AOP report: Development of an adverse outcome pathway for oxidative DNA damage leading to mutations and chromosomal aberrations

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Abstract

The Genetic Toxicology Technical Committee (GTTC) of the Health and Environmental Sciences Institute (HESI) is developing adverse outcome pathways (AOPs) that describe modes of action leading to potentially heritable genomic damage. The goal was to enhance the use of mechanistic information in genotoxicity assessment by building empirical support for the relationships between relevant molecular initiating events (MIEs) and regulatory endpoints in genetic toxicology. Herein, we present an AOP network that links oxidative DNA damage to two adverse outcomes (AOs): mutations and chromosomal aberrations. We collected empirical evidence from the literature to evaluate the key event relationships between the MIE and the AOs, and assessed the weight of evidence using the modified Bradford-Hill criteria for causality. Oxidative DNA damage is constantly induced and repaired in

Abbreviations: AOP, adverse outcome pathway; AO, adverse outcome; AP, apurinic/apyrimidinic; BER, base excision repair; BH, Bradford-Hill; DSB, double strand break; GTTC, Genetic Toxicology Technical Committee; HESI, Health and Environmental Sciences Institute; HR, homologous recombination; IATA, integrated approaches to testing and assessment; KE, key event; KER, key event relationship; MIE, molecular initiating event; MOA, mode of action; NER, nucleotide excision repair; NHEJ, non-homologous end joining; ROS, reactive oxygen species; RNS, reactive nitrogen species; SSB, single strand break; WOE, weight of evidence.

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cells given the ubiquitous presence of reactive oxygen species and free radicals. However, xenobiotic exposures may increase damage above baseline levels through a variety of mechanisms and overwhelm DNA repair and endogenous antioxidant capacity. Unrepaired oxidative DNA base damage can lead to base substitutions during replication and, along with repair intermediates, can also cause DNA strand breaks that can lead to mutations and chromosomal aberrations if not repaired adequately. This AOP network identifies knowledge gaps that could be filled by targeted studies designed to better define the quantitative relationships between key events, which could be leveraged for quantitative chemical safety assessment. We anticipate that this AOP network will provide the building blocks for additional genotoxicity-associated AOPs and aid in designing novel integrated testing approaches for genotoxicity.

1 | INTRODUCTION

The Adverse Outcome Pathway (AOP) framework organizes biological knowledge into a linear sequence of events, starting from a molecular initiating event (MIE) and ultimately leading to an adverse outcome (AO: an endpoint of regulatory concern) (Ankley et al., 2010; OECD, 2018). AOPs are purposefully simplified descriptions of toxicological pathways that are chemical-agnostic (i.e., molecular interaction-specific, not chemical-specific) allowing for broad applications (Villeneuve et al., 2014). AOPs provide an effective method for applying new and existing knowledge to demonstrate the relationships between measurable biological events (Key events: KEs) and AOs at all levels of organization: molecular, cellular, tissue, organism, and population. During the process of developing an AOP, all relevant information is considered for assessing biological plausibility and building empirical evidence to support the relationships between the events (key event relationships: KERs). Typically, data are collected from a survey of the literature and vetted against the modified Bradford-Hill (BH) criteria for causality to evaluate the weight of evidence (WOE) supporting the AOP (OECD, 2018).

The Health and Environmental Sciences Institute (HESI)’s Genetic Toxicology Technical Committee (GTTC) is currently working to develop a series of AOPs ending in potentially heritable genomic damage (e.g., AOs of mutations, chromosomal aberrations, and aneuploidy) to facilitate and promote the application of mechanistic understanding in genotoxicity assessment (Sasaki et al., 2020). The MIEs investigated by the GTTC thus far include oxidative DNA damage, topoisomerase inhibition, interactions with tubulin, and aurora kinase inhibition.

Herein, we describe an AOP network (Figure 1) developed as part of the HESI-GTTC project (AOP #296 in the AOPOWiki; URL: https://aopwiki.org/aops/296) (OECD, 2018). The AOP network links oxidative DNA damage to two regulatory endpoints in genetic toxicology: the induction of chromosomal aberrations and/or gene mutations. We anticipate that this AOP network will be valuable for designing novel Integrated Approaches to Testing and Assessment (IATA) aimed at identifying oxidative DNA damage as MIE and will be useful for providing key modules when building future AOPs (OECD, 2020a).

2 | ADVERSE OUTCOME PATHWAY (AOP #296)

AOP #296 (Figure 1) links oxidative DNA damage to mutations and chromosomal aberrations. We note that oxidative stress and oxidative DNA damage, as well as DNA strand breaks, mutations, and chromosomal damage, are intermediate processes in diverse pathways. Thus, the modules within this AOP network can be bridged into other AOPs. Indeed, this AOP network already shares KEs and KERs with AOP #15: Alkylation of DNA leading to heritable mutations (URL: https://aopwiki.org/aops/15; [Yauk et al., 2015]) and AOP #272: Direct deposition of ionizing energy onto DNA leading to lung cancer (URL: https://aopwiki.org/aops/272; [Chauhan et al., 2020]).

Strictly speaking, AOP #296 is a network because it contains two separate AOs: mutations and chromosomal aberrations. Table 1 summarizes the KEs that constitute the network and available methods for measuring each KE. Detailed descriptions of each KE are available online in the AOPOWiki. The MIE of the network is increases in oxidative DNA damage. This event encompasses the broad range of lesions caused by oxidizing agents including strand breaks caused by direct electrophilic attack on the phosphate backbone and/or oxidatively damaged nitrogenous bases (Cadet et al., 2017; Melis et al., 2013). If the quantity and/or type of oxidative damage exceeds the DNA repair capacity of the cell (both high- and low-fidelity repair pathways), the damage will not be repaired efficiently, completely, and/or accurately, leading to inadequate repair (KE1). Inadequately repaired lesions can lead to an increase in DNA strand breaks (KE2) or insertion of an incorrect base during replication, resulting in elevated numbers of mutations (AO1). Inadequate repair of DNA strand breaks (e.g., joining of two incorrect ends, unincorporated DNA fragment, lack of rejoicing) can then lead to structural chromosomal damage and changes in the DNA sequence, resulting in an increase in chromosomal aberrations (AO2) and mutations (AO1), respectively.
The GTTC AOP sub-team developed the AOP network in accordance with the OECD AOP Users’ Handbook supplement for guidance in AOP development (OECD, 2018). Below, we provide background relevant to the AOP, summarize the evidence supporting the KERs and KEs, provide an overall AOP assessment, and discuss uncertainties, inconsistencies, and major knowledge gaps.

