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Grouping of histone deacetylase inhibitors and other toxicants disturbing neural crest migration by transcriptional profiling

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A B S T R A C T

Functional assays, such as the "migration inhibition of neural crest cells" (MINC) developmental toxicity test, can identify toxicants without requiring knowledge on their mode of action (MoA). Here, we were interested, whether (i) inhibition of migration by structurally diverse toxicants resulted in a unified signature of transcriptional changes; (ii) whether statistically identified transcript patterns would inform on compound grouping even though individual genes were little regulated, and (iii) whether analysis of a small group of biologically relevant transcripts would allow the grouping of compounds according to their MoA. We analyzed transcripts of 35 'migration genes' after treatment with 16 migration inhibiting toxicants. Clustering, principal component analysis and correlation analyses of the data showed that mechanistically related compounds (e.g. histone deacetylase inhibitors (HDACi), PCBs) triggered similar transcriptional changes, but groups of structurally diverse toxicants largely differed in their transcriptional effects. Linear discriminant analysis (LDA) confirmed the specific clustering of HDACi across multiple separate experiments. Similarity of the signatures of the HDACi trichostatin A and suberoylanilide hydroxamic acid to the one of valproic acid (VPA), suggested that the latter compound acts as HDACi when impairing neural crest migration. In conclusion, the data suggest that (i) a given functional effect (e.g. inhibition of migration) can be associated with highly diverse signatures of transcript changes; (ii) statistically significant grouping of mechanistically related compounds can be achieved on the basis of few genes with small regulations. Thus, incorporation of mechanistic markers in functional in vitro tests may support read across procedures, also for structurally un related compounds.

1. Introduction

The capacity of neural crest cells (NCC) to migrate correctly to different parts of the fetus where they differentiate to various tissues (e.g. bone, cartilage, neurons, melanocytes, etc.) is essential for normal human development (Dupin and Sommer, 2012). Defects in neural crest function and differentiation can lead to severe birth defects and diseases such as cleft palate, Hirschsprung disease or CHARGE syndrome (Keyte and Hutson, 2012). Several chemicals are known to disturb the NCC development and migration and thereby cause developmental defects (Di Renzo et al., 2007; Fuller et al., 2002; Menegola et al., 2000). A comprehensive hazard assessment approach requires therefore test systems that detect disturbances of NCC function.

To monitor whether chemicals disturb the migration capacity of NCC, the MINC (migration of neural crest cell) assay has been developed. This test system assesses, how many cells re migrate

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into a cell free area (scratch) within a neural crest cell monolayer. Several toxicants known to affect neural crest cells in vivo have been tested positive in this test. They include triadimefon, mercury, lead and the antiepileptic drug valproic acid (Zimmer et al., 2012). Using the inhibition of migration capacity as an endpoint for toxicity testing seems promising for detection of chemicals potentially hazardous to human early development. Although some biological pathways (e.g. actin polymerization or src signaling) have been identified to be required for NCC migration, it is not clear, which of them is affected by toxicants or whether all toxicants that affect NCC migration, converge on the same biological pathway. Thus the underlying mode of action (MoA) and the associated adverse outcome pathways (AOP) remain unknown and the assay, as most of the current in vitro and in vivo toxicity assays, is still a black box approach.

First attempts have been undertaken to combine traditional assays that are based on so called apical endpoints with measurements of intermediate events, such as changes of mRNA, proteins or metabolites. For instance, it has been shown that morphological deficits triggered by triazoles in the rat whole embryo culture were accompanied by characteristic transcriptome signatures (Robinson et al., 2012), and neuronal cell death triggered by the toxicant 1 methyl 4 phenylpyridinun was preceded by pronounced changes of metabolites and transcripts (Krug et al., 2014).

Such approaches might allow the detection of key events of AOPs, definition of biomarkers (Kuegler et al., 2010) or delineation of an entire MoA/AOP (Bal Price et al., 2015b). On this basis relevant assays could be combined into to a more efficient test battery (Piersma, 2014; Piersma et al., 2013; Zimmer et al., 2014).

One unsolved issue in this process is how a final adverse outcome (e.g. disturbed migration in the MINC) links to intermediate changes inside the cells. One assumption is that toxicants causing the same type and degree of complex adverse outcome, also lead to similar changes of relevant transcripts, proteins or other potential biomarkers of toxicity (Blaauboer et al., 2012).

An opposite hypothesis would be that each toxicant triggers its own particular set of pathway alterations, and that many different such changes of cell function eventually lead to the same adverse outcome. Signatures of biological disturbances would in such cases be different for each toxicant. In between these extremes, a realistic situation could be that a limited number of key events of different biological pathway disturbances lead to the same adverse outcome.

Thus, toxicants sharing a mode of action would trigger similar changes of intermediate cellular markers. Several toxicity path ways may lead to the same adverse outcome (Patlewicz et al., 2014), so that one final outcome could be linked to different (more than one, but most likely not hundreds) signatures of intracellular changes. This would allow grouping of compounds along their specific disturbance patterns or biomarkers. Such groups of up to 20 compounds have for instance been observed for the 148 compounds tested in the TG GATES project (Grinberg et al., 2014). Grouping along mechanistic markers has also been done for the ToxCast phase I chemicals (Kleinstreuer et al., 2014). Such information could guide biologically driven read across proce dures in hazard assessment (Bal Price et al., 2015a) to group compounds based on similarities of MoA. This would complement traditional structure based approaches.

In this study we first identified diverse groups of toxicants that disturb the migration of neural crest cells in the MINC assay. Using a set of 35 migration related genes, we monitored, by RT qPCR, transcriptional changes triggered by these toxicants. Notably, highly significant grouping and correlation of transcript signatures was identified on the basis of small transcript changes, not significant on the single gene level. Grouping of toxicants according to their gene expression profiles identified the group of HDAC inhibitors as hitherto unrecognized drug class affecting NCC migration. The study shows, that the test system gives highly reproducible results across different biological replicates, not only for the primary apical endpoint (i.e. inhibition of migration capacity), but also for transcript changes, as mechanistic end points. In general, the study suggests that some mechanistically related compounds can be grouped on the basis of their transcriptome signature.

2. Materials and methods

2.1. Cell culture and neural differentiation

The reporter hES cell line H9 Dll1 (GFP under Dll1 promoter) was provided by Mark Tomishima from the Memorial Sloan Kettering Cancer Center (MSKCC, NY, USA). Import of the cells and all experiments were carried out according to German legislation under license 1710 79 1 4 27 of the Robert Koch Institute (Berlin, Germany). The cell line was maintained on inactivated murine fibroblasts in DMEM/F12 medium supple mented with 20% serum replacement, HEPES (1M Gibco), L gluthamine (Glutamax, Gibco), non essential amino acids (MEM NEAA, Gibco), β Mercaptoethanol (Gibco) and fibroblast growth factor 2 (10 ng/ml, Invitrogen).

H9 hESC differentiation into neural crest cell was initiated on Mitomycin C treated murine bone marrow derived stromal MS5 cells and continued as described previously in and Fig. 1A Lee et al. (2010) and Zimmer et al. (2012). Frozen stocks of three in vitro amplified NCC from three independent differentiations were used for all the experiments. Each stock was carefully quality controlled based on previous defined parameters such as purity, differentia tion potential and migration capacity.

2.2. Cell migration analysis

Cell migration was assessed with minor changes as described in Zimmer et al. (2012, 2014). On day 2 NCC were thawed and 50,000 cells/cm^2 were seeded in 48 well plates and were grown to a monolayer. A confluent layer of cells is typically reached 2 days after plating. Once the cells have reached 100% confluence a cell free gap (scratch) was created using a 20 μl pipette tip. After scratching, the medium was changed to fresh media containing the test compounds for 48 h. Immediately after scratching the width of the cell free area was determined in a control plate. After 48 h cytotoxicity was analyzed using the resazurin reduction assay as described previously (Zimmer et al., 2012). In order to determine migrated cells, the nuclei were stained with the DNA dye H 33342 and unbiased images (3 4) along the scratch were taken using an Olympus IX81 at 4× magnification. The number of cells in the region of interest (ROI) was counted automatically by Konstanz information miner (KNIME) workflow (Berthold et al., 2008).

2.3. Chemical exposure during migration

hESC derived neural crest cells were exposed to chemicals in N2 medium containing (Lee et al., 2010) EGF (20 ng/ml) and FGF2 (20 ng/ml) for 48 h. For a detailed list of the chemicals and their concentration used in this study see Fig. 2. The concentration of each chemical was chosen after assessing its general toxicity in NCC after 48 h of exposure. Each compound was tested over a wide concentration range (several logs) using the resazurin reduction assay. For each individual toxicant, the highest determined non toxic concentration was then used in the MINC assay.

