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Profiling of the Tox21 Chemical Collection for Mitochondrial Function to Identify Compounds that Acutely Decrease Mitochondrial Membrane Potential

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Abstract

Background: Mitochondrial dysfunction has been implicated in the pathogenesis of a variety of disorders including cancer, diabetes, and neurodegenerative and cardiovascular diseases. Understanding whether different environmental chemicals and drug-like molecules impact mitochondrial function represents an initial step in predicting exposure-related toxicity and defining a possible role for such compounds in the onset of various diseases.

Objectives: To identify individual chemicals and general structural features associated with changes in mitochondrial membrane potential (MMP).

Methods: We used a multiplexed [two endpoints in one screen; MMP and adenosine triphosphate (ATP) content] quantitative high throughput screening (qHTS) approach combined with informatics tools to screen the Tox21 10,000 compound library (~8300 unique chemicals) at 15 concentrations in triplicate to identify chemicals and structural features that are associated with changes in MMP in HepG2 cells.

Results: Approximately 11% of the compounds (913 unique compounds) decreased MMP after 1 h treatment without affecting cell viability (ATP content). Additionally, 309 compounds decreased MMP over a concentration range that also produced measurable cytotoxicity [half maximal inhibitory concentration (IC_{50}) in MMP assay/ IC_{50} in viability assay] ≤ 3 , $p < 0.05$]. Over 11% of the structural clusters that constitute the Tox21 library (76 of 651 clusters) were significantly enriched for compounds that decreased the MMP.

Conclusions: Our multiplexed qHTS approach allowed us to generate a robust and reliable dataset to evaluate the ability of thousands of drugs and environmental compounds to decrease MMP. The use of structure-based clustering analysis allowed us to identify molecular features that are likely responsible for the observed activity.

Introduction

Mitochondria are intracellular organelles found in most eukaryotic cells (Bereiter-Hahn and Voth 1994). In general, these organelles have an outer membrane and an inner membrane characterized by protrusions into the interior matrix. The shape and number of mitochondria vary extensively among different cell types (Bereiter-Hahn and Voth 1994; Sjostrand 1953). Mitochondria play a central role in maintaining cellular homeostasis, being involved in a variety of critical cellular processes from macromolecular synthesis to the regulation of programmed cell death (Dykens et al. 1999). Most of the cell energy is generated as adenosine triphosphate (ATP) within the mitochondria through two complementary processes: the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (Barnes and Weitzman 1986; Dykens et al. 1999; Mitchell 1961). Other metabolic processes, including parts of the urea cycle and porphyrin and steroid synthesis and maturation, also occur in the mitochondrial matrix (Gamble and Lehninger 1973; Lambeth et al. 1982; Lill et al. 1999). Mitochondria play a critical role in the maintenance of calcium homeostasis in the cytoplasm. The ability of mitochondria to take up and release Ca^{+2} , and to regulate the generation of other intracellular messengers such as reactive oxygen species (ROS), makes this organelle an essential part of cell signaling (McBride et al. 2006). Mitochondria are also involved in controlling cell fate by regulating cell cycle progression and the initiation of apoptosis (Lemasters et al. 2002; Pacher and Hajnoczky 2001).

Mitochondrial dysfunction (due to either genetic or environmental factors) has been related to many disorders including cancer and diabetes, and neurodegenerative and cardiovascular diseases (Lesnefsky et al. 2001; Pieczenik and Neustadt 2007). A bioenergetic imbalance, an increase of mitochondrial-related oxidative stress, or a deregulation of the intrinsic apoptotic

pathway seems to play a role in the onset of many of these diseases at a molecular level (Pieczenik and Neustadt 2007). A similar role for mitochondrial-mediated oxidative stress has been proposed in a model for the aging process (Lenaz 1998). Moreover, many clinically approved drugs have been removed from the market due to cardiovascular and liver toxicities associated with impaired mitochondrial function (Nadanaciva and Will 2011). Understanding how different chemicals can affect mitochondrial activity constitutes the first step in predicting possible exposure-related toxic effects from such compounds. Following the generation of mitochondrial toxicity profiles, the next challenge is defining a potential role for these chemicals in the onset of various syndromes or diseases linked to mitochondrial dysfunction (Schmidt 2010).

The Tox21 collaboration, which includes the National Toxicology Program (NTP), the U.S. Environmental Protection Agency (EPA) National Center for Computational Toxicology (NCCT), the NIH Chemical Genomics Center (NCGC, now part of the National Center for Advancing Translational Sciences), and the U.S. Food and Drug Administration (FDA), has a set of goals that includes identifying mechanisms of chemically induced biological activity, prioritizing untested chemicals for more extensive toxicological evaluation, and developing predictive models of *in vivo* biological responses (Kavlock et al. 2009; Tice et al. 2013). The ultimate objective of the Tox21 program is to incorporate advances in molecular systems and computational biology into toxicity evaluations to study the ever increasing number of chemicals present in the environment and to accelerate the transition of toxicology into a predictive science focused on mechanism-based, biological observations (NTP 2004). To rapidly and efficiently evaluate thousands of compounds across many biologically relevant targets, we implemented an

automated robotic system in the Tox21 compound screening process (Attene-Ramos et al. 2013b). During Tox21 Phase I (proof of principle), many biochemical and cell-based assays were successfully developed and miniaturized in a 1536-well plate format for screening against the initial Tox21 collection of approximately 3,000 compounds in a quantitative high-throughput screening (qHTS) platform (Shukla et al. 2010; Tice et al. 2013). In Phase II (production phase), the Tox21 compound library has been expanded to over ten thousand samples including ~8300 unique chemical entities selected by each of the Tox21 partners based on public health and toxicological relevance (Attene-Ramos et al. 2013b).

