THE "DIRTY DOZEND" POPS & OTHER POLLUTANTS: TOXICOLOGICAL PROFILING BY CALUX® PANEL

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Introduction

Traditionally, toxicity testing is performed using a combination of whole animal bioassays and chemical analytical methods. Whole animal bioassays are particularly suitable for hazard identification while chemical analytics can be used to assess exposure, or for the identification of known toxic compounds in complex mixtures. Animal models are often too expensive and time consuming to keep up with current needs, demanding toxicity profiles of an increasing number of compounds and chemical mixtures. Additionally, due to the absence of sufficient toxicity data on pure chemicals, chemical analytical assessment has limited predictive value for human risks especially in complex mixtures. In the current study, a panel of standardized stable human reporter cell lines has been developed that allows rapid screening of hazards of pure chemicals and complex mixtures on multiple toxicological pathways. The ultimate goal is a simple and fast method to accurately predict human and environmental hazard for unknown compounds or complex mixtures, based on CALUX profiles.

Automation of the assay procedure will help to increase the throughput and reproducibility, and reduce errors. Apart from the increase in throughput, down-scaling of the assay from 96-wells to 384-wells format offers the additional advantage that less sample material is required to perform an analysis. Therefore, the current study focuses on automation and miniaturisation of the CALUX screening method.

Materials and methods

CALUX bioassays

The CALUX battery of stable reporter gene assays consisted of the following human U2OS cell based lines: ERα-, PR-, GR-, AR-, PPARα-, PPARγ-, Cytotoxicity-, TRβ-, RAR-, LXR-, ERβ-, p53-, p21-, Nrf2-, ESRE-, TCF-, AP1-, NFκB- and HIF1α-CALUX [1, 2]. Two additional lines were included based on rat H4IIE liver cells expressing the aryl hydrocarbon receptor (AhR) endogenously: the DR- and PAH-CALUX [3].

The CALUX cells were cultured essentially as described before [3]. U2OS CALUX cells were routinely subcultured every 3-4 days in growth medium consisting of DMEM (Gibco) supplemented with 7.5% fetal calf serum, 1x non-essential amino acids (Gibco) and 10 U/ml penicillin and 10 µg/ml streptomycin. H4IIE CALUX cells were routinely subcultured every 3-4 days in growth medium consisting of αMEM (Gibco) supplemented with 10% fetal calf serum. All cell types were maintained at 37 °C and 5% CO2 at all time. All CALUX assays were performed in assay medium, consisting of DMEM without phenol-red indicator (Gibco) supplemented with 5% DCC-stripped fetal calf serum, 1x non-essential amino acids (Gibco) and 10 U/ml penicillin and 10 µg/ml streptomycin.

Cells were seeded in transparent 96- or white 384-wells microplates. After 24h, exposure medium was prepared by adding 2% of a test compound dilution series (16 individual concentrations) in DMSO to a plate with assay medium. Of this exposure mixture, 1 volume was added in triplicate to the assay plates containing the CALUX cells, resulting in final DMSO concentrations of 1%. Positive and negative controls were included on each plate. After 24h exposure (PAH-CALUX: 4h exposure) the exposure medium was removed and cells were lysed with triton-lysisbuffer. Subsequently, the luciferase signal was measured in a luminometer (Berthold), essentially as described before [2].

Automation and HTP-screening

To increase the throughput, reduce errors and improve reproducibility, the CALUX assay procedure has been automated in (standard) 96-wells format. After initial validation of an automated protocol in 96-wells format,

this automated CALUX procedure was adapted for 384-wells format. In brief, cells were seeded using a MicroFlo Select dispenser (BioTek), and subsequent test compound dilution and CALUX exposure were performed using a Hamilton Starlet liquid handling robot coupled to a Cytomat incubator. After exposure, medium was removed and lysis mix added using a BioTek EL406 washer-dispenser. The plates were measured in a luminometer coupled to a stacker (Berthold).

Results and discussion

HTP-screening on the CALUX panel

After the validation of the procedure, a list of approximately 150 reference compounds with known toxicological properties has been screened on 28 different CALUX assays. The list includes the 'dirty dozen' POPs, PCBs, heavy metals, pesticides from the ToxCast program [4], reprotoxic compounds and genotoxins.

The results of this screening could be used to identify CALUX profiles for specific toxicological endpoints. The pesticides showed activity mainly in the endocrine assays ERα-, anti-AR- and anti-PR-CALUX, suggesting endocrine disrupting activities. This is a well-known mode of action of many pesticides. For heavy metals, activity is observed in the cytotoxicity- and stress/signaling pathway assays Nrf2-, ESRE- and AP1-CALUX. This is in line with the known acute toxicity of heavy metals (figure 1).

