

Chemistry & Biology

Mapping Proteome-Wide Targets of Environmental Chemicals Using Reactivity-Based Chemoproteomic Platforms

Highlights

- We used a chemoproteomic platform to map the reactivity of environmental chemicals
- Several proteins involved in metabolism are targets of environmental electrophiles
- A major fungicide inhibits fatty acid metabolism leading to altered lipid profiles

Authors

Daniel Medina-Cleghorn, Leslie A. Bateman, Breanna Ford, Ann Heslin, Karl J. Fisher, Esha D. Dalvie, Daniel K. Nomura

Correspondence

dnomura@berkeley.edu

In Brief

Medina-Cleghorn et al. employ a chemoproteomic platform to map the direct protein targets of several environmental chemicals, and show that these chemicals possess common reactivity with enzymes involved in lipid metabolism.



Mapping Proteome-Wide Targets of Environmental Chemicals Using Reactivity-Based Chemoproteomic Platforms

Daniel Medina-Cleghorn,¹ Leslie A. Bateman,¹ Breanna Ford,¹ Ann Heslin,¹ Karl J. Fisher,¹ Esha D. Dalvie,¹ and Daniel K. Nomura^{1,*}

¹Program in Metabolic Biology, Department of Nutritional Sciences and Toxicology, University of California, Berkeley, Berkeley, CA 94720, USA

*Correspondence: dnomura@berkeley.edu

<http://dx.doi.org/10.1016/j.chembiol.2015.09.008>

SUMMARY

We are exposed to a growing number of chemicals in our environment, most of which have not been characterized in terms of their toxicological potential or mechanisms. Here, we employ a chemoproteomic platform to map the cysteine reactivity of environmental chemicals using reactivity-based probes to mine for hyper-reactive hotspots across the proteome. We show that environmental contaminants such as monomethylarsonous acid and widely used pesticides such as chlorothalonil and chloropicrin possess common reactivity with a distinct set of proteins. Many of these proteins are involved in key metabolic processes, suggesting that these targets may be particularly sensitive to environmental electrophiles. We show that the widely used fungicide chlorothalonil specifically inhibits several metabolic enzymes involved in fatty acid metabolism and energetics, leading to dysregulated lipid metabolism in mice. Our results underscore the utility of using reactivity-based chemoproteomic platforms to uncover novel mechanistic insights into the toxicity of environmental chemicals.

INTRODUCTION

Many complex human diseases cannot be explained by genetics alone and likely have environmental or gene-environment causes (Hemminki et al., 2006; Hunter, 2005). We are exposed to an ever-growing number of chemicals, many of which have been linked to adverse health effects, and most of which have not been characterized in terms of their toxicological potential or mechanisms (Bookman et al., 2011; Rappaport, 2011). It is therefore likely that there are more chemicals in our environment than previously anticipated that act as potential drivers of disease. These agents may go unnoticed due to adverse health effects that occur through yet unknown mechanisms.

While there have been many efforts made to characterize the toxicology of environmental chemicals, ranging from traditional long-term toxicity assessments in animal models and human

epidemiological studies to high-throughput screening and modern systems biology approaches to map gene and protein expression changes, these approaches are often correlative or provide insufficient detail on toxicological mechanisms (Bucher, 2013; Collins et al., 2008; Dix et al., 2007; Huang et al., 2011; Shukla et al., 2010), because gene and protein expression, epigenetic, or metabolite changes that manifest as cellular and systemic phenotypes arising from chemical exposure are indirect outcomes that result from direct chemical interactions with specific molecular targets. Deconvoluting these indirect effects to predict the direct molecular targets has been very challenging. We believe that understanding the direct chemical-protein interactions of environmental chemicals is critical in informing our understanding of downstream molecular events and linking them to pathological effects, providing a direct approach to identifying environmental drivers of disease.

Of particular concern among chemicals in our environment are reactive electrophiles to which we are directly exposed or that form through bioactivation (Rappaport et al., 2012). These environmental electrophiles have the potential to covalently react with nucleophilic amino acid hotspots within the proteome, leading to protein dysfunction and subsequent pathological effects (Rappaport et al., 2012). These “hyper-reactive” nucleophilic hotspots include cysteine, serine, and lysine residues that are often involved in important biological functions such as catalysis, regulation, post-translational modifications, redox balance, metal binding, and protein-protein interactions (Shannon and Weerapana, 2015). Although we are exposed to a large array of potentially reactive chemicals, we have little to no understanding regarding their interactions with hyper-reactive proteome hotspots and the resulting effects on protein function, downstream biochemistry, or ensuing pathophysiological consequences. While there have been many innovative approaches to test the mutagenicity of DNA-damaging reactive electrophiles (Araya and Fowler, 2011; Meier and Gartner, 2014), there has been a considerable lag in technologies that enable global assessment of chemical reactivity with the proteome. At present there is a major gap in our knowledge of how reactive chemicals elicit adverse health effects through chemical-protein interactions.

Historically, the toxicological mechanisms underlying many reactive electrophiles have been thought to occur through indiscriminate covalent modifications on proteins or DNA leading to mutations or non-specific toxicities. However, recent studies

have revealed that even highly reactive endogenous electrophiles preferentially and selectively react with certain sites on specific protein targets, indicating that the interactions of electrophilic compounds with the proteome are driven not only by their reactivity but also by their affinity to certain protein microenvironments (Codreanu et al., 2014; Wang et al., 2014; Weerapana et al., 2008, 2010). Here, we have employed a chemoproteomic platform that uses reactivity-based probes to comprehensively map the proteome-wide targets of reactive environmental chemicals in complex biological systems. We show that certain environmental chemicals show specific reactivity with a set of previously unrecognized protein targets, particularly enriched in enzymes that play key roles in metabolism. Taking the widely applied fungicide chlorothalonil (CTN) as a representative example, we use this reactivity-based chemoproteomic platforms to directly identify specific enzymes and transporters involved in fatty acid metabolism and energetics as *in vivo* targets of CTN in mice, leading to distinct downstream biochemical alterations in the lipidome.

RESULTS AND DISCUSSION

Using Reactivity-Based Probes to Map Proteome-Wide Interactions of Environmental Chemicals

Among amino acid side chains that may be adducted by reactive electrophiles, cysteines are particularly sensitive to modification due to their inherent nucleophilicity (Pace and Weerapana, 2013; Shannon and Weerapana, 2015; Weerapana et al., 2010). This nucleophilicity can be enhanced by local protein microenvironments that lower the inherent pK_a of certain cysteines (Poole, 2015). These hyper-reactive cysteines have been previously shown to be important in diverse protein functions, including catalysis, redox balance, protein-protein interactions, metal binding, and functional regulation (Wang et al., 2014; Weerapana et al., 2010). Weerapana et al. (2008, 2010) described a powerful chemoproteomic platform that utilized a bioorthogonal cysteine-reactive probe, iodoacetamide-alkyne (IAyne), to globally profile these hyper-reactive cysteines across thousands of protein targets directly in complex proteomes. They showed that hyper-reactive cysteines identified by this method were highly enriched in sites important to protein function, indicating that this strategy could be used to assess proteome-wide hyper-reactive and functional sites (Wang et al., 2014; Weerapana et al., 2010).

In this study, we set out to address the knowledge gap in reactive chemical toxicities and to identify potentially novel mechanisms of action by mapping the proteome-wide interactions of a representative and diverse set of highly used reactive environmental electrophiles. We adapted the chemoproteomic strategy put forth by Weerapana et al. (2010), whereby we competed these chemicals against the binding of the cysteine-reactive IAyne probe to hyper-reactive sites in native tissue proteomes (Figures 1A–1C). We have employed two complementary strategies to achieve this goal: a gel-based fluorescence assay for rapid chemical screening and a proteomic-based approach for detailed target identification. In brief, we treated proteomes with a panel of representative reactive environmental chemicals to allow covalent binding to sensitive sites. Proteomes were then treated with the cysteine-reactive probe IAyne to competitively label available hyper-reactive cysteines, followed by appending

an azide-linked rhodamine or biotin enrichment handle using copper-catalyzed azide-alkyne cycloaddition or “click chemistry,” for fluorescent or proteomic-based detection, respectively. Fluorescently tagged proteomes were separated by SDS-PAGE, and biotin-tagged proteomes were enriched and digested to tryptic peptides for liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomic analyses. We then determined the reactivity of the environmental chemicals by either loss of fluorescent labeling or loss of MS signal.

