Integrated Compound Profiling Screens Identify the Mitochondrial Electron Transport Chain as the Molecular Target of the Natural Products Manassantin, Sesquicillin, and Arctigenin

Kevin Lai,[†,§] Douglas W. Selinger,[†] Jonathan M. Solomon,[†] Hua Wu,[†] Esther Schmitt,[‡] Fabrizio C. Serluca,[†] Daniel Curtis,*[,†] and John D. Benson[†,‖]

[†]Novartis Institutes for BioMedical Research, Cambridge, Massachusetts 02139, United States
[‡]Novartis Institutes for BioMedical Research, Basel, Switzerland

Ⓢ *Supporting Information*

ABSTRACT: Phenotypic compound screens can be used to identify novel targets in signaling pathways and disease processes, but the usefulness of these screens depends on the ability to quickly determine the target and mechanism of action of the molecules identified as hits. One fast route to discovering the mechanism of action of a compound is to profile its properties and to match this profile with those of compounds of known mechanism of action. In this work, the Novartis collection of over 12,000 pure natural products was screened for effects on early zebrafish development. The largest phenotypic class of hits, which caused developmental arrest without necrosis, contained known electron transport chain inhibitors and many compounds of unknown mechanism of action. High-throughput transcriptional profiling revealed that these compounds are mechanistically related to one another. Metabolic and biochemical assays confirmed that all of the molecules that induced developmental arrest without necrosis inhibited the electron transport chain. These experiments demonstrate that the electron transport chain is the target of the natural products manassantin, sesquicillin, and arctigenin. The overlap between the zebrafish and transcriptional profiling screens was not perfect, indicating that multiple profiling screens are necessary to fully characterize molecules of unknown function. Together, zebrafish screening and transcriptional profiling represent sensitive and scalable approaches for identifying bioactive compounds and elucidating their mechanism of action.
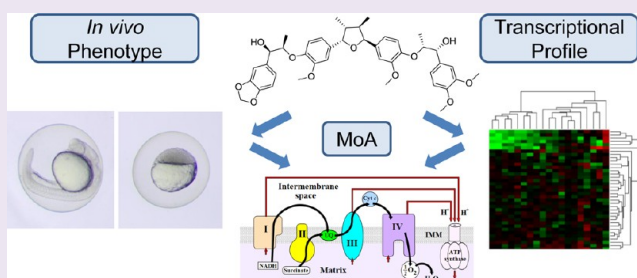
The zebrafish is a model organism that, while traditionally used to study the embryonic development of a vertebrate,[1] is well-suited for chemical compound screens. The optical clarity of the embryos, which develop outside of the mother, permits observation of developing organs and body structures with the aid of a simple dissecting microscope.[2] Embryos develop quickly, becoming free-swimming larvae only five days after fertilization, thus allowing for detection of a wide range of developmental phenotypes in a short span of time. The ability of adult females to lay several hundred eggs per mating enables screening of a large number of compounds at once. Moreover, chemical screening is particularly simple due to the fact that zebrafish embryos are permeable to most compounds added directly to the water in which they develop.[3]

Several screens of synthetic chemical libraries in zebrafish have already identified compounds with specific mechanisms of action.[3,4] For example, a screen for compounds that affect heart patterning identified concentramide, which induces a phenotype similar to the zebrafish PKCλ mutant *heart-and-soul*.[5] Dorsomorphin was identified as an inhibitor of BMP signaling based on its ability to dorsalize early zebrafish embryos.[6] In addition, screens have been performed to identify chemical suppressors of genetic mutants in zebrafish. For example, a

novel compound, persynthemide, was identified as a suppressor of the mitotic arrest phenotype of a zebrafish *bmyb* mutant.[7]

Unlike traditional cell-based screens, which are each designed to assay for a relatively narrow range of activities, phenotypic screens in complex systems allow for interrogation of a wide range of biological pathways, which has led to the identification of compounds with novel, interesting properties.[8−10] Phenotypic screens are relatively unbiased, since compounds affecting *any* interesting phenotype may be selected for further study. With such potential diversity among the hits, however, the greatest challenge in following up a phenotypic screen is identifying the target and mechanism of action (MoA) of compounds of interest. This remains a major hurdle for drug discovery based on phenotypic screens. A number of systematic MoA discovery approaches have been described that attempt to address this challenge, including strategies based on yeast genetics, cell line viability screening, transcriptional profiling, cellular imaging, chemical proteomics, and data mining.[11−21]
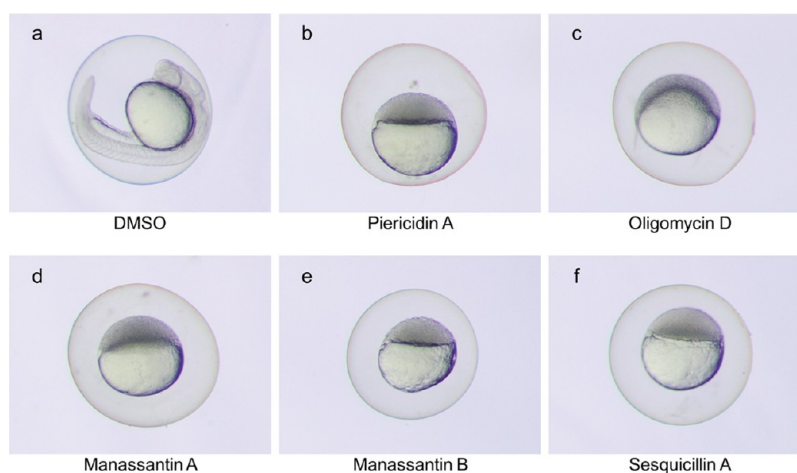
Figure 1. A zebrafish screen identifies natural products that arrest/delay development. Wild-type zebrafish embryos were treated with compounds at 4 hpf and photographed at 24 hpf. Embryos were treated with (a) 0.5% DMSO or ~1 μM of the following: (b) piericidin A, (c) oligomycin D, (d) manassantin A, (e) manassantin B, (f) sesquicillin A.

Table 1. Natural Products That Induce Developmental Arrest of Zebrafish Embryos

(a)

Compound or Class	No. of Cmpds.	Mechanism of Action
Known mechanism of action:		
Piericidins	10	Complex I inhibitor
Antimycins	7	Complex III inhibitor
Rotenoids	5	Complex I inhibitor
Oligomycins	3	Complex V (F_0F_1 ATPase) inhibitor
Nonactins	2	Cyclic K^+ ionophore
Valinomycin	1	Cyclic K^+ ionophore
Other	9	Complex I, II, or III inhibitors

(b)

(a) Compounds with known mechanism of action that induce developmental arrest of zebrafish embryo. (b) Compounds with previously unknown mechanism of action that induce developmental arrest of zebrafish embryos. All were identified in the zebrafish phenotypic screen, except for arctigenin, which was identified by LMF profiling.

