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Triazole induced concentration-related gene signatures in rat whole embryo culture

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ABSTRACT

Commonly used as antifungal agents in agriculture and medicine, triazoles have been shown to cause teratogenicity in a diverse set of animal models. Here, we evaluated the dose-dependent impacts of flusilazole, cyproconazole and triadimefon, on global gene expression in relation to effects on embryonic development using the rat whole embryo culture (WEC) model. After 4 h exposure, we identified changes in gene expression due to triazole exposure which preceded morphological alterations observed at 48 h. In general, across the three triazoles, we observed similar directionality of regulation in gene expression and the magnitude of effects on gene expression correlated with the degree of induced developmental toxicity. Significantly regulated genes included key members of steroid/cholesterol and retinoic acid metabolism and hindbrain developmental pathways. Direct comparisons with previous studies suggest that triazole-gene signatures identified in the WEC overlap with zebrafish and mouse, and furthermore, triazoles impact gene expression in a similar manner as retinoic acid exposures in rat embryos. In summary, we further differentiate pathways underlying triazole-developmental toxicity using WEC and demonstrate the conservation of these response-pathways across model systems.

1. Introduction

One of the more established in vitro methods for investigating embryotoxicity of chemicals is the rat postimplantation whole embryo culture (WEC) model. In WEC, embryos are cultured from embryonic day 10–12 with morphological development [1] and underlying gene expression [2] similar to the in vivo system. During this window in development, the embryo progresses through neurulation and early organogenesis. Embryos are evaluated extensively using a scoring system [3] derived from previous studies [4,5] which accounts for multiple morphological parameters related to growth and development of the embryo. Unlike traditional gestational studies in which pregnant dams are exposed, in WEC, embryos can be cultured under specific conditions, randomized, and processed separately. Therefore, the use of WEC in toxicity testing reduces animal use and discomfort as compared to classical testing in vivo. On the other hand, the lack of maternal metabolism and the assessment of a limited developmental time window in WEC may restrict its use. Validation studies [6,7] have provided mixed results in the predictive nature of WEC for developmental toxicity testing. Further definition of the applicability domain of the WEC model and the addition of mechanistic-based endpoints should enhance the predictability of WEC for developmental toxicity testing.

Within the past decade, a variety of genomic-based methodologies which enable evaluation of multiple molecular markers have been developed. Gene expression profiling (toxicogenomics) allows the evaluation of RNA expression in the majority of genes expressed in a biological sample. The implementation of toxicogenomic-based approaches in WEC may improve prediction of developmental toxicity potential by providing a molecular "signature" that can be more objectively evaluated as compared to current morphological assessments. Recently, we have conducted a series of studies aimed at determining the use of toxicogenomic-based approaches in the WEC in terms of protocol development and application [2,8–12]. These initial studies suggest that the integration of toxicogenomic approaches in the WEC provides a robust output, linking relevant mechanistic information regarding particular compounds with developmental effects [11]. Furthermore, changes on the molecular level can be used to characterize dose/time dependent effects [9,12]. And additionally, these effects are comparable across model systems despite differences in classical developmental toxicity endpoints [10,12].

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Triazoles are largely used as antifungal agents in agriculture and medicine due to their inhibition of the Cyp51 enzyme [13]. Cyp51 mediates conversion of lanosterol to ergosterol, the latter being critical for fungal cell wall integrity. In mammalian cells, ergosterol is not produced, however Cyp51 is critical for sterol biosynthesis. Additionally, triazoles may inhibit other p450-mediated activities resulting in mammalian toxicity. In liver of male rats, triazoles disrupted p450-xenobiotic, lipid and steroid metabolism pathways in association with decreased liver weight and hepatocyte hypertrophy [14,15]. Also, in male adult rats, triazoles caused alterations in steroidogenesis, resulting in increased serum testosterone [16].

