Integration into risk assessment of open source human omics data from *in vitro* studies

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Introduction

A recent milestone in toxicity research was the emergence of the field of toxicogenomics, resulting from the application of knowledge gained from genomics science into conventional toxicology. This field specifically tackles the complex interactions between toxic effects and the structure and activity of the genome [1, 2]. The question now is if the detection of chronic and systemic toxicity is possible via these alternative testing strategies to reduce and replace animal testing.

As part of the ToxBank project (www.toxbank.net) [3] in the SEURAT-1 program, *ab initio* case studies have been proposed in which the toxicity of four compounds are assessed using available human *in vitro* data only. These compounds are the reference compounds doxorubicin, valproic acid and methotrexate with well-defined modes of actions as well as the test compound piperonyl butoxide (PBO). The latter was chosen as a compound relevant for the cosmetics industry.

Here, an approach using the combination of omics data with information extracted from adverse outcome pathways (AOPs) to identify areas of concern and support an evidence-driven risk assessment is presented with the example PBO (Fig. 1)

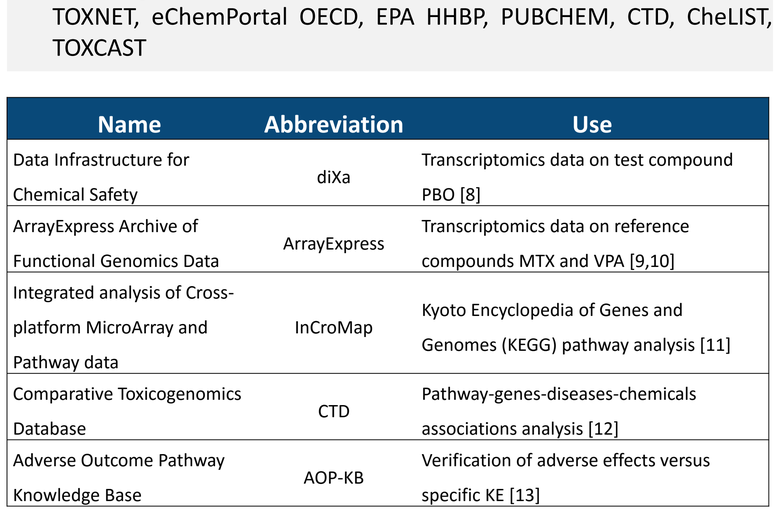


Figure 1. Analysis workflow

Test compound: Piperonyl Butoxide (PBO)

- PBO belongs to a class of chemicals known as the methylenedioxyphenyl compounds and is an insecticide synergist [4]
- PBO was also included in cosmetic products for skin protection [5]
- PBO is classified as a non-genotoxic carcinogen, following *in vitro* and *in vivo* investigations [5]
- A PBO metabolite binds to Cytochrome P450 enzymes, thus reducing the ability of the enzymes in breaking down accompanying pesticides
- PBO's effect on Cytochrome P450 enzymes is biphasic - it both inhibits and induces enzymatic activity; the inhibition of P450 enzymes occurs rapidly, followed by a slow induction process. The rapid inhibition contributes to PBO's effectiveness as a synergist [7]
- Information and downloadable data on PBO were found in the following databases: diXA, Leadscope, COSMOS, ECHA CHEM, ChEBI, TOXNET, eChemPortal OECD, EPA HHBP, PUBCHEM, CTD, CheLIST, TOXCAST

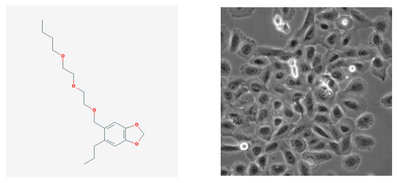
Name	Abbreviation	Use
Data Infrastructure for Chemical Safety	diXa	Transcriptomics data on test compound PBO [8]
ArrayExpress Archive of Functional Genomics Data	ArrayExpress	Transcriptomics data on reference compounds MTX and VPA [9,10]
Integrated analysis of Cross-platform MicroArray and Pathway data	InCroMap	Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis [11]
Comparative Toxicogenomics Database	CTD	Pathway-genes-diseases-chemicals associations analysis [12]
Adverse Outcome Pathway Knowledge Base	AOP-KB	Verification of adverse effects versus specific KE [13]

Omics Data

- In the case study performed here, an *ab initio* risk assessment is modelled, i.e. it is assumed that no information on the toxicity of the compound is available
- To quickly identify areas of concern, omics data form a good starting point, since they show general adaptations of the cell to the exposure
- Vinken et al. [14] used transcriptomics measurements on three different human liver cell lines, for the development of omics-based *in vitro* carcinogenicity screening assays:
  - HepaRG (human hepatoma-derived cells),
  - HepG2 (hepatocellular carcinoma-derived cell line), and
  - hES-DE-Hep (hepatocyte-like cells derived from embryonic stem cells)