We performed a phenotypic screen in zebrafish embryos using the Novartis library of natural products, a large collection of pure compounds derived from a variety of sources, including plants, fungi, and microbes. At approximately 12,000 compounds, this collection was of reasonable size to be screened in its entirety in zebrafish. The largest phenotypic class among the hits was developmental arrest without necrosis. Many of the compounds that induced this phenotype were known inhibitors of the mitochondrial electron transport chain (ETC), which suggested that the target of the remaining

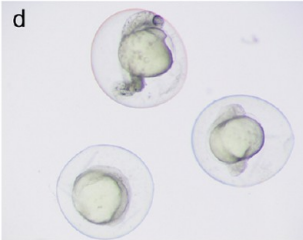| Dose (µM)	Piericidin A	Oligomycin D	Manassantin A	Manassantin B	Sesquicillin A
10.00	++++	++++	++++	++++	++++
3.333	++++	++++	++++	++++	++++
1.111	++++	+++	++++	++++	++++
0.370	++++	+++	+++	+++	++++
0.123	+++	+	++	+++	+++
0.041	+	-	-	++	+
0.014	-	-	-	-	-
0.005	-	-	-	-	-

DMSO

Manassantin A, 0.370µM

Manassantin B, 0.123µM

Figure 2. Dose−response of selected developmental arrest hits *vs* known mitochondrial inhibitors in zebrafish embryos. (a) Wild-type zebrafish embryos were treated with compound at the indicated doses at 4 hpf and observed at 26 and 72 hpf. Effects ranged from complete developmental arrest (++++) to mild delay (+) to no effect (−). For example, +++ indicates embryos that by 72 hpf have developed to a stage normally observed at 28 hpf or earlier, whereas + indicates embryos that by 72 hpf have developed to the equivalent of ∼60 hpf. Embryos were photographed at ∼72 hpf after treatment at 4 hpf with (b) 0.5% DMSO, (c) 0.370 µM manassantin A, and (d) 0.123 µM manassantin B.

compounds, some of which were of unknown function, was also the mitochondrial ETC. A cell-based transcriptional profiling analysis corroborated these predictions, and assays probing cellular metabolism and measuring the function of specific complexes of the ETC were used to investigate the compounds of previously unknown function. These experiments confirmed that all of the molecules in this class were mitochondrial inhibitors. Integrated analysis of these data demonstrates the utility of mapping compound phenotypes to MoAs as a method to elucidate compound function.

■ RESULTS AND DISCUSSION

A Screen of a Natural Product Library in Zebrafish Embryos Identifies a Large Phenotypic Class. Wild-type zebrafish embryos were treated at 4 h post fertilization (hpf) with a 1 µM dose of natural products in 96-well format. At 1 and 3 days post fertilization (dpf), embryos were observed for phenotypic defects. A compound that induced the same phenotype in all 3 of the embryos in a well was considered a hit. Of the 12,200 unique compounds screened, approximately 2% induced necrosis or otherwise nonspecific defects. The effects of these toxic compounds was generally observed by 1 dpf, with severity ranging from patches of slightly necrotic brain tissue, to full-body necrosis, to embryos that died within hours of treatment and rapidly decomposed (Supplementary Figure 1b). Distinct from these, there were a total of 114 compounds (∼1%) that induced highly specific phenotypes. Of these hits, 50 induced developmental arrest without necrosis, comprising the largest phenotypic class. The remaining hits included 18 compounds that induced pericardial edema (Supplementary Figure 1d) and 46 compounds that induced other specific and generally distinct phenotypes, including complete loss of posterior structures, lack of pigmentation, and an undulating notochord (Supplementary Figure 1e−h).

Since the 50 compounds that induced developmental arrest represented such a large proportion of the hits, we decided to characterize them in further detail. The arrest induced by these compounds was nearly always immediate, halting the embryos

at a developmental stage ranging from sphere to partway through epiboly, or within 0−4 h after treatment at 4 hpf (Figure 1). There was no evidence of necrosis at 24 hpf, even though embryos had been immersed in compound for 20 h (Figure 1). Most of the embryos did, however, eventually die by 72 hpf without progressing any further in development (data not shown).

In order to investigate the mechanism of this arrest, we first examined the effects of known arresters of cell growth on zebrafish embryos. Embryos were treated at 4 hpf with a panel of cytotoxic compounds (carboplatin, docetaxel, doxorubicin, gemcitabine, and topotecan), each of which was selected to probe a distinct mode of toxicity. Docetaxel, doxorubicin, and topotecan each induced death or widespread necrosis at the highest doses (up to 100 µM) (Supplementary Table 1). At intermediate doses of these compounds, more moderate or localized necrosis was observed (Supplementary Table 1). Developmental arrest was not observed at any dose. The other two compounds tested gave more subtle phenotypes: The highest doses of gemcitabine caused abnormal tail curvature first observed at 3 dpf, whereas the 100 µM dose of carboplatin prevented hatching at 5 dpf with no evidence of other effects (Supplementary Table 1). Again, developmental arrest without necrosis was not observed and was therefore a phenotype that was relatively specific to the 50 compounds identified in the screen.

Among the compounds that induced developmental arrest, we noticed that many were known inhibitors of mitochondrial function. These include a number of piericidins and rotenoids, which inhibit Complex I of the electron transport chain (ETC),[22] as well as antimycins, which inhibit Complex III (Table 1a).[23] In addition, there are 3 oligomycins that inhibit the F_oF_1 ATPase (also known as Complex V)[24] and 3 cyclic K^+ ionophores that disrupt the mitochondrial membrane potential.[25] In total, 37 of the 50 compounds have a known function, and all of them are inhibitors of mitochondrial function (Table 1a). This finding strongly suggested that the remaining 13 compounds (Table 1b) may also modulate mitochondrial activity. Six of these 13 compounds are sesquicillins, which have