2.4. Konstanz information miner (KNIME) workflow

The consecutive analysis, i.e. the detection and positioning of the scratch followed by the counting of the cells inside a scratch works as follows: in order to detect the scratch after 48 h of incubation, three wells were scratched and images of the three wells were taken directly after scratching. Prior observations allowed us to assume, that the scratch intersects a horizontally centered line parallel to the *y* axis. Starting from a horizontally centered 1 pixel wide box the algorithm iteratively extends the box to the left by a certain width *S* in each iteration. In case the amount of the cells inside the newly added box of width *S* exceeds a threshold predefined *T*, the growing process stops and starts growing to the right. In this way, for each untreated image the scratch is identified and located. The minimum width of a box, i.e. the minimum width of the detected scratches, is used for the succeeding analysis, where a box of this width is shifted over the images from the left to the right. After each shift the number of cells in the box is determined. The position of the box, where the minimal number of cells is found is assumed to be the location of the scratch. This procedure is necessary as the location of the scratch may vary in its horizontal position. Fig. 1C illustrates this process (Berthold et al., 2008).

2.5. Quantitative real time PCR (qPCR)

qPCR samples were obtained after cells were treated for 48 h with the indicated toxicants at the indicated concentrations. Cells were lysed in TriFast™ (Peqlab, Germany). Total RNA was isolated according to the manufacturer's instruction, and transcribed into cDNA using the iScript Kit from BioRad (iScript™ Reverse Transcription Supermix for RT qPCR, BioRad). Quantitative

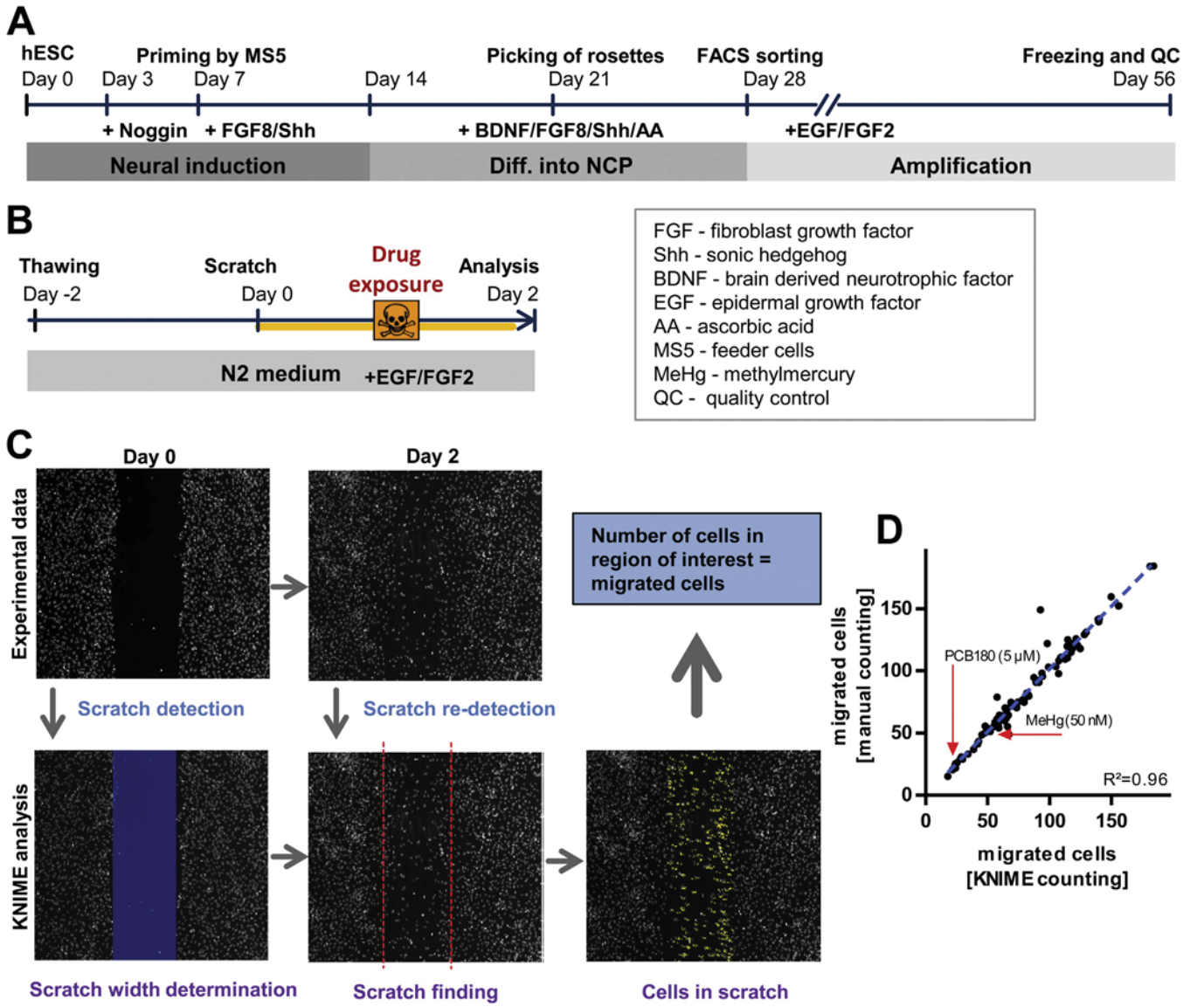


Fig. 1. "Migration inhibition of neural crest cell" (MINC) assay combined with automatic analysis. (A) Schematic illustration of the differentiation protocol of human embryonic stem cells (hESC) to neural crest cell (NCC). (B) Treatment protocol of the MINC assay. (C) Image analysis by the KNIME workflow. On day 0 the scratch was detected and the average width of the scratch was determined from three test wells. After a 2 day incubation period unbiased pictures were taken. The software automatically redetected the scratch in the pictures by using the information from day 0 and determined the position of the scratch and thereby the region of interest (ROI). The cells in the ROI were detected and counted automatically by the software. Finally, the number of cells migrated into the ROI was defined as the total number of migrated cells. (D) Validation of the KNIME image analysis vs. manual counting: the MINC assay was performed in the absence and presence of eight compounds at different concentrations. The number of cells migrating into the scratch was analyzed either manually (as described by Zimmer et al. (2012)) or by using the KNIME analysis tool, based on the same recorded images ($n = 85$).

Compound class or compound	CAS number	Conc. [μM][a]	Experience from MINC or general MoA	Literature evidence for dev. Toxicity (human data is italicized)	References[b]
Heavy metals					
Methyl mercury	22967-92-6	0.05	Migration inhibitor [c]	*Cognitive deficit*	(1,2)
Thimerosal	54-64-8	0.05	Migration inhibitor [c]		
Lead acetate (Pb(Ac))	7758-95-4	2.5	Migration inhibitor [c]	*Prematurity; fetal hypotrophy; malformations; retarded mental development; muscular and behavior disorders; Attention deficit-hyperactivity disorder, cognitive deficits*	(3) (4)
Cadmium chloride ($CdCl_2$)	10108-64-2	0.1	(Known as developmental neurotoxciant)	*Reduced birth weight; reduced IQ*	(5, 6)
Arsenic trioxide (As_2O_3)	7778-39-4	1	(Known as developmental neurotoxciant)	*Verbal and full scale IQ deficits; reduced body weight (in girls only)*	(7, 8)
PCBs					
PCB 153	(35065-27-1)	5	Non-planar PCBs have multiple targets (e.g Tyr - Kinase and various nuclear receptors and ryanodine receptor)	*Behavioral impairments that share significant commonalities with ADHD; Dendrite effects*	Reviewed in (9,10,11,12,13)
PCB 170	(35065-30-6)	0.5			
PCB 180	(35065-29-3)	5			
Pesticides					
Triadimefon	43121-43-3	200	Migration inhibitor [c]	Supernumery ribs[d]	
Triadimenol	55219-65-3	200	Migration inhibitor [c] (Metabolite of triadimefon)	Rats: reduced weight gain in offspring; reduced placental weight; increase in number of ribs; Rabbits: decrease in litter size	(14, 15)
Cyproconazole	113096-99-4	200	Triazole pesticide (P450 inhibition)	Rats: increase in resorption rate; embryoletalithy and cleft palate	(16, 17)
Rotenone	83-79-4	0.001	Mitochondrial inhibitor	Inconclusive, teratogenic effects such as supernumary ribs and unossified sternebrae have been observed in rats and mice, but always at doses accompanied with some maternal toxicity	(18)
HDAC inhibitors					
Valproic acid	99-66-1	1000	Migration inhibitor [c] (Multiple targets including HDAC inhibition)	*Major malformation or developmental delay; behavior disorders; congenital malformation; Asperger syndrome; Autistic disorder*	(19,20 ,21 22)
Trichostatin A	58880-19-6)	0.01	Broad HDAC inhibitor	TSA induced axial skeleton malformations comparable to VPA in mice	(23)
SAHA	149647-78-9	0.5	Broad HDAC inhibitor	In the absence of maternal toxicity, developmental toxicity was observed when 50mg/kg/day were administered to rats between GD6-20. This dose decreased fetal weight while increasing the number of fetuses with skeletal variations such as additional ribs as well as incomplete ossification e.g. of the skull. NOEL: 15 mg/kg/day	(24)
Tubacin	537049-40-4	0.1	Inhibitor of HDAC6		(25)

