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A concentration response modelling approach for deriving embryotoxicity point of departures for Next Generation Risk Assessment

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Encouraged by the successful application of **New Approach Methodologies (NAMs)** in an **exposure-driven Next Generation Risk Assessment (NGRA)** approach for systemic toxicity (Baltazar et al., 2020; Middleton et al., 2022), we created a **developmental and reproductive toxicity (DART) framework** that includes additional *in vitro* assays covering specific DART-related biology (Rajagopal et al. 2022). One of the DART-specific assays included in the framework was **ReproTracker® (Toxys).** ReproTracker® is a human stem cell-based assay that rapidly and reliably identifies **developmental toxicity hazards of chemicals** (Jamalpoor et al., 2022). The assay captures changes in the key cellular events of **stem cell differentiation** into cardiomyocytes, hepatocytes and neural rosettes and upon exposure to the chemical of interest in a **dose dependent manner.**

ReproTracker is designed to output **teratogenicity binary classifications** for each chemical (is a teratogen or is not a teratogen) based on concentrations tested. **Binary classifications do not allow an exposure-based assessment** of the chemical and therefore do not lend themselves for use in NGRA. To use ReproTracker as a NAM in an NGRA approach, a **point of departure** (the concentration at which bioactivity of the assay occurs) needs to be calculated to be compared with a given exposure in a bioactivity exposure ratio (BER). Various **dose response modelling** can derive a POD given a concentration/dose response data but adaptations to the experimental design of ReproTracker are needed to apply such methods efficiently and reliably. This work presents and evaluates the use of the ReproTracker assay to derive PODs with intended use in NGRA and compares methods to calculate PODs from this data.

Ambition

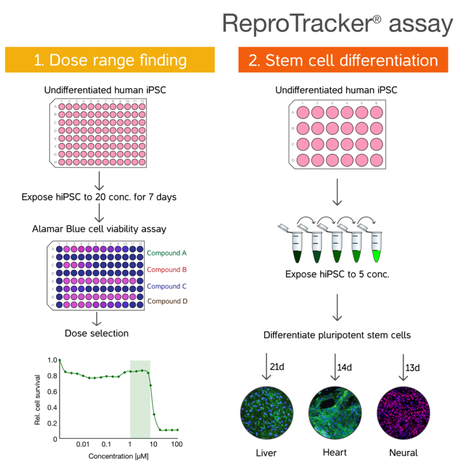
The aim of this work was to adapt the ReproTracker® assay for use in our DART-specific exposure based NGRA framework by:

1) assessing and adapting its experimental design to generate fit for purpose dose response curves
2) assessing different modelling approaches to derive points of departure (POD) from generated dose responses.

Multiple POD modelling methods are evaluated to assess ReproTracker's utility in risk assessment:

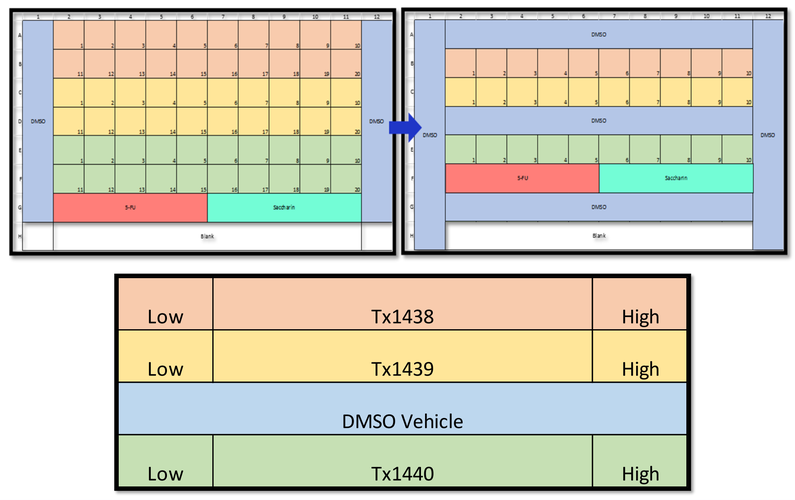
1) Modelling cell viability using Bayesian modelling
2) Modelling time independent responses using BMDExpress2
3) Modelling concentration response over time using state space models

Assay Development



To **optimise the POD modelling**, adaptations to the ReproTracker experimental design have been made from the standard assay (left).

