



Review

An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis



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ARTICLE INFO

Article history:

Received 28 July 2014

Received in revised form

12 September 2014

Accepted 7 October 2014

Available online 16 October 2014

Keywords:

Retinoic acid

Embryogenesis

Developmental toxicity

Malformations

Neural tube patterning

Axial patterning

Biomarkers

Gene expression

ABSTRACT

Developmental toxicity can be caused through a multitude of mechanisms and can therefore not be captured through a single simple mechanistic paradigm. However, it may be possible to define a selected group of overarching mechanisms that might allow detection of the vast majority of developmental toxicants. Against this background, we have explored the usefulness of retinoic acid mediated regulation of neural tube and axial patterning as a general mechanism that, when perturbed, may result in manifestations of developmental toxicity that may cover a large part of malformations known to occur in experimental animals and in man. Through a literature survey, we have identified key genes in the regulation of retinoic acid homeostasis, as well as marker genes of neural tube and axial patterning, that may be used to detect developmental toxicants in *in vitro* systems. A retinoic acid–neural tube/axial patterning adverse outcome pathway (RA–NTA AOP) framework was designed. The framework was tested against existing data of flusilazole exposure in the rat whole embryo culture, the zebrafish embryotoxicity test, and the embryonic stem cell test. Flusilazole is known to interact with retinoic acid homeostasis, and induced common and unique NTA marker gene changes in the three test systems. Flusilazole-induced changes were similar in directionality to gene expression responses after retinoic acid exposure. It is suggested that the RA–NTA framework may provide a general tool to define mechanistic pathways and biomarkers of developmental toxicity that may be used in alternative *in vitro* assays for the detection of embryotoxic compounds.

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Contents

1. Introduction	105
2. Methods	106
3. Results	107
3.1. Retinoic acid balance	107
3.2. Cellular proliferation, migration, and differentiation	107
3.3. Neural tube patterning	107
3.4. Axial patterning	107
3.5. Flusilazole embryotoxicity	108
3.6. WEC	108
3.7. EST	108
3.8. ZET	109

Abbreviations: AOP, adverse outcome pathway; BMD, benchmark dose; CNS, central nervous system; EST, embryonic stem cell test; ESTn, embryonic stem cell test–neural; GMS, general morphology score; GO, gene ontology; MIE, molecular initiating event; NCBI, National Center for Biotechnology Information; NTA, neural tube/axial; NTD, neural tube defects; OECD, organization for economic cooperation and development; RA, retinoic acid; RARE, retinoic acid responsive element; WEC, rat whole embryo culture; ZET, zebrafish embryotoxicity assays.

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3.9. Comparison of flusilazole and RA effects in developmental assays.....	110
4. Discussion	110
Conflict of interest	112
Acknowledgments	112
References.....	112

1. Introduction

Neural tube and axial defects of the vertebrate embryo are among the most common developmental malformations in mammalian species including man. They include neural tube defects (NTD), which are among the most prominent birth defects in the human population. Birth defect registries have reported a prevalence of around 35 cases of spina bifida, 20 cases of anencephaly, and 10 cases of encephalocele per 100,000 births [1]. In the US, the total number of new neural tube defect cases annually amounts to 2500. Moreover, disruption of axial development may result in a variety of craniofacial, limb as well as cardiac and other malformations. Also in experimental animal studies for chemical and pharmaceutical hazard assessment, neural tube and axial defects are frequently observed findings [2]. Human teratogens such as anticonvulsants (e.g. valproate and carbamazepine), cytostatic agents (e.g. cyclophosphamide and methotrexate) and retinoids have been shown to increase the risk for such defects [3–5]. The fetal alcohol syndrome has also been hypothesized to be mediated by changes in retinoic acid homeostasis [6]. Neural tube and axial defects are a consequence of perturbation of rostrocaudal growth and differentiation in the very early stages of vertebrate embryogenesis. It is evidently important to be able to predict a compounds' capacity to induce such malformations before deciding on its targeted use and release on the market.

The assessment of developmental toxic potential of chemicals is classically derived from experimental animal studies according to protocolled, globally harmonized test guidelines [7]. These studies have proven useful in providing overall hazard information based on apical endpoints such as resorptions, prenatal death, growth retardation and malformations. Decades of experience with these studies have shown that the nature of apical endpoints cannot readily be extrapolated between species and not even between strains of the same species [2,8,9]. The relevance of apical animal findings for the human situation is therefore not always clear-cut. However, the basic physiological regulation of embryogenesis is highly conserved among vertebrates and especially among mammalian species. Therefore, it is anticipated that mechanistic information on the interference of chemicals with embryogenesis on the molecular level would provide a more informative background for hazard and risk assessment for man. Such data can be collected either in animal studies or from animal or human cell and tissue culture models. Thus, such approaches not only allow a more detailed insight into mechanisms of dysmorphogenesis in animals, but also facilitate direct comparison with the human situation. The US National Academy of Sciences spread this notion in their report on Toxicity Testing in the 21st century [10]. This report proposes to define a limited number of essential toxicity pathways that could be covered by a limited number of animal-free alternative test systems. Interference at a certain magnitude with these pathways would be indicative of toxicity at the level of the organism. The Organization for Economic Cooperation and Development (OECD) followed up on this proposal by their initiative to define Adverse Outcome Pathways (AOP) [11–13]. These were defined as linear cascades of effects leading from a Molecular Initiating Event (MIE) via subsequent consequences at the cellular and organ level to an overt adverse health effect. AOPs are thought to become instrumental in a future of innovated hazard and risk assessment applying molecular-based alternative test models,

reducing cost, time and animal use, and enhancing mechanistically based prediction of human hazard and risk. Several preliminary AOPs have been generated for further optimization [13,14]. A linear AOP, as proposed by OECD, may indeed suffice for mechanisms where a clear stepwise linear cascade of events describes all the critical stages of the AOP, such as has been envisaged for genotoxic carcinogens or for sensitizers. However, for complex processes such as embryogenesis, where the internal balance of a wealth of competing factors and interacting cell types changes continuously with time and location in the developing embryo, a simple linear approach would undoubtedly miss major players within the AOP, which would significantly reduce its usefulness.

