Quantitative Support for the Adverse Outcome Pathway “Oxidative DNA Damage Leading to Chromosomal Aberrations and Mutations”

By

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Thesis submitted in partial fulfillment of the requirements for the degree:

Master of Science in Biology

with a specialization in

Chemical and Environmental Toxicology

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Abstract

Adverse outcome pathways (AOPs) provide a framework to organize and weigh the evidence linking a toxicant's initial interactions with molecules in the cell to adverse outcomes of regulatory concern. AOPs are constructed in modules that include key events (KEs) and key event relationships (KERs). Quantitative understanding of the KERs is critical for the development of predictive toxicological models. The objective of this project was to investigate the ability to define the quantitative associations of the KERs upstream, and contained in, an existing AOP (#296): "Oxidative DNA Damage Leading to Chromosomal Aberrations and Mutations". The data supporting quantitative associations between these KERs was gathered through literature review and experimental methods. I first used systematic literature review tools to develop and apply a pragmatic and transparent method to search the literature for AOP evidence. A broad search, covering all of the KERs of interest, was initially conducted. This search, which retrieved more than 230 thousand articles, demonstrates the data-rich nature of the AOP. An artificial intelligence informed prioritization of the top 100 articles were then examined in detail. This approach identified 39 articles containing qualitative empirical support for the AOP, but limited quantitative evidence of the KERs. A second search was conducted to address the need for quantitative evidence as well as the lack of evidence for the KER between and increase in reactive oxygen species (ROS) and oxidative DNA damage. The second search retrieved 12 articles that could be used to define a quantitative relationship between cellular ROS and oxidative DNA damage. To begin to address gaps in quantitative understanding, I then conducted experiments in the laboratory to measure oxidative DNA damage, DNA strand breaks, chromosomal aberrations, and mutations in TK6 cells after exposure to a range of concentrations of 4-Nitroquinoline 1-oxide (4NQO: a prototype ROS producing agent). An increase in both oxidative DNA damage and DNA strand breaks was observed after 2, 4, and 6 h exposures with the high throughput comet assay (CometChip). An increase in the incidence of micronuclei was observed after a 24 h exposure to a low concentration of 4NQO, as measured with the

flow cytometry micronucleus assay, while high cytotoxicity was found at higher concentrations. Lastly an increase in mutation frequency was observed with Duplex Sequencing, an error-corrected sequencing technology. Additionally, an increase in the proportion of C>A transversions was observed, consistent with the expected mutations following oxidative DNA lesions. Overall, my work contributes to the quantitative understanding of AOP #296 and this project serves as a key example of AOP-informed study design, highlighting notable challenges in characterizing quantitative relationships.

Dedication

To my Great Aunt Andrea MacLean who was passionate about science, health, and community.

Your impact on the world continues to be felt by those you helped and loved.

Acknowledgements

I have so many people to thank for supporting me through these past two years. I'm grateful for this experience, what started as my undergrad thesis project turned into a master's and an exciting three years researching genetic toxicology.

First, a huge thank you to my supervisors, Dr. Carole Yauk and Francesco Marchetti. Thank you for the opportunity to work on this project and for your support when we had to pivot (due to COVID-19) and pivot (due to lab closures) and pivot (due to instrument failure) again. Thank you for your guidance and encouragement. I would also like to thank my thesis advisory committee members Dr. James Gomes and Dr. William Willmore. Dr. Willmore, thank you for allowing me to use your lab space, taking the time to train and troubleshoot with me.

Next, thank you to the students of the GReAT lab, I am lucky to have had the opportunity to work with such a positive and supportive group of students. Eunnara, thank you for your mentorship and for convincing me to work with Carole for my honour's thesis. Anne-Marie, Annette, Tatiana, I am so glad I had you with me for this very unique master's experience! Géronimo, Emmanuel, and Dave, keep up the GReAT work (and play), good luck finishing your projects! And to the other friends and graduate students I have met along the way, whether we met on a Friday night in Gather, in (zoom) classes, or actually in person, thanks for being a part of this journey and reminding me to enjoy life outside of school.

Thanks to all the people I worked with at Health Canada, the Genomics-GeneTox group for your help in the lab and to my co-authors: Jason O'Brien, Vinita Chauhan for help with my literature review and for your feedback on the manuscript.

I want to thank those in my personal life for supporting me through this past year especially. To my extended family for asking so many questions and helping me improve my elevator pitch for this

project. Thanks to the Feline Cat Café for allowing me to take care of Frank, George, and Apollo. I absolutely loved taking care of those boys and they helped me as much as I helped them. Frank was so shy but rewarded my patience; George kept me warm and made me sit in one spot for hours on end; and Apollo made sure I always took the time to play. To Carl and Marion for letting me park at your house during my long days at the lab.

Last and most importantly thank you to my parents, your passion for education has led me here. You've been there for me through all of the ups, the downs, and the presentation practices. I cannot thank you enough for your endless support and for your encouragement everyday.

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

List of Tables

Chapter 2

Chapter 3

List of Abbreviations

4NQO	4-Nitroquinoline 1-Oxide
8-oxodG	8-Oxo-2'-Deoxyguanosine
AO	Adverse Outcome
AOP	Adverse Outcome Pathway
B-H	Bradford Hill
BMD/BMC	Benchmark Dose/Benchmark Concentration
CEPA	Canadian Environmental Protection Act
CMP	Chemicals Management Plan
COSMIC	Catalogue Of Somatic Mutations in Cancer
DCFH-DA	Dichlorofluorescein Diacetate
DCS	Duplex Consensus Sequence
DMSO	Dimethylsulfoxide
DS	Duplex Sequencing
DSL	Domestic Substances List
EAGMST	Extended Advisory Group on Molecular Screening and Toxicogenomics
FaPydG	2,6-Diamino-4-Hydroxy-5-Formamidopyrimidine
Fpg	Formamidopyrimidine DNA Glycosylase
GPx	Glutathione Peroxidase
GSH	Glutathione
GST	Glutathione-S Transferase
GTTC	Genetic Toxicology Technical Committee
HESI	Health And Environmental Sciences Institute
HPLC	High Pressure Liquid Chromatography
hprt	Hypoxanthine-guanine phosphoribosyl transferase
KE	Key Event
KER	Key Event Relationship
LAC	Luminol-Amplified Chemiluminescence
LMP	Low Melting Point
MIE	Molecular Initiating Event
mito-Ro-GFP	Mitochondrial Matrix Localized Oxidant-Sensitive Ratiometric Probe

MLA	Mouse Lymphoma Assay
NAM	New Approach Methodologies
NGS	Next Generation Sequencing
NRF2	Nuclear Factor (erythroid-derived 2)
OECD	Organisation For Economic Co-Operation and Development
PBS	Phosphate-Buffered Saline
PECO	Population, Exposure, Comparator, and Outcome
PEG	Polyethylene Glycol
qAOP	Quantitative Adverse Outcome Pathway
RPTECs	Renal Proximal Tubular Epithelial Cells
RFU	Relative Fluorescence Units
ROS	Reactive Oxygen Species
RS	Relative Survival
SBS	Single-Base Substitution
SEM	Systematic Evidence Mapping
SR	Systematic Review
SSCS	Single-Strand Consensus Sequence
tk	Thymidine Kinase
TK6	Human Lymphoblastoid TK6
TGR	Transgenic Rodent
WNT	Working Group of The National Coordinators for the Test Guidelines Programme
WPHA	Working Party on Hazard Assessment

Statement of Contributions

Chapter 2: A Case Study on Integrating a New Key Event into an Existing Adverse Outcome Pathway on Oxidative DNA Damage: Challenges and Approaches in a Data-Rich Area

This chapter was published in Frontiers in Toxicology: *in vitro* Toxicology, 4:827328-827328, April 2022

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Chapter 3: Developing Quantitative Key Event Relationships (KERs) between Oxidative DNA damage, DNA Strand Breaks, Mutations and Chromosomal Aberrations.

This chapter has been written as a manuscript for the journal *Environmental and Molecular Mutagenesis*.

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Chapter 1: Introduction

1.1 Chemical Assessment and Genotoxicity Assessment

Before new chemicals are approved for use in commerce in Canada, they must undergo assessment through the Government of Canada. This process is mandated under the Canadian Environmental Protection Act (CEPA), 1999, which requires that an evidence-based approach be used for the assessment of chemicals for their potential impacts to human health (Health Canada) and environmental health (Environment and Climate Change Canada) (Government of Canada, 1999; Barton-Maclaren et al., 2017). In accordance with CEPA, the Chemicals Management Plan (CMP) was put in place to manage the prioritization and assessment of chemicals currently being used on a commercial scale in Canada (those on the Domestic Substances List (DSL)) in addition to chemicals that are being newly manufactured and imported into Canada (Government of Canada, 2007). There are more than 28,000 substances on the DSL, thousands of which are still in need of assessment (Barton-Maclaren et al. 2017). This chemical assessment process is imperative to protect the environment, the general population, as well as vulnerable members of our community.

Among human health endpoints, chemicals must be evaluated for their potential to cause permanent DNA damage (genotoxicity). Genotoxic effects, such as mutations, chromosomal aberrations, or aneuploidy, can lead to genetic disease (Caldecott 2008), cancer, or accelerate the aging process (Hoeijmakers 2009). Standard testing to determine the genotoxic potential of a chemical includes the Ames test and one of the following tests: the *in vitro* metaphase chromosome aberration assay, the *in vitro* micronucleus assay, or a gene mutation assay in a mammalian cell line (ECCC, 2022; ICH, 2012). Depending on the results of these initial tests, an *in vivo* assay in rodents (e.g., the *in vivo* micronucleus assay or the transgenic rodent mutation assay) may be required as a follow up (ECCC, 2022; ICH, 2012).

Current genotoxicity assessment methods require a considerable amount of resources, time to complete, and provide limited information regarding the mode of action of chemicals (Dearfield et al. 2017). Thus, it is not feasible to apply these methods to screen the large numbers of both existing

chemicals and novel compounds that need assessment (Ankley et al. 2010). To effectively address the volume of chemicals awaiting prioritization and assessment, *in vitro* high-throughput screening methods need to be combined with assays that produce mechanistic information. Such a modernized testing strategy would be more cost-effective and provide improved prediction of *in vivo* outcomes than current methods. It is also essential to identify and develop a pragmatic interpretation framework with which to use mechanistic information to predict adverse genetic effects.

One widely acknowledged approach to using mechanistic data to evaluate mode of action, develop test paradigms and predict potential *in vivo* effects is the Adverse Outcome Pathway (AOP) Framework (Ankley et al. 2010; OECD 2018; Sakuratani et al. 2018; Sasaki et al. 2020) from the Organisation for Economic Co-operation and Development (OECD). The AOP framework was developed with the goal to enable the use of new approach methodologies (NAM) that do not have internationally endorsed test guidelines in different regulatory decision-making contexts.

1.2 Adverse Outcome Pathways (AOPs)

The AOP framework organizes and weighs evidence linking a toxicant's initial interactions with molecules in the cell to adverse outcomes of regulatory concern (Ankley et al. 2010; OECD 2018). The framework includes a flow chart of the pathway of events alongside a structured summary of the collected evidence. The components of an AOP are measurable biological events, called key events (KEs), and the causal relationships between them, called key event relationships (KERs). The first and last KEs are specialized: the first KE is the initial interaction between a chemical and a biomolecule, called the molecular initiating event (MIE), and the last KE is an endpoint of regulatory concern, called an adverse outcome (AO). AOPs are built to follow the measurable events of a process regardless of stress, thus KEs and KERs can be used in multiple AOPs. A KER can be either adjacent or non-adjacent; an adjacent KER describes the relationship between two KEs that are next to each other and a non-adjacent KER relates two KEs that may be one or more KEs apart in the AOP (OECD 2018). Each KE contains details

of new and existing test methods enabling the use of mechanism-based methodologies to measure early KEs to predict later KEs (and AOs). By leveraging the predictive power of AOPs, the time and resources required for chemical evaluation can be reduced.

An AOP and its KEs and KERs must be supported by specific types of evidence that follow the modified Bradford-Hill considerations for causal relationships (OECD 2018). These considerations evaluate biological plausibility, essentiality of each KE to pathway progression, and concordance between KEs in dose, concentration, temporal, and incidence response, (Meek et al., 2014). The consideration of biological plausibility questions whether the hypothesized pathway and KERs are conceivable and consistent with established biological knowledge. The essentiality criterion questions whether there is empirical evidence to demonstrate that the KEs are necessary for the AOP's occurrence (Meek et al. 2014). Essentiality of a KE can be tested, for example, by determining whether the pathway continues if that KE is prevented, or whether the events are reversible after dosing is stopped. All KERs must be supported with empirical evidence demonstrating concordance between two KEs. Dose/concentration concordance refers to when an upstream KE is observed at doses/concentrations equal to or lower than a downstream KE (Meek et al. 2014). Temporal concordance refers to evidence supporting that an upstream KE is impacted at an earlier time point than a downstream KE (Meek et al. 2014). Incidence concordance refers to evidence that an upstream KE occurs more frequently than a downstream KE at equivalent doses and exposure times (Meek et al. 2014). Another aspect of the empirical evidence is the quantitative understanding, which refers to how much an upstream KE needs to change before a downstream KE is observed. The quantitative aspect of the empirical evidence is most important for the use of AOPs in a predictive context. Empirical evidence that meets these criteria provide weight of evidence for the AOP.

1.2.1 Literature review & reproducible search methods

There are currently no established guidelines on how to gather (literature) evidence during AOP development. AOP authors often use data from the literature to support weight of evidence evaluation, as well as data from their own laboratory experiments, to address evidence not gathered from the literature. Due to the substantial amount of time, literature, and data review required to gather evidence to support AOPs, it has been proposed that KERs could be developed and reviewed independently in lieu of full AOPs (Svingen et al. 2021). The approach of developing KERs is more amenable to the use of literature/systematic review processes. Given the modular nature of AOPs, KER development is also useful for building on existing AOPs through the introduction of new KEs. As the AOP knowledgebase expands and development becomes more focussed, using a systematic approach to literature searches would be more transparent, reproducible, and potentially more efficient for weight of evidence collection and could provide more meaningful information on uncertainties and inconsistencies in the database.

A variety of search methods can be applied for AOP evidence collection including systematic reviews (SR), scoping reviews, and systematic evidence mapping. SR synthesize all available data to answer a specific research question by using reproducible search methods to synthesize all available data (Grant and Booth 2009). Scoping reviews identify available evidence to answer a broad question, reveal knowledge gaps, summarize the types of available evidence in a given field or clarify key concepts/ definitions in the literature (Grant and Booth 2009). Systematic evidence mapping provides a visual summary of collected research, often to summarize evidence within a broad scope (Wolffe et al., 2019). These methods can be used on their own or in conjunction with one another. With the increasing use of such approaches, there are also a variety of SR tools available, including software tools to facilitate database creation, filtering, review, and prioritization (e.g., AOP-helpFinder (Jornod et al. 2022), SWIFT-Review (Howard et al. 2016), Covidence (Babineau 2014)).

As more institutions recognize the importance of SR for regulatory evaluations (Whaley et al. 2016), it has been proposed that aspects of the systematic review process be used to develop AOPs (Leist et al. 2017). The use of a documented approach to literature searches increases transparency and understanding of the overall weight of evidence to the resulting AOP and KER components including potential uncertainties and inconsistencies in the database.

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

A small number of AOPs have been developed in the area of genotoxicity (Cho et al., 2022; Chauhan et al., 2021; Marchetti et al., 2016; Yauk et al., 2015) to support the use of data derived from new test methods (Sasaki et al. 2020). Included in the genotoxicity AOPs is AOP #296: "Oxidative DNA damage leading to chromosomal aberrations and mutations" (Cho et al., 2022; https://aopwiki.org/aops/296) (Figure 1.1), for which my thesis focuses on expanding and gathering quantitative evidence. This AOP begins with an increase in oxidative damage to DNA (MIE) that overwhelms the DNA repair capacity leading to inadequate repair of the damage (KE1a). Inadequate repair of the oxidative DNA damage (KE1a) then branches into two paths. It can lead to an increase in mutations (AO1), arising from replication of damaged template DNA, and to an increase in DNA strand breaks (KE2), occurring from the DNA repair process removing oxidative lesions. An increase in DNA strand breaks (KE2) can overwhelm the repair capacity and cause inadequate repair (KE1b), leading to an increase in chromosomal aberrations (AO2) (Cho et al. 2022) or mutations (AO1) through error-prone repair of DNA strand breaks.

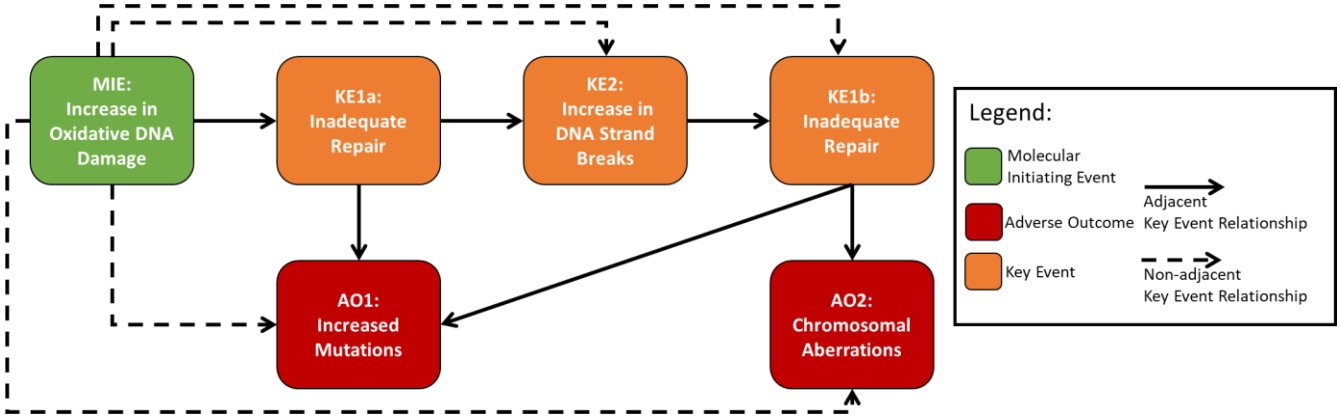

Figure 1.1. Flow diagram of the adverse outcome pathway (AOP) "Oxidative DNA damage leading to mutations and chromosomal aberrations" (modified from Cho et al. 2022).

There is strong biological plausibility and moderate empirical evidence in support of the KERs in AOP #296, but the quantitative understanding of all adjacent and non-adjacent KERs is low (Cho et al. 2022). This means that although the pathway is highly biologically plausible, there is a lack of collected evidence to quantify the response-response relationships across time and dose. Specifically, there is a lack of evidence showing what level of oxidative DNA damage must occur before there is progression to the later KEs (inadequate repair and DNA strand breaks) or progression to the AOs (chromosomal aberrations and mutations). This is primarily due to a lack of studies that use multiple stressor concentrations and time points within a single experimental design (i.e., to establish dose, incidence, and temporal concordance within a model system), which is required to build quantitative understanding. Extrapolation across model systems and experiments is not possible because endogenous levels of Reactive Oxygen Species (ROS) vary between cell lines and cell types, as does the level of oxidative DNA damage that a cell must incur before repair capacity is overwhelmed (Milkovic et al., 2019; Ray et al., 2012). This lack of quantitative understanding is a knowledge gap occurring in many AOPs that limits potential use for predictive toxicology. In the present work, strengthening the base of

quantitative evidence for this pathway would allow for better use of ROS or oxidative DNA damage measurements to predict the occurrence of chromosomal aberrations and mutations.

Oxidative stress is the imbalance of oxidants and antioxidants within a cell, occurring from either an increase in oxidants, a depletion of antioxidants or an impairment of the antioxidant response process (Birben et al., 2012). When oxidative stress occurs, the oxidants can cause cellular damage, such as DNA lesions (Cadet et al., 2017). Oxidants can be produced from an endogenous source or introduced from an exogenous source. Endogenous oxidants are produced from metabolic reactions and are present in cells at steady state. These oxidants are essential for cell signalling processes and the electron transport chain (Birben et al. 2012), but do not pose a threat to the cell at steady state as they are regulated by the cell's antioxidant response and endogenous antioxidants (Jean Cadet et al. 2017). Exposure to chemicals such as 4-Nitroquinoline 1-oxide (4NQO), potassium bromate (Platel et al., 2009), heavy metal ions such as cadmium (Kocadal et al., 2020), or ionizing radiation (Jean Cadet et al. 2017) can result in the increased production of ROS. In excess, ROS become harmful to the cell by causing an accumulation of oxidative damage to DNA, proteins, and cellular structures. An excess of oxidative DNA damage overwhelms the repair pathways (Birben et al. 2012).

1.4 DNA damage, chromosomal aberration, and mutation measurement methods

1.4.1 Oxidative DNA damage and DNA strand break measurement with the CometChip assay

Oxidative DNA damage includes a wide variety of DNA lesions such as modifications to or removal of nitrogenous bases, modifications to sugar components, and cross-linking of DNA with proteins (Birben et al. 2012). When a cell is overwhelmed with oxidative DNA damage, repair pathways are no longer able to effectively repair oxidative lesions (Birben et al. 2012). Incomplete repair can result in DNA strand breaks arising from extensive amounts of base excision repair (Slupphaug et al., 2003). DNA

Strand breaks can also lead to DNA replication fork stalling that may be inadequately repaired (Caldecott 2008).

The CometChip assay can be used to measure oxidative DNA damage and DNA strand breaks (Ge et al. 2014). The comet assay measures DNA strand breaks by seeding cells in an agarose gel, lysing them, and electrophoresis is performed (Collins 2004). Strand breaks and damaged DNA, travel further than undamaged DNA, creating the characteristic comet shape. Then a fluorescent dye is used to stain DNA and the percentage of DNA in the tail of the comet is compared to the DNA in the head of the comet to quantify the DNA damage (Collins 2004). The CometChip assay uses High Throughput Single-Cell Gel Electrophoresis to run the standard alkaline comet assay in a 96-well plate format and measure DNA strand breaks (Figure 1.2). By treating cells with the enzyme Formamidopyrimidine DNA Glycosylase (Fpg), which creates strand breaks at oxidative DNA lesions, the CometChip assay can be used to measure oxidative DNA damage. The 96-well plate format of the CometChip assay provides an advantage over the standard comet assay by allowing for a greater throughput, as well as automated image processing, both of which reduce subjectivity, and variability between slides (Weingeist et al. 2013) and laboratories (Forchhammer et al. 2010).

