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A high throughput screening system for predicting chemically-induced reproductive organ deformities

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ABSTRACT

There is a great need for alternative testing methods for reproductive toxicants that are practical, fast, cost-effective and easy to interpret. Previously we followed a pragmatic approach using readily available tests, which was successful in predicting reproductive toxicity of chemicals [13]. This initial battery still contained apical tests and is fairly complex and low in its throughput. The current study aimed to simplify this screening battery using a mechanistic approach and a panel of high throughput CALUX reporter gene assays. A mechanistic approach was taken to validate this high throughput test battery. To this end it was challenged with two preselected sets of chemicals addressing two major apical effect classes relevant in reproductive toxicity. We found selectivity in this battery in that 82% of the compounds inducing reproductive organ deformities were predicted correctly, while for compounds inducing neural tube defects this was the case in 47% only. This is consistent with the mechanisms of toxicity covered in the battery. The most informative assays in the battery were ERalpha CALUX to measure estrogenicity and the AR-anti CALUX assay to measure androgen receptor antagonism.

1. Introduction

Since there is a huge demand to assess potential toxicity of new and existing chemicals, the world of toxicity testing of chemicals is changing rapidly. A major bottleneck in the process of evaluating chemicals is the use of animal experiments, which is costly, ethically debatable, resource- and time-consuming. To modernize this process the European Framework 7 project ChemScreen (www.chemscreen.eu) aimed to select a minimal panel of assays that can be used to predict reproductive toxicity of chemicals in integrated testing strategies. Recent advance in our understanding of the mechanisms of action of toxicants has allowed development of relatively simple cellular assays that allow measurement of activation of toxicity pathways by chemicals. Using this knowledge it may be feasible to predict toxicity of chemicals through establishing their impact on such pathways *in vitro*, thereby reducing and even replacing animal experimentation [1].

Reproductive toxicity testing requires most experimental animals in chemical safety testing [2]. Because of the complexity of the reproductive cycle many *in vitro* models have been developed, but none of them recapitulate the complexity of the entire reproductive cycle. Although single tests are not able to capture this complexity it has been shown in the context of the FP6 ReProTect project that a battery of tests covering only part of this cycle can successfully predict the reproductive toxicity of a range of potent toxicants [3]. The assays used in this study in general were rather complex with very few molecular assays that can be used in high throughput. Recently the US-EPA ToxCast program has focussed on the use of alternative testing approaches based on high throughput screening assays. As a result, it was found that using 662 high throughput screening assays developmental toxicity in the rat can be related to 12 molecular pathways, and toxicity could be predicted with about 70% accuracy [4]. This approach using molecular screening assays

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has received considerable attention in recent years, and methods are being developed to couple the read-out of such assays to the more apical endpoints used in animal tests, to facilitate the use of such screenings data in chemical risk assessment. As such, the development of so-called adverse outcome pathways [5] coupling molecular initiating events to apical effects in animals have become an important area of research.

The ChemScreen panel of assays to predict reproductive toxicity aimed to be cost-effective and to be disseminated widely. Therefore preferentially simple and straightforward methods were included. An important element in our approach was to start with the use of assays that are well underway of being accepted as alternatives to animal experiments, and build up the screening battery from there. This involved the use of receptor based reporter gene assays, such as CALUX® assays for androgenic and estrogenic compounds that are evaluated in an ECVAM/OECD guided validation effort [6–9]. We expect that inclusion of validated tests in the core of a screening battery can pave the way for a growing platform to which new mechanism-based assays can be included when knowledge increases, possibly even without going through the entire process of time consuming validation, because the basic technology for each assay is the same.

A second part of our strategy was to use assays that are highly selective for specific molecular pathways, as has been typically the approach in the setup of the CALUX battery of U2-OS based reporter gene assays [10,11]. This is attained by using minimal promoter elements coupled to multimerized, highly selective response promoter elements in these assays driving luciferase expression. Since the U2-OS cells have extremely low expression levels of endogenous receptors, through introduction of the cognate receptor/reporter gene combination highly selective and responsive assays were generated. In our current effort to assess the activity profile of a chemical using multiple assays this has the potential advantage of a very low level of false-positives. Though this, we aim to avoid the use of multiple assays per single endpoint as has been chosen in the ToxCast program [12], thereby reducing the amount of tests and simplifying the testing and data analysis.