In the current study, we constructed an AOP framework, linking a variety of possible MIEs to yet another variety of adverse outcomes, and thus containing a combination of possible AOPs. The framework is based on the essential role of retinoic acid (RA) in the formation of, and differentiation within the neural tube and body axis. RA is the active form of vitamin A, and the conversion of vitamin A in the body is carefully limited to the extent that the active form is necessary within time- and place-dependent physiological homeostasis. As its diversity of functions in embryogenesis became apparent, RA has been named a morphogen, indicating the fundamental importance of this molecule in embryonic development [15]. The guiding role of retinoic acid is critically dependent on finely tuned tissue concentrations that vary with the location within the embryo as well as with time during embryogenesis. Subtle disturbances of retinoic acid levels can have grave developmental consequences, as has been shown in extensive animal developmental toxicity studies with retinoids as well as in human subjects after the intake of retinoic acid in multivitamin preparations during pregnancy [16–19]. Conversely, RA depletion has similar untoward effects on embryogenesis. The dysmorphogenic effects of the disturbance of RA balance are not limited to the body axis. In addition, all tissues and organs that receive a contribution from neural crest cells migrating into peripheral tissues are vulnerable to retinoic acid embryopathy. This includes facial structures such as the branchial arches and the ears, the heart, and the limbs. The complexity of RA embryopathy stipulates the central role of RA in embryogenesis. As a consequence, we anticipate that an AOP framework based on MIEs interfering with RA homeostasis and related adverse outcomes regarding (dys)morphogenesis of the body axis could allow the detection of a major subset of developmental toxicants. Some of these embryotoxicants may interfere with components of this RA–neural tube/axial (RA–NTA) framework directly, whereas others may have an MIE outside this domain but show secondary effects mediated by components of the framework and may thus be detected through interference with this AOP framework anyway.

The effectiveness of retinoic acid at a given time and location in the embryo is dependent on a combination of factors, all of which need to be considered when constructing an RA–NTA AOP framework. In addition to time in development and location in the embryo, these factors include the RA concentration, and the presence of other regulators that stimulate or suppress the effect of RA. In the following sections, we have mapped critical molecular components of retinoic acid regulated neural tube and axial morphogenesis, based on existing literature data. The multidimensional cascade starts with retinoic acid balance, the concentration of which is regulated by synthesizing and metabolizing enzymes.

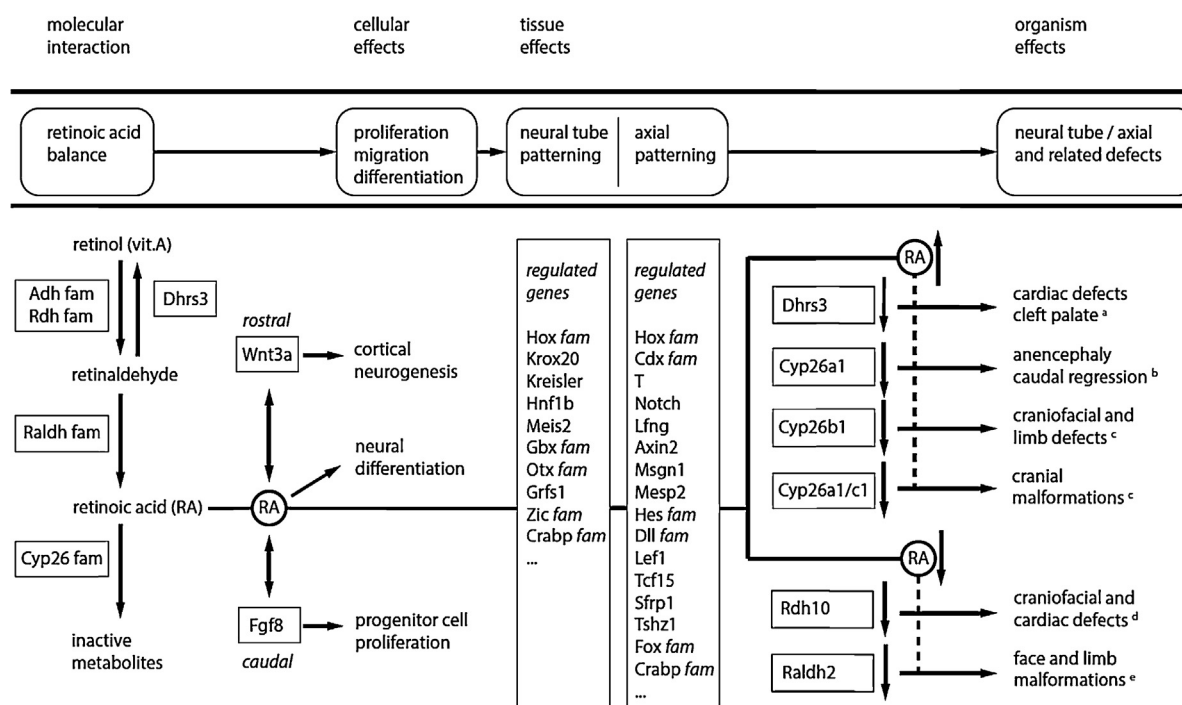


Fig. 1. Schematic representation of the proposed retinoic acid–neural tube/axial patterning adverse outcome pathway (RA–NTA AOP) framework. References for indicated defects: a [34], b [37], c [38], d [32], and e [30]. For detailed explanation, see text.