3 | BACKGROUND

Oxidative stress generally refers to an imbalance between oxidants and antioxidants within a cell, where oxidants overwhelm the antioxidants. Elevated levels of cellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been associated with genomic instability (Hanahan & Weinberg, 2011; Panieri & Santoro, 2016; Roszkowski et al., 2011). Indeed, the ability to induce excessive ROS and other free radicals is a common characteristic of carcinogens (Krewski et al., 2019; Smith et al., 2016). Therefore, the potential for chemicals to induce oxidative DNA damage can be an important consideration in chemical genotoxicity and carcinogenicity safety assessment.

Oxidants can arise from both endogenous and exogenous sources. Many endogenous biochemical processes involve redox reactions; at steady state, cellular respiration and various metabolic activities (e.g., oxidative phosphorylation in the mitochondria, NADPH oxidase, monoamine oxidase, and CYP450 monooxygenase activities) continuously generate ROS and other free radicals (Liou & Storz, 2010). Furthermore, the role that ROS and RNS play as secondary signaling molecules in various pathways indicates that certain amounts of these reactive species (e.g., H$_2$O$_2$) are essential for proper cellular functions (D’Autreaux & Toledano, 2007; Finkel, 2011; Weidinger & Kozlov, 2015). Healthy cells are capable of tightly regulating oxidant levels at steady state using endogenous antioxidants (e.g., N-acetylcysteine, glutathione) and enzymes (e.g., glutathione-S-transferases) and have antioxidant responses for countering elevations in ROS and other oxidants to a certain extent (e.g., via catalase), to maintain non-cytotoxic levels of oxidants.
### TABLE 1  Summary of key events in the AOP network (AOP #296) and methods of measurement

| Type                          | Event title                        | Description                                                                                                                                                                                                 | How to measure                                                                                                                                 |
|-------------------------------|------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------|
| **Molecular Initiating Event (MIE)** | Increases in Oxidative DNA damage | This KE includes:                                                                                                                                                                                          | Relative quantification methods                                                                                                              |
|                               |                                     | • A broad range of lesions including direct breakage of the phosphate backbone of DNA causing strand breaks                                                                                             |  | Modified comet assay (Fpg or hOGG1 enzyme digestion of DNA prior to electrophoresis) (Moller et al., 2017)                                                                                           |
|                               |                                     | • Oxidation of nitrogenous bases of the DNA. Notably, oxidative guanine lesions (e.g., 8-Oxo-2′-deoxyguanosine (8-oxo-dG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG)) are the most extensively studied and known to be the most abundant compared to other oxidative base lesions. |  | Enzyme-linked immunosorbant assays (ELISA) (Breton et al., 2003)                                                                                                                                       |
|                               |                                     | • Absolute quantification methods                                                                                                                                                                           |  | ³²P-labelling (Collins, 2000) (has generally been replaced with newer assays)                                                                                                                        |
|                               |                                     | • Relative quantification methods                                                                                                                                                                          |  | High performance liquid chromatography coupled to electrochemical detection (HPLC-EC) (Chepelev et al., 2015)                                                                                           |
|                               |                                     | • Liquid chromatography (LC) coupled with mass spectrometry (MS) (Collins, 2000)                                                                                                                           |  | Liquid chromatography (LC) coupled with mass spectrometry (MS) (Collins, 2000)                                                                                                                        |
| **Key Event (KE)**           | Inadequate DNA repair               | This KE includes:                                                                                                                                                                                          | The KE can be inferred from retention of DNA lesions or increase in mutations and chromosomal aberrations (AO1 & AO2) which would indicate lack of repair, incomplete repair, or incorrect repair. Thus, methods of measuring this KE are mostly indirect (e.g., time-course measurement of DNA lesions following exposure, dose–response experiments). Direct methods would include assays that measure a cell's capability to repair DNA damage (e.g., introducing fluorescent reporter construct containing a specific lesion [Mao et al., 2011; Chaim et al., 2017; Nagel et al., 2019]). Models of DNA repair deficiency such as knock-out cell lines and rodent models (e.g., Agg−/− mice [Kay et al., 2021]) can be used to determine which pathway is critical for repairing the genotoxic lesion (Olivieri et al., 2020). |
|                               |                                     | • Lack of DNA repair (accumulation of unrepaired damage)                                                                                                                                                     |  | Transgenic rodent mutation assays (e.g., bacterial transfection with reporter gene, fluorescence tag expression in transgenic mice) (Lambert et al., 2005; OECD, 2020). |
|                               |                                     | • Incorrect or error-prone DNA repair (insertion of incorrect base, joining two incorrect ends by NHEJ)                                                                                                    |  |  |                                                                                                                                         |
|                               |                                     | • Incomplete DNA repair (accumulation of repair intermediates)                                                                                                                                               |  |  |                                                                                                                                         |
| **Key Event (KE)**           | Increases in DNA strand breaks      | This KE includes:                                                                                                                                                                                          |  | Comet assay (neutral and alkaline) (Collins, 2004; Ge et al., 2021; Ngo et al., 2021; OECD, 2014b)                                                                                                       |
|                               |                                     | • Increase in both single strand breaks (SSB) and double strand breaks (DSB)                                                                                                                                |  | γ-H2AX foci detection methods (e.g., fluorescent immunostaining and detection by flow cytometry, microscopy, in-cell Western, and ELISA) (Bryce et al., 2016; Garcia-Canton et al., 2013; Khoury et al., 2013) |
|                               |                                     | • Strand breaks can arise during excision repair (e.g., BER, NER), during replication and transcription (e.g., topoisomerase), or directly due to chemical insult (e.g., collapsed replication fork)                         |  | Pulsed field gel electrophoresis (Kawashima et al., 2017)                                                                                                                                         |
|                               |                                     | • Two SSBs in close proximity to each other on opposite strands can lead to a DSB                                                                                                                        |  | Transcriptomic biomarkers of DNA damage (e.g., TGx-DDI biomarker [Li et al., 2017])                                                                                                                  |
| **Adverse Outcome (AO)**     | Increases in Mutations              | This KE includes:                                                                                                                                             |  | Various gene mutation assays (e.g., HPRT, TK, PIG-A/O assays) (Dobrovolsky et al., 2014; Gollapudi et al., 2015)                                                                                         |
|                               |                                     | • Permanent alterations in DNA sequence                                                                                                                                                                       |  | Transgenic rodent mutation assays (e.g., bacterial transfection with reporter gene, fluorescence tag expression in transgenic mice) (Lambert et al., 2005; OECD, 2020b) |
|                               |                                     | • The alteration may involve a few bases (e.g., single nucleotide variants or small insertions/deletions) or a larger segment of DNA (e.g., incorrect rejoining of DSB, loss or insertion of large fragments)                     |  |  |                                                                                                                                         |

