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Systems modeling of developmental vascular toxicity

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Abstract

More than 80,000 chemicals in commerce present a challenge for hazard assessments that toxicity testing in the 21st century strives to address through high-throughput screening (HTS) assays. Assessing chemical effects on human development adds an additional layer of complexity to the screening, with a need to capture complex and dynamic events essential for proper embryo-fetal development. HTS data from ToxCast/Tox21 informs systems toxicology models, which incorporate molecular targets and biological pathways into mechanistic models describing the effects of chemicals on human cells, three-dimensional organotypic culture models, and small model organisms. Adverse outcome pathways (AOPs) provide a useful framework for integrating the evidence derived from these *in silico* and *in vitro* systems to inform chemical hazard characterization. To illustrate this formulation, we have built an AOP for developmental toxicity through a mode of action linked to embryonic vascular disruption (Aop43). Here, we review the model for quantitative prediction of developmental vascular toxicity from ToxCast HTS data and compare the HTS results to functional vascular development assays in complex cell systems, virtual tissues, and small model organisms. ToxCast HTS predictions from several published and unpublished assays covering different aspects of the angiogenic cycle were generated for a test set of 38 chemicals representing a range of putative vascular disrupting compounds. Results boost confidence in the capacity to predict adverse developmental outcomes from HTS *in vitro* data and model computational dynamics for *in silico* reconstruction of developmental systems biology. Finally, we demonstrate the integration of the AOP and developmental systems toxicology to investigate the unique modes of action of two angiogenesis inhibitors.

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Adverse outcome pathway, Angiogenesis, 5HPP-33, TNP-470, ToxCast, Tox21.

Introduction

The cardiovascular system is the first organ system to function in the vertebrate embryo, reflecting its critical role during development and pregnancy [1–3]. Stepwise events in vascular patterning commence in the early embryo with nascent vessel assembly from angioblasts (vasculogenesis) and expand by sprouting new vessels from the pre-existing network (angiogenesis) into an arborized system of arteries, veins, and lymphatics (angioadaptation) shaped by hemodynamic forces and microphysiological signals [4]. Vascular insufficiency is tied to many disease processes (stroke, diabetes, preeclampsia, neonatal respiratory distress, Alzheimer's, and so on [4]) and adverse pregnancy outcomes [5], including preterm labor [6], low birth weight [7], birth defects [8], and miscarriage [9]. The interplay between maternal exposure and fetal effects is reflected in the dynamics of vascular development.

Thalidomide, for example, disrupts immature blood vessel development in the rudimentary limb bud, leading to altered mesenchymal outgrowth and, in turn, phocomelia [10]. Taken together, the evidence linking disruption of vascular development to adverse birth outcomes supports the hypothesis that gestational exposure to vascular disrupting chemicals is one of the major modes of action in teratogenesis [5].

A predictive signature for putative vascular disrupting compounds (pVDCs) was developed using information from genetic mouse models (Jackson Labs Mammalian Phenotype Browser) to map high-throughput screening (HTS) *in vitro* data (ToxCast™) to critical vascular developmental signaling pathways, and analyzed against the backdrop of *in vivo* legacy data from pregnant animal bioassays (ToxRefDB) [11,12]. The U.S. Environmental Protection Agency Toxicity Forecaster (ToxCast™) program was initiated to investigate the molecular targets of environmental contaminants using cell-free and cell-based HTS assays [13]. Computational models are built upon the results of these screening data to rapidly predict chemical hazards to human health [14,15]. The *in vitro* data generated through the ToxCast™ program originally included 309 environmental chemicals tested across a diverse suite of >600 HTS assays [5,11]. Many, although not all, chemicals associated with developmental toxicity *in vivo* had an *in vitro* bioactivity signature for vascular disruption [11]. For example, the antiangiogenic thalidomide analog, 5HPP-33, scored high for predicted vascular disruption in the ToxCast™ data set [11]. Its vascular disrupting potential was further demonstrated in a computational agent-based model with emergent features that recapitulated the *in vitro* effects of 5HPP-33 on human umbilical vein endothelial cell (HUVEC) vasculogenesis [16]. 5HPP-33 was subsequently confirmed to be embryolethal in rat whole embryo culture (WEC) [17], consistent with evidence of embryotoxicity in rodents, and in contrast to its teratogenicity in rabbits when administered orally [8,18,19]. TNP-470 is an additional antiangiogenic reference compound that is embryotoxic in rodents [20] and also scored high for predicted vascular disruption in the ToxCast™ data set [11].