As retinoic acid is primarily a patterning factor by cell differentiation induction [20], we subsequently mapped critical interactions between RA and directly competing factors that affect the cellular decision to proliferate or differentiate. This decision also affects the migration of cells, which differs with the differentiation stage. In very general terms, embryonic stem cells and mature differentiated cells tend to be more attached in their local environment whereas progenitors in intermediate stages of differentiation are more mobile [21]. At the tissue level, we mapped positional information represented by localized gene expression patterns that modulate the local cellular response to retinoic acid, affecting tissue development. We have limited this morphogenetic survey to neural tube and axial patterning, providing a workable framework size without attempting to be exhaustive. We collected a series of molecular markers of dysmorphogenesis from the scientific literature. Finally, morphogenetic changes causally linked to neural tube and axial defects were collected, completing the cascade from initiating events at the molecular level to adverse outcomes at the level of the organism.

We illustrate the applicability of the RA–NTA AOP framework using our extensive database on concentration dependent flusilazole-mediated effects in developmental models [22–25]. Flusilazole is a triazole compound that modulates RA balance and this is thought to be an important mechanism underlying its developmental toxicity [26]. Triazoles inhibit the activity of many cytochrome P450 (Cyp) enzymes. The inhibiting effect on Cyp51 that plays a role in the conversion of lanosterol to ergosterol in fungi and yeast gives triazoles their antifungal capacity [27]. However, the developmental toxic effects of triazoles are likely related to Cyp26 inhibition [22]. Dysmorphogenic effects observed after triazole exposure include skeletal defects (vertebral transformations), craniofacial malformations, altered hindbrain patterning and defects in the caudal region [28].

Not only did this analysis confirm that crucial elements of the RA–NTA AOP framework are actually detectably responsive and can be used as (components of) a molecular biomarker for developmental toxicity. Moreover, these analyses illustrate the diverging niches

of the different assays within the developmental landscape. Thus, this approach did not only allow dedicated detection of compounds that affect neural tube and axial development. It also assisted in defining in more detail the biological applicability domains of alternative assays, a crucial exercise in determining the optimal place of a given alternative assay within a testing strategy of combined assays.

2. Methods

A theoretical RA–NTA AOP framework was designed based on existing literature data (Fig. 1). The framework was based on retinoic acid balance, followed by its roles in central nervous system development and axial patterning, the identification of critical genes that might be used as biomarkers of developmental changes, and culminating in RA-dependent adverse developmental effects. The framework was used as a starting point to look for RA-mediated gene expression changes in flusilazole-challenged developmental model systems, rat whole embryo culture (WEC), the embryonic stem cell test (EST), and the zebrafish embryotoxicity test (ZET). Potential RA–NTA biomarker genes were identified from the Gene Ontology Biological Process *Anterior–posterior pattern formation* [29], supplemented with genes involved in RA biosynthesis and metabolism, genes with retinoic acid responsive elements (RAREs) [30] and genes selected based on survey of relevant scientific literature. (The quoted GO term name is somewhat of a misnomer as it covers neural tube and axial developmental genes as well and contains the majority of NTA genes mentioned in this manuscript.) Gene homologs for other species (rat, zebrafish) were matched to the corresponding mouse gene using NCBI homologue and official gene symbols.

From our own previous studies in the model systems mentioned above [22–25], dose–response data for the selected genes were analyzed using the Benchmark Dose (BMD) approach using PROAST software ([31]; www.rivm.nl/proast). For each individual gene, the optimal model was selected from the nested exponential model family using a likelihood ratio test. In addition, a goodness

of fit test was applied by comparing the log-likelihood of the fitted model with that associated with the 'full model'. For each assay the dose–response curve for the classical endpoints was determined using the same approach (WEC: malformation rate; EST: inhibition of differentiation; ZET: general morphology score (GMS)). Subsequently, genes were identified as potential biomarkers if the BMD associated with a 5% benchmark response for that gene was below the BMD for the classical endpoints of the particular assay.

3. Results

3.1. Retinoic acid balance

RA is produced from the dietary vitamin A (retinol) supply through a 2-step process *via* retinaldehyde (Fig. 1). Although several enzymes, such as the ubiquitously expressed alcohol dehydrogenase *Adh7*, are able to oxidize retinol into retinaldehyde, genetic studies show that retinol dehydrogenase *Rdh10* plays an important role in this critical, rate-limiting step in the synthesis of RA. Loss of *Rdh10* activity leads to abnormalities that are characteristic of an RA-deficiency phenotype [32], including craniofacial, cardiac and forelimb defects, culminating in embryonic death. The local RA concentration in a given tissue is primarily driven by the balance between a family of retinaldehyde dehydrogenases (*Raldh*) and a family of *Cyp26* enzymes. *Raldh* produce RA from retinaldehyde. *Cyp26* enzymes metabolize RA into inactive derivatives. The expression of *Raldh* and *Cyp26* enzymes is tissue dependent and strictly regulated within the developmental program. The *Dhrs* enzyme family converts retinaldehyde back to retinol, thereby regulating the amount of available substrate for RA production by *Raldh* enzymes. RA downregulates *Raldh2* and *Rdh10* and upregulates *Dhrs3*, *Cyp26* and the retinoic acid binding protein *Crabp2* [33], by which self-enhanced degradation is achieved. Along with *Cyp26*, *Dhrs3* plays an important role in prevention of excess RA formation and *Dhrs3* ablation leads to defects typically associated with exposure to high levels of RA during embryogenesis or *Cyp26* deficiency, such as cardiac malformations and cleft palate [34]. This provides a complex feedback system by which RA levels in tissues are carefully controlled locally. *Raldh* enzymes are primarily expressed in mesodermal tissues, and stimulate RA-mediated neural differentiation from progenitor cells in the neuroectoderm [30]. *Raldh2* knockouts show malformations of face and limbs reminiscent of lack of differentiation, due to the absence of the differentiating stimulus that RA offers [30], which is accompanied by a dysregulation of a host of patterning genes [35]. Studies using knockout mice have shown that *Raldh2* is most important for the conversion of retinaldehyde into RA during early embryogenesis [36]. *Raldh1* and *Raldh3* have specific functions at later stages during eye and nasal development. *Cyp26* family members are also location-specifically expressed for a fine-tuned regulation of RA levels. For instance, *Cyp26a1* is expressed in the anterior neural plate at rhombomere 2, and *Cyp26a1* knockouts display anencephaly. In addition, *Cyp26a1* knockouts display caudal regression, caused by an overload of RA that stimulates precocious differentiation at the expense of caudal growth [37,38]. *Cyp26b1* is expressed in the hindbrain and limb buds, and consequently *Cyp26b1* knockouts show craniofacial malformations and limb defects [38]. *Cyp26c1* is expressed in the anterior neural plate, and its knockout together with *Cyp26a1* ablation results in cranial malformations [38].