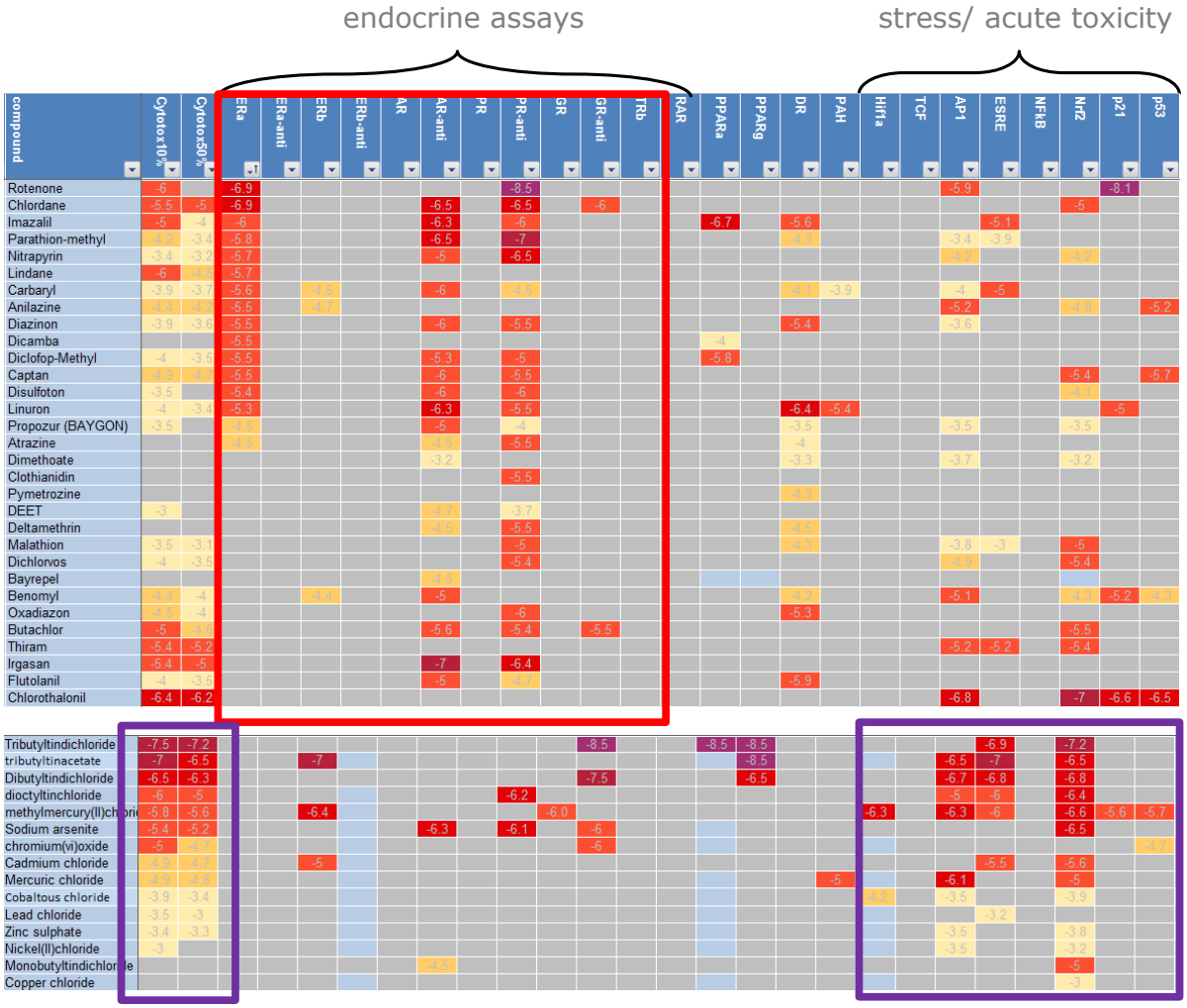
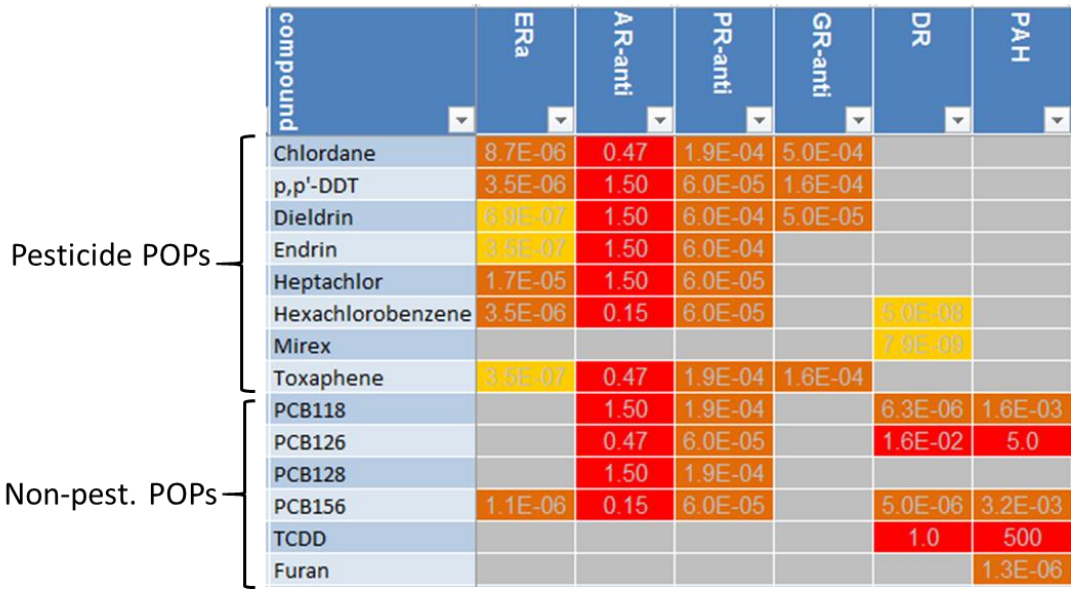