Fig. 2. Table of all compounds used for this study. The table indicates the concentration used for migration analysis as well as for gene expression analysis for each compound used in this study. We chose tool compounds known from previous migration studies as well as compounds with similar structure or mode of action. [a]Nominal concentration used in this study; [b]Association of figure reference to paper reference list number; [c]in in-house NCC migration assay (Zimmer et al., 2012); [d]NCC give rise to rib cage (Henderson et al., 1999); (1) (Grandjean et al., 1997); (2) (Grandjean and Landrigan, 2006); (3) (Opler et al., 2008); (4) (Ha et al., 2009); (5) (Tian et al., 2009); (6) (Kippler et al., 2012); (7) (Hamadani et al., 2011); (8) (Saha et al., 2012); (9) (Eubig et al., 2010); (10) (Patandin et al., 1999); (11) (Valvi et al., 2012); (12) (Lai et al., 2002); (13) (Chen et al., 1992); (14) (Zarn et al., 2004); (15) (Di Renzo et al., 2007); (16) (Machera, 1995); (17) (Giavini and Menegola, 2010); (18) (Krieger, 2004); (19) (Dean et al., 2002); (20) (Arndt et al., 2005); (21) (Meador et al., 2006); (22) (Rasalam et al., 2005); (23) (Menegola et al., 2005b); (24) (Verbois, 2006); (25) (Valenzuela-Fernandez et al., 2008).

real time PCR (qPCR) was performed on a BioRad CFX96 Thermal Light Cycler (Biorad, München, Germany). In order to keep technical variability low, the RNA of all samples of each replicate was prepared, and transcribed into cDNA in one working day. Also, qPCR of samples of one replicate was performed in parallel to avoid bias introduced by the sequence of processing and analysis.

For quantification, qPCR threshold cycles were normalized in a first step to the housekeeping gene GAPDH. qPCR data of treated samples were normalized by subtraction of the untreated control and displayed as fold change relative to controls ($2^{(-\text{delta delta } C(t) \text{ method})}$) (Livak and Schmittgen, 2001), as described in detail earlier (Zimmer et al., 2012, 2014). The sequences of specific primers are given in Fig. S1.

2.6. Biostatistics and data display algorithms

The R software for statistical computing version 3.1.0, was used as main tool for the statistical analysis.

Expression profiles and individual compounds were hierar chically clustered using Euclidian distance and average linkage method. Additionally, *K* means clustering was performed in order to identify groups of genes with similar expression patterns. The optimal clustering parameters were selected using within groups sum of squares and resulted in three clusters. Matrices of gene expression values were visualized as heatmaps produced using *R* package pheatmap (Kolde, 2012). Empirical *p* values for correla tions of gene expression of compound pairs were derived from 10,000 permutations of the data sets and ratioing of those runs with better correlations than the Pearson's *r* values indicated vs. the total number of runs.

Principal component analysis (PCA) was performed on the original data set of 17 compounds. The first three principal components covered 78% of the variance in the data. In order to visualize expression data in three dimensions, representing the first three principal components (PC), the 'Excel 3D scatterplot' macro was used.

For further analysis, 10 compounds with three replicates were selected from the original data set. In order to investigate further the separation of the selected compounds, linear discriminant analysis (LDA) was performed. LDA (Cheok et al., 2003; Rencher, 2003) assumes predetermined groups (in contrast to PCA) and thus belongs to the class of supervised techniques testing the validity of the assumption (=grouping). However, LDA cannot be applied on 'ill defined problems', i.e. when the number of features is larger than the number of observations (Rencher, 2003). Therefore, we first applied PCA on the selected part of the original data set to reduce dimensionality, as proposed in Belhumeur et al. (1997). The first 13 principal components that covered 95% of the variance were selected for further analysis.

The leave one out cross validation method (LOOCV) was used to evaluate the LDA model performance with respect to model accuracy (Acc_{LOOCV}). The *R* package caret (Kuhn et al., 2008) was used for model building and performance evaluation. In order to assess the quality of separation achieved by discriminant functions in the given data set, a permutation test was used. The labels of the individual samples were randomly permuted 1012 times and used for model building. The model accuracy (AccRP) was then calculated on each permutation step and used to calculate an empirical *p* value under the assumption of the null hypothesis H0: $Acc_{LOOCV} = AccRP$ and of the alternative hypothesis H1: $Acc_{LOOCV} > AccRP$.

The calculation and display of toxicity curves were done using GraphPad Prism 5.0 (Graphpad Software, La Jolla, USA).

3. Results

3.1. Unbiased data acquisition for neural crest migration inhibition by automated imaging of scratch assays

Migration of neural crest cells (NCCs) is a key biological process in vertebrate development and a potential target of developmental toxicants. In line with this, it has been shown that the migration of neural crest cell assay (MINC) (Zimmer et al., 2012) is able to identify inhibitors of NCC migration in a sensitive and specific manner. The NCCs used for our assay are derived from human embryonic stem cells (hESC), and stored as frozen stocks until immediate use in the MINC (Fig. 1A). Several acceptance criteria such as purity >98% and proper migration behavior were defined as quality measures for each of the batches used. For the present study, we followed the established protocol and probed migration capacity of NCCs in the time window of 48 96 h after thawing and plating (Fig. 1B).

The main endpoint of the assay requires manual, time consuming counting of migrated cells, and an estimation of the original scratch width (Zimmer et al., 2012). To make the test system more operator independent, and to automate the data acquisition and processing pipeline, an automated digital imaging based analysis of the scratch was developed using the open source KNIME (Konstanz information miner) software (Berthold et al., 2008). A protocol was developed, which measures the initial scratch width (day 0), then uses an algorithm to identify and re locate the scratch after 48 h, and to quantify the migrated cells automatically (Fig. 1C).

To compare the new approach (automated counting using KNIME) to manual counting, cell migration was quantified in 84 test conditions (five independent experiments, nine compounds at various concentrations) using both quantification methods. The results on migration inhibition did not differ significantly between both methods (Fig. 1D). Our data suggest that KNIME analysis may be slightly more conservative (smaller inhibition values) than manual counting, but definitely does not lead to oversensitivity of the assay. Since the new KNIME based quantification method resulted in reliable and unbiased results, it was used to test a larger set of potential toxicants in the MINC (Fig. 2, Suppl. Fig. 2).

3.2. Inhibition of neural crest cell migration by different groups of environmental toxicants

To test whether the new setup of the MINC assay was able to confirm positives known from previous studies (e.g. VPA, MeHg), and to identify new hits we compiled different groups of well known environmental toxicants (metals, pesticides and poly chlorinated biphenyls (PCBs)) to be tested in the MINC. Initially, we determined the appropriate test concentration for each new compound and positive controls. The aim of this pilot testing was to use a concentration with no significant cell death and at least 25% inhibition of migration. Therefore we performed the MINC assay with increasing concentrations of the chemical and determined cell viability by resazurin reduction and migration inhibition after 48 h exposure by our new test set up, as described above (Suppl. Fig. 3A).

Compounds not inhibiting migration at a non cytotoxic concen tration were excluded from further testing. We felt that this pre selection of test concentrations and compounds was useful for comparing different endpoints of interest (mRNA changes vs. migration inhibition) within this in vitro cell system.

After the preliminary tests, compounds were tested in observer blinded fashion on 4 5 different cell lots in independent experiments. Non cytotoxicity was confirmed for the known MINC positive controls valproic acid (1 mM) and MeHg (50 nM), as well as for the new potential toxicants As_2O_3 (1 μM), $CdCl_2$ (100 nM), cyproconazole (200 μM), rotenone (10 nM), PCB 170 (0.5 μM), PCB153 (5 μM) PCB 180 (5 μM) (Suppl. Fig. 4A, Fig. 2). We compiled a comparison with in vivo doses or in vitro concentra tions found/used in other studies of the same compounds. The concentrations used here by us were in concordance with such literature data (Suppl. Fig. S2).

All compounds (at the chosen concentration) blocked migra tion significantly across all test rounds. Treatment with MeHg reduced migration to 63 ± 2.3%, and VPA reduced migration to 49 ± 6% (means ± SEM, as all following data in this paragraph). Inhibition by As_2O_3 was to 58 ± 5.4% of control, for $CdCl_2$ to 65 ± 4.7%, for cyproconazole to 69 ± 5.4%, for rotenone to 70 ± 7.5%, for PCB 170 to 73 ± 10.1%, for PCB 153 to 69 ± 5.5%, for PCB 180 to 44% ± 12% (Fig. 3A; Suppl. Fig. 4B). In a control study, cells were also treated with the negative control compounds acetylsalicylic acid (250 μM) and acetaminophen (250 μM) (Kader eit et al., 2012). Migration was not impaired by the negative controls (Fig. 3B).