3.2. Cellular proliferation, migration, and differentiation

Rostro-caudal patterning of the neural tube is influenced by at least three morphogen gradient systems, RA, *Wnt3a* and *Fgf8* [20]

that determine location-specific cell proliferation, migration and/or differentiation (Fig. 1). All three are produced in the paraxial mesoderm during gastrulation where they regulate rostral and caudal expression of genes involved in rhombomere specification. *Wnt3a* dominates in the forebrain region, where it induces, e.g., cortical neurogenesis, whereas *Fgf8* shows an increasing concentration gradient rostro-caudally and supports progenitor cell proliferation and caudal extension [20]. RA is an important morphogen with local effects throughout the body axis, inducing, e.g., neural cell differentiation. The regulation of RA biosynthesis and metabolism is therefore region-specific. In addition, RA is thought to be a diffusible morphogen [36] able to act as a graded signal over long distances [39]. This RA gradient is also controlled by local RA degradation in which *Cyp26a1* plays an important role. *Fgf8* and *Wnt3a* also exhibit gradients along the rostro-caudal axis [40]. These morphogen gradient systems are interdependent and linked at the level of RA gradient formation [33,41]. For instance in hindbrain *Fgf8* and *Wnt3a* inhibit the upregulation of *Cyp26a1* by RA and during caudal body development *Fgf8* antagonizes RA signaling by repressing *Raldh2* expression [42]. RA signaling appears mutually antagonistic as transient RA treatment during somitogenesis restricts *Fgf8* and *Wnt3a* signaling. The ultimate functions of morphogen gradient systems are to specify distinct, spatial domains of gene expression and cell differentiation [33].

3.3. Neural tube patterning

Fig. 1 lists genes identified as important players in neural tube patterning. In the hindbrain, RA initiates early expression of the *Hox* gene family and patterning [30,43]. *Hoxa1*, *Hoxb1*, *Hoxa4*, *Hoxb4*, and *Hoxd4* genes all contain retinoic acid responsive elements (RAREs) and their expression is initiated in response to RA. Auto- and cross-regulatory loops between *Hox* genes themselves and rhombomere-specific transcription factors *Krox20* (*Egr2*), *Kreisler* (*Mafk*), and *Hnf1b* (*vHnf1*) provide additional regulatory input to modulate *Hox* genes expression during segmental regulation [43]. This regional diversity results in segment-specific developmental programs regulating the identity of the hindbrain neurons and hindbrain-derived neural crest cells, which play a role in the development of the brain stem, but also influence the development of inner ear, branchial arches, and heart and large vessels [30]. Retinoic acid induced hindbrain segmentation defects are attributed to alterations in *Hox* gene expression [44]. *Raldh2* loss of function and vitamin A-deficiency result in misspecification of the caudal hindbrain in which the putative rhombomere 4–8 (r4–r8) regions are reduced and/or respecified toward more anterior (r3- and r4-like) identity. Thus, r3–r4 markers *Hoxb1* and *Krox20* (*Egr2*) spread posteriorly, whereas r5–r7 markers (*Kreisler* (*Mafk*), *Hoxd4*) are reduced or abolished [30,37]. *Cyp26a1* loss of function results in misspecification of a region of the hindbrain positioned just rostrally to the region affected by retinoid deficiency [37]. Although the overall rhombomeric organization appeared unaltered, further analysis of the consequences of *Cyp26a1* loss of function revealed an anterior expansion of r4 (*Hoxb1* expression) and a partial posterior transformation of r2–r3 (ectopic *Hoxb1* expression and lower expression *Meis2* in r2) [37]. Besides *Hox* genes, the *Gbx* and *Otx* gene families have important roles in body segmentation throughout the animal kingdom [45]. *Gbx2* particularly determines the mid-hindbrain boundary in mice [46].

3.4. Axial patterning

RA is also needed during caudal elongation and plays a role in controlling mesodermal segmentation and differentiation, neurogenesis, and regional patterning of the spinal cord [36]. As the vertebrate embryo enters neurulation, body axis elongation