a

b
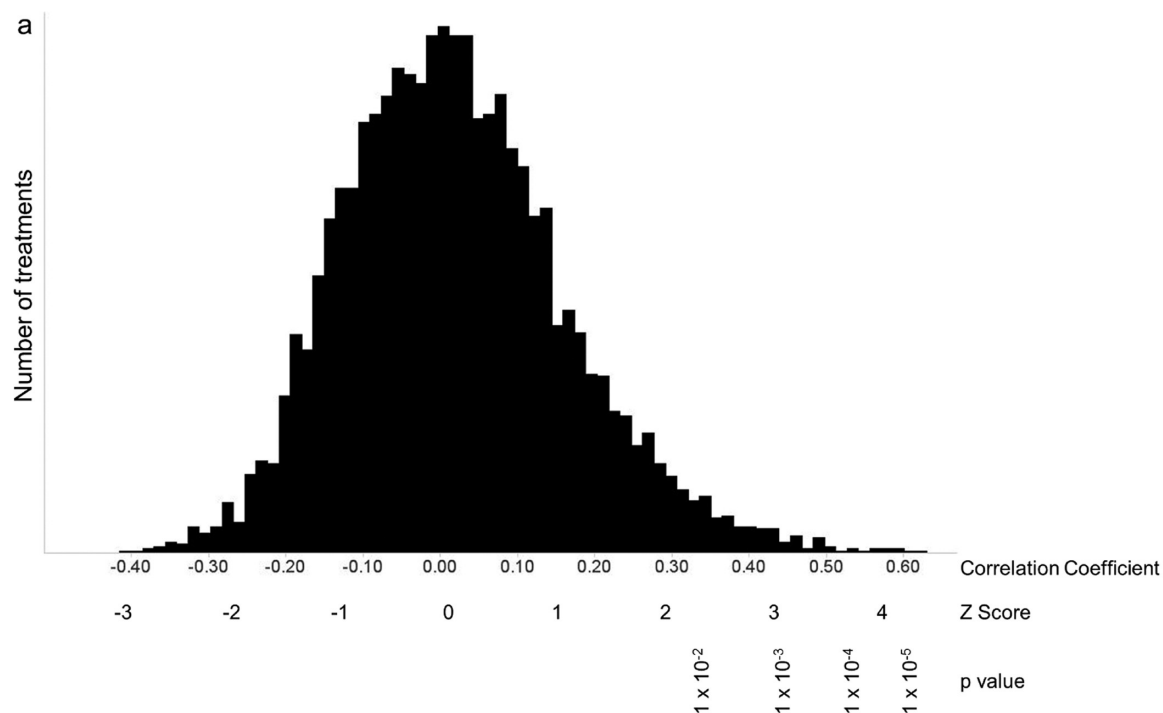
Pearson	FDR	p-value	Concentration	Compound Name	Known Use or Mechanism of Action
0.630	0.023	4.84E-06	10000 nM	Manassantin A	Previously unknown
0.611	0.023	9.05E-06	10000 nM	SB 224289 hydrochloride	Selective 5-HT$_{1B}$ receptor inverse agonist
0.597	0.023	1.40E-05	10000 nM	Manassantin B	Previously unknown
0.592	0.023	1.65E-05	10000 nM	Mundulone	Previously unknown
0.580	0.023	2.37E-05	10000 nM	C2	Previously unknown
0.579	0.023	2.47E-05	10000 nM	Rotenoid 1	ETC complex I inhibitor
0.564	0.028	3.82E-05	10000 nM	KBio2_003312	Previously unknown
0.563	0.028	3.96E-05	10000 nM	Rotenoid 2	ETC complex I inhibitor
0.551	0.035	5.70E-05	10000 nM	Piericidin A	ETC complex I inhibitor
0.536	0.048	8.71E-05	10000 nM	PD 102807	Selective muscarinic M4 receptor antagonist
0.528	0.055	1.08E-04	10000 nM	Evodiamine	Anti-proliferative effect on human prostate cancer cell lines, *in vivo* antiallergic effect
0.524	0.056	1.21E-04	10000 nM	Sorafenib	b-Raf, VEGFR, PDGFR, c-kit inhibitor
0.506	0.080	2.00E-04	10000 nM	Venturicidin A	ETC complex V (F$_o$F$_1$ ATPase) inhibitor
0.504	0.080	2.11E-04	10000 nM	Venturicidin	ETC complex V (F$_o$F$_1$ ATPase) inhibitor
0.501	0.080	2.28E-04	10000 nM	C3	Previously unknown
0.497	0.080	2.57E-04	10000 nM	C4	Previously unknown
0.495	0.080	2.69E-04	10000 nM	Dequalinium dichloride	Blocker of the apamin-sensitive small conductance Ca^{2+}-activated K$^+$ channel
0.495	0.080	2.70E-04	100 nM	Piericidin A	ETC complex I inhibitor
0.494	0.080	2.75E-04	100 nM	21-hydroxy-oligomycin A	ETC complex V (F$_o$F$_1$ ATPase) inhibitor
0.491	0.082	2.94E-04	10000 nM	C5	Previously unknown
0.489	0.084	3.17E-04	10000 nM	C6	Previously unknown
0.486	0.085	3.37E-04	10000 nM	Cruentaren A	ETC complex V (F$_o$F$_1$ ATPase) inhibitor
0.481	0.094	3.88E-04	10000 nM	Rimcazole dihydrochloride	σ receptor antagonist; inhibitor of dopamine transporter
0.469	0.121	5.23E-04	10000 nM	C7	Previously unknown
0.464	0.131	5.91E-04	10000 nM	C8	Previously unknown
0.462	0.132	6.20E-04	10000 nM	C9	Previously unknown
0.457	0.132	7.11E-04	10000 nM	C10	Previously unknown
0.457	0.132	7.15E-04	10000 nM	Niclosamide	Uncoupler of oxidative phosphorylation

Figure 3. continued

b

Pearson	FDR	p-value	Concentration	Compound Name	Known Use or Mechanism of Action
0.455	0.132	7.36E-04	10000 nM	C11	Previously unknown
0.454	0.132	7.53E-04	10000 nM	C12	Previously unknown
0.454	0.132	7.53E-04	10000 nM	Nalanthalide (sesquicillin)	Previously unknown
0.454	0.132	7.57E-04	100 nM	Verucopeptin	Antibiotic
0.451	0.138	8.18E-04	10000 nM	Oligomycin D	ETC complex V (F_oF_1 ATPase) inhibitor
0.450	0.138	8.45E-04	10000 nM	Halocarban	Used in soaps, lotions, and toothpaste
0.449	0.138	8.68E-04	10000 nM	Arctigenin	Inhibitor of IκBα phosphorylation; inhibitor of MKK1
0.445	0.145	9.38E-04	10000 nM	C13	Anti-bacterial
0.441	0.157	1.04E-03	100 nM	C14	Previously unknown
0.436	0.170	1.19E-03	100 nM	Myxothiazol	ETC complex III inhibitor
0.435	0.170	1.20E-03	10000 nM	Purvalanol A	Cyclin-dependent kinase inhibitor
0.432	0.170	1.29E-03	10000 nM	A 77636 hydrochloride	Dopamine D1-like receptor agonist
0.430	0.170	1.36E-03	10000 nM	C15	Previously unknown
0.430	0.170	1.36E-03	10000 nM	C16	Previously unknown
0.428	0.170	1.41E-03	10000 nM	Rotenoid 3	ETC complex I inhibitor
0.428	0.170	1.41E-03	10000 nM	C17	Previously unknown
0.428	0.170	1.41E-03	10000 nM	Deguelin	ETC complex I inhibitor
0.428	0.170	1.43E-03	100 nM	Stigmatellin	ETC complex III inhibitor
0.428	0.170	1.43E-03	100 nM	Myxothiazol	ETC complex III inhibitor
0.424	0.180	1.56E-03	10000 nM	C18	Previously unknown
0.422	0.184	1.62E-03	10000 nM	Thuggacine	Previously unknown
0.416	0.195	1.87E-03	10000 nM	C19	Previously unknown