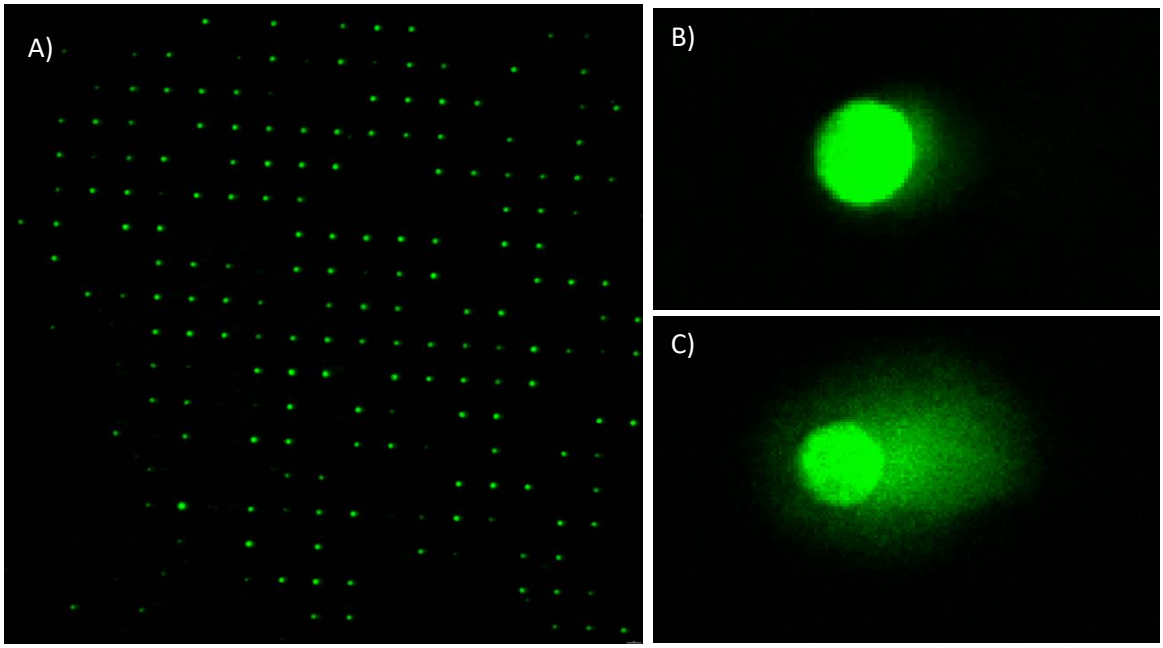

Figure 1.2. A) Comets in the microwells of one well on a 96-well plate, imaged at 5X with Lecia DMi8 confocal microscope. B) Single comet with a short tail, indicating fewer DNA strand breaks. C) Single comet with a long tail, indicating a greater amount of DNA strand breaks.

1.4.2 Chromosomal aberration detection using the microflow micronucleus assay

Lack of repair of DNA strand breaks, or repair by non-homologous end joining, can lead to translocations, inversions, insertions and deletions of chromosomal pieces (Pfeiffer 2000). Chromosomal fragments that lag during anaphase are not incorporated into either nuclei and form a micronuclei within the cell (Thomas et al., 2003) (Figure 1.3, column 2). Which can be measured by flow cytometry using the microflow micronucleus assay (Avlasevich et al., 2006; Bryce et al., 2008). The microflow micronucleus assay is a high throughput format of running the (traditional) micronucleus assay; two dyes are used to differentiate between dead or dying cells and healthy or micronucleated nuclei (Figure 1.3). The size of the nuclei is used to differentiate between nuclei and micronuclei. With flow cytometry, the micronucleus assay can be run in a 96-well plate, increasing the number of cells analyzed per treatment, throughput, and efficiency. The microflow micronucleus assay is also a reliable tool for characterizing the dose-response relationship of genotoxic compounds (Bryce et al., 2010).

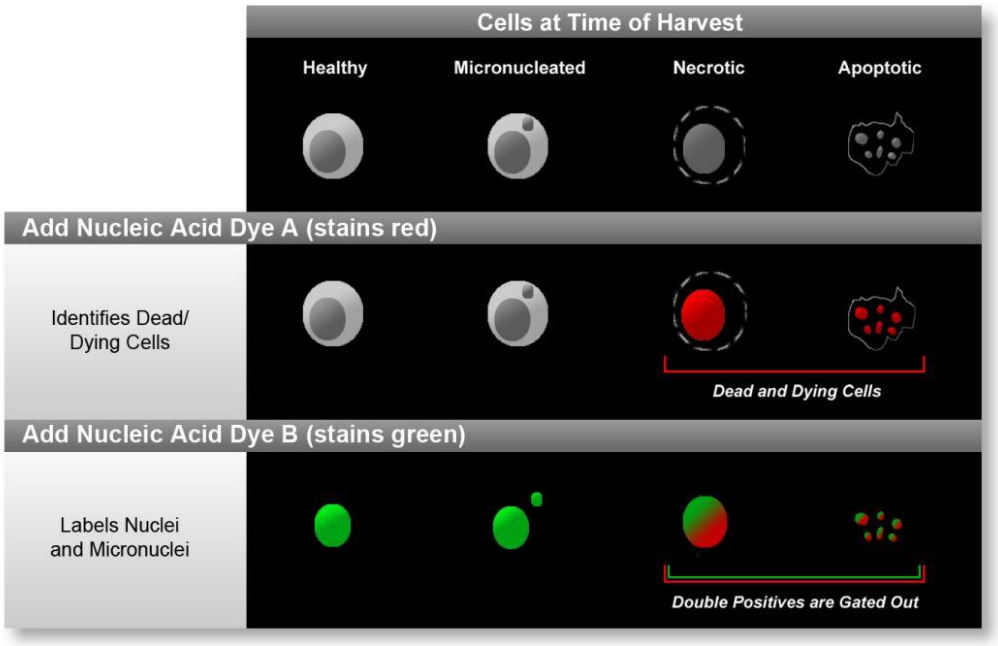

Figure 1.3. Visualization of the microflow micronucleus assay demonstrating how the two nucleic acid dyes are used to differentiate between healthy or micronucleated cells and dead or dying cells (Litron Laboratories, 2022). Presented with permission from Litron Laboratories.

1.4.3 Mutation detection using Duplex Sequencing

Lack of oxidative DNA damage repair can result in mutations arising from the replicative polymerase or a translesion synthesis polymerase inserting the wrong base opposite the lesion (Boiteux et al., 2017). Conventional assays for the measurement of mutations include *in vivo* methods such as the transgenic rodent (TGR) gene mutation assay and *in vitro* mammalian cell gene mutation assays such as the mouse lymphoma assay (MLA). The TGR gene mutation assay involves incorporating bacterial reporter genes into a rodent genome, then recovering these reporter genes and analyzing the phenotypic response of a bacterial host (Lambert et al., 2005). The mouse lymphoma assay using the thymidine kinase gene, involves culturing L5178Y $TK^{+/-}$-3.7.2C cells and plating them in media that selects for mutations (OECD 2016b). These methods of *in vitro* and *in vivo* mutagenicity testing have limitations, such as that specific rodents and cell lines must be used, so the methods cannot be integrated into other tests, and may be limited to certain tissue types. The mutations observed in these assays are limited to bacterial reporter

genes or a single mammalian gene and are assessed as mutations that cause a phenotypic change. Also, it is not possible to characterize the mutation spectrum, without extensive further work. Therefore, these methods are not well suited for quantitative measurement of global levels of mutations caused by oxidative DNA damage.

Error-corrected next generation sequencing (NGS) technologies, such as Duplex Sequencing (DS), are able to identify and eliminate sequencing artifacts, providing an unprecedented level of accuracy for mutation analysis across endogenous loci in the genome (Kennedy et al. 2014). DS uses a unique barcoding process paired with bioinformatic filtering, to overcome the error-prone nature of conventional NGS (1 in 10^3-10^4) and accurately quantify mutation frequencies as low as 1 in 10^8 sequenced bases (Kennedy et al. 2014; Schmitt et al. 2012). The technology can be summarized in three steps (Figure 1.4) (Schmitt et al. 2012). First, adapter synthesis (Figure 1.4 A): starting with a sequencing adapter with two unique arms, a randomized Duplex tag (unique barcode), and a fixed end sequence, the complement of the Duplex tag and the fixed sequence is extended, and an A tail is added to the complement strand. Second, PCR amplification (Figure 1.4B): the adapters are then ligated with prepared DNA fragments and amplified through PCR. The PCR products then have two unique tag sequences, one from each adapter that is ligated to the double stranded DNA (represented in Figure 1.4B as α and β). Thus, the PCR products can be grouped into two families, the αβ family and the βα family. Third, error correction (Figure 1.4 C): a single-strand consensus sequence (SSCS) is determined for each family and a duplex consensus sequence (DCS) is determined from both SSCSs. The resulting mutation data have a high sensitivity and high content data with the ability to characterize the mutation spectra, which is suitable for quantitative measurement of global levels of mutations caused by oxidative DNA damage.

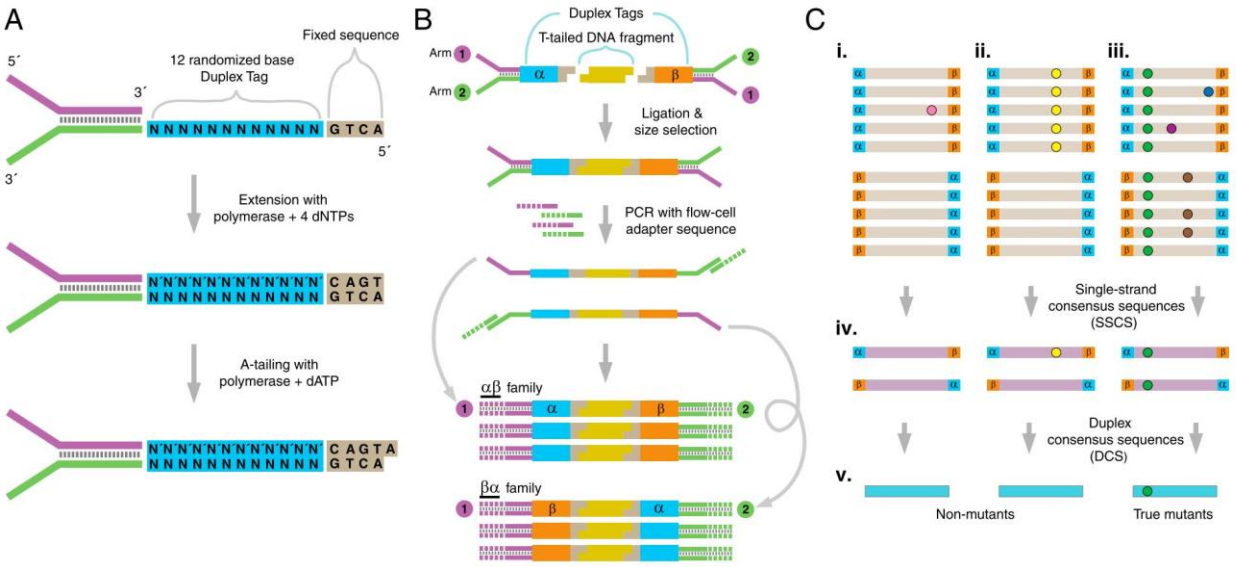

Figure 1.4. Summary of how the uniquely barcoding each strand of a double stranded DNA molecule prior to sequencing allows Duplex Sequencing to detect a mutation in greater than 1x10^{7} sequenced bases (Schmitt et al. 2012)**.** Presented with permission through Proceedings of the National Academy of Sciences of the United States of America (PNAS) exclusive License to Publish.

1.5 Objectives

The objective of my thesis was to investigate the quantitative associations of the KERs included in and upstream of AOP #296 "Oxidative DNA damage leading to chromosomal aberrations and mutations" (Cho et al. 2022), through literature review and experimental methods. Directly upstream of the pathway, data were collected to build KERs between a KE 'Increase in cellular ROS' and AOP #296. Empirical data were gathered to quantify the KERs between observed oxidative DNA damage and the KEs "Increase in DNA strand breaks", "Increase in chromosomal aberrations", and "Increase in mutations". Data were also gathered to support the adjacent KER between increase in cellular ROS and observed oxidative DNA damage to build on AOP #296.

The literature review component of this project was conducted to explore freely available literature review tools as a case study for the use of a transparent literature review within AOP development. The approach to this literature review was to focus on the KERs, in alignment with the suggestion by Svingen

et al. (2021) that KERs should be the main AOP modules that are developed and reviewed (as opposed to entire AOPs).

The experimental component of the project was conducted to address a deficiency found in the literature of quantitative data linking the MIE, oxidative DNA damage, to the two adverse outcomes, chromosomal aberrations, and mutations, both in my own literature search as well as in Cho et al. (2022). For these experiments, 4NQO was chosen as the prototype stressor as it is well established to cause oxidative DNA damage, chromosomal aberrations and mutations *via* the formation of ROS (Arima et al. 2006; Brüsehafer et al. 2016; Nunoshiba and Demple 1993).

Specifically, the objectives for this thesis were as follows,

Chapter 2: Conduct a documented literature review to:

i. Investigate and demonstrate the use of an SR review tool called Covidence and systematic evidence maps to expand on the existing AOP #296
ii. Examine the supporting evidence with a focus on quantitative understanding, describing how increases in ROS can lead to chromosomal aberrations and mutations

Chapter 3: Measure oxidative DNA damage, DNA strand breaks, chromosomal aberrations, and mutations in TK6 cells exposed to a known ROS producing agent, using modern quantitative *in vitro* tests and use the resulting data to:

iii. Establish *in vitro* study designs and produce data to define the quantitative associations of the KERs in the existing AOP #296
iv. Test the performance of an innovative error-corrected sequencing technology known as Duplex Sequencing

The completion of these objectives will expand AOP #296 and will build a base of quantitative data in support of the KE and KERs. This work will demonstrate the use of documented literature review, and DS data that can be used in the future development of AOPs.

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Chapter 2: A Case Study on Integrating a New Key Event into an Existing Adverse Outcome Pathway on Oxidative DNA Damage: Challenges and Approaches in a Data-Rich Area

2.1 Abstract

Adverse outcome pathways (AOPs) synthesize toxicological information to convey and weigh evidence in an accessible format. AOPs are constructed in modules that include key events (KEs) and key event relationships (KERs). This modular structure facilitates AOP expansion and network development. AOP development requires finding relevant information to evaluate the weight of evidence supporting each KER. To do this, the use of transparent/reproducible search methods, such as systematic review (SR), have been proposed. Applying SR to AOP development in a data-rich area is difficult as SR requires screening each article returned from a search. Here we describe a case study to integrate a single new KE into an existing AOP. We explored the use of SR concepts and software to conduct a transparent and documented literature search to identify empirical data supporting the incorporation of a new KE, increase in cellular reactive oxygen species (ROS), upstream of an existing AOP (#296): 'Oxidative DNA Damage Leading to Chromosomal Aberrations and Mutations'. Connecting this KE to the AOP is supported by the development of five new KERs, the most important being the first adjacent KER (increase in ROS leading to oxidative DNA damage). We initially searched for evidence of all five KERs and screened 100 papers prioritized by the search software to develop a preliminary evidence map. After removing papers not containing relevant data based on our Population, Exposure, Comparator and Outcome statement, 39 articles supported one or more KERs; these primarily addressed temporal or dose concordance of the non-adjacent KERs with limited evidence supporting the first adjacent KER. We thus conducted a second focused set of searches using search terms for specific methodologies to measure these first two KEs. After filtering, 12 articles were identified that contained quantitative evidence supporting the first adjacent KER. Given that integrating a new KE into an existing AOP requires the development of multiple KERs, this approach of building a preliminary evidence map, focusing evidence gathering on the first adjacent KER, and applying reproducible search strategies using specific

methodologies for the first adjacent KER, enabled us to prioritize studies to support expansion of this data-rich AOP.

2.1 Introduction

2.1.1 Adverse Outcome Pathways: general background and challenges

Adverse Outcome Pathways (AOPs) synthesize toxicological information from various sources to convey and weigh evidence in an accessible and transparent format (Ankley et al., 2010; Sakuratani et al., 2018). The AOP format provides a framework for organizing mechanistic information that describes the chain of events from a molecular initiating event (MIE) through a series of intermediate key events (KEs) to an adverse outcome (AO) (Perkins et al., 2011). Key event relationships (KERs) explain the causal linkages between KEs and form the basis of using AOPs for predictive toxicology. By focusing on mechanistic information, AOPs are generally referred to as chemically agnostic. They can thus be applied broadly as a predictive tool for AOs induced by chemicals tested using high-throughput and high-content mechanistic data sources. They are also used to support test method development, identify knowledge gaps, and direct priority research.

A central premise of the AOP program is that AOPs are modular, with KE and KER units that can be used in multiple pathways, facilitating collaboration and the development of AOPs relevant to multiple stressors. This modular design also enables the expansion of existing AOPs and the creation of AOP networks that more thoroughly explain toxicological effects. Thus, a foundational principle is that the creation of duplicate or similar KEs is to be avoided. KEs, which are measurable biological events, should be developed such that they are broadly applicable while specifics, such as timescale (acute or chronic) or cell type, should be described in the KER or in the overall AOP. However, the AOP-Wiki is open for submissions and AOP authors have varying levels of familiarity with AOP conventions. Moreover, the AOP framework is relatively new and the standardization of AOPs is ongoing. The creation of many virtually identical KEs in the AOP-Wiki by many different authors represents a critical challenge at present.

KERs summarize the available empirical data and biological knowledge to describe the causal relationships between KEs using the modified Bradford-Hill (B-H) criteria. These criteria include: (a) biological plausibility (i.e., the relationship's consistency to current accepted mechanistic knowledge); (b) empirical evidence of dose, temporal, and incidence concordance between the two KEs; and (c) essentiality of the KEs (i.e., evidence that an upstream KE must occur in order to observe a downstream KE). A summary of the quantitative understanding of the relationship between the two KEs and consideration of uncertainties and inconsistencies is also provided in KER development. The overall weight of evidence of an AOP is supported by KERs that are both adjacent and non-adjacent (OECD, 2018); indeed, empirical evidence linking non-adjacent KEs in an AOP often provides some of the greatest support if intermediate KEs are less routinely measured.

To weigh the evidence supporting KERs and AOPs, AOP authors are required to summarize the current knowledge, find data meeting B-H criteria in support of the relationships, and identify uncertainties and inconsistencies within the literature. This evidence is often found across diverse sources and, for a well-studied research area, the amount of literature and data review required to identify evidence to support KERs and AOPs can be substantial. Moreover, empirical evidence and quantitative understanding are often difficult to mine from the literature. Given the extensive work required for AOP development in general, it has recently been proposed that KERs could be developed and reviewed independently, rather than the current process wherein entire AOPs are developed and reviewed (Svingen et al., 2021). These authors also propose that where possible pragmatic, transparent and documented methods, including the use of systematic review (SR) tools and approaches, be applied for KER and AOP development, as opposed to the narrative approach that is currently used in AOP literature review. They note, however, that this may not be practical for data-rich fields. The proposal by Svingen et al. (2021) is highly relevant to our interest to build on an existing series of AOPs in the area of genotoxicity assessment (described in more detail below).

Within AOP development there are no strict guidelines on how to gather literature evidence for a KER. However, as more institutions recognize the importance of SR for regulatory evaluations (Whaley et al., 2016), it has been proposed that transparent search methods also be used to develop AOPs (Leist et al., 2017). Reproducible search methods transparently describe how literature was searched and reviewed when weighing the collected evidence. There are a variety of search methods applied today including scoping reviews, systematic evidence mapping (SEM), and SR; SR is the most widely used. SEMs provide a visual summary of systematically gathered research. They are often conducted to characterize gaps and summarize evidence with a broad scope and do not require risk-of-bias analysis before focusing a SR on a specific question (Wolffe et al., 2019). SR is a framework that aims to answer a specific research question by using reproducible search methods to synthesize all available data. A variety of SR tools are now available, including software tools to facilitate database creation, filtering, review, and prioritization. Using a transparent and documented approach to literature searches provides a stronger overall weight of evidence to the resulting AOP and KER components and informs on uncertainties and inconsistencies in the database.

Below we present a case study to investigate approaches for creating and integrating a single KE upstream of an existing AOP and applying SR tools to collect evidence supporting KERs to 'network' into this existing AOPs. We anticipated challenges in the literature search because this is an extremely data-rich area, and the work was to be done by a single researcher (SRs require a team). Thus, we explored the use of a transparent and documented approach to search the literature using a SR software tool to screen the collected articles with defined inclusion/exclusion criteria. Then, an evidence map was built to visualize the evidence collected.

2.1.2 The use of AOPs in genotoxicity assessment

Genotoxicity assessment determines the ability of chemicals to cause genetic damage, such as chromosomal aberrations and mutations, which can lead to cancer (Hoeijmakers, 2009) and genetic disease (Caldecott, 2008). Products in commerce are required to be assessed for their genotoxic potential in order to protect human and ecological health. At present, conventional tests typically classify chemicals as genotoxic or non-genotoxic but provide limited to no mechanistic information about how the test chemicals leads to genotoxicity-associated AO (Dearfield et al., 2017).

To facilitate the development of mechanism-based testing and its use in genotoxicity assessment, AOPs corresponding with mechanisms of genotoxicity are under development (Sasaki et al., 2020; Yauk et al., 2013). There is thus an expanding number of genotoxicity-focused AOPs (e.g., Chauhan et al. 2021, https://aopwiki.org/aops/272; Marchetti et al. 2016, https://aopwiki.org/aops/106; Yauk et al. 2015, https://aopwiki.org/aops/15) in various phases of development. At present, the AOP-Wiki (https://aopwiki.org/aops) includes three genotoxicity AOPs that use the KEs “inadequate repair”, “mutations”, or “chromosomal aberrations”. One of these AOPs terminates in permanent genomic damage in offspring, reflecting interest in the use of genotoxicity as an endpoint of regulatory concern (Heflich et al. 2020); whereas, the other two AOPs lead to cancer outcomes. One of these AOPs is endorsed by the Organisation for Economic Co-operation and Development (OECD), the other two are presently under internal review by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). Endorsement indicates that an AOP has been subject to external review, approved by the Extended Advisory Group for Molecular Screening and Toxicogenomics, and finally ‘endorsed’ by the Working Group of the National Coordinators for the Test Guidelines Programme (WNT) and the Working Party on Hazard Assessment (WPHA). Endorsement by the WNT and WHPA indicates that the appropriate scientific review process was followed and the AOP is deemed to be worthy of public dissemination (OECD, 2021). Additional AOPs that will be merged with this genotoxicity

network have been described by the Genetic Toxicology Technical Committee (GTTC) of the Health and Environmental Sciences Institute (HESI) and are in various stages of development (Sasaki et al., 2020). With the expanding genotoxicity AOP network (Figure 1) there is a need to further harness the modular nature of the AOP framework and collaborate to develop additional KEs applicable to multiple pathways that inform genotoxic mechanisms and outcomes.