To identify environmental chemicals and drugs that might affect mitochondria dysfunction, we used a qHTS approach to evaluate the compounds from the Tox21 10K compound library that potentially reduce the mitochondrial membrane potential (MMP) in human liver carcinoma (HepG2) cells. Compounds affecting mitochondrial function and structural integrity by different mechanism would likely change the MMP, making this measure a suitable comprehensive first endpoint for screening. Furthermore, the MMP screen was multiplexed with a viability assay to identify compounds that induced concurrent cytotoxicity, a potential confounder based on the assay format. Using this approach, we identified a group of known and novel compounds with the ability to reduce MMP, and we identified several structural features associated with the observed biological activity.

Materials and Methods

Reagents and Tox21 10K compound library

Human HepG2 (hepatocellular carcinoma) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The Mitochondrial Membrane Potential

indicator (m-MPI) was from Codex Biosolutions (Montgomery Village, MD, USA). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was from Sigma-Aldrich (St. Louis, MO, USA). Intracellular ATP content, an indicator of cell viability, was measured using the CellTiter-Glo® luminescence cell viability assay (Promega, Madison, WI, USA). Detailed compound library information is described in the supplemental material section (see Supplemental Material, compound library).

Quantitative high throughput screening (qHTS) using a multiplex mitochondrial membrane potential and cell viability assay

The multiplex assay consisted of two endpoints, MMP and total intracellular ATP, measured in HepG2 cells in the same assay well after a 1 hr treatment. The assay was optimized using m-MPI (Sakamuru et al. 2012) to measure changes in MMP and the CellTiter-Glo® reagent to measure intracellular ATP levels (Crouch et al. 1993). The assay protocol is described in supplemental materials and summarized in Table S1. The assay plate map is shown in Figure S1.

qHTS data analysis

Analysis of compound concentration–response data was performed as previously described (Inglese et al. 2006; Huang et al. 2011) and the details can be found in the supplemental materials. Throughout the manuscript, we used the following nomenclature: antagonist compounds are defined as those that depolarized the mitochondria (decreased the MMP; showed inhibitory curves) and agonist compounds are those that hyperpolarized the mitochondria (increased the MMP; showed activation curves). The schematic workflow of the activity assignment is shown in Figure S2.

Activity assignments based on triplicate runs

As described in Figure S2, each curve class was first converted to a curve rank as previously described (Huang et al. 2011), such that more potent and efficacious compounds with higher quality curves were assigned a higher rank. Curve ranks should be viewed as a numerical measure of compound activity. Curve ranks from replicate runs of a compound were then averaged. The activity outcome of each compound for each of four readouts (ratio, rhodamine, FITC, and cell viability) was assigned based on its average curve rank and reproducibility call as shown in Table S2. The final MMP activity outcome of each compound was determined based on its multi-channel readout activity as shown in Table S3.

Evaluation of curve class reproducibility

For each sample, all of the replicate curves were compared pairwise for consistency. For each pair of curves, if one was inactive and the other was active, or if one was agonist and the other one was antagonist, an inconsistency count was added to the class for each of the two curves. This process was repeated for all the samples. The inconsistency counts were tallied for each curve class and divided by the total number of comparisons made to yield the curve reproducibility value.

Chemical structure cluster analysis

For the purpose of structure clustering, MMP assay results were aggregated to the unique DSSTox substance (GSID, CASRN) level. Approximately 200 unique compounds without structures were excluded from this analysis. Compounds in the structured portion of the unique DSSTox compound library (Version 3a) were clustered based on structural similarity (512-bit

ChemAxon® fingerprints Version 6.2.1) using the Self-Organizing Map (SOM) algorithm (Kohonen 2006) yielding 651 clusters. Each cluster was evaluated for its enrichment of active antagonists (compared to the library average) using Fisher's exact test.

Results

qHTS performance and replicate reproducibility

As shown in Figure 1 (red curves) and Figure S3A, the positive control FCCP decreased MMP in a concentration dependent manner in all 459 plates with an average IC_{50} of $0.11 \pm 0.07 \mu\text{M}$ [mean \pm standard deviation (SD)]. For the entire screen, the signal to background (S/B) ratio for the MMP assay was 5.8 ± 2.9 and Z' factor averaged 0.64 ± 0.15 . The S/B ratio for the cell viability assay was 67.2 ± 16.8 and Z' factor was 0.80 ± 0.10 .

As an additional measure of the assay's technical performance, the reproducibility was evaluated for both the MMP and viability readouts against the 88 compounds present in duplicate in each assay plate (Tox21-88) and against the three copies of the Tox21 10K library with compounds plated in different well locations in each copy (Attene-Ramos et al. 2013b). The reproducibility measures of the Tox21-88 as well as the triplicate assay runs in terms of active match, inactive match, inconclusive, and mismatch rates (see the details in the Supplemental Material, reproducibility call) and potency differences are listed in Table 1. For both assays the reproducibility was high, with mismatch rates less than one percent of the library and AC_{50} (concentration at half-maximal activity) fold differences around 1.5 (Table 1).

