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Chem. Res. Toxicol., **Just Accepted Manuscript** • DOI: 10.1021/acs.chemrestox.7b00290 • Publication Date (Web): 20 Nov 2017

Just Accepted

ACS Publications

Chemical Research in Toxicology is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

The Toxmatrix: Chemo-genomic profiling identifies interactions that reveal mechanisms of toxicity

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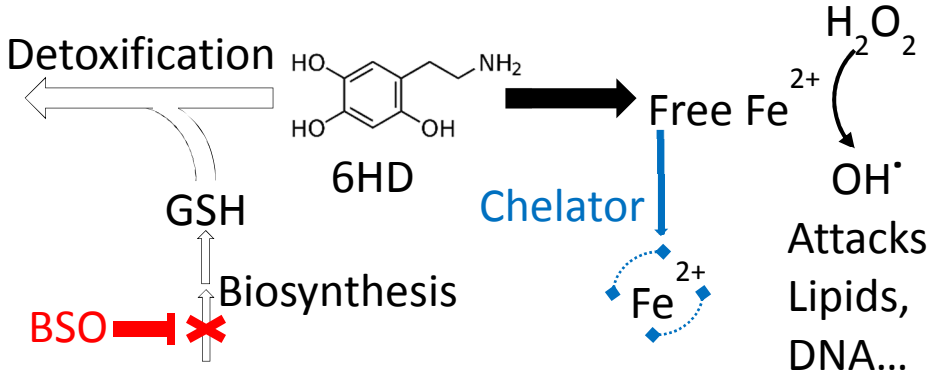

ABSTRACT

A chemical genomics 'Toxmatrix' method was developed to elucidate mechanisms of cytotoxicity using neuronal models. Quantitative high-throughput screening (qHTS) was applied to systematically screen each toxicant against a panel of 70 modulators, drugs or chemicals that act on a known target, to identify interactions that either protect or sensitize cells to each toxicant. Thirty-two toxicants were tested at 10 concentrations for cytotoxicity to SH-SY5Y human neuroblastoma cells, with results fitted to the Hill equation to determine an IC_{50} for each toxicant. Thirty-three toxicant:modulator interactions were identified in SH-SY5Y cells for 14 toxicants, as modulators that shifted toxicant IC_{50} values lower or higher. The target of each modulator that sensitizes cells or protects cells from a toxicant suggests a mode of toxicant action or cellular adaptation. In secondary screening, we tested modulator-toxicant pairs identified from the SH-SY5Y primary screening for interactions in three differentiated neuronal human cell lines: dSH-SY5Y, conditionally-immortalized dopaminergic neurons (LUHMES), and Neural Stem Cells. Twenty toxicant-modulator pairs showed pronounced interactions in one or several differentiated cell models. Additional testing confirmed that several modulators acted through their primary targets. For example, several chelators protected differentiated LUHMES neurons from four toxicants by chelation of divalent cations; and buthionine sulphoximine sensitized cells to 6-hydroxydopamine and 4-(Methylamino)phenol hemisulfate by blocking glutathione synthesis. Such modulators that interact with multiple neurotoxicants suggest these may be vulnerable toxicity pathways in neurons. Thus, the Toxmatrix method is a systematic high-throughput approach that can identify mechanisms of toxicity and of cellular adaptation.

Keywords: neurodegeneration, high-throughput screening, neuronal models

INTRODUCTION

Toxic environmental chemicals that have traditionally been identified by animal testing, are increasingly being identified by *in vitro* assays. For example, *in vitro* quantitative high throughput screening (qHTS) has been performed by the Tox21 Consortium for ten thousand such chemicals.[1-3] Specialized qHTS methods were developed by the Tox21 partners to robotically screen thousands of compounds at multiple concentrations in 1536-well microplates to identify those that activate a variety of cellular-stress events. Chemicals that activate such events, *e.g.* disruption of mitochondrial membrane potential, TP53 activation, cytotoxicity, *et al*.,[4] demand further evaluation as putative toxicants. We evaluated whether one could extend this qHTS approach to systematically elucidate mechanisms for toxicants identified by qHTS by identifying interactions between each toxicant and toxicity pathways within the cell model. Thus, toxicants were tested using a systems-biology approach for interaction with a panel of modulators, chemicals that each perturb a cell stress-related pathway, to determine if they protect or sensitize cells to each toxicant.

Many drugs and drug metabolites are selectively toxic to a specific human cell type, either through selective pharmacokinetic properties (PK), or selective toxicodynamics (TD).[5] PK may determine selective toxicity to neurons, *e.g.* when MPTP is metabolized into toxic MPP+ by astrocytes.[6] In turn, the "Toxicity Pathways" approach[7] describes TD toxicity mechanisms as normal cellular pathways that result in adverse events when disrupted. This approach implies that the sensitive cell type has a unique pathway, or is especially sensitive to disruption of the toxicity pathway. Smirnova *et al.* have suggested that cellular resilience, the manner in which a cell adapts to resist toxicity, may be more important than the cells' direct susceptibility to toxicity in determining which cell types survive.[8] The toxicity pathway and resilience approaches are not conflicting, but rather complementary descriptions of how the toxicant insults the cell, and how the cell attempts to adapt to the insult, respectively.

Neurons are protected from most toxicants by the blood-brain barrier, yet neurons must have unique TD pathways that make these cells particularly sensitive to toxicants. For example, neurotoxicants may be

toxicodynamically selective for neurons by disrupting the microtubules that support the long axons of neurons, or by disrupting mitochondria that are used intensively to repolarize membranes in electrically-active neurons. Characterization of neurotoxicants that kill mature neurons is urgently needed in order to identify neurotoxic chemicals that are suspected to cause Parkinson's disease (PD), Alzheimer's disease (AD), and other neurodegenerative disorders.[9]

Neuronal cytotoxicity models were investigated in a previous study from this laboratory that compared 3 human neuronal models: SH-SY5Y neuroblastoma cells,[10] LUHMES conditionally-immortalized dopaminergic neurons,[11] and Neural Stem Cells (NSC)[12] that differentiate into a mix of neuronal, astrocytic, and oligodendrocytic cell types.[13] For neurotoxicological screening the LUHMES model was preferred due to its ability to differentiate nearly 100% of cells within seven days, to its high-level expression of neuronal markers, and to its greater sensitivity to 21 -out-of- 32 known or candidate neurotoxicants tested,[12] These three models, and particularly dLUHMES cells, are further applied in this study to elucidate the mode or mechanisms by which neurotoxicants may kill neurons. Thus, LUHMES cells seem to recapitulate *in vitro* the selective sensitivity of *in vivo* neurons to neurotoxicants in many cases. Nevertheless, some neurotoxicants will demand a model system that includes metabolic activation, neuronal interactions with other cell types, or the 3-dimensional microenvironment found within the brain.

Toxicants have previously been characterized to elucidate their mechanisms of action, or classify modes of toxicity, by identifying a second chemical factor that either sensitizes or protects the cell from death. Wolpaw *et al.* demonstrated a systematic "modulatory profiling" screen to identify pairs of drugs that showed synergy or antagonism in killing tumor-derived cells.[14] Drugs that showed similar modulatory profiles were grouped in order to find outlier anticancer drug candidates that implied a novel mode of action.

