Temporal and incidence concordance between oxidative DNA damage and DNA strand break levels

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Introduction

Genotoxicity Testing

- Genotoxicity assessment surveys chemicals for their ability to cause genomic damage that can lead to cancer and genetic disease. Examples of this genomic damage (Figure 1) are:
  - DNA damage
  - Mutations
  - Chromosomal aberrations
  - Aneuploidy

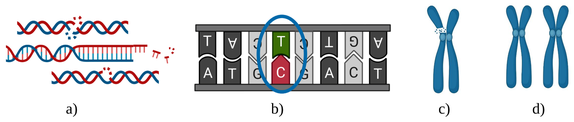

Figure 1. Diagrams showing types of DNA damage that genotoxicity assessment surveys chemicals for, a) DNA damage, double and single strand breaks, b) mutations, G to A transversion, c) chromosomal aberration, d) aneuploidy. Created with BioRender.com

- Current methods of genotoxicity testing require considerable resources and time, and do not provide mechanistic information for the tested chemicals. These assays include:
  - Ames assay
  - Micronucleus assay *in vivo* and *in vitro*
  - Mutation assays *in vivo* and *in vitro*
- For effective prioritization and assessment of chemicals, there is a need to develop methods that are less resource-intensive (higher-throughput) and provide mechanistic information to facilitate the prediction of adverse outcomes.

Adverse Outcome Pathways (AOPs)

- Adverse Outcome Pathways (AOPs) provide a framework for organizing mechanistic information to describe the chain of events from the initial interactions of a chemical with cellular molecules to an adverse effect (Organisation for Economic Co-operation and Development; OECD).
- AOPs can facilitate the use high-throughput mechanistic testing data to predict adverse outcomes.
- AOPs are built from measurable biological events called key events (KEs) and descriptions of the causal relationships between them.
- AOPs start and end with specialized KEs. The first KE is called the molecular initiating event (MIE) and is the first interaction between a toxicant and a biomolecule. The last KE is called the adverse outcome (AO) and is generally an endpoint of regulatory concern (Figure 2).
- To support the key event relationships (KERs), the modified Bradford-Hill considerations are used (Meek et al., 2014), which include:
  - Biological plausibility
  - Essentiality of KEs
  - Temporal, dose and incidence concordance up the upstream and downstream KEs
- In order to use AOPs as predictive models, a quantitative understanding of the pathway and KERs is required to determine how much the MIE and KEs need to change to cause the following KEs and ultimately the AO.

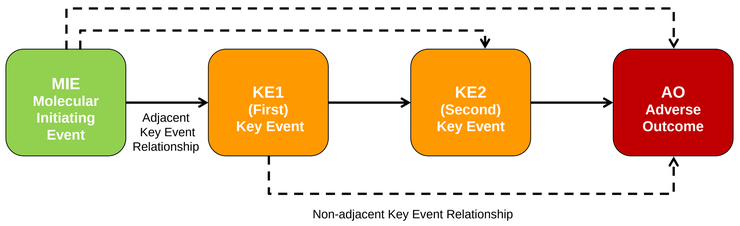

Figure 2. Flow diagram of the components of an Adverse Outcome Pathway (AOP)

Reactive Oxygen Species (ROS)

- Reactive oxygen species (ROS) are cellular oxidants that are essential for cell signalling processes and the electron transport chain. ROS can also be introduced from exogenous sources, such as exposure to:
  - chemicals
  - heavy metals
  - ionizing radiation
- ROS do not pose a threat to the cell at steady state as they are regulated by the cell's antioxidant response. ROS become harmful to the cell when there is an imbalance of oxidants and antioxidants, which is known as oxidative stress.
- During oxidative stress, oxidants can cause cellular damage such as DNA lesions; inadequate repair of these lesions can lead to mutations or chromosomal aberrations.

AOP#296: Oxidative DNA damage leading to chromosomal aberrations and mutations

- This project collected empirical evidence to support a specific AOP, developed by Cho *et al.*, which describes oxidative DNA damage leading to chromosomal aberrations and mutations (Figure 3).

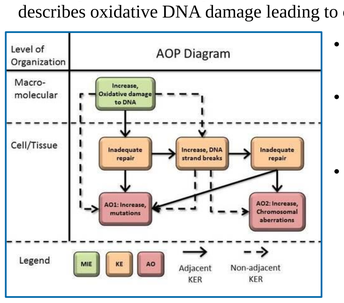

Figure 3. Flow diagram of AOP#296: Oxidative DNA damage leading to chromosomal aberrations and mutations. Cho *et al.*, 2020, aopwiki.org/aops/296

- Oxidative DNA damage includes a wide variety of DNA lesions.
- When a cell is overwhelmed with oxidative DNA damage, repair pathways are unable to effectively repair oxidative lesions.
- Inadequate repair can result in mutations, or it can result in DNA strand breaks that lead to chromosomal aberrations.

Objectives

1. To collect quantitative data to measure the MIE, increase in oxidative DNA lesions, with the Fpg modified CometChip assay and the second KE, DNA strand breaks, with the standard CometChip assay (Figure 4) .
2. Use this empirical data to inform concordance in dose/concentration, temporality, and incidence of these two KEs, according to the modified Bradford-Hill considerations.

