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Prediction of embryotoxic potential using the ReProGlo stem cell-based Wnt reporter assay

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ABSTRACT

The ReProGlo assay was developed in 2009 to predict embryotoxic potential of drugs and chemicals by use of a stem cell-based *in vitro* system. It utilizes a luciferase reporter to detect drug-induced alterations in the canonical Wnt/β-catenin signaling pathway, which is involved in regulation of early embryonic development. It allows the simultaneous determination of cell viability and luciferase reporter activity in a high throughput format. The present study was conducted within the framework of the EU *ChemScreen*-project. It (1) enlarges the original number of test-compounds from 17 to now 80, (2) introduces a new classification scheme and (3) anchors the results against a prediction scheme based on structural features of chemicals. The assay is applicable as stand-alone for priority setting or in a test battery.

1. Introduction

The process of embryonic development is stringently regulated by several signaling pathways that are evolutionary conserved from the fruit fly to humans [1]. Among these is the so-called canonical Wnt/β-catenin-pathway (hereafter referred to as "Wnt pathway"), which governs axis specification in the developing embryo [2]. It is well documented, that genetic manipulation of the Wnt pathway results in abnormal development [3,4]. Chemicals may also interact with signaling molecules within the pathway and thereby alter gene expression patterns downstream of the pathway. It is also well documented, however, that many other cellular components, including several nuclear receptors, indirectly affect the activity of the Wnt pathway, in that they interact with molecules of the pathway like β-catenin or TCF/LEF transcription factors (*e.g.* see [5]). Since these nuclear receptors are themselves targets for certain drugs and chemicals, a wide spectrum of chemically unrelated agents may alter Wnt downstream signaling and thereby affect early embryonic development.

We have recently developed a rather simple *in vitro* test system, the so-called ReProGlo assay, which utilizes mouse embryonic stem (ES) cells stably expressing a luciferase reporter for the Wnt-signaling pathway [6]. In our previous study we had tested 17 different drugs and chemicals in the ReProGlo assay. We have now considerably extended this number and report in this paper on the experience obtained with the assay based on the results of now 80 chemicals, including the previous ones.

The application of a new test requires knowledge about its predictive power. Test results are normally validated against or "anchored" on results from animal experiments or human experience, if available. In particular in the case of embryotoxicity of drugs and chemicals this procedure has its limitations, however, because of the well-documented strong species differences in response, rendering the animal experiment as a "gold standard" highly questionable [7]. We have therefore abstained in the present paper from anchoring our results for individual chemicals solely on animal experimental data collected for exactly the respective chemical in question; we have rather categorized the test chemicals that we have used in our study according to a previously published classification scheme [8], which is based on chemical structure and was constructed using a large fraction of the animal developmental toxicity literature that is available, so there is relevance in comparing the results of our assay with the tree as a way of comparing it to the mammalian developmental toxicity literature.

Abbreviations: BMC, benchmark concentration; CS1/2, Chemscreen feasibility study 1/2 test set; DART, developmental and reproductive toxicity; DEP, diethylphthalate; DES, diethylstilbestrol; DMSO, dimethyl sulfoxide; EAA, ethoxyacetic acid; ES cells, embryonic stem cells; MAA, methoxyacetic acid; PFOSA, perfluorooctane sulfonamide; INI, initial ReProGlo test set; RPT, ReProTect feasibility study test set.

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Fig. 1. Schematic representation of the sequential experimental steps of the ReProGlo assay.

Finally, we have learned during this second phase of test development, that a classification of test chemicals based on ReProGlo results into the two categories "positive" and "negative" may not be sufficiently describe what we observe in the assay. In particular, there are "gray-zone" chemicals, where the "specific" effect (significant alteration of the activity of the Wnt reporter) is close to the concentration at which "unspecific" toxicity (significant decrease in cell viability) becomes also detectable. One may see this in analogy to the animal experiment, where for some chemicals teratogenic effects are seen in the offspring but at a dose level where maternal toxicity is also evident. We therefore introduce in this paper a third category, "unspecific positive", to label chemicals which behave in the assay as described above.

2. Materials and methods

2.1. Test chemicals

In this paper, we present the results obtained with 80 chemicals tested with the ReProGlo assay. Several parts of this ReProGlo data set have previously been published: The first 17 test chemicals were selected by ourselves as an initial test set to investigate the usefulness of the assay (INI [6]). 10 chemicals were tested as a part of the ReProTect feasibility study (RPT [9]) and 12 chemicals were tested during the ChemScreen feasibility study part 1 (CS1 [10]). The second part of the ChemScreen feasibility study added a set of 48 chemicals (CS2), which are discussed here for the first time. Due to some overlap of chemicals in the individual test sets, the total number of chemicals sums up to 80. For a complete list of chemicals tested so far in the ReProGlo assay see Tables 1a–c. Information about the source of chemicals is given in supplementary Table 1. Chemicals of test sets INI and RPT were dissolved as described in Uibel, 2010 [6]. Chemicals of test sets CS1 and CS2 were handled as follows: all water-soluble compounds were dissolved in water at a concentration of 1 M. If the compound was not soluble at 1 M, the concentration was decreased to the maximum solubility of the compound in steps of 0.25 log units. If the final concentration turned out to be too low for use in the ReProGlo assay, the compound was dissolved in dimethyl sulfoxide (DMSO). All other compounds were directly dissolved in DMSO, following the same procedure.