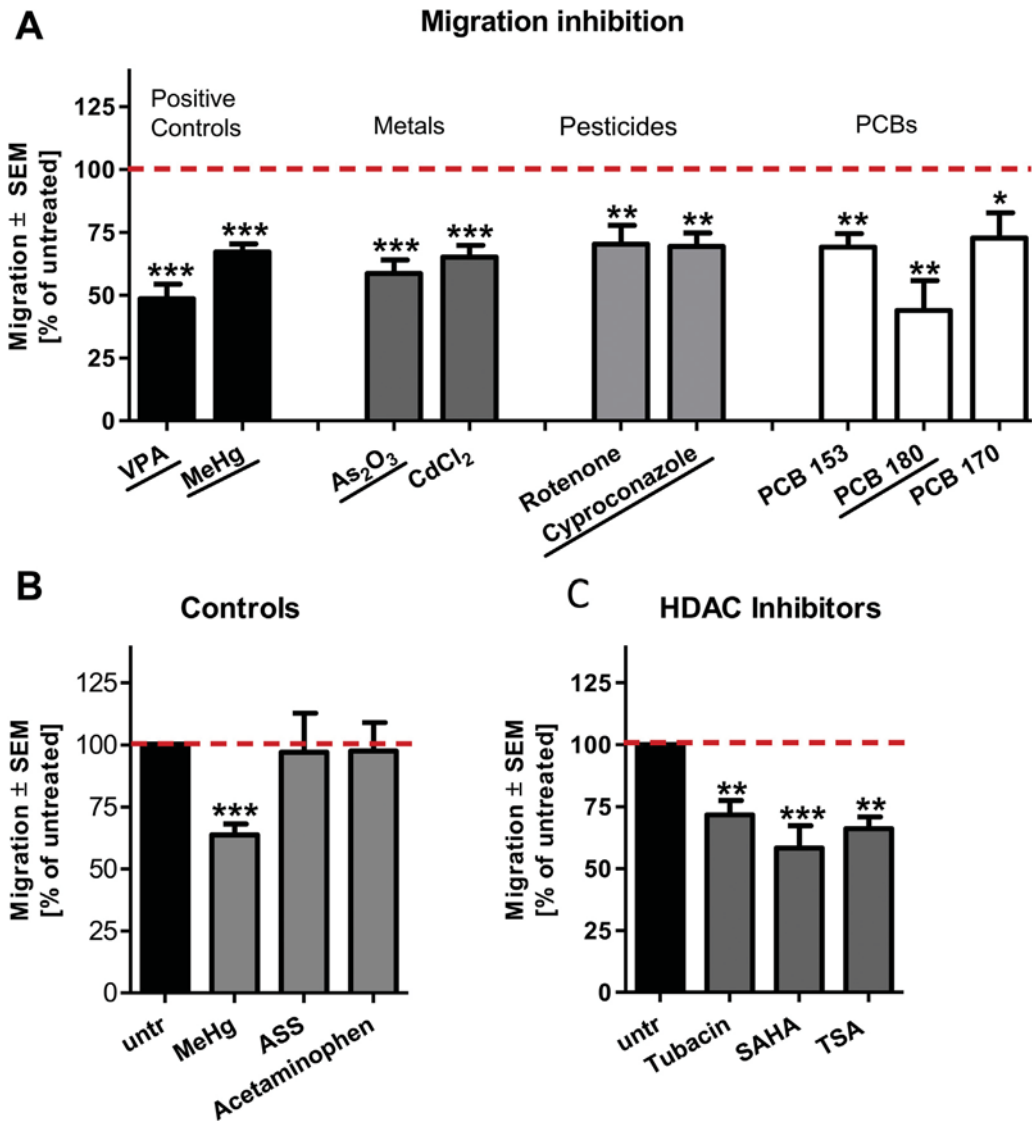


Fig. 3. Inhibition of neural crest cell migration by different compounds. Neural crest cells were treated for 48 h with the indicated compounds; migration into region of interest (ROI) was assessed by the MINC assay and compared to an untreated control as described in the method section. The MINC assay was performed using the indicated compounds in non-cytotoxic concentration, which were determined by viability testing using resazurin reduction. For all indicated compounds viability was ≥100% at the chosen concentration (see Suppl. Fig. S4). (A) Migration was inhibited by the following compounds VPA (1000 μM), MeHg (0.05 μM), As_2O_3 (1 μM), $CdCl_2$ (0.1 μM), rotenone (0.001 μM), cyproconazole (200 μM), PCB 153 (5 μM), PCB 180 (5 μM), PCB 170 (0.5 μM), PCB 138 (0.5 μM). (B) The MINC assay was performed using negative (ASS, acetaminophen) and positive (MeHg) control compounds using the following concentrations: acetylsalicylic acid (250 μM; ASS), acetaminophen (250 μM) and MeHg (0.05 μM). MeHg was tested along in this set of experiments as acceptance control for general assay function. (C) Inhibition of migration after treatment with the HDAC inhibitors tubacin (0.1 μM), SAHA (0.5 μM) and TSA (0.01 μM). $^*p \leq 0.05$, $^{**}\leq 0.01$ $^{***}\leq 0.001$. Data are means of 4–5 independent experiments ±SEM compared to untreated control (set to 100%). Dotted red lines indicate the 100% level of the untreated control for better visual orientation.

3.3. Upregulation of three exemplary migration related genes (CAV1, ITGA4, MYLK) by valproic acid

As a first approach to explore a potential correlation between gene regulation and altered migration, we selected three transcripts for RT qPCR analysis. The choice of these markers for this preliminary analysis was driven by an easily available in house quantification method. We decided to use the signaling component CAV1 (caveolin) (Lentini et al., 2008; Park and Han, 2009), the adhesion component ITGA4 (integrin) (Deakin et al., 2009; Liu et al., 1999; Testaz and Duband, 2001) and the Rho Kinase (Groysman et al., 2008) effector MYLK (myosin light chain kinase) (Miao et al., 2010), as they have been used in earlier studies, but for different cell types, and they contribute to different migration related processes. Their differential gene expression after toxicant treatment (same concentration as for migration data) was then compared for selected representatives of each toxicant group (As_2O_3, cyproconazole, PCB 180) and two positive controls (VPA, MeHg). Assay conditions corresponded exactly to the MINC assay as described above (48 h exposure, same concentration and culture condition).

We found that the anti epileptic compound VPA up regulated all three transcripts, compared to negative controls or untreated cells. MeHg did not clearly alter expression of these three genes; As_2O_3 and cyproconazole, at concentrations inhibiting migration approxi mately to the same extent, down regulated the three genes and PCB 180 showed a mixed response pattern (CAV1 and ITGA4 were slightly down regulated and MYLK was not regulated) (Fig. 4).

3.4. Inhibition of NCC migration by diverse HDAC inhibitors

VPA, a clinically used drug, had originally only been used as very robustly functioning positive control with no particular relationship

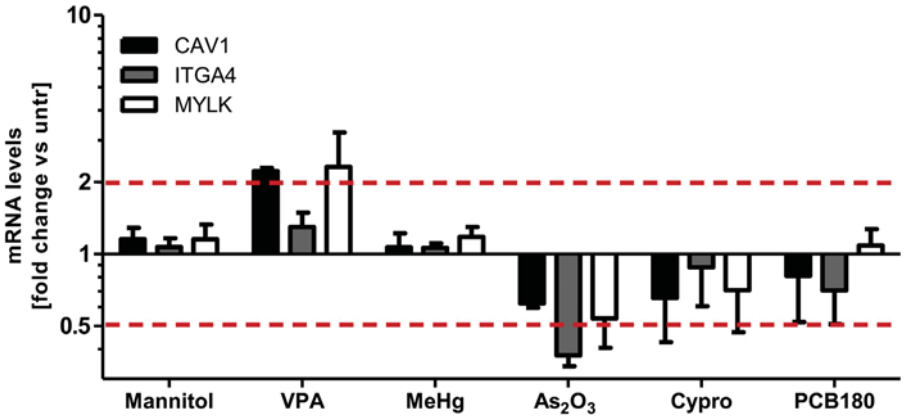


Fig. 4. Gene expression changes of the migration related genes CAV1, ITGA4, MYLK. Neural crest cells were incubated for 48 h with the indicated compounds exactly as done for the MINC assay using the same concentrations shown to reliably inhibit NC cell migration: (Mannitol (negative control; 250 μM), VPA (1000 μM), MeHg (0.05 μM), As_2O_3 (1 μM), cyproconazole (200 μM) and PCB 180 (5 μM)); mRNA was isolated from NCC after 48 h treatment with the toxicants and analyzed by RT-qPCR for CAV1 (black), ITGA4 (gray) and MYLK (white). Gene expression levels are presented relative to untreated controls, and normalized to GAPDH. Data are means of three independent experiments ±SEM.

