate solution containing 12.5 ng/μl modified sequencing-grade trypsin (Promega, Madison, WI) at 4°C. After 45 min, the excess trypsin solution was removed and replaced with 50 mM ammonium bicarbonate solution to just cover the gel pieces. Samples were then placed in a 37°C room overnight. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. The extracts were then dried in a speed-vac (~1 hr). The samples were then stored at 4°C until analysis.

On the day of analysis the samples were reconstituted in 5 - 10 μl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 2.6 μm C18 spherical silica beads into a fused silica capillary (100 μm inner diameter x ~30 cm length) with a flame-drawn tip²¹. After equilibrating the column each sample was loaded via a Famos auto sampler (LC Packings, San Francisco CA) onto the column. A gradient was formed and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid).

As peptides eluted they were subjected to electrospray ionization and then entered into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein databases with the acquired fragmentation pattern by the software program, Sequest (Thermo Fisher Scientific, Waltham, MA)²². All databases include a reversed version of all the sequences and the data was filtered to between a one and two percent peptide false discovery rate.

Analysis of Proteomics Data

p75 and p25 were identified in separate proteomics experiments. The methods of identification of each species is detailed below.

Identification of p25—To identify p25, Coomassie stained gel sections of dithionite eluates were analyzed by LC-MS/MS as described in the previous section (n=1 gel section for each experimental condition). For data analysis, all proteins present at less than 5 total peptides were excluded. Next, proteins were sorted by the ratio of “sum intensity” in the probe + UV condition as compared to the probe – UV and probe + UV + competitor conditions. Proteins present at a higher degree in the probe + UV sample as compared to the

other groups sorted to the top of the list. The top five proteins from this list are presented in Figure 3c.

Identification of p75—To identify p75, silver stained gel bands of p75 from boiled eluates were analyzed by LC-MS/MS as described in the previous section (n=1 gel slice for each experimental condition). For data analysis, all proteins present at less than 10 total peptides were excluded initially. The remaining proteins were ranked by degree of enrichment in the probe + UV treated samples as compared to the other two experimental conditions by % total intensity. This parameter reflects the peptide ion signal corresponding to a particular protein in the sample as a percentage of the total ion signal of all proteins in the sample. The top 5 proteins from this list are presented in Figure 3d.

SDS-PAGE and Immunoblots

Cells were lysed in 1% SDS Buffer A (50 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl₂) supplemented with 1:10000 benzonase nuclease (Sigma E1014). Protein concentration was measured by A₂₈₀ and lysates were normalized. Samples were supplemented with 4x Laemmli Sample buffer containing 50 mM 2-mercaptoethanol. Samples were boiled for 3 minutes at 95°C and immediately loaded on a Tris-Glycine gel (10%). Gels were run at a constant voltage of 120V. Gels were transferred onto 0.45 micron nitrocellulose membrane by wet transfer in a standard Towbin transfer buffer for 75 minutes at 400mA constant current. An ice pack was placed in the transfer chamber to prevent overheating.

Membranes were stained with Ponceau S (0.1% w/v in 5% acetic acid) immediately after transfer to visualize total protein content on membranes. Ponceau was removed by washing membranes in Phosphate Buffered Saline with 0.025% Tween 20 (PBST). Following removal of ponceau stain, membranes were blocked for 45 minutes in 5% non-fat dry milk (NFDM) diluted in PBST. Membranes were then incubated with primary antibody overnight at 4°C with gentle rocking in 5% NFDM diluted in PBST. The following day, membranes were washed three times for five minutes per wash in PBST. Secondary antibody (HRP-conjugate) was added to membranes in PBST alone (no milk) for 1 hour at room temperature with gentle rocking. Membranes were washed three times for ten minutes per wash and then incubated for 1 minute with 2-3 mL of Pierce™ ECL Western Blotting Substrate (ThermoFisher 32209). Blots were imaged using a Bio-Rad Chemidoc™ imaging system. The following are the dilutions and product numbers for the primary antibodies used in this study: anti-HADHA 1:1000 (Abeam ab203114). The HADHA antibody was raised in rabbit and was subsequently probed with 1:5000 dilution of Goat anti-rabbit HRP (Bio-Rad 170-6515).