commences and the developing trunk extends along the rostro-caudal axis. During this process, there is a caudal growth zone where RA clearance is required and Cyp26a1 is expressed, and a rostral differentiation zone with high RA bioactivity due to Raldh2 expression in the (newly formed) somites. Similar to the situation in neural tube development, axis elongation is influenced by at least three morphogen gradient systems RA, Wnt3a and Fgf8 [20,42]. Cells in the caudal stem zone are in continuous proliferation as the embryo elongates, with Fgf8 as a key signal for the maintenance of this process [47]. Wnt3a also prevents premature growth arrest by upregulating Cyp26 expression through Cdx and Hox transcription factors. During the termination of body axis elongation Hox13 paralogs reduce Cyp26 expression and precocious Hox13 expression can therefore lead to a premature arrest of axial growth and axial truncation. Additional regulating genes may provide relevant markers for development (Fig. 1). Caudal-like homeobox genes (Cdx) are important during AP axis development as targets of both Wnt and RA signaling [40]. They also directly activate Hox gene expression. Another Wnt-target which plays a role in maintaining Wnt signaling in the posterior part of the growing embryo is T (Brachyury) [48]. Transcription factors T and Cdx, as well as Wnt and Fgf signaling, all were shown to be affected by loss or gain of RA biosynthesis [42]. In Cyp26^{-/-} mice, Wnt3a and T both were downregulated or their expression domains were spatially reduced [37,49]. Body axis elongation involves somitogenesis, a periodic arrangement during which somites are rhythmically produced from the paraxial mesoderm. This process relies on a 'clock and wavefront' mechanism. The 'segmentation clock' is a molecular oscillator driven by Wnt, Notch and Fgf signaling and generates cyclic waves of gene expression that progress rostrally along the presomitic mesoderm [30]. Presomitic mesodermal cells experience alternating cycles of Wnt and Notch signaling which are further enhanced by complementary cyclic expression of Notch signaling inhibitors (Lunatic fringe Lfng) and Wnt signaling inhibitors such as Axin2 [50]. The establishment of the first segmental prepattern occurs when presomitic mesodermal cells become competent to respond to the oscillations. This occurs at the level of the 'determination' or 'wave' front. The position of this front is determined by antagonistic gradients of Fgf/Wnt and RA [35,40,51]. The 'determination' front can be visualized using Mesogenin1 (Msn1) which is downregulated preceding the activation of Mesp2 [51]. Mutations in genes involved in somitogenesis like Lfng, Mesp2, Hes7 and Dll3 lead to the generation of skeletal and muscular deformities. Furthermore, RA has an important role in the bilateral symmetry of the left and right somite columns. RA deficient mouse embryos often exhibit fewer somites on one side (most often right) [30] and left–right asymmetric expression of Hes7 and Lfng was observed in the presomitic mesoderm [50]. RA is also essential for normal axial turning and spinal column development as RA-deficient mouse embryos are unable to undergo axial turning to achieve the normal fetal position and disruption of RA may be associated with human vertebral defects such as scoliosis [50].

3.5. Flusilazole embryotoxicity

To identify possible transcriptomic biomarkers of flusilazole embryotoxicity in the RA–NTA framework we analyzed concentration-dependent gene expression responses in alternative developmental toxicity assays carried out in earlier work from our laboratory. They included the rat whole embryo culture (WEC), the embryonic stem cell test (EST), and the zebrafish embryotoxicity assay (ZET) [25,52,53]. Genes related to neural tube and axial patterning were identified as potential biomarkers if the BMD associated with a 5% benchmark response for that gene was below the BMD for the classical endpoint of the particular assay. Fig. 2 combines the results in a Venn diagram showing unique and common

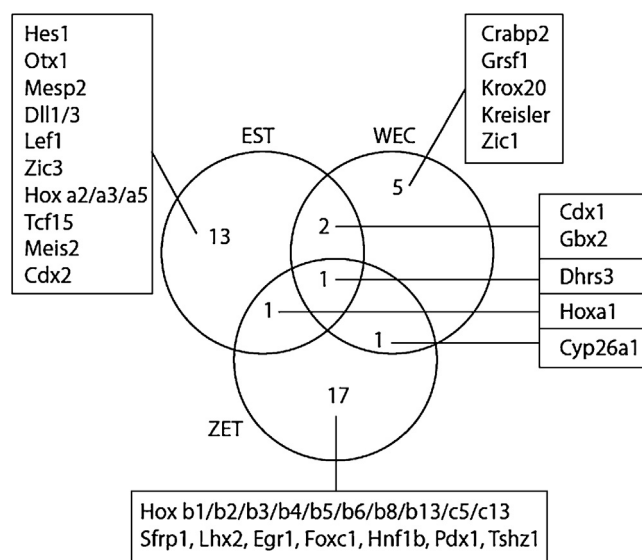


Fig. 2. Venn diagram showing neural tube and axial patterning developmental genes that showed a 5% effect size at lower concentrations than the classical endpoint in WEC, EST and ZET after flusilazole treatment.

genes regulated. Fig. 3 shows profiles of the dose-response curves for the expression of the genes identified as potential biomarkers together with the classical endpoints in the WEC, EST and ZET assays. In WEC, Dhhrs3 and the Wnt-target Grsf1 were responsive at relatively low exposure levels. In EST, Otx1, Gbx2, and Hoxa5 were responsive at relatively low exposure levels. Hoxa2 and Tcf15 also showed sensitive expression patterns. Tshz1 and Hoxb2a were responsive at relatively low exposure levels in ZET. In each model, additional genes showed concentration–response curves that showed 5% effect size levels at lower concentrations as compared to the classical readout in each model system.

3.6. WEC

In WEC, flusilazole-affected genes play a role in RA biosynthesis and metabolism (Dhhrs3, Cyp26a1, Crabp2) and in hindbrain patterning (Krox20, Kreisler). Other relevant genes affected by flusilazole in WEC were Cdx1, Gbx2, Grsf1 and Zic1. Cdx1 is mainly known for its role in trunk specification. In hindbrain Cdx1 also transiently represses Kreisler (Mafb) restricting its expression anterior of the r6/r7 boundary [54]. Gbx2 is a Wnt-target that plays an important role in the formation of the mid–hindbrain border and also specifies the anterior hindbrain [46]. Besides its role in axial elongation and axial mesoderm specification, the Wnt target gene Grsf1 is also involved in mid/hindbrain development, where it is necessary for maintaining Fgf8 and Gbx2 expression [55]. Zic (zinc finger of the cerebellum) transcription factors play a role in neural development and are suggested to be involved in the initiation and maintenance of RA metabolism gene expression during development [44,56]. Loss of function of Zic1 leads to forebrain and cerebellum defects and a decrease of neuronal differentiation in the spinal cord [57].