- **Increasing throughput of dose range finding**
  - By reducing number of concentrations tested per chemical. Adjusted plate layout shown top right.
- **Increasing dose response modelling suitability**
  - By increasing number of concentration tested and the increasing concentration dilution steps.
- **Increasing protectiveness for risk assessment**
  - By including AlamarBlue as an additional endpoint on differentiating cells and calculating cytotoxicity POD.
- **Improving baseline estimation:**
  - By increasing number of controls for better baseline estimations; reducing experimental variability between treated samples compared to previous design where controls were on separate treatment plates. Adjusted plate layout shown bottom right

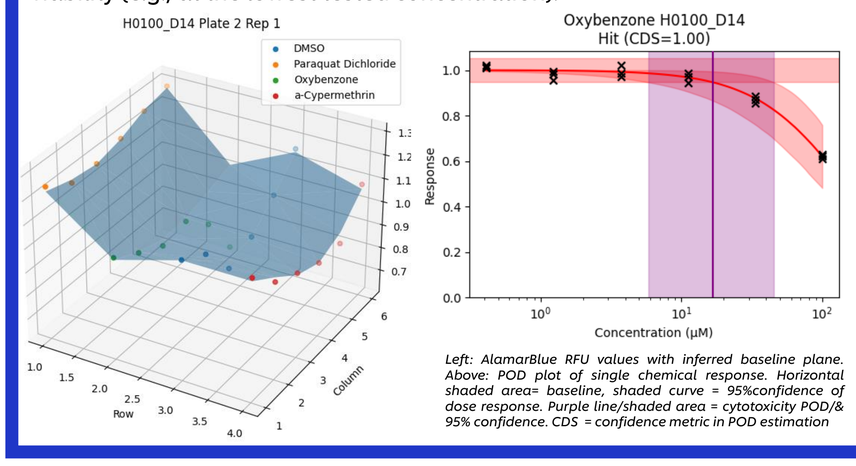


Low	Tx1438	High
Low	Tx1439	High
DMSO Vehicle		
Low	Tx1440	High

POD Modelling

The **AlamarBlue assay** provides measurement of **cell viability** on the differentiating lineages. Effects of chemical concentrations are modelled using **Bayesian methods** to **account for local variations** in fluorescence. Baseline RFU values are inferred based on observed plate effects and this inferred baseline is used to compare with treated samples. **PODs are defined as a decrease in viability from the baseline** inferred per row. Model assumptions state that baseline **RFU response between rows correlated** but can have different means allowing for row dependent offset.

Defining a baseline from solvent controls only (one row) resulted in frequent increase in viability (RFU values offset higher than controls) and false positives at very low concentrations (RFU values offset below controls). **Modelling with respect to changes in rows' baseline results in more robust cytotoxicity PODs** which **reflect observed cell morphology** also. The output provides uncertainty across the POD estimation but observed cell morphology should also be checked to ensure a baseline calculated by the model isn't already showing a reduction in viability (e.g., at the lowest tested concentration).



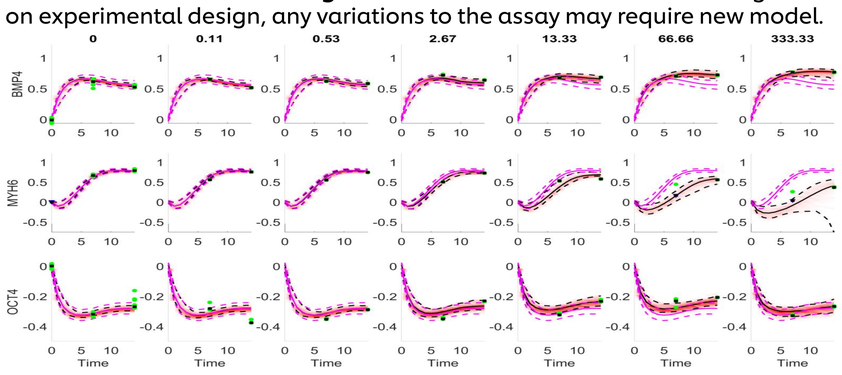
Left: AlamarBlue RFU values with inferred baseline plane. Above: POD plot of single chemical response. Horizontal shaded area= baseline, shaded curve = 95%confidence of dose response. Purple line/shaded area = cytotoxicity POD/& 95% confidence. CDS = confidence metric in POD estimation