Cellular oxygen consumption measurements

An Agilent Seahorse XF96e Analyzer was used to perform oxygen consumption measurements in cells. NSCLC cells were plated at 20,000 cells per well in 80μL media. Cells were not plated in corner wells of the Seahorse plates. Cells were kept in the tissue culture hood for 1h after plating and then allowed to adhere overnight at 37°C/5% CO₂. The following day, cells were washed twice with 200μL/well of Seahorse medium and then put in 150μL/well Seahorse medium for the assay. Seahorse medium was made from DMEM

(Sigma D5030) without phenol red and sodium bicarbonate, supplemented with 10 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate.

After being washed and transferred to Seahorse medium, cells were moved to a 37°C incubator free of CO₂ for 45 minutes. Compounds were diluted appropriately in Seahorse medium and then loaded into the Seahorse cartridge. The loaded Seahorse cartridge was placed into the Seahorse XF Analyzer for calibration. After calibration was completed, the cell plate was loaded and oxygen consumption measurements were recorded.

For Seahorse assays involving permeabilized cells, NCI-H157 cells were plated at 20,000 cells per well in 80µL media. Cells were not plated in corner wells of the Seahorse plates. Cells were kept in the tissue culture hood for 1h after plating and then allowed to adhere overnight at 37°C/5% CO₂. The following day, cells were washed once with 200 µL/well of 1X Mitochondrial Assay Solution (MAS) buffer (220 mM mannitol, 70 mM sucrose, 10 mM KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES, 1 mM EGTA, 0.6% w/v fatty acid free BSA). The pH of the MAS buffer was adjusted to 7.4 using KOH (cannot use NaOH due to the potential for activating the mitochondrial Na⁺/Ca²⁺ exchanger). After washing, cells were placed in 150 µL MAS buffer containing 2.0 nM XF Plasma Membrane Permeabilizer (XF-PMP) (Agilent 102504-100) and either 10 mM pyruvate + 2 mM malate + 4 mM ADP or 10 mM succinate + 2 µM rotenone + 4 mM ADP. Pyruvate (Sigma 107360), succinate (Sigma S3674) and malate (Sigma M6413), were supplemented in MAS buffer in the free acid form. ADP (Sigma A5285) was added as a potassium salt. Following mitochondrial substrate supplementation, the pH of the MAS buffer was adjusted to 7.4 using KOH. Once cells were placed in MAS buffer containing XF-PMP and mitochondrial substrates, cells were immediately assayed for oxygen consumption without any intervening incubation in a CO₂-free chamber.

Generation of knockout cell lines using CRISPR/Cas9

HADHA was genetically silenced in NCI-H157 cells using CRISPR/Cas9. H157 cells were plated in 6-well plates at 100,000 cells per well. The following day, cells were transfected with 900ng px330 (Addgene 42230) HADHA sgRNA plasmid and 100ng pSF-CMV-PGK-Puro (Oxford Genetics OG394R1) using a 3:1 ratio of Fugene® HD (Promega E2311) to plasmid DNA (3µL Fugene: 1µg plasmid). Transfection mixes were diluted in Optimem® serum-free medium (ThermoFisher 31985070). 24 hours after transfection, cells were selected for 48h using 2µg/mL puromycin. Puromycin was removed from cells and the transfectants were allowed to grow for one week. sgRNA transfected cells were then trypsinized, diluted and plated at 300-1000 cells per plate in 10 cm dishes. After 2 weeks, approximately 20 individual clones were picked and propagated for validation of HADHA knockout (by western blot).