Figure 3. Multiplex transcriptional profiling identifies compounds positively correlated with manassantin B. (a) Distribution of compound treatments with transcriptional profiles correlated with that of 10,000 nM manassantin B, ordered by Pearson coefficients. (b) Top 50 compounds with transcriptional profiles similar to that of manassantin B. MCF7 cells were treated with compounds at either 100 or 10,000 nM concentration and compared with a 10,000 nM dose of manassantin B. FDR = false discovery rate.

been shown to arrest cells in G1 phase of the cell cycle at relatively high doses (∼40 μM);[26] however, the mechanism of this arrest is as yet unknown. Manassantin B, one of two manassantins in this group, has also been described as an inducer of G1 arrest (∼5 μM).[27] At lower doses, manassantin B has been variously described as an inhibitor of HIF-1α (10 nM),[28,29] NF-κB activation (2.5 μM),[30] and PMA-induced ICAM-1 expression (50 nM).[31] No specific activity toward mitochondria has been previously ascribed to the manassantins nor to the remaining 5 compounds in this group (Table 1b).

To further investigate their putative anti-mitochondrial activity, we compared the phenotype of these compounds in zebrafish to that of known mitochondrial inhibitors, over a range of doses. As in the original screen, embryos were treated continuously with compound at 4 hpf and observed at 1 and 3 dpf. Similar to the known mitochondrial inhibitors piericidin A and oligomycin D, the highest doses of the putative mitochondrial inhibitors induced complete developmental arrest (Figure 2a). At lower doses, each appeared to induce developmental delay rather than complete arrest (Figure 2a,c,d). The degree of this delay was similar in embryos treated with known mitochondrial inhibitors (Figure 2a). Similar profiles of dose-dependent delay were observed when other known mitochondrial inhibitors and other developmental arrest hits from the screen were tested (data not shown). Additionally, when the compound was washed out after varying durations of treatment, the phenotypic effects were similar for all compounds, that is, the developmental arrest was generally irreversible at the highest doses tested, even after treatment for as little as 1 h (data not shown). Thus, the phenotypic similarity to known mitochondrial inhibitors raised the

possibility that the mitochondrion is the target of sesquicillins, manassantins, and other compounds that arrest zebrafish development.

Multiplex Transcriptional Profiling Corroborates and Extends Predictions of Compounds That Have Anti-mitochondrial Activity. To further characterize the compounds that induce developmental arrest without necrosis, we employed a cell-based transcriptional profiling approach based on the LMF method (multiplex ligation-mediated amplification with the Luminex FlexMAP optically addressed and barcoded microsphere and flow cytometric detection system).[32] Approximately 100 genes with highly variable expression were selected for their ability to classify the MoAs of a wide range of compounds, similar to the approach described by Lamb *et al.*[15] About 2,800 reference and investigational compounds were profiled in MCF7 cells to build a database of comparators. MoA predictions were made for compounds of unknown function based on the similarity of their expression profiles to the profiles of reference compounds with known activities (Supplementary Table 2).

The expression profile of manassantin B was compared to that of approximately 2,800 reference and investigational compounds by calculating Pearson correlations. When plotted as a histogram, a tail is observed on the right side of the distribution, indicating that there are compounds among those analyzed whose profiles are significantly correlated with that of manassantin B (Figure 3a). Among the most highly correlated compound treatments are rotenoids, oligomycins, piericidin A, and other known inhibitors of mitochondrial function (Figure 3b). While there are additional known mitochondrial inhibitors that are significantly correlated to manassantin B, we selected

the top 50 compounds, displayed in Figure 3b, for further analysis. At the top of this list is manassantin A, a close structural relative of manassantin B. A sesquicillin derivative (nalanthalide) was also among the compounds related to manassantin B, thus corroborating the prediction in zebrafish that these compounds have a similar function. Interestingly, there are a number of compounds that were not previously known to have inhibitory activity in mitochondria. Thus, like the zebrafish screen, LMF profiling could allow us to define the MoA of additional compounds with previously unknown activity. We selected several of these to test whether, in fact, the MoA of these compounds was inhibition of mitochondrial activity.

Identifying the Specific Mitochondrial Complex Targeted by Selected Compounds. In order to confirm the predicted anti-mitochondrial MoA of compounds from the zebrafish screen and LMF profiling, we used a cell-based assay to assess the impact of these compounds on mitochondrial function. Cells cultured in media containing glucose derive ATP primarily from glycolysis.[33] When galactose is substituted for glucose, however, cells rely nearly exclusively on oxidative phosphorylation for ATP synthesis.[33] Thus, cells grown in galactose media are more sensitive to compounds that block mitochondrial function than cells grown in media with glucose.

As expected, many piericidins, rotenoids, and other known mitochondrial inhibitors are particularly toxic to cells cultured in galactose media, while they have virtually no effect on cells in glucose media, resulting in high glucose-galactose (Glu-Gal) IC_{50} ratios (Table 2). Compounds predicted to be mitochondrial inhibitors from zebrafish screening were also found to display preferential toxicity in galactose media, including both manassantins and sesquicillins (Table 2).

While the glucose-galactose assay confirmed that many of the compounds we identified by zebrafish screening indeed inhibit mitochondrial function, several did not score in this assay, including some of the positive controls. For example, 2 of the 4 piericidins tested had Glu-Gal IC_{50} ratios that were high, as expected, but 2 of them did not, for reasons unknown (Table 2). Additionally, valinomycin and nonactin, both known uncouplers of electron transport, were toxic in both glucose and galactose media (Table 2). In such cases, other activities of the compound outside of mitochondrial inhibition may account for its more general toxicity.