(Continues)
All biomolecules, including nuclear and mitochondrial DNA, are susceptible to oxidative damage (Cadet et al., 2017; Markkanen, 2017). Oxidative stress occurs when free radicals overwhelm the cellular antioxidant capacity, which can occur under certain physiological conditions, such as inflammation, and via exogenous stressors that are direct oxidants and/or induce oxidative species. Perturbation of cellular respiration (e.g., electron transport chain disruptors [Wang et al., 2015]), depletion of endogenous antioxidants (e.g., glutathione [Beddowes et al., 2003]), generation and/or amplification of reactive species (e.g., redox cycling of quinones [Penning, 2017], Fenton reaction of metals (Cu$^{2+}$, Fe$^{2+}$, Ni$^{2+}$) with molecular oxygen [Lloyd & Phillips, 1999]), and direct oxidation of cellular components (e.g., ionizing and non-ionizing radiation, oxidants such as potassium bromate [Murata et al., 2001]) are ways in which toxicants cause oxidative stress and damage in the cell.

ROS and other free radicals cause a broad range of oxidative damage to DNA. Direct electrophilic attack on the phosphate backbone can induce DNA strand breaks. Additionally, all nitrogenous bases are vulnerable to oxidative damage (Cadet & Wagner, 2013; Cooke et al., 2003). Oxidative adducts form mainly on C5 and less frequently on C6 of thymine and cytosine (e.g., thymine glycol, 5-hydroxy-2′-deoxyctydine [5-OHdC]), and on C8 of guanine and adenine (e.g., 8-oxo-deoxyguanine [8-oxo-dG], 8-oxo-deoxyadenine,2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), 8-nitroguanine, 4,6-diamino-5-formamidopyrimidine (FapyA) (Berquist & Wilson III, 2012; Hiraku, 2010; Whitaker et al., 2017). Guanine is most prone to oxidation due to its low oxidation potential (i.e., most easily loses electrons) (Jovanovic & Simic, 1986). In fact, 8-oxo-dG is the most mutagenic and abundant oxidative DNA lesion; between 2400 and 10,000 8-oxo-dG are estimated to be present in the genome at steady state due to endogenous oxidants (Kalam et al., 2006; Ohno et al., 2006; Swenberg et al., 2011). In addition, abasic (AP; apurinic or apyrimidinic) sites are abundant in dsDNA at steady state as they can arise from spontaneous hydrolytic reactions and the removal of nitrogenous bases by glycosylases during base damage repair (Swenberg et al., 2011). Oxidative degradation of certain cellular components can also give rise to toxic products and exacerbate the damage in the cell during oxidative stress; for example, lipid peroxidation generates DNA-reactive aldehydes, such as malondialdehyde and acrolein, that form mutagenic malondialdehyde-2′-deoxyguanosine (M1G) and γ-hydroxy-1,N2-propano-2′-deoxyguanosine adducts, respectively (Gentile et al., 2017; Zhou et al., 2005).

For practical reasons, AOP #296 equates 8-oxo-dG formation with ‘oxidative DNA damage’, and the MIE. The fate of guanine lesions has been most extensively researched and is well understood (Cadet et al., 2017; Markkanen, 2017; Roszkowski et al., 2011; Whitaker et al., 2017). In human biomonitoring studies, 8-oxo-dG is an accepted biomarker of oxidative stress and oxidative DNA damage (Cooke et al., 2008; Li et al., 2014). We note that 8-oxo-dG is not a terminal product of oxidative damage; 8-oxo-dG can be further oxidized to additional mutagenic intermediates such as spiroiminodihydantoin and guanidinohydantoin (Jena & Mishra, 2012). However, as with many other oxidative lesions on pyrimidines and adenine, these

### TABLE 1

| Type                  | Event title                     | Description                                                                 | How to measure                                                                 |
|-----------------------|---------------------------------|-----------------------------------------------------------------------------|-------------------------------------------------------------------------------|
| Adverse Outcome (AO)  | Increases in Chromosomal        | This KE includes:                                                           | • PCR methods (Allele-specific competitive blocker-polymerase chain reaction [ACB-PCR] for using mutant-specific primers to amplify point mutations in the target allele; single molecule PCR (Banda et al., 2013) • Error-corrected next generation sequencing (Salk & Kennedy, 2020) and similar technologies |
|                       | aberrations                     | Chromatid-type and chromosome-type structural aberrations such as translocations, inversions, rings, and fragmentation. | • Micronucleus detection (scoring by light or fluorescent microscopy and flow cytometry) (Bryce et al., 2014; OECD, 2014a; OECD, 2016b) • Chromosomal aberration test (metaphase spread examined by microscopy and aberrations are scored) (OECD, 2016a; OECD, 2016c; OECD et al., 2015) • Indirect detection methods such as γH2AX foci and p53 protein expression assays (flow cytometric and in-cell Western blotting), and fluorescent protein reporter assays for stress signaling pathway activation (Bryce et al., 2016; Hendriks et al., 2012; Khoury et al., 2013) |
lesions are estimated to be small fractions compared to 8-oxo-dG (Cooke et al., 2008; Yu et al., 2005).

When DNA damage is caused by uncertain mechanisms, oxidative stress is often suspected because it is observed under diverse chemical insults such as tert-butylhydroquinone (Eskandani et al., 2014; Ramadan & Suzuki, 2012), heavy metals (Patlolla et al., 2009a; Patlolla et al., 2009b; Skipper et al., 2016), and carbon tetrachloride (Beddowes et al., 2003). Oxidative DNA base damage has been implicated in genomic instability, which is one of the hallmarks of cancer (Hanahan & Weinberg, 2011). Furthermore, oxidative DNA damage is observed in many non-neoplastic diseases including neurodegenerative (Coppedè & Migliore, 2015), cardiovascular (Ishida et al., 2014; Shah et al., 2018), and inflammatory airway diseases (Tzortzki et al., 2012). Thus, measurement of 8-oxo-G is an effective strategy to assess a chemical's potential to induce oxidative DNA damage (through direct and indirect processes) and can provide insight into the causal genotoxic mechanism.