Plasmids

HADHA knockout—sgRNAs to knock out HADHA (hydroxyacyl-CoA dehydrogenase) were cloned into the BbsI site of px330 (Addgene 42230) using the following pairs of annealed oligonucleotides (bold letters denote sgRNA targeting sequences):

sgHADHA-1: CACCGGGGACTGGTTGACCAACTGG/
AAACCCAGTTGGTCAACCAGTCCCC

sgHADHA-3: CACCGATGTGCTAACACTTACCCAG/
AAACCTGGGTAAGTGTTAGCACATC

sgHADHA-5: CACCGTTCTTGAAGGGTCTTGCAAG/
AAACCTTGCAAGACCCTTCAAGAAC

Oligonucleotide pairs were diluted at a concentration of 3 μ M per oligo in 1x NEB Buffer 2 (New England BioLabs B7002S) and then annealed by boiling at 95°C for 5 minutes followed by incubation in a 75°C water bath that was slowly allowed to equilibrate to room temperature. The oligos were then diluted 1:100 in water, and 1 μ L of the diluted oligos were ligated into 30-50 ng of BbsI digested px330 with T4 DNA ligase. Ligation reaction was carried out in a total volume of 10 μ L at room temperature for 1 hour. 1-2 μ L of the ligation reaction was transformed into 50 μ L of DH5 α cells. Individual bacterial colonies were propagated in LB-Amp liquid culture after which the isolated plasmid DNA was subject to restriction digest and Sanger sequencing for validation of sgRNA incorporation into px330.

Hydroxyacylcarnitine analysis of cells

H157 cells were plated in 6cm dishes at 70% confluence and allowed to adhere overnight. The following day, cells were washed once with DPBS and then exchanged to RPMI media, 5% FBS, 4 mM glutamine without glucose and with 10 μ M BSA-oleate. At the time of media change, compounds were added to cells. Cells were incubated at 37°C/5% CO₂ for 24h. After compound incubation, cell dishes were placed on wet ice, washed in DPBS and scraped into 1 mL of DPBS. Cells were pelleted at 1200rpm/4°C for 3 minutes, after which DPBS was aspirated. Pellets were stored at -80°C until the time of metabolite extraction and analysis.

Acylcarnitines were quantitated as previously described with modifications²³⁻²⁴. Briefly, cell pellets were suspended in 100% ethanol and an internal standard containing d3-labeled C0, C2, C3, C4, C5, C5-OH, C5-DC, C8, C14, and C16 was added (Cambridge Isotopes NSK-B and NSK-B-G1-1). The sample was sonicated for 5 seconds and 10 μ L of the sample was used to determine protein concentration using the DC Protein assay (Bio-Rad). The remaining sample was centrifuged at 13,000g for 5 minutes and dried under gently flowing nitrogen at 37°C. To butylate the samples, 3N HCl in butanol (Regis Chemical Co. Morton Grove IL, #201009) was added and the sample was incubated at 65 to 70°C for 15 minutes. Once the derivatized sample was cooled and dried, the sample was dissolved in 80% acetonitrile for quantification by flow injection analysis on a Sciex 4500 QTrap mass spectrometer with Agilent 1260 binary pump using an isocratic gradient of 80% acetonitrile. Results were normalized for protein content.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The quinazoline dione compound is a selective non-small cell lung cancer toxin.

(a) Chemical structure of the lead quinazoline dione compound SW069087 (**1**). **(b)** Concentration-toxicity IC₅₀ (μM) values of compound **1** across 96 non-small cell lung cancer lines demonstrates selective toxicity. Four non-transformed cell lines (HBEC34KT, HBEC3KT, HBEC13KT and HBEC30KT) are insensitive.

Figure 2. Two proteins of 25kD (p25) and 75kD (p75) are putative targets of the quinazoline dione compound.