We thus employed an alternative approach to measure mitochondrial inhibition more directly. In order to determine specifically which complex of the electron transport chain, if any, is affected by each of these compounds, we performed biochemical assays that independently measure the activity of Complex I, IV, or V, each isolated individually from detergent-solubilized bovine heart mitochondria by immunocapture.[34] To measure the combined activity of Complexes II and III, chemical inhibitors of Complexes I and IV were added to solubilized mitochondria prior to addition of the compounds to be tested. In each assay, the rate of product formation or reagent depletion was measured spectrophotometrically.

A panel of compounds was tested in each assay at a concentration of 10 μM to survey their inhibitory activity toward each of the electron transport complexes. Whereas the Glu-Gal assay was unable to detect the anti-mitochondrial activity of some of the piericidins, these biochemical assays demonstrated that all 4 piericidins tested are indeed inhibitors of Complex I, as expected (Table 3a). Manassantins A and B were identified as specific inhibitors of Complex I, whereas the

Table 2. Metabolic Analysis of Selected Novel Mitochondrial Inhibitors Identified by Zebrafish Screening: Comparison of Activity with Known Mitochondrial Inhibitors

compound	glucose media IC_{50} (nM)	galactose media IC_{50} (nM)	Glu-Gal IC_{50} ratio
Known Mitochondrial Inhibitors			
Piericidin A	>20000	0.006723	>2975040
Piericidin B1 N-oxide	>20000	2.240	>8929
Piericidin B1	>20000	>20000	1
Piericidin (actinopyrone derivative)	>20000	>20000	1
Rotenoid 1	>20000	35.54	>562
Antimycin A4a	>20000	4.963	>4030
Ascochlorin	>20000	218.9	>91
Stigmatellin	>20000	69.59	>287
Oligomycin D	>20000	0.4819	>41505
Valinomycin	18.77	21.54	0.871
Nonactin	20.92	31.06	0.674
Putative Novel Mitochondrial Inhibitors Identified in Zebrafish Screen			
Manassantin A	>20000	0.2471	>80935
Manassantin B	>20000	0.5699	>35094
Sesquicillin A	>20000	239.8	>83
Colletochin (sesquicillin derivative)	>20000	6.321	>3164
Nalanthalide (sesquicillin derivative)	>20000	12.99	>1539
Sesquicillin derivative 1	>20000	47.91	>417
Sesquicillin derivative 2	>20000	385.2	>51
Sesquicillin derivative 3	12500	35	357
Mundulone	11030	926.6	11
KBio2_003312	12730	2074	6
C1	>25000	25000	1
2,4-diacetyl-phloroglucinol	>25000	>25000	1
Fumigachlorin	20000	50.45	396

sesquicillins inhibited Complex II or III (Table 3b). Nonactin, a K^+ ionophore and known disrupter of the mitochondrial membrane potential, did not score in any of these assays. On the other hand, valinomycin, another K^+ ionophore, showed activity in several of the assays (Table 3a). These biochemical assays may therefore be susceptible to false negatives in their detection of anti-mitochondrial activity. Notably, the zebrafish screen detected both valinomycin and nonactin as developmental arresters (Table 1), and valinomycin was also significantly correlated with manassantin B by LMF, even though it did not appear among the top 50 compounds (Pearson correlation = 0.32, $p = 0.014$, false discovery rate = 0.38). Nonactin was not analyzed by LMF.

Compounds that displayed activity at 10 μM in one or more of the biochemical assays were retested over a range of doses in order to determine their relative potency (Table 3 and Supplementary Figure 2). The manassantins were confirmed to be potent inhibitors of Complex I, with IC_{50} values of less than 25 nM (Table 3b). Most of the other compounds predicted by the zebrafish assay to inhibit mitochondrial function had IC_{50} values of less than 1 μM (Table 3b and data not shown). Thus, these assays confirmed the molecular target

Table 3. Biochemical Assays for Inhibition of Individual Complexes of the Electron Transport Chain[a]

(a):

Compound	cI	cII/III	cIV	cV	IC$_{50}$ (nM)	Fish Arrest?
Positive Controls:						
Rotenone	0.00001	ND	ND	ND	ND	Yes
Antimycin	ND	0.00002	ND	ND	ND	Yes
Potassium cyanide	ND	ND	0.00001	ND	ND	ND
Oligomycin	ND	ND	ND	0.00461	ND	Yes
Other Known Mitochondrial Inhibitors:						
Piericidin A	0.00002	NS	NS	NS	ND	Yes
Piericidin B1	0.00001	0.00117	NS	NS	ND	Yes
Piericidin B1 N-oxide	0.00001	0.00001	NS	NS	ND	Yes
Piericidin (actinopyrone derivative)	0.00001	NS	NS	NS	ND	Yes
Ascochlorin	NS	<0.00001	NS	0.00433	cII/III: 7.4	Yes
Valinomycin	0.00377	0.00895	0.00070	NS	ND	Yes
Nonactin	NS	NS	NS	NS	ND	Yes

(b):

Compound	cI	cII/III	cIV	cV	IC$_{50}$ (nM)	Fish Arrest?
Manassantin A	0.00001	NS	NS	0.00659	cI: 21	Yes
Manassantin B	0.00001	NS	NS	0.00698	cI: 4.5	Yes
Sesquicillin A	NS	<0.00001	NS	0.00966	cII/III: 550	Yes
Nalanthalide (sesquicillin)	NS	<0.00001	NS	NS	cII/III: 140	Yes
Mundulone	<0.00001	NS	NS	NS	cI: 2.6	Yes
KBio2_003312	<0.00001	NS	NS	NS	ND	Yes

(c):

