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RESEARCH ARTICLE

Advancing Genotoxicity Assessment by Building a Global AOP Network

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ABSTRACT

Current genotoxicity testing strategies face several challenges, including a high incidence of misleading positive results that lead to unnecessary animal testing, limited mechanistic insights, insufficient integration of innovative methodologies, and a lack of quantitative assessment. Despite rapid advancements in technology and scientific understanding, genotoxicity testing batteries have remained largely unchanged for years. To modernize genotoxicity assessment and incorporate innovative approaches, the development of Integrated Approaches for Testing and Assessment (IATAs) is essential. These frameworks combine existing knowledge with data from New Approach Methodologies (NAMs) aiming to reduce or eliminate reliance on in vivo testing. Genotoxicity is particularly well-suited for IATA development as numerous cutting-edge, non-animal methods have emerged in recent years, including 3D test systems, Prediscreen, MultiFlow, ToxTracker, and transcriptomic-based biomarkers such as GENOMARK and TGx-DDI. However, the integration of NAMs into IATAs must be systematic and scientifically robust. In this process, the Adverse Outcome Pathway (AOP) framework plays a crucial role by linking molecular-level events to adverse health effects, thereby supporting the structured selection of NAMs. This article explores the key challenges and gaps within the current European regulatory frameworks for chemical compound genotoxicity assessment and discusses how an AOP-based IATA can address these issues. Additionally, we present a global AOP network for permanent DNA damage, designed to guide IATA development and improve regulatory decision-making. This integrated

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approach has the potential to enhance the accuracy, efficiency, and ethical standards of genotoxicity assessment while reducing reliance on animal testing.

1 | Current Strategies for Genotoxicity Assessment and Limitations

Genotoxicity, the ability to induce damage to the genetic material of living organisms, is a fundamental concept in the field of toxicology. Understanding genotoxicity is pivotal in assessing the potential risks posed by chemicals, drugs, environmental pollutants, and other agents (e.g., ionizing radiation) that humans might be exposed to. A genotoxic agent has the ability to alter the structure, information content, or segregation of DNA, including by interfering with normal replication processes (European Chemicals Agency. 2017). In humans, genetic damage in somatic cells is linked to severe health outcomes such as cancer, premature aging, and cardiovascular diseases. In contrast, DNA damage to germ cells can result in infertility or inheritable genetic disorders (Phillips and Arlt 2009; Hanahan and Weinberg 2011; Choudhuri et al. 2021). Consequently, genotoxicity testing forms a cornerstone of modern toxicological risk assessment, aiding in the identification of substances that have the potential to induce DNA damage (i.e., gene mutations, chromosomal aberrations) which can significantly impact human health (Phillips and Arlt 2009).

Current genotoxicity testing of chemical compounds typically targets three main endpoints: gene mutations, structural chromosome aberrations (clastogenicity) and numerical chromosome aberrations (aneugenicity) (Steiblen et al. 2020). There is no single test that covers all three genotoxic endpoints, and consequently, a battery of tests is used (OECD 2017). Although the composition of the test batteries varies between different regulatory domains (e.g., chemicals, cosmetics, pharmaceuticals, plant protection products, biocides and pharmaceuticals) and jurisdictions, they consistently follow the same principles. Genotoxicity testing of chemicals in Europe generally starts with a set of in vitro assays. Depending on the outcome of the in vitro tests and the legislative context, in vivo testing will be performed as a second step. The in vitro test battery includes a bacterial reverse gene mutation test (also called "Ames test") and/or a mammalian gene mutation assay to evaluate the chemical's potential to induce gene mutations, as well as a mammalian cell test to detect chromosomal damage such as the in vitro micronucleus test or the in vitro chromosomal aberration test (OECD 2017; Fortin et al. 2023; USFDA 2007). Selection of the in vivo follow-up tests is based on the in vitro results. If these are positive, the selected in vivo test should address the same endpoint as the positive in vitro test (OECD 2017; Fortin et al. 2023; USFDA 2007; European Chemicals Agency. 2017, 2022; EFSA Scientific Committee 2011).