In humans, the classification of triazole compounds as developmental toxicants remains debated, despite studies indicating positive associations with teratogenic outcomes [17]. In animal models, triazoles are teratogenic at high dose levels. For example, in rat, gestational exposure to triazoles, either flusilazole (FLU), cyproconazole (CYP) or triadimefon (TDI) resulted in a spectrum of malformations which commonly included both craniofacial and axial defects [18–20]. Recent experimental studies in rat [21] and zebrafish [22,23] suggest that triazole compounds may alter retinoic acid (RA) levels in association with teratogenicity due to high similarities with RA-induced effects on the morphological and/or mechanistic level [17]. In WEC, at teratogenic concentrations both RA and triazoles (e.g. TDI, FLU) disrupted branchial arch development, neural crest cell migration and hindbrain segmentation [24,25].

In this study, we examine the concentration-dependent effects of the triazoles, FLU, CYP, TDI on global gene expression in relation to developmental toxicity in WEC. We identify pathways of triazole-induced developmental toxicity and demonstrate the conservation of these effects across model systems.

2. Methods

2.1. Animal care

Animal studies were approved and conducted in agreement with the National Institute for Public Health and the Environment (RIVM) Animal Care facility under federal regulations. Wistar rats (HsdCpd:WU) (Harlan, the Netherlands) were housed within the RIVM facility under climate controlled conditions with a 12 h light cycle (4:00–16:00, dark). Water and food were provided ad libitum. After acclimating for at least a 2 week period, virgin female rats were housed with adult male rats for a 3 h mating period (9:00–12:00). Upon evidence of a copulatory plug, we declared females pregnant (gestational day 0) and placed them into separate cages. Throughout the study, all animals were monitored daily for general health.

2.2. Whole embryo culture

As described previously [3,6], on gestational day (GD) 10, pregnant dams were euthanized via intracardiac injection of T61^R between 9:00 and 12:00. Rat embryos were immediately removed from the uterus with the yolk sac and ectoplacental cone left intact. Embryos with 1–5 somites were cultured and used for morphological and gene expression assessments. Following our previous studies investigating the use of WEC as a screening tool [8,12], embryos with 2–4 somites were used for microarray studies. Placed in separate flasks, embryos were cultured in a serum mixture (90% pregnant bovine serum, 10% rat serum (Biochrom, Berlin, Germany)) diluted by 14% Hank's solution supplemented with 1.57 mg/mL D-glucose and 75 μg/mL L-methionine (Gibco). Using increasing concentrations of oxygen, gassing occurred during culture; at 9:00 and 16:00 for 30 s on the first day (~−1 h and ~7 h) (5% O_2, 5% CO_2, 90% N_2), 9:00 and 16:00 (~23 h and ~30 h) for 30 s on the second day (20% O_2, 5% CO_2, 75% N_2) and 9:00 (~47 h) for 10 s on the third day of culture (40% O_2, 5% CO_2, 55% N_2).

2.3. Triazole exposure–WEC

Flusilazole (FLU; CAS# 85509-19-9, Sigma–Aldrich, Zwijndrecht, The Netherlands) at concentrations of 3, 10, 30 and 60 μg/mL, cyproconazole (CYP; CAS# 94361-06-5, Sigma–Aldrich) at 300 and 500 μg/mL, triadimefon (TDI; CAS# 43121-43-3, Sigma–Aldrich) at 100 and 300 μg/mL, all-*trans*-retinoic acid (RA; CAS# 302-79-4, MP Biomedical, Irvine, CA) at 0.5 μg/mL or vehicle control (dimethyl sulfoxide (DMSO, 1 μl/mL)) were added to 2 mL of culture medium prior to culturing (8:00–9:00). Concentrations were based on previous studies in our lab assessing the impact of triazoles [26] or RA [12] on embryonic morphology. The concentration of DMSO in this study does not significantly alter morphology (4 h and 48 h) and has limited effects on gene expression (4 h) [11].