2.2. ReProGlo assay procedure

The ReProGlo assay as previously described [6] uses murine embryonic stem cells, stably transfected with a Wnt-signaling responsive reporter vector containing a luciferase gene. The principal steps of the assay are depicted in Fig. 1. Cells are seeded on 96-well plates on day 1 and treated with different concentrations of the test chemicals on day 2. On day 3, Luciferase activity and cytotoxicity (*via* Alamar Blue) are assessed. When we introduced the ReProGlo assay, it was conducted by manual pipetting. We now developed it to a semi-automatic assay, as day 3 is almost completely managed by a liquid handling platform (Tecan Freedom EVO 100). Unfortunately, the automation of the steps performed at day 2, which was tested with the CS1 chemicals [10], turned out to be too error-prone. These chemicals were later re-evaluated in the semi-automated version. As another improvement to the original ReProGlo protocol, we now investigated two chemicals per plate instead of only one, using 4 technical replicates for every concentration. At least four, in some cases up to nine experiments were conducted for each test compound.

2.3. Data analysis

Cytotoxicity and Luciferase activity were calculated for each concentration relative to the solvent control. The mean value of the four wells was used to calculate a dose–response curve using OriginPro 8 (sigmoidal dose-response curve fit). For Luciferase activity, the concentrations at which a doubling or bisection of the signal occurred were calculated using the curve fit. These benchmark concentrations were termed BMC_2 or $BMC_{0.5}$. The benchmark concentration for 80% cell viability was termed $BMC_{0.8}$. A valid outcome was defined as an effect in more than half of the experiments conducted for a single compound. Test compounds were classified in three categories: (1) **positive**: chemicals showing a specific effect on Wnt signaling, but effects on cell viability were not detected or occurred at a much higher concentration ($BMC_{0.8} > 2 \times BMC_{0.5/2}$). (2) **negative**: chemicals showing no effect on Wnt signaling, regardless of cytotoxic effects. (3) **unspecific**: chemicals showing a specific effect on Wnt signaling but also on cell viability ($BMC_{0.8} < 2 \times BMC_{0.5/2}$).

2.4. DART assessment

The work of Wu and co-authors [8] presents a framework for assessment of developmental and reproductive toxicity (DART) of chemicals based on their structure. Based on an extensive study of available *in vivo* data, they selected a training set of chemicals and used it to develop a decision tree. Any given structure can now be run through this decision tree, which leads to a two-category classification of its DART potential (known precedent DART potential and no known precedent DART potential). There is some overlap between the ReProGlo test set and the training set for the decision tree (chemicals marked in Tables 1a–c). The remaining chemicals were assessed with kind assistance from Dr. George Daston and Dr. Cathy Lester, both co-authors of the original work [8], partly with the help of an automated screening tool.

3. Results

Out of 80 chemicals tested, 25 were clearly positive in the ReProGlo assay, which are depicted in Table 1a. Of these, 22 were also positive in the DART assessment decision tree. Three chemicals (**DMSO**, **ciclosporin**, **molinate**) were positive in the ReProGlo, but showed no structural alert in the DART assessment.

Table 1b shows 30 out of the 80 test chemicals that were classified by the ReProGlo assay as negative. Five of these 30 compounds were also negative in the DART assessment (**D-mannitol**, **clopyralid**, **sodium thioglycolate**, **2-methylhexanoic acid** and **sodium chloride**), whereas 25 of the chemicals showed structural DART alerts.

Out of the 80 chemicals tested, the 25 chemicals depicted in Table 1c showed unspecific results in the ReProGlo assay, meaning that apart from the specific effect on the Wnt signaling pathway ($BMC_{2/0.5}$), they also produced cytotoxicity ($BMC_{0.8}$) within the same concentration range. Of these 25 compounds, 23 were also positive in the DART assessment leaving only two chemicals of this group (**cyanazine** and **bis(trichloromethyl) sulfone**) with a negative DART prediction. A summary of the results obtained with the 80 chemicals in the ReProGlo assay in comparison with the DART prediction is graphically displayed in Fig. 2A. The pie chart in Fig. 2B is used to indicate the presumed reasons for the discrepancy between