In the present study we evaluate a systematic and quantitative matrix screening method called the Toxmatrix, to determine whether it can yield insights into toxicant mechanisms. The Toxmatrix method uses modulator chemicals that address known targets, to systematically identify those that promote or inhibit toxicity of known or suspected neurotoxicants. Seventy modulators were selected from the published literature to target pathways for cellular adaptation to stress, or for pathways that regulate necrosis or apoptosis, cell survival, or regulation of energy metabolism. In brief, master plates for toxicants and modulators are prepared using robots and barcoding to enable automated sample-tracking. qHTS uses cells cultured in 5 μL volumes in 1536-well microplates, dosed with precise 100 nL volumes using an acoustic dispenser. Concentration-response cytotoxicity data for each toxicant and toxicant + modulators are recorded in a database and automatically analyzed by curve-fitting, derivation of IC_{50} values and quality scores, and visualization. This qHTS infrastructure enabled us to formulate and systematize the Toxmatrix method and to evaluate its suitability to determine mechanisms of cytotoxicity.

METHODS

Chemicals and Compound Management The 32 toxicant chemicals and 70 modulators were included in this study were purchased from Sigma-Aldrich Chemical Co., Selleck Chemicals LLC, R & D/Tocris, or synthesized at NCATS (see supplementary Table 1). The compounds were dissolved in dimethyl sulfoxide (DMSO) at either 20 mM (200X) or 50 mM (500X) for the toxicants. Modulators were diluted in 3 five-fold steps to generate 200x master solutions for primary screening in SH-SY5Y cells, to include a middle dosage selected from published studies. For secondary screening in three cell lines, toxicants were diluted to 9 concentrations in three-fold steps and modulators were also diluted to 9 concentrations in three-fold steps based on the minimum toxic concentration from primary screening.

Cell Culture and Differentiation Human cell lines including neuroblastoma cell line SH-SY5Y, neuronal stem cell NSC, and a conditionally-immortalized human fetal mesencephalic cell line LUHMES cells were cultured and differentiated as described before.[6, 12] Briefly, SH-SY5Y cells (ATCC,

Gaithersburg, MD) were cultured in 1:1 mixture (EME/F12) of Eagle's Minimum Essential (EMEM, ATCC) and F12 (Invitrogen) containing 10% fetal bovine sera (FBS). SH-SY5Y cell differentiation was induced by 10 μM all-trans-retinoic acid in 3% FBS culture medium. Gibco® Human Neural Stem Cells (NSC; Invitrogen/Thermo-Fisher, Waltham, Massachusetts, USA) were adherently cultured in vessels coated with Geltrex and grown in the defined StemPro NSC SFM complete medium consisting of KnockOut D-MED/F-12 with 2% StemPro Neural Supplement, 2 mM GlutaMax-I Supplement, and human recombinant bFGF and EGF (20 ng/ml each). NSC differentiation was carried out in StemPro NSC SFM medium without bFGF and EGF.[12] LUHMES cells were grown in culture vessels coated with poly-L-ornithine and human fibronectin (50 μg/ml each) in Advanced DMEM/F12 medium containing 2 mM L-glutamine, N2-supplement (1X) and 40 ng/ml bFGF. LUHMES cells were subsequently differentiated by adding 1 μM cAMP and 1 μg/ml tetracycline in advanced DMEM/F12 medium with 2 mM L-glutamine, 1X N2-supplement, and 2ng/ml GDNF.[6] Both proliferating and differentiating cells were transferred into tissue culture-treated 1536-well plates with the same coatings using a Multidrop instrument (Thermo-Fisher Scientific, Waltham, MA USA) in a volume 5 μL containing 2000 cells per well at one day before the modulators and toxicants were applied. After 9 passages, cell lines were discarded and replaced from frozen stocks. Identities of the three cell lines were verified following each experiment by short tandem repeat profiling (WiCell Research Inst. Madison, Wisconsin, USA). Cell lines were also checked following each experiment for mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel Switzerland).

qHTS Toxmatrix Screening Undifferentiated cells, or cells allowed to differentiate in T225 flasks, were distributed in 1536-well plates one day before applying chemical treatments, using 2,000 cells per well in 5 μL medium. The primary Toxmatrix consisted of 32 chemical toxicants and 70 modulators (supplementary Table 1). The toxicants and modulators were made in separate 1536-well compound plates in appropriate concentrations as 200X master plates. Each of the toxicants was applied to cells at 10

concentrations and no-toxicant controls from a maximum of 100 μM in three-fold steps; while each of the modulators was applied to cells at 3 concentrations in five-fold steps, designated "low", "middle" and "high". For this "10x3" primary screening, each compound was loaded in 25 nL of DMSO using a 1536 pintool instrument (V&P Scientific). In the secondary 9x9 Toxmatrix screening, 36 pairs of toxicants (13) and modulators (22) with appropriate controls were selected, based on data from the primary screening. Each compound was serially diluted (1:3) to make compound source plates at 500-fold the final concentration, including 9 concentrations of the toxicants and 9 concentrations for the modulators, plus no toxicant and no-modulator samples. Each dosage was loaded in 10 nL into cultured cells using an ATS-100 acoustic dispenser (EDC Biosystems, Fremont, CA, USA). In both primary and secondary screenings cells were cultured in 5 μL of medium in 1536-well plates. The modulators were loaded four h before the toxicants were applied, and every permutation of modulator- and toxicant concentrations was tested. The first 4 rows of cells in each 1536-well plate were treated with DMSO only as negative controls for cell survival, or tetra-octyl ammonium bromide as positive controls for 100% cell killing. Cytotoxicity assays were carried out at 24 and 48 h after the modulators and toxicants were applied.

Cell Viability and Caspase 3/7 Assays Cells were assessed for viability using a CellTitreGlo® luminescence assay, Promega, Madison Wisconsin, USA). After compound treatment, 5 μL of these detection reagents were added into each well of the 1536-well assay plates, and the assay plates were incubated for 30-60 min at room temperature. Luminescent intensity was measured on a Viewlux instrument (PerkinElmer, Waltham MA, USA) to indicate cell viability.

qHTS Data Analysis Analysis of compound concentration–response data was performed as previously described.[4, 15] Briefly, raw plate reads were first normalized relative to the positive control compound (-100%) and DMSO-only wells (0%) as follows: % Activity = $((V_{compound} - V_{DMSO})/(V_{pos} - V_{DMSO})) \times 100$, where $V_{compound}$ denotes the compound well values, V_{pos} denotes the median value of the positive control wells, and V_{DMSO} denotes the median values of the DMSO-only wells, and then corrected by applying a NCATS in-house correction algorithm.[16] Concentration–response titration points for each

toxicant at fixed modulator concentration were fitted to a four-parameter Hill equation[17] using an iterative grid algorithm.[18] These curve-fitting results yielded concentrations of half-maximal inhibition (IC_{50}) and maximal response (efficacy) values. Modulator concentrations that caused ≥10% cytotoxicity were excluded. Modulator:toxicant:cell-type combinations that yielded a significant IC_{50} shift, exceeding 3-fold either leftward (+), or rightward (-) were verified by visual inspection from the curve fits. The maximum IC_{50} shift is reported among non-cytotoxic modulator concentrations.

Validation of Toxmatrix Screenings Further experiments to validate the selected pairs of toxicant-modulator functional interactions were carried out manually in 384 well plates using differentiated human LUHMES cells. Briefly, 10,000 LUHMES cells/well were loaded in 50 μL to 384 well plates and differentiation was induced for 7 days. Modulators PHE, M30, or BSO were used to treat cells for a four h interval prior to treatment with a toxicant concentration that caused approximately 50% killing. Supplements GSH or NAC were also added for this 4 hour interval as indicated. After treatment, cell viability was assayed at 24 and 48 h to determine whether a modulator or modulator + supplement affected toxicant-induced cytotoxicity.