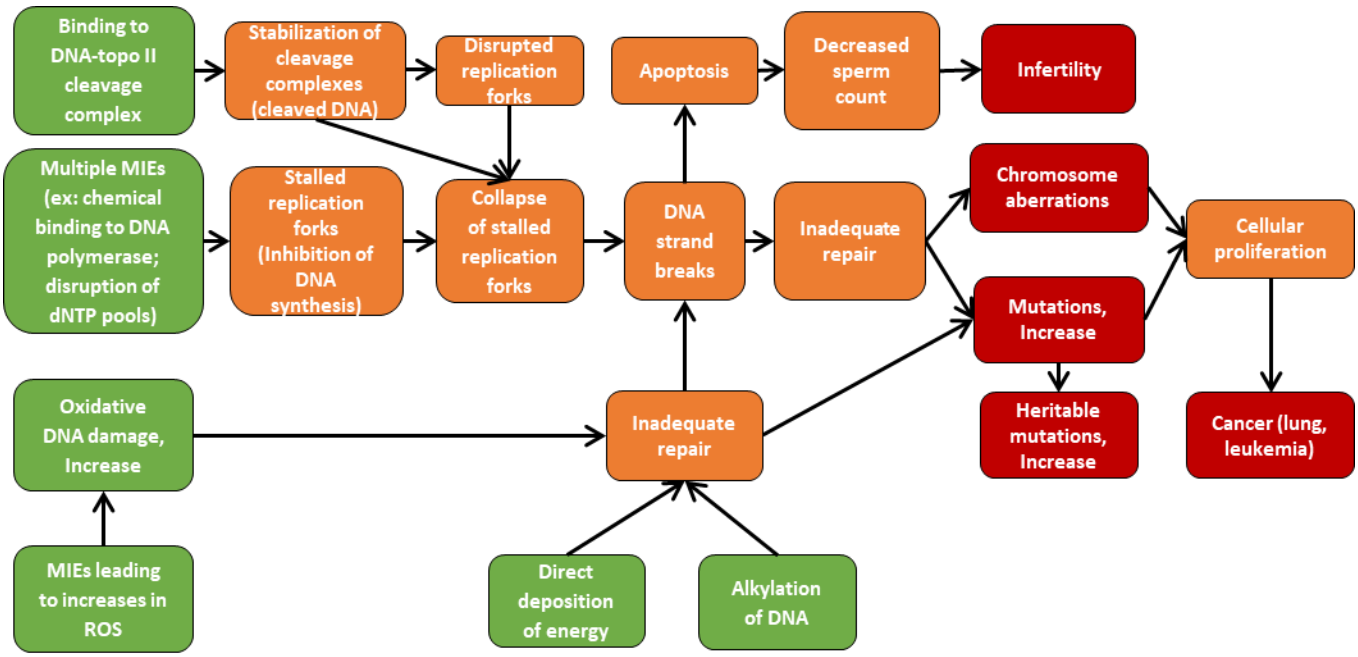

Figure 2.1. Adverse outcome pathways that converge on key events associated with genotoxicity were extracted in the AOP-wiki and from Sasaki et al., 2020. Select AOPs were chosen to demonstrate the growing network of AOPs that converge on shared KEs to form a network leading to chromosome damage, mutations. A network of KEs leading to aneuploidy is being developed separately. MIEs: molecular initiating events.

2.1.3 Expansion of an existing AOP on oxidative DNA damage: this project

Initial work within the GTTC developed AOP #296: 'Oxidative DNA damage leading to chromosomal aberrations and mutations' (Cho et al., 2022) (Figure 2). This pathway was first suggested

during a GTTC workshop in 2017, where the pathway was used to demonstrate how AOPs could be harnessed to advance mode of action analysis in genetic toxicology (Sasaki et al., 2020). The AOP begins with an increase in oxidative damage to DNA (MIE) that overwhelms the repair capacity leading to inadequate repair of oxidative DNA damage (KE1). Inadequate repair of the oxidative DNA damage (KE1) then branches into two paths. It can lead to an increase in mutations (AO1), arising from replication of damaged template DNA, and/or to an increase in DNA strand breaks (KE2), occurring from the DNA repair process removing oxidative lesions. An increase in DNA strand breaks (KE2) can overwhelm the repair capacity and cause inadequate repair (KE1), leading to an increase in chromosomal aberrations (AO2) (Cho et al., 2022).

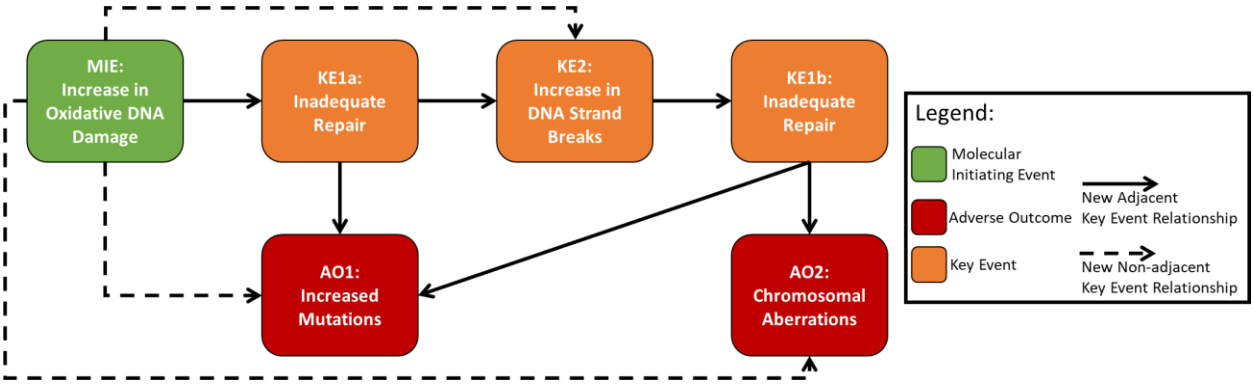

Figure 2.2. Flow diagram of the adverse outcome pathway 'Oxidative damage leading to mutations and chromosomal aberrations' (modified from Cho et al., 2022).

AOP #296 indicates that, in addition to direct oxidation of DNA by chemicals like hydrogen peroxide and other radicals, there are other upstream MIEs that indirectly cause oxidative DNA damage. Thus, the MIE for AOP #296 could also be considered an intermediate KE for other AOPs. Oxidative DNA damage includes a wide variety of DNA lesions such as modifications to, or removal of, nitrogenous bases, modifications to sugar components, and cross-linking of DNA with proteins (Birben et al., 2012).

Oxidative DNA damage can be caused by exposure to a toxicant either directly by interaction between the toxicant and DNA, or indirectly through an increase in reactive oxygen species (ROS). Indeed, there are many direct and indirect mechanisms by which toxicants themselves or exposure to toxicants can produce ROS and subsequently lead to oxidative DNA damage.

The overarching aim of the present work is to build on AOP#296 through networking in an upstream KE reflecting increases in ROS and developing the relevant KERs for evaluation, aligned with the proposal of Svingen et al. (2021). The approach taken toward this objective was to: (a) identify the relevant new KE needed through a search of the AOP-Wiki; (b) determine if there are existing KERs that could be used to connect to AOP#296; and (c) apply freely available SR tools to facilitate collection of evidence to support the new KERs required.

2.2 Methods

2.2.1 KE and KER identification

We first aimed to identify a KE that described increases in cellular levels of ROS. The AOP-wiki (https://aopwiki.org/) was searched using the terms 'reactive oxygen species' and 'oxidative stress' on the KE search page (https://aopwiki.org/events; using the 'search key events' box), to identify existing KEs that might be suitable for incorporation in our pathway. Each KE was reviewed to examine the extent of development, its review status (e.g., part of an 'endorsed' AOP), and whether KERs already exist to link the KE to other events in AOP#296.

2.2.2 Broad Literature Search

In this case study, there were two rounds of literature searches and screenings performed using an SR management tool. The first search was broad; the terms were chosen such that they encompassed all five KERs and the search was conducted to assess the available data and to determine more specific questions or data gaps. The second set of searches was conducted to address a specific KER that was underrepresented in the previous search.

The broad search was performed in the University of Ottawa online Library, using the OMNI search tool (ExLibris Primo, 2020). Two terms were chosen: "reactive oxygen species" to capture the proposed new KE increase in ROS and "DNA damage" to capture the KEs of AOP#296. The terms "reactive oxygen species" and "DNA damage" (see Table S1 for search syntax) were searched and filtered for articles. The 236,505 results were sorted by relevance according to OMNI's Intelligent Ranking Technology. The Intelligent Ranking Technology ranks results based on word match, value score (query words in author, title, subject, plus date) and word proximity.

The 100 most 'relevant' results as ranked by the software were chosen as the starting place to evaluate the types and quality of evidence available for this data-rich area. These results were uploaded to the SR management tool Covidence.org (Covidence, 2021) for screening. Covidence was used to track included and excluded articles at each stage as well as prioritize studies by using artificial intelligence (active learning) so that studies that were more likely to be included were screened first (Miwa et al. 2014). Covidence was also used to remove duplicates. The titles and abstracts of the remaining articles were screened using a modified Population, Exposure, Comparator and Outcome (PECO) statement. The standard PECO statement was modified to include Population, Exposure, and Endpoints (Table 1) to better fit the empirical evidence and quantitative understanding needed for our AOP. The population and exposure categories were broad, and the endpoints category included the KEs of our AOP: increase in ROS, oxidative DNA damage, inadequate repair, mutations, DNA strand breaks, and chromosomal aberrations. During the title and abstract screening, articles not meeting the modified PECO statement were excluded. After the title and abstract screening, the full texts of the remaining articles were screened for the measurement of cellular ROS, the measurement of one or more of the downstream KEs and for empirical evidence (dose, temporal, or incidence concordance between the measured KEs) or quantitative understanding (the inclusion of a control, or more than one dose or timepoint measured). During the full text screening, articles that did not measure cellular ROS, one or more downstream KE, and lacked empirical or quantitative evidence were excluded. Articles that passed screening were included as evidence for our proposed KERs, and data were extracted from these articles. The data extracted included the toxicant, model used, the relevant KEs, the assays used to measure the KEs, whether essentiality was measured, and the type of evidence that was contained (temporal, dose/concentration, incidence or conflicting).

Table 2.1. Population, Exposure, Endpoint statement, for the inclusion/exclusion of full text screening.

Modified PECO element	Evidence
Populations	Animal (all levels), human, *ex vivo*, *in vivo*, cell, cell-lines, epidemiological, cohort, population, organ, tissue, cellular, molecular, cellular components, biologically based models
Exposures	Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), Chemicals or Stressors causing an increased production of ROS/RNS (directly or indirectly)
Endpoints	All KEs in AOP: Cellular Reactive Oxygen Species (ROS), Oxidative DNA damage, Inadequate repair, Mutations, DNA strand breaks and Chromosomal aberrations

2.2.3 Specific Literature Search

Once the collected data from the broad search were assessed, a second focused search was conducted to specifically collect empirical data supporting the direct KER linking 'increase in ROS' to 'increase in oxidative DNA damage. The second search was performed on October 28, 2021, in the uOttawa online Library, using the OMNI search tool (ExLibris Primo, 2020). Terms (Table 2) were selected to represent reliable and quantitative assays for the measurement of cellular ROS and of oxidative DNA damage (see Table S1 for search syntax). Some selected methods for the measurement of oxidative DNA damage are not specific to the detection of oxidative DNA lesions and, therefore, the common oxidative lesion 8-oxodG was added to these searches. These terms were searched in all combinations, for a total of 40 searches. The titles and abstracts of these articles were then screened using a modified PECO statement. The modified PECO statement was the same as the broad search (Table 1), except the only used endpoints were cellular ROS and oxidative DNA damage.

Table 2.2. Quantitative and reliable methods measuring cellular ROS or oxidative DNA damage used in search terms for focused search.

Methods measuring cellular ROS	**Methods measuring oxidative DNA damage**
Electron paramagnetic resonance spectroscopy	FPG-modified comet
Electrochemical detection of ROS	ELISA AND 8-oxodG*
HyPer	LC-MS AND 8-oxodG*
Hydroethidine	HPLC-EC AND 8-oxodG*
Mito-SOX	
Mito-HE	
Electrode detection of ROS	
Boronate probes	
CellROX	

* 8-oxodG was added to indicated search terms because these methods are not specific to the detection of oxidative DNA damage.

2.3 Results

2.3.1 Identification of relevant KE and KERs

A search of the AOP-Wiki revealed that many KEs addressing alterations in ROS already existed. There were sixteen potential KEs found in the AOP-Wiki search, with names spanning "reactive oxygen species", "reactive oxygen species production", "reactive oxygen and nitrogen species", "mitochondrial reactive oxygen species", and "oxidative stress". These KEs are separately included in 60 AOPs as either MIEs or intermediate KE, and their corresponding AOP-wiki pages are in various stages of development, ranging from having all fields completely blank, having most fields blank, to having all fields filled out. There were no existing KERs that contained content to link these KEs to mutations and chromosomal aberrations for AOP#296 or other AOPs.

Having recognized redundancy of KEs in the AOP-Wiki as a general challenge for AOP development that needs to be addressed, a group of AOP 'gardeners' (individuals that have been recruited by the OECD to curate the information in the AOP-Wiki) is working on eliminating blank or unused KE. Because cellular ROS alteration is central to many toxicological pathways, an expert working group including these gardeners within the OECD has been created to develop a hub of consolidated KEs for this area that can be used by the broader community. The formation of this expert Working Group occurred following the launch of the present study. Thus, given our knowledge of, and participation in, this exercise, we decided to focus our work on the development of the new KERs that would be required to link the consensus ROS KE(s) upstream of AOP #296's MIE, "increase in oxidative DNA damage" (Figure 3). We determined that this would require investigating and potentially developing five new KERs: one adjacent KER – increases in ROS leading to oxidative DNA damage; and four non-adjacent KERs - ROS leading to inadequate DNA repair, ROS leading to DNA strand breaks, ROS leading to mutations, and ROS leading to chromosomal aberrations. In order to develop the KERs, we are

tentatively using the name “increase in ROS” for this study but will follow the recommendations of the OECD working group when a consensus is reached regarding the consolidated ROS KE(s).

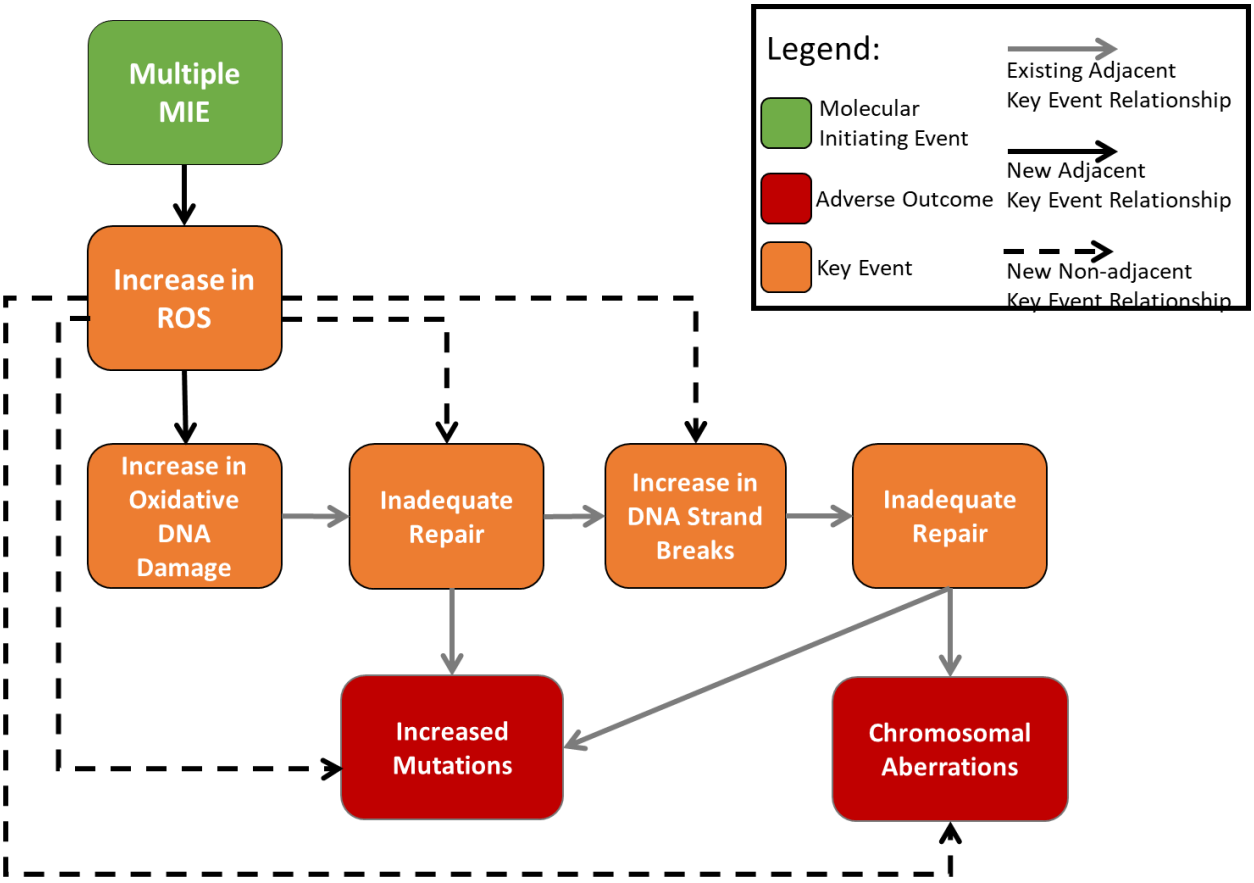

Figure 2.3. Flow diagram of the adverse outcome pathway ‘Oxidative damage leading to mutations and chromosomal aberrations’ (Cho et al., 2022, https://aopwiki.org/aops/296) with the inclusion of an upstream KE “increase in cellular ROS”, which represents the eventual consensus ROS KE, occurring after the multiple MIEs by which ROS are produced after toxicant exposure, and the resulting new KERs.

2.3.2 Broad Literature Search Results

The initial broad literature search led to the retrieval of 236,505 records, which is clearly beyond scope or scale of what is feasible and necessary to screen. We thus used a screening strategy to explore the top 100 articles. From these 100 papers, duplicate removal, title and abstract screening, and full text

screening led to the elimination of 61 articles (Figure 4). Specifically, from the 100 most 'relevant' results that were uploaded to COVIDence.org for screening, 4 duplicates were removed. The titles and abstracts of the remaining 96 articles were screened using a modified PECO statement (Table 1); 18 articles were excluded, and 78 articles remained for full text screening. The full texts of the remaining 78 articles were screened for the measurement of cellular ROS, the measurement of one or more of the downstream KEs, and the availability of empirical evidence (dose, temporal, or incidence concordance between the measured KEs) or quantitative data (the inclusion of a control, or more than one dose or timepoint measured). This resulted in the exclusion of 39 articles. The data were extracted from the remaining 39 articles.

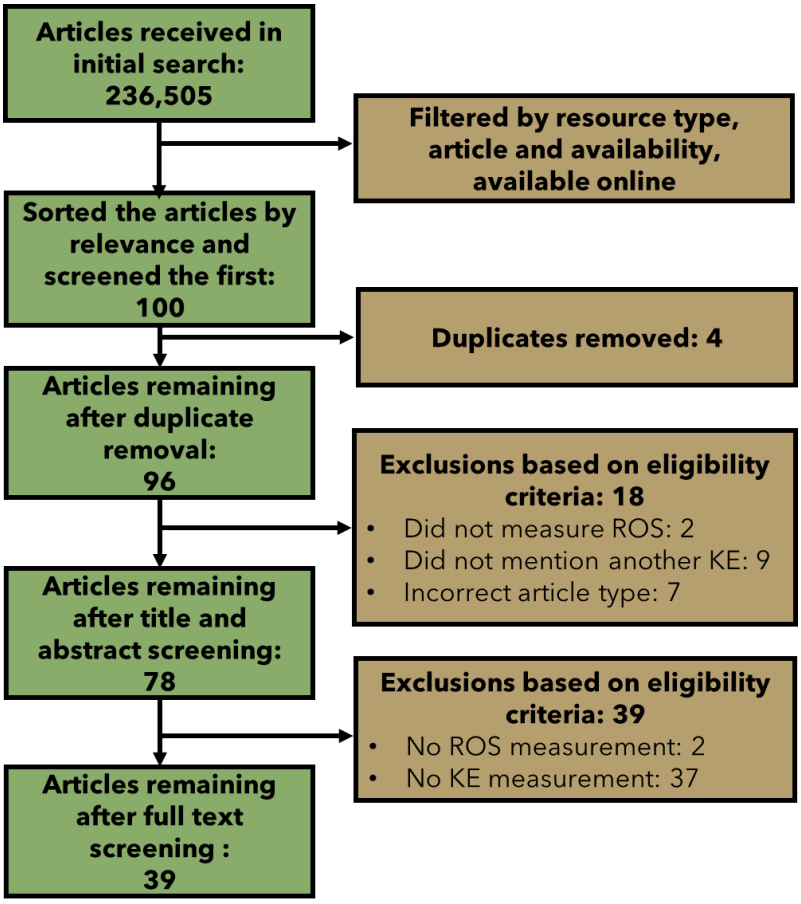

Figure 2.4. Flow chart showing the number of articles included and excluded at each step of the broad literature review.

The articles were tagged as to which KEs they measured and which KERs they supported to develop a 'preliminary' evidence map (Table 3). As it was part of the selection criteria, all of the selected articles measured cellular ROS. The overwhelming majority of the articles also measured DNA strand breaks; 35/39 articles. Within these 35 articles, four also measured oxidative DNA damage, one measured inadequate repair and one measured mutations. The next most measured KE was oxidative DNA damage with 5 out 39 articles. Within these five articles measuring oxidative DNA damage, four also measured DNA strand breaks and one measured mutations. The remaining three KEs, inadequate repair, mutations and chromosomal aberrations were measured in three, two, and one of the selected articles, respectively.

Table 2.3. Evidence map describing the number of articles identified through the screening that measured each key event (KE) in the "Oxidative DNA damage leading to chromosomal aberrations and mutations" pathway as well as increases in ROS.

	KE1: Oxidative DNA damage	KE2: Inadequate repair	KE3: DNA strand breaks	AO1: Mutations	AO2: Chromosomal aberrations
KE1: Oxidative DNA damage	**5**	**1**	**4**	**1**	**0**
KE2: Inadequate repair		**3**	**1**	**1**	**1**
KE3: DNA strand breaks			**35**	**1**	**0**
AO1: Mutations				**2**	**1**
AO2: Chromosomal aberrations					**1**

The articles were also tagged according to which of the B-H criteria empirical evidence types they supported (Table 4). Temporal concordance was measured in 32 articles, 16 of which also measured dose concordance. Dose concordance was measured in 21 articles, 16 of which also measured temporal concordance. Incidence concordance was not found in any articles. There was conflicting evidence of dose concordance in one article.

Table 2.4. Evidence map describing the number of articles retrieved in the screening that addresses each type of empirical evidence required to evaluate the weight of evidence of a KER.