2.4. Whole embryo RNA isolation

Following previous studies [11], whole embryos were scored for total somites and the development of the neural tube and further isolated from the yolk sac and ectoplacental cone. Separately, embryos were immediately stored in RNAlater RNA stabilization solution (Ambion, Austin, TX) and placed at 4 °C for one week and then ≤−20 °C until further processing. After thawing on ice, each embryo was homogenized by passing through a 26G needle and 1 mL syringe at least ten times. RNA was isolated from homogenized tissue using the RNeasy Micro Plus RNA Isolation Kit (Qiagen). Final volumes of RNA were obtained by eluting with 13 μl of nuclease-free water. RNA quantity and quality were determined using the Nanodrop (Nanodrop Technologies Inc., Wilmington, DE) and the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA extracts with an absorbance value between 1.9 and 2.2 (260 nm/280 nm) and RNA integrity number (RIN) > 7 were used for microarray analyses. RNA was stored at −80 °C.

2.5. Microarray hybridization

RNA hybridization and microarray experimentation were performed by ServiceXS B.V. (Leiden, Netherlands). Using 100 ng of purified total RNA as a template for the Affymetrix 3′IVT Express Labelling Kit (901229), RNA targets were prepared. Fragmentation reactions were performed using 7.5 μg cRNA for each biological sample. At a concentration of 0.0375 ng/μl fragmented/denatured cRNA, hybridization was conducted using Affymetrix HT RG-230 PM Array Plate (Affymetrix Inc., Santa Clara, CA) according to the manufacturer's instructions. The Affymetrix HWS Kit (901530) was used for hybridization, washing and staining of chips. In total, for all experimental groups, 72 arrays were used ($n = 8$ arrays for each experimental group representing eight single embryos, derived from at least 7 separate dams).

2.6. Microarray analysis-data processing

The quality of microarray images was inspected visually and raw values were examined for average background scale factors, 3′/5′ ratios of glyceraldehydes 3-phosphate dehydrogenase and 3′/5′ ratios of Bactin, and NUSE and RLE signal quality metrics (http://arrayanalysis.org, BiGCaT Maastricht University). One out of the 72 arrays was eliminated from our analysis. Affymetrix CEL files were normalized using the Robust Multichip Average (RMA) algorithm [27] using the Brainarray custom CDF version 14 (http://brainarray.mbni.medumich.edu/Brainarray/Database/CustomCDF) [28]. Affymetrix internal controls ID were not used in further analyses, leaving a total of 12,032 probe sets for EntrezGeneID mapped genes.

2.7. Identification of differentially expressed genes

Log transformed data was imported into BRBarraytools (National Cancer Institute [29]). Fold change values were calculated using the arithmetic mean of each exposure group and determining ratios between each group in comparison with the vehicle control group. Using 71 arrays, representing 71 embryos, we employed three linear models (one-way ANOVA) to determine the significance of triazole effects. To determine genes significantly altered by one of the three triazoles, we used a cutoff of $p < 0.001$ (p-value). The corresponding false discovery rate (FDR) for genes identified to be significantly regulated by any of the three triazoles was approximately 30% [30].

2.8. Functional enrichment analysis of differentially expressed genes

All genes significantly altered by at least one of the three triazole compounds ($p < 0.001$) were examined for enrichment of functional associations (Gene Ontology (GO) biological process terms) using DAVID [31] and MAPPFinder [32]. Significantly enriched terms were identified to have at least 2 genes within each term and either a 10% increase in fold enrichment and $p < 0.05$ (DAVID) or Z-score < 2 and $p < 0.05$ (MAPPFinder). Enrichment analyses were conducted for all levels of terms, however, only biological processes Levels 3 and 4 were shown in this study to reduce the redundancy of terms of similar function. Generally, "Levels 3 and 4" represent categories of genes with moderate-high specificity of function within the GO hierarchical system [31]. Furthermore, biological process terms were grouped based on GO classification ([33], http://geneontology.org) to demonstrate themes.

2.9. Quantitative functional analyses

We determined the dose-dependent effect of genes within selected enriched pathways (cholesterol metabolism, lipid metabolism, steroid metabolism, retinoic acid/retinol metabolism, hindbrain development and neurogenesis) by calculating the geometric (mean) average absolute fold change of gene changes of genes within selected pathways. Identification of gene-GO biological process associations was conducted using DAVID [31].