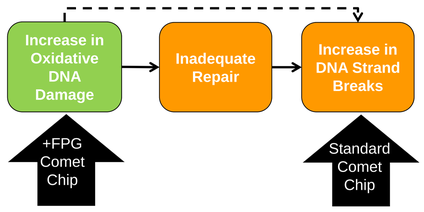

Figure 4. Flow diagram of the first three events of AOP#296, with arrows indicating the assays that will be used to test for them.

Methods & Results

Cytotoxicity assay

- A cytotoxicity assay was run to determine the top concentration for this experiment – the concentration of potassium bromate ($KBrO_3$) that induces 50% cytotoxicity in TK6 cells.
- $KBrO_3$ exposed cells were dyed with propidium iodide (PI), to which live cells are impermeable and were analyzed with a flow cytometer.
- After a 4-hour exposure and a 20-hour recovery, $KBrO_3$ induced TK6 cytotoxicity greater than 50% at 3.75 mM (Figure 5). Based on these results the top concentration for the CometChip analysis should be 3 mM $KBrO_3$.

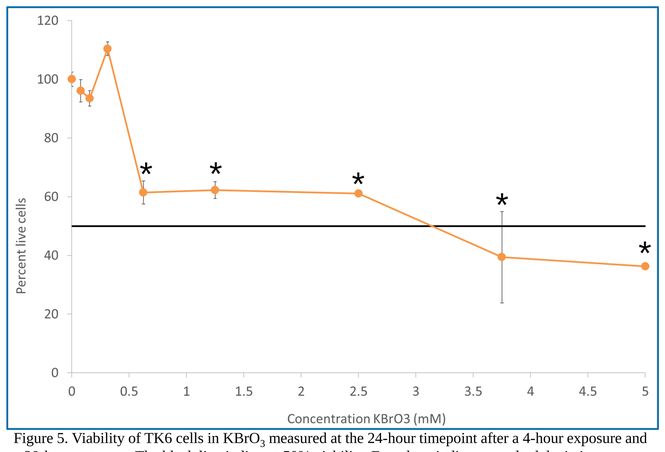

Figure 5. Viability of TK6 cells in $KBrO_3$ measured at the 24-hour timepoint after a 4-hour exposure and a 20-hour recovery. The black line indicates 50% viability. Error bars indicate standard deviation between three technical replicates. The statistical significance (p-value<0.05) between each treatment and the negative control was determined using the one-way analysis of variance (ANOVA) and post hoc Dunnett's test, and is indicated by an *.

CometChip assay

- The CometChip assay, also known as High-Throughput Single-Cell Gel Electrophoresis, allows the standard alkaline comet assay to be run in a 96-well plate format (Ge *et al.*, 2014).
- Briefly, cells are seeded into an agarose gel, then they are exposed to the test chemical. Cells are lysed and the gel is then submerged in an alkaline solution to allow for the DNA to unwind. Electrophoresis is performed, then the comets are imaged and analyzed to identify strand breaks (Figure 6, Figure 7).
- The Fpg modification uses the enzyme Formamidopyrimidine DNA Glycosylase, to create strand breaks at oxidative DNA lesions allowing their detection.
- In this experiment, TK6 human lymphoblastoid cells were exposed for 1-hour to increasing concentrations of $KBrO_3$ to induce cellular oxidants and two CometChip assays were run:

  (a) the Fpg modified CometChip assay to determine oxidative DNA damage

  (b) the standard CometChip assay to quantify DNA strand breaks.

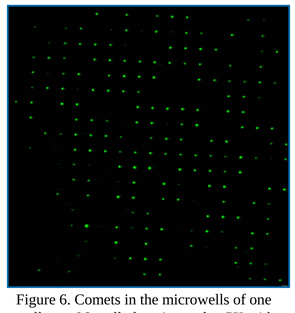

Figure 6. Comets in the microwells of one well on a 96-well plate, imaged at 5X with Lecia DMi8 confocal microscope.

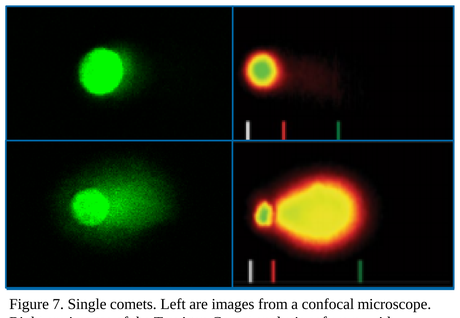

Figure 7. Single comets. Left are images from a confocal microscope. Right are images of the Trevigen Comet analysis software, with indicators of the start of the comet head (white), end of the comet head (red) and end of the comet tail (green). Top are comets with short tails, indicating fewer strand breaks; bottom are comets with large tails, indicating more strand breaks. Right images from Syorka *et al.*, 2018.