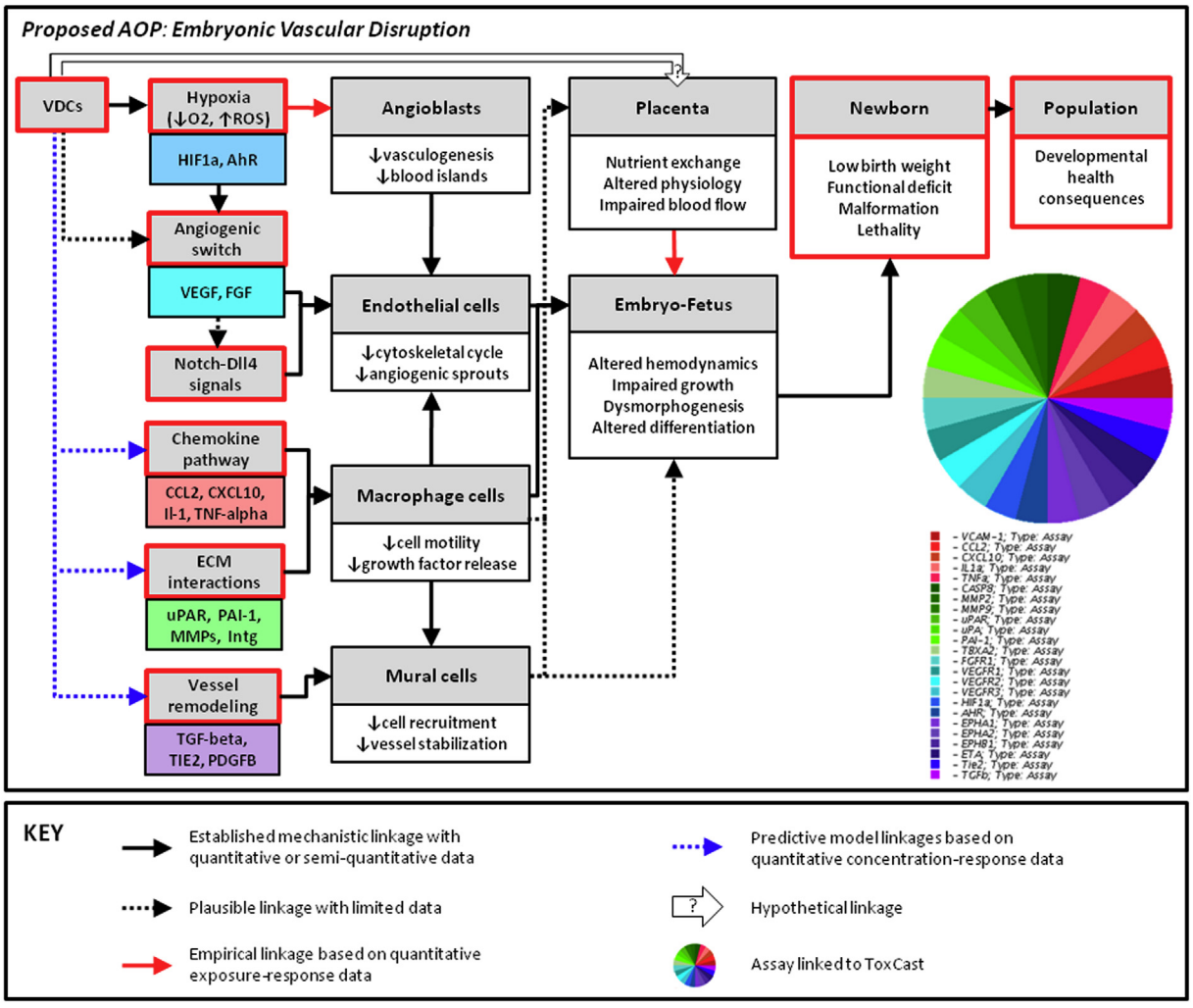
Adverse outcome pathways (AOPs) provide a useful framework to link molecular initiating events (MIEs) to key cellular- and tissue-level changes resulting in adverse outcomes, and to inform chemical prioritization for hazard characterization. We have built an AOP for developmental toxicity through a mode of action linked to embryonic vascular disruption for the Organization for Economic Cooperation and Development Workplan. This AOP, referred to as Aop43 in the Organization for Economic Cooperation and Development Wiki [https://aopwiki.org/aops/43], centers on the ligand activation of vascular endothelial growth factor receptor 2 (VEGFR2) by vascular endothelial growth factor A, which initiates angiogenic sprouting, growth, and fusion during early developmental stages [21]. Inhibition of VEGFR2 activity is the MIE, which may occur through modification of local vascular endothelial growth factor A levels, VEGFR2 expression or molecular function, or impacts on downstream signal transduction pathways [22–25]. Here, we review the construction of Aop43 and examine its usefulness as a framework to understand and predict developmental toxicity relevant to prioritization for hazard characterizations and human risk assessment decisions for cases that consider exposures and uses. To develop an AOP for embryonic vascular disruption (Figure 1), we searched the gene ontology and mammalian phenotype (MP) browsers of the Mouse Genome Informatics database [http://www.informatics.jax.org/] for terms affiliated with the disruption of vascular development. Terms for abnormal vasculogenesis [MP:0001622] and abnormal angiogenesis [MP:0000260] were captured and linked to ToxCast™ assays. This list had 65 target genes with bona fide roles in vasculogenesis or angiogenesis, 50 of which had evidence of abnormal embryonic vascular development based on genetic mouse models [5]. The broader AOP framework links multiple, complementary studies that serve as weight of evidence supporting the AOP and from which developmental toxicity predictions are possible.