In the absence of a complete understanding of the complex regulatory network involved in reproductive toxicity, the FP7 ChemScreen project has started with the selection of a pragmatic testing battery that was based on the earlier work of Schenk et al. [3]. It covers several important processes in the reproductive cycle but is a simplification of the ReProTect battery avoiding some of the more complex tests involving primary tissues or cells and ones which were less informative [3,13]. Through inclusion of a CALUX panel of reporter gene assays and cyp17 and cyp19 assays it focused more on underlying molecular endpoints [13]. Interestingly, when evaluated with a range of reproductive toxicants the predictivity of this pragmatic testing panel equalled that of the ReProTect panel, being able to predict reproductive toxicity of all but one chemical [13].

Although the pragmatic ChemScreen test battery showed very promising results, it has the drawback that it still contains a fairly large number of assays, while only the CALUX panel of reporter gene assays is run in a high throughput mode. Therefore in the current study we explored the possibility of using the high throughput screening battery only to predict reproductive toxic effects, as a further step of improvement of this battery. Interestingly, we noted before that this CALUX panel alone already predicted 8/12 reproductive toxic chemicals correctly using a simple prediction model. When PBPK modeling was used the CALUX battery was equally predictive compared to the entire battery of ChemScreen tests and predicted the same 11/12 chemicals correctly [13]. With this limited number of chemicals tested this of course needs further substantiation. We also noticed that the battery of high throughput tests seemed biased toward measuring hormonal activities of chemicals. Several of the assays involved already have been found to have a good correlation with specialized rodent and rabbit *in vivo* bioassays, such as the Allen-Doisy-, Hershberger-, and McPhail assays, specifically designed to measure hormonal activities of chemicals [14,15]. It is unknown, however to what extent these assays are predictive to apical effects in multigenerational reproductive toxicity tests as carried out in regulatory testing of chemicals. The current study was designed to test this. The uncertainties associated with animal testing with respect to variability and relevance to humans has led to the notion that the establishment of simple correlations between animal data and alternative tests is not a very suitable way to validate the latter [16]. This is particularly the case when alternatives have a strong mechanistic basis and increased attention to the scientific background of a test and the plausibility of the relevance to human toxicology should then receive more attention. Therefore, in the current study we took a mechanistic approach to validate our test battery. To this end the battery was challenged with two preselected sets of chemicals addressing two major apical effect classes relevant in reproductive toxicity in rats, reproductive organ deformities (RO) and neural tube defects (NTD). Because of the assays present in the battery we anticipated that the former class would be predicted better by the battery than the latter. Our results indeed show that the current battery is of limited value predicting NTD, but is particularly successful in predicting RO, which is consistent with the current composition of the battery and the mechanisms of toxicity covered.

2. Materials and methods

2.1. Test chemicals

Halosulfuron-methyl (CAS no: 100784-20-1), cadmium chloride (10108-64-2), fenachlorazol-ethyl (103112-35-2), nonylphenol (104-40-5), carbendazim (10605-21-7), 2-methoxyethanol (methyl cellosolve) (109-86-4), cellosolve acetate (111-15-9), diethylene glycol dimethyl ether (Diglyme) (111-96-6), endosulfan (115-29-7), spiroxamine (118134-30-8), hydroquinone (123-31-9), tributyltinchloride (1461-22-9), 2-ethylhexanoic acid (149-57-5), benomyl (17804-35-2), prothioconazole (178928-70-6), cyanazine (21725-46-2), molinate (2212-67-1), carbamazepine (298-46-4), acetylsalicylic acid (50-78-2), triclopyr (55335-06-3), diethylstilbestrol (56-53-1), 5-(4-chlorophenyl)-6-ethyl-(pyrimethamine) (58-14-0), dibromoacetic acid (631-64-1), dibutyltindichloride (683-18-1), D-mannitol (69-65-8), fluazifop-butyl (69806-50-4), fenoxycarb (72490-01-8), p,p′-DDE (p,p′-dichlorodiphenyldichloroethylene) (72-55-9), NaCl (7647-14-5), glycolic acid (79-14-1), bisphenol A (80-05-7), mancozeb (8018-01-7), diisobutyl phthalate (84-69-5), butyl benzyl phthalate (85-68-7), pentachlorophenol (87-86-5), cyproconazole (94361-06-5), sulfluramid (4151-50-2), vinclozolin (50471-44-8) all were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Bis(trichloromethyl) sulfone (3064-70-8) was from Chemos (Regenstauf, Germany) and 2,4-D-iso-propylamine salt (5742-17-6) and di(*n*-hexyl)phthalate (84-75-3) from Dr. Ehrenstorfer (Augsburg, Germany).

2.2. Compound handling

All compounds were dissolved in DMSO in Teflon lined glass vials at a concentration of $1E^{-1}$ M. If this concentration was not soluble, the concentration was decreased step-wise by 0.5 log unit until the compound was completely dissolved. To assess the solubility of the compounds in assay medium prior to addition to the cells, the DMSO stock solution was added to the assay medium at