(a) Structure of SW218360 (2), a quinazoline dione photoreactive probe (QDC-probe). (b) Concentration-toxicity curve of the QDC-probe in K-562 cells demonstrates biological activity. Points and error bars represent mean $\pm$ SEM of three biological replicates. Error bars are not drawn if SEM is too small, (c) SDS-PAGE and fluorescence scan of K-562 cells treated with increasing concentrations of QDC-probe with and without UV treatment. The probe crosslinks four major proteins in a UV and dose-dependent manner, which are referred to by their estimated molecular weights as p25, p32, p48 and p75. (d) SDS-PAGE and fluorescence scan of K-562 cells UV-crosslinked with 300 nM of compound 2 in the presence of increasing concentrations of the active analog SW218336 (3). Compound 3 (active) competes p25 and p75. (e) SDS-PAGE and fluorescence scan of K-562 cells UV-crosslinked with 300 nM of compound 2 in the presence of increasing concentrations of the inactive analog SW217973 (4). Compound 4 (inactive) does not compete any protein crosslinked by the QDC-probe. (f) Nine analogs were tested for the ability to compete probe binding to p25. Log₁₀ for p25 competition EC_{50} and cellular toxicity (IC_{50}) was plotted for each analog. Competition of p25 correlates strongly with analog toxicity ($R^2=0.91$). (g) Nine analogs were tested for the ability to compete probe binding to p75. Log₁₀ for p75

competition EC_{50} and cellular toxicity (IC_{50}) was plotted for each analog. Competition of p75 correlates modestly with analog toxicity ($R^2=0.63$).

Figure 3. Purification and identification of p25 and p75.

(a) Three 1L cultures of K-562 cells were treated with QDC-probe (2) and competitor (3) as follows: probe – UV, probe + UV and probe + UV + competitor, lysed and then clicked with diazo biotin-azide. Probe crosslinked proteins were then enriched using streptavidin resin, eluted, separated by SDS-PAGE and analyzed by mass spectrometry, (b) Silver stain gel of streptavidin enriched probe crosslinked proteins eluted by sodium dithionite or boiling in SDS. Boxes are drawn around the enriched p75 and p25 protein bands, (c) Proteomics analysis of total dithionite eluates of QDC-probe crosslinked proteins clicked to diazo biotin-azide and enriched with streptavidin. Sum intensity is the aggregate signal of all peptides corresponding to a protein. MT-ND1 and NDUFS7 are top candidates for p25. (d) Proteomics analysis of silver stained p75 gel slices highlighting HADHA as top candidate for p75. % total sample reflects the summed intensity of all peptides corresponding to a given protein as a percentage of the summed intensity of all peptides detected in the sample.

Figure 4. p75 is HADHA, and is an off-target binding partner of the QDC.

(a) Western blot of cell lysates from parental H157 cells and three independent HADHA knockout clones generated by CRISPR/Cas9. (b) Chemical structure of SW217433 (12), a quinazoline dione probe with a diazine photo-crosslinker group, (c) SDS-PAGE and fluorescence scan of H157 parental and HADHA knockout clones treated and crosslinked with 30 nM compound 12. The active quinazoline analog 3 and piericidin are used in single dose as competitors for each cell line. HADHA knockout abolishes probe crosslinking to p75. (d) SDS-PAGE and fluorescence scan of H157 parental and HADHA knockout clones treated and crosslinked with 100 nM compound 2. Compound 3 and piericidin are used in single dose as competitors for each cell line. HADHA knockout abolishes probe crosslinking to p75. (e) Concentration-toxicity curves of parental and HADHA knockout H157 cells with compound 3). HADHA loss does not alter sensitivity to the quinazoline dione. Points and error bars represent mean $\pm$ SEM of three biological replicates. Error bars are not drawn if SEM is too small.

Figure 5. The quinazoline dione compound does not inhibit HADHA activity.

(a) Chemical structure of the quinazoline dione analog SW217808 (**13**). (b) Chemical structure of the quinazoline dione analog SW213110 (**14**). (c) SDS-PAGE and fluorescence scan of H157 cells UV-crosslinked with 100 nM QDC-probe (**2**) in the presence of a single dose of the analogs **13**, **14**, **3** (active quinazoline analog), and piericidin. Compound **13** competes p75 but not p25. (d) Concentration-toxicity curves of H157 cells treated with Compounds **3**, **13**, and **14**. **13** and **14** are both significantly more inactive than **3**. Points and error bars represent mean $\pm$ SEM of three biological replicates. Error bars are not drawn if SEM is too small, (e) LC-MS/MS analysis of hydroxyacylcarnitine species in parental H157 cells treated with **13** and **14** along with untreated HADHA knockout H157 cells. Compound **13**, an analog that binds only p75, does not inhibit HADHA activity in cells. Bars represent mean $\pm$ SEM of three biological replicates.