	Temporal concordance	Dose/concentration concordance	Incidence concordance	Conflicting
Temporal concordance	**32**	**16**	**0**	**0**
Dose/concentration concordance		**21**	**0**	**0**
Incidence concordance			**0**	**0**
Conflicting				**1**

2.3.2.1 Adjacent KER: ROS leading to oxidative DNA damage

There were five papers that contained quantitative and empirical evidence of the relationship between an increase in ROS and an increase in oxidative DNA damage (Table S2). Two of these papers measured endpoints that could be used to demonstrate that an increase in ROS occurs at lower or equal concentrations of the tested toxicants to those concentrations that induce an increase in oxidative DNA damage (De Iuliis et al., 2009; Jacobsen et al., 2008). One of the papers presents conflicting evidence; the endpoints measured show an increase in ROS at a higher concentration than an increase in oxidative DNA damage (Mittal et al., 2014). Such types of conflicting data can occur because of differences in the dynamic ranges and sensitivity of the different methodologies used. Conflicting data could also occur because there is a certain increase in the level of ROS that would not be detected as a cell is able to control the ROS using antioxidants; an increase in ROS would only be detected once the cell is overwhelmed and cellular antioxidants are unable to control the ROS. Three of these papers provide empirical data to show that an increase in ROS occurs at earlier or the same timepoints to those

timepoints that induce an increase in oxidative DNA damage (Babbar et al., 2006; Beattie et al., 2013; Mittal et al., 2014).

2.3.2.1 Nonadjacent KERs

The empirical evidence collected for the non-adjacent KERs was enriched in papers supporting the relationship between an increase in ROS and an increase in DNA strand breaks. Indeed, there were 35 papers providing empirical evidence supporting an increase in ROS leading to DNA strand breaks (Table S3), while there were only three papers supporting an increase in ROS leading to inadequate repair (Table S4), two linking cellular ROS leading to mutations (Table S5), and one linking cellular ROS to chromosomal aberrations (Table S6). Within the 35 papers that supported an increase in ROS and an increase in DNA strand breaks, there were 30 papers demonstrating temporal concordance and 21 demonstrating dose/concentration concordance. Of the three papers that supported an increase in ROS leading to inadequate repair, all three addressed temporal concordance (Renée L. Flaherty et al. 2017; Preston et al. 2009; Shih et al. 2021) and two provided data for dose/concentration concordance (Renée L. Flaherty et al. 2017; Preston et al. 2009). There were no instances of incidence concordance or conflicting evidence. The two papers that supported an increase in ROS leading to mutations provided empirical evidence of temporal concordance (Jacobsen et al. 2008; Shih et al. 2021),while only one (Jacobsen et al. 2008) supported dose/concentration. Finally, one paper that supported an increase in ROS leading to chromosomal aberrations showed temporal concordance (Shih et al. 2021).

2.3.3 Specific Literature Search Results

The specific literature searches resulted in a total of 307 retrieved articles (Figure 5). 100 articles were found to be duplicates across the searches. After title and abstract screening, 27 articles were found have measured ROS and oxidative DNA damage and were eligible according to the modified Population, Exposure, Comparator and Outcome (PECO) statement (Table 1). After full text screening

and data extraction, 12 articles were found to have support for the quantitative relationship between ROS and oxidative DNA damage (Table S7). The only searched method for oxidative DNA damage measurement that did not return a paper in the final data extraction was LCMS. Out of the 12 collected articles: four measured oxidative DNA damage with the Fpg-modified comet assay, four measured oxidative DNA damage with an ELISA and four measured oxidative DNA damage with HPLC. The methods for ROS measurement were varied, three articles measured ROS with the oxidation of homovanillic acid, two articles measured ROS with HPLC, and one article measured ROS with each of the following methods: MitoSox fluorescence assay, chemiluminescence, glutathione-S transferase (GST) activity, mitochondrial matrix localized oxidant-sensitive ratiometric probe (mito-Ro-GFP), luminol-amplified chemiluminescence (LAC), HPLC and glutathione peroxidase (GPx) activity.

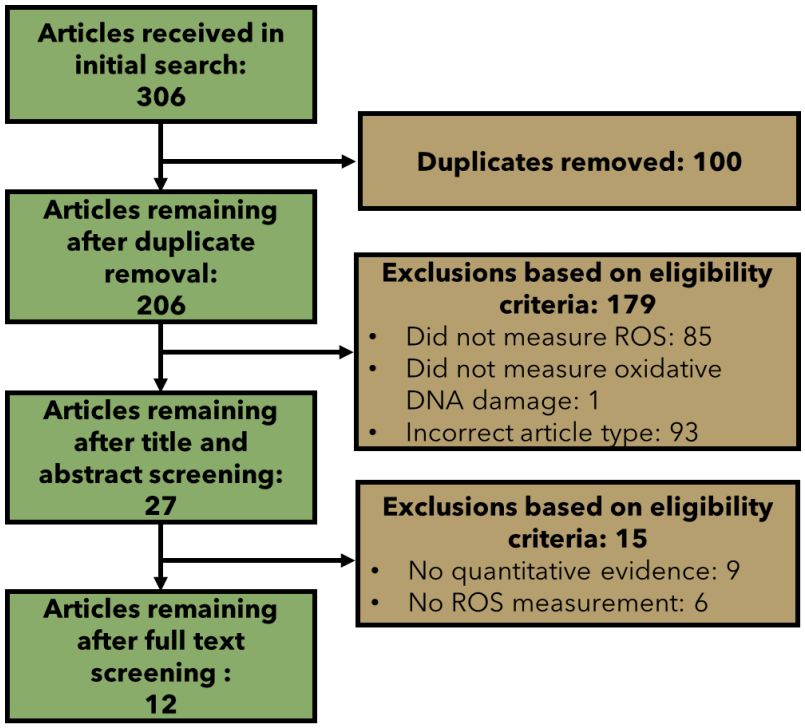

Figure 2.5. Flow chart showing the number of articles included and excluded at each step of the specific literature search.

2.4 Discussion

Our case study explored the integration of a single KE into an existing AOP in a data-rich area. An initial challenge encountered was the existence of multiple KEs in the AOP-Wiki related to increases in cellular ROS that contained similar information. As KEs are purposefully modular to readily enable the development of AOP networks, this represents a significant challenge. Rather than choosing or developing the appropriate KEs, we decided to focus instead on KER development because a parallel activity to address redundancy in KEs is already underway within the OECD. Given that we identified five KER that would be useful to bridge increases in ROS into AOP #296, we invested efforts in identifying an efficient, transparent, and reproducible search strategy to collect relevant empirical evidence to support these KERs.

Our initial broad search retrieved over 200,000 articles, emphasizing the scale of research already conducted in this area and the extent of biological knowledge. It was clearly not feasible to screen and review these studies. Nevertheless, this initial broad search suggested that a SR may be beyond scope for our purposes (even narrowing by prototypical stressors), as suggested for data-rich AOPs by Svingen et al. (2021). Literature review tools were then used to retrieve and review the top 100 most 'relevant' articles meeting stringent B-H criteria. This provided the basis to begin to build a small database for this data-rich area that could be used to support the KERs and identify those with the most data even within this small pilot study. Indeed, from the 39 articles that made it through the screening process, our evidence map suggests there is extensive empirical evidence (35 articles; 90% of articles retrieved) for the relationship between an increase in ROS leading to DNA strand breaks. This also suggests that this is likely one of the strongest KERs supporting the overall weight of evidence that an increase in ROS can lead to mutations and chromosomal aberrations. However, while empirical evidence aligned with the B-H criteria was identified within these top 39 articles, there were limited data to develop a quantitative understanding linking the extent to which ROS levels increase prior to the manifestation of downstream

KEs. This is a typical challenge identified by AOP authors, where quantitative understanding is generally one of the main knowledge/data gaps.

The KERs describing an increase in ROS leading to oxidative DNA damage (the adjacent KER) and inadequate repair (non-adjacent KER) were also insufficiently supported based on this preliminary screen. However, detailed literature reviews exist that link these events, providing strong biological plausibility of the relationships between ROS, oxidative DNA damage, and inadequate repair relationships. Thus, an extensive database of empirical evidence for these KERs is likely not necessary, as biological plausibility is considered the most important evidence criterion and is given a very strong weight (OECD, 2018).

Unfortunately, we did not find strong data to support the quantitative relationships of the KERs within the 39 papers retrieved. There was some evidence of a quantitative dose or temporal response for all of the KERs, which could be developed into a quantitative understanding (e.g., linking ROS to oxidative DNA damage and ROS to DNA strand breaks). However, the amount of ROS measured in these papers was reported in different ways: relative fluorescence units (RFUs), units/hour, percent positive cells, and percent ROS generation of control. This results in the inability to translate the amount of ROS in one study to the amount of ROS in another study. Critically for this area, the role of endogenously produced ROS varies between cell types, cell culture media, and cellular environments (Milkovic et al., 2019); therefore, the ability to scavenge ROS will vary depending on the protocol and the cell type (Ray et al., 2012). Also, there was very little overlap in the cells or cell lines that were used, limiting the comparison of ROS production and scavenging ability between cell types. Most of the collected papers (30/39) used dichlorofluorescein diacetate (DCFH-DA) fluorescence to measure ROS. However, DCFH-DA cannot be reliably used to measure intracellular H_2O_2, as it tends to autofluoresce and to leak out of cells when deacetylated by endogenous deacetylases (Kalyanaraman et al., 2012). Since DCFH-DA is not suitable for the quantification of H_2O_2 or ROS molecules, and there are other more reliable fluorescent

dyes for the quantification of ROS, the majority of the collected papers were not suitable for the purposes of developing quantitative understanding of the KERs.

Our preliminary mapping of the literature suggests that priority KERs for future literature searches or research would be those linking cellular ROS to chromosomal aberrations and mutations. The literature search found the least amount of evidence for these KERs; within the 39 papers, two provided evidence for ROS leading to mutations and one paper provided evidence for ROS leading to chromosomal aberrations. A secondary and tertiary screen could be implemented to review these gap areas if deemed necessary. For example, a tiered approach could be used where search terms are successively eliminated to focus in on the areas requiring further data (i.e., subsequent searches to focus exclusively on papers that measure mutations and chromosomal aberrations).

Finally, because we deemed the first adjacent KER to be the most important, and identified the need for quantitative evidence, we conducted specific searches to fill this gap. To do this we decided to do a set of 36 searches pairing each of what we determined were suitable methodologies to derive quantitative measures of the two KEs. These searches retrieved 12 articles following screening that measured a quantitative relationship between cellular ROS and oxidative DNA damage. The specific literature search was able to capture more empirical evidence as well as evidence of a quantitative understanding for the prioritized KER. The 12 papers retrieved provide evidence of the timing between events and the relationship between incidence/magnitude of increased ROS and oxidative DNA damage in different model systems. This evidence could be used in future studies to build predictive relationships

2.4.1 Conclusions and Recommendations

As the OECD's AOP program matures, an abundance of new AOPs are in development. A significant challenge is ensuring the re-use of existing KEs and KERs within the AOP-Wiki to facilitate both AOP development by reducing work and the creation of AOP-networks that provide a more thorough understanding of toxicological effects. Indeed, our initial challenge in this project was the existence of multiple KEs related to ROS and oxidative stress in the AOP-Wiki (16 KEs relating to ROS and oxidative stress that are included in 60 AOPs). We encourage authors to carefully review existing KE(R)s before creating new ones and for authors to remove KE(R)s from the AOP-Wiki that they have created but do not intend to develop.

A second challenge in AOP development is the need for more robust and transparent evidence collection to foster reproducibility and support the overall weight of evidence in evaluating AOPs. We explored concepts and tools in SR for this purpose to refine our search strategies. As non-experts, we found this approach to be highly useful and encourage further use in AOP development, as it provides a more transparent weight of evidence evaluation than the traditional narrative approach and more readily enables identification of inconsistencies in the literature. Our recommendations based on our experiences herein are as follows:

1) For data-rich areas, we propose that one approach might be a tiered literature review to produce evidence maps wherein the initial screen involves selecting a sub-set of the top papers (e.g., the most relevant), with search terms successively narrowed until sufficient evidence (as deemed by the team of authors) is collected. In the future, machine learning tools may also be deployed to facilitate use of SR tools for more efficient and documented literature searches in AOP development. Such tools already exist but were beyond the scope of our present work (e.g., Jornoud et al., 2021).

2) In addition, filtering for specific, reliable assays that produce quantitative data could provide an alternative strategy to both reduce the collected articles and improve the likelihood of developing a quantitative understanding with the collected articles.

3) Other strategies to narrow searches to retrieve the most relevant articles that were not applied in this study and would be useful include filtering for specific well-studied stressors or for specific well-studied models to narrow searches.

A third challenge is the extensive amount of time required to develop AOPs. Our work demonstrates that even the development of a single (or a small selection) of KERs can require a significant amount of work. Thus, the research herein supports that providing the option of having the KER as a module for development and review is logical step forward as the AOP program evolves. Allowing KER review, paired with developing more efficient and systematic approaches to evidence collection and review, should streamline AOP development and increase confidence in their application.

Overall, we found that the application of SR review concepts and tools to undertake more transparent and documented literature search methods was an improvement over narrative approaches in KER development. It will be important for the community of experts to recommend best practices that balance the strengths of SR tools in AOP development against the depth of analysis needed, in particular for data-rich areas where there is extensive biological understanding. In anticipation of achieving that broader consensus, we encourage dialogue between experts in SR tools and AOP developers to advance this area.

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Chapter 3: Developing Quantitative Key Event Relationships (KERs) between Oxidative DNA damage, DNA Strand Breaks, Mutations and Chromosomal Aberrations.

3.1 Abstract

Adverse outcome pathways (AOPs) provide a framework to organize and weigh the evidence linking a toxicant's initial interactions with molecules in the cell to adverse outcomes of regulatory concern. Quantitative understanding is critical for the development of predictive toxicological models. The objective of the present study was to investigate the ability to define the quantitative associations of the KERs in AOP #296 through the measurement of oxidative DNA damage, DNA strand breaks, chromosomal aberrations, and mutations in TK6 cells after exposure to a range of concentrations of 4-Nitroquinoline 1-oxide (4NQO). An increase in both oxidative DNA damage and DNA strand breaks was observed after 2, 4, and 6 h exposures with the high throughput comet assay (CometChip). Oxidative DNA damage was observed at a lower concentration than DNA strand breaks. As measured with the flow cytometry micronucleus assay, an increase in the incidence of micronuclei was observed after a 24 h exposure to a low concentration of 4NQO, while high cytotoxicity was found at higher concentrations. Micronuclei were observed at the same concentration and at a later timepoint (24 h) than oxidative DNA damage. Similarly, an increase in mutation frequency at 48 h was observed using Duplex Sequencing, an error-corrected Next Generation Sequencing technology. An increase in the proportion of C>A transversions was observed, consistent with the expected substitutions induced by oxidative DNA lesions. These findings contribute to the quantitative understanding of AOP #296 and this study serves as a key example of AOP-informed study design and highlights notable challenges in characterizing quantitative relationships. Moreover, our research advances the application of Duplex Sequencing as a new approach to the measurement of chemically induced mutagenesis for genetic toxicology.

3.2 Introduction

Adverse outcome pathways (AOPs) provide a framework to organize and weigh the evidence linking a toxicant's initial interactions with molecules in the cell to adverse outcomes of regulatory concern (Ankley et al. 2010; OECD 2018). AOPs synthesize toxicological information from various sources into an organized summary that includes a flow chart of the pathway and narrative descriptions of the available methodologies and supporting evidence (Becker et al. 2015). AOPs are built to be stressor agnostic; the broader goal is increasing the utility of mechanistic data in predicting mode of action or hazard for use in various regulatory applications, informing knowledge and testing gaps, and developing modern test paradigms that effectively harness *in vitro* data (OECD 2018; Sakuratani et al., 2018).

AOPs are described by a series of measurable and essential key events (KEs), with specialized KEs at the start and end of the pathway (Perkins et al. 2011):

(1) The molecular initiating event (MIE), which is the initial interaction between a chemical and a biomolecule.

(2) The adverse outcome (AO), which is an endpoint of regulatory concern.

Connecting each of the KEs are key event relationships (KERs); these describe the causal relationship between two KEs and can be adjacent or non-adjacent. The weight of evidence supporting the KERs, and the pathway as a whole, is evaluated using modified Bradford-Hill (B-H) criteria: biological plausibility, essentiality of the KE, empirical evidence, uncertainties and inconsistencies, and quantitative understanding (Becker et al. 2015; Meek et al. 2014). Quantitative understanding is critical for the development of predictive toxicological models. In AOP descriptions, the evidence base supporting each B-H criterion is evaluated to indicate a KER's weight of evidence as strong, moderate, or weak.

A variety of AOPs have been developed in the area of genetic toxicology to promote the use of new test methods (Yauk et al., 2015, https://aopwiki.org/aops/15; Marchetti et al., 2016,

https://aopwiki.org/aops/106; Chauhan et al. 2021, https://aopwiki.org/aops/272). The present study focuses on increasing the empirical evidence for AOP #296: "Oxidative damage leading to chromosomal aberrations and mutations" (Cho et al., 2022; https://aopwiki.org/aops/296) (Figure 1). This AOP begins with an increase in oxidative damage to DNA (MIE) that overwhelms the repair capacity leading to inadequate repair of the damage (KE1a). Inadequate repair of the oxidative DNA damage (KE1a) then branches into two paths. It can directly lead to an increase in mutations (AO1) arising from replication of damaged template DNA, and to an increase in DNA strand breaks (KE2), occurring from the DNA repair process removing oxidative lesions. Inadequate repair of DNA strand breaks (KE1b) can lead to an increase in chromosomal aberrations (AO2) or mutations (AO1) downstream (Cho et al. 2022).

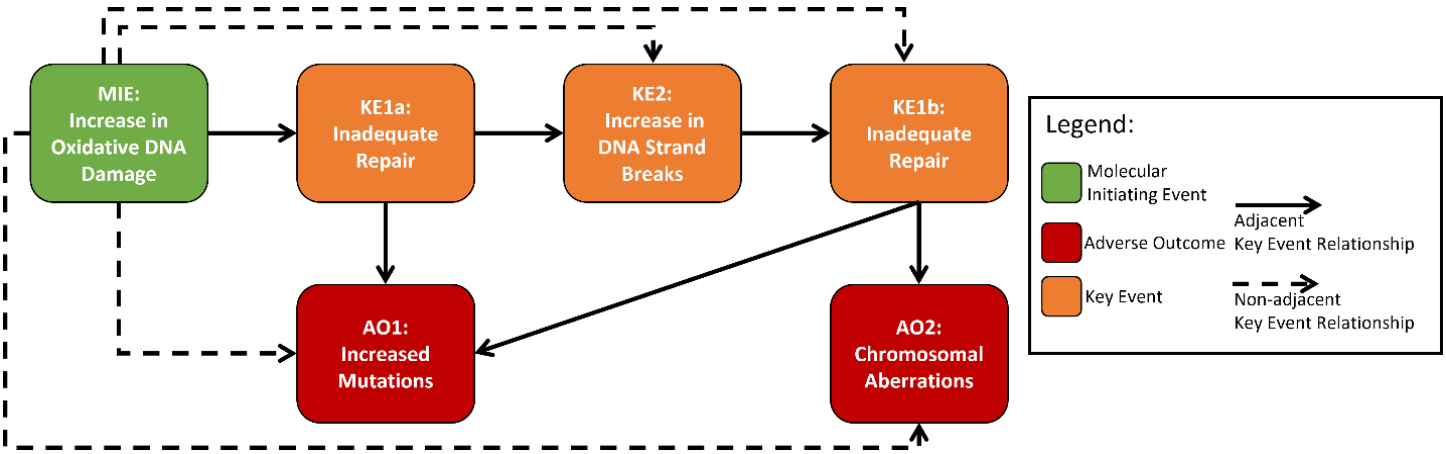


Figure 3.1. Flow diagram of the adverse outcome pathway (AOP) "Oxidative DNA damage leading to mutations and chromosomal aberrations" (modified from Cho et al. 2022).

There is strong biological plausibility and moderate empirical evidence in support of the KERs in AOP #296, but quantitative understanding of all the (adjacent and non-adjacent) KERs is low (Cho et al. 2022). This means that although the pathway is highly biologically plausible, and there is evidence to support that the earlier KEs occur at earlier timepoints and lower doses than later KEs, there is a lack of evidence showing what level of oxidative DNA damage must occur before there is progression to the

later KEs (inadequate repair and DNA strand breaks) or AOs (chromosomal aberrations and mutations). This is primarily due to a lack of studies that use multiple stressor concentrations and time points within a single experimental design (i.e., to establish dose, incidence, and temporal concordance within a model system), which is required to build quantitative understanding. Extrapolation across model systems and experiments is difficult - endogenous levels of reactive oxygen species (ROS) vary between cell lines and cell types and thus so does the level of oxidative DNA damage that a cell can incur before repair capacity is overwhelmed (Milkovic et al. 2019; Ray et al., 2012). This lack of quantitative understanding is a gap in many AOPs that limits utility and development of predictive models. Strengthening the base of quantitative evidence for AOP #296 would allow for better use of oxidative DNA damage and DNA strand break measurements to predict the occurrence of chromosomal aberrations and mutations.

The objective of the present study was to define the quantitative associations of the KERs in AOP #296 through the measurement of oxidative DNA damage, DNA strand breaks, chromosomal aberrations, and mutations in TK6 cells after exposure to a range of concentrations of 4-Nitroquinoline 1-oxide (4NQO). 4NQO was chosen as the prototype stressor as it is well established to cause oxidative DNA damage and chromosomal aberrations *via* the formation of ROS (Arima et al. 2006; Brüsehafer et al. 2016). 4NQO is thought to contribute to an increase in cellular reactive oxygen species (ROS) through the depletion of glutathione (GSH) via 4NQO-GSH conjugation (Stanley and Benson 1988). GSH directly scavenges oxidants and is important for maintaining appropriate cellular levels of ROS (Pizzorono 2014). This increase in cellular ROS is associated with an increase in the oxidative DNA lesion 8-oxo-2'-deoxyguanosine (8-oxodG) (Arima et al. 2006).

To accomplish our objectives, we took advantage of two of the most modern, quantitative *in vitro* genetic toxicology assays available: the high-throughput comet assay (CometChip) (Ge et al. 2014; Sykora et al. 2018) and flow cytometry micronucleus assay (Avlasevich et al. 2006; Bryce et al. 2010). In

addition, we used an innovative error-corrected sequencing technology known as Duplex Sequencing (DS) to quantify and characterize mutations (Kennedy et al. 2014; Salk and Kennedy 2020; Schmitt et al. 2012). By individually barcoding each strand of each double stranded DNA molecule prior to sequencing, DS readily allows the identification and elimination of sequencing artifacts, providing an unprecedented level of accuracy for mutation analysis across endogenous loci in the genome (Kennedy et al. 2014). This is the first application of DS in profiling mutation frequencies and spectra following exposure to an agent that causes oxidative DNA damage. Thus, this study also provides proof of concept and validation data for the use of DS for *in vitro* mutagenicity assessment of ROS-producing agents.