We tested representative classes of widely used or commonly exposed reactive environmental chemicals, including metals, chemical breakdown products, and several pesticide classes, for their concentration-dependent cysteine reactivity in mouse liver and kidney proteomes against IAyne labeling of cysteine-reactive sites. Using the gel-based detection method, we observed dose-dependent reactivity of certain chemicals such as monomethylarsonous acid (MMA), a highly toxic bioactivated arsenical (Watanabe and Hirano, 2013); hydroquinone (HQ), a bioactivated metabolite of benzene (McHale et al., 2012); the widely used fungicide CTN (Wilkinson and Killeen, 1996); and the broad-spectrum pesticide chloropicrin (Oriel et al., 2009) (Figures 1D and S1). In contrast, chemicals such as the chloroacetanilide herbicide acetochlor, the organophosphorus insecticide chlorpyrifos oxon (CPO), and the common industrial chemical and food contaminant acrylamide showed little to no observable reactivity at the tested concentrations (Figures 1D and S1). These seemingly unreactive chemicals may still bind to protein targets, but their reactivity may be masked in gel-based approaches or they may target other amino acid hotspots in the proteome. For example, our previous studies using activity-based protein profiling have shown that CPO and triphenyl phosphate (TPP) react with the catalytic serines of several serine hydrolases (Medina-Cleghorn et al., 2014; Morris et al., 2014; Nomura et al., 2005, 2008, 2011). Alternatively, these chemicals may not form stable protein adducts, may be rapidly oxidized by detoxifying enzymes, or may react with other non-protein biological nucleophiles such as glutathione or nucleic acids.

To identify the direct protein targets of the chemicals that showed reactivity by our gel-based detection methods, we first applied our LC-MS/MS-based competitive chemoproteomic analysis to MMA, CTN, and chloropicrin at 100 μ M *in vitro* in mouse liver proteomes (Figure 2A and Table S1). Based on literature and chemical properties, these chemicals were determined as likely to directly and covalently interact with protein targets in complex proteomes when compared with potentially indirect mechanisms of HQ such as generation of oxidative stress. Out of 339 total targets enriched by IAyne labeling (>5 spectral counts, >2-fold above no-probe DMSO controls), we found that CTN, MMA, and chloropicrin interact with 37, 69, and 119 protein targets, respectively (Figure 2B and Table S1). First, we note that by using this chemoproteomic approach, we were able to enrich cysteine-reactive protein targets across hundreds of proteins spanning diverse protein classes (Table S1). Second, while each reactive agent shows a distinct protein target profile, CTN, MMA, and chloropicrin collectively bind 31 protein targets in common, spanning many unrelated protein classes that include metabolic enzymes, cell-structure proteins, chaperones, and translation-related proteins. Third, we also show that the targets of individual chemicals are not merely

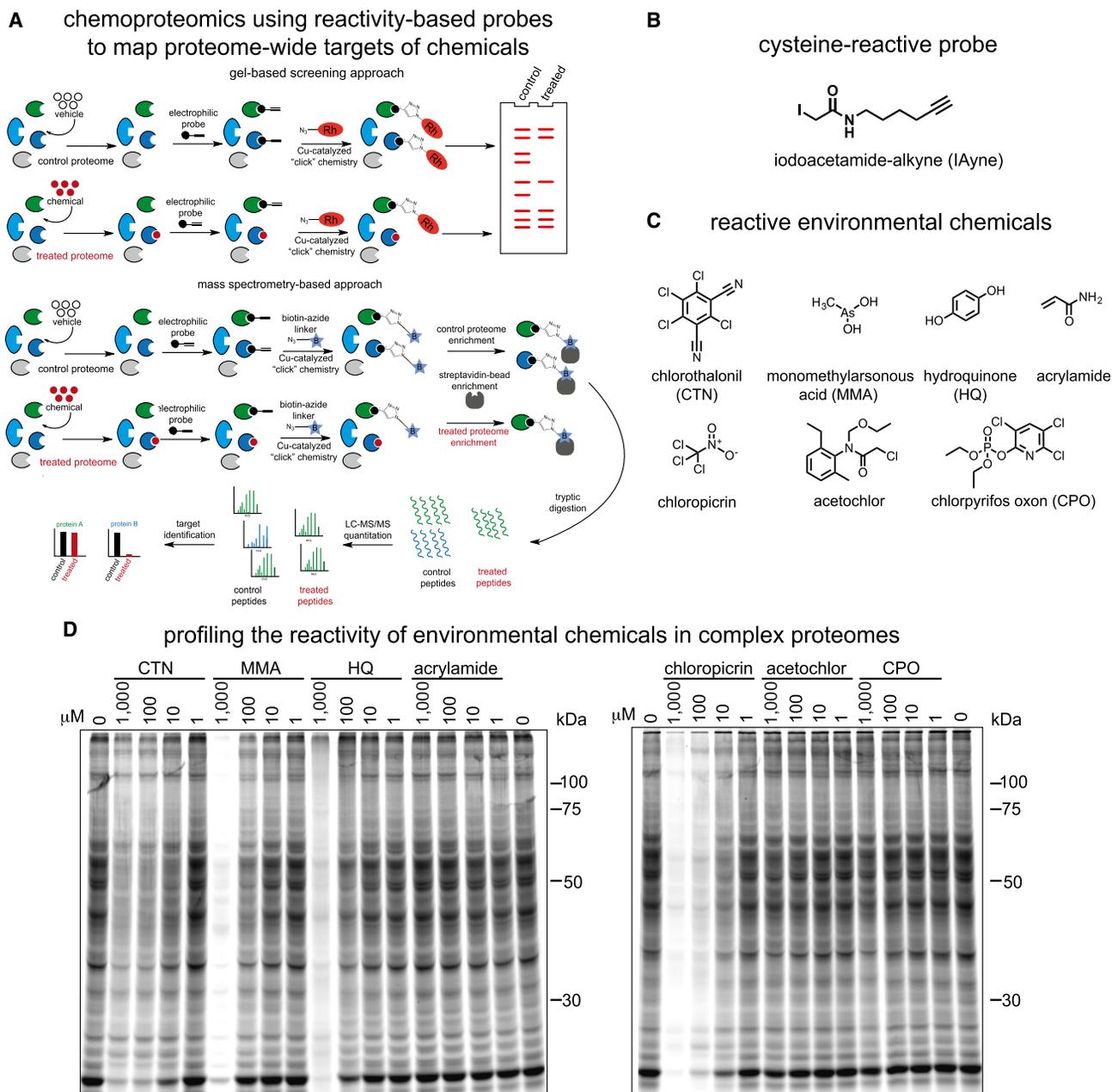
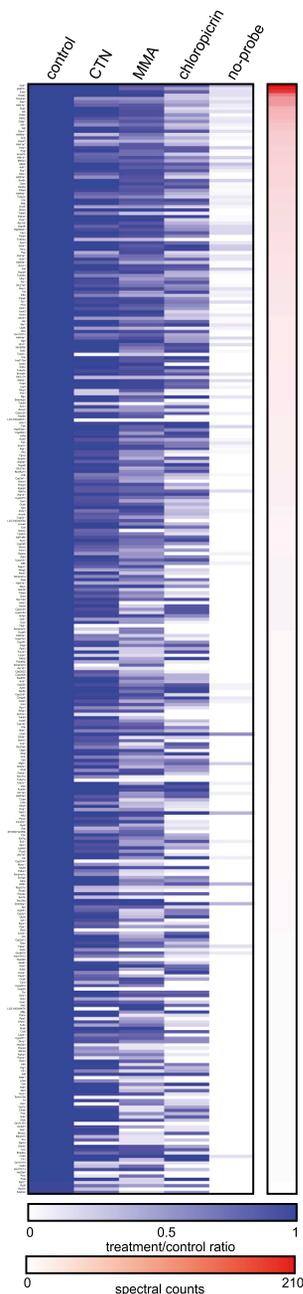


Figure 1. Mapping Proteome-Wide Interactions of Reactive Environmental Chemicals Using Reactivity-Based Probes and Chemoproteomic Profiling

(A–C) Here we mapped the direct chemical-protein interactions of reactive environmental chemicals, including metals (MMA), chemical breakdown products (HQ, acrylamide), and pesticides (CTN, acetochlor, chloropicrin, CPO) directly in complex proteomes. We employed a gel-based fluorescence assay for screening and a proteomic approach for target identification. Proteomes were pre-treated with either vehicle or environmental electrophiles, competitively labeled with the cysteine-reactive iodoacetamide-alkyne (IAyNe) probe, after which an azide-linked rhodamine or biotin enrichment handle was appended using “click chemistry” for fluorescent or proteomic detection, respectively. Fluorescently tagged proteomes were separated by SDS-PAGE, and biotin-tagged proteomes were enriched and digested to tryptic peptides for liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomic analyses. Reactivity of environmental chemicals was determined by loss of fluorescent labeling or MS signal.

