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Discriminating classes of developmental toxicants using gene expression profiling in the embryonic stem cell test

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ABSTRACT

The embryonic stem cell test (EST) has been shown to be a promising *in vitro* method for the prediction of developmental toxicity. In our previous studies, we demonstrated that the implementation of gene expression analysis in the EST may further improve the identification of developmental toxicants. In the present study, we investigated if gene expression profiling could be used to discriminate compound classes with distinct modes of action (MoA) using the EST protocol. Gene expression data of our previous study were used and were analyzed of embryonic stem cell (ESC) differentiation cultures exposed to six compounds belonging to two classes with distinct MoA, namely phthalates and triazoles. We used three approaches to study class-characteristic gene regulation that may be useful for discrimination of compound classes. First, at the individual gene level, gene signatures characteristic for each class were identified that successfully discriminated both classes using principal component analysis. Second, at the functional level, enriched gene ontology (GO) biological processes showed their usefulness for class discrimination via hierarchical clustering. Third, two previously identified gene sets, which we designed to predict developmental toxicity, appeared successful in separating phthalate from triazole compounds. In summary, we established the possibility to discriminate between compound classes in the EST system using three different specific transcriptomics-based approaches. Differential gene expression information specific for the class of compound under study may be employed to optimize prioritization of compounds within that class for further testing.

1. Introduction

According to international legislation, current toxicity testing is primarily assessed using laboratory animals. *In vivo* studies are expensive, requiring a considerable number of experimental animals, and raise important ethical concerns. For these reasons, European policy is promoting alternative testing methods and assessment strategies to reduce the use of laboratory animals and whenever possible, replace animals employed for toxicological studies (Lilienblum et al., 2008). Although none of the existing *in vitro* methods that have been identified is capable of replacing the existing classical *in vivo* approach, a select few have been recognized as potential alternative methods for the assessment of developmental toxicity (Genschow et al., 2002).

One of the most studied *in vitro* alternatives for developmental toxicity screening is the murine Embryonic Stem cell Test (EST). Our transcriptomic studies suggest that the implementation of toxicogenomic-based assessments into the EST may lead to improved prediction, by providing detailed information regarding modulation of embryonic stem cell (ESC) differentiation in relation to concentration and time (van Dartel et al., submitted for publication, 2010b,d) and furthermore, enabling the prediction of toxicity based on pre-determined gene lists related to ESC differentiation (van Dartel et al., 2010b).

Diverse studies have shown that discrimination of compounds with similar modes of action is possible using biological activity profiling patterns. Compound discrimination has been used pre-

Abbreviations: EB, embryoid body; ESC, embryonic stem cells; EST, embryonic stem cell test; GO, Gene Ontology; GOID, Gene Ontology identifier; PCA, principal component analysis; MBP, monobutyl phthalate; MMP, monomethyl phthalate; MEHP, monoethylhexyl phthalate; FLU, flusilazole; HEX, hexaconazole; TDI, triadimefon.

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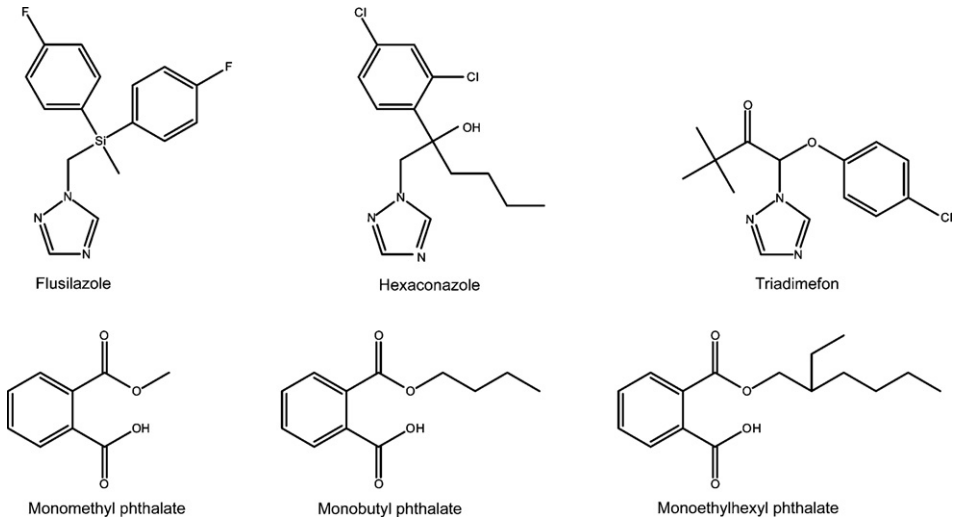