4 | KEY EVENT RELATIONSHIPS (KERs)

KERs describe the causal relationships between upstream and downstream KEs. The WOE analysis of KERs involves an assessment of biological plausibility, empirical evidence including evidence supporting the essentiality of each KE in the AOP, and consideration of uncertainties and inconsistencies. In AOP #296, two sets of KERs connect the KEs in the network – adjacent and non-adjacent (Figure 1). Adjacent KERs link two KEs that are directly upstream and downstream of each other in the pathway. Non-adjacent KERs link two KEs that are separated by one or more intermediate KEs; these KERs are particularly useful for strengthening the evidence for the overall pathway when there is a limited number of studies supporting the adjacent KERs (e.g., when one KE is not routinely measured). Tables 2 and 3 summarize the adjacent and non-adjacent KERs within the AOP network, respectively. These tables describe each of the KERs and provide examples of empirical evidence supporting the relationships. Extended sets of empirical evidence for each KER are available online in the AOPWiki, and collectively, the empirical evidence for both types of KERs provides WOE for the overall AOP (OECD, 2018).

To develop this AOP, the team members were assigned modules (i.e., KEs or KERs) and reviewed the literature to identify key methodologies for measuring the KEs within their modules. For each KER, a literature search was conducted independently of other KERs to maintain modularity. The modified BH criteria for causality guided the selection of studies that were used as empirical evidence. Initially, the BH criteria were created to facilitate the evaluation of causal associations in epidemiological studies. The BH criteria used herein have been modified for applications in WOE assessment of AOPs (Becker et al., 2015).

The three main criteria considered when evaluating empirical support for the KERs include: concordance in concentration/dose, temporality, and incidence of the KEs (Becker et al., 2015; OECD, 2018). Concentration or dose concordance indicates that the upstream KE is observed at the same or lower stressor concentrations or doses than the downstream KE within the KER. Similarly, incidence concordance indicates that the upstream KE occurs more frequently (or at the same frequency), or at a higher (or the same) incidence, than the downstream KE. Temporal concordance is demonstrated by the detection of the upstream KE at an earlier (or identical) time point to the downstream KE. Both in vivo and in vitro data were considered and diverse experimental methods were used within these studies (Tables 2 and 3). Thus, to evaluate empirical support for the AOP, studies that measured more than one KE from the AOP were considered.

5 | BIOLOGICAL PLAUSIBILITY

Proper repair of oxidative DNA lesions is critical for maintaining genomic integrity. It is well-established knowledge that DNA repair capacity in a cell is limited and that an increase in oxidative DNA lesions above a cell's threshold for effective repair can lead to damage of the genome (Brenerman et al., 2014; Cadet & Wagner, 2013; Yang et al., 2006). Overall, based on the current mechanistic understanding of mutation and chromosomal aberration induction by ROS and other oxidizing agents, biological plausibility of this AOP network is strong.

The mutagenicity of certain oxidative base lesions has been extensively studied (Riva et al., 2020). Many of the oxidative base lesions can form stable base pairs with incorrect dNTPs during replication via translesion synthesis (TLS), giving rise to base substitutions (Cadet et al., 2017; Maddukuri et al., 2014; Shimizu et al., 2007). For example, FapyA and 8-oxo-dA can base pair with C, giving rise to A to C transversions (Kalam et al., 2006). Similarly, dA can be inserted opposite of 8-oxo-dG or FapyG by replicative polymerases and TLS polymerases, η and κ, leading to G to T transversions (Markkanen et al., 2012; Shimizu et al., 2007; Taggart et al., 2014). Since 8-oxo-G is the predominant oxidative DNA lesion, G to T transversions are the most predominant base substitutions observed in cells exposed to various oxidative stress inducers (Arai et al., 2002; Klungland et al., 1999; Unfried et al., 2002).

Oxidative damage to nitrogenous bases is primarily repaired by base excision repair (BER) and, to a lesser extent, by nucleotide excision repair (NER) (Scott et al., 2014; Shafirovich et al., 2016; Whitaker et al., 2017). Several different DNA glycosylases initiate the repair of oxidative base lesions: OGG1, MYH (MUTYH), NTHL1, NEIL1, NEIL2, and NEIL3 (Markkanen, 2017; Shafirovich et al., 2016). BER glycosylases are either mono-functional (glycosylase only) or bifunctional (glycosylase and lyase). DNA glycosylases remove oxidized nitrogenous bases by hydrolyzing the N-glycosidic bond, giving rise to an abasic site (AP). The lyase function of bifunctional glycosylases creates a SSB 3' to the AP site after the removal of the oxidized nitrogenous base (Jacobs & Schar, 2012; Minko et al., 2016).