(D) Gel-based analyses of environmental chemical reactivity with cysteine-reactive sites on proteins in mouse liver proteomes. Proteomes were pre-treated in vitro with chemicals at the designated concentrations (30 min) and then competed with IAyNe labeling (10 μM, 60 min), rhodamine conjugation, and cysteine reactivity visualized by SDS-PAGE and in-gel fluorescence. The gel-based analyses reveal the substantial cysteine reactivity of MMA, HQ, CTN, and chloropicrin. Shown are representative gels from $n = 3$. Cysteine reactivity in kidney is shown in [Figure S1](#).

A cysteine reactivity of environmental chemicals



B direct cysteine-reactive targets of CTN, MMA and chloropicrin in vitro in mouse liver

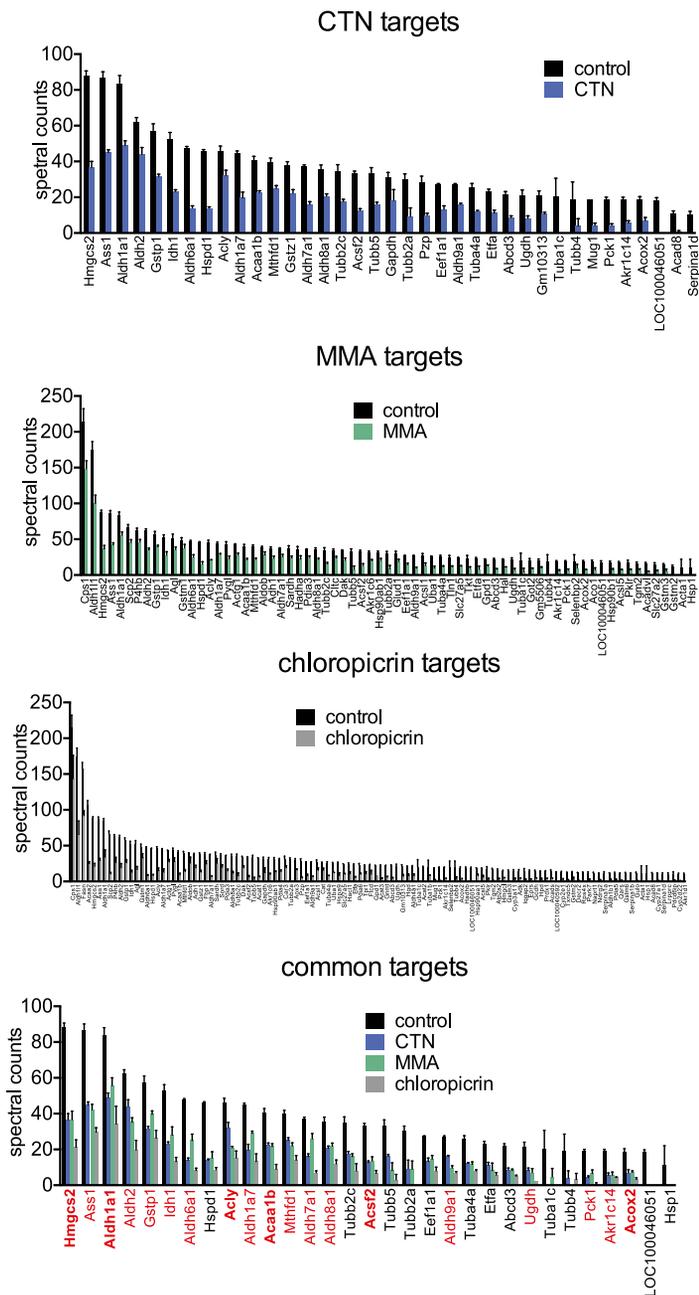


Figure 2. Target Identification of Reactive Environmental Chemicals

(A) Competitive chemoproteomic analysis for direct protein target identification of CTN, MMA, and chloropicrin (reactive chemicals from Figure 1D) was performed using mouse liver proteome treated with vehicle or chemical (100 μ M, 30 min) prior to IAYne labeling (10 μ M, 30 min) or vehicle (no-probe control). Each biological replicate uses a liver homogenate from a different 6-week-old male C57BL/6 mouse. Subsequent biotin conjugation, avidin enrichment of targets, and analysis of trypsin-digested peptides by LC-MS/MS revealed enrichment of 339 total proteins by IAYne labeling spanning diverse protein classes (>5 spectral counts, >2-fold above no-probe DMSO controls). Data are expressed as heatmaps showing ratios of treatment compared with control. IAYne-enriched targets are sorted by their spectral counts.

(B) MMA, CTN, and chloropicrin significantly displace IAYne labeling of protein targets in vitro in mouse liver ($p < 0.05$ compared with vehicle-treated controls using two-way ANOVA with Tukey's multiple comparisons test). MMA, CTN, and chloropicrin possess 31 overlapping targets. Highlighted in red are metabolic enzyme targets, and in bold red are those enzymes involved in lipid metabolism. Data are presented as mean \pm SEM; $n = 3$ per group.

Raw proteomic data and statistical analyses are shown in Table S1. HQ targets are shown in Figure S2.

restricted to the most abundant IAyne-enriched targets, but instead span the entire range of detected protein abundances, suggesting that these interactions result from a combination of affinity and reactivity toward these targets and not just through non-specific reactivity with the most abundant IAyne-enriched targets (Figures 2A and 2B). Conversely, there are many high-abundance IAyne-enriched proteins that were not bound by CTN, MMA, or chloropicrin. Most of the targets of CTN, MMA, and chloropicrin identified by our chemoproteomic approach have not been previously identified as targets of these agents (Figure 2B and Table S1). Interestingly, 19 of the 31 shared targets are enzymes involved in key metabolic processes (highlighted in red in Figure 2B), and six of these enzymes are involved in different aspects of lipid metabolism (in red and bold in Figure 2B). *Hmgcs2*, *Acaa1b*, *Acsf2*, and *Acox2* are all involved in energetic fatty acid oxidation, *Acly* is involved in producing acetyl-coenzyme A (CoA) for de novo lipogenesis, and *Aldh1a1* catalyzes the oxidation of retinaldehyde to form the critical signaling molecule retinoic acid (Kanehisa and Goto, 2000). These agents binding covalently to reactive nucleophiles on their target enzymes may disrupt lipid metabolism in particular, representing a novel mechanism of toxicity not previously associated with these chemicals. Thus, we show that structurally dissimilar reactive environmental chemicals share certain specific and overlapping targets predominantly involved in metabolism. These protein targets may be particularly susceptible to disruption by environmental electrophiles, and exposure to multiple agents may synergistically affect protein function to exert significant toxicities.

In addition to CTN, MMA, and chloropicrin, we also profiled HQ and acrylamide reactivity at 100 μ M in mouse liver proteome in vitro. We identified 12 protein targets of HQ, most of which do not overlap with those of CTN, MMA, and chloropicrin (Figure S2 and Table S1). This lack of overlap may be due to the indirect mechanism of HQ in generating reactive oxygen stress that may be leading to cysteine oxidation (Kalf, 1987), compared with the potentially covalent reactive mechanisms of CTN, MMA, and chloropicrin. Consistent with the gel-based approaches, we observed no direct targets of acrylamide (Table S1). It is possible that this apparent lack of HQ and acrylamide reactivity may be due to insufficient concentration or the need for bioactivation, or that these compounds are rapidly oxidized by detoxifying enzymes, may form transient or unstable protein adducts, or may react with other biological nucleophiles. We also note the possibility that we may be masking hyper-reactive cysteines present in living cells that may become oxidized during tissue proteome preparation.