BMDExpress dose response example from data from a single chemical, biomarker and timepoint. BMDL, BMD and BMDU are marked by vertical lines from x axis. Red points = biomarker expression mean and standard deviation.

BMDExpress2 is software **implementing parametric modelling** methods and is used to derive PODs from biomarker **response across tested concentrations**. A **benchmark response factor (BMR) of 1.349** is used to calculate BMD as **10% transcriptomic change** from control baseline. BMDs are only calculated from decreasing monotonic dose responses. A lower bound (BMDL) is taken as PODs. BMD modelling is a **well-recognised approach** and is used for various dose response data, particularly for transcriptomics. This approach is **easily transferable** to data from varying experiments due **to lack of consideration of experimental design**, which allows some flexibility in the assay set up (e.g., sample layout) but does mean **batch effect cannot be accounted for**. **Constant variance across samples** is also assumed using this methods which we know is not the case with qPCR data (lower expression = higher Ct variability).

A **State space approach** (developed internally) is used to incorporate **biomarker responses over time**. This **Bayesian hierarchical model** determines a POD as the concentration that changes the gene response with respect to time. A **baseline profile** for each biomarker is derived from solvent control **response over time** and is used to compare treatment profiles. Being a **probabilistic model**, **uncertainty is given** around baseline and treatment dose responses.

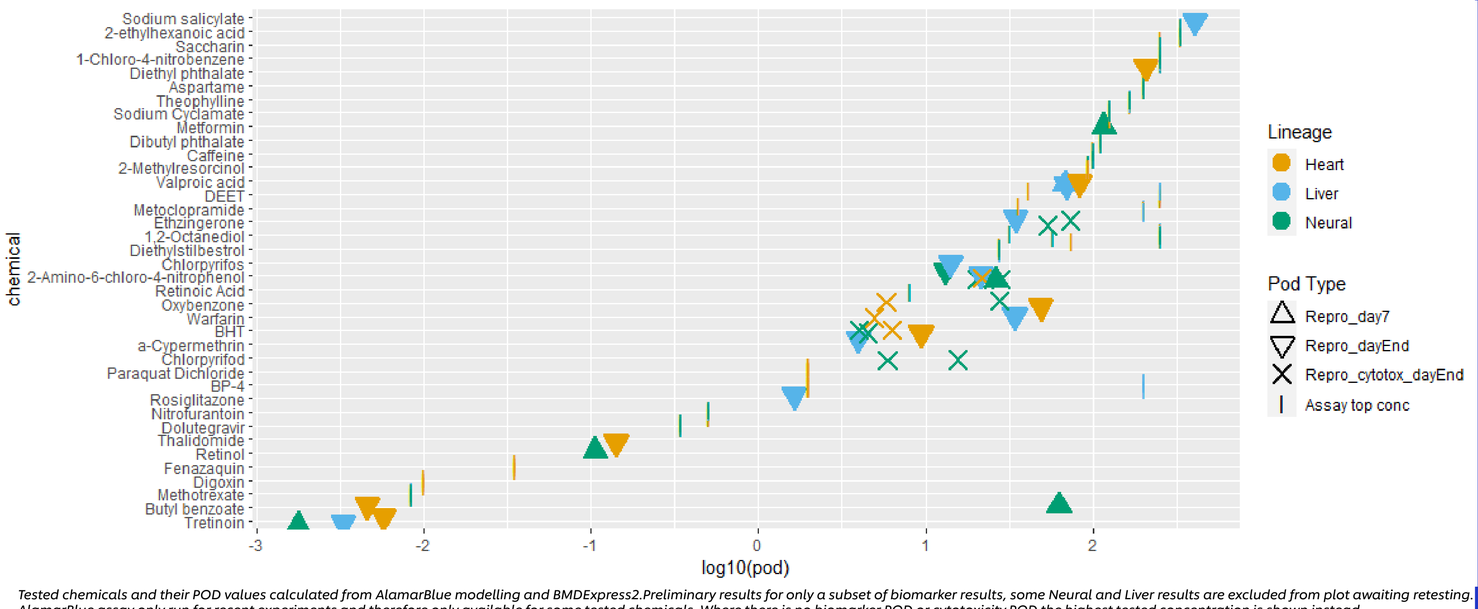
This approach is used to visualise changes to differentiation induced by chemical treatment as a function of time, which allows us to assess expected biomarker responses in control samples and gage the treatment response as a fluid **measure over the course of differentiation** (rather than using a timepoint snapshot). The hierarchical model is **based around experimental set up and reflects experimental replicate batches and treatment plates**. The analyses are run as an entire experiment and all data (not just a single chemical) is used to estimate parameters allowing **POD calculations more robust to outliers and biological variation**. Due to the model being based on experimental design, any variations to the assay may require new model.