Assessing the weight of evidence in support of Aop43

First-generation predictive models for prenatal developmental toxicity built from the ToxCast™ data revealed a complex web of biological processes with many connections to vasculogenesis and angiogenesis [11,26]. To assess the *in silico* pVDC predictions [5], 38 chemicals representing a range of pVDC scores were selected to represent roughly equal numbers of pVDCs and non-pVDCs ranging from known developmental toxicants to chemicals that have not demonstrated overt developmental outcomes [11]. These compounds were then tested across ten *in vitro* platforms from laboratories addressing different aspects of the vasculogenic/angiogenic cycle. Some of the platforms used traditional HUVECs, while others used human-induced pluripotent stem cells, rat fetal aortic explants [16,27], or transgenic reporter zebrafish lines [28,29]. The platforms covered different stages of the vascular cycle, from angiogenic sprouting and network assembly in various matrices [30,31] to coculture models of tubulogenesis [32,33], microvessel outgrowth, and patterning [17,28]. In several cases, a link between key event relationships (KERs) is evident through chemical toxicity profiles. For example, chemicals that disrupted angiogenic sprouting [30] also inhibited endothelial tubular network formation [31].

The weight of evidence for KERs in Aop43 is summarized in Figure 2, with ToxCast™ pVDC scores in the