Evaluation of curve class reproducibility

The replicate data provided a good opportunity to evaluate the robustness of the process for selecting actives based on curve class. The reproducibility of each curve class, calculated using the ratio readout, is shown in Figure 2A. The expectation is that high quality curves (i.e., class 1.1, 1.2, 2.1, 2.2) are more reproducible and such compounds are less likely to be classified as inactive in repeated tests. Figure 2A shows that this expectation is true for the antagonist curves (negative curve classes), as class -1.1 curves showed the highest reproducibility (98%), followed by the class -2.1 curves (95% reproducibility). The other two high quality curve classes, class -1.2 and -2.2, were also more reproducible than the low quality curves, class -1.3, -1.4, -2.4 and -3, with the exception of class -2.3 curves, which appeared to be more reproducible than the class -1.2 and -2.2 curves. This may be due to the small sample size (only 50 class -2.3 curves) while the average number of other curves was >1000 per curve class. A similar pattern was found for the agonist curves (positive curve classes); however, the agonist curve classes showed overall lower reproducibility than their antagonist counterparts. For example, the class 1.1 and 2.1 curves were still the most reproducible (75% reproducibility) among the agonist curves, but were much less reproducible than the corresponding antagonist curves (-1.1 and -2.1), both of which had >95% reproducibility. The decreased reproducibility seen for the agonist curves might be the direct consequence of assay design. In this assay, the amount of m-MPI dye that enters the mitochondrial matrix and forms red fluorescence aggregates is proportional to the MMP. We used the uncoupler FCCP to optimize the assay signal window to detect a decrease in red fluorescence (i.e., a decrease in the MMP). An increase in red fluorescence can be detected but the signal window is much smaller. The class 4 (inactive) curves were found to be fairly reproducible as well with 80% reproducibility.

Identification of environmental compounds and drug-like molecules that decrease the MMP

Decisions on compound activity were made taking into account triplicate runs of not only the ratio (red/green) readout in the MMP assay but also each fluorescence channel individually and the cytotoxicity results (see Materials and Methods, and Tables S1 and S2). Of the 8312 unique compounds evaluated, 913 (11%) decreased the MMP at 1 hr without apparent cytotoxicity (Figure 2B). Sixty-five percent of the tested compounds (5366) did not alter the MMP, and 21% (1778 compounds) produced responses that were considered inconclusive for a variety of reasons, including 309 of the compounds that were cytotoxic [IC_{50} in MMP assay/ IC_{50} in viability assay ≤ 3 , $p < 0.05$]. Conversely, 254 compounds (3%) appeared to increase the MMP. These compounds will be evaluated in a separate manuscript. The 913 compounds that decreased the MMP without affecting cell viability had IC_{50} values ranging from 9.6 nM to 72 μ M with 79 compounds having an IC_{50} lower than 1 μ M. The protein kinase C (PKC) modulator Bryostatin 1 was the most potent compound with an IC_{50} of 9.6 nM. Other potent compounds included carbocyanine with an IC_{50} of 14.1 nM, basic blue 7 with an IC_{50} of 18.5 nM, and triethyltin bromide with an IC_{50} of 21.7 nM. The 20 most potent compounds are listed in Table S4.

SAR analysis of compounds that decreased the MMP

The ~8300 unique compounds within the Tox21 10K compound library were clustered based on their structural similarity using the self-organizing maps (SOM) algorithm (Kohonen 2006), yielding 638 structural clusters and 13 singletons. Of these clusters, 76 (11.7%) were significantly enriched with compounds that decreased the MMP ($p < 0.05$), with 31 clusters having a p-value lower than 0.001 (Figure 3, Table S5). The most significantly enriched cluster

(Figure 3, Row 22, column19; $p=2.04 \times 10^{-16}$, Table S5) contained 20 of 25 active compounds. Active compounds in this cluster had either an anthraquinone or a benzophenone core with one or more hydroxyl groups. As shown in Figure 4A, 3-hydroxybenzophenone ($IC_{50} = 27.7 \mu\text{M}$) was slightly more potent than the 4-hydroxybenzophenone ($IC_{50} = 41.3 \mu\text{M}$). The 2-hydroxybenzophenone analogue was inactive in the MMP assay. Two other hydroxybenzophenones present in the cluster, 2-hydroxy-4-methoxybenzophenone and mexenone, were not active. Both of these inactive compounds have only one hydroxyl group (in position 2) and one methoxy group (in position 4).

Some structural motifs emerged repeatedly in the clusters enriched for MMP actives. Many of these clusters contained compounds with a phenol moiety, a few had a nitrobenzene core, and three shared a thiazole substructure (Figure 3 red boxes). The majority of the chlorinated organic insecticides present in the library including toxaphene and aldrin were active as antagonists in the MMP assay. However, chlorendic acid and its anhydride were inactive. Other classes of MMP-active compounds derived from this cluster analysis included parabens, flavonoids, triarylmethane dyes, thiazolidinediones, and ionic liquids, among others (Figure 3 and Table S5). Interestingly, the potencies of parabens seem to correlate with the length of the aliphatic side chain, such as pentylparaben ($IC_{50} = 8.7 \mu\text{M}$) and propylparaben ($IC_{50} = 38.6 \mu\text{M}$). Methylparaben, which has a shorter side chain, was inactive in the MMP assay (Figure 4B).