Example output from an initial implementation the ReproTracker state space modelling approach. 3 heart gene response (rows) are given for a chemical treatment where columns are various concentration of a single chemical tested. Solvent control response is given in column 1 and carried through all concentration plots. Pink solid curve and dotted curves give the control time response and uncertainty. Black curve and dotted curve give the treated response and uncertainty.

Results

Cytotoxicity and **gene expression** PODs (BMDExpress2) are calculated are visualised below:



Tested chemicals and their POD values calculated from AlamarBlue modelling and BMDExpress2.Preliminary results for only a subset of biomarker results, some Neural and Liver results are excluded from plot awaiting retesting. AlamarBlue assay only run for recent experiments and therefore only available for some tested chemicals. Where there is no biomarker POD or cytotoxicity POD the highest tested concentration is shown instead.

Next Steps

- **Refine state space model**
  - Incorporate amplification efficiencies into data normalisation
  - Evaluate methods for handling missing datapoints
- **Compare PODs to exposure scenario cmax values**
  - Evaluate the utility and protectiveness of each POD methods
- **Apply ReproTracker PODs in a full NGRA approach**
  - Use ReproTracker PODs along with other assay POD and exposure modelling to determine risk
- **Run inter-lab transferability and reproducibility trial**
  - Testing 10 blinded compounds across 2 labs

More on our DART NGRA approach

- **Practical Application of New Approach Methods in Developmental and Reproductive Toxicity Testing** (OS03-03)
  - Predrag Kukic
  - Tuesday 12th September, Short Orals Session 3, 16.00-18.00
- **Development and validation of Mother-foetus PBK model: A case study of PBK read-across for Valproic acid and 2-Ethyl Hexanoic Acid** (P08-18)
  - Gopal Pawar

Baltazar et al. (2020). A Next-Generation Risk Assessment Case Study for Coumarin in Cosmetic Products. Toxicological sciences: an official journal of the Society of Toxicology. 176, 236–252 DOI:10.1093/toxsci/kfaa048.

Jamalpoor A & Hartvelt S et al. (2022). A novel human stem cell-based biomarker assay for in vitro assessment of developmental toxicity. Birth Defects Res. 2022 Nov 15;114(19):1210-1228. DOI: 10.1002/bdr2.2001

Rajagopal et al.(2022). Beyond AOPs: A Mechanistic Evaluation of NAMs in DART Testing. Front. Toxicol. 4:838466 DOI: 10.3389/ftox.2022.838466

Middleton et al. (2022). Are non-animal systemic safety assessments protective? A toolbox and workflow. Toxicological Science. 189, 124-147 DOI: 10.1093/toxsci/kfac068

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