Figure 1

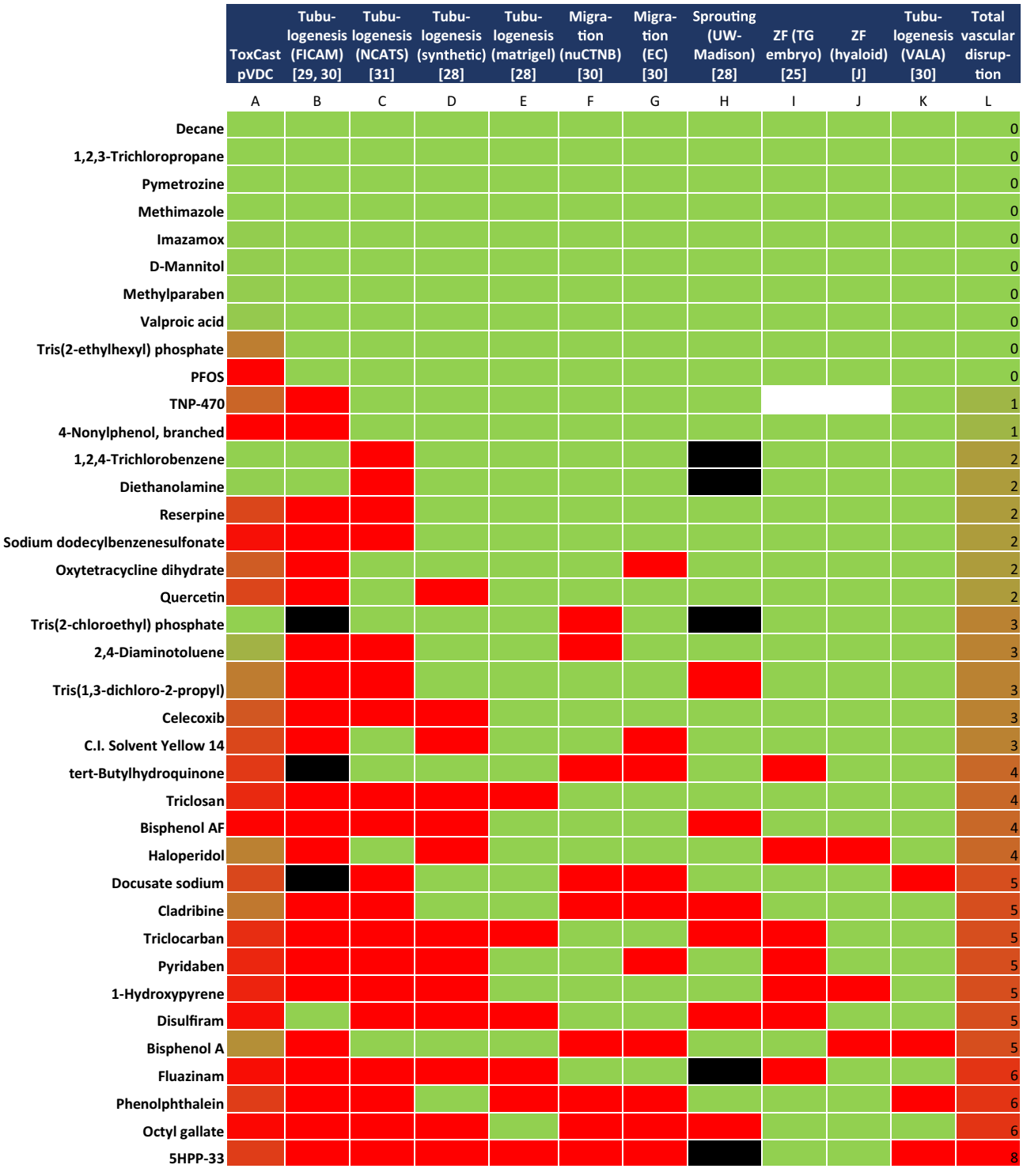


Aop43 for embryonic vascular disruption (reproduced with permission from Knudsen and Kleinstreuer [5]). AOP, adverse outcome pathway; VDCs, vascular disrupting compounds; VEGF, vascular endothelial growth factor.

first column, followed by columns indicating chemical effects for each of the ten data sets on endothelial cell morphology [21,28–34]. Shades of red indicate chemicals that were predicted positive (column A) or for which any *in vitro* assay measured a positive effect (column L), with higher scores indicated by darker shades of red. In contrast, a gradient color scheme was not used for columns B through K (positive = red, negative = green, black = cytotoxic, white = not tested). While no single study confirmed all the pVDC predictions, the combined vascular disrupting effects across all studies (column L) aligned well with the *in silico* predictions (column A). Based on these data, the *in silico* predictions included two false positives (tris(2-ethylhexyl) phosphate and perfluorooctanesulfonic acid (PFOS)) and three false negatives (1,2,4-trichlorobenzene; diethanolamine, and tris(2-chloroehtyl) phosphate) compared to column L. Overall, the *in silico* prediction sensitivity and specificity were 0.89 and 0.80, respectively; prediction accuracy was 87%, with greater predictivity of true positives (positive predictive value 93%) compared to predictivity of true negatives (negative predictive value 73%).

The Finnish Center for Alternative Methods assay (Figure 2; column B) [32] showed the greatest alignment with the pVDC predictions. This was one of the six *in vitro* tubulogenesis assays represented. Briefly, the assay utilizes primary HUVECs and adipose-derived stromal cells to measure tubule formation [32]. In this study, the 38 chemicals tested were assigned tubulogenesis scores to derive a concentration response and predict half-maximal activity (AC_{50}) values [33]. An additional tubulogenesis assay (National Center for

Figure 2



Vascular disruption potential of 38 ToxCast™ chemicals selected to represent a range of pVDC scores from 0% (green) to 100% (red). ToxCast™ pVDC prediction scores are shown in column A, with 10 platform endpoints represented in columns B–K. Chemical endpoints in each of columns B–K were assigned a score of 1 (red) or zero (green), with cumulative *in vitro* scores (i.e., number of assay positives) indicated in column L. Gradient colors used in columns A and L correspond to pVDC scores (A) or cumulative *in vitro* scores (L). Black = cytotoxicity; white = not tested. Compared to total *in vitro* outcomes (L), the *in silico* (A) prediction sensitivity and specificity were 0.89 and 0.80, respectively; prediction accuracy was 87%. pVDC, putative vascular disrupting compound.

Advancing Translational Sciences; column C) that utilizes commercially available co-culture of hTERT-immortalized mesenchymal stem cells and aortic endothelial cells [34] was also highly correlated with the *in silico* predictions. A third tubulogenesis assay comparing growth of commercially available HUVECs in synthetic medium (column D) or Matrigel (column E) predicted several antiangiogenic compounds [31], most of which aligned with Finnish Center for Alternative Methods predictions, albeit with less sensitivity. An additional tubulogenesis assay (column K) aligned poorly with the *in silico* pVDC predictions (only four of the 28 positive chemicals in column L were detected in this assay); however, endothelial cell migration assays (columns F and G) aligned better with the predictions (nine and ten of the 28 positive chemicals were detected in these assays, respectively) [33]. In addition to the *in vitro* assays, a VEGFR2 reporter assay using transgenic zebrafish picked up seven pVDC compounds (column I) [28], while an additional zebrafish screen assessing hyaloid bone development picked up three pVDCs (column J) [29]. Taken together, the cumulative results from the ten assays had a high correlation with the pVDC predictions. This example supports a weight of evidence approach on the basis of an AOP framework that can better inform hazard evaluations than any study taken alone. While the growing evidence supporting key events in Aop43 is strong, gaps remain in our understanding of the molecular signaling events linking them (i.e., KERs). Integrated systems models (e.g., WECs) are useful for investigating the molecular biology underlying KERs and constructing AOP-based ontologies to elucidate the developmental toxicity of environmental contaminants.