A brief overview of the different genotoxicity testing requirements per chemical domain within the EU is provided in Table 1. Depending on the legislative context, in vivo genotoxicity testing for chemicals is thus either prohibited (e.g., cosmetics), required in all conditions (e.g., active ingredients of plant protection products and pharmaceuticals), or only performed in case of a positive in vitro test result (e.g., REACH). However, according to the European Directive 2010/63/EU on the protection of animals, wherever possible, methods or strategies should be used that do not entail living animals. Moreover, the REACH regulation requires that new testing of a substance involving vertebrate animals is only conducted as a last resort.

As we already have in vitro genotoxicity tests, why do we, in many cases, still need to rely on in vivo testing? The most important reason is that the current in vitro testing battery is facing different limitations (Buick et al. 2021; Adeleye et al. 2015):

1. *The high rate of "misleading" in vitro positive results*: Traditional in vitro genotoxicity tests often have low specificity, meaning that a positive result in the in vitro test does not always correlate with a confirmed genotoxic outcome in an appropriate in vivo follow-up test (Adeleye et al. 2015; Hartung 2009; Thienpont et al. 2023, 2024). These false positives can lead to unnecessary animal testing, wasting resources, or prematurely halting the development of promising compounds. However, with the introduction of a maximum testing concentration, the use of cell types that are p53 competent, and a stronger emphasis on taking into account cytotoxicity in the evaluation of test results, the rate of false positives has dropped substantially (Whitwell et al. 2015).
2. *The lack of quantitative analysis of the collected genotoxicity data*: While in vitro and in vivo genotoxicity assays are primarily used for hazard identification, they have been minimally utilized for more advanced hazard and risk assessment (Phillips and Arlt 2009). Quantitative analysis of genotoxicity data would, however, offer several opportunities such as potency ranking of compounds, determination of a reference point from genetic dose–response data, calculation of a margin of exposure, or derivation of a health-based guidance value. Although over recent years, important achievements have been made in the quantitative analysis of genotoxicity data (Beal et al. 2023; Nicolette et al. 2021; Chepelev et al. 2023; Luijten et al. 2020; White et al. 2020; Thienpont et al. 2024), several major challenges still need to be addressed, including a better understanding of how the size of effects measured in in vitro assays relates to the occurrence of adverse human health effects (Choudhuri et al. 2021; Hanahan and Weinberg 2011).
3. *The limited mechanistic information provided*: Traditional in vitro tests often provide no or only limited mechanistic information. Obtaining more insights into the underlying mode of action (MoA) of a chemical could help to identify whether a chemical operates through a threshold mechanism and provides more meaningful data for quantitative risk assessments (Choudhuri et al. 2021; Hanahan and Weinberg 2011).
4. *The throughput of the assays*: Current genotoxicity tests have a limited throughput, making them insufficient to handle the large number of naturally occurring, newly developed, or commercially available chemicals that need to be tested (Buick et al. 2021; OECD 2017). Indeed, the chemical space potentially encompasses billions of natural and synthetic molecules (Medina-Franco

TABLE 1 | Brief overview of the different mutagenicity tests required per chemical domain within the EU.

	In vitro testing battery			In vivo testing		
Regulatory framework	**Gene mutation assay in bacteria**	**Mammalian cell gene mutation assay**	**Cytogenicity study in mammalian cells**	**Somatic cells**	**Germ cells**	**Role of NAMs**
Chemicals *Regulation (EC) No. 1907/2006 on the Registration, Evaluation, Authorisation and restriction of CHemicals (REACH)*	***Requirement***: Always ***Test***: OECD TG 471	***Requirement***: ≥10 t/year (*waiving possible in certain scenarios*) ***Tests*** **(*one out of the two*)**: OECD TG 476 OECD TG 490	***Requirement***: ≥10 t/year ***Test***: OECD TG 487	***Requirement***: Only in case of positive results in one or more in vitro tests. ***Tests*** **(*to be selected based on positive* in vitro *results*)**: OECD TG 474 OECD TG 489	***Requirement***: Only for substances ≥100 t/year if positive results in in vivo tests in somatic cells and no clear conclusion on germ cell mutagenicity. ***Tests*** **(*one out of the two*)**: OECD TG 483 OECD TG 488	Supporting information (e.g., QSAR models for gene mutations in bacteria)
Plant protection products *Regulation (EC) No. 1107/2009 concerning the placing of plant protection products on the market* *Regulation (EU) No. 283/2013 outlining the specific data requirements for active ingredients of plant protection products* *Guidance provided by*EFSA Scientific Committee (2011) and EFSA (2021)	***Requirement***: Always ***Test***: OECD TG 471	***Requirement***: No legal requirement	***Requirement***: Always ***Tests***: OECD TG 487	***Requirement***: Always ***Tests*** **(*at least one of the following if* in vitro *results are negative, otherwise depending on the positive* in vitro *results*)**: OECD TG 474 OECD TG 488 OECD TG 489	***Requirement***: On a case-by-case basis	Supporting information; additional in vitro testing may be needed for active substances bearing structural alerts, if the standard tests have not been optimized for these alerts