CTN Shows Selective Reactivity with Protein Targets In Vivo in Mouse Kidney

Building on our in vitro profiling, we next wanted to validate our chemoproteomic strategy using an in vivo model of exposure in mice. We treated mice with the maximally tolerated doses of CTN, MMA, HQ, acrylamide, and chloropicrin at 100, 1, 50, 100, and 4 mg/kg intraperitoneally (i.p.), respectively, and subsequently labeled liver and kidney proteomes ex vivo with IAyne to detect in vivo cysteine reactivity (Figure 3A). While we did not observe reactivity using gel-based methods in liver, CTN showed considerable in vivo reactivity in mouse kidney (Fig-

ure 3A). We thus selected CTN for further study. CTN is one of the most widely used pesticides, with 9 million pounds (\sim 4 million kg) applied per year in United States agriculture alone (Grube et al., 2011). Despite its heavy usage, CTN has also been shown to cause renal toxicity and kidney carcinogenesis in multiple animal models, although the underlying toxicological mechanisms remain poorly understood (Wilkinson and Killeen, 1996). Thus, our finding that CTN showed selective reactivity in mouse kidney compared with liver was interesting (Figure 3A).

Encouraged by these results, we conducted a comprehensive assessment of CTN reactivity in vivo in mouse liver and kidney by developing a tailored bioorthogonal probe, CTNyne (Figures 3B and S3). The CTNyne probe is a structural analog of CTN that contains a bioorthogonal alkyne handle for click chemistry conjugation after labeling in proteomes. We competitively labeled liver and kidney proteomes from mice treated in vivo with vehicle or CTN with the CTNyne probe, and appended a rhodamine reporter for in-gel fluorescence analysis. We confirmed that CTNyne labels multiple targets in kidney, and that many of these targets were competed by in vivo CTN treatment, demonstrating the increased specificity for CTN targets using the CTNyne probe (Figure 3C). While CTNyne labeled many protein targets in liver, most of these targets were not displaced by in vivo CTN treatment, recapitulating our previous observation with IAyne labeling (Figure S3).

CTN Affects Metabolic Enzymes Involved in Energetics and Fatty Acid Metabolism

Based on the findings from our in-gel fluorescence analysis, we next sought to identify the in vivo targets of CTN in mouse kidney using our CTNyne probe and gain mechanistic insight into CTN toxicity. We used the kidney proteomes of mice exposed in vivo to vehicle or CTN for an ex vivo competitive labeling by our CTNyne probe, followed by LC-MS/MS-based chemoproteomic analysis. Through this approach, we identified 216 protein targets that were significantly enriched by the CTNyne probe compared with no-probe controls (Figure 3D and Table S2). From these enriched proteins, we identified 44 targets that were significantly competed by in vivo CTN treatment more than 2-fold and were detected at >20 spectral counts in abundance (Figure 3E and Table S2). In contrast to our findings in mouse kidney in vivo, we identified only five in vivo targets in mouse liver (Figure S3 and Table S2).

Of the 44 in vivo kidney targets of CTN that we identified, we were intrigued to find that a substantial proportion of these targets (19 proteins) were enzymes involved in the metabolism of small-molecule metabolites (red emphasis, Figure 3E). The majority of these enzymes (12 targets) are involved in energetics, participating in glycolytic, gluconeogenic, or fatty acid metabolic pathways (bold red emphasis in Figure 3E). These enzymes include the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), triosephosphate isomerase 1 (*Tpi1*), and lactate dehydrogenases (*Ldha*, *Ldhb*), and the gluconeogenic enzymes fructose-1,6-bisphosphatase 1 (*Fbp1*) and pyruvate carboxylase 1 (*Pck1*). Enzyme targets involved in fatty acid transport or oxidation include fatty acid transport protein 2 (*Fatp2*), 3-ketoacyl-CoA thiolase 1b and 2 (*Acaa1b* and *Acaa2*), peroxisomal acyl-CoA oxidase 1 (*Acox1*), and carnitine palmitoyltransferase 1a (*Cpt1a*), as well as the ketone

body-generating enzyme 3-hydroxymethyl-3-methylglutaryl-CoA lyase (Hmgcl). Collectively, our results indicated that CTN might alter renal energetics and fatty acid metabolism.

To validate these metabolic enzymes as true CTN-specific hits, we recombinantly overexpressed representative targets from each pathway in HEK293T cells and subsequently performed *in vitro* competition studies with CTN against IAyne or CTNyne labeling. Indeed, we show that Acaa1b, Acaa2, Gapdh, Hmgcl, and Pck1 were labeled by IAyne, and this labeling was displaced in a concentration-dependent manner by CTN, indicating that CTN specifically bound to one or more hyper-reactive cysteine on these proteins (Figure 4A). While we found that CTN displaced binding of CTNyne to Fatp2 (Figure 4A), Fatp2 was not labeled by IAyne (data not shown), suggesting that CTN may bind to another hyper-reactive residue on this protein besides cysteine. We calculated 50% inhibitory concentration (IC₅₀) values of 0.9–24 μM for CTN binding to Acaa1b, Acaa2, Gapdh, Hmgcl, Pck2, and Fatp2 (Figure 4A). We note that the IC₅₀s are a rough estimate of relative reactivity, since the reaction is covalent and there is no equilibrium. Furthermore, we demonstrate that CTN is binding to catalytic cysteines on key energetic enzymes through blunted or eliminated IAyne labeling upon mutagenesis of Acaa2 and Gapdh catalytic cysteines to C92A and C150A, respectively (Figures 4B and S4).

Although we were unsuccessful at recombinantly overexpressing Cpt1a, we confirmed that the endogenous kidney carnitine palmitoyltransferase activity from *in vivo* CTN-treated mice was significantly inhibited compared with vehicle-treated controls, as measured by an activity assay using trimethyl-d⁹-L-carnitine and C16:0 free fatty acid (C16:0 FFA) substrates measuring C16:0-trimethyl-d⁹-carnitine product formation by LC-MS/MS (Figure 4C). We also wanted to determine whether CTN inhibits the activity of Fatp2, an enzyme with both peroxisomal fatty acyl-CoA synthetase activity and cytosolic fatty acid transport activity (Falcon et al., 2010). We indeed show that acyl-CoA synthetase activity was completely inhibited in the kidneys of mice treated *in vivo* with CTN compared with vehicle-treated controls, as measured by an activity assay using [U-¹³C]C16:0 FFA substrate and measuring [U-¹³C]C16:0 fatty acyl-CoA (FA-CoA) product formation by single-reaction monitoring (SRM)-based LC-MS/MS (Figure 4D). Next, to determine whether fatty acid transport might be impaired *in vivo* due to inhibition of Fatp2, we performed an *in vivo* isotopic fatty acid tracing study and showed that *i.p.* administered isotopically labeled [U-¹³C]C16:0 FFA incorporation into representative complex lipids in mouse kidney was significantly lower in CTN-treated mice, including isotopically labeled species of acyl carnitine, lysophosphatidylcholine, and triacylglycerols (Figure 4E).

While we cannot directly attribute these activities to Fatp2, these data taken together suggest that fatty acid transport and acyl-CoA synthetase activities attributable to Fatp2 are inhibited *in vivo* by CTN in the kidney.

We next wanted to assess the downstream biochemical effects that may arise from inhibition of the metabolic enzyme targets of CTN, especially those involved in fatty acid metabolism. We performed SRM-based LC-MS/MS metabolomic analyses on kidneys from mice treated *in vivo* with CTN to identify alterations in the lipidome conferred by CTN treatment. Consistent with our results suggesting that CTN disrupts fatty acid transport both into cells through Fatp2 inhibition and into mitochondria through Cpt1a inhibition, and causes impairments in fatty acid oxidation, we observe expected increases in FFA levels and depletions in the levels of FA-CoAs (Fatp2 and β-oxidation products) and acyl carnitines (Cpt1a product) in mouse kidney from *in vivo* CTN treatment (Figures 4E and S4; Table S3). CTN has previously been shown to react directly with glutathione, causing glutathione depletion and toxicity (Wilkinson and Killeen, 1996). However, we show that, at the doses used here, both liver and kidney glutathione levels were unchanged upon CTN administration (Figure S4).

