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Embryonic vascular disruption adverse outcomes: Linking high throughput signaling signatures with functional consequences

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ABSTRACT

Embryonic vascular disruption is an important adverse outcome pathway (AOP) as chemical disruption of cardiovascular development induces broad prenatal defects. High throughput screening (HTS) assays aid AOP development although linking *in vitro* data to *in vivo* apical endpoints remains challenging. This study evaluated two anti-angiogenic agents, 5HPP-33 and TNP-470, across the ToxCastDB HTS assay platform and anchored the results to complex *in vitro* functional assays: the rat aortic explant assay (AEA), rat whole embryo culture (WEC), and the zebrafish embryotoxicity (ZET) assay. Both were identified as putative vascular disruptive compounds (pVDCs) in ToxCastDB and disrupted angiogenesis and embryogenesis in the functional assays. Differences were observed in potency and adverse effects: 5HPP-33 was embryolethal (WEC and ZET); TNP-470 produced caudal defects at lower concentrations. This study demonstrates how a tiered approach using HTS signatures and complex functional *in vitro* assays might be used to prioritize further *in vivo* developmental toxicity testing.

1. Introduction

Embryonic vascular disruption is an important adverse outcome pathway (AOP) given the knowledge that chemical disruption of early cardiovascular system development leads to broad prenatal defects. The cardiovascular system is the first functional organ system to develop in mammals [1]. The primary vasculature differentiates from angioblasts to form the primitive vascular plexus in the early embryo and visceral yolk sac (vasculogenesis). These systems undergo subsequent remodeling and expansion (angiogenesis) to form the mature cardiovascular system [1]. Since numerous biological processes are linked to vascular development, *in utero* vascular disruptions are thought to be associated with a variety of birth defects and pregnancy complications [2] ranging in nature and severity from embryolethality [3] to preeclampsia [4], microphthalmia [5], and limb defects [6] to name a few. Vascular disruption was identified as one of 6 teratogenic mechanisms linked with medications taken by women of childbearing potential [7]. In humans, the most common apparent cause of limb deficiencies was found to be vascular disruption defects [8]. Susceptibility to thalidomide was linked to the disruption of immature angiogenic network at time of exposure [9]. Predicted vascular disrupting chemicals in ToxCast correlate with developmental toxicity [10]. Finally, many genetic and environmental factors can alter molecular pathways regulating angiogenesis [11].

Development of high-throughput screening (HTS) and *in vitro* profiling assays in recent years has generated advances towards providing the experimental throughput, target specificity, and computational capabilities necessary to thoroughly investigate vascular developmental toxicity. For example, the US EPA's ToxCast program and the Tox21 consortium generate *in vitro* HTS data on 1000′s of chemicals against 100′s of molecular targets and biological pathways [12,13]. These HTS data facilitate chemical hazard identification to support chemical prioritization efforts for further evaluation under existing regulatory requirements (e.g. Endocrine Disruptor Screening Program), establishment of AOPs for toxicity mechanisms, and development of computational predictive models for these mechanisms. Predictive models for prenatal developmental toxicity [14] as well as disruptions in embryonic vascular development [11,15] were recently developed, using this large *in vitro* dataset to build mechanistic understanding of adverse developmental outcomes.

One potential challenge with building predictive models from the existing HTS dataset is that the vascular disruption assays

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within the ToxCast and Tox21 programs (ToxCastDB, http://epa.gov/ncct/toxcast/data.html) consist mainly of cell-based or cell-free biochemical endpoints targeting specific molecular regulators of vasculogenesis or angiogenesis [10]. In contrast, most *in vivo* developmental toxicity assays, particularly regulatory guideline studies such as those compiled in US EPA's Toxicity Reference Database (ToxRefDB, http://actor.epa.gov/toxrefdb/faces/Home.jsp), are based on descriptive, apical endpoints which have little depth or detail for specific mechanisms of action. To evaluate the predictive capability of the ToxCast and Tox21 program assays, there is a need for an intermediate tier of functional assays that provide more specific information on vascular disruption and resulting adverse outcomes. In vitro functional assays of intermediate complexity, while lower in throughput, may retain many or all the cells of the tissue with intact cell-matrix interaction and complexity and therefore are considered as a bridge between in vivo and cell culture systems. Examples where intermediate functional assays have shown utility include improved drug liability in safety assessments for cardiovascular [16] and drug-induced liver injury [17].