Exploring KERs via transcriptomics analysis of integrated systems models

Vascular disrupting compounds, 5HPP-33 and TNP-470, cause lethality and dysmorphogenesis in rat WEC

Embryonic zebrafish clearly shows the quantitative linkages in Aop43 from MIE (VEGFR2 inhibition by PTK787) to dysmorphogenesis and viability later in life [21]. Because species differences may underlie some of the insensitivity of the zebrafish model towards the 38-chemical list in Figure 2, it is useful to identify mammalian developmental systems models for exploring KERs. Ellis-Hutchings et al. [17] used rat WEC focused on two known angiogenesis inhibitors with high pVDC bioactivity scores from ToxCast™, yet differing *in vitro* activity scores (i.e., 5HPP-33, a synthetic thalidomide analog [35], had the highest *in vitro* activity score, while TNP-470, a synthetic fumagillin analog [17,36], had the lowest positive *in vitro* activity score (Figure 2)). Endothelial cells are a primary target for both compounds based on the potency of 5HPP-33 (AC50 = 4.9 μM) and TNP-470 (AC50 = 0.3 μM) on inhibiting endothelial cell proliferation over any of the other ToxCast™ assays used for the vascular disruption signature [11,17].

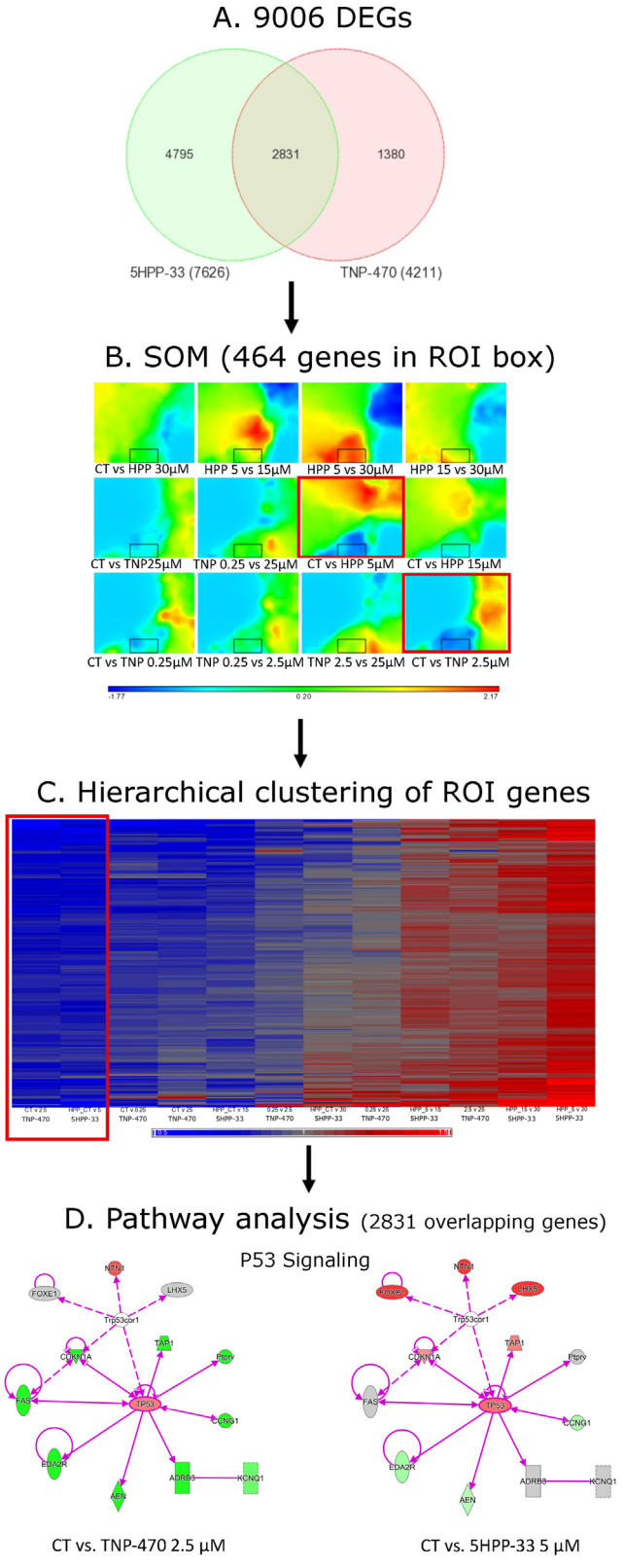
Ellis-Hutchings et al. [17] next evaluated vascular aberrations in an intact conceptus (rat WEC) and *ex vivo* angiogenesis model (rat aortic explant assay) exposed to each of the two vasculogenesis/angiogenesis inhibitors. Gestation day 10 (GD10) rat embryos isolated from the uterus and cultured in the presence of these inhibitors showed major effects at 48 h compared to dimethylsulfoxide (DMSO)-exposed vehicle controls: 5HPP-33 (≥15 μM) caused embryolethality and TNP-470 (≥0.25 μM) caused dysmorphogenesis. These effects were also observed in a zebrafish embryotoxicity assay for 5HPP-33 (>0.1 μM) and TNP-470 (>0.01 μM) [17]. Both compounds also inhibited microvessel outgrowth from rat aortic explants. Taken together, both compounds consistently inhibited angiogenesis in all assays reported by Ellis-Hutchings et al. [17], with TNP-470 potency 10—100× greater than that of 5HPP-33 and with 5HPP-33 effects appearing at earlier stages of the angiogenic cycle.