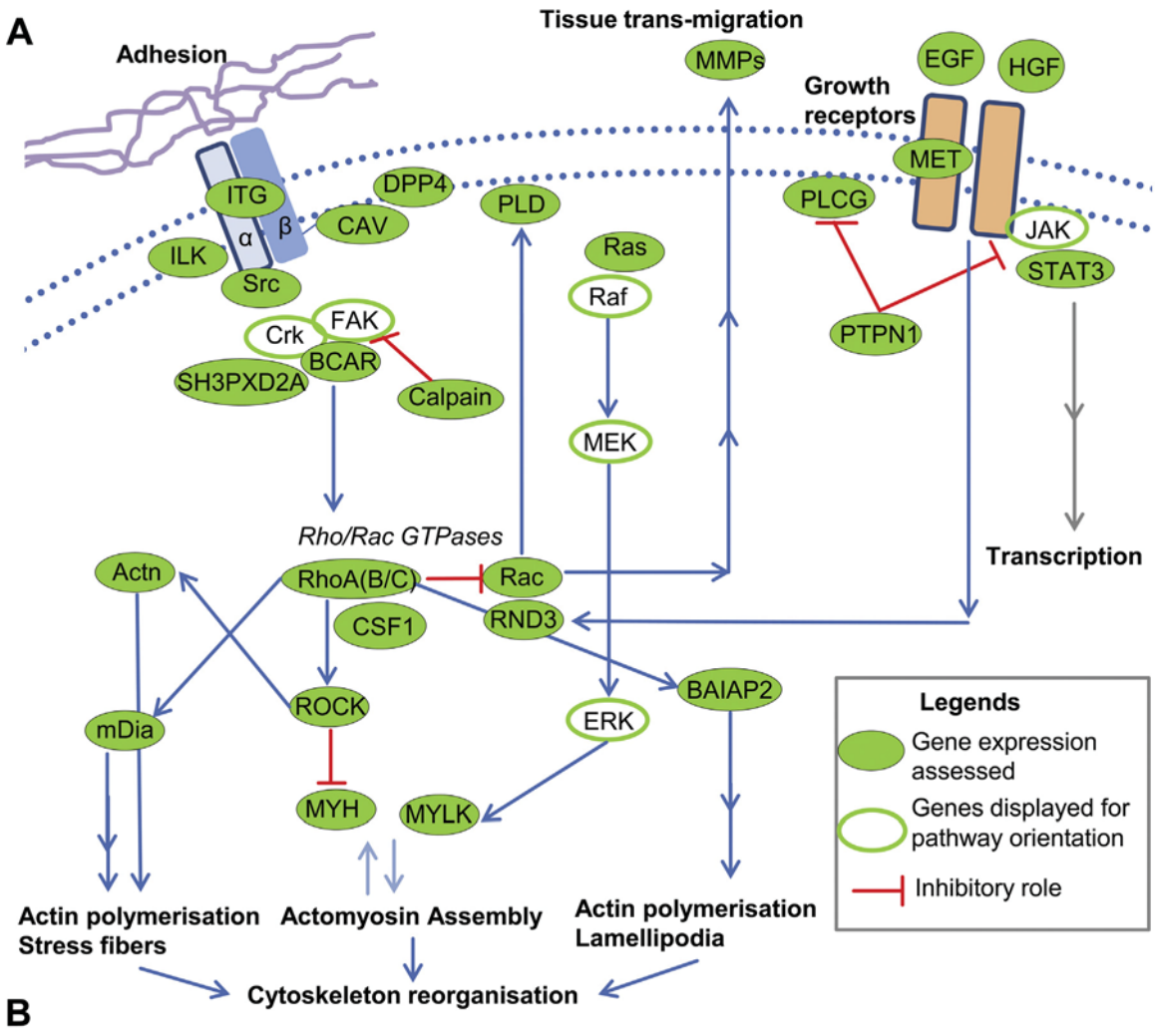
to environmental toxicants. However, we were intrigued by the significant, and unidirectional up regulation of the three migration related genes MYLK, ITGA4 and CAV1. One of the many known modes of action of VPA is inhibition of histone deacetylases (HDAC), and HDAC inhibition often leads to altered transcriptional activity. Therefore we wondered whether inhibition of neural crest migration is a general toxicological feature of HDAC inhibitors (HDACi) or specific for VPA. Three additional, and more specific, HDACi, namely trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA) and tubacin were therefore tested in the MINC assay. VPA, TSA and SAHA inhibit all class I+II HDACs (Dietz and Casaccia, 2010), while tubacin is specific for HDAC 6 (Ding et al., 2014). The exposure to SAHA (500 nM), TSA (10 nM) and tubacin (100 nM) lead to a strong decrease in NCC migration to 66 ± 9%, 58 ± 5% and 72 ± 6% of control (Fig. 3C). From these data, we concluded that HDACi do represent a new group of toxicants with potential adverse effects on neural crest during development indeed due to HDAC inhibition and not due to other off targets of VPA. Therefore we were interested in seeing whether they showed a uniform gene regulatory response.

3.5. Confirmation of up regulation pattern by HDAC inhibitors

The preliminary data on gene expression changes suggested specific patterns of transcript changes for different toxicant groups. To obtain more information on transcript changes within one group of toxicants, and to expand the number of transcripts studied, we extended our analysis to 35 migration related genes (Fig. 5) and a larger set of compounds (Fig. 2). The genes were selected based on migration related functions and changes observed during migration as well as altered expression in response to a variety of toxicants that alter migration. However it is not clear whether changes in these genes play roles in causing migration, respond to migration events, or were simply correlated with cell migration. The study now included the pilot compounds VPA, MeHg, As_2O_3, cyproconazole, and PCB 180. It also included the additional metals $CdCl_2$, additional PCBs PCB170 and PCB 153 (PCBs), as well as more positive controls from previous studies, i.e. triadimenol, triadimefon (triazoles), Pb acetate and thimerosal (metals) (Zimmer et al., 2012). NCCs were incubated for 48 h with non cytotoxic, migration inhibitory concentrations of these compounds, before mRNA was isolated and analyzed. As a negative control, mannitol was included. Although the observed regulations did not reach the 5% significance level on the individual gene level (after false discovery rate correction), clustering and color coding in a heat map suggested co regulation patterns. Moreover, hierarchical clustering clearly indicated two major groups of compounds according to their gene regulation pattern (Fig. 6, Suppl. Fig. 5). The first group included the general HDAC inhibitors VPA, TSA and SAHA, and it was characterized by upregulation of >50% of the analyzed transcripts. The second cluster consisted of two subgroups of compounds. The first contained cyproconazole, the PCBs 170 and 180, As_2O_3 and $CdCl_2$, and was characterized by an overall down regulation of tran scripts. The second group contained the remaining compounds and showed very little transcriptional change. This analysis confirmed the unique properties of HDACi that distinguished them from other migration inhibitors. It also clearly showed that the same apical endpoint (impaired migration) can be related to largely different changes of intracellular markers.

To compare the phenotypic response (migration deficit) and the corresponding gene expression changes we chose three com pounds (TSA, As_2O3 and PCB180) and analyzed the concentration dependency of gene regulation for a set of 5 7 genes chosen from Fig. 6. Gene expression analysis revealed that the mRNA response curves are mostly monotonic. This suggests that the expression data are not random numbers (although changes are small), but biologically consistent. The toxicant concentrations used in this study were right in the range when transcripts started changing and the change in RNA expression scaled with toxicant concentra tion. This suggests that transcript changes and altered migration may be biologically connected, despite the small overall changes (Suppl. Fig. 3B).

To get initial information on co regulation of the migration related genes, we performed *k* means clustering of the 35 markers across all test compounds. This resulted in three distinct clusters (Suppl. Fig. 6A). For instance, the initial markers CAV1 and MYLK co segregated in cluster 1, and this cluster very strongly distinguished general HDAC inhibitors from other compounds. Cluster 2 was enriched in genes such as calpain or PTPN, whose cognate proteins have inhibitory roles in the migration network (Suppl. Fig. 6B). Cluster 3 contained the initial marker ITGA4 and many signal transduction components. Clear pathways or biologi cal principles did not emerge from this analysis across all compounds. An alternative approach, i.e. analysis of the genes co regulated by one given group of compounds (e.g. HDACi) by search for overrepresented KEGG pathways and gene ontology terms yielded the obvious migration processes, but no information beyond this.



ACTN	Actinin	FAK	Protein-tyrosin kinase	PLD1	Phospholipase D
BAIAP2	Brain specific angiogenesis inhibitor	HGF	Hepatocyte growth factor	PTPN1	Protein tyrosin phosphatase
BCAR1	Breast cancer anti Estrogen resistance	ILK	Integrin linked kinase	RAC	Small GTP binding protein
CAPN1	Calpain (Protease)	ITGA	Integrin (β/α)	RHO	Small GTP binding protein
CAV1	Caveolin (couples integrins)	JAK	Janus tyrsosin Kinase	RND3	Rho family GTPase
Crk	V-crk Oncogene homolog	MEK	MAPK Kinase	ROCK1	Rho associated kinase
CSF	Colony stimulating factor	MET	Hepatocyte growth factor receptor	RAF	MAP kinase
DIA	Diaphanous Homolog 1 (modeling of scytoskeleton)	MMP	Matrix metallopeptidase	SH3PXD2A	Adapter protein (SH3 and PX domains)
DPP4	Dipeptidyl-Peptidase	MYH	Myosin heavy chain	SRC	V-src homolog
EGF	Epidermal growth factor	MYLK	Myosin light chain kinase	STAT3	Signal transducer of Transcription
ERK	MAP Kinase	PLCG1	Phospholipase C		

Fig. 5. Cell migration regulation pathways. (A) Schematic representation of selected genes and their functional relationship among each other and to migration. Genes tested on gene expression level in this study are depicted in green. Additional genes, shown in white, are displayed for pathway orientation. (B) Abbreviations and full names of the genes analyzed in this study (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

3.6. Correlation of gene expression profiles of structurally and mechanistically related compounds

Clustering gives equal weight to both low information components (i.e. minor regulations) and to biologically more meaningful information (regulated genes). Therefore, we applied principal component analysis (PCA) to the transcript data set as alternative approach. We were interested whether compounds of the same mechanistic group (HDACi) would cluster together, and separate from other groups of toxicants. The first two principal