Figure 1. Activity profile of a selection of pesticides (top panel) and heavy metals (bottom panel). Values indicate PC10 values compared to the reference compound activity. Red border: endocrine assays. Purple border: general toxicity/stress pathways. Pesticides are predominantly active on endocrine assays, while heavy metals act on general tox/stress pathway assays.

The dirty dozen POPs can be sub-divided into pesticides and non-pesticides. The pesticide POPs showed a similar 'endocrine' profile as the ToxCast pesticides, while the non-pesticide POPs (PCBs, PAHs, furan, TCDD) mainly showed very high activity in the AhR-receptor mediated DR- and PAH-CALUX (figure 2). These PCBs/PAHs are indeed known to activate the dioxin (AhR)-receptor.



	compound	ERa	AR-anti	PR-anti	GR-anti	DR	PAH
Pesticide POPs	Chlordane	8.7E-06	0.47	1.9E-04	5.0E-04		
	p,p'-DDT	3.5E-06	1.50	6.0E-05	1.6E-04		
	Dieldrin	6.9E-07	1.50	6.0E-04	5.0E-05		
	Endrin	3.5E-07	1.50	6.0E-04			
	Heptachlor	1.7E-05	1.50	6.0E-05			
	Hexachlorobenzene	3.5E-06	0.15	6.0E-05		5.0E-08	
	Mirex					7.9E-09	
	Toxaphene	3.5E-07	0.47	1.9E-04	1.6E-04		
Non-pest. POPs	PCB118		1.50	1.9E-04		6.3E-06	1.6E-03
	PCB126		0.47	6.0E-05		1.6E-02	5.0
	PCB128		1.50	1.9E-04			
	PCB156	1.1E-06	0.15	6.0E-05		5.0E-06	3.2E-03
	TCDD					1.0	500
	Furan						1.3E-06

Figure 2. Activity profile of the dirty dozen-POPs for the most relevant CALUX assays. Values indicate relative potency (REP) values compared to the reference compound activity. Yellow -> red = increasing relative potency. Reference compounds: ERa; E2. AR-anti; flutamide. PR-anti and GR-anti; Ru486. DR; TCDD. PAH; Benzo-a-pyrene.

In conclusion, the compound screening showed a different CALUX activity profile for different classes of compounds. A clearly distinct profile was observed for pesticides, heavy metals, and non-pesticide POPs. The observed CALUX profiles can be explained on the basis of the known toxicological properties of the different compounds, which demonstrates the physiological relevance of the CALUX results. Moreover, because of recent automation- and downscaling activities, the throughput of the CALUX assays has been increased dramatically. This enables the screening of large sample numbers in a short period of time, while requiring only small amounts of sample material. This makes the CALUX panel a promising fast, efficient and economical tool for toxicological profiling with multiple areas of application, including incidental or routine monitoring and large epidemiological studies.

References:

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