Collectively, we show that the major fungicide CTN inhibits metabolic enzymes in kidney that are involved in energetic metabolism, leading to downstream biochemical alterations in glycolysis and fatty acid metabolism.

Conclusions

Here, we have used a chemoproteomic platform employing reactivity-based chemical probes to map the proteome-wide interactions of environmental chemicals and uncover novel mechanisms through which these chemicals may cause adverse health effects. We show here that individual environmental electrophiles engage specific protein targets with privileged reactivity, and do not non-specifically target all proteins or merely react with the most abundant targets. This indicates that these agents possess affinities to highly reactive nucleophiles on specific proteins, suggesting that the mechanisms of toxicity may be through interactions with these selected proteins to cause distinct downstream adverse biochemical events. By competing these chemicals against a cysteine-reactive probe, we show that seemingly dissimilar reactive environmental agents including the fungicide CTN, the arsenical MMA, and the insecticide chloropicrin possess both distinct and overlapping targets in the proteome, suggesting that these protein targets may be particularly susceptible to reactive electrophiles. It will be of future interest to examine whether prolonged or combined exposure to these and other reactive chemicals causes sustained and cumulative

(C) CTNyne labels multiple protein targets in mouse kidney (10 μM, 30 min), and is displaced by *in vivo* CTN treatment (100 mg/kg *i.p.*, 2 hr).

(D and E) *In vivo* CTN-specific protein targets were identified by labeling *in vivo* vehicle- and CTN-treated mouse kidney proteomes with CTNyne (50 μM, 30 min), followed by biotin conjugation, avidin enrichment, and tryptic peptide analysis by LC-MS/MS. (D) 216 protein targets were significantly enriched by the CTNyne probe compared with no-probe control. Data are presented as a heatmap of total CTNyne-enriched targets normalized to the average spectral counts of CTNyne control for each protein. (E) 44 proteins were identified as CTN targets that were >20 spectral counts in abundance and significantly ($p < 0.05$ by two-tailed t test) competed >2-fold by *in vivo* CTN treatment. 19 of these targets are metabolic enzymes (red emphasis), 12 of which are involved in glycolytic, gluconeogenic, or fatty acid metabolic pathways (bold red emphasis).

Data in (A) and (C) are representative gels from $n = 3$ –4 mice/group. Data in (D) and (E) are from $n = 3$ –4 mice/group; raw proteomic data are shown in Table S2. Data in (E) are presented as mean ± SEM, $n = 3$ per group. *In vivo* liver targets of CTN are shown in Figure S3.

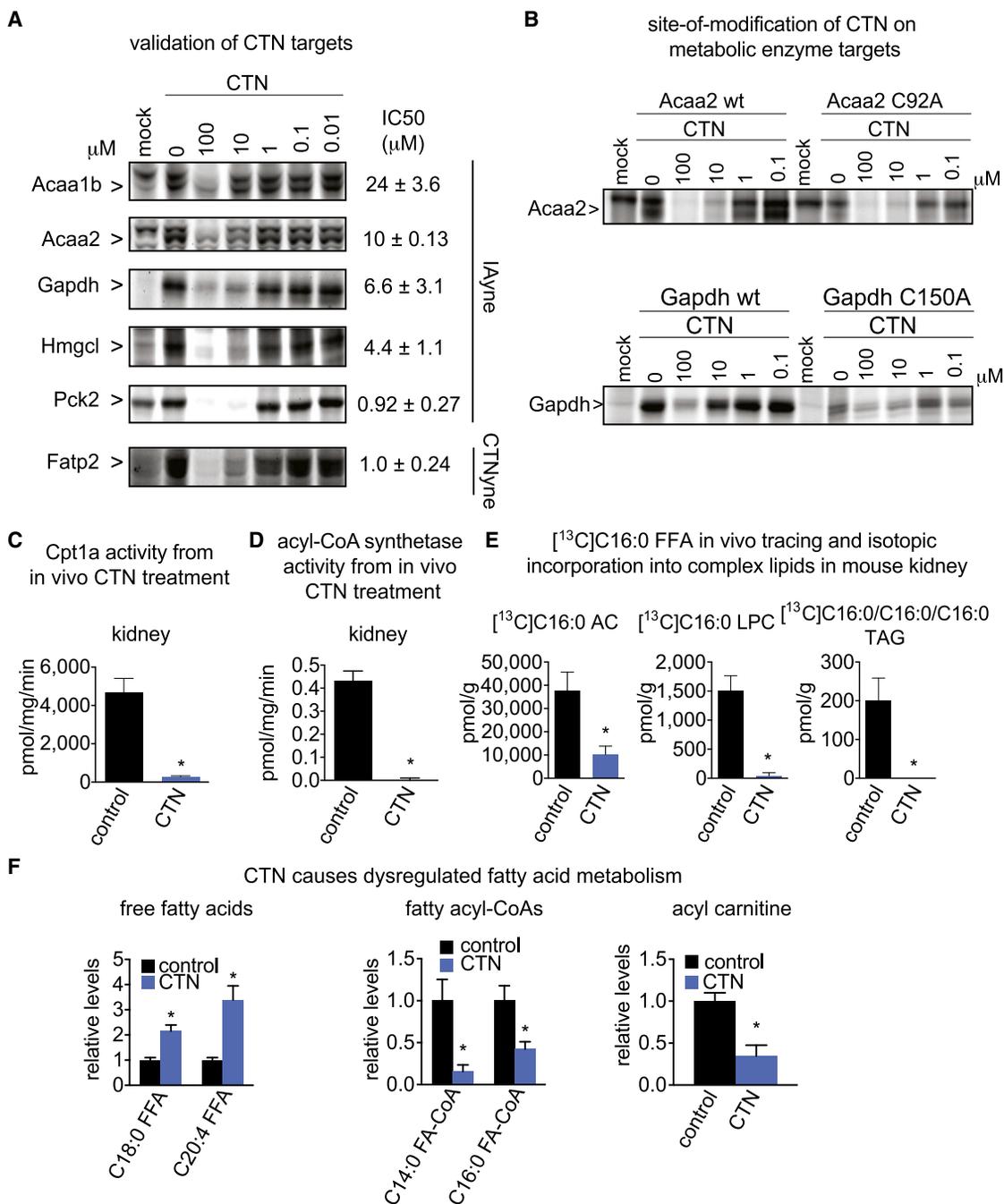


Figure 4. CTN Inhibits the Activity of Several Metabolic Enzymes Involved in Energetics and Fatty Acid Metabolism Leading to Dysregulated Lipid Metabolism

(A) Representative metabolic enzyme targets of CTN in vivo in mouse kidney were validated by recombinant target overexpression in HEK293T and COS7 cells. Lysates from mock- or target-overexpressing cells were pre-incubated with vehicle or 0.01–100 μM CTN (30 min), followed by labeling with IAYne or CTNyne (10 μM, 30 min). 50% inhibitory concentration (IC₅₀) values are reported.

(B) CTN binds the catalytic cysteine on Acaa2 (C92) and Gapdh (C150), as confirmed by CTN-sensitive IAYne labeling of Acaa2, and Gapdh is substantially lower in Acaa2 C92A and Gapdh C150A-overexpressing compared with wild-type (wt) Acaa2 and Gapdh-overexpressing HEK293T lysates. Expression controls for wild-type and mutant enzymes are shown in Figure S4, ascertained by qPCR analysis of RNA harvested alongside these experiments showing equivalent expression.

(C and D) Carnitine palmitoyltransferase (C) and fatty acyl-CoA synthetase (D) activity are significantly inhibited in vivo CTN-treated (100 mg/kg i.p., 2 hr) mouse kidney proteomes compared with vehicle-treated controls. Carnitine palmitoyltransferase activity was assessed ex vivo using C16:0 free fatty acid (FFA) and trimethyl-d⁹-L-carnitine measuring C16:0-d⁹-carnitine formation by SRM-based LC-MS/MS. Acyl-CoA synthetase activity was assessed ex vivo using [U-¹³C]C16:0 FFA and CoA, measuring [U-¹³C]C16:0-CoA formation by SRM-based LC-MS/MS.