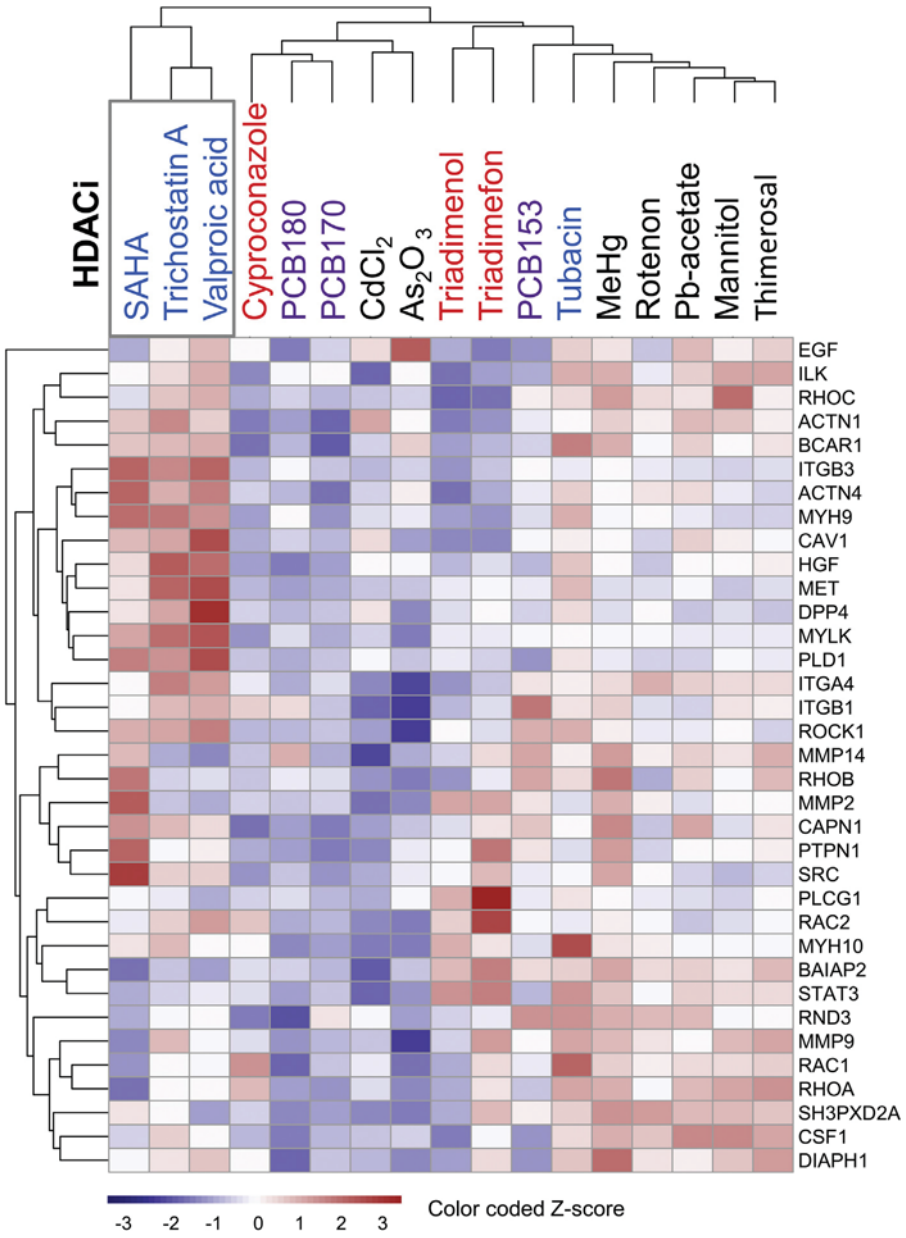
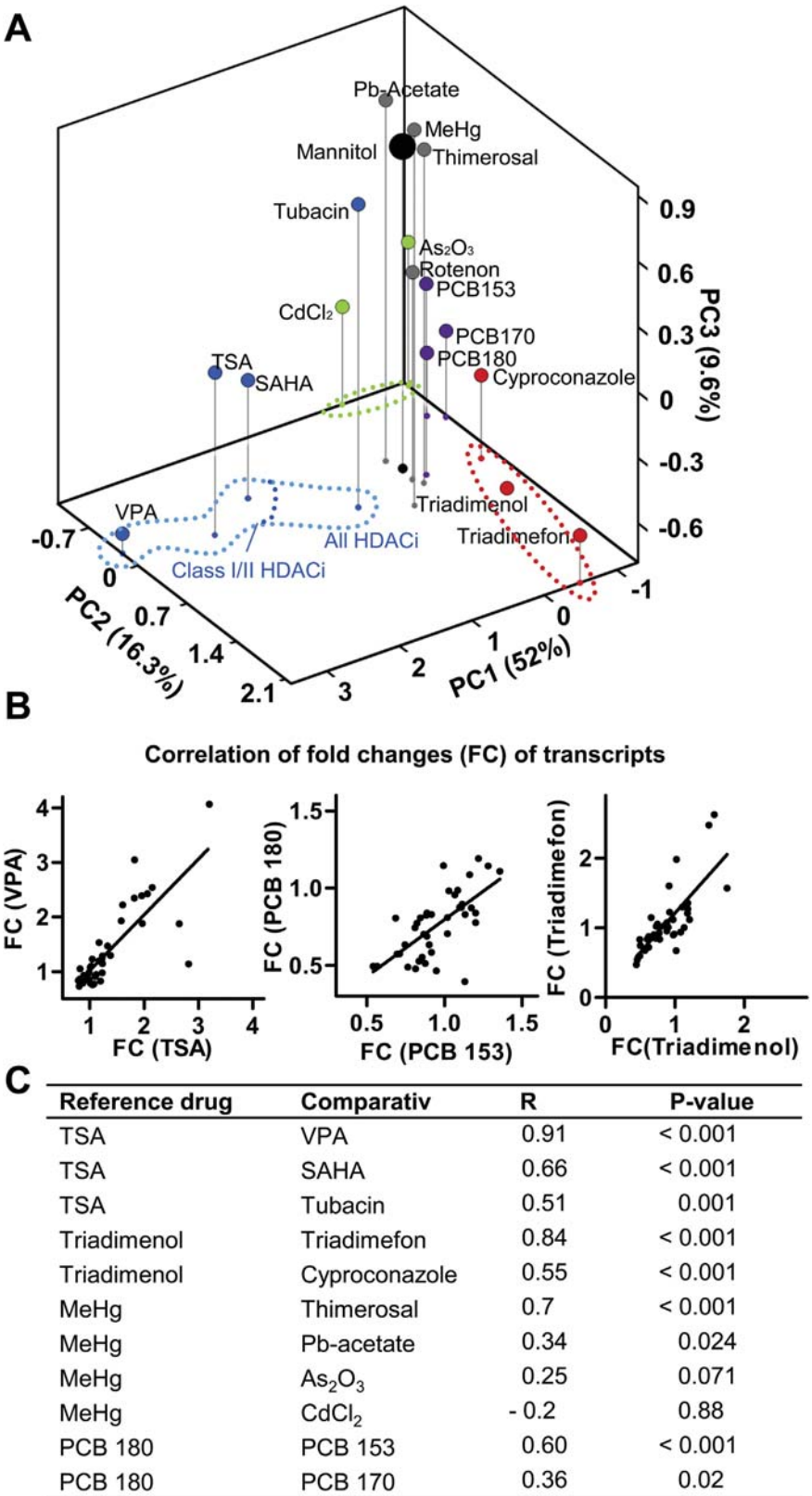


Fig. 6. Differential gene expression of 35 migration related genes over individual compound treatments. Neural crest cells were exposed to the indicated compounds for 48 h (equal concentration as in the previous MINC assay; indicated in Fig. 2). RNA was isolated and gene expression was assessed by RT-qPCR. Gene expression levels are indicated relative to untreated control and the reference gene GAPDH. Expression levels were scaled and color-coded (*z*-score). Dark red and blue colors indicate high and low levels of expression respectively. Expression profiles were hierarchically clustered using Euclidian distance and average linkage. Data are means of three independent experiments (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

components (PC1/PC2) covered 68% of the total variance in the data, and PC3 additional 10%. Therefore analysis was limited to the first three PC dimensions (Fig. 7A). The class I/II HDAC inhibitors VPA, TSA and SAHA were clearly separated from all other compounds, even when only PC1 and PC2 were considered. On the projection to the 2D plane, the HDAC 6 inhibitors tubacin lay right between the group of HDACi and the negative control mannitol. Another group that separated well on the first two dimensions was the triazoles cyproconazole, triadimefon and triadimenol, as well as the small group of light metals (As_2O_3 and $CdCl_2$). The heavy metal compounds Pb acetate, MeHg and thimerosal did not clearly separate from the control. The PCBs did not clearly separate from other groups, but they clustered together as one group. Thus, the PCA appeared to show a higher power of separation, than the standard clustering algorithms. It was interesting that some clear grouping emerged already with the use of a relatively small number of marker genes.