Following BER initiation by glycosylases, the AP endonuclease (APE1) then creates a SSB 5' to the AP site. A single nucleotide gap will form if a bifunctional glycosylase preceded APE1, as there will be a nick 3' to the AP site created earlier by the lyase function. If BER is initiated by a mono-functional glycosylase, the AP site will remain after the APE1 reaction. Flap endonuclease (FEN1) is then required to excise the AP site by creating a nick 3' to the site, giving rise to a single nucleotide gap. DNA polymerase β then synthesizes over the gap.
| Key event relationship (KER) title | Description | Examples of empirical evidence |
|-----------------------------------|-------------|--------------------------------|
| Increases in Oxidative DNA damage leads to Inadequate DNA repair | At steady state, oxidative lesions generated by endogenous free radicals are readily repaired by basal repair mechanisms, mainly base excision repair (BER), to maintain baseline levels. However, if the level of oxidative DNA lesions (i.e., oxidized bases, abasic sites, strand breaks) increases above a cell's ability to detoxify, an exceeded repair capacity can lead to lack of, or faulty repair (i.e., increase in unrepaired lesions, repair intermediates, mispaired bases) - all indicators of inadequate DNA repair. | Concentration/Dose Concordance  
• Threshold dose–response curve of 8-oxo-dG observed in the liver and urine of mice exposed to increasing doses of X-rays; exceeded threshold indicates exceeded repair capacity leading to the observed increase in oxidative damage (Li et al., 2013)  
Temporal Concordance  
• The 8-oxo-dG levels in Ogg1-overexpressing Chinese hamster ovary cells returned to baseline within 4 hours following UV exposure, while wild type cells retained 70% of the lesions (Dahle et al., 2008)  
Other types of evidence  
• Enrichment of 8-oxo-dG and γH2AX (DSB marker) in the same genomic regions was observed in human cells in culture by chromatin immunoprecipitation sequencing (ChIP-seq) (Amente et al., 2019; Murata et al., 2012) |
| Inadequate DNA repair leads to Increases in DNA strand breaks | Exceeded BER capacity due to an increase in oxidative lesions can lead to an accumulation of repair intermediates, including AP sites and SSBs. Increase in the number of SSBs elevates the risk of two SSBs occurring in close proximity to each other; if two SSBs occur on opposite strands, it may be converted into a DSB, exacerbating the damage. Increase in unrepaired lesions and repair intermediates due to inadequate repair can further impede the repair of other damaged sites nearby. BER intermediates are known to be replication blocks that can cause a replication fork to stall and collapse. Collapsing of replication forks can cause DSBs, the most toxic type of DNA lesion. | Concentration/Dose and Incidence Concordance  
• OGG1-deficiency causing concentration-dependent increase in strand breaks (Wu et al., 2008)  
• BER-proficiency leading to increase in strand breaks due to BER imbalance or interference with replication (Ensminger et al., 2014; Wang, Li, et al., 2018)  
Dose and Temporal Concordance  
• Dose-dependent increase in DSBs measured in primary human dermal cells after a 16-h recovery following ionizing irradiation (Rydberg et al., 2005) |
| Increases in DNA strand breaks leads to Inadequate DNA repair | Increase in the number of strand breaks can exceed the repair capacity (DSB: NHEJ or HR; SSB: SSBR), resulting in prolonged presence of strand breaks (lack of repair). Increase in the occurrence of NHEJ may also increase the incidence of two incorrect ends being joined, altering the DNA sequence. DSBs may also lead to mutagenic salvage DNA repair pathways such as break-induced replication (BIR) and microhomology-mediated break-induced replication (MMBIR). | Concentration and Incidence Concordance  
• In the Rydberg et al. (2005) study above, dose-dependent increase in misrejoined DSBs was also observed in primary human dermal cells after a 16-h recovery following irradiation (Rydberg et al., 2005)  
• In several studies, dose-dependent increase in unrepaired DSBs was detected in irradiated mammalian cells after varying recovery periods, indicating exceeded repair capacity (Kuhne et al., 2000; Kuhne et al., 2005; Lobrich et al., 2000)  
Temporal Concordance  
• In the studies above, the levels of DSBs were measured immediately after exposure and after recovery; inadequate repair was indicated by unrepaired or mis-rejoined DSBs after the recovery period. |
TABLE 2 (Continued)

| Key event relationship (KER) title | Description | Examples of empirical evidence |
|-----------------------------------|-------------|--------------------------------|
| Inadequate DNA repair leads to Increases in Mutations | Higher incidences of NHEJ and mutagenic salvage repair can increase the chance of incorrect joining of two broken ends, altering the DNA sequence. Unrepaired base lesions can lead to point mutations, especially if they are able to form stable base pairs with incoming nucleotides during replication (e.g., 8-oxo-dG base pairing with adenine). | Incidence and Concentration Concordance • Concentration-dependent increase in DSB misrepair and mutation frequency in cells exposed to ionizing radiation (McMahon et al., 2016) • A larger fold increase in KBrO3-induced mutations in Ogg1 −/− mice vs. wild type mice (Arai et al., 2002) Temporal Concordance • A larger fold increase in spontaneous mutations in Ogg1 −/− mice at 14 weeks of age than at 9 weeks, compared to wild type (Minowa et al., 2000) |

| Inadequate DNA repair leads to Increases in Chromosom al Aberrations | If DSBs are not repaired in a timely manner, the ends can shift away from the original position, reducing the likelihood of error-free repair. Unrepaired strand breaks and mis-joined ends by incorrect repair can result in translocation, inversion, deletion of sections (unincorporated fragments), and other structural aberrations of the chromosome (e.g., ring and loop formation). | Concentration/Dose and Incidence Concordance • Dose-dependent increase in incorrect rejoining of DSBs in irradiated cells (McMahon et al., 2016) • Clear, positive correlation between radiation dosage and clastogenic endpoints (CA, MN, copy number variants) reported by several studies (Arlt et al., 2012; Balajee, 2014; George et al., 2009; Suto et al., 2015) Temporal Concordance • DNA-PK (DNA-dependent protein kinase) was chemically inhibited and DSBs and MN were measured in gamma-irradiated cells at 3 h and 24 h, respectively, post-irradiation; irradiation dose-dependent increase in DSBs and inhibitor concentration-dependent increase in MN were observed (Chernikova et al., 1999) |

and DNA ligase forms a new phosphodiester bond that seals the SSB (Whitaker et al., 2017). Overwhelmed BER capacity may cause an imbalance in the initiating steps of BER, resulting in incomplete repair of oxidative lesions (Cabelof et al., 2004; Parrish et al., 2018; Wang, Li, et al., 2018; Yang et al., 2006). If BER is disrupted before completion, intermediates such as AP sites and SSBs can persist in the genome (Cabelof et al., 2004; Parrish et al., 2018; Sheng et al., 2012).

Both strand breaks and AP sites can arise directly due to reactive species and as BER intermediates (Cadet et al., 2017). SSBs and AP sites are known to block DNA replication; if a replication fork encounters such lesions, it can stall and collapse, resulting in a DSB (Ensinger et al., 2014; Kidane et al., 2014; Kuzminov, 2001). Furthermore, unrepaired oxidative base lesions, SSBs, and AP sites are more toxic and difficult to repair when in closer proximity to each other (Pearson et al., 2004; Sedletska et al., 2013; Yang et al., 2004). Oxidative base lesions, especially 8-oxo-dG, can impede the repair of neighboring SSBs, increasing the risk of DSB formation (Calsou et al., 1996; Lomax et al., 2004). Thus, if such base lesions persist in the genome due to inadequate repair, the repair of existing and new strand breaks can be impaired, leading to a net increase in strand breaks (Caglayan & Wilson, 2015). In addition, if two SSBs occur on opposite strands within a sufficiently small distance during BER, the SSBs may be converted to a DSB. Some studies suggest that multiple DNA lesions within one or two helical turns can increase the rate of DSB formation (Cannan & Pederson, 2016).