Discussion

The strategy reported here is a comprehensive utilization of a multiplexed qHTS approach combined with SAR analysis as a first step in the identification of environmental chemicals/drugs and structural features that decrease MMP. Specifically, we measured MMP

changes using a fluorescent dye (Sakamuru et al. 2012) and changes in intracellular ATP content as a proxy for cytotoxicity using a bioluminescence assay (Crouch et al. 1993) in HepG2 cells in triplicate to profile the Tox21 10K compound library. The results of the triplicate runs for each channel [red and green fluorescence, ratio (red/green), cytotoxicity and compound auto-fluorescence] were used to make compound activity calls (see Materials and Methods, and Tables S1 and S2). Having at least 3 dose-response curves for each compound allowed us to evaluate the technical performance of the assay, thereby increasing our confidence in the compound activity calls. Only 0.55 % of the curves had mismatched activities for the MMP assay and even fewer mismatches (0.03%) were seen with the viability assay. The AC_{50} fold change between replicates was generally 1.5 or less, providing further evidence for high reproducibility in responses. The triplicate data also provided us with the opportunity to evaluate the rationale for the activity assignments based on curve class. High quality curve classes were more reproducible than low quality curve classes. This observation provides quantitative evidence supporting the categorization of compounds with high quality curve classes as actives.

Over 11% (913) of the unique compounds in the Tox21 10K library decreased the MMP at 1 hr without apparent cytotoxicity (Figure 2B). An additional 309 compounds significantly decreased both MMP and cell viability (ATP content) over the same concentration range (cytotoxicity is considered excessive when the $MMP\ IC_{50}/viability\ IC_{50} \leq 3$, $p < 0.05$). These compounds were classified as inconclusive antagonists (Figure 2B). The CellTiter Glo® assay is widely used to assess cell viability by measuring intracellular ATP content, and is especially well suited, by virtue of its homogeneous assay format, and luminescence signal output, to multiplexing with assays generating fluorescence readouts. Assessing cell viability could be useful for

differentiating compounds that decreased MMP from those that caused cytotoxicity (and thus a decrease in the fluorescent signal associated with MMP) and, in general, cytotoxic compounds were excluded from further data analysis. However, when assessing mitochondria function, caution must be exercised when interpreting the data from this cell viability assay. Compounds that perturb normal mitochondria function could cause a decrease in intracellular ATP content. Our data analysis mainly focused on the active antagonist compounds but the “inconclusive antagonist (cytotoxic)” category of compounds should be further investigated. In fact, Rotenone (CASRN 83-79-4) and papaverine (CASRN 61-25-6), two known inhibitors of mitochondrial respiration and the uncoupler dinitrophenol (CASRN 51-28-5) and a few of its derivatives, fall into this category. The exact duration of compound treatment prior to application of the MMP detection dye will likely have an effect on the above activity assignments. Based on our previous studies (Attene-Ramos et al. 2013a; Sakamuru et al. 2012), we chose a one-hour treatment to maximize the detection of compounds that affect mitochondria without triggering cytotoxicity.

Of the 913 active compounds, 79 had an IC_{50} less than 1 μ M for the MMP assay. The most potent compound for the MMP assay was the PKC modulator Bryostatin 1 with an IC_{50} of 9.6 nM. Bryostatin 1 has been reported to induce cell cytotoxicity and/or apoptosis through several mechanisms including PKC and Bcl-2 modulation, and alterations in the mitochondria lipid composition that lead to a decrease in the MMP (Cartee et al. 2000; Liu et al. 1999; Wang et al. 2003). There are several triarylmethane dyes present among the most potent compounds for this assay including basic blue 7, methyl violet, and basic red 9. These dyes have been shown to preferentially accumulate in the mitochondria and act as protonophores and/or inhibitors of the electron chain transport resulting in the initiation of apoptosis through the opening of the

mitochondrial permeability transition pore (Attene-Ramos et al. 2013a; Kowaltowski et al. 1999). Many organotin compounds including triethyltin bromide, tributyltin methacrylate, bis(tributyltin)oxide, tributyltin chloride, triphenyltin acetate, and triphenyltin hydroxide are also among the most potent compounds identified in this study. Organotins are able to accumulate in biological membranes and interact with many different proteins and lipids (Pagliarani et al. 2013; Saxena 1987). These chemicals are known to inhibit oxidative phosphorylation and induce uncoupling of mitochondrial energy transduction (Aldridge and Street 1964; Sone and Hagihara 1964), causing mitochondrial swelling and opening of the permeability transition pore (Bragadin and Marton 1997; Nishikimi et al. 2001). In the MMP assay, we observed that triorganotins were in general more potent than tetraorganotins or diorganotins, and aryl substituted organotins were less potent than alkyl substituted ones. This was consistent with previous observations about organotin toxicity (Saxena 1987).