Compound	cI	cII/III	cIV	cV	IC$_{50}$ (nM)	Fish Arrest?
SB 224289 hydrochloride	0.00002	NS	NS	0.00496	cI: 2700; cV: 4300	No
C2	0.00001	NS	NS	NS	cI:1300	Yes
PD 102807	0.00004	NS	NS	NS	ND	No
Evodiamine	NS	NS	NS	NS	ND	No
Sorafenib	0.00168	0.00003	NS	0.00030	cI: >10000; cII/III: 1300; cV: 1200	No
C3	0.00001	0.00691	NS	0.00291	ND	No
C4	<0.00001	NS	NS	0.00029	ND	Yes
Dequalinium dichloride	0.00020	NS	NS	0.00421	ND	No
21-hydroxy-oligomycin A	0.00464	NS	NS	0.00444	ND	Yes
C5	0.00001	NS	NS	0.00147	ND	No
C6	0.00028	NS	NS	0.00105	ND	No
Rimcazole dihydrochloride	NS	NS	NS	NS	ND	No
C7	NS	NS	NS	0.00193	cV: 860	No
C8	NS	0.00045	NS	0.00045	ND	No
C9	NS	NS	NS	NS	ND	No
C10	0.00001	NS	NS	0.00043	ND	No
C11	0.00002	0.00308	NS	0.00005	ND	No
C12	NS	NS	NS	NS	ND	No
Verucopeptin	0.00170	NS	NS	NS	ND	No
Halocarban	NS	0.00027	NS	0.00075	cII/III: 9800	No
Arctigenin	0.00001	NS	NS	NS	cI: 340	Yes
C13	NS	NS	NS	NS	ND	No
C14	NS	NS	NS	0.00051	ND	No
Purvalanol A	NS	NS	NS	0.00330	ND	No
A 77636 hydrochloride	0.00007	0.00198	NS	0.00038	cI: >10000, cV: 9700	No
C15	NS	NS	NS	0.00125	ND	No
C16	NS	NS	NS	0.00100	ND	No
C17	NS	NS	NS	NS	ND	No
C18	NS	NS	NS	NS	ND	No
Thuggacine	NS	NS	NS	0.00327	ND	No
C19	NS	NS	NS	0.00079	ND	No

[a]Each compound was tested for inhibition of Complex I (cI), Complex II and III (cII/III), Complex IV (cIV), and Complex V (cV) at a dose of 10 μM. The assay was performed in triplicate, and a one-tailed unpaired t test was performed to determine if the inhibitory activity was significantly greater than that of a DMSO control. Compounds were re-analyzed in selected assays at doses of 10^{-5} to 10^5 nM to calculate IC$_{50}$ (Supplementary Figure 2). "Fish Arrest" column indicates whether developmental delay or arrest is seen in zebrafish embryos treated with doses of up to 10 μM of compound. (a) Positive controls and other known mitochondrial inhibitors. (b) Compounds identified by zebrafish screening as putative mitochondrial inhibitors. (c) Additional compounds identified as putative mitochondrial inhibitors by LMF profiling, but not by zebrafish screening. Numbers indicate p-values. NS = not significant ($p \geq 0.01$); ND = not determined. Red shading indicates $p < 0.001$; pink shading indicates $p < 0.01$.

of a number of compounds as specific complexes of the mitochondrial electron transport chain.

Zebrafish Screening and Transcriptional Profiling Approaches Complement One Another. In the case of the manassantins and sesquicillins, both the zebrafish screen and transcriptional profiling suggested that the target of these compounds is the mitochondrion, a prediction that was confirmed by several independent assays. There were a number of compounds, however, that were predicted to be mitochondrial inhibitors by LMF profiling but were not found as hits in the zebrafish screen, even though they were present in the natural product library that was screened. These compounds were subsequently tested in the biochemical assays for mitochondrial toxicity, as well as in zebrafish embryos at varying doses to determine whether they induce a developmental delay/arrest phenotype. For example, 21-hydroxy-oligomycin A was identified by LMF profiling as a mitochondrial inhibitor based upon its similarity to manassantin B and other reference mitochondrial inhibitors (Figure 3b). When it was tested in the biochemical mitochondrial toxicity assays, it was indeed found to be an inhibitor of Complex V, as might be expected, being a derivative of the known Complex V inhibitor oligomycin (Table 3c). When zebrafish embryos were treated with 21-hydroxy-oligomycin A at doses up to 30 μM, the highest doses induced developmental arrest without necrosis, similar to the other confirmed mitochondrial inhibitors (Figure 4). The zebrafish screen, therefore, was susceptible to false negatives due to the fact that the compound collection was screened at only one dose, 1 μM. Note that even a potent mitochondrial inhibitor such as manassantin A, with an IC$_{50}$ in the Complex I activity assay of ~5 nM (Table 3b), induces developmental delay in zebrafish embryos only at a dose of ~120 nM or higher (Figure 2a). Compound permeability or metabolism may account for the relatively lower sensitivity of the zebrafish assay compared to that of cell-based or biochemical assays.

In addition, since the number of compounds that could be screened in zebrafish was limited by the extensive time and effort required by this approach, transcriptional profiling identified additional putative mitochondrial inhibitors that were simply not screened in zebrafish. For example, arctigenin was confirmed to be a Complex I inhibitor (Table 3c) and was also found to induce developmental delay in zebrafish embryos (Figure 4). Note, however, that this compound, like 21-hydroxy-oligomycin A, would not have scored as a hit in the zebrafish screen since embryos were normal when treated with a 1 μM dose and only showed mild developmental delay with doses of at least 30 μM (Figure 4). Additionally, the collection of compounds profiled by LMF included not only natural products but also synthetic small molecules from the Novartis compound collection. Many of these synthetic compounds were also confirmed to be mitochondrial inhibitors in the Glu-Gal and biochemical assays (Tables 2 and 3c), and several also induced developmental delay or arrest in zebrafish embryos (Table 3c and Figure 4).

While transcriptional profiling is able to compensate for the false negatives of the zebrafish screening approach and has the potential to generate many more MoA predictions because of its high throughput, it may be susceptible to false positives. For example, evodiamine was predicted by LMF to be a mitochondrial inhibitor but did not score in any of the biochemical assays for the individual mitochondrial complexes (Table 3c). Unlike some of the known electron transport
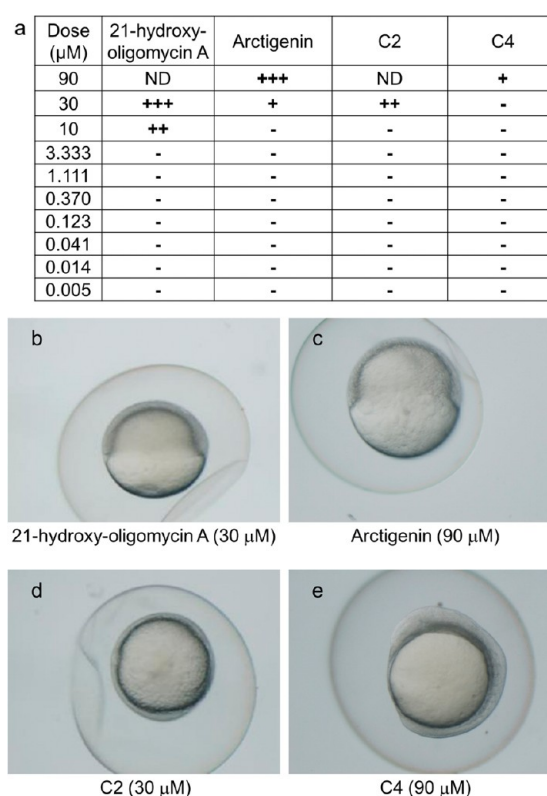