While BER is essential for maintaining genomic integrity, it is known to have both protective and damaging effects on the genome during oxidative stress. For example, MUTYH, a glycosylase that removes dA opposite 8-oxo-dG, may undergo futile cycles of dA removal and reinserterion by polymerases, potentially increasing AP sites in the genome (Hashimoto et al., 2004). Moreover, knock-out of OGG1, a bifunctional glycosylase that primarily targets 8-oxo-dG lesions opposite dC, prevented AP site accumulation in human cells infected with H. pylori monitored over a 72-h period (Kidane et al., 2014). AP sites are electrophilic and chemically unstable and, thus, are prone to strand breakage (Greenberg, 2014). In addition, an increase
## TABLE 3  Summary of non-adjacent key event relationships (KER)

| Key event relationship (KER) title | Description | Empirical evidence |
|-----------------------------------|-------------|--------------------|
| **Increases in Oxidative DNA damage leads to Increases in DNA strand breaks** | Biologically plausible mechanisms linking increase in oxidative DNA lesions to increase in strand breaks include: 1) Incomplete BER due to an imbalance in the glycosylase and AP site endonuclease (APE) activities resulting in AP site and SSB accumulation; 2) increase in oxidative lesions impeding the repair of neighboring lesions (possibly SSBs); 3) occurrence of SSBs in close proximity to each other during BER; 4) collision of replication fork with BER proteins and intermediates. |  |
| **Increases in Oxidative DNA damage leads to Increases in Mutations** | It has been extensively demonstrated that 8-oxoG, the most abundant oxidative DNA lesion, preferentially forms base pairs with incoming dA during replication causing G to T transversions, which are characteristic of oxidative DNA damage. | Concentration/Dose Concordance  
In Fpg-modified comet assay, significant increase in Fpg-sensitive sites (e.g., 8-oxo-dG, AP sites) occurred at lower concentrations than strand breaks in HepG2 cells exposed to a nodularin (Lankoff et al., 2006). |
| **Increases in DNA strand breaks leads to Increases in Mutations** | Increases in SSBs and DSBs can lead to a higher incidence of erroneous repair by NHEJ and mutagenic salvage repair pathways. | Concentration/Dose and Incidence Concordance  
Concentration-dependent increases observed in strand breaks after 1 h and in mutant frequency measured after 23 and 72 h after bleomycin exposure in TK6 cells (Platel et al., 2011). |
| **Increases in DNA strand breaks leads to Increases in Chromosomal Aberrations** | Mechanistically, DSBs must occur for chromosomal aberrations to occur. If DSBs are not rejoined in a timely manner, the ends may shift away from their original position, resulting in loss of segments or rearrangement of sections. | Temporal Concordance  
Several in vivo and in vitro studies demonstrated the occurrence of strand breaks (via comet assay and γH2AX foci detection) at early time points (1–4 h) post exposure and micronucleus, chromatid break, or translocation at later time points (24–40 h) (Dertinger et al., 2019; Platel et al., 2009; Trenz et al., 2003; Turner et al., 2015). |
in the number of damaged sites where BER is in progress may increase the risk of DSB generation due to collision with the replication fork (Enslinger et al., 2014). Other studies have shown exacerbated strand breaks in BER-proficient cells following acute chemical exposures (Banda et al., 2017; Sheng et al., 2012; Wang, Li, et al., 2018). However, a compromised BER capability (due to glycosylase variants or knock-out) leads to an increase in spontaneous mutation frequency and structural damage (DSBs and MN formation) under chronic chemical exposures (Aral, 2002; Gallick et al., 2013; Hu et al., 2005; Minowa et al., 2000; Ondovcik et al., 2012). Collectively, these studies suggest that elevated BER glycosylase activity in response to increases in oxidative lesions can give rise to higher incidences of incomplete repair but a timely clearance of oxidative base lesions is ultimately critical for maintaining genomic stability.

 Failed repair of oxidative lesions (e.g., replication fork stalling and collapsing due to BER intermediates or unrepaird SSBs during the S phase) can lead to the formation of DSBs. The active repair pathway and the efficiency of repair can vary depending on the cell cycle stage, and these pathways can be error-prone. Non-homologous end joining (NHEJ), the fastest DSB repair pathway, is an error-prone process that ligates two broken ends generally based on microhomologies (≤4 nt) of single-strand overhangs and is active across all cell cycle stages (Chang et al., 2017; Lieber et al., 2010; Rodgers & McVey, 2016). The level of NHEJ activity has been observed to increase with the progression in cell cycle, reaching the highest level in the G2 phase; consequently, the efficiency of DSB repair has been reported to be the highest in the G2/M phase (Mao et al., 2008). Comparatively, NHEJ is often a longer process than homologous recombination (HR), which requires homologous sequences in the sister chromatid or homologous chromosome as a template to ensure fidelity of the reconstructed strands; HR is mostly restricted to the S and G2 phases of the cell cycle and is most active in the S phase (Brandsma & van Gent, 2012; Fugger & West, 2016; Gandhi et al., 2012).

 As with oxidative base lesions, the repair capacity for DSBs is limited and repair kinetics are compromised with increasing DSBs; thus, the risk of faulty or lack of repair and, consequently, structural chromosomal damage (AO2) increases with the number of DSBs (Calsou et al., 1996; Cannan & Pederson, 2016). The presence of DNA strand breaks over a prolonged period of time increases the chance of the two broken ends diffusing away from the original position, hampering restoration of the original structure (Schipler & Ililakis, 2013). In a cell under chemical insult, DSBs may also lead to the induction of saline DNA repair pathways with high error rates such as microhomology-mediated end joining (MMEJ), break-induced replication (BIR), and microhomology-mediated break-induced replication (MMBRIR) (Kramara et al., 2018; Sakofsky et al., 2015; Seol et al., 2018). While salvage repair pathways and NHEJ promote survival of the cell, the inadequate repair of DSBs can ultimately result in structural aberrations (AO2) (i.e., large deletions and insertions, translocations, ring formation) and mutations (AO1) (Ferguson & Alt, 2001; Ililakis et al., 2004). In addition, chromosomal aberrations can also arise from HR, which is typically a highly accurate repair pathway; for example, a crossover event between the damaged site and a region of sequence homology on another chromosome can result in exchange-type structural aberrations causing translocations, radial formation, and/or dicentric chromosomes with accompanyingacentric fragments (Obe et al., 2002). Thus, DSBs in areas of repetitive sequences have a higher risk of misalignment with an incorrect chromosome region during homologous recombination (Brandsma & van Gent, 2012).