3.3 Methods

3.3.1 Cell Culture and Chemicals

TK6 human lymphoblastoid cells were obtained from the American Type Culture Collection (ATCC# CRL-8015; ATCC, Manassas, VA). Cells were cultured and maintained at $1x10^5$ to $1x10^6$ cells/mL in RPMI 1640 medium supplemented with 10% heat inactivated horse serum, 2 mM of L-glutamine and 1 mM of sodium pyruvate, at 37°C and 5% CO2. 4-Nitroquinoline-N-oxide, ≥98% purity, (CAS No: 56-57-5), purchased from Sigma-Aldrich, was dissolved, and diluted in dimethylsulfoxide (DMSO) (1% v/v), which served as the vehicle control. The concentration range selected in this study was initially based on similar experiments using 4NQO in the literature (Brüsehafer et al. 2016).

3.3.2 High-Throughput Single-Cell Gel Electrophoresis

High-Throughput Single-Cell Gel Electrophoresis, i.e., the CometChip assay, was conducted using the Trevigen CometChip 96-well system (catalogue number: 4260-096-CS, Trevigen, Maryland, United States) with and without the Formamidopyrimidine DNA glycosylase (Fpg) enzyme modification to measure oxidative DNA damage and DNA strand breaks, respectively, as per the manufacturer's instructions. Methods are briefly described below. Cells were treated with a range of 4NQO concentrations (0.015625, 0.03125, 0.0625, 0.125, 0.25, 0.5, or 1 µg/mL) or an equivalent volume of DMSO (1% v/v). Each treatment was performed in biological and technical triplicate.

3.3.2.1 Preparation and Cell Loading

CometChips, which are glass plates covered with agarose gel that contain 30 µm in diameter single-cell microwells (catalogue number: 4260-096-01, Trevigen, Maryland, United States), were equilibrated in room temperature phosphate-buffered saline (PBS), then placed into the 96-well macrowell former. The cell suspension was filtered through a 40 µM cell strainer to achieve a single cell suspension and the cells were diluted to a concentration of 1.5×10^5 cells/mL. PBS was aspirated from

each well and 100 µL of the cell suspension was added to each well. The plate was covered and incubated at 37℃ for 15 minutes, rocked back and forth a few times, and then incubated at 37℃ for another 5 min.

3.3.2.2 Chemical Exposure and Fpg treatment

Remaining cells and culture media were aspirated from all wells, then 99 µL of media and 1 µL of treatment (to achieve the concentrations described above) were added to each well. Plates were incubated at 37℃ for 2, 4, or 6 hours. After the chemical exposure, excess media were aspirated and the surfaces of the CometChips were washed gently with PBS to remove excess, unloaded cells. A layer of molten low melting point (LMP) agarose was applied on the surface of the CometChip to cover the microwells loaded with cells. Chips were then submerged in lysis buffer (Trevigen catalogue number: 4250-050-01, Trevigen, Maryland, United States) and allowed to lyse for 1 h at 4℃ under light occlusion. Chips were exposed in sets of two, one treated with Fpg and the other not treated with Fpg, but both chips were equilibrated in the enzyme buffer to ensure equal conditions. Both chips were submerged in Fpg enzyme reaction buffer (HEPES 40 mM, KCl 0.1 M, EDTA, 0.5 mM KOH, Bovine serum albumin 0.2 mg/ml, pH 7.5) to equilibrate at room temperature for 15 minutes; this step was repeated twice using fresh buffer. The chips were then placed either in (a) Fpg enzyme reaction buffer with the Fpg enzyme (1:10,000 dilution, New England Biolabs, Ipswich, Massachusetts), or (b) in Fpg enzyme reaction buffer only. Chips were then incubated at 37℃ for 20 minutes. Chips were transferred to cold alkaline electrophoresis buffer (0.2 M NaOH, 0.2 M EDTA, 0.1% TritonX) to stop the enzyme reaction.

3.3.2.3 Electrophoresis

Chips were rinsed then submerged in cold alkaline electrophoresis buffer (0.2 M NaOH, 0.2 M EDTA, 0.1% TritonX) at 4℃ for 20 minutes under light occlusion; this step was repeated with fresh buffer. Then chips were secured in a CometAssay® electrophoresis chamber and covered with 700 mL alkaline electrophoresis buffer. Electrophoresis was performed at a constant voltage of 22 V and

variable current at 280 mA for 50 minutes at 4°C. The chips were then removed from the electrophoresis chamber and submerged in the first neutralization buffer (400 mM Tris, pH 7.4) for 15 min at 4°C under light occlusion; this step was repeated using fresh buffer, then chips were submerged in the second neutralization buffer (20mM Tris, pH 7.4) for 30 min at 4°C under light occlusion.

3.3.2.4 Imaging and Data Analysis

Prior to imaging, the chips were stained with 0.2X SYBR gold diluted in the second neutralization buffer (20mM Tris, pH 7.4) overnight. Then chips were placed in a clean one-well plate and imaged with the 5X objective of a Lecia DMi8 automated confocal fluorescence microscope (Leica Microsystems, Wetzlar, Germany). The resulting TIFF images were analyzed with Trevigen Comet Analysis software (Bio-Techne, Devens, MA, USA). This software scans each image, identifies analyzable comets, and determines the percentage of DNA in the comet tails by comparing the fluorescence intensity of the tail to the total fluorescence intensity of the comet. The median percent tail DNA of all the comets found in each well was calculated by the software.

3.4 Microflow Micronucleus Assay

Chromosomal aberrations were quantified using the MicroFlow micronucleus assay. The cells were exposed to a range of 4NQO concentrations (0.00781, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5, or 1 µg/mL) or an equivalent volume of DMSO (1% v/v). The MicroFlow micronucleus assay was performed after a 24 hour exposure. Each treatment was performed in quadruplicate. These experiments were performed at Integrated Laboratory Systems (ILS, now Inotiv, Indiana, United States).

Cells were treated in 12-well plates, at a cell suspension volume of 3.0 ± 0.1 mL and a density of 2.0 ± 0.25 x 10^5 cells/mL. Cells were exposed to DMSO or 4NQO for 24 hours at 37°C and 5% CO_2. After the 24 hour exposure, cells in control wells were counted to confirm that at least 1.5 cell cycles had occurred. Once cell counts were sufficient, cells were harvested, and flow cytometry analysis was

conducted following the Litron Labs *in vitro* MicroFlow™ kit instruction manual (Avlasevich et al., 2006). Briefly, cells were isolated then dyed with Nucleic Acid Dye A to identify dead or dying cells and incubated for 30 minutes under a light source. Cells were lysed with the MicroFlow™ kit's proprietary Complete Lysis Solution 1 and incubated for one hour in the dark at 37°C, then cells were lysed with the MicroFlow™ kit's Complete Lysis Solution 2 and incubated for 30 minutes in the dark at room temperature. Both Complete Lysis Solutions contain Nucleic Acid Dye B, to identify nuclei and micronuclei. Samples were stored in the dark at room temperature for 24 hours before the fluorescence of both nucleic acid dyes were read using a Becton-Dickinson FACSCantoII™ flow cytometer (BD Biosciences, San Jose, CA) using FACSDiva 8.0.1 software.

3.5 Error-Corrected Sequencing

Error-corrected DS was conducted using TwinStrand DS kits to measure the mutation frequency after a 24 hour exposure to 4NQO (0.006, 0.008 and 0.016 µg/mL) or an equivalent volume of DMSO (1% v/v). Each treatment was performed in duplicate. The 4NQO concentration range was chosen according to the relative survival observed in the micronucleus assay; the top concentration, 0.016 µg/mL, induced a 45% decline in relative survival at 24 hours and was selected to be aligned with the top concentration recommended in the Organisation for Economic Co-operation and Development (OECD) test guideline for micronucleus frequency induction (OECD Test Guideline 487; OECD 2016). The libraries were built using the TwinStrand DuplexSeq Human Mutagenesis™ kit that uses the TwinStrand v1.0 Human Mutagenesis panel (TwinStrand Biosciences, Washington, United States). The panel consists of 20 target sites that are 2,400 base pairs in length, for a total target region of 48 kb. The target sites are distributed across 20 chromosomes, spanning genic and intergenic regions, and are representative of the complete genome with respect to the GC-content and proportions of coding and non-coding regions (TwinStrand Biosciences). The target regions were chosen to exclude regions where mapping quality

could be compromised (highly repetitive elements or pseudogenes), and genes reported to have a significant role in cancer (based on the and Catalogue of Somatic Mutations in Cancer (COSMIC) database) or under positive selective pressure (Valentine et al. 2020).

3.5.1 Cell Pellet Preparation and Chemical Exposure

Cell pellets were prepared at Integrated Laboratory Systems (ILS, now Inotiv, Indiana, United States), before being shipped to Health Canada for DS Library preparation. Cells were treated in a 12-well plate, at a cell suspension volume of 3 ± 0.1 mL and a density of 2.0 ± 0.25 x 10^5 cells/mL. Cells were exposed to DMSO or 4NQO in duplicate for 24 hours at 37°C and 5% CO2. After the 24 hour exposure, cells in control wells were counted to confirm that at least 1.5 cell cycles had occurred. Then, cells were washed with 1X PBS pH 7.4, resuspended in fresh culture medium at a density of 2.0 ± 0.25 x 10^5 cells/mL, and returned to the same incubator for another 24 hours. After a total incubation period of 48 hours, cells were washed with 1X PBS, centrifuged, and PBS was removed. The remaining pellet was flash frozen in liquid nitrogen and stored at -80°C before being shipped to HC.

3.5.2 DNA Extraction and Library Preparation

DNA was extracted from the cell pellets using the Qiagen DNeasy blood and tissue kits (Catalogue number: 69504, Qiagen, Hilden, Germany) according to the Qiagen user manual. DNA concentration was measured using a Qubit™ 4 Fluorometer (Invitrogen, Waltham, MA, USA). DNA integrity was measured using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Inc., Santa Clara, USA). Samples were confirmed to have a DNA integrity number greater than 7.

Libraries were prepared using Twinstrand's Human Mutagenesis DS Kit according to the manual (Human Mutagenesis Kit Twinstrand Biosciences Inc., Washington, United States). Briefly, 500 ng of DNA per sample was fragmented enzymatically to about 300 base pairs (confirmed on an Agilent TapeStation). DNA was then treated with polymerases and nucleotides to repair the ends and add A-tails. Illumina adapters and unique molecular identifiers were ligated to the DNA. Samples were then

cleaned using paramagnetic beads coated with magnetite (iron) and carboxyl molecules, polyethylene glycol (PEG) and salt. A first round of PCR was run for 10 cycles to label the samples with unique indices. Samples were then cleaned and hybridized to the Human Mutagenesis panel probes. DNA was captured using iron beads containing streptavidin binding sites to bind to biotin on the mutagenesis panel probes. The second round of PCR contained probes for the target regions and was run for 14 cycles. Samples were cleaned, hybridized, and captured in the same process as followed the first round and PCR. Then a third round of PCR was run for 6-8 cycles. DNA concentration was measured using a Qubit™ 4 Fluorometer (Invitrogen, Waltham, MA, USA). DNA fragment size was measured using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Inc., Santa Clara, USA). Libraries were then pooled to a final concentration of 10 nM per sample, frozen, and sent to Psomagen (Maryland, United States) for sequencing.

3.5.3 Sequencing and Data Interpretation

Libraries were sequenced on a NovaSeq 6000 and the resulting data were uploaded to the DNAnexus platform (Valentine et al., 2020), as demultiplexed FASTQ files, and processed through the TwinStrand Biosciences DS Mutagenesis App™ (Version 3.18.0). The application processing methods contained in the application were described in detail previously (Valentine et al., 2020). Briefly, the application determines consensus between the duplex reads based on their unique molecular identifying tags, and removes duplicate observations and interspecies contamination. Read pairs were error-corrected and bases with low quality were masked as “N” for ambiguous base assignment, then duplex consensus reads were created. In order to eliminate biases from double counting bases in overlapping paired-end reads, the read pairs then went through balanced overlap hard clipping. Variants were called using VarDictJava with all parameters optimized (Zhongwu et al., 2015). The application pipeline produced a summary of sequencing quality metrics, mutation frequencies, mutation spectra, and trinucleotide spectra (Twinstrand Biosciences Inc., Washington, United States).

3.6 Statistical Analysis

Statistical analyses were conducted in the R environment for Statistical Computing (R Core Team 2017). Analysis of variance was conducted on the median % tail DNA using the aov() function. For the Comet assay analysis, the model consisted of main effects of dose, time point, Fpg with all the 2 way and 3 way interactions. The ANOVA Table was estimated using the anova() function. Pairwise comparisons were conducted using the doBy R package. The p-values from the hypothesis tests comparing the percent DNA in tail at each concentration to controls were adjusted for multiple testing using the Holm-Sidak correction. This multiple testing correction was applied within each time point and Fpg treatment independently. For the micronucleus analysis, generalized linear models were fit to the data using the glm() function assuming an over dispersed binomial error distribution. The overall dose effect was tested using the anova() function using the Likelihood ratio statistic. Pairwise comparisons were conducted using the doBy R package (Hojsgaard & Halekoh, 2021). The p-values from the hypothesis tests comparing the percent DNA in tail at each concentration to controls were adjusted for multiple testing using the Holm-Sidak correction. This multiple testing correction was applied within each time point.

3.6.3 Duplex Sequencing Trinucleotide Mutation Spectra and Catalogue of Somatic Mutations in Cancer (COSMIC) signature analysis

Cosine similarities between the trinucleotide mutational spectra and COSMIC signatures was evaluated in the R environment for Statistical Computing (R Core Team 2017) using the MutationalPatterns 3.8.0 package (Manders et al., 2021). Observed mutations at each concentration were pooled. The average of the vehicle control spectra was used as the control signature. The combination of the control and the COSMIC signatures that had the highest cosine similarity to the spectra observed after 4NQO exposure was determined using the fit_to_signatures_strict() function. The fit_to_signatures_strict() function also determined the number of mutations in each of the 4NQO exposure spectra that contributed to each signature in the mutation spectrum.

3.3 Results

3.3.1 Oxidative DNA damage and DNA strand breaks measured by the Comet Assay

The CometChip assay was performed after a 2, 4, or 6 hour exposure (n = 3) to seven concentrations (0.01565 µg/mL – 1 µg/mL) of 4NQO alongside concurrently exposed DMSO solvent controls. Cells were exposed in chips in duplicate: one chip was treated with the Fpg enzyme (+Fpg) to measure oxidative DNA damage; and the other chip was treated without the Fpg enzyme (-Fpg) to measure DNA single strand breaks.

We first examined the concentration and temporal responses within each of the +/-Fpg assays separately. There was a significant increase in oxidative DNA damage observed using the +Fpg modified CometChip compared to solvent controls starting from the lowest concentration of 4NQO at all three timepoints (Figure 3.2). Baseline level of damage (average of 10.0 ± 1.5% tail DNA) increased to a maximum response of 74.0 ± 11.1% tail DNA at the 2 hour 0.5 µg/mL 4NQO concentration. Levels of oxidative DNA damage following 4NQO at 2 hours were significantly higher than levels at 4 or 6 hours at the lowest three concentrations (0.0156 µg/mL – 0.0625 µg/mL 4NQO). There was no difference between the oxidative DNA damage at 4 and 6 hours at any concentration.

There was a significant increase in DNA strand breaks observed in the standard (-Fpg) CometChip compared to solvent control after treatment with 0.0625 µg/mL 4NQO at 2 and 4 hours, and with 0.125 µg/mL – 1 µg/mL 4NQO concentrations (i.e., from 3rd lowest concentration) at all three timepoints (Figure 3.2). Baseline levels of DNA strand breaks increased from an average of 11.0 ± 1.6 % tail DNA to a maximum of 50.0 ± 7.5 % tail DNA at the 2nd highest concentration at 2 hours, consistent with responses observed in the +Fpg CometChip. There were no differences between the DNA strand break levels between 2, 4 and 6 hour time points for any concentrations.

We then investigated the relationship between levels of DNA damage in the +/- Fpg assays at matched time points and concentrations. We confirmed a significantly higher incidence of oxidative DNA damage (i.e., +Fpg) than DNA strand breaks (i.e., -Fpg) at 2 hours across all concentrations (0.015625 µg/mL - 0.25 µg/mL 4NQO) except at the top two concentrations of 0.5 and 1 µg/mL (Figure 3.3A). The largest difference between % tail DNA was at 0.125 ug/mL, where there was a 2.2-fold increase from 28.9 ± 4.3 to 63.2 ± 9.4% tail DNA at 2 hours for –Fpg relative to +Fpg. In contrast, margins between median % tail DNA became narrower at the later time points (Figure 3.3B, C) and there was no significant difference in the incidence of oxidative DNA damage (i.e., +Fpg) and DNA strand breaks (i.e., -Fpg).

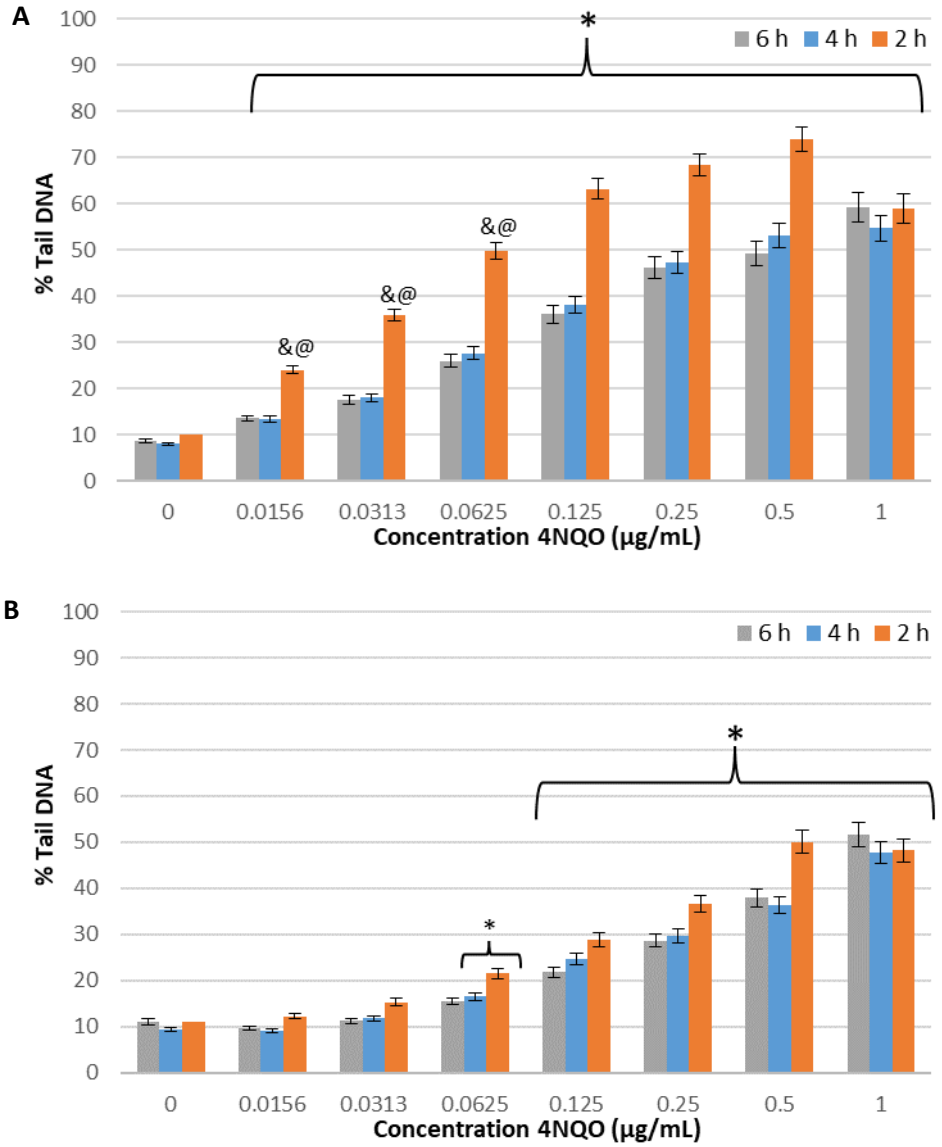


Figure 3.2. Percent DNA observed in comet tails after 2 (orange), 4 (blue), or 6 (grey) hour exposures to 4NQO with Fpg enzyme treatment (A) and without Fpg enzyme treatment (B). Each bar represents three biological replicates that consisted of three technical replicates. The error bars represent the standard error of the mean of the biological replicates. Statistically significant (adjusted $p < 0.05$) increases from the solvent is indicated by an *. Statistically significant (adjusted $p<0.05$) increases at 2 hours compared to 4 or 6 hours is indicated by a & or @, respectively.

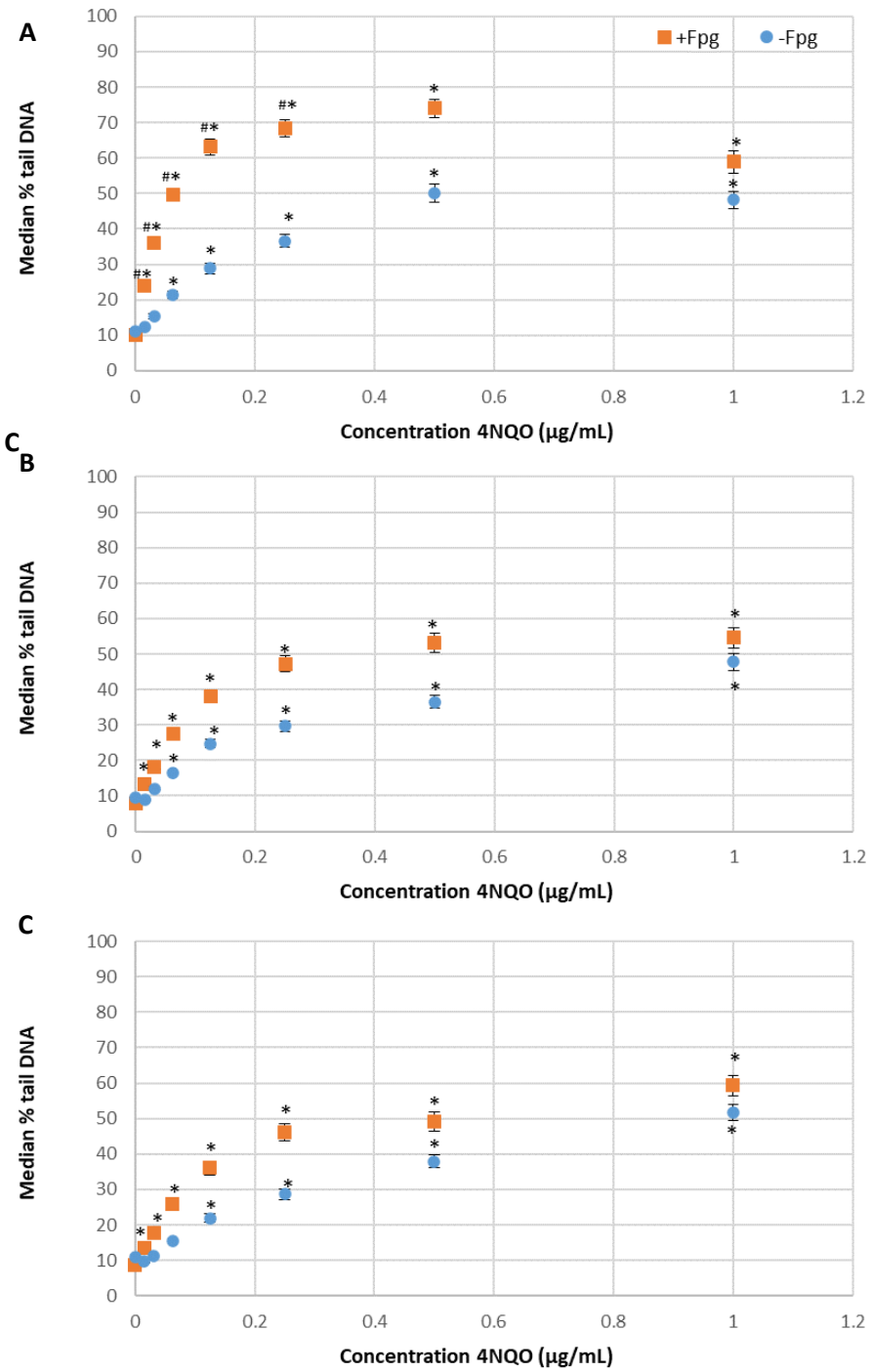


Figure 3.3. Comparison of oxidative DNA damage (+Fpg) and DNA strand breaks (-Fpg) after a 2 (A), 4 (B), or 6 (C) hour exposure to 4NQO. Each point represents three biological replicates that consisted of three technical replicates. The error bars represent the standard error of the mean of the biological replicates. Statistically significant (adjusted $p < 0.05$) increases from the solvent is indicated by an *. Statistically significant (adjusted $p < 0.05$) increases from +Fpg treatment as compared to the non-Fpg treatment at the same concentration is indicated by a #.