Methods & Results

CometChip assay (continued)

- After a 1-hour exposure to $KBrO_3$, there was an increase in oxidative DNA damage (+Fpg) from 10.7 % tail DNA in controls to a peak of 19.1 % tail DNA at 1 mM. In contrast, there was no increase in DNA strand breaks (-Fpg), which had an average of 7.6 % tail DNA across all concentrations (Figure 8).

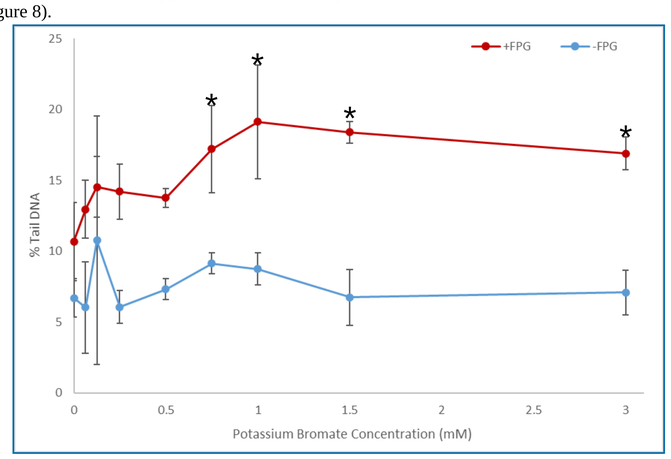

Figure 8. Percent tail DNA observed in comets after 1-hour exposure to $KBrO_3$. The red points are results of the Fpg modified comet and represent oxidative lesions. The blue points are the results of the standard comet and represent DNA strand breaks. Error bars indicate standard deviation between three technical replicates. The statistical significance ($p<0.05$) between each treatment and the negative control was determined using the one-way analysis of variance (ANOVA) and post hoc Dunnett's test, and is indicated by an *.

Conclusions

- 3mM $KBrO_3$ induced about 50% cytotoxicity in TK6 cells and was selected as the top concentration for subsequent experiments.
- After a 1-hour exposure, we observed an increase in oxidative DNA lesions (MIE) with no effect on DNA strand breaks (KE2), supporting that the MIE occurs first and that there is a higher level of oxidative DNA lesions plus DNA strand breaks (Fpg modified CometChip) than DNA single strand breaks on their own (CometChip).
  - Thus, we observed the expected responses that were consistent with the Bradford-Hill considerations for temporal and incidence concordance.

Future Work

- Future work will investigate longer exposure to $KBrO_3$ and other chemicals that cause oxidative Dna damage to further define temporal, concentration, and incidence concordance.
- This work will also establish a quantitative threshold of cellular ROS that leads to the downstream effects, increase in oxidative DNA damage, DNA strand breaks, mutations and chromosomal aberrations. Establishing a quantitative threshold will improve the utility of this AOP in predicting potential adverse outcomes associated with a chemical exposure.
- Further experiments will also include Duplex Sequencing™ to measure mutations and the MicroFlow micronucleus assay to measure chromosomal aberrations (Figure 9).

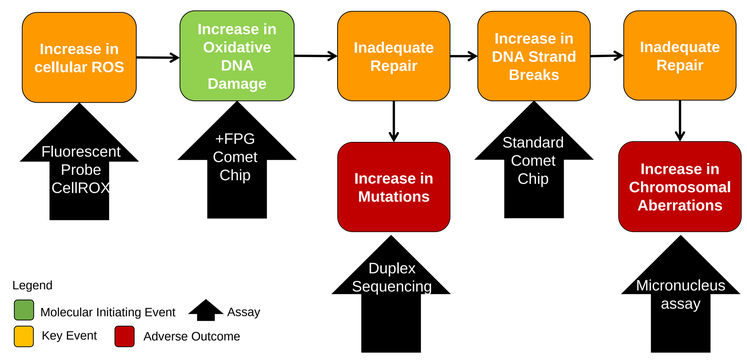

Figure 9. Flow diagram of AOP#296: Oxidative DNA damage leading to chromosomal aberrations and mutations (Cho *et al.*, 2020, aopwiki.org/aops/296), with arrows indicating the assays that will be used in future work to test for the events.

References & Acknowledgements

References

Cho, E *et al.* (2020). Oxidative DNA damage leading to chromosomal aberrations and mutations. AOP Wiki, https://aopwiki.org/aops/296.

Ge, J. *et al.* (2014). Cometchip: A high-throughput 96-well platform for measuring DNA damage in microarrayed human cells. *Journal of Visualized Experiments*, 92, 8–15. doi: 10.3791/50607

Meek, M. E *et al.*, (2014). Mode of action human relevance (species concordance) framework: Evolution of the Bradford Hill considerations and comparative analysis of weight of evidence. Journal of Applied Toxicology, 34(6), 595–606. doi: 10.1002/jat.2984

OECD. (2018). "Users" Handbook supplement to the Guidance Document for developing and assessing Adverse Outcome Pathways", OECD Series on Adverse Outcome Pathways, No. 1, OECD Publishing, Paris." 1, 1–62. doi: 10.1787/5jlv1m9d1g32-en

Sykora, P. *et al.* (2018). Next Generation High Throughput DNA Damage Detection Platform for Genotoxic Compound Screening. *Scientific Reports*.

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