Dose (μM)	21-hydroxy-oligomycin A	Arctigenin	C2	C4
90	ND	+++	ND	+
30	+++	+	++	-
10	++	-	-	-
3.333	-	-	-	-
1.111	-	-	-	-
0.370	-	-	-	-
0.123	-	-	-	-
0.041	-	-	-	-
0.014	-	-	-	-
0.005	-	-	-	-

Figure 4. Dose−response of selected LMF hits in zebrafish embryos. (a) Wild-type zebrafish embryos were treated with compound at the indicated doses at 4 hpf and observed at 26 hpf and 72 hpf. Effects ranged from complete developmental arrest (++++) to mild delay (+) to no effect (−), as in Figure 2a. ND = not determined. Embryos were photographed at ~24 hpf after treatment at 4 hpf with (b) 30 μM 21-hydroxy-oligomycin A, (c) 90 μM Arctigenin, (d) 30 μM C2, and (e) 90 μM C4.

decoupling agents such as nonactin that likewise do not score in these assays, evodiamine induced necrosis in zebrafish embryos rather than developmental delay or arrest (data not shown). Thus, our evidence appears to indicate that evodiamine is not a mitochondrial inhibitor. In such cases, the zebrafish assay serves as a useful tool to limit potential false positives among the predictions made by LMF profiling.

In many cases, however, compounds that were identified by LMF as putative mitochondrial inhibitors, but not by zebrafish screening, were found not to induce developmental delay or arrest upon subsequent testing in zebrafish embryos (Table 3c). Dose−response analysis for selected compounds revealed that while these compounds have some activity against particular ETC complexes, the IC$_{50}$ values are generally in the 1 to 10 μM range. For example, SB 224289 hydrochloride inhibits Complex I with an IC$_{50}$ of 2.7 μM and Complex V with an IC$_{50}$ of 4.3 μM. Thus, not only did LMF identify mitochondrial inhibitors that were also found in the zebrafish screen, it yielded additional compounds with more moderate anti-mitochondrial activity. Whereas the zebrafish assay detects potent and selective mitochondrial inhibitors, LMF profiling more broadly detects anti-mitochondrial activity at higher compound doses. Therefore, these results demonstrate the power of the zebrafish system and LMF profiling as complementary tools for defining the primary MoA of novel compounds.

In summary, the zebrafish system was used to conduct a chemical genetic screen to identify compounds that specifically

perturb development. Of the 114 hits among 12,200 natural products screened, the most commonly observed phenotype was developmental arrest without necrosis, a specific phenotype that was not detected among a panel of known cytotoxic compounds. In this phenotypic class, all of the compounds with a known MoA were inhibitors of mitochondrial function, which implicated the remaining compounds, including some of unknown function, as mitochondrial inhibitors. Transcriptional profiling and subsequent biochemical and cell-based assays confirmed that many of these compounds, including the manassantins, sesquicillins, and arctigenin, target specific complexes of the mitochondrial ETC. Consistent with our results, inhibition of mitochondrial respiration by arctigenin has also been recently demonstrated.[35]

The heavy representation of mitochondrial inhibitors among the hits identified in the phenotypic screen may be due to the particular dose at which the natural product collection was screened. We selected the relatively low dose of 1 μM in order to identify especially potent compounds with specific activities. Many natural products that have been reported in the literature to induce specific defects in zebrafish have minimally active doses higher than 1 μM. For example, at least 4 μM cyclopamine is required to see any effect on interocular distance in zebrafish embryos,[36] and at least 50 μM is required to induce cyclopia (data not shown). Thus, it is not surprising that our screen yielded such a large proportion of hits that inhibit a critical cellular process such as mitochondrial electron transport. Rescreening the collection at a higher dose, while certainly increasing the rate of toxicity, might yield other hit compounds that affect specific developmental pathways or cellular processes.

The LMF transcriptional profiling approach was a useful method of corroborating the predictions made by the zebrafish screen and identifying additional compounds that may share the same MoA. Whereas screening large compound collections at multiple doses in zebrafish can be quite labor-intensive, the throughput of the LMF method is potentially much higher. As we have shown, some natural products identified by LMF to be mitochondrial inhibitors were not detected in the zebrafish screen, either because the dose was not high enough or because the compound was part of a larger collection that was not screened in zebrafish. It is important to note, however, that the accuracy of the LMF predictions depends on the particular set of compounds with known MoAs that are selected as references. Prior to the discovery of the link between these novel compounds and mitochondrial inhibition in zebrafish, few known mitochondrial inhibitors had been analyzed by LMF. When more of these inhibitors were added to the reference set, it became clear that the compounds identified in zebrafish clustered with this MoA over many others. Thus, the results of the zebrafish screen strengthened the predictions made by the LMF method. In addition, being a complex biological system, the zebrafish also appears to have advantages over the cell-based and biochemical assays we used, which were sometimes unable to detect certain classes of mitochondrial inhibitors, such as K^+ ionophores that disrupt the mitochondrial membrane potential.

It is notable that LMF predicted anti-mitochondrial activity for a number of compounds that have been described in the literature as having other specific activities. For example, SB 224289, a selective 5-HT$_{1B}$ (serotonin) receptor inverse agonist, had a transcriptional profile that matched that of manassantin B very closely. While the biochemical assays

confirmed that SB 224289 indeed has some mild anti-mitochondrial activity (IC$_{50}$ values in the Complex I and V assays between 2 and 5 μM), much lower doses have been shown to have activity toward the 5-HT$_{1B}$ receptor (10 nM).[37] In fact, the LMF signature of SB 224289 also shared significant similarity to that of other serotonin modulators (data not shown). Thus, LMF appears to be able to detect multiple activities. Notably, zebrafish treated with SB 224289 do not undergo developmental delay or arrest; rather, high doses (~90 μM) are lethal, and no specific effects are observed at lower doses (data not shown). In contrast, all of the established mitochondrial inhibitors tested to date induce the developmental delay/arrest phenotype. It is possible, then, that this phenotype is only associated with the most potent and selective mitochondrial inhibitors. Thus, the zebrafish may be useful as an accurate method of screening for strong mitochondrial toxicity among potential drug candidates.