(legend continued on next page)

adduction of these common targets, leading to adverse biochemical and pathophysiological effects.

We note that the other reactive environmental chemicals we screened that did not show cysteine reactivity by our gel-based fluorescence method may still react with protein targets. These chemicals may potentially bind to hyper-reactive cysteines on particular targets that are simply poorly resolved by SDS-PAGE, may bind at even higher concentrations, or may bind to other hyper-reactive amino acid side chains. For example, while the flame retardant TPP and the bioactivated insecticide metabolite CPO do not show cysteine reactivity, we have previously shown that these chemicals covalently bind to and inhibit catalytic serines of several serine hydrolases (Medina-Cleghorn et al., 2014; Morris et al., 2014; Nomura et al., 2008; Nomura and Casida, 2011). Nonetheless, the enzyme targets that we identify here represent multiple, potentially novel mechanisms of toxicity that may even be in common across broad classes of environmental electrophiles. We also note that we may still be missing some direct protein targets of the chemicals that we showcase in this study. The cysteine-reactive probe may be labeling multiple sites on each protein, and thus we may be obscuring chemical-protein interactions when we quantify enriched proteins based on total peptides for each protein. In addition, certain chemicals may also require bioactivation in specific tissues *in vivo* before they may bind highly reactive nucleophilic residues. It will be of future interest to complement our study with a more comprehensive quantitative proteomics approach for identifying sites of probe modification and sites of chemical displacement of probe binding.

We also demonstrate the ability to apply the chemoproteomic strategies described in this study to assess *in vivo* reactivity of environmental chemicals, which enables assessment of target organs that may be particularly susceptible to toxicity from environmental agents. Using the fungicide CTN as an example, we showed that CTN exhibits particular reactivity with protein targets *in vivo* in mouse kidney compared with liver. Through the compound-specific CTNyne probe, we identified 44 direct protein targets of CTN *in vivo* in mouse kidney. We found that this target list was enriched in metabolic enzymes involved in glycolytic and fatty acid metabolism and that the activities of these enzymes were seemingly inhibited. Through subsequent metabolomic profiling, we observed a dysregulation of lipid metabolism, consistent with our target list. The inhibition of glycolytic and gluconeogenic metabolism, fatty acid transport, and fatty acid oxidation enzymes, potentially leading to altered levels of key metabolites and perturbed metabolic networks, represents a novel mechanism of toxicity for CTN.

While the doses of CTN used in this study to identify direct *in vivo* targets were high, the apparent IC_{50} values of CTN against the targets that we have validated here are within an order of

magnitude of the concentration of CTN that has been observed in human serum. Barr et al. (2010) previously reported up to 25 ng/g of CTN found in human maternal and cord sera, which equates to ~ 100 nM. Acaa2, Acaa1b, Gapdh, Fatp2, and Pck1 showed $9.4\% \pm 1.2\%$, $3.4\% \pm 3.4\%$, $20\% \pm 8.4\%$, $13\% \pm 6.7\%$, and $6.8\% \pm 6.8\%$ reduction, respectively, in IAYne labeling compared with vehicle-treated controls. Acaa2 and Gapdh were significantly inhibited ($p < 0.05$ compared with vehicle-treated controls using a two-tailed t test) at 100 nM.

Indeed, studies in animal models have linked CTN to selective kidney toxicity and carcinogenesis, although the mechanisms have remained unclear (Wilkinson and Killeen, 1996). Here, we suggest that the kidney-specific toxicity of CTN may be due to the inhibition of energetic and fatty acid metabolism enzymes, which may impair cell function, causing cellular necrosis and subsequent kidney dysfunction. When considered in the context of chronic exposure, these toxicities may develop into pathophysiologicals such as inflammation, fibrosis, and cancer. Because CTN presumably acts through covalent modification of these targets, cumulative exposure may cause an accumulation of protein adducts on these targets, leading to their eventual inhibition.

While we have identified novel potential toxicological mechanisms of CTN, we still do not understand the basis for the observed selective reactivity of CTN in kidney compared with liver. Previous studies have postulated that CTN may cause kidney-specific toxicity through glutathione conjugation and further bioactivation by β -lyase, an enzyme highly expressed in renal proximal tubules (Wilkinson and Killeen, 1996). Thus, while we show here that CTN itself directly interacts with the observed *in vivo* CTN targets, CTN may also undergo further kidney-specific bioactivation to inhibit the observed targets. It will be of future interest to investigate the role of CTN metabolites in inhibiting the CTN targets, and perform long-term and low-dose chronic treatment studies to determine whether CTN may cause renal toxicity or carcinogenesis through inhibiting these targets.

Many of the metabolic enzyme targets inhibited by CTN and other reactive agents used in this study involve active-site cysteines that directly contribute to the catalytic mechanisms. For example, Gapdh uses the active-site cysteine to form a hemithioacetal intermediate, and many of the fatty acid oxidation enzymes (e.g. Acaa1b and Acaa2) form thioester intermediates using their active-site cysteines (Mathieu et al., 1997; Poirier et al., 2006; Seidler, 2013). The enrichment of metabolic enzymes among the observed targets may suggest that enzymes that use cysteines in their mechanism are particularly susceptible to reactive environmental electrophiles.

In this study, we have demonstrated that chemoproteomic profiling using reactivity-based probes enables the mapping of direct proteome-wide interactions of reactive environmental

(E) Isotopic incorporation of [U - ^{13}C]C16:0 FFA into complex lipids *in vivo* in kidney was measured *ex vivo* by SRM-based LC-MS/MS from mice treated with vehicle or CTN (100 mg/kg *i.p.*, 1 hr), followed by [U - ^{13}C]C16:0 acid (100 mg/kg *i.p.*, 1 hr). AC, acyl carnitine; LPC, lysophosphatidylcholine; TAG, triacylglycerols. (F) Consistent with inhibition of CTN target fatty acid transport and oxidation enzymes, *in vivo* CTN treatment (100 mg/kg *i.p.*, 2 hr) leads to elevated FFA, decreased C14:0 and C16:0 fatty acyl-CoA (FA-CoA), and decreased C12:0 acyl carnitine levels in mouse kidney, compared with vehicle-treated controls. Data in (A) and (B) are representative gels from $n = 3$ /group. Data in (C–E) are presented as mean \pm SEM; $n = 3$ –5 mice/group. Significance is presented as * $p < 0.05$ compared with vehicle-treated controls using two-tailed tests. Data in (F) show fatty acid-derived metabolites with significantly (* $p < 0.05$) altered abundances in two independent experiments. Raw metabolomic data are shown in Table S3 and a more detailed overview of the metabolomics data is shown in Figure S4.

chemicals, which provides mechanistic detail for downstream toxicities. We also show that metabolomic profiling can be coupled with chemoproteomic-based target identification to help demonstrate the biochemical outcomes associated with interactions of these chemicals with specific protein targets. Looking forward, we anticipate that metabolomics approaches will enable identification of robust biomarkers of exposure and toxicity for CTN and other environmental chemical agents. Furthermore, we foresee that using chemoproteomic platforms to screen emerging industrial, environmental, or pharmaceutical chemicals against a suite of reactivity-based probes for other hyper-reactive amino acids will reveal potential off-targets and toxicological liabilities early on in the development pipeline. The advance knowledge of specific reactivities and detailed mechanistic insights revealed by this approach can be used to direct efforts toward optimizing the chemical structures and ensuring the selectivity and safety of future chemicals.

SIGNIFICANCE

We are exposed to a growing number of chemicals in our environment, most of which have not been characterized in terms of their toxicological potential or mechanisms. Research into understanding how chemicals may produce adverse effects has primarily focused on DNA-mutating or reversible endocrine-disrupting mechanisms, and has largely ignored potential alternative toxicological mechanisms arising from chemical interactions with the >20,000 protein targets in the human proteome. Particularly concerning among chemicals in our environment are reactive electrophiles that have the potential to covalently react with amino acid hotspots and lead to protein dysfunction and pathological effects. Here, we have used a reactivity-based chemoproteomic platform to map the cysteine reactivity of environmental chemicals, and show that contaminants such as monomethylarsonous acid and widely used pesticides such as chlorothalonil and chloropicrin possess common reactivity with a distinct set of sensitive proteins. Many of these proteins are involved in key metabolic processes, suggesting that these targets may be particularly sensitive to environmental electrophiles. We also show that the widely used fungicide chlorothalonil specifically inhibits several metabolic enzymes involved in fatty acid metabolism and energetics, leading to dysregulated lipid metabolism in mice. Our results highlight the utility of using reactivity-based chemoproteomic platforms to uncover novel mechanistic insights into the toxicity of environmental chemicals, and provide a potential framework for testing emerging chemicals.