3.3.2 Micronucleus Frequency Analysis

The Microflow Micronucleus assay was performed to measure the percentage of micronuclei observed after a 24 hour exposure across an eight-concentration range (0.00781 µg/mL – 1 µg/mL) of 4NQO alongside the solvent control (DMSO) in triplicate. Note that lower concentrations were used in this analysis as the exposure duration was longer. There was a significant increase in the induction of micronuclei at all tested concentrations, except for the highest and lowest concentrations (Figure 3.4). However, there was a steep decline in the relative survival (RS) of the cells from 100% RS in the vehicle control to 83% at 0.00781 µg/mL 4NQO, 55% at 0.0156 µg/mL, 25% at 0.0313 µg/mL, to only 10% survival by 0.0625 µg/mL 4NQO. Therefore, the micronucleus frequency results may be confounded by cytotoxicity at concentrations above 0.0313 µg/mL. Consequently, only the results at the lowest two concentrations (0.00781 µg/mL and 0.0156 µg/mL) were considered in our analysis. The percentage of cells with micronuclei significantly increased from 0.331 ± 0.0003% in the solvent controls, to 3.6 ± 0.037% at 0.0156 µg/mL ($p < 0.05$).

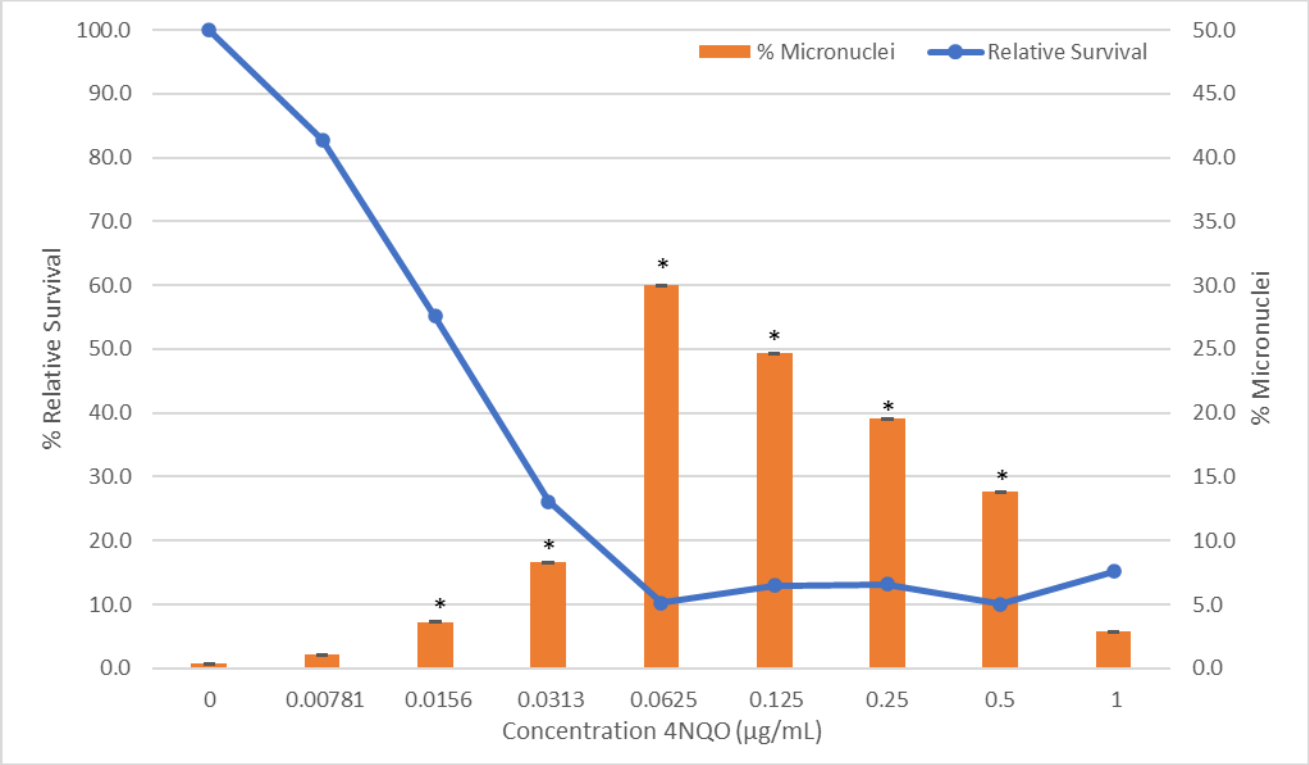


Figure 3.4. Percentage of micronuclei (orange bars) and relative survival (blue line) observed after a 24 hour exposure to 4NQO. Each orange bar represents the results of four technical replicates with error bars representing the standard error of the mean. Statistically significant (adjusted $p < 0.05$) increases from the solvent are indicated by an *.

3.3.3 Mutation Frequency and Spectrum Analysis by Duplex Sequencing

DS was performed to measure and characterize the mutations occurring after a 24 hour exposure to 4NQO (0.006 – 0.016 µg/mL) and a 24 hour recovery in fresh media. Note that lower concentrations were used in this analysis based on the results of relative survival observed in the micronucleus test. The average number of duplex bases sequenced per sample was ~ 1 billion, for a total of ~15 billion duplex bases across all samples. The sample with the fewest duplex bases sequenced was 0.75 billion, therefore all samples met a minimum target of 0.5 billion duplex bases. Mutation frequency was calculated by dividing the number of unique mutant bases by the total number of duplex bases sequenced. For the following analyses we focused on the mutation spectra and therefore only single nucleotide variants (SNVs) were counted.

The overall mutation frequency observed in controls was 2.2×10^{-7}; after exposure to 4NQO, the overall mutation frequency increased in a concentration-dependent manner (Figure 3.5A). All 4NQO concentrations induced a significant increase in mutation frequency compared to control, reaching a maximum of 3.1-fold above controls at the highest concentration (6.8×10^{-7}).

In addition to determining mutation frequency, the use of DS enables characterization of mutation spectrum. We thus also classified the mutations by base substitution type. C>T (39%) and C>A (28%) mutations were the most prevalent in TK6 cell controls. 4NQO treatment caused a significant increase in the proportion and frequency of C>A mutations by a 1.7 fold (48%) and 5.5 fold, respectively, at the highest concentration (Figure 3.5).

Mutation spectrum was also considered within the trinucleotide context. We did not observe notable differences in the trinucleotide spectra for each treatment (Figure 3.6); indeed, there was a high degree in concordance of the trinucleotide mutation spectrum across all of the concentrations, with only a minor difference of enrichment in C>A mutations compared to control spectra. We compared our trinucleotide frequencies to single-base substitution (SBS) signatures in the COSMIC Mutational Signatures database (version 3.3) using cosine similarity. SBS1 (spontaneous or enzymatic deamination of 5-methylcytosine to thymine), SBS6 (defective DNA mismatch repair), SBS87 (Thiopurine chemotherapy treatment) and SBS5 (clock-like signature) had similarities (a cosine similarity greater than 0.5) to our control spectrum. We then used our control signature as an additional SBS in a cosine similarity analysis of our 4NQO exposed samples against the COSMIC mutational signatures database. A concentration dependent increase in the number of mutations contributing to the control signature was observed in this analysis (Figure 3.7). In addition, after 4NQO exposure, SBS39 (unknown etiology) was enriched in the medium and high concentrations in a concentration dependent manner.

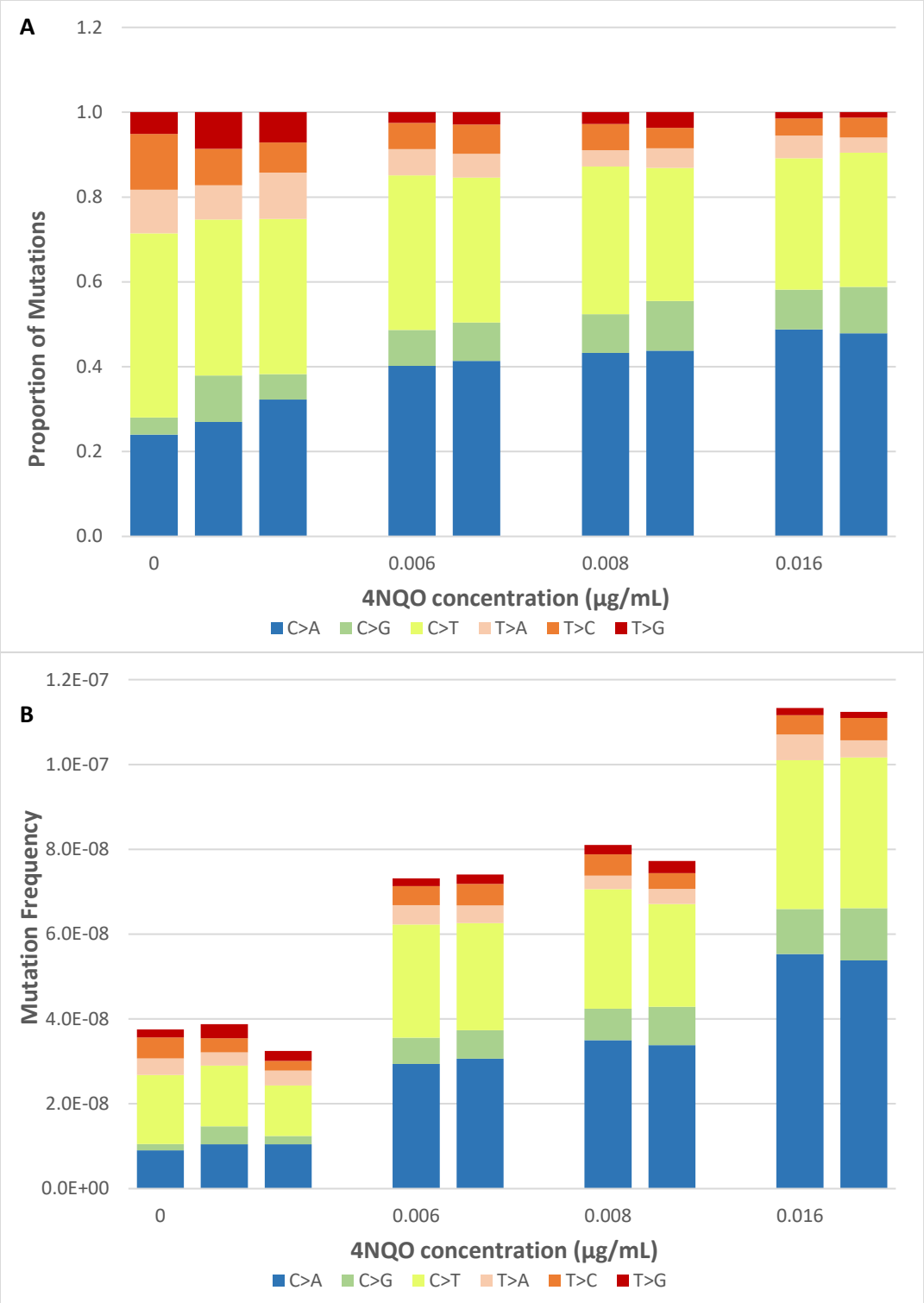


Figure 3.5. a) Mutation frequency per base pair and b) proportions of individual base substitutions in TK6 cells after exposure 4NQO or vehicle control (VC, DMSO) for 24 h. Cells were sampled 24 h after the conclusion of the exposure. Each bar represents a technical replicate.

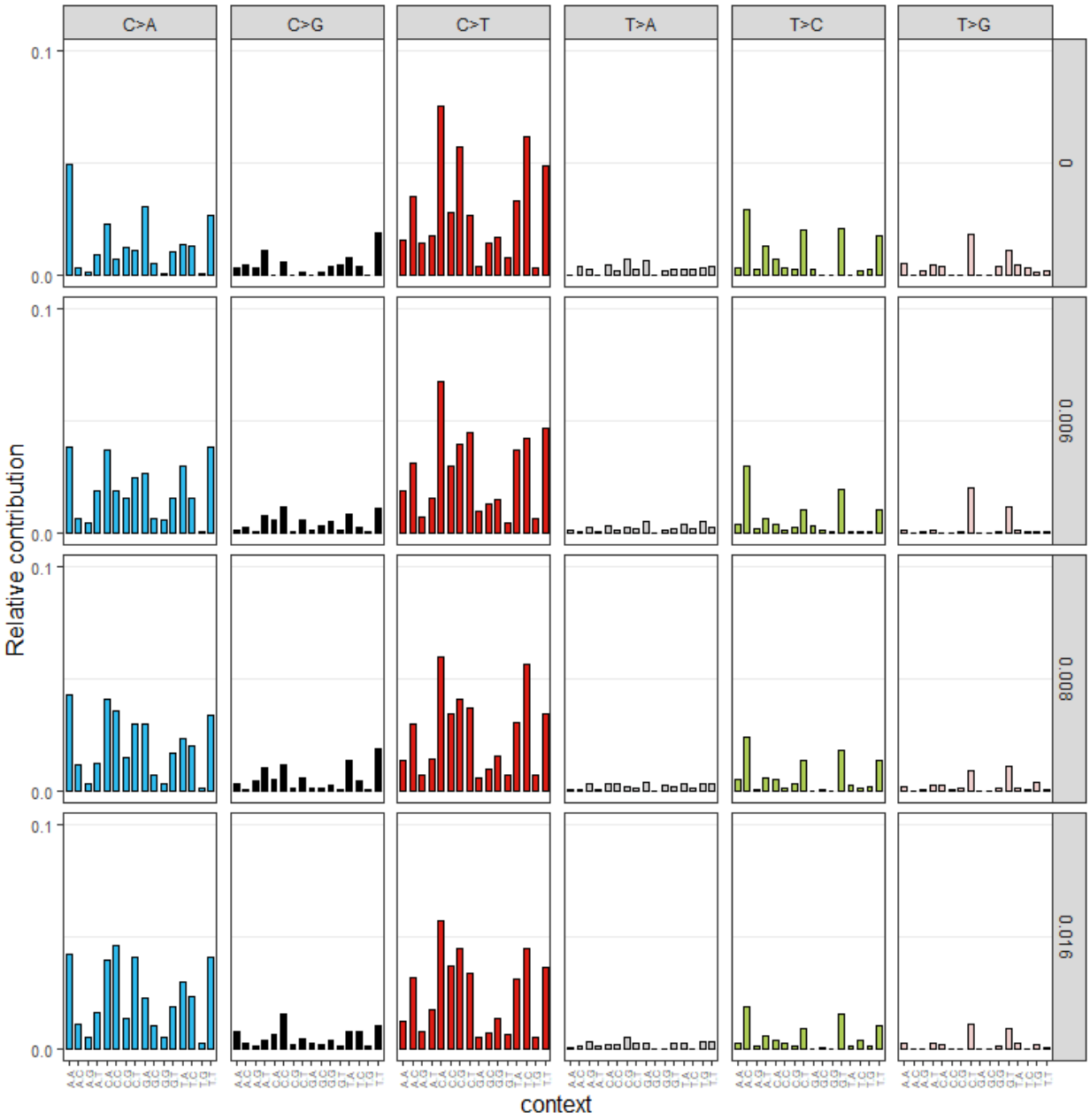
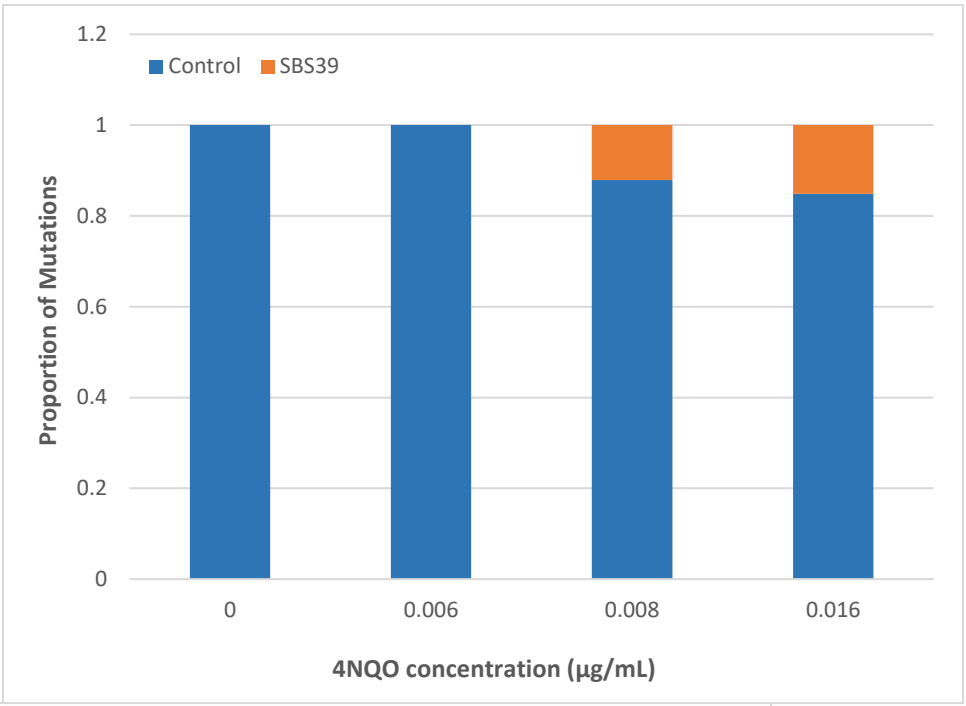


Figure 3.6. Trinucleotide spectra in TK6 cells after exposure to 4NQO or vehicle control for 24h. Cells were sampled 24 h after the conclusion of the exposure. Mutation frequencies were averaged for each exposure concentration. The substitution subtype is listed at the top, with the two flanking nucleotides shown along the bottom The Y axis indicates the proportion of each substitution type within the entire population of mutations recovered. Gray bars on the right indicate concentration in µg/mL.



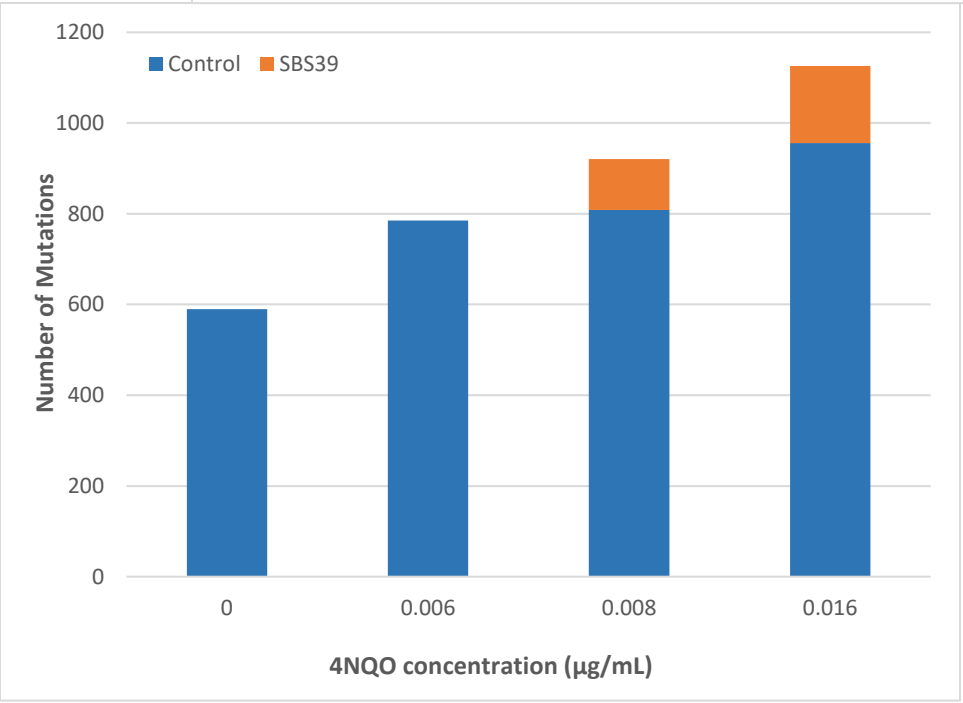


Figure 3.7. A) Proportion of mutations and b) Number of mutations in the observed trinucleotide signature contributing to the reconstructed signature.

3.4 Discussion

ROS are essential for cell signaling processes and are always present in the cell; therefore, oxidants are constantly being regulated by the cell's antioxidant response and endogenous antioxidants (Cadet et al., 2017). However, an imbalance in this process can lead to oxidative stress, which would trigger AOP #296 "Increase in oxidative DNA lesions leading to chromosomal aberrations and mutations." This AOP is critical in genetic toxicology as oxidative DNA damage is a primary mechanism of action associated with many xenobiotic exposures. Herein, we characterized quantitative relationships for KERs in AOP #296 and demonstrate use of an innovative sequencing technology to inform quantitative relationships associated with mutagenicity.