(Continues)

TABLE 1 | (Continued)

Regulatory framework	In vitro testing battery: Gene mutation assay in bacteria	In vitro testing battery: Mammalian cell gene mutation assay	In vitro testing battery: Cytogenicity study in mammalian cells	In vivo testing: Somatic cells	In vivo testing: Germ cells	Role of NAMs
Biocides *Regulation (EU) No. 528/2012 on the placing on the market of biocidal products* *Commission Delegated Regulation (EU) 2021/525* *ECHA Guidance Vol III Part A* *ECHA Guidance Vol III Parts B + C*	***Requirement***: Always ***Test***: OECD TG 471	***Requirement***: Always ***Tests*** **(*one out of the two*)**: OECD TG 476 OECD TG 490	***Requirement***: Always ***Tests***: OECD TG 487 (preferred) OECD TG 473	***Requirement***: Only in case of positive results in one or more in vitro tests ***Tests*** **(*to be selected based on positive* in vitro *results*)**: OECD TG 474 OECD TG 475 OECD TG 488 OECD TG 489	***Requirement***: Only in case of positive results in in vivo tests in somatic cells and lack of toxicokinetic evidence on the capacity of the substance to reach germ cells. ***Tests*** **(*one out of the two*)**: OECD TG 483 OECD TG 488	Supporting information
Pharmaceuticals *ICH guideline S2 (R1)*	***Requirement***: Always ***Test***: OECD TG 471	***Requirement***: if cytogenetic study is not available (option 1). ***Test***: OECD TG 490	***Requirement***: if mammalian gene mutation assay not available (option 1). ***Tests*** **(*one out of the two*)**: OECD TG 487 OECD TG 473	***Requirement***: One assay on chromosomal damage (Options 1 and 2). Second in vivo assay (Option 2). ***Tests***: OECD TG 474 or 475 (Options 1 and 2) One extra in vivo assay typically detecting DNA damage in the liver (Option 2)	***Requirement***: No legal requirement	Supporting information

(Continues)

TABLE 1 | (Continued)

Regulatory framework	In vitro testing battery			In vivo testing		Role of NAMs
	Gene mutation assay in bacteria	**Mammalian cell gene mutation assay**	**Cytogenicity study in mammalian cells**	**Somatic cells**	**Germ cells**	
Cosmetics *SCCS 12th Rev of Notes of Guidance*	***Requirement***: Always ***Test***: OECD TG 471	***Requirement***: Only if for some reason, OECD TG 471 is not applicable ***Tests***: OECD TG 476 OECD TG 490	***Requirement***: Always ***Tests***: OECD TG 487 (preferred) OECD TG 473	***Animal ban testing***	***Animal ban testing***	NAM toolbox can be applied in case of inconclusive in vitro results; the toolbox includes for example indicator assays (e.g., ToxTracker, γH2Ax/pH 3 assays, in vitro (modified) comet assay, ...), toxicogenomics, new gene mutations assays (e.g., in vitro Pig-a-test, ecNGS, ...), comet and micronucleus on 3D reconstructed skin models and high-information content methods.