EXPERIMENTAL PROCEDURES

Mice

Male C57BL/6 mice (6–8 weeks old) were acutely (2 hr) exposed by i.p. injection to 100 mg/kg CTN (Tocris Bioscience) in a vehicle of 42:2.5:1:1 saline/DMSO/emulphor/ethanol (10 μ l/g mouse). For isotopic tracing studies, mice were first exposed to 100 mg/kg CTN by i.p. injection for 1 hr, then exposed to 100 mg/kg [12 C]16:0 FFA (Sigma) or [13 C]16:0 FFA (Cambridge Isotopes) dissolved in undiluted PEG40 castor oil (Spectrum) by i.p. injection for 1 hr. Following exposure, mice were euthanized by cervical dislocation, and livers

and kidneys were immediately removed and flash-frozen in liquid nitrogen. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of California, Berkeley.

Processing of Mouse Brain, Liver, and Kidney Proteomes

Tissues were homogenized in Mg^{2+}/Ca^{2+} -free 1 \times PBS (Corning Life Sciences), followed by a 1,000 \times g centrifugation of the homogenate. The resulting supernatant was collected and used for subsequent assays. Protein concentrations were determined by Bradford assay (Bio-Rad).

Cell Culture, Recombinant Overexpression, and Site-Directed Mutagenesis

HEK293T and COS7 cells were cultured in DMEM media containing 10% fetal bovine serum and maintained at 37°C with 5% CO₂. Recombinant cDNA constructs containing enzymes of interest in the SPORT6 vector were purchased from Thermo Open Biosystems and transiently transfected into HEK293T (Acaa1b, Acaa2, Fatp2, Gapdh) cells using Fugene HD (Promega) or into COS7 cells (Hmgcl, Pck1) using Lipofectamine 2000 (Life Technologies). Site-directed mutagenesis was performed using a QuikChange II XL kit (Agilent Technologies) and mutant plasmids were transiently transfected into HEK293T cells. Further details can be found in [Supplemental Methods](#).

Electrophile Competition and Click Chemistry Procedures to Append Analytical Handles to IAyne- or CTNyne-Treated Mouse Proteomes

Proteome samples diluted in PBS (10, 25, or 50 μ g in 50 μ l of PBS for gel-based analysis; 0.5 mg liver homogenate in 0.5 ml of PBS or 1 mg of kidney homogenate in 0.5 ml of PBS) were subjected to vehicle or electrophile treatment for 30 min at 37°C. Then, IAyne (1 μ M or 10 μ M, gel-based assays; 10 μ M, MS-based assays; CHES) or CTNyne (10 μ M, gel-based assays; 50 μ M, MS-based assays) labeling was performed for 30 min (gel-based analysis) or 1 hr (MS-based analysis) at room temperature. Copper-catalyzed azide-alkyne cycloaddition (click chemistry) was performed using previously described methods ([Nomura et al., 2010](#); [Weerapana et al., 2010](#)). Fluorescent and proteomic detection of IAyne- or CTNyne-labeled bands were performed as previously reported ([Nomura et al., 2010](#); [Weerapana et al., 2010](#)). Further details can be found in [Supplemental Methods](#).

Metabolomic Profiling

Metabolomic profiling was performed using previously reported procedures ([Benjamin et al., 2013](#)). Additional details are reported in [Supplemental Methods](#).

Activity Assays

Carnitine palmitoyltransferase activity was assayed by incubating kidney lysate from in vivo exposed mice at 10 μ g in 400 μ l of PBS with 100 μ M C16:0 FA-CoA lithium (Sigma-Aldrich) and 100 μ M trimethyl-d⁹-L-carnitine (Cambridge Isotopes) for 5 min at 37°C. Reactions were quenched by addition of 1.2 ml of 2:1 chloroform/methanol with dodecylglycerol (10 nmol, internal standard) and mixed by vortexing. The organic layer was collected and dried under a stream of N₂ then resuspended in 120 μ l of chloroform for SRM-based LC-MS/MS analysis targeting palmitoyl-d⁹-trimethyl-carnitine, and analyzed as previously described.

Acyl-CoA synthetase activity was assayed as using a modified protocol based on [Falcon et al. \(2010\)](#). In brief, 5 mg of kidney lysate from mice treated in vivo with CTN or vehicle were incubated in 200 μ l of Tris-HCl (pH 8.5) buffer containing 10 mM ATP (Sigma), 10 mM MgCl₂ (Sigma), 200 μ M CoA (Sigma) and 10 μ M [13 C]16:0 FFA (Cambridge Isotopes) was added for 30 min at 37°C. Reactions were quenched with 750 μ l of Dole reagent (20:5:1 2-propanol/n-heptane/2M H₂SO₄), vortexed, and dried under a stream of N₂. The product was then resuspended in a buffer of 4 ml of 2:1:1 chloroform/methanol/PBS + 0.1% formic acid, containing 10 nmol of pentadecanoic acid standard. The product was mixed by vortexing and the organic layer was collected, dried under a stream of N₂, resuspended in 120 μ l of chloroform, and analyzed by SRM-based LC-MS/MS analysis targeting [13 C]16:0 FA-CoA as previously described.

Statistical Analyses

For proteomic experiments comparing proteome-wide interactions of several environmental chemicals, a two-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis. For proteomic and metabolomics analyses of in vivo CTN targets comparing vehicle with CTN treatment in liver or kidney, we used a two-tailed t test coupled with a >2-fold difference to identify significant ($p < 0.05$) CTN targets or CTN-induced changes in metabolite levels was used (Bourgon et al., 2010; McCarthy and Smyth, 2009).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Methods, three tables, and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.chembiol.2015.09.008>.

ACKNOWLEDGMENTS

We thank Dr. Kathy Collins of the University of California, Berkeley for use of the Typhoon flatbed scanner. We thank the UC Davis Proteomics Core for MS-based proteomics detection. This work was supported by the Searle Scholar Award, the Center for Environmental Research on Toxics, the NIH (P42ES004705; R01CA172667), the American Cancer Society Research Scholar Award (RSG-14-242-01-TBE), and BASF-CARA Award.