6 | Essentiality of Key Events

The essentiality of each KE in the pathway can be demonstrated by modulating one KE (e.g., via overexpression or inhibition of an enzyme) and observing concordance in the downstream KEs. The essentiality of the MIE, “Increase, Oxidative DNA Damage”, has been demonstrated by studies inhibiting or exacerbating ROS by treating cells with an antioxidant (e.g., N-acetylcysteine) or depleting endogenous glutathione (using buthionine sulphoximine). For example, pretreatment with an antioxidant reduced the quantity of oxidative base lesions (MIE) that are formed following exposure to a ROS inducer, with concordant decreases in DNA strand breaks (KE2: Increase, DNA Strand Breaks) and MN (AO2: Increase, Chromosomal Aberrations) observed at later time points (Dong et al., 2014; Federici et al., 2015; Kopp et al., 2020; Reliene et al., 2004). Similarly, depletion of glutathione resulted in elevated levels of both 8-oxo-dG and DNA strand breaks (Beddowes et al., 2003).

The essentiality of KE1, “Inadequate DNA Repair”, has been demonstrated by studies that modulated DNA repair capacity by knocking out various BER glycosylases (Hu et al., 2005; Nallanthighal et al., 2017; Suzuki et al., 2010; Wang, Li, et al., 2018). For example, steady-state levels of oxidative lesions (e.g., 8-oxo-dG, FapyG, FapyA) and spontaneous G to T transversions (AO1: Increase, Mutations) increased in mammalian cells deficient in various BER glycosylases (Hu et al., 2005; Nallanthighal et al., 2017; Suzuki et al., 2010). OGG1 deficiency had different effects on cells exposed for different durations. After a 30 min-exposure to hydrogen peroxide, significantly fewer strand breaks (KE2) were observed in OGG1-deficient cells compared to wild type cells, suggesting an accumulation of SSBs due to incomplete BER in wild type cells (Wang, Li, et al., 2018). A seven-day continuous exposure to silver nanoparticles in mice induced significant concordant increases in 8-oxo-dG, DSBs (KE2), and MN (AO2) in Ogg1-deficient mice compared to wild type mice (Nallanthighal et al., 2017). An increase in OGG1 activity may lead to incomplete BER and an acute exacerbation of strand breaks; however, persistent oxidative DNA lesions resulting from aberrant glycosylase activity may induce MN in daughter cells.

The essentiality of KE2, “Increase, DNA Strand Breaks”, has been shown by studies that examined both mutations (AO1) arising from incorrect rejoining of DSBs and chromosomal damage indicated by MN induction (AO2) (Kurashige et al., 2017; Tatsumi-Miyajima et al., 1993). Tatsumi-Miyajima et al. (1993) introduced plasmids with or without a DSB in the supF gene to five different human fibroblast cell lines and monitored supF gene mutation frequency in a colony formation assay. The plasmids that contained a DSB produced a higher frequency of supF mutants than the undamaged plasmids; up to 50% of the analyzed colonies were supF gene mutants indicating mutagenic repair of the DSB by human fibroblasts. To demonstrate the
essentially of KE2 in inducing structural damage (AO2), studies examining strand breaks and MN were considered. In a study by Kurashige et al. (2017), rat thyroid cells were irradiated with X-rays with or without NAC pre-treatment; a reduced number of strand breaks and a concordant reduction in MN frequency were observed in NAC-treated cells. Together, these two studies demonstrate the effect of modulating DNA strand breaks on the two AOs of the AOP network.

7 | UNCERTAINTIES AND KNOWLEDGE GAPS

During the process of developing this AOP network, we identified areas requiring further understanding and empirical evidence. Most importantly, the quantitative relationships between the KEs within the AOP network require development. In order to facilitate predictive toxicology, the level to which oxidative DNA damage occurs in order to result in mutations and chromosomal aberrations must be determined. Currently, empirical evidence to establish quantitative relationships is limited; there is a lack of studies specifically measuring DNA strand breaks, chromosomal aberrations, and mutations in relation to the quantities of oxidative DNA lesions detected at early time points. Fully evaluating the modified BH criteria was a challenge for certain KEs because the methods used to measure the MIE, KEs, and AOs differ vastly in sensitivity and analytical sampling times (e.g., comet assay for strand breaks vs. gene mutation and MN assays). Thus, a better understanding of the dose–response curves (thresholds and shapes) of different commonly used assays and the quantitative relationships between the responses measured by these assays are critical for establishing stronger causal relationships between the KEs.

Current knowledge of the consequences of oxidative base lesions is largely based on studies focusing on 8-oxo-dG. Indeed, the literature on the mutagenicity and repair of oxidative base lesions is overrepresented by studies on 8-oxo-dG, while the mutagenic and clastogenic potential of other oxidative lesions, such as 8-oxo-dA and thymine glycol, are not as well defined (Bellon et al., 2009; Kalam et al., 2006). Furthermore, the mechanisms through which oxidative base lesions cause clastogenicity and mutagenicity involve several different repair pathways. While BER is the primary repair pathway for oxidative lesions, MMR and NER may also play important roles in repairing oxidative damage (Brierley & Martin, 2013; Melis et al., 2012). For example, MMR removes oxidized bases that are inserted into DNA during replication, predominantly 8-oxodGMP paired to dC, as well as non-oxidized bases inserted opposite oxidatively damaged bases, such as dAMP inserted opposite 8-oxodG (Bridge et al., 2014; Colussi et al., 2002). NER is responsible for repairing bulkier, helix-distorting lesions induced by free radicals, such as 8,5-cyclopurine-2-deoxynucleosides and intra- and inter-strand crosslinks (Melis et al., 2012). In cells with defective BER glycosylases, NER has been observed to provide additional support in repairing oxidatively altered bases that do not disturb the helical structure (Melis et al., 2012). Furthermore, there are genetic factors that may modulate a cell’s susceptibility to oxidative stress and associated DNA damage. For example, cells that are deficient in BRCA1 or BRCA2, proteins involved in genome maintenance, have been observed to have elevated levels of intracellular ROS and to be more sensitive to oxidative DNA damage in the S phase due to compromised HR (Fridlich et al., 2015; Renaudin et al., 2021). Overall, the interplay of different repair pathways in modulating the KEs downstream of the MIE requires further research to better define both the mechanistic and quantitative understanding of the AOP.