Figure 6. The quinazoline dione compound binds to and inhibits mitochondrial Complex I.

(a) SDS-PAGE and fluorescence scan of K-562 cells treated and UV-crosslinked with 300nM of QDC-probe (2) in the presence of two Complex I inhibitors, piericidin and rotenone. Both Complex I inhibitors compete p25, but not p75. (b) SDS-PAGE and fluorescence scan of K-562 cells treated and UV-crosslinked with 300 nM of QDC-probe 2 in the presence of Complex III (antimycin) and Complex V (oligomycin) inhibitor. Neither antimycin nor oligomycin competes p25. (c) Oxygen consumption of H157 cells treated with the active quinazoline dione analog SW218336 (3). Compound 3 causes a dose-dependent inhibition of oxygen uptake in H157 cells. Antimycin is a positive control. Points and error bars represent mean $\pm$ SEM of 6-8 biological replicates, (d) Oxygen consumption of permeabilized H157 cells in the presence of pyruvate and malate to assay Complex I linked respiration. Compound 3 causes a dose-dependent inhibition of Complex I activity. Points and error bars represent mean $\pm$ SEM of 6-8 biological replicates, (e) Oxygen consumption of permeabilized H157 cells in the presence of succinate and rotenone to assay Complex II linked respiration. Compound 3 does not inhibit Complex II activity. Points and error bars represent mean $\pm$ SEM of 6-8 biological replicates.

Figure 7. Complex I inhibition is the on-target effect of the quinazoline dione compound.

(a) Concentration-toxicity curve of H157 cells treated with the piericidin in the presence and absence of pyruvate. Pyruvate rescues piericidin toxicity. Points and error bars represent mean $\pm$ SEM of three biological replicates. Error bars are not drawn if SEM is too small.

(b) Concentration-toxicity curve of H157 cells treated with the active quinazoline dione SW218336 (3) in the presence and absence of pyruvate. Pyruvate rescues quinazoline dione toxicity. Points and error bars represent mean $\pm$ SEM of three biological replicates. Error bars are not drawn if SEM is too small. (c) Concentration-toxicity curves of H157, DFCI-024, H1581, H1437, H1693 and H2882 cells treated with compound 3 (quinazoline dione) and (d) piericidin demonstrates that Complex I inhibition is selectively toxic. Points and error bars represent mean $\pm$ SEM of three biological replicates. Error bars are not drawn if SEM is too small.

Figure 8. Six scaffolds with a similar selectivity profile to QDC bind Complex I.

(a) Correlation of the selectivity profiles of 220 NSCLC toxins with the quinazoline dione compound SW069087 (**1**). (b-g) Structures of the 6 scaffolds with selectivity profiles similar to QDC compound **1** with $r > 0.6$ (h) SDS-PAGE and fluorescent scan of K-562 cells treated with 300 nM QDC-probe **2** and single doses of active QDC analog SW218336 (**3**), Piericidin, lead QDC SW069087 (**1**), and compounds 15-20

Figure 9. Pyruvate rescues toxicity of compounds 15-20.

(a-f) Concentration-toxicity curves of compounds **15-20** in H157 cells in the presence or absence of 1mM pyruvate. Points and error bars represent mean $\pm$ SEM of three biological replicates. Error bars are not drawn if SEM is too small.

Table 1.

Quinazoline dione analogs.

SW ID	Structure	K-562 IC ₅₀ (μM)
069087 (1)		0.65
218336 (3)		0.23
218973 (4)		>50
217865 (5)		0.25
217727 (6)		1.26
217981 (7)		3.16
218335 (8)		>50
218363 (9)		7.94
217912 (10)		1.00
218343 (11)		1.26