In the present study, we conducted an intermediate tier of assays to help bridge our understanding of vascular disruption effects, using vascular outgrowth and embryonic development assays as a proof of concept. The rat aortic explant assay (AEA) was included as a prototypical angiogenesis assay [18] while the rat whole embryo culture (WEC) assay and zebrafish developmental toxicity (ZET) assay were included to assess embryo development [19,20]. The WEC assay has played a pivotal role in advancing our knowledge of normal embryonic development evidenced by the role it played in studies of normal gastrulation [21], neural tube formation [22], craniofacial development [23,24], and cardiac development [25], just to name a few. The WEC and ZET intact embryo assays have been shown to have test accuracies for in vivo teratogencity prediction of 68–80% and 75–92% [26], respectively, and been previously used to assess vascular remodeling, endothelial cell proliferation, placental development, and vessel disruption [27–31]. Results obtained in the AEA, WEC, and ZET assays can be related back to the HTS data to support further assessment of how well the ToxCast data may predict vascular disruption. Dose-response data derived from this study may assist interpretation of HTS assay results, particularly in benchmarking for positive responses. We tested the hypothesis that compounds predicted by ToxCast and Tox21 HTS assays to be putative vascular disrupting compounds (pVDCs) as a result of their high vascular bioactivity toxicological prioritization index (ToxPi) score [10,11,15], would demonstrate developmental toxicity and vascular disruption in the selected intermediate tier assays mentioned above. An additional goal of the research was to understand if the developmental toxicity was a consequence of vascular disruption or an alternative mechanism of action. Here, we evaluated two anti-angiogenic compounds with high ToxPi pVDC scores and differing mode(s) of action, 5HP*P*-33 and TNP-470, in the three functional assays mentioned above.

The two compounds selected as a proof of concept for embryonic vascular disruption include a synthetic thalidomide analogue (5HP*P*-33) and a synthetic fumagillin analogue (TNP-470). Although *in vivo* data in mammalian systems or humans are not available for 5HP*P*-33, thalidomide is well known both for its anti-angiogenic [32] and teratogenic [33] properties. Across a number of synthetic thalidomide analogues, 5HP*P*-33 showed the most potent anti-angiogenic activity in HUVEC cells [32]. Other thalidomide derivatives are well studied for their vascular developmental toxicity (e.g., CPS49), with primary findings being the prevention of blood vessel outgrowth and remodeling, loss of angiogenic vessels, and induction of limb reduction defects in chick embryos [9]. Proposed modes of action of 5HP*P*-33 include alteration of tubular polymerization [34–36], inhibition of mitosis [35], induction of apoptosis [37], extracellular matrix remodeling [15,34], and inhibition of NF-κB [38]. TNP-470 was developed as an anti-angiogenic cancer therapeutic agent with a reduced preclinical toxicity profile to replace the antifungal, fumagillin [39]. Tumor growth and angiogenesis is inhibited by TNP-470 in mice bearing Lewis lung carcinoma cells [40] and while *in vivo* developmental data in mammalian systems is not available from regulatory guideline toxicity studies, TNP-470 administered to mice caused microphthalmia and reduced hyaloid vessel length in one study [5] and disrupted placental development resulting in complete embryonic resorptions in another [41]. In the latter study, decreased blood vessel density was observed in E9 (embryonic day 9) following TNP-470 administration on E7. TNP-470′s suspected mode of action includes disruptions of methionine aminopeptidase II (MetAP2) in vascular endothelial cells resulting in their cytostatic growth arrest [42]. Experiments with microtubule-associated protein 2 (MAP2) conditional knockout mice and MAP2-null lines further indicate a role for MetAP2 in early embryonic development as well as in a p53-dependent gastrulation checkpoint [43]. While it was hypothesized that both 5HP*P*-33 and TNP-470 would induce developmental toxicity and vascular disruption in the selected intermediate tier assays, the separate anti-angiogenic mechanisms of action for these pVDCs should result in distinct effects on embryonic development. This paper is part of a larger research program focusing on validation/qualification of pVDC signatures in varying model systems including: evaluation of the ToxCast Phase I [44] and ToxCast Phase II chemical libraries using zebrafish, human endothelial cells [45], and an RNAseq analysis of 5HP*P*-33 and TNP-470 in culture rat embryos [46] with the parallel goal of an AOP Wiki developed for this pathway (https://aopwiki.org/wiki/index.php/Aop:43).

2. Materials and methods

2.1. Chemicals

5HPP-33 (H9415, Lot number 09K46131; CASRN 105624-86-0) and TNP-470 (T1455, Lot number 021M4609 V; CASRN 129298-91-5) were obtained from Sigma-Aldrich Chemical Company (St. Louis, Missouri) and had purities of ≥ 98%.

2.2. Animal husbandry

All animal studies described in this paper were performed at The Dow Chemical Company (Midland, MI). The laboratory is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and all animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at The Dow Chemical Company. Nulliparous adult female Crl:CD(SD) rats (Charles River Breeding Laboratories, Portage, MI) were used in AEA and WEC experiments while New Zealand White rabbits (Covance Research Products, Kalamazoo, MI) were used in AEA experiments. Rats used in the WEC experiments were time-mated at the breeders' facilities. In order to promote uniformity in stage of embryo development, mating was restricted to approximately three hours (4–7 p.m.) and the following day was considered gestation day (GD) 0. The animals were housed singly in stainless steel cages in rooms designed to maintain environmental conditions for temperature (22 ± 3 °C for rats, 20 ± 1 °C for rabbits), humidity (40–70% for rats, 40–60% for rabbits), and photocycle (12-h light/dark). Rats were housed either in wire bottom cages suspended above catch pans or in solid bottom cages with absorbent bedding. Cages contained a hanging feeder and pressure-activated lixit valve-type watering system. Environmental enrichment for the rats included non-woven gauze placed in the cages (wire bottom cages) or use of ground corn cob and shredded Aspen bedding and open areas on the cage sides