Identification of Relevant Pathways

- The transcriptomics data described was analysed using the program InCroMAP to identify pathways, which are influenced by the treatment of the cells with PBO at different concentrations and time points
- BH FDR correction was applied and fold changes were regarded as significant if the Q-values <0.05
- Relevant pathways were identified using KEGG pathway enrichment approach (Fig. 2)



PBO (CAS: 51-03-6) **Human liver cells**

#	Name	Observation 1 [Fo...	Observation 2 [Fo...	Observation 3 [Fo...	Observation 4 [Fo...	Observation 5 [Fo...
1	DDR1	-0.1484	0.2077	-0.0552	0.0781	-0.1476
2	RFC2	0.2067	0.0576	0.0812	0.2074	-0.2214
3	HSPA6	0.0	0.3789	-0.3491	0.3164	-0.5243
4	PAX8	-0.2768	0.3862	6.885E-3	-0.0907	-0.1478
5	GUCA1A	0.0	0.0	0.0	0.0	0.0
6	UBA7	0.0593	0.2412	-0.0768	-0.0115	-0.0656
7	THRA	0.0353	-0.1627	-0.2139	-0.503	0.1639
8	PTPN21	-0.3862	0.0	0.0	0.4931	0.0
9	CCL5	0.0	0.0	0.0	0.0	0.0
10	CYP2E1	0.0508	0.3687	0.4542	0.0714	-0.5979
11	EPHB3	0.0	0.0	0.0	0.0	0.0
12	ESRRA	0.1552	0.41	0.1881	0.3628	0.1921
13	CYP2A6	1.1508	1.258	0.6781	0.8939	0.891
14	GAS6	-0.094	-0.0483	-0.1617	8.106E-3	-0.0735
15	MMP14	0.1582	0.3125	-0.1453	0.4794	-0.2498
16	TRADD	-0.0159	-0.2934	-0.2295	0.0716	-0.1865
17	AL139022.1	-0.0526	0.1672	-0.2055	0.0595	-0.1458
18	PLD1	0.3473	-0.1168	0.7028	0.1314	0.222

Differential Expression

#	ID	Name	List r...	BG ratio	P-value	Q-value	Genes/Compounds
1	path:hsa00982	Drug metabolism - cytochrome P450	4/213	18/14868	1.028E-4	8.273E-3	CYP2A6, CYP3A7, CYP2B6,
2	path:hsa00830	Retinol metabolism	5/213	36/14868	1.402E-4	8.273E-3	CYP2A6, CYP3A7, CYP2B6,
3	path:hsa00980	Metabolism of xenobiotics by cytochrome P450	4/213	26/14868	4.484E-4	0.0132	CYP2A6, CYP3A7, CYP2B6,
4	path:hsa05204	Chemical carcinogenesis	4/213	26/14868	4.484E-4	0.0132	CYP2A6, CYP3A7, CYP2B6,
5	path:hsa05321	Inflammatory bowel disease (IBD)	4/213	50/14868	4.913E-3	0.1159	STAT3, MAF, NFATC1, IFN
6	path:hsa00140	Steroid hormone biosynthesis	3/213	33/14868	0.0103	0.2029	CYP3A7, AKR1D1, HSD3B1
7	path:hsa04670	Leukocyte transendothelial migration	4/213	73/14868	0.0167	0.2823	MLLT4, RAPGEF4, PTPN11,
8	path:hsa00565	Ether lipid metabolism	2/213	19/14868	0.0274	0.404	ENPP2, LPCAT2
9	path:hsa00983	Drug metabolism - other enzymes	2/213	22/14868	0.0354	0.4648	CYP2A6, CYP3A7
10	path:hsa00300	Lysine biosynthesis	1/213	3/14868	0.0424	0.4999	ALDH7A1
11	path:hsa00232	Caffeine metabolism	1/213	4/14868	0.0561	0.505	CYP2A6
12	path:hsa00071	Fatty acid metabolism	2/213	29/14868	0.0564	0.505	ALDH7A1, ADH1A
13	path:hsa04630	Jak-STAT signaling pathway	4/213	122/14868	0.0672	0.505	SOCS1, STAT3, PTPN11, I
14	path:hsa00561	Glycerolipid metabolism	2/213	33/14868	0.0692	0.505	ALDH7A1, GK
15	path:hsa04650	Natural killer cell mediated cytotoxicity	3/213	75/14868	0.0701	0.505	NFATC1, PTPN11, IFNGR1
16	path:hsa05142	Chagas disease (American trypanosomiasis)	3/213	77/14868	0.0738	0.505	CD3D, CFLAR, IFNGR1