In the case of the mitochondrial inhibitors, the ability to compare the phenotypic effects of compounds highlights the utility of zebrafish as a tool for defining MoA. In other cases, however, the correlation may not be as clear. For example, the 18 compounds from the zebrafish screen that induced pericardial edema are not obviously related by MoA. Pericardial edema is a commonly observed phenotype among unhealthy wild-type embryos (data not shown). While it is clear that these compounds are hits, in that they induced pericardial edema in all 3 of the embryos in the well, this phenotype may have various causes. For the remaining 46 hits, it will also be difficult to identify their MoA based upon similarity to phenotypes induced by other compounds, since their phenotypes are generally unique. However, we can be guided by the extensive collection of mutants identified by genetic screens, in order to make predictions about which pathway the compound might impinge upon, based on similarity of the phenotype to that of a genetic mutant. Additionally, as more compound screens are performed in zebrafish and more compound-induced phenotypes are documented, it will become easier to make predictions, similar in principle to the LMF transcriptional profiling method we employed.

In this work, we have demonstrated a path for elucidating MoA from phenotypic *in vivo* screening of compounds and compound profiling to defining biochemical function in cellular and *in vitro* assays. The combination of profiling technologies we employed can also identify novel compounds with MoA similar to those of known compounds that target particular pathways of interest, thus potentially yielding new chemical scaffolds around which improved therapeutics may be designed.

■ METHODS

Zebrafish Screen and Dose−Response Analysis of Hits. Multiple pairs of wild-type zebrafish of the AB strain were synchronously mated, and embryos were pooled from several clutches. Using a pipet, three embryos were dispensed with 200 μL of E3 medium (5 mM NaCl, 0.17 mM KCl, 0.44 mM CaCl$_2$, 0.68 mM MgSO$_4$) into each well of a black-walled 96-well plate (BD Falcon). At 4 hpf, 1 μL of 200 μM compound in DMSO was added to each well for a final concentration of ~1 μM. On each plate, 88 compounds were screened at once, with the last column of the plate containing embryos treated with 1 μL of DMSO as a control or left untreated. All 12,200 compounds screened came from a Novartis internal collection of pure natural products. Embryos were kept at 28.5 °C and were observed manually with a stereomicroscope at 1 and 3 dpf. Wells in which all 3 embryos displayed the same phenotypic defect were recorded as hits. For dose−response analysis of the hits, serial dilutions of the

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compounds were made from 10 mM stock solutions in DMSO. The final concentration of DMSO in the E3 medium containing embryos did not exceed 2%.

Glucose-Galactose Assay for Mitochondrial Toxicity. Hep3B cells were obtained from ATCC and were grown and maintained in DMEM with 10% FBS. Cells were washed with PBS, trypsinized, and resuspended in either glucose medium or galactose medium, made essentially as described.[38] Glucose medium contains DMEM without glucose (Invitrogen), 10% dialyzed FBS (Invitrogen), 25 mM glucose, 1 mM sodium pyruvate, 5 mM HEPES (pH 7.2–7.5), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Galactose medium contains DMEM without glucose, 10% dialyzed FBS, 10 mM galactose, 1 mM sodium pyruvate, 5 mM HEPES (pH 7.2–7.5), an additional 2 mM of glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin.

The cell concentration was adjusted to 1.33×10^5 cells per mL, and 30 μL of medium containing 4,000 cells was plated into each well of Nunc 384-well plates. Compounds to be tested were serially diluted in DMSO in 384-well screen plates (Matrix). A Wellmate Matrix 2 × 2 robot was used to dilute the compounds 1:25 in either glucose or galactose medium and to dispense 10 μL of the diluted compounds into the wells containing cells. Cells were incubated for approximately 20 h at 37 °C before cell viability was measured using CellTiter-Glo (Promega). IC$_{50}$ values were determined using the DMP IC$_{50}$ calculator. This tool, using the R statistical software package (http://www.r-project.org/), determines the appropriate fit for the data: either a 4- or 3-parameter nonlinear regression model, or a linear model. All IC$_{50}$ calculations were confirmed by visual analysis.

Mitochondrial Toxicity Assays. MitoTox kits MT-OX1, MT-OX2, MT-OX4, MT-OX5 (Mitosciences, Inc.) were used to measure the inhibitory activity of compounds on Complex I, II/III, IV, and V, respectively. The manufacturer's protocol was generally followed. DMSO was used as a negative control, and positive controls for each of the kits were rotenone, antimycin, potassium cyanide, and oligomycin, respectively. Spectrophotometric data were collected with SpectraMax software and analyzed with GraphPad Prism software.

Transcriptional Profiling (LMF). A gene signature was derived from analysis of the "Connectivity Map" data set[15] and was found to be broadly useful for MoA classification. Briefly, CEL files were normalized using Affymetrix's MAS5 algorithm, quantile normalized, and floored to 1% of the signal range. Log$_2$ ratios were calculated using as a denominator the median of plate- or batch-matched vehicle controls. We found that the 100 most variable probe sets could be used to accurately co-cluster compounds with shared MoAs for a number of different mechanisms, including, but not limited to, inhibitors of HDAC, HSP90, and PI3K/mTOR. (Note that here we define expression variability of a probe set as the standard deviation of its log$_2$ ratios across all compound-treated MCF7 cells.) For our clustering analysis we used hierarchical clustering with correlation coefficients and complete linkage in Spotfire DecisionSite v9.1. The 100 probe sets derived from microarrays were then converted into an LMF signature[32] by filtering for a median expression level of 600 and consolidating redundant probe sets. The final 100 probe signature included probes for 96 variable mRNAs, 1 probe for beta actin, and 3 probes for GAPDH.

MCF7 cells were treated with compounds at either 100 nM or 10 μM doses for 6 h. At least 2 biological replicates were run for each treatment and dose. The 100 probe LMF signature was then measured essentially as described.[32] Approximately 2,800 reference and investigational compounds were tested to build a database of comparators. Query compounds, such as the putative mitochondrial inhibitors described here, were tested, and Pearson correlation coefficients for each treatment were calculated relative to the other treatments in the database. Correlations were calculated using a 77-probe subsignature, which we found maximized the correlation of biological replicates and removed failed/noisy probes. P-values for these correlations were calculated by assuming their normal distribution and robustly estimating the center and spread using the median and MAD, respectively. Statistical calculations were carried out

using the R statistical software package. Benjamini and Hochberg false discovery rates were calculated using the R package "multtest."[39]

A strong correlation between compound treatments was taken as an indication that the compounds may share a common MoA. We found that roughly 40% of the 10 μM compound treatments were better correlated to their biological replicates than to any other treatment in the database, indicating that the platform was sensitive to a diverse set of bioactivities but also likely to be insensitive to many others. The construction, validation, and utility of this gene signature-based MoA discovery platform will be described in more detail elsewhere.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: daniel.curtis@novartis.com.

Present Addresses

§Fate Therapeutics, San Diego, CA 92121.

‖David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139.

Notes

The authors declare no competing financial interest.

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