Of the 651 structural clusters that comprise the Tox21 10K compound library, 76 were significantly enriched with compounds that decreased the MMP ($p < 0.05$). Some structural motifs emerged repeatedly within the enriched clusters (Figure 3), and these may constitute mitochondrial toxicophores. The most common motif was a substituted phenol that appeared in many clusters. These compounds are known to act as protonophores, facilitating the translocation of protons back into the mitochondrial matrix. The potency and efficacy observed among all the compounds depend, most likely, on the nature of the substituent groups and how they affect the hydroxyl proton acidity and the lipophilicity of the entire molecule (Spycher et al. 2008). Interestingly, when our results were cross-referenced with the EPA's Chemical Data Reporting database (CDR <http://epa.gov/cdr/>, accessed 22 September, 2014), the three compounds in the

CDR database with the highest use that are also active in the MMP assay are all substituted phenols (4-aminophenol, CASRN 123-30-8; 4-(1-methyl-1-phenylethyl)phenol, CASRN 599-64-4; and 2,3,4,5,6-pentachlorophenol, CASRN 87-86-5). Other common structural motifs within active clusters identified by this structural analysis were nitrobenzenes (including nitrophenols) and thiazoles. Nitrophenols are known protonophores and have been used in structural alert models to define uncouplers (Naven et al. 2013).

The most significantly enriched cluster (Figure 3 row 22, column 19, $p= 2.04 \times 10^{-16}$) contained 20 active compounds (out of a total of 25 compounds) that either had an anthraquinone or a benzophenone core with one or more hydroxyl groups. Hydroxyanthraquinones have been reported to be uncouplers of cellular respiration and their potencies depend on the number and location of the hydroxyl groups (Betina and Kuzela 1987). The effects of benzophenone derivatives on mitochondrial function have not been extensively studied. Our data suggest that the presence of at least one hydroxyl group is necessary (but not sufficient) for the molecule to decrease the MMP. Benzophenone, 4-methylbenzophenone, and 4,4'-dichlorobenzophenone were inactive in the MMP assay. On the other hand, most of the hydroxylbenzophenones were active (or inconclusive due to cytotoxicity). In general, compounds with two hydroxyl groups in each ring were more potent than compounds with one or three hydroxyl groups per aromatic ring. The location of the hydroxyl group seems to play an important role in compound potency. Of the monohydroxylated benzophenones, 3-hydroxybenzophenone was more potent than 4-hydroxybenzophenone, and 2-hydroxybenzophenone was inactive in the MMP assay (Figure 4A). This is not true for the dihydroxylated benzophenones, however, where a compound with a

hydroxyl in the ortho position (2,2'-dihydroxybenzophenone, IC_{50} = 14.1 μ M) was active and a compound with a hydroxyl in the para position (4,4'-dihydroxybenzophenone) was inconclusive.

The cluster analysis also revealed many categories of compounds capable of decreasing the MMP, including cardiac glycosides, flavonoids, ionic liquids, chlorinated organic insecticides (e.g., toxaphene), parabens, quinolone-based dyes, thiazolidinedione-based drugs (e.g., rosiglitazone), anthracycline-derived antineoplastic drugs (e.g., pirarubicin) and triarylmethane dyes, among others (Figure 3). Some of these structural groups have been previously reported to interact with mitochondria and affect mitochondrial functions. Cardiac glycosides like ouabain and proscillaridin were among the most potent compounds in the screen. These compounds affect mitochondrial energy production, in part, by decreasing mitochondrial calcium concentration (Liu et al. 2010). Flavonoids have been previously reported to decrease MMP and induce ROS-mediated apoptosis in a variety of cell types, but the specific mechanism is still unknown (Attene-Ramos et al. 2013a; Wang et al. 1999). Triarylmethane dyes accumulate in the mitochondria, and inhibit cellular respiration leading to mitochondrial swelling and apoptosis (Kowaltowski et al. 1999). Rosiglitazone and troglitazone are anti-diabetic thiazolidinedione drugs that exert their therapeutic effects by activating peroxisome proliferator-activated receptors (PPARs). These drugs have been linked to liver toxicity, in part, by disrupting MMP, which in turn leads to opening of the mitochondrial permeability transition pore and eventually to cell death (Tirmenstein et al. 2002). Parabens are esters of para-hydroxybenzoic acid used as preservatives in many industries. They have been linked to mitochondrial mediated cytotoxic effects (Nakagawa and Moldeus 1998). The cytotoxic effect is related to the length of the aliphatic side chain that increases the hydrophobicity of the compound. A similar effect is also

observed in the MMP assay, where compounds with longer side chains, such as dodecylparaben ($IC_{50}= 4.2 \mu M$) and pentylparaben ($IC_{50}=8.7 \mu M$), are more potent than those with shorter side chains, like propylparaben ($IC_{50} = 38.6 \mu M$) and methylparaben (inactive) (Figure 4B). Finally, four of five anthracycline-based chemotherapy drugs decreased the MMP. Pirarubicin ($IC_{50} = 0.6 \mu M$) and valrubicin ($IC_{50} = 1.9 \mu M$) were the most potent compounds in this group. Aclarubicin hydrochloride, plicamycin, and the structurally related chromomycin A3 were also active while adriamycin hydrochloride was inactive. Anthracyclines are considered to cause cardiotoxicity at least in part, by affecting mitochondrial function and generating oxidative stress, although the molecular mechanisms is not well understood (Mordente et al. 2012)