Studies on oxidative damage to nuclear DNA (nDNA) were examined exclusively when developing this AOP network. However, we note that oxidative damage occurs not only in nDNA but also in the deoxynucleotide (dNTP) and ribonucleotide (rNTP) triphosphate pools and in mitochondrial DNA (mtDNA). mtDNA is more susceptible to oxidative damage than nDNA due to its location and crosstalk exists between the nucleus and mitochondria during oxidative stress; DNA glycosylases that initiate BER such as OGG1 are encoded by nDNA and the enzymes translocate to the mitochondria to repair oxidative mtDNA damage (Cha et al., 2015; Saki & Prakash, 2017). BER is essential for maintaining mitochondrial genome stability as it is for the nuclear genome (Cha et al., 2015). Oxidized dNTPs and rNTPs, especially 8-oxodGTP and 8-oxoG, can be inserted into both genomes during replication and excision repair, giving rise to mismatches and impeding the repair of existing damage, respectively; both scenarios can directly lead to inadequate DNA repair, promoting the progression of this AOP network (Caglayan et al., 2017; Colussi et al., 2002; Malfatti et al., 2017; Russo et al., 2004). Moving forward, KEs addressing oxidative damage to the dNTP pool and mtDNA are necessary to build a more complete map of oxidative stress-related genotoxicity and to expand the AOP network to other related AOs.

8 | OVERALL WEIGHT OF EVIDENCE ASSESSMENT (SUMMARY) OF THE AOP NETWORK

8.1 | Biological plausibility: Strong

Rationale: Considering the current literature and mechanistic understanding of oxidative DNA lesions, the repair pathways, and the induction of the two AOs, the biological plausibility of the AOP network is strong. Extensive research has been performed to investigate the toxicity of 8-oxo-dG; this guanine lesion is known to be abundant and is an accepted biomarker of oxidative stress and DNA damage. Many studies have reported a strong positive correlation between the levels of 8-oxo-dG and G to T transversions. Mechanistically, 8-oxo-dG is known to base pair with both dC and dA, but with a stronger preference for dA. Indeed, G to T transversions are the most prevalent base substitutions observed under oxidative stress-inducing chemical exposures and in BER glycosylase knock-out cells and rodents. Moreover, BER, the primary repair pathway for oxidized base lesions, and strand break repair pathways (HR and NHEJ) have been extensively studied and the existence of a limit in repair capacity of DNA repair is well known. It was established that inadequate BER and strand break...
repair can both lead to increased strand breaks downstream. Finally, inadequate repair of strand breaks leading to structural genomic damage has been observed in numerous studies and is a mechanistically plausible event.

8.2 | Essentiality of the KEs: Strong

Rationale: The essentiality of the MIE and the two KEs has been demonstrated by studies that modulated these events in both in vitro and in vivo models. An increase or decrease in oxidative DNA lesions, DNA repair capacity (via gene knock-out), or DNA strand breaks led to concordant changes in the downstream KEs and the AOs in a variety of model systems.

8.3 | Empirical evidence supporting the KERs: Moderate

Rationale: Empirical evidence supporting individual KERs varies between weak and strong. The two KERs with strong WOE, Increases in Oxidative DNA Damage leading to Mutations and Inadequate DNA repair leading to Mutations, are supported by data from both in vivo and in vitro studies (e.g., OGG1 deficiency or overexpression in human cells and rodents) that demonstrate concentration/dose, incidence, and temporal concordances in the two events. However, the extent of evidence available to evaluate the BH criteria for certain KERs (especially strand breaks leading to the two AOs) was low because most studies are not designed to take this into consideration and do not measure all the necessary endpoints to support a KER within a single study. Moreover, concentration and temporal concordance analysis was not feasible in many cases because of the use of methods to measure strand breaks that differed in sensitivity, dynamic range, and measurement time points.

8.4 | Quantitative understanding: Weak

Rationale: There is very limited quantitative understanding of the amount of oxidative DNA damage that exceeds repair capacity and leads to mutations and chromosomal aberrations; few studies have specifically investigated these relationships using a quantitative approach. In order to address the current gaps in quantitative understanding, future studies should be designed to concurrently measure the KEs of this AOP over increasing stressor concentrations and time in different model systems. Such experiments would allow quantitative analysis of the changes in the KEs in relation to one another.

9 | CONCLUDING REMARKS

Oxidative stress is a cellular hazard that contributes to DNA damage during chemical insult; indeed, the ability to induce oxidative stress and genotoxicity are two prevalent characteristics of carcinogens. The AOP network described here provides WOE that links early oxidative DNA damage to regulatory endpoints in genetic toxicology (Heflich et al., 2020). As the genotoxicity testing paradigm moves toward integrating more quantitative and predictive in vitro methods, incorporation of mechanistic information will become necessary in chemical safety assessment. The AOP framework provides an effective method for organizing mechanistic knowledge and identifying knowledge gaps. As demonstrated in the development of the AOP network, the framework can be used to coordinate information from various mechanism-based assays to assess the probability that a chemical will cause oxidative DNA damage leading to mutations and/or chromosomal aberrations. As such, AOPs that describe genotoxic pathways can provide the foundation for designing IATA for genotoxicity. With new mechanistic assays measuring non-traditional genotoxicity endpoints emerging, AOPs will help contextualize their utility in applied genetic toxicology. Incorporating AOPs in genotoxicity assessment will facilitate the transition toward predictive, mechanism-based testing paradigms for more streamlined and more efficient assessment of chemical safety.

10 | STATEMENT OF AUTHOR CONTRIBUTIONS

All authors contributed to determining the KEs in the AOP and the structure of the flow diagram. EC provided first drafts of KER(R) and the overall assessment of the AOP, received and consolidated input from co-authors, revised accordingly, and managed all information in the AOPWiki. All authors provided content during KE(R) development, reviewed and agreed to the final AOP, and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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