One fundamental challenge encountered by most AOP developers is the lack of suitable empirical data to support the qualitative and quantitative associations between KEs. The ideal study design for AOP development involves the use of a single model system that measures multiple KEs from the AOP in the same study to establish dose, temporal, and incidence concordance (OECD 2018) . Moreover, data informing the quantitative relationships to establish predictive toxicology models are lacking. We used the TK6 cell model to conduct a concentration-response and time-series analysis applying methodologies to measure four KEs in AOP #296 using 4NQO as the prototypical oxidative DNA damage-inducing agent. Our results provide strong qualitative data to support the KER studied. However, quantitative association between the MIE and KE2 with the AOs could not readily be established because of different sensitivities, dynamic ranges, and resolution of these methodologies. Nonetheless, the study serves as a key example of AOP-informed study design and highlights notable challenges in characterizing quantitative relationships. Moreover, our research advances the application of DS as a new approach to the measurement of chemically induced mutagenesis for genetic toxicology. A clear and robust concentration response was observed in mutation frequency by DS alongside the

expected changes in mutation spectrum induced by oxidative DNA damage, supporting its use in detecting this important mechanism of action genetic toxicology.

Using the high throughput Comet assay, we demonstrate that an increase in oxidative DNA damage occurred at lower concentrations of 4NQO than DNA strand breaks in TK6 cells at all exposure time points. This is consistent with the B-H criterion that early KEs should occur at lower equal concentrations to later KEs. Oxidative lesions were significantly increased from DNA strand breaks at 2 h, but not at 4 or 6 h (Table 3.1). The data suggest that most of the oxidative lesions had been repaired by 4 h and only DNA strand breaks remained. In addition, DNA strand breaks occurred early and were similar between 4 and 6 hours, suggesting DNA repair mechanisms may have been saturated (i.e., KE1b, inadequate repair). These data support that a portion of the DNA strand breaks observed are due to the presence of 8-oxodG lesions measured by the high throughput comet +Fpg. This is empirically consistent with the results of other studies comparing the Fpg modified comet assay and the standard comet assay. Azqueta et al. 2013 exposed TK6 cells for 4 hours to 0.0047 µg/mL 4NQO and found an increase in oxidative DNA damage, while at 0.038 µg/mL 4NQO they observed an increase in DNA strand breaks. Møller et al. 2018 exposed THP-1 cells for 3 hours to 0.038 µg/mL 4NQO and found an increase in oxidative DNA damage as well as an increase in DNA strand breaks. Together these results show that the use of the high throughput comet assay with and without Fpg provides empirical support for the KER between the MIE and KE2.

We next attempted to relate levels of oxidative DNA lesions and strand breaks to chromosomal aberrations measured using the micronucleus test. We noted that there was an increase in oxidative DNA damage and stand breaks at earlier timepoints than an increase in micronuclei, supporting the B-H criterion of temporal concordance of the KER. At the lowest concentration of 4NQO, a 2.4-fold increase in oxidative DNA damage and an 11-fold increase in micronuclei were observed. However, a strong quantitative association in incidence (a critical element of quantitative understanding) between these

events is difficult to establish because the unit of measurement for the two assays are disparate: oxidative DNA damage was measured using percent tail DNA that reflects the relative amount of DNA damage occurring in a single cell; whereas, the incidence of individual micronuclei containing pieces of chromosomes (assuming a clastogenic mechanism) are represented in the micronucleus assay. Another challenge in establishing a quantitative association are differences in the utilized study design and assay sensitivity/dynamic range. Specifically, cells were exposed continuously for 24 h to 4NQO for the purpose of the micronucleus assay. This was done because cell division is needed to produce micronuclei (TK6 cells have a 12-24 h doubling time). The more prolonged exposure may lead to overt cytotoxicity across a large portion of a higher concentrations used in the micronucleus assay. Alternatively, the high incidence of strand breaks is likely triggering cell death that is manifested at 24 hours. Overall, the discrepancies in assay detection limits make it very difficult to quantitatively compare the endpoints.

Table 3.1. Observed oxidative DNA damage, DNA strand breaks, micronuclei, and mutations summarized as fold change (FC)

Method	Comet +Fpg	Comet +Fpg	Comet +Fpg	Comet -Fpg	Comet -Fpg	Comet -Fpg	Micronucleus	DS
Key event (KE)	Oxidative DNA damage	Oxidative DNA damage	Oxidative DNA damage	DNA strand breaks	DNA strand breaks	DNA strand breaks	Chromosomal aberrations	Mutations
Time point	2 h	4 h	6 h	2 h	4 h	6 h	24 h	24 h + 24 h
FC compared to	DNA strand breaks	DNA strand breaks	DNA strand breaks	Control	Control	Control	Control	Control
Concentration (µg/mL)								
0.006	-	-	-	-	-	-	-	2.04
0.00781	-	-	-	-	-	-	3.14	2.20
0.01563	1.95	1.48	1.39	1.23	0.96	0.88	10.98	3.13
0.03125	2.35	1.52	1.56	1.54	1.26	1.02	25.18*	-
0.0625	2.32	1.67	1.68	2.16	1.76	1.40	90.52*	-
0.125	2.19	1.54	1.65	2.90	2.64	1.98	74.57*	-
0.25	1.87	1.59	1.61	3.67	3.17	2.59	58.90*	-
0.5	1.48	1.46	1.30	5.02	3.89	3.43	41.77*	-
1	1.22	1.14	1.15	4.84	5.11	4.68	8.57*	-

Significant ($p < 0.05$)

*Cytotoxicity > 55%

Investigation of oxidative DNA lesions, strand breaks, and chromosomal aberrations at different exposure and recovery time points would increase the quantitative understanding of their relationships and potentially address decreases in overt cytotoxicity occurring at 24 hours. Conducting the micronucleus analysis after shorter exposures, such as 6 h exposure and 18 h recovery to align with the 6 h exposure timepoint used for the oxidative DNA and DNA strand break analyses could more effectively enable comparison of these endpoints. Several previously published studies used similar timepoints in their study design. One such study showed that exposure of CCD-18Co cells to 1 µM 4NQO for 1 h resulted in an increase in DNA strand breaks; exposure for 2 h with a 24 h recovery resulted in an increase in the frequency of micronuclei (Tan et al. 2020). Another such study showed that exposure of HL-60 cells to 0.53 µM 4NQO for 1 h resulted in an increase in DNA strand breaks; the same exposure for 4 h with a 20 h recovery resulted in an increase in the frequency of micronuclei (Abraham et al. 2007). These two studies also showed that 4NQO induced an increase in ROS levels: Tan et al. (2020) found an increase in nitric oxide and Abraham et al. (2007) found an increase in total cellular ROS and a decrease in GSH levels. The results implicate ROS in the mechanism of DNA damage induced by 4NQO. Furthermore, Brüsehafer et al. (2016) found that longer recovery periods reduced the cytotoxicity of 4NQO measured by the micronucleus assay. At longer timepoints stalled cells would have the time to progress and at shorter timepoints these stalled cells would be counted as damaged cells, contributing to the observed cytotoxicity (Sobol et al. 2012). Later time points for oxidative DNA damage would likely not result in an increase in oxidative DNA damage or DNA strand breaks, as demonstrated by the decreasing trend from our results from 2 h to 4 and 6 h. Additionally, a similar exposure and recovery period (2 h exposure and 18 h recovery) for the comet analysis of T cells after exposure to 4NQO showed a significant decline in DNA strand breaks from a 2 h exposure to the 18 h recovery time point (Bausinger and Speit 2014). Future studies should investigate these types of DNA damage at other exposure time points to add weight of evidence and precision to the quantitative database.

DS revealed a concentration-dependent increase in mutation frequency in response to 4NQO, supporting its utility as a potentially powerful tool for mutagenicity assessment. Even the lowest tested concentration showed a 2-fold increase in mutation frequency from controls, suggesting high sensitivity. As with the micronucleus frequency analysis, applying the DS data to define the quantitative associations of the KERs was complicated by the different concentrations required in the assays. However, we note that a 2-fold increase in oxidative DNA damage at 0.01563 µg/mL 4NQO is associated with a 3-fold increase in mutation frequency. Additional studies designed to define these relationships more clearly should quantify per nucleotide levels of damage (e.g., by HPLC) rather than DNA damage levels per cell.

Contrary to the more conventional *hprt* and *tk* gene mutation assays, DS provides mutation spectrum data in parallel with mutation frequency. We found that the greatest change in the basic mutation spectra between the control and high-concentration 4NQO exposed samples was a 5-fold increase in C>A mutation frequency. This is an expected mutation as 8-oxodG and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPydG) are common oxidative lesions that can stably pair with adenine, causing a G:C to T:A transversions (J. Cadet and Wagner 2013). Work by Downes et al. (2014) found G>T(C>A) mutations to be among the most frequent after 4NQO exposure. G:C>T:A mutations were most frequent in Big Blue Rat2 cells (Ryu et al. 1999) and in 4NQO induced tumours (Ide et al. 2001). 4NQO is a known tobacco mimetic and signatures rich in C>A mutations are also associated with bulky adducts formed from tobacco smoking (Alexandrov et al. 2013) and related compounds (Kucab et al. 2019). Oxidative stress plays a role in smoking-associated cancers (Kanojia and Vaidya 2006; Miranda et al. 2011) and the presence of oxidative DNA damage. Thus, the basic mutation spectrum measured by DS is concordant with the work of previous studies and supports that oxidative DNA lesions are the drivers of the increase in mutation frequencies observed.

In addition to looking at a simple mutation spectrum, we investigated the mechanistic insight provided through analysis of trinucleotide mutation spectra. The COSMIC signatures associated with ROS producing agents are SBS36 and SBS18, neither of which had a high cosine similarity to the control or 4NQO exposed spectra. However, both SBS36 and SBS18 have predominantly C>A mutations, which were increased in all samples exposed to 4NQO. The pattern of C>A mutations is also consistent with another trinucleotide signature derived from human iPSCs exposed to a ROS producing agent (Kucab et al. 2019). Overall, our results support a mechanistic association between oxidative DNA lesions and mutations via the production of a characteristic mutation, C>A, observed by DS in our data set. Therefore, our findings are also concordant with other ROS producing agents, supporting that the mutations observed are due to oxidative DNA damage. Studies such as the one herein can produce novel signatures of mutagens that can be used to inform mutational spectra observed in humans, including in cancers, to advance understanding of the role of environmental exposures to agents such as ROS-producers in causing human disease. Future study designs should include another method of oxidative DNA damage analysis that measures a per nucleotide count of oxidative lesions, for example the measurement of 8-oxodG by High Pressure Liquid Chromatography (HPLC). These results would be more easily comparable to the mutation frequency measured by DS and would be the ideal experimental design to determine a quantitative relationship in support of the AOP.

Our data demonstrate the potential advantages of DS over conventional mutagenicity assessment techniques that rely on phenotypic selection-based mutations in reporter genes. The DS target panel was chosen to be representative of the complete genome, covering a broader spectrum of endogenous DNA versus conventional approaches that count cellular phenotypic changes that occur because of mutations in individual reporter genes. The DS approach is more efficient (can be done over the span of a couple of weeks) and yields detailed results about the frequency as well as the characteristics of the mutations that occurred as described below.

In conclusion, we used an AOP-informed study design in TK6 cells to examine the qualitative and quantitative relationships between oxidative DNA damage, strand breaks, micronuclei and mutations. Although our analysis was challenged by different resolutions and sensitivities of the assays, our results suggest that a 2-fold increase in +Fpg comets at 2 hours leads to a 3-fold increase in mutation frequency at 24 hours and a 10-fold increase in micronucleus frequency at 24 hours in TK6 cells. Alternative study designs and methodologies should be considered to clarify this quantitative relationship further. In addition to more quantitative analysis of oxidative lesions in DNA at the per nucleotide level, future studies should precisely match exposure duration/concentrations used to quantify relationships between oxidative DNA lesions, chromosomal aberrations, and mutations. Future work should also characterize the quantitative relationships between these endpoints using other oxidative stressors that cause an increase in cellular ROS through diverse mechanisms. The use of diverse cell-lines is also important to elucidate the quantitative relationship between oxidative DNA damage, DNA strand breaks, chromosomal aberrations, and mutations as extrapolation across model systems and experiments is not possible. Endogenous levels of ROS vary between cell lines and cell types, as does the level of oxidative DNA damage that a cell must incur before repair capacity is overwhelmed (Milkovic et al., 2019; Ray et al., 2012). Characterizing the quantitative response of DNA damage in multiple cell lines, will increase the predictive utility of the AOP. Predictive AOPs will help to create testing strategies that can identify the mode of action of chemicals, simplifying their prioritization and assessment. Finally, the study clearly demonstrates the potential for the utility of DS for mutagenicity assessment. We recommend extensive additional proof of concept studies to define optimal study designs and to validate this technology for this application.

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Chapter 4: Discussion

The AOP framework is a useful tool for organizing mechanistic data to evaluate stressor mode of action, develop test paradigms and predict potential *in vivo* effects. By informing test paradigms that make use of *in vitro* mechanistic information to predict potential *in vivo* effects from AOPs, this framework is important in advancing efforts to increase efficiencies and reduce animal use in the screening, prioritization, and assessment of chemicals. Quantitative understanding of an AOP is necessary for their use in the prediction of potential *in vivo* effects for use in regulatory applications. Quantitative understanding is a gap in many AOPs that limits potential development of predictive toxicological models (example: Villeneuve et al. 2018). My thesis focused on developing quantitative understanding of AOPs in the area of genetic toxicology to protect the population from exposure to stressors that can lead to genetic disease (Caldecott 2008), cancer, or accelerate the aging process (Hoeijmakers 2009).

The overall objective of this thesis was to collect quantitative evidence in support of AOP #296 "Oxidative DNA damage leading to chromosomal aberrations and mutations". Evidence was collected through literature review and experimental methods. The literature review gathered articles containing empirical evidence in support of KERs between an increase in cellular ROS and AOP#296. The experiments were designed to address the lack of data that was found in support of the KERs between an increase in cellular ROS and the two AOs, chromosomal aberrations, and mutations.

4.1 Fulfilment of Thesis Objectives

Chapter 2: Conduct a documented literature review to:

i. Investigate and demonstrate the use of a systematic review tool called Covidence and systematic evidence maps to expand on the existing AOP #296

A case study was presented and published in which Covidence was used to facilitate the documented collection of evidence to support KERs empirically and quantitatively in AOP #296. In addition, SEMs were created to summarize, visualize, and interpret the evidence acquired by the literature review. The SEMs showed that the results of the original broad search were concentrated on one KER and that the identified priority KER was lacking support from the original search. This finding provided the rationale for a second focused search that was conducted to collect evidence for the adjacent KER linking an increase in cellular ROS to an increase in oxidative DNA damage. An SEM was also created to illustrate that all but one of the collected articles contained temporal or dose/concentration concordance.

ii. Examine the supporting evidence with a focus on quantitative understanding, describing how increases in ROS can lead to chromosomal aberrations and mutations

The results of the broad search revealed ample qualitative evidence to support the KERs but a lack of quantitative evidence. Thus, in the second focused literature review, search terms were chosen such that only quantitative assays were used. This strategy was successful in producing examples of data from the literature that can be used to quantitatively define the relationship between increases in ROS and oxidative DNA damage.

Chapter 3: Measure oxidative DNA damage, DNA strand breaks, chromosomal aberrations, and mutations in TK6 cells exposed to a known ROS producing agent, using modern quantitative *in vitro* tests and use the resulting data to:

iii. Establish *in vitro* study designs and produce data to define the quantitative associations of the KERs in the existing AOP #296

The objectives were achieved to produce a single integrated experiment measuring multiple KEs to quantify the KERs qualitatively and quantitatively. For example, we found that a 2-fold increase in +Fpg comets (representing levels of oxidative DNA damage) at 2 hours is associated with a 3-fold increase in mutation frequency at 24 hours measured by DS and a 10-fold increase in micronucleus frequency at 24 hours measured by flow cytometry in TK6 cells. However, quantitative associations were difficult to define due to 4NQO cytotoxicity, differences in required assay timepoints, and assay sensitivity/dynamic range. For example, the high cytotoxicity observed at 24 hours resulted in the use lower concentrations in the assays measuring the AOs (mutations and chromosomal aberrations) than in the assay measuring the MIE (oxidative DNA damage). Nevertheless, the B-H criteria of temporal concordance were supported across all of the events in the order of the pathway. The +/-Fpg analysis also supports that DNA repair saturation occurs, as strand breaks continue to be retained at later time points once oxidative lesions have been repaired. Micronuclei were observed at the same concentration as oxidative DNA damage and lower concentrations than DNA strand breaks. However, the micronuclei were observed in samples that were exposed to 4NQO for much longer (24 h compared to 2, 4, or 6 h). The same observation can be made for mutations.

iv. Test the performance of an innovative error-corrected sequencing technology known as Duplex Sequencing

DS demonstrated a high level of sensitivity, as a significant change in overall mutation frequency was quantifiable with exposure to very low concentrations of 4NQO. DS also provided high content data that supported the pathway and mechanism of toxicity for 4NQO. The simple base substitution spectra showed an increase in C>A mutations (both proportion and frequency), which is the characteristic mutation observed in response to oxidative DNA damage.

4.2 Contribution to Scientific Knowledge

This thesis contributes to scientific knowledge in three important areas: (a) increasing understanding of the qualitative and quantitative association between increases in ROS, oxidative DNA damage, strand breaks, mutations and chromosomal aberrations; (b) demonstrating a pragmatic, systematic and transparent literature review approach that can be used in AOP development; and (c) demonstrating the utility of DS as a potential tool to transform mutagenicity assessment for regulatory toxicology.

My first study details an approach to transparent literature review for AOP development in a data-rich field or when SR methods are otherwise unsuited. Transparent literature review can provide AOPs with greater reproducibility, more efficient evidence collection, contribute to less biased evidence collection and provide more meaningful information on uncertainties and inconsistencies. The findings and recommendations from this study can be used as a reference when developing guidelines on how AOP authors should search the literature for evidence. The second study serves as an example of a study design to define quantitative relationships between endpoints in AOPs. The prediction of *in vivo* effects from the *in vitro* assays using AOPs is important for the future of chemical assessment. This study demonstrates the use of three modern, quantitative *in vitro* genetic toxicology assays aligned with AOP #296 and highlights challenges in quantitatively comparing the measured endpoints.

4.3 Future Directions/Recommendations

While conducting the literature review, three main challenges were encountered. The first was the existence of multiple ROS and oxidative stress related KEs. While a current effort is underway to consolidate the existing ROS and oxidative stress related KEs (Tanabe 2022), AOP authors should take care to review existing KE(R)s before creating new ones. The second challenge was how to incorporate the concepts of SR into a literature review in a data-rich area. In this study, we utilized a tiered approach to initially screen available data, then the search was narrowed to address the identified priorities. Some strategies that can be used to narrow a search are filtering for specific assays, stressors, or model organisms. The third challenge was the extensive amount of time that AOP development takes. Therefore, we recommend that KERs be recognized as a module for development of review in the AOP program.

To continue the work of literature review in AOP development there now exists a variety of SR tools available, including software tools that use machine learning to facilitate database creation, filtering, review, and prioritization. AOP-helpFinder is a webserver that searches the literature for results relevant to a specified AOP, KE(s) and stressors (Jornod et al. 2022). SWIFT-Review is an application that reduces the manual prioritization and categorization of a collected database (Howard et al. 2016). These more automated tools will encourage AOP developers to incorporate SR strategies, reduce the time needed to produce AOPs, find relevant evidence efficiently, and support a more transparent and unbiased approach to AOP development.

The main hub of the AOP knowledge base is contained in the AOP wiki website (aopwiki.org). The AOP wiki allows authors to create AOPs, KEs and KERs, and update them as necessary, supporting the AOPs as living documents and facilitating collaboration (OECD 2018). Next steps from this project are to summarize the data from both the literature review and experimental results in order to add them into the appropriate KERs in the AOP wiki. This will also include creating the new KER between an

increase in cellular ROS and an increase in oxidative DNA damage. Within the AOP wiki the evidence of the KERs between an increase in oxidative DNA damage, DNA strand breaks, chromosomal aberrations and mutations is high. However, the quantitative understanding of the KERs in AOP #296 is currently low, and incorporating the data gathered in this project will help to increase the weight of evidence for these quantitative relationships.

Future work in the AOP wiki could continue to develop the AOP network upstream from AOP #296, by exploring potential upstream events that cause an increase in cellular ROS. Potential MIEs and KEs leading to increased levels of ROS include electron transport chain inhibition (Hwan Han et al. 2008; Lin et al. 2012) and reduction of glutathione (Kocadal et al. 2020; Murata et al. 2001). However, the ways in which ROS are induced by chemical species are diverse and the chemical species that are encapsulated by the term ROS are also diverse (Kalyanaraman et al. 2012; Murphy et al. 2011), such that there are many KEs and KERs that could be studied. ROS are implicated in the progression and pathology of numerous diseases (Benzi and Moretti 1995; Kirkinezos and Moraes 2001; Sugamura and Keaney 2011) and their oxidative effects are not limited to the damaging of DNA. Therefore, the study and development of these KERs would be impactful to not only the field of genotoxicity, but to the study of human health as a whole.

In order to develop stronger quantitative understanding, future experiments should use study designs that measure mutations and chromosomal aberrations with shorter exposures, to match the exposures done for oxidative DNA damage and DNA strand breaks measurement, and longer recovery periods, as necessary for the occurrence of sufficient cell cycles. An additional advantage to testing longer recovery is that it allows stalled cells to progress and form micronuclei instead of being counted as damaged cells (Sobol et al. 2012). This is a possible reason for the observed overt cytotoxicity and aligns with the observation of lower 4NQO cytotoxicity with longer recovery periods by Brüsehafer et al., 2016. While recovery periods are necessary for the measurement of mutations and chromosomal

aberrations at shorter timepoints, recovery periods in the measurement of oxidative DNA and DNA strand breaks could be used to quantify DNA repair. In this study, the KE of inadequate DNA repair was not directly addressed; previous CometChip work has demonstrated the utility of the assay in conjunction with repair inhibitors or post-exposure recovery periods (Weingeist et al. 2013).

Besides the modifications to study design described above, other approaches could be taken to develop stronger quantitative relationships for oxidative DNA damage leading to mutations and chromosomal aberrations. One such approach is benchmark dose/concentration (BMD/BMC) modelling (Slob 2002). BMD/BMC modelling could be used to relate the endpoints regardless of the concentrations at which they are measured as it uses mathematical modelling of dose-response relationships to identify the dose at which a pre-determined change in response occurs. This approach uses numerous statistical models to fit dose/concentration-response data; the model that best fits the data is used to identify the BMC/BMD and upper and lower confidence intervals (Wills et al. 2016). The BMD/BMCs of each endpoint can be statistically compared and can be ranked from lowest (most potent) to highest (least potent) to determine a relationship between endpoint measurements (Slob 2002).

Another approach to quantitatively understanding AOPs is to model the KERs using statistical and mathematical models. AOPs that are developed this way are called quantitative AOPs or qAOPs (Conolly et al., 2017). Due to the large base of data that is needed to create a statistical model, multiple omics (transcriptomic, proteomic, and metabolomic) experiments are often needed. Some such models have been developed for cellular ROS levels. Hamon et al. (2014) modelled the pharmacokinetic activity of a nuclear factor (erythroid-derived 2) (Nrf2) pathway by integrating omic data derived from Nrf2 pathway related mRNA, proteins, and metabolites in renal proximal tubular epithelial cells (RPTECs). Another model was similarly developed by Hiemstra et al. (2022) using HepG2 cells. The resulting models can be used to predict cellular levels of ROS over time following exposure to a stressor. Further investigation of

omics data related to other KEs, such as DNA repair pathways, could provide a quantitative understanding of the relationship between oxidative stress and oxidative DNA damage as well as its repair.