Conclusions

A fundamental objective of the Tox21 collaboration is the generation of a robust, reliable dataset for use in prioritizing chemicals for more in-depth but lower throughput toxicological studies and for the generation of reliable computational models. Using a multiplexed qHTS-based assay strategy, we profiled over 8300 environmental and drug-like compounds for their ability to change MMP in HepG2 cells. Monitoring changes in MMP is a suitable first step to screen for mitochondrial dysfunction since compounds that directly or indirectly disrupt a diverse set of mitochondrial and cellular events can potentially affect MMP. The combination of testing of each compound as three positional-varied 15-point dilution series and the inclusion of 88 duplicate controls within each plate resulted in a robust screening platform, which in turn allowed us to identify more than 800 compounds that decreased the MMP in HepG2 cells. Furthermore, we defined several chemical structural motifs that were frequently associated with a decrease in MMP that could represent putative toxicophores for mitochondrial toxicity. The

information generated here may be used to construct computational models to predict new chemical structures capable of disrupting MMP and also to generate a tiered approach for selecting compounds for more costly, lower throughput mechanistic studies (e.g., changes in oxygen consumption and inhibition of protein complexes in the respiratory chain). The interpretation of the information obtained using these additional approaches is expected to lead to the prioritization of compounds for *in vivo* testing, providing enhanced understanding of the toxicokinetic and toxicodynamic properties of the compounds including organ specific toxicity.

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Table 1. Reproducibility for the MMP and cell viability assays.

Assay reproducibility	Active match %	Inactive match %	Inconclusive %	Mismatch %	AC₅₀ fold change
MMP (Tox21-88)	41.88	36.65	19.09	2.39	1.46
Cell viability (Tox21-88)	8.81	80.63	10.23	0.34	1.41
MMP (10K triplicate run)	17.57	67.52	14.33	0.55	1.53
Cell viability (10K triplicate run)	4.78	90.80	4.39	0.03	1.42

For each assay the reproducibility was calculated for the Tox21-88 compounds (duplicates in each plate) and for the 10K library (three copies) with compounds plated in different well locations.

Figure legends

Figure 1. qHTS concentration response data binned into curve classes 1-4. Concentration response [ratio (590 nm/535 nm)] curves for the FCCP control titrations and over 10,000 substances tested, including all the replicates (red, FCCP positive control; blue, curve classes 1.1, 1.2, or 2.1; green, curve classes 1.3, 1.4, 2.2, 2.3, 2.4, and 3; gray, curve class 4)

Figure 2. Screening statistics. (A) Stability analysis. The reproducibility of each curve class, calculated using the ratio readout. High quality curves (i.e., class 1.1, 1.2, 2.1, 2.2) are more reproducible than other curve types for antagonist activity (inhibitory curve classes). A similar pattern with lower reproducibility was observed for the agonist curves. Class 4 inactive curves were fairly reproducible. (B) Distribution of the results (% of the total library) based on the combined call using the MMP assay (ratio and independent channels), cell viability, and compound auto-fluorescence assays.

Figure 3. Structure activity relationship (SAR) of compounds that decrease MMP. All compounds with associated structures present in the library were clustered, based on structural similarity using the Self-Organizing Map (SOM) algorithm (Kohonen 2006). Then, each cluster was evaluated for enrichment for active antagonists (compared to the library average) using Fisher's exact test. Enriched clusters are shown in red and deficient clusters, blue (scale values represent the log p value for each cluster). Representative scaffolds are shown for some of the more enriched clusters. The red boxes define regions of molecules that share a common substructure.

Figure 4. (A) Changes in the ability of hydroxybenzophenone isomers to decrease MMP. 3-Hydroxybenzophenone was the more potent isomer while 2-hydroxybenzophenone was inactive in this assay. (B) Decreases in MMP for different paraben molecules. Activity increased with increasing length of the side chain (and subsequent increase in hydrophobicity).

Figure 1.

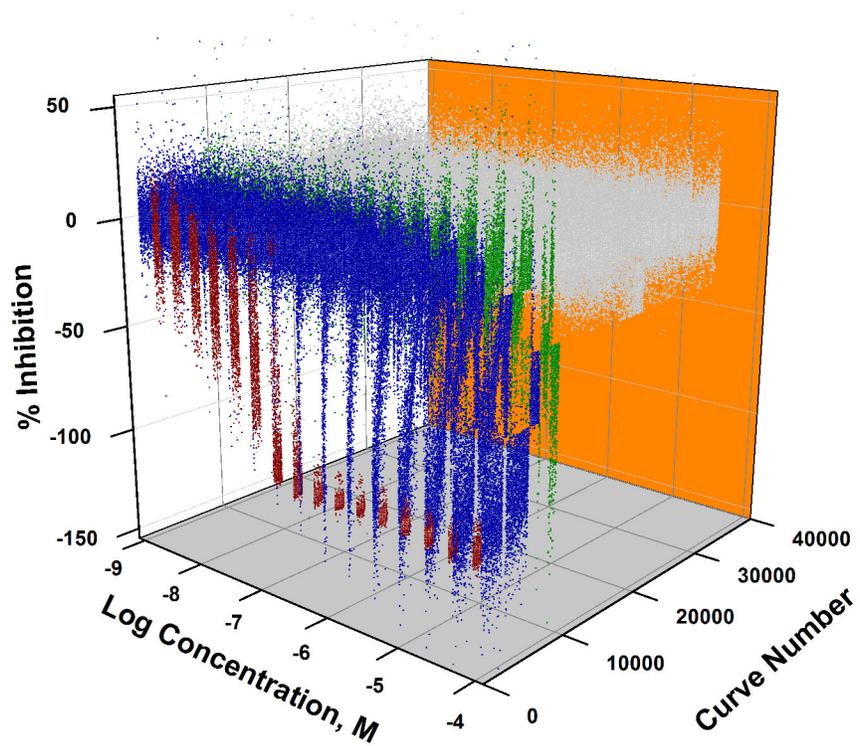


Figure 2A.