RESULTS AND DISCUSSION

Identification of Toxicant:Modulator Pairs in SH-SY5Y Cells We sought to build a matrix screening tool that can reveal mechanisms of toxicity or adaptation by identifying modulators that protect or sensitize cells to the toxicant. Seventy modulators were selected from the literature to target pathways related to necrosis or apoptosis, cell survival, regulation of energy metabolism, as well as adaptation to stress including: detoxification, oxidative stress, DNA damage and repair, cell cycle regulation, innate immune responses, ER stress, autophagy, proteolysis, and disruption of enzyme cofactors. This Toxmatrix approach was designed to identify modulators for each toxicant that shifted the concentration-response curve of a toxicant left or right relative to the modulator-free controls (sensitization or

protection, respectively). The goal was to identify a modulator concentration that shifted the IC_{50} maximally without exhibiting cytotoxicity by itself. Cells were allowed to respond to modulator chemicals for four hours prior to toxicant treatments. SH-SY5Y cells were used initially to screen of 32 toxicants x 70 modulators. The SH-SY5Y cell line neuronal model was selected because it was used to identify several candidate neurotoxicants,[1, 4, 12] and because SH-SY5Y cells were readily cultured for high-throughput screening[1] in 1536-well microplates. The qHTS screening format of Inglese *et al.*[19] was adopted to assay cytotoxicity in 1536-well microplates using 10 concentrations of each toxicant from 100 μM to 5 nM in three-fold increments, and no-toxicant controls; as well as 70 modulators at three concentrations each, and no-modulator controls, for each query toxicant.

This survey using SH-SY5Y cells examined combinations of 32 toxicants at 10 concentrations each (plus the vehicle no-toxicant control), with 70 modulators at 3 concentrations each (plus modulator-only controls); comprising 98,560 nominal sample-data points. For each toxicant:modulator pair, a curve was drawn to fit the Hill equation[18] for the 10 toxicant concentrations without modulator, and an additional curve for each modulator concentration. Representative plots are shown for three toxicant:modulator pairs effects on SH-SY5Y cells in Figure 1. Figure 1 shows the cytotoxic concentration-response for the toxicant MAP, left-shifted hence sensitized by modulator $CoCl_2$ in Fig1A, unchanged by treatment with modulator necrostatin 5 in Fig 1B, and right-shifted hence protected by treatment with modulator NAC in Fig 1C. Among 64 microplates, the median % Coefficient of Variation for DMSO controls was 3.9% with a standard deviation (SD) of 0.71%, the median signal/background ratio was 31 with a SD of 1.7, and the median Z' score[4] was 0.90 with a SD of .02. These reproducible data and robust curve-fits resulted in reliable automated classification of protection or sensitization events for nearly all toxicant:modulator pairs. Modulators that caused cytotoxicity without a toxicant at high concentrations (viability <90%, *e.g.* the high concentration of $CoCl_2$ in Fig 1A) were excluded from results. In several cases automatic calls of sensitization or protection appeared to result from an upward- or downward- shift exceeding 10% relative to the 100% baseline for particular modulators at low toxicant concentration. Such curves were also

excluded from results as likely artefacts. This process enabled us to identify discrete and substantial protective or sensitizing interactions for further studies, and to exclude false-positive or weak interactions.

Figure 2 summarizes 33 significant interactions among pairwise combinations of 32 toxicants with 70 modulators, in which the modulator increased toxicity (sensitization) in 23 pairs, and the modulator decreased toxicity (protection) in 10 pairs. The significant toxicant:modulator pairs indicated in Figure 2 were evaluated by examining the curves for consistent sensitization or protection by the modulator pairs (modulator caused >3-fold shift of the IC_{50} at a concentration that caused <10% cytotoxicity) as illustrated in Fig 1A and Fig 1B. Figure 2 indicates several generalizable results. First, a particular modulator tended to function either in protection or sensitization, but rarely in both modes; whereas several toxicants were subject to both effects by modulators. Among the 70 modulators tested, five were protective whereas sixteen acted as sensitizers. Two modulators functioned in both modes, the divalent cation chelator DMPS, which protected SH-SY5Y cells from 5 toxicants, but sensitized cells to HCP; and KU0063794, a TOR inhibitor drug that both protected SH-SY5Y cells from OTZ, and sensitized them to MHG. Unexpectedly, several modulators that were designed to protect cells from TNFalpha-driven necrosis, necrostatins 1, 5, and 7, instead sensitized SH-SY5Y cells to killing by HCP or MALG under these conditions (Figure 2).

Toxicant:Modulator Interactions in 3 Neuronal Models From a matrix of 32 toxicants and 70 modulators, we identified 33 pairwise interactions in SH-SY5Y cells (Figure 2), including combinations of 14 known or suspected neurotoxicants and 19 modulators. These 33 pairs were selected for secondary screening using SH-SY5Y, LUHMES, and NSC cells, after differentiation protocols that increased the neuronal characteristics of these models.[12] Conditions were developed for each cell line, extending previous work using 384-well microplates,[12] to enable differentiation and culture in 1536-well format for high-throughput Toxmatrix cytotoxicity assays. In these experiments, cells were differentiated for 7 days,[12] plated, and allowed to re-elaborate neurites (termed dSH-SY5Y, dLUHMES, and dNSC), before addition of modulators and toxicants. These were performed in 1536-well microplates using 9

concentrations of each toxicant and no-toxicant DMSO controls, as well as 9 concentrations of each modulator and no-modulator controls. For Figure 3, cytotoxicity assays were performed at both 24 h and 48 h to observe both transient interactions that manifested at 24 h and slowly developing interactions that manifested at 48 h. Cytotoxicity results from this matrix of 9,240 data points were then examined to identify combinations of toxicants and modulators that interacted selectively and robustly in these differentiated cells. The maximum fold-shift in IC_{50} is reported in Figure 3 for each interaction that resulted in a significant shift in IC_{50} (>3-fold increase or decrease) at a modulator concentration that resulted in <10% cytotoxicity measured as intracellular [ATP].

Figure 3 illustrates two notable trends in toxicant:modulator:cell line interactions. Each modulator characteristically either protected or sensitized cells with multiple toxicants; and toxicant:modulator interactions tended to occur in more than one model. Specifically DMPS, NAC, nicotinamide, and SP-600125 showed only protective actions, whereas $CoCl_2$, BSO, necrostatin 7, WYE-354, CNF-2024, SRT1720, GSK-1904529A and TW-37 showed only sensitizing actions. DMPS protected cells from five toxicants, MAP, 6HD, CAP, DTCM, and MHG; and N-acetyl-L-cysteine (NAC) protected cells from MAP, DTCM, and MHG. Conversely, necrostatin 7 sensitized cells to three toxicants: 6HD, MALG, and MHG. In fact, several modulators exhibited consistent effects for multiple toxicants, particularly DMPS, NAC, and necrostatin 7. Similarly, interactions almost invariably occurred in treatments with the same toxicant in multiple cell types, the exceptions being GSK-1904529A that sensitized only dSH-SY5Y from MALG, and nicotinamide that protected only dLUHMES from VIN. Note that in Figure 3 IC_{50} fold change is indicated for changes exceeding 3-fold, whereas changes between 2-3-fold are colored red or blue without a number to reveal trends that did not reach the 3-fold significance standard. Interactions were often but not always observed at both 24 h and 48 h time points. For example, at both time points DMPS protected: dLUHMES from 6HD, all three cell lines from CAP, and dSH-SY5Y as well as dLUHMES from DTCM. Conversely, enhanced cytotoxicity was sometimes observed at only one time point; *e.g.* dLUHMES was selectively sensitized to 6HD by both BSO and necrostatin 7 at 24 h but only

weakly or not at all at 48 h. Many toxicant:modulator:cell line interactions gave rise to testable hypotheses; these are discussed below.