The Aop43 framework guides exploration of KERs

Although modes of action for TNP-470 (noncanonical Wnt inhibition [37]) and 5HPP-33 (tubulin polymerization inhibition [38]) are known, the various entry portals to Aop43 by these and other structurally diverse compounds can be inferred with the Aop43 framework. For example, Ellis-Hutchings et al. (2017) referred to the HTS data to posit several MIEs that may underlie the embryolethality of 5HPP-33 and the dysmorphogenesis effects (e.g., abnormal caudal extension and somite patterning) of TNP-470 [17]. They noted that 5HPP-33's primary reported effects on microtubule destabilization could impact cell growth through PI3K inhibition, p53 accumulation, and estrogen receptor signaling, among others, based on the ToxCast™ bioactivity data. For TNP-470, they highlighted that the reported mode of action is methionine aminopeptidase II (MetAP2) inhibition, possibly through the Wnt signaling pathway, among other ToxCast™ hits that were not discussed. While ToxCast™ bioactivity assay results may identify putative molecular targets for a given chemical, these data are based on cell-free or cell-based assays that cover a finite set of biological space and are not expected to capture the full suite of dynamic interactions among the numerous tissue types involved in embryonic development.

Another approach to identifying the signaling pathways and molecular events for an observed phenotype in intact organisms (*ex vivo* or *in vivo*) is to look at global gene expression at a time point at the beginning of the exposure period that may capture MIEs or key events

Figure 3



Transcriptomic analysis of rat whole-embryo culture at 4 days after exposure to angiogenesis inhibitors, 5HPP-33 (0–30 µM) or TNP-470 (0–25 µM). **(a)** 9006 total differentially expressed genes (DEGs) at any exposure concentration compared to dimethylsulfoxide (DMSO) controls included 2831 that were significantly affected by both compounds. **(b)** Self-organizing map (SOM) comprising all 9006 DEGs ranked 5HPP-33 (HPP) and TNP-470 (TNP) treatments by similarities in gene expression changes. Each of the 12 boxes represents a comparison between two treatments. SOM analysis identified a group of 464 genes (black box) that were similarly differentially expressed following exposure to 2.5 µM TNP-470 or 5 µM 5HPP-33. **(c)** Fold-change comparison of 464 genes from region of interest (ROI) in part **(b)**. **(d)** Pathway analysis exploring the effects of 2.5 µM TNP-470 and 5 µM 5HPP-33 on p53 signaling.

leading to embryotoxicity. Here, we describe previously unpublished results of a transcriptomics study connected to the embryology previously reported [17] to further understand how disruption of embryonic vascular development leads to developmental toxicity. The methods are described in detail in the Supplemental Materials, and the data have been deposited to the Gene Expression Omnibus under accession number GSE132375. Briefly, we used RNA sequencing analysis of whole, 10-day-old rat embryos at an early *ex vivo* culture time point (4 h) following exposure to three concentrations of 5HPP-33 or TNP-470, which were selected to cover *ex vivo* effects ranging from 0% to 100%. We next conducted differential gene expression analysis to identify molecular and cellular signaling pathways potentially underlying the *ex vivo* effects.