The disadvantage of PCA is that the descriptive data (visual inspection) are hard to quantify, i.e. the apparent clustering of groups is difficult to test for statistical significance. Therefore, we tested, whether other quantitative approaches would support these findings. First, we explored whether gene expression profiles of related compounds showed better correlation than unrelated compounds. The scatter plots of gene expression for VPA vs. TSA, PCB153 vs. PCB180, and triadimefon vs. triadimenol suggested that there is in fact a good correlation of transcript changes, even though the regulations of individual genes as such were moderate only, and none of the single changes were significant at the 5% level after performing *t* tests and FDR corrections (Fig. 7B).



Reference drug	Comparativ	R	P-value
TSA	VPA	0.91	< 0.001
TSA	SAHA	0.66	< 0.001
TSA	Tubacin	0.51	0.001
Triadimenol	Triadimefon	0.84	< 0.001
Triadimenol	Cyproconazole	0.55	< 0.001
MeHg	Thimerosal	0.7	< 0.001
MeHg	Pb-acetate	0.34	0.024
MeHg	As_2O_3	0.25	0.071
MeHg	$CdCl_2$	- 0.2	0.88
PCB 180	PCB 153	0.60	< 0.001
PCB 180	PCB 170	0.36	0.02

Fig. 7. Correlation of compound treatments on their gene expression profile. Neural crest cells were exposed to the indicated compounds for 48 h (equal concentration as in the previous MINC assay; indicated in Fig. 2). RNA was isolated and gene expression was assessed by qPCR; differential expression was calculated by subtraction of untreated control. (A) Based on the gene expression data obtained by qPCR analysis of 35 migration related genes principal component analysis (PCA) was performed. First three components cover correspondingly PC1: 52%, PC2: 16.3%, PC3: 9.6% of the variance in the data. Projections of the individual experiments to the two-dimensional planes reflect the separation by the corresponding PCs. (B) Comparison of gene expression profiles of structural or functional similar compounds (TSA/VPA, PCB180/153, triadimenol/triadimefon) by scatter plot correlation. (C) Pearson correlation of the gene expression profiles of the same group of compounds (HDAC inhibitors, triazoles, metals). The *p*-values were determined using a permutation test: the fold-change values ($n = 35$) associated with one of the compounds were shuffled 10,000 times among the genes. The empirical *p*-value indicated shows the fraction of reshufflings for which a correlation coefficient larger than the original coefficient was found.

In order to quantify this, correlation coefficients (r) and the significance of correlation (empirical p value) were calculated for relevant compound couples (Fig. 7C). Using TSA as a reference compound of HDACi, VPA and SAHA showed high correlation, while tubacin correlated somewhat less. Within the triazoles, triadimefon correlated significantly with its metabolite triadimenol and with cyproconazole. MeHg correlated with the very related organomercurial thimerosal but not with Pb acetate, As_2O_3 and $CdCl_2$. PCB 180 correlated with PCB 153, and to a smaller extent ($p = 0.02$) with PCB 170.

3.7. Robustness of gene expression changes and of HDAC group clustering

As second statistical quantification approach following the qualitative grouping observed in the PCA, we chose linear discriminant analysis (LDA). This method assumes already given groups (in contrast to PCA), but allows the quantification of significant group differences. Like PCA, it leads to dimension reduction onto linear vectors (linear discriminants). These separations can then subsequently be used for statistical testing.

Three replicates of 10 compounds (VPA, SAHA, TSA, triadimefon, tubacin, $CdCl_2$, mannitol, cyproconazole, triadimenol and As_2O_3), i.e. 30 data samples (each based on 35 transcript measurements by PCR) were used for the analysis, and the replicates of a given compound were considered to form a group. The LDA model was then constructed as described in methods. The model accuracy (Acc_{LOOCV}) determined by leave one out cross validation (LOOCV) was 63% with a 95% confidence interval ranging from 44% to 80%. In a second step, a permutation analysis (altogether 1012 permutations) was used to calculate an empirical p value for the classification accuracy. A p value of 2×10^{-12} was obtained, suggesting that the accuracy of the LDA model is significant.

All toxicant replicates clustered very closely to one another, and many compounds separated from one another, when the first 3 linear discriminants (LD) were considered (Fig. 8). Visual inspection shows that the first LD separated five compounds (VPA, SAHA, TSA, triadimenol, and As_2O_3) clearly from the others. The second LD separated triadimefon and the third one was not sufficient alone for separation of additional compounds. Performance of the LDA classifier was examined by its ability to correctly classify individual experiments into the compound groups. For 7 out of 10 compounds $\geq$2 of the three replicates were correctly assigned (Suppl. Fig S7). This final analysis confirmed, that the intra compound variation (replicates) was significantly lower than the inter compound variation, at least for the HDACi and e.g. triadimenol and As_2O_3. The resulting findings are in line with our suggestion that the compound specific transcript signatures were statistically significant, even though individual gene variations were not.

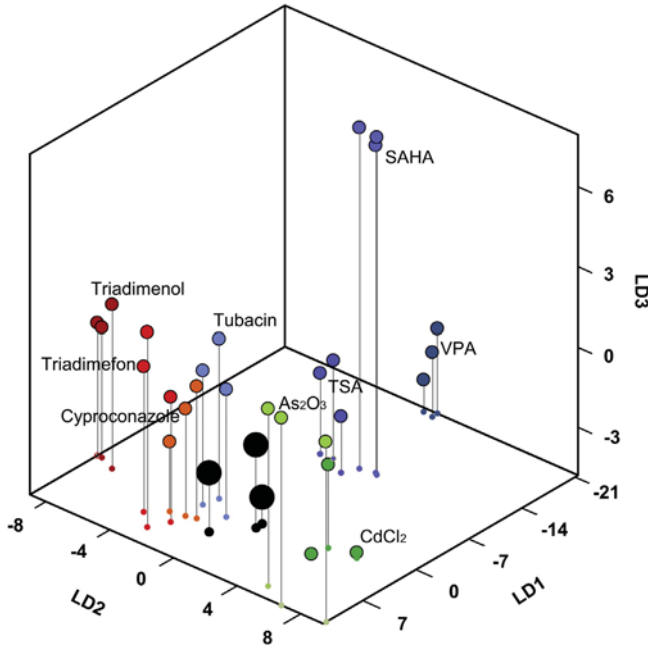


Fig. 8. Linear discriminant analysis (LDA) to visualize separation of gene expression profiles due to toxicant treatment. The linear discriminant analysis (LDA) describes the separation of the gene expression profiles of nine selected compounds plus mannitol as negative control (black). The values of the discriminant functions for each individual experiment are shown on the axes. Depicted are the single experiments ($n = 3$) for the 10 selected treatments.

4. Discussion

The MINC assay has been established initially to provide a test system for neural crest function based on human cells (Zimmer et al., 2012). This assay was then used in a feasibility study for an in vitro test battery in the area of developmental toxicology (Zimmer et al., 2014). In the present study, the test system was developed further for general applicability by exchanging the manual evaluation of migrated cells for a new unbiased computer based analysis method. Moreover, expression levels of about three dozen migration related genes were quantified as additional endpoint. This data set was generated within the MINC test system to explore whether the additional biological information layer would allow grouping and sub classification of toxicants that inhibit NCC migration. We found that some groups of chemicals with supposedly related modes of action did cluster to gene expression signatures. For instance, statistical approaches based on the overall pattern of altered gene expression showed that compounds (VPA, TSA and SAHA) belonging to the class of HDACi clearly clustered together. Also PCBs or triazoles tended to form clusters, although not as sharply defined as HDACi. Thus, incorporation of a relatively small number of mechanism based endpoints into the cell function based MINC assay allowed for grouping of compounds along their toxic mode of action. This finding suggests that incorporation of gene expression patterns as additional endpoints in phenotypic/functional cell based assays can support biological read across. By using such approaches on the background of data on many well characterized toxicants in the same assay, additional toxicological information can be derived for new toxicants that would not be available from the primary endpoint of the tests.

Cell migration is a key process during development, as well as in various diseases (Franz et al., 2002). Migration of neural crest cells from the neural tube to their final destination is a key event during the development of many tissues, including the peripheral nervous system, the heart and facial bones (Dupin and Sommer, 2012). Disturbance of such migration events can lead to severe malformations or diseases including Hirschsprung disease, Teratology of Fallot or DiGeorge syndrome in humans (Keyte and Hutson, 2012; Menendez et al., 2013). Impairment of NC development and function after exposure to a variety of chemicals, including pesticides, planar PCBs or anticonvulsant drugs has been observed in different vertebrate test systems (Di Renzo et al., 2007; Fuller et al., 2002; Grimes et al., 2008; Menegola et al., 2005a; Papis et al., 2006). Such animal based test systems are very limited in their throughput. Therefore, the data provided often compare only few compounds and doses. Test systems able to explore a larger set of compounds including groups of structurally and/or mechanistically related compounds useful for QSAR or read across approaches are rare. An additional limitation for mechanistic studies can be that a particular system evaluates an integrated endpoint affected by cell

migration, cell division and cell differentiation at the same time (Moors et al., 2007). Since mechanistic conclusions are difficult or impossible, if several different biological processes contribute to the overall readout, we have shown earlier that the MINC assay faithfully assesses cell migration independent of differentiation and cell division (Zimmer et al., 2012). The incorporation of the unbiased KNIME plugin for quantification of migration data in the MINC now further supports the evaluation of toxicant effects on migration only.