Sensitizing Interactions in Differentiated Cell Models Toxicant:modulator:cell line interactions that sensitized neuronal cells implied that the modulator acted upon a target-pathway that enhanced toxicity or interfered with adaptation. The diversity of significant toxicant:modulator combinations generated a variety of hypotheses regarding toxicant modes of action. For example, sensitization of dLUHMES and dNSC to MAP by $CoCl_2$, an inducer of the HIF1 hypoxia pathway, suggests that HIF1 activation stimulated a pro-apoptotic or pro-necrotic phenotype that made dLUHMES susceptible to MAP cytotoxic challenge. Necrostatin 7 sensitized several cell lines to 6HD, MALG, and MHG. This was surprising, since the necrostatins were developed for their ability to protect various cell types from the cytotoxic effect of Tumor Necrosis Factor.[20] There is precedent for the presumed target of the necrostatins, RIP1K, acting on the NFkB pathway, either sensitizing or protecting cells dependent on cell type and cytotoxic stimuli.[21] Nevertheless, it was shown recently that necrostatin 7 does not inhibit RIP1 Kinase, the known target of necrostatin 1, and -5, hence necrostatin 7 may work through a different, unknown, target.[20] In support of the possibility that necrostatin 7 works *via* a unique target, necrostatins 1 and 5 coincided in sensitizing SH-SY5Y cells to HCP; whereas necrostatin-7 differed in sensitizing this cell line to MALG and CR2. The sensitizing activity of necrostatin 7 to 6HD, MALG, and MHG in these neuronal models suggested a shared pathway of toxicity among these three toxicants, but the mode or mechanism awaits further characterization. BIIB021, an inhibitor of HSP90 that inactivates NFκB,[22] also sensitized dSH-SY5Y and dLUHMES to CLM. SRT1720, a Sirtuin activator, sensitized dSH-SY5Y, dLUHMES, and dNSC cell lines to MPP. This was surprising given that SIRT1 activators as well as SIRT2 inhibitors have been proposed as therapeutic treatments for PD.[23] Since SRT1720 has been shown to increase mitochondrial metabolism, and MPP is thought to attack mitochondrial electron transport, we hypothesize that SRT1720 increases electron flow into an electron transport chain that is blocked at Complex I by MPP, resulting in increased electron flow to H_2O_2 and increased toxic radical formation.[24] GSK-

1904529A, an IGF1 Receptor inhibitor, was observed to sensitize dSH-SY5Y cells to MALG, an antifungal used in aquaculture; possibly by blocking unknown survival factor(s) that are dependent on IGF1 action.[25] TW-37, an inhibitor of the antiapoptotic BCL2 protein, sensitized dSH-SY5Y cells to TUNC, suggesting that BCL2 mitigates an apoptotic activity of TUNC.[26]

Protective Interactions in Differentiated Cell Models A variety of modulators were identified that protected cells from known or suspected neurotoxicants, including NAC and DMPS. Because NAC and DMPS protected neuronal models from a variety of neurodegenerative toxicants, interactions involving these two modulators were selected for further studies aimed at confirming or refuting the expected targets of NAC and DMPS. NAC protected dSH-SY5Y from MAP, DTCM, and MHG, as well as protecting dLUHMES from DTCM (Figure 3). This protective activity indicates that these toxicants cause oxidative stress, originating either from reactive drug metabolites or oxidative byproducts of H_2O_2 resulting from disrupted electron transport in the mitochondria.[27] NAC can act either by feeding GSH synthesis or by acting directly as a scavenger, a question that is addressed below in Figure 4. DMPS also protected cells from multiple toxicants; protecting dSH-SY5Y and dNSC from MAP, dLUHMES and dNSC from 6HD and MHG, dSH-SY5Y and dLUHMES from DTCM, and all three cell lines from CAP, Figure 3. These interactions reveal that cytotoxic mechanisms of OTZ, MAP, 6HD, CAP, DTCM, and MHG involve liberation of divalent cations, likely to be free iron, which is known to generate toxic hydroxyl radical from H_2O_2.[28] Nicotinamide protected dLUHMES cells from the toxicity of MHG, acting either as a SIRT inhibitor, or as an antioxidant. JNK 1/2/3 inhibitor SP-600125 mitigated the toxicity of VIN towards both dLUHMES and dNSC cells, suggesting that the JNK stress pathway functions as a toxicity pathway downstream of VIN treatment.

Modulator interactions with toxicants 6HD, CAP, MAP, and DTCM were of particular interest to study in dLUHMES cells, since dLUHMES are more sensitive to these toxicants than either undifferentiated LUHMES, or SH-SY5Y cells.[12] These results suggested that toxicants 6HD, CAP, MAP, and DTCM may be selectively neurotoxic because of neuronal- or dopaminergic-neuronal characteristics of the

dLUHMES cells. dSH-SY5Y and dLUHMES cells were protected from these toxicants by DMPS (except 6HD in SH-SY5Y and MAP in dLUHMES did not meet the 3-fold threshold), which led us to evaluate DMPS action using additional chelators. Additionally, dLUHMES and dSH-SY5Y were protected from DTCM by NAC and sensitized to 6HD by BSO, which causes depletion of glutathione, which led us to additional study of the role of glutathione in adaptation to oxidative stress. These additional evaluations of BSO and DTCM were performed in dLUHMES neurons to determine whether interactions arose from the generally-accepted target of the specific modulator, revealing pathways impacted by each toxicant, or arose from off-target activities that obscure these pathways. In these experiments, fixed concentrations of toxicant and modulator were selected to cause a level of cytotoxicity intermediate between 0% and 100%, in order to look for increased or decreased toxicity in response to either a supplement or a substitute modulator.

The Glutathione Pathway in dLUHMES BSO markedly sensitized dLUHMES cells, as well as dNSC and dSH-SY5Y, to 6HD (Figure 3). Since BSO is known to block glutathione biosynthesis by inhibiting γ-glutamylcysteine synthetase,[29] we hypothesized that cells were using GSH to detoxify 6HD, and that BSO interfered with this detoxification by depleting cellular glutathione. We tested this hypothesis by a simple supplementation strategy. dLUHMES cells were supplemented with GSH or with the cysteine precursor NAC, to ask whether these supplements restore the dLUHMES cells' ability to resist cytotoxicity from 6HD. Figure 4A shows that NAC or GSH partially alleviated the toxicity of 6HD + BSO to dLUHMES cells, supporting the hypothesis that GSH allays the cytotoxicity of 6HD. GSH at 100 μM was particularly effective at decreasing cytotoxicity at an intermediate BSO concentration of 5 μM. In cells, 6HD is thought to give rise to a quinone, with production of H_2O_2. Previous work has demonstrated that 6HD applied to SH-SY5Y cells is converted into a quinone that gives rise to H_2O_2, causing oxidative stress.[30] The quinone is detoxified by conjugation to glutathione[27, 30] such that GSH is consumed and regenerated in this process.[35, 38, 31] Our result is also in agreement with a previous experiment that demonstrated GSH can partially mitigate toxicity of 6HD to SK-N-SH neural cells, the

precursor to SH-SY5Y cell.[27] Similarly, BSO increased the sensitivity of dLUHMES cells to MAP, and supplementation with NAC or GSH partly alleviated this sensitivity (Figure 4B). In the cases of 6HD and MAP, NAC likely acted at least partly by replenishing GSH, since GSH supplementation was demonstrated to partly restore the sensitizing activity of BSO (Figure 4). Since GSH is not known to penetrate cells intact, it is not clear whether GSH acted inside cells, or exerted antioxidant activity extracellularly. Taken together, these results suggest that GSH mitigates cytotoxicity by relieving oxidative stress caused by both 6HD and MAP.