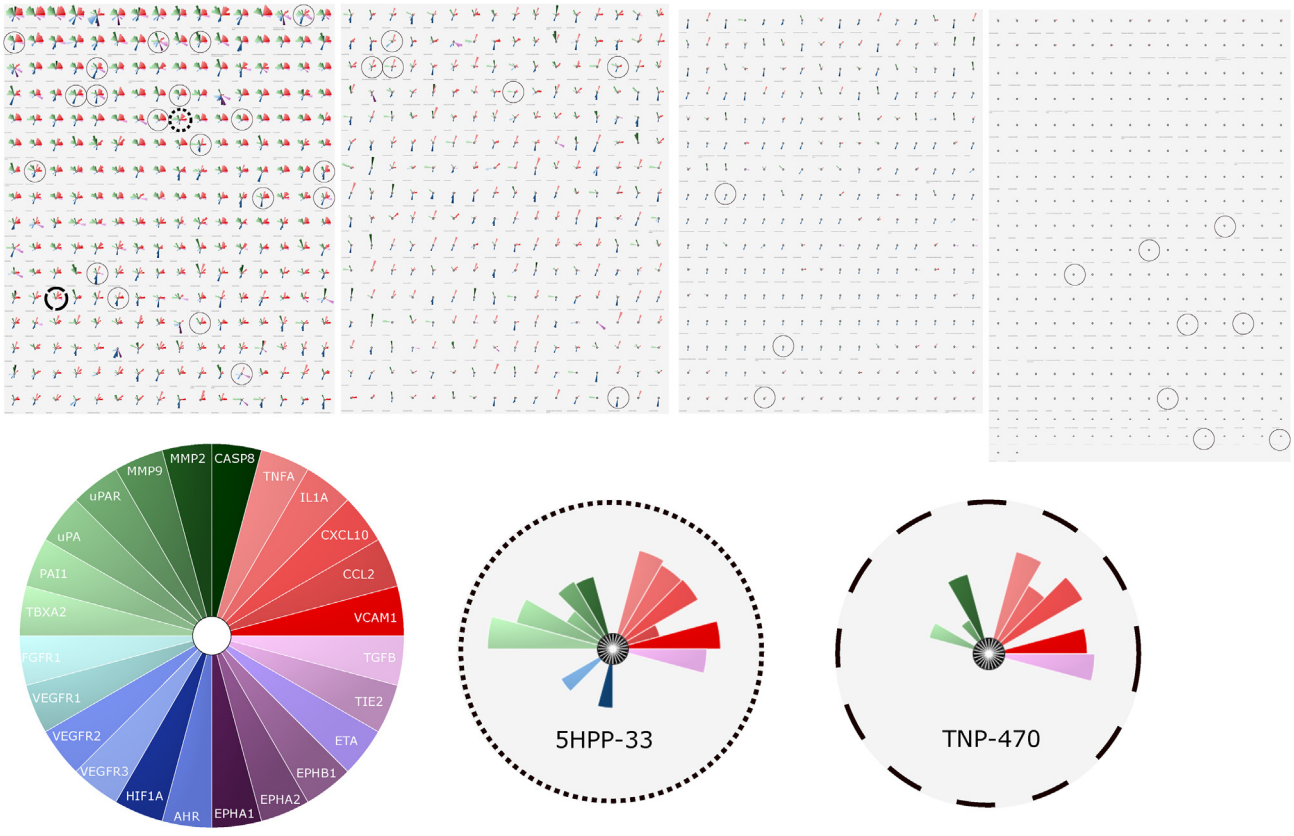
Pathway analysis of the 9006 differentially expressed genes (DEGs) for TNP-470 and 5HPP-33 identified several pathways as being unique to 5HPP-33 versus TNP-470 embryotoxicity, which could explain the higher potency at which TNP-470 causes vascular defects or the specific nonvascular effects such as somite defects and caudalization observed in WECs at 48 h. While differences in gene expression changes were expected on the basis of different phenotypic outcomes, we also hypothesized that similarities in DEG patterns would be tied to disruption of vascular patterning. Unsupervised hierarchical clustering of embryonic DEGs indicated transcriptional responses to the highest concentration of 5HPP-33 (30 µM), which were similar to responses elicited by TNP-470 at any concentration (Figure 3). TNP-470 invoked concentration-dependent changes in the subset of DEGs at 4 h of exposure, consistent with the concentration-dependent dysmorphogenesis observed at 48 h. Three canonical pathways were consistently implicated in both TNP-470 and 5HPP-33 toxicity: farnesoid X receptor (FXR)/retinoid X receptor (RXR) activation, liver X receptor (LXR)/RXR activation, and p53 signaling. FXR and LXR activation were among the top pathways for TNP-470 2.5 µM and 25 µM and 5HPP-33 30 µM. Based on gene expression changes, inhibition of FXR and LXR pathways may be key events via RXR heterodimerization. Similarly, inhibition of p53 signaling was implicated as a shared mechanism for TNP-470 2.5 µM and 5HPP-