3.7. EST

In EST, flusilazole responses included Dhhrs3, involved in RA biosynthesis, as well as the patterning genes Cdx1 and Gbx2 (also regulated in WEC). Other identified genes involved in somitogenesis and/or axial elongation and patterning were Mesp2, Dll3, Cdx2, Hoxa1 (also regulated in ZET), Hoxa2, Hoxa3, and Hoxa5. Genes involved in somitogenesis that were also identified in EST were

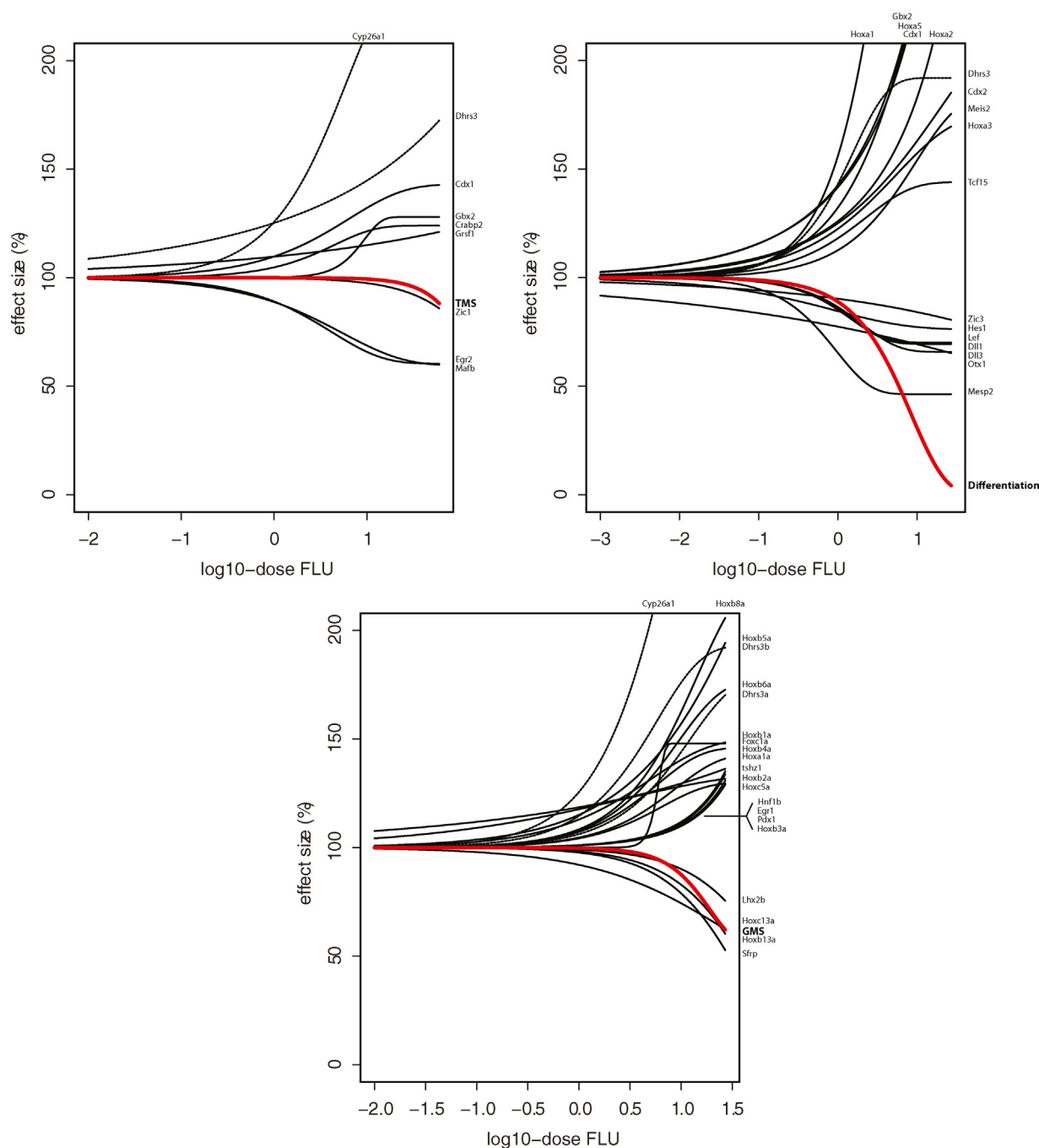


Fig. 3. Response curve clusters of marker genes (black) that showed a 5% effect size at lower concentrations than the classical end point (red) in WEC (A), EST (B) and ZET (C) after flusilazole exposure. Concentration given as log 10-dose in mg/l (WEC) or μ M (EST/ZET) in culture. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Lef1, Tcf15, Dll1, and Hes1. Lef/Tcf transcription factors have been identified as nuclear mediators of Wnt signaling). Dll1 is a Notch ligand which was shown to be regulated by Lef1, thereby linking the Wnt and Notch signaling during somitogenesis [58]. Hes1 is a downstream target of the Notch pathway and is regulated by the clock linked to segmentation. The segmentation clock however remains functional in Hes1 null mouse indicating Hes1 is not part of the clock mechanism, but rather represents a read-out of the clock [59]. Other patterning genes that were identified in EST were Meis2, Otx1, and Zic3. Meis2 is also a co-factor for Hoxa1 and Pbx1 in the regulation of mesodermal Raldh2 expression levels during hindbrain development [60]. Meis2 also plays a role in Krox20 activation in r3 [61]. Otx1 is involved in anterior neural patterning and

loss of function results in epilepsy other neurological defects. As mentioned previously, Zic transcription factors play a role in neural development and are linked to RA metabolism regulation. Zic3 loss of function leads to neural tube defects (closure defects in hindbrain/exencephaly and *spina bifida*) and laterality defects affecting left–right asymmetry [62].

3.8. ZET

Flusilazole responsive genes identified in ZET include retinoic acid metabolism genes Dhhrs3 (regulated in all three models), Cyp26a1 (also regulated in WEC), Hox genes (Hoxa1 (also regulated in EST), Hoxb1, Hoxb2, Hoxb3, Hoxb4, Hoxb5, Hoxb6, Hoxb8,

Table 1
Comparison of flusilazole and retinoic acid responsiveness of selected RA-regulated genes in three alternative models. Note similar directionality for all genes that show regulation.