Protective Divalent Cation Chelation in dLUHMES The action of DMPS, a relatively cell-permeable chelator of divalent cations,[32] in protecting dLUHMES cells from 6HD, MAP, CAP, DTCM, and MHG, was also further evaluated. Patients diagnosed with heavy metal poisoning are often treated with a chelator such as DMPS to mobilize accumulated toxic metals; however it is possible that DMPS also serves as a scavenger of reactive oxidants.[33] To resolve this question additional divalent cation chelators, 1,10,-phenanthroline (PHE) and M30[34] were tested to determine whether they can substitute for DMPS to protect dLUHMES cells from two of these five toxicants. Interestingly, chelator PHE, like DMPS, significantly mitigated the toxicities of 6HD and of MAP to dLUHMES cells (Figure 5A and Figure 5B, respectively); whereas chelator M30 increased the toxic effects of both 6HD and MAP. Since M30 is designed to be a scavenger of radicals as well as a chelator, but did not protect LUHMES cells, these results suggest that it is the metal chelating activity of DMPS that protects LUHMES cells from toxicants, rather than any oxidant scavenger activity. Although all three chelators can tie up divalent cations such as Fe^{2+} or Zn^{2+}, such chelators differ in their abilities to penetrate cell membranes and partition into subcellular organelles as well as their affinities for ligands, hence may have different effects in intoxicated cells. Interestingly, whereas DMPS also protected dLUHMES from these five compounds in these follow-up experiments, neither PHE nor M30 protected dLUHMES cells from 20 μM CAP, 4 μM DTCM, or 1 μM MHG (not shown). Previous work has shown that chelators can protect neuronal models from 6HD; however different chelators were protective in different cell models. For example, Kobayashi

et al. showed that the widely studied chelator desferroxamine was protective for SH-SY5Y neurons treated with 6HD.[35] Desferroxamine and M30 have also shown neuron-protective ability in 6HD-treated rat models;[34, 36] however neither desferroxamine nor M30 were protective in the dLUHMES model (M30 Figure 5A, desferroxamine not shown). Chelators have been tested in Parkinson's disease patients with intriguing, but not definitive results, and relatively narrow therapeutic margins.[37, 38] Thus, chelators are diverse in their actions on different toxicants, revealing in this study that the five toxicants 6HD, MAP, CAP, DTCM, and MHG are related but not identical in their cytotoxic mechanisms.

It is also useful to note that several modulators that yielded interactions in primary screening had "sister modulators", that is, modulators with the same primary target (Supplementary table S2). For example, necrostatin 1 and necrostatin 5 both inhibit RIP1K, and both sensitized SH-SY5Y cells to HCP; and DMPS and PHE both protected dLUHMES from 6HD and MAP. Other sister modulators failed to yield similar interactions, *e.g.* mTOR inhibitors GSK-1904529A, KU0063794, and WYE-354; HSP90 inhibitors BIIB021 and Alvespimycin; or Nitric Oxide Synthase inhibitors L-NAME and L-NMMA (Figure 2 and Table S2); suggesting that modulators are often promiscuous or divergent in their pharmacological properties.

Toxicants with Shared Mechanisms Toxicants may be grouped based on being sensitized or protected by shared modulators, suggesting shared mechanisms of toxicity. For example, DMPS protected dLUHMES cells from 6HD, MAP, CAP, DTCM, and MHG (Figure 3), indicating a shared mechanism of toxicant action *via* release of divalent cations; likely iron(II).[28] Similarly, BSO sensitization of dLUHMES cells to 6HD, and MAP (Figure 4) revealed that these toxicants deplete reduced glutathione that can be partly ameliorated by NAC (which also protected dLUHMES from DTCM, Figure 3) or by GSH supplements. Taken together, these results suggest similar modes of toxicity for 6HD, MAP, and DTCM, in causing cytotoxicity via both oxidative stress and divalent cation release. These two events are consistent with cytotoxic mechanisms previously studied for 6HD in models of Parkinsonism in animals. In these models, 6HD is thought to disrupt mitochondrial Complex I of the electron transport chain,

increasing H_2O_2 production and releasing iron from iron-sulfur centers and heme-iron in Complex I.[28] The interactions of chelators and of BSO with several neurotoxicants each suggests that disruption of iron cofactor complexes and depletion of reduced glutathione may be vulnerable toxicity pathways in neurons, and should be investigated further.

A very simple schematic cytotoxicity model is proposed in the Table of Contents graphic to describe 6HD (or MAP) toxicity *via* both release of iron and by oxidative stress. This schematic shows adaptation of dLUHMES, by detoxification of 6HD using GSH, as open arrows. 6HD auto-oxidizes to a quinone, which may be detoxified by conjugation to glutathione.[30] These results do not rule out additional action of GSH to detoxify peroxides or further oxidation products generated by 6HD disruption of the mitochondrial electron transport chain, since supplementation with GSH or NAC only partially mitigated toxicity of 6HD. Modulator BSO inhibits GSH synthesis, and thus blocks detoxification. The solid arrow in the Table of Contents schematic illustrates 6HD liberation of free Fe^{2+} from Complex I of the mitochondrial electron transport chain, enabling Fe^{2+} catalyzation of hydroxyl radical formation which oxidizes lipids and mitochondrial DNA.[28] Certain chelators, DMPS and PHE, partially protected dLUHMES cells from 6HD presumably by tying up free Fe^{2+}.[28]

CONCLUSIONS

The Toxmatrix method was effective in identifying toxicant:modulator interactions for a variety of toxicants. This method assayed 9 or 10 concentration levels of each toxicant to identify a quantitative "tipping point", the toxicant concentration that overwhelmed the cells ability to adapt; then examined each modulator for the ability to shift the IC_{50} left or right. Each interaction yielded a hypothesis indicating crosstalk between a toxicity pathway perturbed by the toxicant, with the pathway addressed by the primary target of the modulator. A variety of toxicants:modulator interactions identified in these studies represent hypotheses that merit further investigation, such as the role of sirtuin activator SRT1720 in promoting toxicity of

MPP+. Given that both activators and inhibitors of sirtuins are proposed as therapeutic treatments for PD[23], further interaction studies may facilitate selection of drugs for clinical study. Several such hypotheses were confirmed for 6HD and MAP toxicity, establishing the liberation of Fe^{2+}, and detoxification by GSH for both toxicants. Modulators such as BSO, DMPS, and necrostatins were particularly useful because they perturbed toxicity pathways impacting a variety of toxicants. These results should enable selection of an optimized set of modulators to improve the efficiency of future Toxmatrix studies by our lab and by others. Thus, this study harnesses and extends qHTS techniques and analytical tools to demonstrate a new method to address mechanistic toxicology. Using this new Toxmatrix method, we demonstrate systematic identification of pathways of toxicity and pathways of cellular adaptation. This is a pathway-based approach which supports the "Toxicology in the 21st century" testing strategy by identifying molecular changes which alter an adverse outcome. By this approach, chemicals that are identified as "actives" from quantitative high-throughput screening for stress pathways, can be further studied to identify toxicity pathways and mechanisms.

ACKNOWLEDGMENT

We are grateful to Craig Thomas, NCATS, for constructive discussion and contribution of thirty-five modulator compounds.

FUNDING SOURCES

This research was supported by the Tox21 Consortium and the NIH Intramural Research Program of NCATS.