Overall, my thesis contributes to the quantitative understanding of AOP #296 and serves as an example of AOP-informed study design. Notable challenges were highlighted in characterizing quantitative relationships both when developing these relationships from evidence gathered from the literature and evidence gathered through experimental methods. Further work is required to strengthen the quantitative relationships so that AOP #296 can be used in regulatory applications. Nevertheless, this project tested a transparent approach to literature review that can be leveraged by future AOP developers and demonstrated an application of genotoxicity assays in AOP-informed study design quantitative interpretation.

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5. Supplementary Materials

5.1 Chapter 2

Table S1. Empirical evidence of the adjacent key event relationship between an increase in cellular ROS and oxidative DNA damage.

Citation	Toxicant	Model	ROS assay	Oxidative DNA damage assay	Evidence Dose/concentration	Evidence Temporal
(De Iuliis et al. 2009)	Radio-frequency electromagnetic radiation (RF-EMR)	human spermatozoa (*in vitro*)	DHE and MitoSOX Red (MSR) fluorescence assays	8-OHdG ELISA	**ROS:** Increased after exposure to 1.8 GHz RF-EMR at specific absorption rate (SAR) 1.0 W/kg for 16 h. **Oxidative DNA damage:** Increased after exposure to 1.8 GHz RF-EMR at specific absorption rate (SAR) 2.8 W/kg for 16 h	
(Jacobsen et al. 2008)	C60	MutaMouse lung epithelial cells (FE1-MML)	DCFH-DA fluorescence assay	Comet assay + FPG	**ROS:** Increased after exposure to 2.78 ug/ml C60 for 3 h. **Oxidative DNA damage:** Increased after exposure to 100 ug/ml C60 for 3 h.	
	SWCNT				**ROS:** Increased after exposure to 2.78 ug/ml SWCNTs for 3 h. **Oxidative DNA damage:** Increased after exposure to 100 ug/ml SWCNTs for 3 h.	
(Mittal and Pandey 2014)	Cerium oxide nanoparticles (CeO_2 NPs)	A549 (lung adenocarcinoma) cells	DCFH-DA fluorescence assay and cellular glutathione content	FPG modified comet assay	**ROS:** Increased after exposure to 10 ug/ml CeO_2 NPs for 6 h. **Oxidative DNA damage:** Increased after exposure to 1 ug/ml CeO_2 NPs for 6 h. **(Conflicting)**	**ROS:** Increased after exposure to 10 ug/ml CeO_2 NPs for 3 h. **Oxidative DNA damage:** Increased after exposure to 1 ug/ml CeO_2 NPs for 6 h.
(Babbar and Casero 2006)	Tumor necrosis factor alpha (TNF-a)	BEAS-2B (Human bronchial epithelial) cells	DCFH-DA fluorescence assay	8-OHdG ELISA		**ROS:** Increased after exposure to 10 ng/ml TNF-a for 30 min. **Oxidative DNA damage:** Increased after exposure to 10 ng/ml TNF-a for 30 min.
		HBEC3KT cells				**ROS:** Increased after exposure to 10 ng/ml TNF-a for 3 h. **Oxidative DNA damage:** Increased after exposure to 10 ng/ml TNF-a for 3 h.

(Beattie et al. 2013)	Luteinizing hormone (LH)	MA-10 Ledig cells	DCFH-DA fluorescence assay	Comet assay + BSO (L-Buthionine-sulfoximine)		**ROS:** Increased after exposure to 100 ng/ml LH for 5 min. **Oxidative DNA damage:** Increased after exposure to 100 ng/ml LH for 2 h.

Table S2. Empirical evidence of the non-adjacent key event relationship between an increase in cellular ROS and DNA strand breaks.

Citation	Model	Toxicant	ROS assay	DNA strand break assay
(Pan et al. 2007)	HL-60 (human cancer)	t 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (5-OH-HxMF)	DCFH-DA DHE and CMFDA fluorescence assays	DNA fragmentation
(Joseph et al. 2014)	Normal human Keratinocytes (HaCaT)	Gamma radiation	DCFH-DA fluorescence assay	Alkaline Comet assay
(Gagnaire et al. 2020)	Zebrafish (Danio rerio)	Tritium, a radioactive isotope of hydrogen	ROS stimulation index	Alkaline Comet assay
(Alsharif and Hassoun 2004)	Mice Peritoneal lavage cells (TCDD sensitive female C57BL/6J mice)	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and Vitamin A or Vitamin E succinate	Reduction of cytochrome C assay	Alkaline elution technique
(Z. Wang et al. 2021)	Nitric acid donors nanocapsules (NO-NCPs)	pulsed laser irradiation	DCFH-DA and DHE fluorescence assays	Chromosomal fragmentation
(Gu et al. 2017)	Shrimp, *Litopenaeus vannamei* with and without LvPHB2 knockout	Vibrio alginolyticus infection	DCFH-DA fluorescence assay	Comet assay

(Russo et al. 2006)	Human spermatozoa	B(a)P	DCFH-DA fluorescence assay	Comet assay
(Jacobsen et al. 2008)	MutaMouse lung epithelial cells (FE1-MML)	Carbon black, C60 fullerenes or SWCNT	DCFH-DA fluorescence assay	Comet assay
(Beattie et al. 2013)	MA-10 Leydig cells	luteinizing hormone (LH)	DCFH-DA fluorescence assay	Comet assay
(Cai et al. 2007)	Bel-7402 cells (Liver Carcinoma)	$Cu(OP)_2$	DCFH-DA fluorescence assay	Comet assay
(Ren et al. 2016)	MCF-7 breast cancer cells.	Psoralidin (PSO)	DCFH-DA fluorescence assay	Comet assay
(Itziou et al. 2011)	Digestive Gland Cells and Haemocytes from land snails *Eobania vermiculata*	Heavy Metals cadmium, lead, or copper	DCFH-DA fluorescence assay	Comet assay
(Luukkonen et al. 2010)	SH-SY5Y neuroblastoma cells	FeCl2	DCFH-DA fluorescence assay	Comet assay (3 hours)
(Yin et al. 2019)	JB6 P+ (mouse epidermal cell line)	Arsenic and UVB radiation	DCFH-DA and DHE fluorescence assays	Comet assay (SSB), γ-H2AX immunofluorescent staining (DSB)
(Ni et al. 2014)	A549 (lung adenocarcinoma) cells	chrysophanol	DCFH-DA fluorescence assay	Comet assay and DAPI staining
(Gil et al. 2019)	A549 (lung adenocarcinoma) cells	2-Hydroxy-3',5,5'-trimethoxychalcone (named DK-139)	DCFH-DA fluorescence assay	Comet assay and γH2AX (12h, 24h)

(Zhang et al. 2015)	Caco-2 cells (differentiated and undifferentiated)	Aflatoxin B1 (AFB1) and aflatoxin M1 (AFM1	DCFH-DA fluorescence assay	CometChip
(Li et al. 2010)	Chondrocytes from juvenile rabbit joint	ofloxacin	DCFH-DA fluorescence assay	CometChip
(Chan et al. 2017)	BEAS-2B (Human bronchial epithelial) cells	house dust mites (HDM)	CellROX and MitoSOX fluorescence assays, Nitrite assay and XO activity assay	CometChip, gamma-H2AX
(Pagoria et al. 2005)	Supercoiled DNA	CQ and CQ-related photosensitizers benzil, bezophenone, and 9-fluorenone (9-F)	TEMPO-9-AC fluorescence	conversion of supercoiled (phi)X-174 RF I double stranded DNA to open circular and linear forms.
(E. Wang et al. 2017)	Human sperm	Silver nanoparticles	DCFH-DA fluorescence assay and standard ROS production kits	DNA fragmentation, sperm chromatin dispersion method
(Weyemi et al. 2012)	Human non-tumoral thyroid cell line (HThy-ori3.1)	doxycycline forced expression of H-Ras	DCFH-DA fluorescence assay	gamma-H2AX immunofluorescence and double strand break kinetics in agarose gel

(Froeling et al. 2019)	MiaPaCa2 Rosa26 cells	Napabucasin (2-acetylfuro-1,4-naphthoquinone or BBI-608)	DCFH-DA fluorescence assay and GSH/GSSG-Glo assay (Promega)	phospho-histone gamma-H2AX
(Renee L Flaherty et al. 2017)	Breast Cancer Cell lines. TNBC: MDA-MB-231 and HCC38, non-TNBC: MCF-7 and MCF10a (TNBC - Triple negative breast cancer)	Stress hormones cortisol and norepinephrine (psychological stress)	Electrochemical sensors	phosphorylated γ-H2AX, Comet assay
(Brar et al. 2012)	A549 (lung adenocarcinoma) cells	bleomycin	Electron paramagnetic resonance (EPR)	qPCR (mtDNA and nDNA measured separately)
(Mittal and Pandey 2014)	A549 (lung adenocarcinoma) cells	Cerium oxide nanoparticles (CeO_2 NPs)	DCFH-DA fluorescence assay, measurement of cellular GSH level	standard Comet assay
(B. Lee et al. 2020)	*Escherichia coli*	Lactoferricin B like peptide (LBLP)	DCFH-DA fluorescence assay	TUNEL assay
(Nie et al. 2019)	Porcine early embryos	Thiamethoxam (TMX)	DCFH-DA and MitoSOX fluorescence assay	TUNEL assay and γH2AX
(De Iuliis et al. 2009)	Human spermatozoa (*in vitro*)	Electromagnetic radiation/Mobile phone radiation 1.8 GHz	DHE and MitoSOX Red (MSR) fluorescence assays	TUNEL Assay for measuring apoptotic DNA fragmentation

(Chen et al. 2020)	Mouse oocytes	Ionomycin	ROS Orange Working Solution fluorescence assay	γH2AX and DAPI staining
(K. Lee et al. 2015)	NT-407 cell, U2OS cells and Huh7 cells	Cyclo(phenylalanine-proline) (cFP)	MitoSOX and DCFH-DA fluorescence assays	γH2AX and Neutral comet assay
(Samara et al. 2021)	Mantle cell lymphoma (MCL) mature B-cell lymphoid neoplasm cells (Jeko-1, REC-1 and Z138 cells)	Deferasirox (DFX)	DCFH-DA fluorescence assay	γH2AX western blot
(Greenshields et al. 2017)	Female C57BL/6 mice injected with ID8 murine ovarian cancer cells, HEY1 and HEY2 ovarian cancer cells	Artesunate (ART)	DCFH-DA fluorescence assay	γH2AX western blot
(Ren et al. 2015)	MCF-7 breast cancer cells	Cucurbitacin B (Cuc B)	DCFH-DA fluorescence assay	γH2AX western blot
(Toduka et al. 2012)	(CHO)-K1 cells	ZnO, CuO, Fe3O4, TiO_2, and Ag nanoparticles	DCFH-DA fluorescence assay	γH2AX

Table S3. Empirical evidence of the non-adjacent key event relationship between an increase in cellular ROS and inadequate repair.

Citation	Toxicant	Model	ROS assay	Inadequate Repair assay	Evidence: Dose/concentration	Evidence: Temporal
(Shih et al. 2021)	Arecoline	OEC-M1 cell line (oral squamous cell carcinoma)	Flow cytometer ROS Assay Kit 520 nm (Invitrogen, Cat. 88-5930)	Host cell reactivation assay		**ROS:** Increased after exposure to 100 ug/ml arecoline for 4 h. **Inadequate Repair:** reduced repair capacity was observed after exposure to 100 ug/ml arecoline for 24 h.
(Renee L Flaherty et al. 2017)	Norepinephrine	MDA-MB231 (triple negative breast cancer (TNBC)) cells	Electrochemical sensors used to detect specific ROS/RNS species (H_2O_2, NO_2, $ONOO^-$ and NO	Comet assay	**ROS:** H_2O_2 was increased after 1 uM norepinephrine after 15 min. **Inadequate repair:** DNA strand breaks occurred after exposure to 1 uM norepinephrine for 30 min, after a 20 min repair period the DNA strand breaks were repaired	**ROS:** $H2O_2$ was increased after 1 uM norepinephrine after 15 min. **Inadequate repair:** DNA strand breaks occurred after exposure to 1 uM norepinephrine for 30 min, after a 20 min repair period the DNA strand breaks were repaired
	Cortisol				**ROS:** NO_2 was increased after 1 uM cortisol after 15 min. **Inadequate repair:** DNA strand breaks occurred after exposure to 1 uM cortisol for 30 min, after a 20 min repair period the DNA strand breaks remained	**ROS:** NO_2 was increased after 1 uM cortisol after 15 min. **Inadequate repair:** DNA strand breaks occurred after exposure to 1 uM cortisol for 30 min, after a 20 min repair period the DNA strand breaks remained
(Preston et al. 2009)	Cisplatin	HEK 293 (Human embryonic kidney) cells	DCFH-DA fluorescence assay	8-OHdG ELISA	**ROS:** Increased after exposure to 30 uM cisplatin for 1 h. **Inadequate repair:** Oxidative DNA lesions were increased after exposure to 30 µM cisplatin for 1 h and a recovery period of 30 min or 1 h post,	**ROS:** Increased after exposure to 30 uM cisplatin for 1 h. **Inadequate repair:** Oxidative DNA lesions were increased after exposure to 30 µM cisplatin for 1 h and a recovery period of 30 min or 1 h post, after a recovery period of

					after a recovery period of 0 h, 2 h, 3 h, and 4 h there was no increase in oxidative DNA damage.	0 h, 2 h, 3 h, and 4 h there was no increase in oxidative DNA damage.

Table S4. Empirical evidence of the non-adjacent key event relationship between an increase in cellular ROS and mutations.

Citation	Toxicant	Model	ROS assay	Mutation assay	Evidence: Dose/concentration	Evidence: Temporal
(Shih et al. 2021)	Arecoline	OEC-M1 cell line (oral squamous cell carcinoma)	Flow cytometer ROS Assay Kit 520 nm (Invitrogen, Cat. 88-5930)	Host cell reactivation assay		**ROS:** Increased after exposure to 100 ug/ml arecoline for 4 h. **Mutation:** Increased after exposure to 100 ug/ml arecoline for 24 h.
(Jacobsen et al. 2008)	C60 or SWCNT	MutaMouse lung epithelial cells (FE1-MML)	DCFH-DA fluoresence assay	cII mutation frequency	**ROS:** Increased after exposure to 2.78 ug/ml C60 for 3 h. **Mutations:** No increase after exposure to 100 ug/ml C60 for 8 passages (total exposure time 576 h, total exposure concentration 8 mg).	**ROS:** Increased after exposure to 2.78 ug/ml C60 for 3 h. **Mutations:** No increase after exposure to 100 ug/ml C60 for 8 passages (total exposure time 576 h, total exposure concentration 8 mg).

Table S5. Empirical evidence of the non-adjacent key event relationship between an increase in cellular ROS and chromosomal aberrations.

Citation	Toxicant	Model	ROS assay	Chromosomal aberration assay	Evidence Temporal
(Shih et al. 2021)	Arecoline	OEC-M1 cell line (oral squamous cell carcinoma)	Flow cytometer ROS Assay Kit 520 nm (Invitrogen, Cat. 88-5930)	Micronucleus assay	**ROS:** Increased after exposure to 100 ug/ml arecoline for 4 h. **Chromosomal aberrations:** Increased after exposure to 100 ug/ml arecoline for 24 h.

Table S6. Quantitative evidence of the adjacent key event relationship between an increase in cellular ROS and oxidative DNA damage.

Citation	Toxicant	Model	ROS assay	Oxidative DNA damage assay	Description of the quantitative understanding
(Justiniano et al. 2017)	zinc pyrithione (ZnPT)	cultured malignant keratinocytes (SCC-25)	MitoSOX Red™ fluorescence assay	FPG-modified comet assay	Exposure to 5 µM ZnPT in SCC-25 keratinocytes, for 10 and 60 min, induced an increase in mitochondrial ROS (measured by the MitoSOX Red™ fluorescence assay) from 1 RFU to 1.5 RFU (10min) and 3.25 RFU (60min). Exposure to 5 µM ZnPT in SCC-25 keratinocytes, for 6 h induced increase in oxidative DNA damage (measured by the +Fpg comet assay) from a relative comet tail moment of 4 to 5.5.
(Gajski et al. 2015)	contaiminated water from abandoned uranimum mining area	Human blood (healthy female donor, age 24)	HPLC - MDA	FPG-modified comet assay	Exposure of human blood to contaminated water for 4 hours resulted in a significant increase in MDA, a marker of oxidative stress, from 10 nmol/g protein to 15 nmol/g protein. Exposure of human blood to contaminated water for 4 hours resulted in a significant increase in oxidative DNA damage (measured by the +Fpg comet assay) from 4% to 7% tail DNA.
(Gomez et al. 2009)	Methionine dietary restriction	Male Wistar Rat heart and liver mitochondria	homovanillic acid oxidation by H_2O_2	HPLC (8-oxodG)	Treatment with pyruvate, malate and methionine supplemented Male Wistar rats resulted in a significant increase of H_2O_2 in liver mitochondria from 0.05 nmol H_2O_2 /min mg protein to 0.1 nmol H_2O_2 /min mg protein. Treatment with pyruvate, malate and methionine supplemented Male Wistar rats resulted in a significant increase of 8-oxo-dG content in liver mitochondria from 3.5 8 OxodG/10^5 dG in mtDNA to 6.5 8 OxodG/10^5 dG in mtDNA.
(Inés Sanchez-Roman et al. 2012)	Methionine dietary restriction and aging	Male Wistar rat liver mitochondria	oxidation of homovanillic acid by H2O2	HPLC (8-oxodG and dG) for mt-DNA	Methionine restriction in the diet of male Wistar rats resulted in a significant decrease of liver mitochondrial H2O2 production from 0.13 nmol H2O2 /min mg protein to 0.06 nmol H2O2 /min mg protein. Methionine restriction in the diet of male Wistar rats resulted in a significant decrease of oxidative DNA damage in the liver mitochondria from 4.75 8-OxodG/10^5 dG in mtDNA to 3.5 8-OxodG/10^5 dG in mtDNA
(Taha et al. 2010)	iron-ascorbate (FE/ASC)-	Intestinal Caco-2/15 Cell Line mitochondria	HPLC - MDA	ELISA	Exposure of Caco-2/15 cells to 0.2 mM Fe and 2 mM ASC for 6 h resulted in a significant increase in mitochondrial MDA, a marker of oxidative stress, from 75 pmol/mg protein to 225 pmol/mg protein. Exposure of Caco-2/15 cells to 0.2 mM Fe and 2 mM ASC for 6 h resulted in a significant increase in mitochondrial 8-OHdG content from 0.2 ng/µg to 0.4 ng/µg.
(Ines Sanchez-Roman et al. 2011)	Methionine dietary restriction	Male Wistar rats, heart mitochondria	oxidation of homovanillic acid by H2O2	HPLC (8-oxodG and dG) for mt-DNA and ELISA for genomic DNA	Methionine restriction in the diet of male Wistar rats for 7 weeks resulted in a significant decrease in the rate or ROS production when treated with pyruvate, malate and rotenone, in heart mitochondria from 3.56 to 3.03 nmoles of H_2O_2/min. Methionine restriction in the diet of male Wistar rats for 7 weeks resulted in a significant decrease in 8-oxo-dG content from 6.5 8-oxo-dG/10^5 dG to 5.75 8-oxo-dG.
(Bruskov et al. 2002)	Heat	salmon sperm DNA	chemiluminescence in a peroxidase–luminol–p-iodophenol	ELISA	75°C heat for 4 hours was found to induce 2.4 nM H2O2 in phosphate buffer. 75°C hear for 24 hours was found in induce 96 8-oxodG/10^5 dG in salmon sperm DNA

(Lajmanovich et al. 2015)	Pesticide 2,4-D	adult male of R. arenarum (toad species)	GST activity	Endo III and FPG-modified comet assays	Dermal exposure of male R. arenarum (toads) to 20mg/L 2,4-D for 48, resulted in significantly increased blood glutathione S-transferase (GST) activity measured. Dermal exposure of male R. arenarum (toads) to 20 mg/L 2,4-D for 48 h, resulted in no significant change to oxidative DNA damage in the blood.
(Soberanes et al. 2012)	PM2.5	male-C57BL/6 mice	mitochondrial matrix localized oxidant-sensitive ratiometric probe (mito-Ro-GFP)	8-oxo-dG ELISA	Exposure of mouse lung epithelial cells to PM for 48 hours resulted in a significant increase in oxidation of the mitochondrial ROS probe mito-Ro-GFP from 5% to 10% (5 µg/cm2 PM) and 40% (10 µg/cm^2 PM). Exposure of mouse lung epithelial cells to concentrated PM for 72 hours resulted in a significant increase in 8-oxodG positve nuclei from 2 positive nuclei/field to 50 positive nuclei/field.
(Vadrot et al. 2012)	Tumour necrosis factor alpha	human hepatoma HepG2 cells	Luminol-amplified chemiluminescence (LAC)	ELISA	Exposure of HepG2 cells to 30ng/ml TNF-alpha for resulted in a a peak of ROS chemilunesence at 10 min, a significant increase of ROS to 170% of the basal chemiluminesence. Exposure of HepG2 cells to 30 ng/ml TNF-alpha for 15 min, 30 min, 1 h and 3h resulted in a peak increase in 8-oxo-dG at 1 h, from 0.3 8-oxo-dG ng/mg DNA to 1 8-oxo-dG ng/mg DNA.
(Espinosa et al. 2007)	hypertension	human subjects with hypertension	HPLC-EC determination of GSH (reduced glutathione) and GSSG (oxidzed glutathione) content	hPLC-EC (8-Oxo-dG and dG)	Blood samples from humans with hypertension had significantly increased oxidized glutathione to reduced glutathione ratios (GSSG nmol mg protein-1 / GSH nmol mg protein-1) from 0.60 to 5.80. Blood samples from humans with hypertension had significantly increased mitochondrial 8-oxo-dG content from 3.97 8-oxo-dG(nmol)/creatine(mmol) to 5.94 8-oxo-dG(nmol)/creatine(mmol) and nuclear 8-oxo-dG content from 5.40 8-oxo-dG(nmol)/creatine(mmol) to 6.65 8-oxo-dG(nmol)/creatine(mmol).
(Mustafa et al. 2011)	hypoxia (hyperoxic and hypoxic conditions)	common carp, Cyprinus carpio L	Glutathione peroxidase (GPx) activity	FPG-modified comet assay	Exposure of carp to hyperoxic conditions for 30 days resulted in an increase in GPx activity from 8*10^2 GPx activity nmol/min/ml to 1.1*10^2 GPx activity nmol/min/ml. Exposure of carp to hyperoxic conditions for 30 days resulted in an increase in % tail DNA in the FPG modified cometchip assay from 30% to 45%.

5.1.1 Supplementary References

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