	FLU	RA		FLU	RA		FLU	RA	
EST			Hoxa5						Cyp26a1
			Hoxa1						Dhrs3
			Cdx1						Cdx1
			Hoxa2						Gbx2
			Dhrs3						Crabp2
			Gbx2						ND
			Hoxa3						Grsf1
			Meis2						Mafb
			Cdx2						Krox20
			Tcf15						Zic1
			Mesp2						
			Lef1						
			Dll1						
			Dll3						
			Otx1						
			Zic3						
			Hes1						
WEC									
ZET									

Hoxc5, Hoxb13, Hoxc13) and Hnf1b (RARE). Furthermore Sfrp1, Tshz1 and Foxc1 were identified. Sfrp1 is a secreted Wnt antagonist that plays a role in axis elongation and somitogenesis [63]. Double homozygous mutations in Sfrp1 and Sfrp2 lead to a reduction of the thoracic region as consequence of rostro-caudal axis reduction and incomplete somite segmentation. The rostro-caudal axis reduction was associated with mesoderm cell migration, and aberrant somite segmentation was correlated with perturbed Notch oscillator cycles [63]. The biological function of Tshz1 during development is not known, however studies revealed that loss of Tshz1-function leads to specific malformations of middle ear components, soft palate defects and Hox-like vertebral malformations and homeotic transformations in the cervical and thoracic regions. Tshz1 may be a downstream target of Hox proteins or may interact with Hox genes to control common target genes during the differentiation of somatic mesoderm [64]. Foxc1 is suggested to play a role in the establishment of paraxial mesoderm fate as Foxc1 and Foxc2 compound null mice embryos completely lack morphological somites. Foxc1-null mice exhibit congenital hydrocephalus and abnormalities of the eye, skull, axial skeleton, kidney-ureter and cardiovascular systems [65]. Other genes identified in ZET were Lhx2 which plays a role in midline axon guidance, patterning of the ventral forebrain and eye morphogenesis [66] and Egr1 which contains a RARE and is expressed in presomitic mesoderm and specific brain areas during development [67]. Lastly, Pdx1 was identified which also contains a RARE and is expressed in a multipotent progenitor population that gives rise to the pancreas [30].

3.9. Comparison of flusilazole and RA effects in developmental assays

The RA-dependent patterning genes that were found to be modulated by flusilazole in the three alternative developmental assays were also studied in existing data of retinoic acid exposure studies (Table 1) [68–70]. Though not always significantly regulated as compared to controls, these genes all showed the same directionality of response after RA and flusilazole exposure in each of the three assays.

4. Discussion

We have attempted to exploit the central role of RA in shaping the vertebrate body plan as a logical starting point for building an RA-NTA AOP framework and for defining molecular biomarkers for developmental toxicity. From the perspective of *in vivo* developmental toxicity, neural tube and axial patterning represent frequently affected morphological parameters. In an analysis of rat and rabbit developmental toxicity studies in the ToxRefDB, Knudsen et al. [2] showed that 150/193 positive studies showed nonlethal developmental toxicity findings that involved axial malformations and variations in the rat. In the rabbit, these numbers amounted to 74/84, showing that axial patterning is prominent as to its vulnerability toward developmental toxicity, and indicating its importance as a group of readout parameters for developmental toxicity. Therefore, biomarkers and AOPs in this area might be

expected to allow detection of a major subset of developmental toxicants. The function of RA in neural tube and axial development is highly conserved throughout vertebrate embryogenesis, which facilitates mechanistic extrapolation among species and particularly toward man [71]. Moreover, crucial genes regulating RA levels as well as patterning genes for neural tube and body axis development have been shown to be modulated by differentiation processes in alternative assays [25,52,68,72], which turns them into promising candidates for biomarkers of developmental toxicity. Robinson et al. [73] described the gene expression changes in central nervous system (CNS) Developmental Gene Ontology terms, and showed that during the 48 h developmental window of rat postimplantation whole embryo culture, *Aldh1a2* and *Cyp26a1* were gradually significantly down regulated. Patterning genes regulated in the same time frame within this GO term were, e.g., *Hoxa10*, *Hoxb8*, *Hoxc10*, *Hoxd10*, *Fgf8*, *Nkx1*, *Nkx2*, *Nkx6-2*, *Sox11*, and *Neurod1*. They also showed that human homologs of these genes were regulated similarly in the same period in human embryogenesis. In the neural embryonic stem cell test (ESTn) protocol designed by Theunissen et al. [74], RA-induced neural differentiation was accompanied by >16-fold down regulation of *Cyp26a1* and by >16-fold up regulation of *Aldh1a2*. Similar profound changes in gene expression were observed for NTA markers such as *Foxd1*, *Hoxa1*, *Hoxa2* and *Pax6*, with numerous additional NTA related genes being significantly regulated at lower fold ratios. Jergil et al. [75] showed regulation of *Cyp26a1* and *Aldh1a2* after exposure of differentiating embryonic stem cells to valproic acid and a teratogenic analog, but not by a non-teratogenic analog. In addition they showed significant regulation of several NTA genes, a.o. *Otx2* and *Wnt3a*. This shows that even in cell culture models in which differentiation occurs, but without spatial patterning as in the embryo, patterning related genes are regulated and can potentially be used as biomarkers of developmental effects.