Fig. 1. Chemical structures of triazoles and phthalates tested in the present study.

dominantly in drug discovery (Fliri et al., 2005; Melnick et al., 2006), and this application has also been studied to assess its potential for toxicological screening (Steiner et al., 2004; Martin et al., 2007). For example, Martin et al. demonstrated successful grouping of compounds with similar toxicological effects using signatures characteristic for specific toxic outcome (i.e. hepatomegaly and PXR activation). Application of compound discrimination analyses is of interest for ranking of structurally related compounds with unknown toxicity for further testing. Many new compounds or compounds with insufficient toxicological data are structurally related to compounds of which a wealth of toxicological data is available. Before deciding on generating a full toxicological profile of the new compound, it may be useful to compare the gene expression profile in an appropriate *in vitro* test of the new with the well-studied compound(s). If a comparable gene expression pattern is observed it may be possible to rely on the data of the tested compound as a surrogate for assessing the toxic properties of the new compound (Daston and Naciff, 2010). This approach has been supported by studies that were able to distinguish (Liu et al., 2005) or rank (Lamb et al., 2006) similarly structured compounds for their toxic potencies using gene expression profiling. Furthermore, discrimination of compounds based on specific signatures is of interest for prioritizing structurally related compounds with unknown toxicity for further testing.

The objective of the present study was to evaluate the ability of the EST to categorize compounds with a similar mode of action by applying a toxicogenomics approach. In our previous study, we evaluated the identification of developmental toxicants using gene expression analysis (van Dartel et al., 2010c). In this study, using this dataset, we assessed common and differential gene expression regulation patterns of triazole and phthalate compounds, and evaluated whether these two classes are discriminated based on their gene expression response in the EST system. We selected these two classes of compounds on the basis of the availability of effect data in the EST, suggested different mechanisms of toxicity, and furthermore, the relevance of their effects on human health.

Triazoles are often used in agricultural settings as well as in pharmaceutical applications for their known antifungal effects. The fungicidal mode of action of triazoles is based on the inhibition of the enzyme lanosterol-14α-demethylase (Cyp51), which plays an essential role in sterol metabolism and is required for the synthesis and integrity of the fungal cell wall (Vanden Bossche et al., 1990). In addition, triazoles may affect also other Cyp-enzymes, which may result in developmental malformations when embryos are exposed prenatally (Menegola et al., 2003; Tiboni et al., 2009). The observed effects include urogenital and skeletal malformations (Knudsen et al., 2009). The triazole compounds that were selected for the present study were flusilazole (FLU), hexaconazole (HEX) and triadimefon (TDI) which all contain the 1,2,4-triazole moiety (Fig. 1).

The second class of compounds included in the present study are phthalates, which are esters of phthalic acid (benzene-1,2-dicarboxylic acid) in ortho configuration. These compounds are widely used as plasticizers to increase the flexibility of a variety of PVC products like toys, vinyl floors and catheters. Developmental toxicity studies have shown that the monoester phthalates containing side-chain lengths of C4–C6 may induce teratogenic effects (Fabjan et al., 2006). In the present study, we included two developmentally toxic phthalates, monobutyl phthalate (MBP; C4), and monoethylhexyl phthalate (MEHP; C6), as well as one non-developmentally toxic phthalate monomethyl phthalate (MMP; C1). Several modes of action have been suggested for the teratogenic properties of phthalates, including intracellular acidification, altered cellular proliferation and induction of DNA damage (Nau and Scott, 1986; Rusyn et al., 2006). Malformations induced by the developmental toxic phthalates tested in the present study include external and skeletal malformations, cleft palate and hydrocephaly (Ema et al., 1995, 1996; Yagi et al., 1980).

In the present study, we evaluated the discriminatory ability of gene expression to classify triazoles and phthalates using multiple approaches based on (1) gene lists characteristic for each class identified in the present study (2) enriched Gene Ontology (GO) processes, and (3) gene lists derived from our earlier toxicogenomics studies that identify developmental toxicants in the EST. We could successfully segregate the tested compounds into two distinct classes using these approaches. It can be concluded that, as a proof-of-principle, our findings show the possibility of using gene expression data obtained from ESC differentiation inhibition for the discrimination of compound classes with different modes of action of developmental toxicity.

2. Materials and methods

Data of our previous study (van Dartel et al., 2010b) were used for the analyses described here. The methods and data regarding culture conditions, compound exposures, assessment of differentiation inhibition, and gene expression profiling have been described in full detail (van Dartel et al., 2010b). The data have been deposited in EBI's ArrayExpress (http://www.ebi.ac.uk/arrayexpress) and are accessible through ArrayExpress accession number E-MTAB-300.