ABBREVIATIONS

Toxicants: 6HD, 6-hydroxydopamine; CAP, Captan; CLM, Chlorambucil; CR2, Sodium dichromate; DGX, Digoxin; DTCM, Dithiocyanatomethane; HCP, Hexachlorophene; MALG, Malachite green oxalate; MAP, 4-(Methylamino)phenol hemisulfate salt; MHG, Methyl mercury (II); MPP, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; OTZ, 2-octyl-4-isothiazolin-3-one; TUNC, Tunicamycin; VIN, Vincristine sulfate

Modulators: BSO, buthionine sulphoximine; DMPS, 2,3-dimercapto-1-propanesulfonic acid; GSH, reduced glutathione; L-NAME, NG-Nitroarginine Methyl Ester; L-NMMA, NG-Methyl-L-arginine acetate salt; NAC, N-acetyl-L-cysteine; PHE, o-Phenanthroline; and TPEN, (N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine

SUPPORTING INFORMATION

A full listing of the 70 modulator chemicals used in this work, SMILE identifiers, and the expected target of each, is provided. This material is freely available via the Internet at http://pubs.acs.org.

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Figure Legends

Table 1. Modulators, CAS # identifiers, and expected targets for each. Abbreviations: BSO, buthionine sulphoximine; DMPS, 2,3-dimercapto-1-propanesulfonic acid; GSH, reduced glutathione; L-NAME, NG-Nitroarginine Methyl Ester; L-NMMA, NG-Methyl-L-arginine acetate salt; NAC, N-acetyl-L-cysteine; PHE, o-Phenanthroline; and TPEN, (N,N,N', N'-tetrakis(2-pyridylmethyl)-ethylenediamine; IGF1R, Insulin-like Growth Factor 1 Receptor; IR, Insulin Receptor; NOS, Nitric Oxide Synthase (-1, -2, and -3); A full list of the 70 modulators used in screenings is available in Supplementary Table S1.

Figure 1. Example plots illustrating interactions between toxicant MAP and indicated modulators in SH-SY5Y cells: sensitization to cytotoxicity **(A)**, no interaction **(B)**, or protection **(C)**. Green spots show that the modulators were not toxic at the three concentrations indicated, except the highest concentration of $CoCl_2$ which killed the cells. Black lines indicate toxicant MAP alone concentration-response; Dark blue-, Light blue-, and grey-, lines indicate MAP plus a modulator at low, medium, and high concentrations, respectively.

Figure 2. Heatmap showing interactions between toxicants[12] and modulators (columns) that alter cytotoxicity in SH-SY5Y cells. Maximal fold-change values are shown comparing IC_{50} value with modulator divided by IC_{50} value without modulator. Cell sensitization to a toxicant by a modulator is indicated by a positive IC_{50} shift value if it exceeded 3-fold increase or decrease at the highest modulator concentration that did not cause $\geq$10% cytotoxicity. Red fill indicates sensitization to a toxicant, with increasing color saturation from 2 to 16-fold. Blue fill indicates a negative IC_{50} shift hence protection from a toxicant, with increasing color saturation from -2 to -16-fold. White fill indicates no interaction. 32 toxicants were tested in 10 concentrations, or with DMSO vehicle, and with 3 concentrations of each of 70 modulators. Toxicant/modulator combinations that yielded significant interactions, are colored red for increased sensitivity, blue for protection by modulator, or white for no significant interaction. The maximum fold-change shift in IC_{50} observed is shown in each colored square.

Figure 3. Heatmap illustrating toxicant:modulator interactions in three differentiated neuronal models. Differentiated human cells are labeled "dSH-SY5Y, "dLUHMES", or "dNSC". Among toxicant:modulator pairs tested, 16 did not show IC_{50} shifts exceeding 3-fold for any of the three differentiated cell types, and are omitted from this Figure. Cell colors are as described in the Figure 2 legend except extended to show 2- to 16-fold IC_{50} shifts. Modulator abbreviations and drug targets are listed in Table 1. "NT" indicates the toxicant did not kill the cells at the highest concentration tested, 100 μM; and "TOX" indicates the toxicant killed the cells at the lowest concentration tested, 15 nM

Figure 4. Sensitization of dLUHMES cells to 10 μM 6HD (**A**), or 4 μM MAP (**B**) by glutathione synthesis inhibitor, BSO, is partially relieved by 100 μM GSH or 100 μM NAC. BSO alone (open circles) was moderately cytotoxic to dLUHMES cells; whereas **A**) 10 μM 6HD + BSO (filled squares) were highly toxic. Addition of 100 μM NAC (grey filled circles) or 100 μM GSH (open triangles) to 6HD + BSO or MAP + BSO moderated cytotoxicity.

Figure 5. Protection of dLUHMES cells from 6HD MAP by PHE, but not by M30. PHE alone (open circles) was not toxic to dLUHMES cells; whereas M30 alone (open squares) was moderately cytotoxic. Addition of PHE (filled circles) to 10 μM 6HD (**A**), or 4 μM MAP (**B**) moderated cytotoxicity; whereas addition of M30 (filled squares) increased cytotoxicity.

Modulator	CAS #	Target
BIIB021	848695-25-0	HSP90 inhibitor
BSO	5072-26-4	Glutamate Cysteine Ligase inhibitor
CoCl2	7646-79-9	HIF1a/hypoxia inducer
DMPS	74-61-3	divalent cation chelator
Eliprodil	119431-25-3	NMDA Antagonist
GSK-1904529A	1089283-49-7	IGF-1R/IR inhibitor
KU0063794	938440-64-3	mTOR inhibitor
L-NAME	50903-99-6	NOS inhibitor
NAC	616-91-1	ROS scavenger
necrostatin 1	4311-88-0	RIP1K inhibitor
necrostatin 5	337349-54-9	RIP1K inhibitor
necrostatin 7	351062-08-3	necroptosis inhibitor
Nicotinamide	98-92-0	SIRT1/2/3/6 inhibitor
o-Phenanthroline	3248-05-3	Chelator of divalent cations
Pazopanib	444731-52-6	VEGFR1/2/3 inhibitor
Saquinavir	149845-06-7	HIV protease- and CYP3A4 inhibitor
SP-600125	129-56-6	JNK1, JNK2, JNK3 inhibitor
SRT1720	1001645-58-4	SIRT1 activator
TPEN	16858-02-9	divalent cation chelator
TW-37	877877-35-5	BCL2-binding inhibitor
WYE-354	1062169-56-5	mTOR inhibitor

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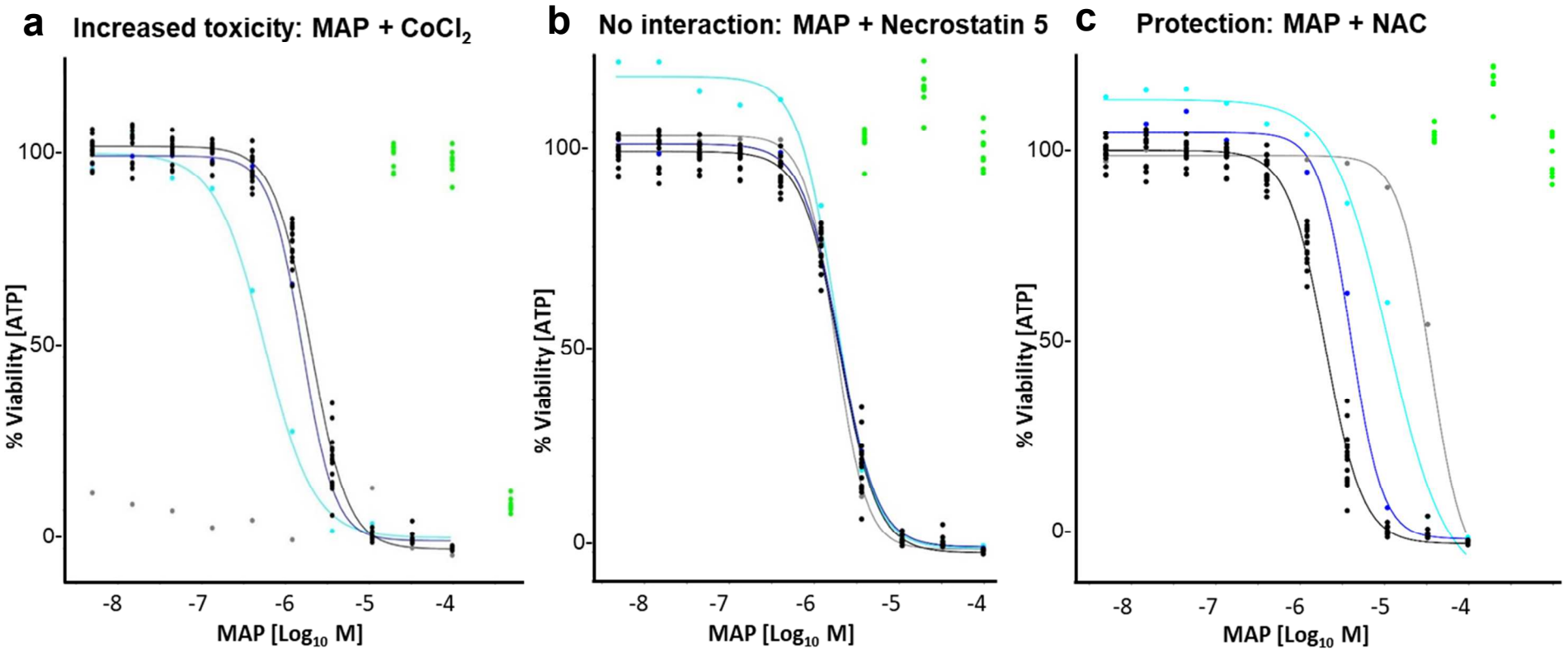