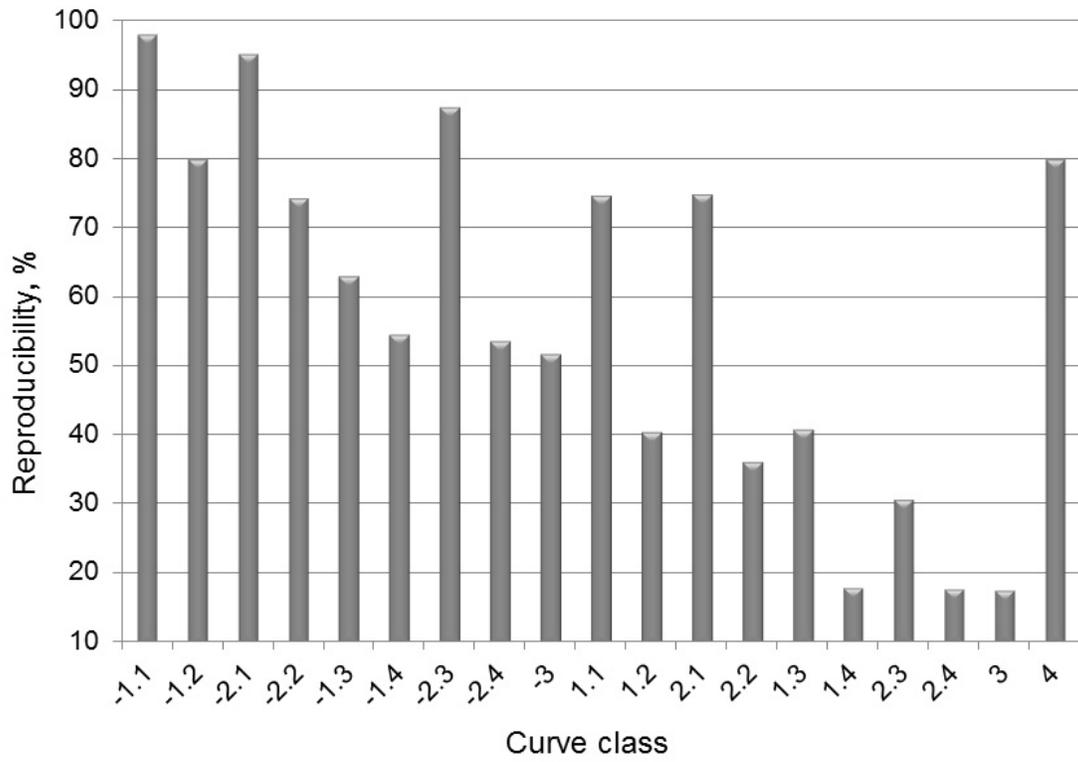


Figure 2B.

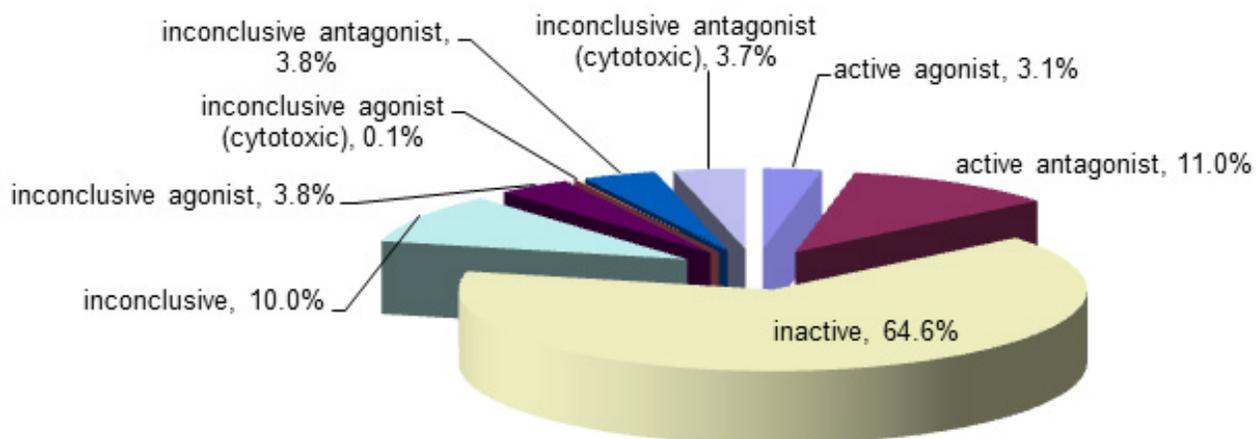


Figure 3.