Received: May 20, 2015

Revised: September 17, 2015

Accepted: September 19, 2015

Published: October 22, 2015

REFERENCES

- Araya, C.L., and Fowler, D.M. (2011). Deep mutational scanning: assessing protein function on a massive scale. *Trends Biotechnol.* *29*, 435–442.
- Barr, D.B., Ananth, C.V., Yan, X.Y., Lashley, S., Smulian, J.C., Ledoux, T.A., Hore, P., and Robson, M.G. (2010). Pesticide concentrations in maternal and umbilical cord sera and their relation to birth outcomes in a population of pregnant women and newborns in New Jersey. *Sci. Total Environ.* *408*, 790–795.
- Benjamin, D.I., Cozzo, A., Ji, X., Roberts, L.S., Louie, S.M., Mulvihill, M.M., Luo, K., and Nomura, D.K. (2013). Ether lipid generating enzyme AGPS alters the balance of structural and signaling lipids to fuel cancer pathogenicity. *Proc. Natl. Acad. Sci. USA* *110*, 14912–14917.
- Bookman, E.B., McAllister, K., Gillanders, E., Wanke, K., Balshaw, D., Rutter, J., Reedy, J., Shaughnessy, D., Agurs-Collins, T., Paltoo, D., et al. (2011). Gene-environment interplay in common complex diseases: forging an integrative model—recommendations from an NIH workshop. *Genet. Epidemiol.* *35*, 217–225.
- Bourgon, R., Gentleman, R., and Huber, W. (2010). Independent filtering increases detection power for high-throughput experiments. *Proc. Natl. Acad. Sci. USA* *107*, 9546–9551.
- Bucher, J.R. (2013). Regulatory forum opinion piece*: Tox21 and toxicologic pathology. *Toxicol. Pathol.* *41*, 125–127.
- Codreanu, S.G., Ullery, J.C., Zhu, J., Tallman, K.A., Beavers, W.N., Porter, N.A., Marnett, L.J., Zhang, B., and Liebler, D.C. (2014). Alkylation damage by lipid electrophiles targets functional protein systems. *Mol. Cell. Proteomics* *13*, 849–859.
- Collins, F.S., Gray, G.M., and Bucher, J.R. (2008). Toxicology. Transforming environmental health protection. *Science* *319*, 906–907.
- Dix, D.J., Houck, K.A., Martin, M.T., Richard, A.M., Setzer, R.W., and Kavlock, R.J. (2007). The ToxCast program for prioritizing toxicity testing of environmental chemicals. *Toxicol. Sci.* *95*, 5–12.
- Falcon, A., Doege, H., Fluitt, A., Tsang, B., Watson, N., Kay, M.A., and Stahl, A. (2010). FATP2 is a hepatic fatty acid transporter and peroxisomal very long-chain acyl-CoA synthetase. *Am. J. Physiol. Endocrinol. Metab.* *299*, E384–E393.
- Grube, A., Donaldson, D., Kiely, T., and Wu, L. (2011). Pesticide Industry Sales and Usage: 2006 and 2007 Market Estimates (Environmental Protection Agency).
- Hemminki, K., Bermejo, J.L., and Forsti, A. (2006). Opinion—the balance between heritable and environmental aetiology of human disease. *Nat. Rev. Genet.* *7*, 958–965.
- Huang, R., Xia, M., Cho, M.H., Sakamuru, S., Shinn, P., Houck, K.A., Dix, D.J., Judson, R.S., Witt, K.L., Kavlock, R.J., et al. (2011). Chemical genomics profiling of environmental chemical modulation of human nuclear receptors. *Environ. Health Perspect.* *119*, 1142–1148.
- Hunter, D.J. (2005). Gene-environment interactions in human diseases. *Nat. Rev. Genet.* *6*, 287–298.
- Kalf, G.F. (1987). Recent advances in the metabolism and toxicity of benzene. *Crit. Rev. Toxicol.* *18*, 141–159.
- Kanehisa, M., and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* *28*, 27–30.
- Mathieu, M., Modis, Y., Zeelen, J.P., Engel, C.K., Abagyan, R.A., Ahlberg, A., Rasmussen, B., Lamzin, V.S., Kunau, W.H., and Wierenga, R.K. (1997). The 1.8 Å crystal structure of the dimeric peroxisomal 3-ketoacyl-CoA thiolase of *Saccharomyces cerevisiae*: implications for substrate binding and reaction mechanism. *J. Mol. Biol.* *273*, 714–728.
- McCarthy, D.J., and Smyth, G.K. (2009). Testing significance relative to a fold-change threshold is a TREAT. *Bioinformatics* *25*, 765–771.
- McHale, C.M., Zhang, L.P., and Smith, M.T. (2012). Current understanding of the mechanism of benzene-induced leukemia in humans: implications for risk assessment. *Carcinogenesis* *33*, 240–252.
- Medina-Cleghorn, D., Heslin, A., Morris, P.J., Mulvihill, M.M., and Nomura, D.K. (2014). Multidimensional profiling platforms reveal metabolic dysregulation caused by organophosphorus pesticides. *ACS Chem. Biol.* *9*, 423–432.
- Meier, B., and Gartner, A. (2014). Having a direct look: analysis of DNA damage and repair mechanisms by next generation sequencing. *Exp. Cell Res.* *329*, 35–41.
- Morris, P.J., Medina-Cleghorn, D., Heslin, A., King, S.M., Orr, J., Mulvihill, M.M., Krauss, R.M., and Nomura, D.K. (2014). Organophosphorus flame retardants inhibit specific liver carboxylesterases and cause serum hypertriglyceridemia. *ACS Chem. Biol.* *9*, 1097–1103.
- Nomura, D.K., and Casida, J.E. (2011). Activity-based protein profiling of organophosphorus and thiocarbamate pesticides reveals multiple serine hydrolase targets in mouse brain. *J. Agric. Food Chem.* *59*, 2808–2815.
- Nomura, D.K., Leung, D., Chiang, K.P., Quistad, G.B., Cravatt, B.F., and Casida, J.E. (2005). A brain detoxifying enzyme for organophosphorus nerve poisons. *Proc. Natl. Acad. Sci. USA* *102*, 6195–6200.
- Nomura, D.K., Blankman, J.L., Simon, G.M., Fujioka, K., Issa, R.S., Ward, A.M., Cravatt, B.F., and Casida, J.E. (2008). Activation of the endocannabinoid system by organophosphorus nerve agents. *Nat. Chem. Biol.* *4*, 373–378.
- Nomura, D.K., Long, J.Z., Niessen, S., Hoover, H.S., Ng, S.W., and Cravatt, B.F. (2010). Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis. *Cell* *140*, 49–61.
- Nomura, D.K., Lombardi, D.P., Chang, J.W., Niessen, S., Ward, A.M., Long, J.Z., Hoover, H.F., and Cravatt, B.F. (2011). Monoacylglycerol lipase exerts dual control over endocannabinoid and fatty acid pathways to support prostate cancer. *Chem. Biol.* *18*, 846–856.
- Oriel, M., Edmiston, S., Beauvais, S., Barry, T., and O'Malley, M. (2009). Illnesses associated with chloropicrin use in California agriculture, 1992–2003. *Rev. Environ. Contam. Toxicol.* *200*, 1–31.
- Pace, N.J., and Weerapana, E. (2013). Diverse functional roles of reactive cysteines. *ACS Chem. Biol.* *8*, 283–296.
- Poirier, Y., Antonenkov, V.D., Glumoff, T., and Hiltunen, J.K. (2006). Peroxisomal beta-oxidation—a metabolic pathway with multiple functions. *Biochim. Biophys. Acta* *1763*, 1413–1426.

- Poole, L.B. (2015). The basics of thiols and cysteines in redox biology and chemistry. *Free Radic. Biol. Med.* 80C, 148–157.
- Rappaport, S.M. (2011). Implications of the exposome for exposure science. *J. Expo. Sci. Environ. Epidemiol.* 27, 5–9.
- Rappaport, S.M., Li, H., Grigoryan, H., Funk, W.E., and Williams, E.R. (2012). Adductomics: characterizing exposures to reactive electrophiles. *Toxicol. Lett.* 213, 83–90.
- Seidler, N.W. (2013). Basic biology of GAPDH. *Adv. Exp. Med. Biol.* 985, 1–36.
- Shannon, D.A., and Weerapana, E. (2015). Covalent protein modification: the current landscape of residue-specific electrophiles. *Curr. Opin. Chem. Biol.* 24, 18–26.
- Shukla, S.J., Huang, R., Austin, C.P., and Xia, M. (2010). The future of toxicity testing: a focus on in vitro methods using a quantitative high-throughput screening platform. *Drug Discov. Today* 15, 997–1007.
- Wang, C., Weerapana, E., Blewett, M.M., and Cravatt, B.F. (2014). A chemoproteomic platform to quantitatively map targets of lipid-derived electrophiles. *Nat. Methods* 11, 79–85.
- Watanabe, T., and Hirano, S. (2013). Metabolism of arsenic and its toxicological relevance. *Arch. Toxicol.* 87, 969–979.
- Weerapana, E., Simon, G.M., and Cravatt, B.F. (2008). Disparate proteome reactivity profiles of carbon electrophiles. *Nat. Chem. Biol.* 4, 405–407.
- Weerapana, E., Wang, C., Simon, G.M., Richter, F., Khare, S., Dillon, M.B., Bachovchin, D.A., Mowen, K., Baker, D., and Cravatt, B.F. (2010). Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* 468, 790–795.
- Wilkinson, C.F., and Killeen, J.C. (1996). A mechanistic interpretation of the oncogenicity of chlorothalonil in rodents and an assessment of human relevance. *Regul. Toxicol. Pharmacol.* 24, 69–84.