The identification of genes that would be specific for NC cell migration is not an easy task. The mechanism of cell migration are very well described in various immune cells, cancer cells (Lauffenburger and Horwitz, 1996), NCC, and central neural precursors (Gong, 2014; Hippenmeyer, 2014; Kuriyama and Mayor, 2008; Kwan et al., 2012; Liu, 2011; Mayor and Theveneau, 2014). Comparison of the different cell types shows that the core migration machinery is evolutionary conserved and shared by all cells, while the integration of signals, the contribution of various pathways, and the combination of final cellular processes might differ dramatically from cell to cell, and even from stimulus to stimulus. Therefore the reaction of NCC to toxicants differs from that of cancer cells or even from that of related central nervous system precursors (Zimmer et al., 2012), even though the overall genetic control machinery is mostly the same. Many 'migration genes' are also involved in entirely different cellular processes. Therefore gene ontology groups (GOs) for migration contain about 1600 genes. Many of them are however only involved in migration in very specific situations, e.g. after activation of a certain receptor on a specialized immune cell. For our study, we selected about 70 of the key machinery genes by choosing few representatives for the major processes controlling cell migration such as cytoskeleton rearrangements (RAC1, RHOA, ROCK), cell adhesion (ITGA4, ITGB1, ITGB3), and tissue invasion (MMP). After examining the expression levels in NCC we compiled a final list of robustly expressed 35 genes, which we used for the analysis.

The usefulness of our approach was most evident from the confirmation of HDACi as neural crest toxicants. The starting point of the study was the use of valproic acid (VPA), as convenient stable and water soluble positive control, as we found previously (Zimmer et al., 2012) that it strongly inhibits NCC migration. The drug is used as antiepileptic and to dampen bipolar disease. The clinical mode of action is supposed to involve inhibition of gamma aminobutyric acid degradation, but it may also involve modulation of ion channels, wnt signaling and altered phosphoi nositol turnover (Rosenberg, 2007; Terbach and Williams, 2009; Williams et al., 2002). Moreover, VPA is a well known develop mental neurotoxicant that induces severe malformations when taken during pregnancy (Ornoy, 2009). When our pilot experiment indicated general gene up regulation by VPA, we considered that histone deacetylase inhibition might play a role as relevant target of VPA in the MINC. VPA is known to broadly inhibit the de acetylation of histones, and therefore, its alternative use in cancer therapy has been considered. The increased acetylation state of histones is known to make DNA more accessible and this chromatin change promotes relatively unspecific gene activation. To test the relevance of HDAC as target in NCC migration, additional compounds were tested that are more selective and potent HDACi, and that lack effects on other VPA targets. These all were found to be hits in the MINC, and they triggered similar gene expression signatures as VPA. This makes it likely that VPA acted in the assay mainly as HDACi. Interestingly, various HDACi have been shown to inhibit invasive motility of cancer cells (Eyüpoglu et al., 2005; Liu et al., 2003). The compound does not seem to affect a target in the core migration machinery, since it only affects migration in some tumor cells, and if reduces migration of some immune cells, but not others (Brogdon et al., 2007). Our data obtained with tubacin, an HDACi that specifically inhibits the isoform HDAC6 (Haggarty et al., 2003), suggest that HDAC6 inhibition may play a role in the MINC. This isoform is mainly located in the cytosol, and it regulates the acetylation state of tubulin (Hubbert et al., 2002), which is important for cell motility (Tran et al., 2007). Our clustering analysis suggested that VPA, TSA and SAHA are much closer related than this group is related to tubacin. This example shows that additional background information (e.g. knowledge on enzyme inhibition pattern) can be useful to further refine the biological read across of our gene expression signature.

The finding that the two light metal salts, $CdCl_2$ and As_2O_3, clearly separated from the heavy metal compounds MeHg, thimerosal and Pb acetate intrigued us. Indeed, $CdCl_2$ and As_2O_3 have been shown earlier to influence the global level of DNA and histone methylation, and thereby strongly affecting gene expres sion (Cui et al., 2006; Zhou et al., 2012). On the other hand, especially the mercurial compounds have been shown to have relatively little influence on gene expression also in other human stem cell based test systems (Krug et al., 2013). The heavy metals may exert their toxic effect directly on the protein level via, e.g. direct enzyme inhibition. For instance, mercury directly binds to free protein thiol groups (Kanda et al., 2012; Rocha et al., 2012). For example, mercury can directly inhibit γ secretase and thereby disrupt the notch pathway during neural development (Alattia et al., 2011). Direct biochemical effects as possible mode of action for the heavy metals is further supported by the fact that the pesticide rotenone clustered in the same group. It is well known that rotenone inhibits the mitochondrial respiratory chain, and thereby depletes intracellular ATP (Li et al., 2003) independent of gene expression changes.

For all the other chemicals used, grouping based on gene expression was less clear than for HDACi. This was somewhat astonishing as, e.g. the groups of triazoles or PCBs contained chemicals that are structurally closely related and are expected to have at least related modes of action. However, different PCBs may have different affinities for the same receptor or have additional targets, which could explain the different grouping. Moreover, the chosen gene set was very limited and a larger set may have shown similarities and differences more clearly. The grouping of chemicals based on gene expression changes is a new approach and few publications are yet available on this topic. Amongst them, two studies expanded the embryonic stem cell test (EST) by an additional transcriptomics endpoint. In the first, six phtalates were compared to six triazoles, with the groups already known at the onset of the study, and with whole transcriptome information provided (van Dartel et al., 2011). In the second study from the same lab (van Dartel et al., 2010), two individual compounds were compared to see whether transcriptome data would distinguish them in an embryonic stem cell assay. In both studies, the groups to be distinguished were given before statistical analysis, while our study performed initial grouping without pre defining the different compound classes.

The inclusion of a higher number of genes in our strategy may eventually lead to the identification of biomarkers of toxicity (BoT). A BoT is defined as "a parameter that provides quantitative information that is mechanistically relevant to and predictive of an adverse effect" (Blaauboer et al., 2012). Biomarkers play an important role especially for in vivo studies to get early and sensitive indications of liver or kidney injury (Dedeoglu et al., 2013; Schomaker et al., 2013). Moreover, they are important for in vitro studies with cell death as primary endpoint. In such assays mechanistic biomarkers allow to obtain more information on the mode of action of a compound that can be translated to the human situation. This goal is less important for the MINC, as it already uses a functional endpoint relevant to human toxicology. In such a system the use of intracellular markers has slightly different priorities. The two applications are (i) mechanistic markers that

give quantitative information on toxicity pathways/adverse outcome pathways (AOP) activated by the compound. This would then help to map AOPs in general for the field of developmental toxicity (Bal Price et al., 2015a), and to judge new potential toxicants from the knowledge of their activation of certain AOPs; (ii) grouping of chemicals within the assay to support read across procedures by additional biological data. Notably, such a grouping might turn out to be dependent on the cell type as well as the toxicity endpoint used for the study. We have recently shown that even with the functional well defined group of HDACi there is a very low overlap of changed genes by HDACi between different test systems (Krug et al., 2013) or even when one compound is applied for different times within one test system (Balmer et al., 2014).

Our approach might support current read across strategies for initial information on developmental toxicity: (i) known compounds would be tested together with the less known compound in question in functional assays such as the MINC. This would already yield first information on the hazard potential. (ii) This information would then be refined by marker sets (e.g. mRNA, miRNA, immunocytochemical changes, protein expression, etc.) that allow further mechanistic grouping. (ii) This may guide a form of "biological read across" (Patlewicz et al., 2014) of unknown compounds to assign them to a group of known toxicants. A variation of this approach would be the general profiling of mechanistically extremely well characterized compounds with only one or few biochemical modes of action in the assay. Then, the similarity of marker changes by an unknown compound could be used to infer the mode of action. The feasibility of this approach has been shown for a diverse set of markers and endpoints by a recent elegant study (Kleinstreuer et al., 2014): 84 endpoints were used for testing of mechanistically well character ized compounds. The patterns obtained from this where then used for grouping of several hundred environmental toxicants. Several studies have shown that mRNA marker sets can be used for the grouping of small molecules according to assumed modes of action (Lamb et al., 2006; Smalley et al., 2010; Zhang and Gant, 2008) but broad application has been limited until recently by the high cost of gene expression profiling. For similar approaches in the MINC, a larger set of migration related genes needs to be investigated by emerging technologies that allow high throughput expression profiling of hundreds to a thousand genes at moderate cost (Duan et al., 2014) Ideally such a larger gene set would be backed up by additional endpoints such as metabolomics or proteomics. Such an approach could be used to identify then again the most predictive set of markers for grouping of chemicals.

We envisage that this approach can be an immediate step to support hazard assessment by read across and for early grouping of potential toxicants, long before the technology and resources are ready to determine full AOPs/toxicity pathways (Leist et al., 2008).

Transparency document

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

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