Figure 2. Data analysis

- The analysis of HepaRG cells confirmed the interaction of PBO with cytochrome P450 but this was not observed in HepG2 and hES-DE-Hep cells
- The pathways identified in HepaRG cells showed concentration- and time-dependent enrichment, as the treatment with the higher concentration showed most significant effects at 72h comparative with 24h
- A similar effect was observed in HepG2 cells but the pathways enriched were different. In the case of of hES-DE-Hep a cell batch-dependent response was seen (Fig. 3)

Correlation between Pathways and Diseases

- To identify adverse outcomes of PBO, we initially followed a consensus approach using information of all cell lines (Fig. 4)
- The top pathways were selected and analysed further using the services of CTD, which correlate the observed pathways with diseases
- The applied query resulted in a large number of associated diseases from which the top ranks are given in the table below

Rank	Disease Name	Disease ID	Number of associated genes
1	Dermatitis, Allergic Contact	MESH:D017449	100
2	Prostatic Neoplasms	MESH:D011471	49
3	Breast Neoplasms	MESH:D001943	48
4	Stomach Neoplasms	MESH:D013274	44
5	Lung Neoplasms	MESH:D008175	40
32	Fibrosis	MESH:D005355	15

- Fibrosis, an adverse effect related to PBO appears on rank 32 of the list of diseases associated with the identified pathways
- Genes of three pathways are associated with this disease. It is also interesting that these genes are mainly related to pathways identified with the HepaRG cells
- A next step included the analysis on separate cell models, showing 21 genes associated with fibrosis in the HepaRG cells (table below), whereas 4 genes were associated with fibrosis in HepG2 cells

Rank	Disease Name	Disease ID	Number of associated genes
4	Drug-Induced Liver Injury	MESH:D056486	50
43	Fibrosis	MESH:D005355	21

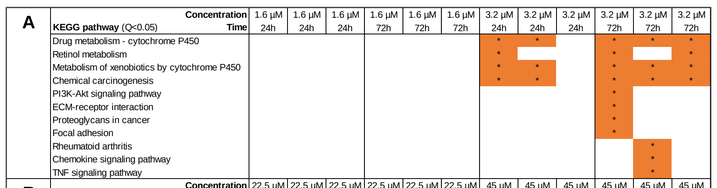


Figure 3. Most relevant pathways identified in HepaRG cells (A), HepG2 (B) and hESC DE-Hep cells (C)

Enriched pathway (Q<0.05)	KEGG ID	HepaRG	HepG2	hESC DE-Hep
ECM-receptor interaction	04512	*		*
Focal adhesion	04510	*		*
PI3K-Akt signaling pathway	04151	*		*
Adherens junction	04520		*	*
Cell cycle	04110		*	*
DNA replication	03030		*	*
Proteoglycans in cancer	05205	*		*

Figure 4. Top pathways enriched at least in two cell lines

Verifying Adverse Effects by Testing for Specific Key Events

- Adverse outcome pathways (AOPs) summarize all available information leading from a molecular initiating event (MIE) to intermediate key events (KE) and finally to the adverse outcome (AO)
- Starting from the adverse outcome (Fig. 5), these relations can now be followed backwards and more specific tests can be performed to verify the occurrence of key events

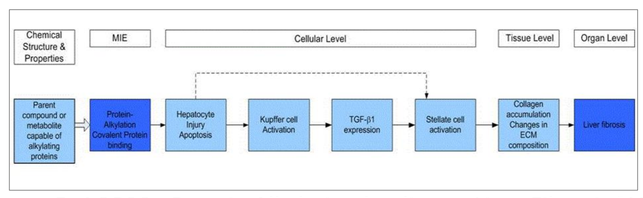


Figure 5. AOP38 - Protein Alkylation leading to Liver Fibrosis [13]

- The transcriptomics data analysis showed that fibrosis could be a potential adverse outcome for exposure to PBO
- The KE preceding the AO is the **collagen accumulation** for which the AOPKB describes three different methods for testing this KE [15]
- The next upstream KE described is **stellate cell activation** for which data does exist (*unpublished data from HeMiBio project*)
- Up-regulation of **TGF-β1 expression** is the next upstream KE, which can be measured by ELISA [13]. Unfortunately, we could not find any such data for PBO. At the transcription level, in none of the cell systems analyzed here (HepaRG, HepG2 and hES-DE-Hep) is any effect on the TGF-β1 gene expression levels observed. However, the AOP does not list gene expression as a valid test for this KE
- Therefore, we propose as next steps to perform additional tests to confirm or exclude these KEs

Conclusions

- We show here that transcriptomics data is able to identify fibrosis as one potential adverse outcome of treatment with PBO
- HepaRG cells seem to be more appropriate to test for this specific effect
- Using information from AOP-KB, we were then able to verify key events and, in this way, strengthen the evidence for this specific adverse effect
- Additional tests are proposed, especially the TGF-β1 measurements by ELISA to see if the TGF-β1 expression is up-regulated

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