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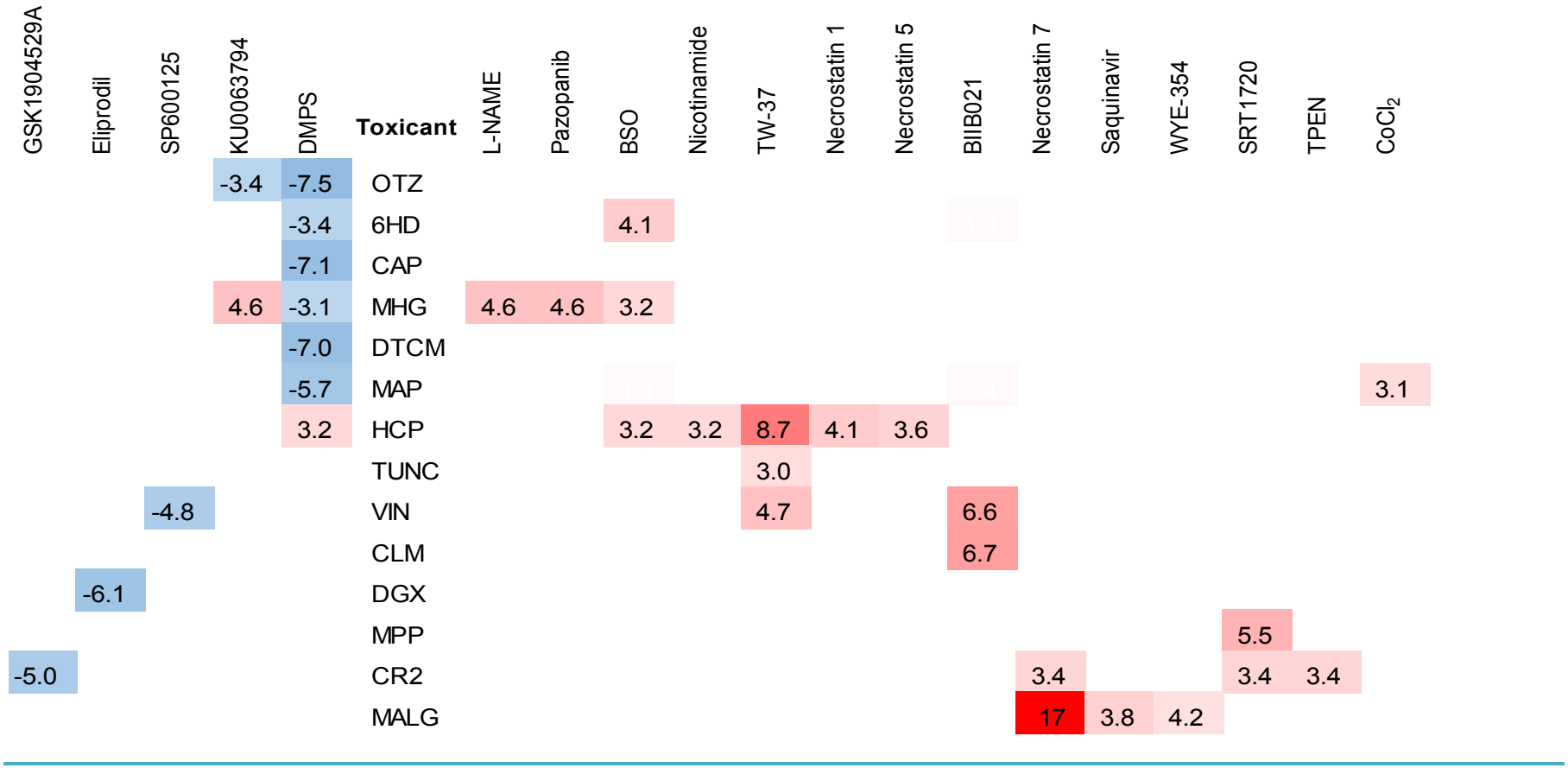

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Toxicant	Modulator	dSH-SY5Y 24h	dSH-SY5Y 48h	dLUHMES 24h	dLUHMES 48h	dNSC 24h	dNSC 48h
MAP	$CoCl_2$			4.5	13		6.3
MAP	DMPS	-3.2	-2.8	-2.5	-2.4	-3.1	
MAP	N-acetyl-cysteine	-3.8	-3.9				
6HD	BSO		4.3	910	3.0	2.6	72
6HD	DMPS	-2.9		-6.1	-3.5	-3.8	
6HD	Necrostatin 7		25	74	2.3		
6HD	WYE-354					4.9	21
CAP	DMPS	-11	-8.7	-8.3	-2.8	-4.2	-3.3
CLM	CNF-2024		3.2	4.5	3.6	2.4	2.5
DTCM	DMPS	-18	-6.4	-10	-8.6	-2.6	
DTCM	N-acetyl-cysteine	-9.7		-9.4	-6.2		-2.3
MPP	SRT1720	4.8		4.2	3.0		3.8
MALG	GSK-1904529A	3.5			TOX		
MALG	Necrostatin 7		3.4		TOX		4.3
MHG	DMPS	-2.8	-2.5	2.6	-9.0	-7.3	
MHG	N-acetyl-cysteine	-3.6	-3.0	-2.2	-2.6	-3.0	-2.3
MHG	Necrostatin 7		10	4.6		2.2	5.9
MHG	Nicotinamide				-4.0		
TUNC	TW-37	10				NT	
VIN	SP-600125			-4.4		-11	TOX

Figure 3. Heatmap illustrating toxicant:modulator interactions in three differentiated neuronal models. Differentiated human cells are labeled "dSH-SY5Y, "dLUHMES", or "dNSC". Among toxicant:modulator pairs tested, 16 did not show IC_{50} shifts exceeding 3-fold for any of the three differentiated cell types, and are omitted from this Figure. Cell colors are as described in the Figure 2 legend except extended to show 2- to 16-fold IC_{50} shifts. Modulator abbreviations and drug targets are listed in Table 1. "NT" indicates the toxicant did not kill the cells at the highest concentration tested, 100 μM; and "TOX" indicates the toxicant killed the cells at the lowest concentration tested, 15 nM.