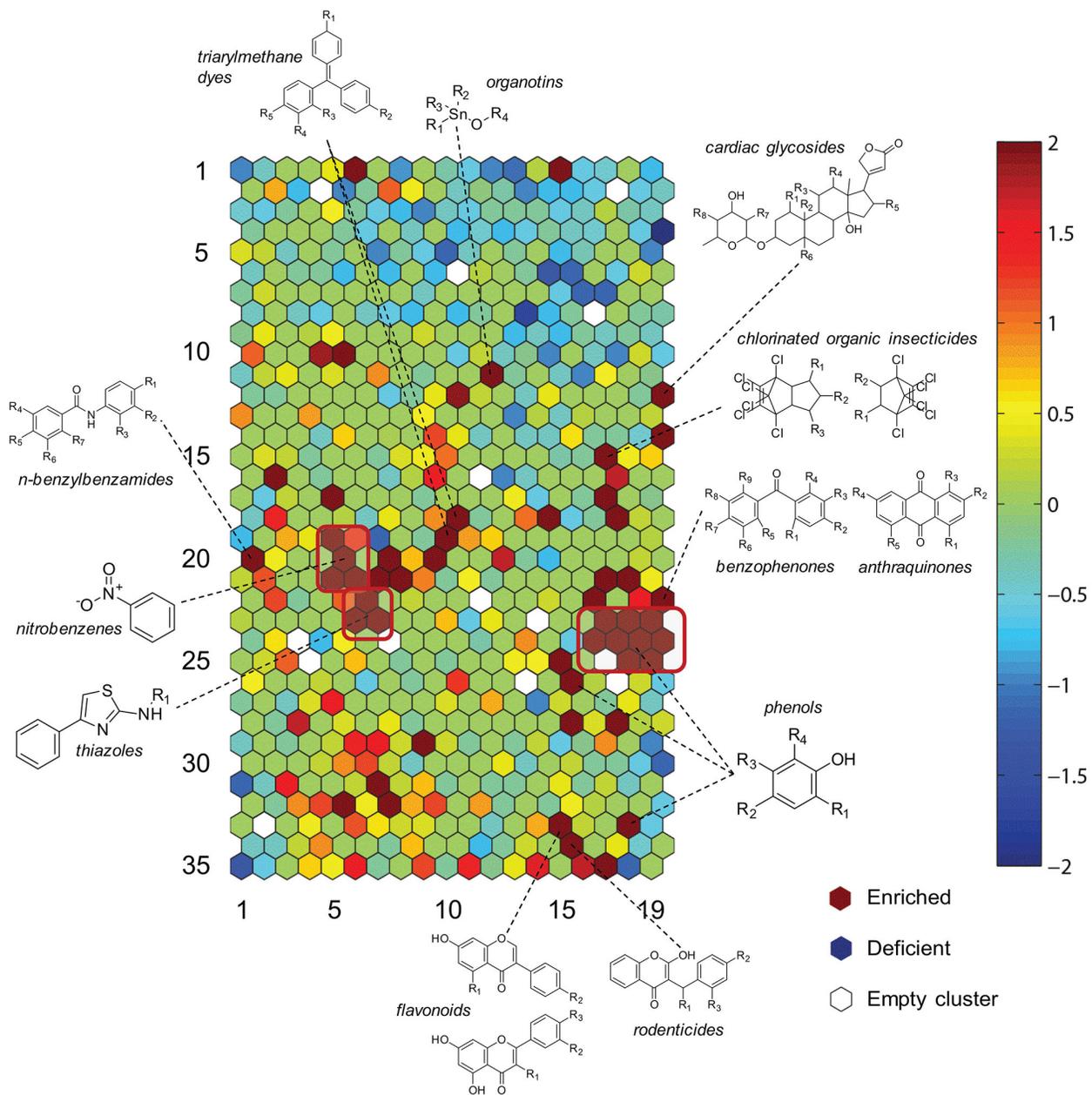


Figure 4A.

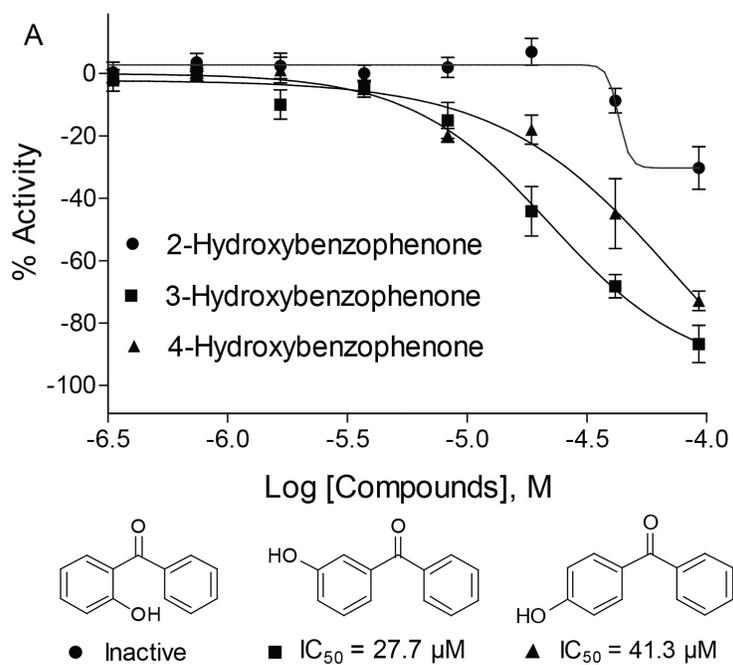


Figure 4B.