33 5 μM versus DMSO controls, consistent with ToxCast™ predictions. P53 signaling was the top canonical pathway for TNP-470 at 2.5 μM. Although it was not a top enriched pathway for 5HPP-33 at 5 μM, TRP53COR1, a corepressor of p53, was implicated as a potential upstream regulator by the increased expression of three downstream genes: *Foxe1*, *Lhx5*, and *Ntn1*. Moreover, the highest concentrations that caused overt phenotypes (5HPP-33, 30 μM; TNP-470 25 μM) were associated with gene expression changes greater than eightfold for p53-regulated genes such as *Ccng1*, *Cknd1*, and *Fas*. It is worth noting that systemic toxicity may mediate the higher concentration effects associated with p53 signaling; however, the lower concentration effects are more likely linked to specific vascular genes involved in this pathway as they are below expected cytotoxicity thresholds identified from the ToxCast™ data set. While p53 signaling is not currently incorporated into Aop43, literature evidence also supports a link between p53 signaling, VEGF repression [39], and TNP-mediated angiogenesis disruption [40]. Overall, the transcriptomics study demonstrates that whole-genome transcriptome profiling of tissues from rat embryos exposed to known VDCs can be used to identify toxicity pathways and biological processes associated with, but not necessarily exclusive to, embryonic vascular disruption. Taken together, the assays described in this article provide some support that *in silico* chemical screening to predict *in vivo* bioactivity while reducing animal experimentation is becoming more realistic.

Application

The *in vivo* tests used for assessing prenatal developmental toxicity for regulatory purposes describe gross apical endpoints but are not designed for mechanistic insights. As such, a challenge for predictive toxicology is characterizing surrogate processes that can be monitored by new approach methodologies such as HTS and interpreted within the context of an adverse developmental outcome. Altered blood vessel formation and remodeling is one potential surrogate, substantiated by results from a large number of HTS and functional assays with strong correlations to *ex vivo* developmental effects. Through whole-genome sequencing, we uncovered additional pathways that bolster our mechanistic understanding of the role of embryonic vascular

Figure 4



Updated pVDC signature ranking the 38 chemicals (circled; selected to represent a range of pVDC scores) among 1058 ToxCast™ phase I and II chemicals. Each ToxPi slice represents a ToxCast™ molecular target included in the Aop43 model as described in the study by Knudsen and Kleinstreuer [5]. 5HPP-33 and TNP-470 rank in the top 25% of chemicals. pVDC, putative vascular disrupting compound.

disruption in developmental toxicity and help refine the vascular disruption AOP.

The WEC transcriptomics study identified molecular signaling pathways that are not currently incorporated into Aop43. These findings, in addition to new RNA-seq results, demonstrate the importance of p53 signaling in developmental angiogenesis and support the hypothesis that chemical disruption of canonical vascular developmental pathways may invoke adverse developmental outcomes of a diverse nature, from embryolethality to morphological defects. These data also provide a more comprehensive list of the early transcriptomic responses following potential MIEs that can be scaled to larger chemical libraries [41].

Since the publication of the pVDC manuscript [5], additional ToxCast™ chemical library phases were tested. We now have 1059 chemicals with HTS bioactivity data covering the pVDC signature that is publicly available in ToxCast™ [InvitroDB_v2] (https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data). Applying the same pVDC signature usingToxPi (v2.1) software [42] resulted in a new chemical ranking with 5HPP-33 and TNP-470 among the top 25% and the other 36 chemicals generally retaining their respective locations on the pVDC spectrum consistent with previous predictions (Figure 4). This analysis also identified several new pVDCs with ToxPi scores greater than those of 5HPP-33. For example, farglitazar (Figure 2, row 1, 9th from left) is a synthetic peroxisome proliferator–activated receptor-gammaligand [43], a group studied as angiogenesis inhibitors and developmental toxicants [44–46]. Additionally, tributyltin chloride (Figure 2, row 1, 2nd from left) is cytotoxic to endothelial cells [47,48] and causes embryolethality when administered during organogenesis [49].

It is important to note that tributyltin chloride might inhibit angiogenesis through more specific modes of action beyond cytotoxicity. Moreover, this and other chemicals may target additional cell types (e.g., neurons [50]) and organ systems beyond the scope of Aop43. For example, tributyltin chloride is a neurobehavioral toxicant [51], implicating the central nervous system as a target organ of toxicity. Thus, while Aop43 is useful for identifying potential vascular disruptors, this endpoint may not always be the most important from a regulatory decision-making perspective. Furthermore, for some chemicals, the primary targets may not always be the vasculature, particularly in the case of exposures resulting in sublethal or subteratogenic adverse outcomes. These caveats support the need for follow-up *in vitro* and *in silico* studies to investigate the angiogenesis disruption potential, as well as other target organ effects, of the high-ranking pVDCs shown in Figure 4. Taken together, mapping HTS features to AOPs brings into context the weight of evidence for critical determinants that potentially invoke the altered phenotype in a self-organizing, integrated system. AOPs provide the necessary structure for quantitative prediction of cellular and tissue responses to molecular perturbation, and such frameworks provide mechanistic insight to chemical hazard assessments in a rapid and robust manner.

Disclaimer

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Conflict of Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cotox.2019.04.004.

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- • of special interest
- •• of outstanding interest

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