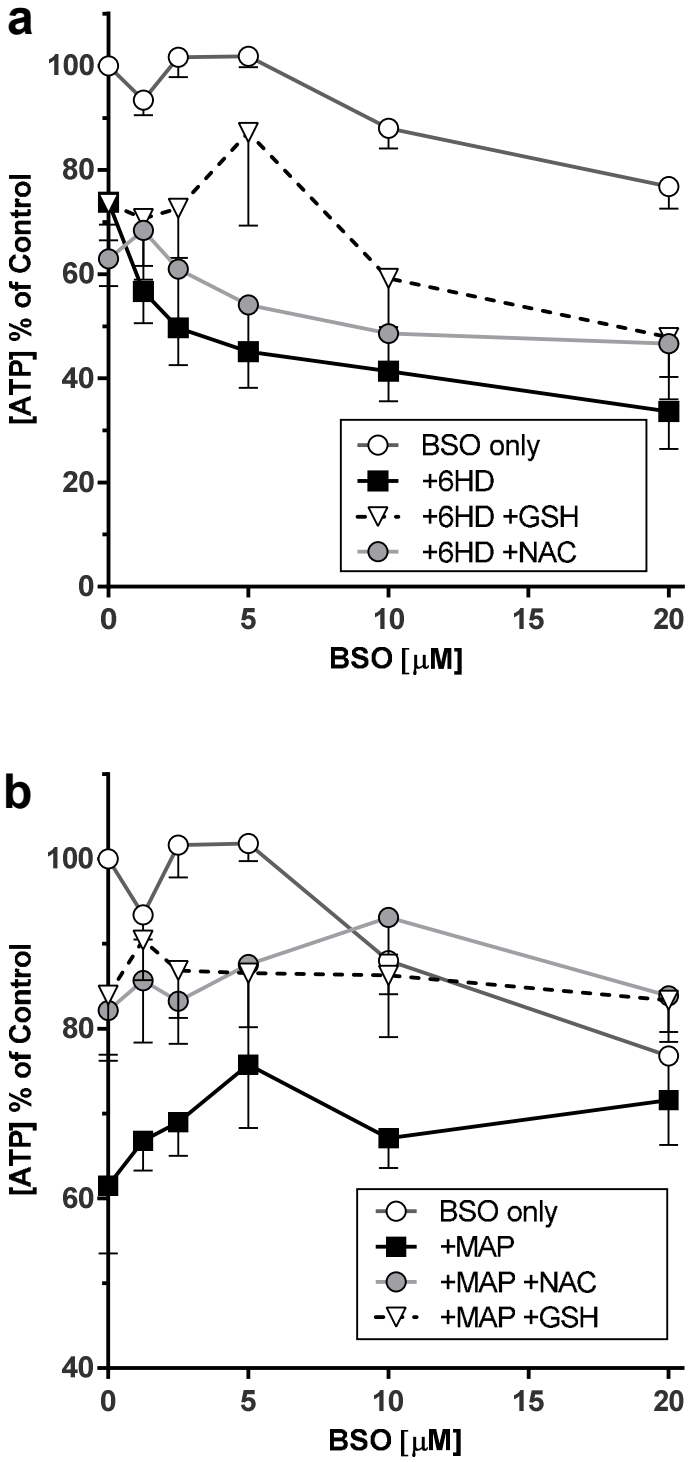

Figure 4. Sensitization of dLUHMES cells to 10 μM 6HD (**a**), or 4 μM MAP (**b**) by glutathione synthesis inhibitor, BSO, is partially relieved by 100 μM GSH or 100 μM NAC. BSO alone (open circles) was moderately cytotoxic to dLUHMES cells; whereas **A**) 10 μM 6HD + BSO (filled squares) were highly toxic. Addition of 100 μM NAC (grey filled circles) or 100 μM GSH (open triangles) to 6HD + BSO or MAP + BSO moderated cytotoxicity.

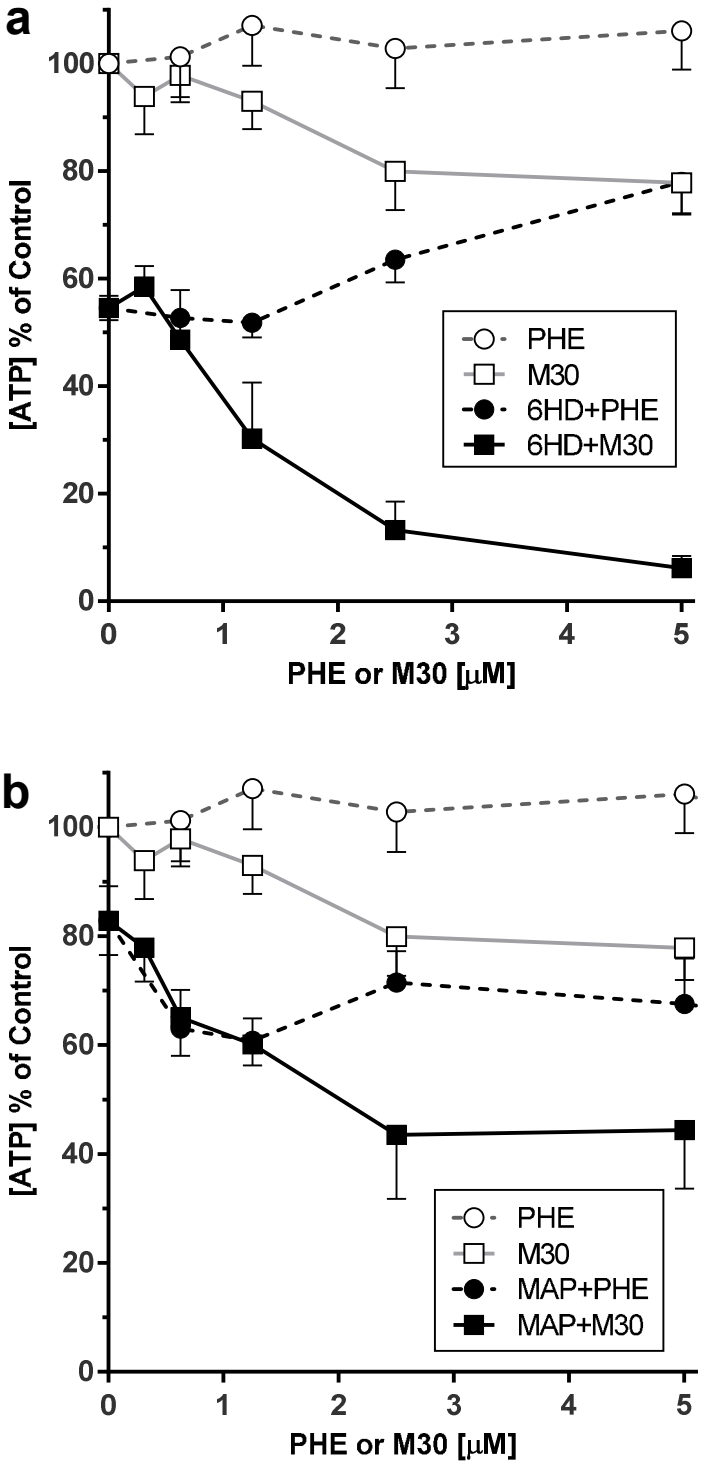

Figure 5. Protection of dLUHMES cells from 6HD MAP by PHE, but not by M30. PHE alone (open circles) was not toxic to dLUHMES cells; whereas M30 alone (open squares) was moderately cytotoxic. Addition of PHE (filled circles) to 10 μM 6HD (**A**), or 4 μM MAP (**B**) moderated cytotoxicity; whereas addition of M30 (filled squares) increased cytotoxicity.