The present analysis of flusilazole concentration–response gene expression data in three alternative assays for developmental toxicity provides a further illustration of the usefulness of the RA–NTA framework for defining biomarkers of developmental toxicity. The directionality of gene regulation by flusilazole and by RA appeared similar, confirming an important role for RA–NTA related genes in flusilazole embryopathy. In addition, the overlap of regulated genes among assays occurred at crucial genes in the framework, *Dhrs3* being regulated in all three assays, and *Cyp26a1*, *Hoxa1*, *Cdx1*, *Gbx2* being regulated in two out of three assays. These are important candidate biomarker genes for developmental toxicity. One of the defining features of vertebrates is the conserved segmented spine, which is patterned during development. Comparison of the mouse, chicken and zebrafish segmentation clock genes revealed a conserved signaling oscillation in Notch, Wnt and Fgf signaling pathways during patterning [76,41]. However, the conservation appeared restricted to the rhythmic activation of these pathways as individual cyclic genes mostly differed between vertebrate classes. Therefore, the key events seem conserved across classes, but evolutionary plasticity is also evident. The differences observed in gene expression modulation between assays may partly be due to this plasticity, but could in addition be a consequence of differences in developmental stage and biological domain between assays. Whereas in EST the entire differentiation route from stem cell to mature differentiated cell occurs in the virtual absence of pattern formation, both other assays contain patterning of the complete embryo. WEC is limited to the two-day organogenesis period roughly between the 4 and 28 somite stage when neural tube formation takes place. It is therefore not surprising that genes identified in the RA–NTA AOP framework using WEC flusilazole data play important roles in hindbrain development. *Gbx2* and *Grsf1* were both upregulated. *Cdx1*, which represses *Kreisler* (*Mafb*), was upregulated while a downregulation of *Kreisler* and *Krox20* (*Egr2*)

was observed. ZET includes patterning from fertilized egg to the larval stage, and genes identified using the ZET indeed included a variety of genes involved in axial patterning, such as *Hox* family genes, *Tshz1* and *Sfrp1* linking to cervical and thoracic axial skeleton defects, but also *Hox13* genes involved in axial extension arrest. Other genes were involved in brain and eye development (*Egr1*, *Lhx2*, *Hnf1b*). In EST, a series of RA–NTA related genes was regulated, indicating that, in spite of the absence of spatial patterning and the difficulty of staging this assay in terms of embryologic time window, this assay proves useful for detecting RA–NTA related effects. The EST unique gene regulation in this assay as compared to the WEC and ZET may partly be a function of the relative homogeneity of this cell culture as compared to whole embryos, which would favor the detection in EST of significant expression changes in genes that have a very restricted localized expression in the embryo. Thus, combining different assays for developmental toxicity provides common as well as complementary information that may aid the interpretation of findings and the prediction of developmental toxicity potential of chemicals.

The RA–NTA AOP framework contains a number of potential MIE, represented by changes in each of the enzymes that regulate retinoic acid balance through its synthesis or metabolism. The multiplicity of MIE collected in this framework led us to use the group term ‘molecular interaction’ in Fig. 1, rather than ‘MIE’, which is reserved for a single AOP defined as connecting one MIE to one adverse outcome [11]. Furthermore, a multitude of morphological adverse outcomes has been identified in the form of a spectrum of malformations. It should be realized that this AOP framework provides a general design only that leaves room for considerable further detail. This is obviously true for the potential biomarker gene lists, for which the data from the model systems that we analyzed here already provided additional candidates. Moreover, markers at different levels of regulation, such as in the proteome and the metabolome, may provide useful sensitive markers of dysmorphogenesis [77]. In addition, the contribution of regulation of the downstream biochemical route of RA action *via* its specific RA receptors and binding protein families (*Rabp* and *Crabp*) may be significant [78]. In the flusilazole data analysis, only *Crabp2* was found regulated and in WEC only, and it remains to be established whether the binding protein family is sufficiently sensitive to exposures in such *in vitro* models and rate limiting in RA action. The current selection of NTA related genes also ignores the action of RA on important processes such as antero-posterior patterning of the neural tube and neuron type regulation [20], limb innervation [20] and germ cell differentiation [38,79]. However, in reductionist *in vitro* assays, one might speculate that compound induced significant regulation of key genes in RA homeostasis could serve as a more general indicator of developmental hazard, covering a larger subset of apical end points than currently incorporated in the framework. Further work is needed on specifying a molecular biomarker set for NTA.

Another crucial remaining issue is the interpretation of biomarker regulation *in vitro* in terms of hazard. In our analysis of flusilazole *in vitro* data we have shown monotonic concentration dependency of the magnitude of biomarker gene regulation. Combinations of functionally related genes regulated similarly strengthen the evidence for perturbation of affected pathways. Concentration response *per se* can be interpreted as a signal for possible developmental toxicity potential of the test compound, determining further testing in animal studies. In case of full replacement of *in vivo* testing, *in vitro* effects need to be interpreted in terms of adaptation *versus* adversity, which requires the definition of thresholds of adversity as is a common issue in animal studies [80]. This definition is more difficult when alternative assays are more reductionist, excluding more aspects of the homeostatic mechanisms that in the intact organism may compensate for the

primary effect. The magnitude of the response, the combination of functionally related biomarkers regulated in synergy and kinetic extrapolation of effective concentrations *in vitro* to *in vivo* effective external doses may combine to provide a useful measure of compound hazard [81]. The advent of the AOP concept has greatly stimulated the mapping of complete trajectories from MIE to adverse outcome [11], and is revealing gaps in knowledge about molecular, cellular and tissue effects underlying the pathogenesis of adverse effects, that need to be filled to fully understand mode of action. On the other hand, irrespective of full understanding of mode of action, if critical steps in the process from exposure toward adversity can be identified and biomoned in *in vitro* systems, this may provide important hazard information. The RA–NTA framework presented here provides suggestions for key events and markers in neural tube and axial patterning that may be employed to allow detection of a major subset of developmental toxicants.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This work was supported by the Netherlands Genomics Initiative (NGI)/Netherlands Organization for Scientific Research (NWO) [grant number 050-060-510] and the Ministry of Infrastructure and the Environment. This work was carried out with financial support from the Commission of the European Communities, the collaborative project ChemScreen (GA244236).

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