Note: OECD TG 471: bacterial reverse mutation test (or "Ames test"); OECD TG 473: in vitro chromosomal aberration test; OECD TG 474: mammalian erythrocyte micronucleus test; OECD TG 475: mammalian bone marrow chromosomal aberration test; OECD TG 476: in vitro mammalian cell gene mutation tests using Hprt and xprt genes; OECD TG 483: mammalian spermatogonial chromosomal aberration test; OECD TG 487: in vitro mammalian cell micronucleus test; OECD TG 488: transgenic rodent somatic and germ cell gene mutation assays; OECD TG 489: in vivo mammalian alkaline comet assay; OECD TG 490: in vitro mammalian cell gene mutation tests using the Thymidine Kinase gene (OECD 2014, 2016a, 2016b, 2016c, 2016d, 2016e, n.d., 2016f, 2020b, 2022).

et al. 2022), which are, for the most part, uncharted in terms of toxicological characterization (Brändli 2023; McCord et al. 2022).

5. *Inadequate metabolic activity of test systems*: A major limitation of many in vitro genotoxicity assays is the absence or insufficient representation of the metabolic activity that occurs in humans. Many chemicals require metabolic activation to become genotoxic (e.g., benzo[*a*]pyrene; acrylamide) (Arlt et al. 2008; Din et al. 1993; Koyama et al. 2011; Henneberger et al. 2024). In vitro test systems may lack the necessary metabolic enzymes or co-factors to simulate this process, leading to false negatives for chemicals that need activation to induce genotoxic effects. Some in vitro assays address this by incorporating liver microsomes or S9 metabolic activation systems (Ames et al. 1973), which contain enzymes like cytochrome P450 (Bak et al. 2024). However, these systems, especially those derived from non-human species, may not fully mimic human metabolism, causing inaccuracies in the safety assessment (Koyama et al. 2011; Dobo et al. 2009; Brendt et al. 2021; Nesslany 2017).

Modern regulatory genotoxicology thus needs tests providing mechanistic and more human-relevant information with higher throughput and higher content for effective chemical evaluation (Buick et al. 2021). Over the last decades, efforts have been made to increase the throughput of standard tests, such as the in vitro micronucleus test (OECD 2023) using automated image analysis and Fluorescence-activated cell sorting (FACS) (Fenech et al. 2013; Rodrigues et al. 2021). On the other hand, several new innovative animal-free methods have been developed. Despite this progress, the integration of these new methods into regulatory decision-making remains limited due to the time-consuming and resource-intensive process of validation and acceptance (van der Zalm et al. 2022). However, some of these methods are included in the work plan of the OECD Test Guideline program (e.g., ToxTracker, pH3/yH2AX and 3D reconstructed human skin micronucleus and comet assay) with the goal of transitioning towards next-generation genotoxicity assessment (European Chemicals Agency. 2023; Nicolette et al. 2021; Luijten et al. 2020; Dearfield et al. 2017). The development and application of these new methods will play an increasingly important role in filling critical data gaps related to the safety of chemical compounds for human health. However, confidence in their use will have to grow through experience, data sharing and continued learning (Krewski et al. 2020, 2010).

The domain of genotoxicity has thus access to a large number of New Approach Methodologies (NAMs). The term NAMs gather any technology, methodology or combination that can provide information on chemical hazard and risk assessment while avoiding the use of animals, and may include in silico, in chemico, in vitro, and ex vivo approaches (Bajard et al. 2023; Jacobs et al. 2020). Nevertheless, as outlined above, in vivo testing is still required in different regulatory settings. One possibility to stimulate the use of NAMs and facilitate the interpretation of their results is to combine them in an Integrated Approach for Testing and Assessment (IATA). Such an IATA represents a framework for integrating information about chemical substances regarding a toxicological endpoint in order to support chemical safety within a regulatory context. IATAs combine already existing information along with newly generated data from NAMs or conventional toxicity testing methods to fill data gaps and ideally not rely on animal testing or only as a last resort. By first focusing on existing data and filling data gaps by using NAMs, IATAs can potentially reduce, or even abolish, animal testing. In addition, IATAs can use high-throughput methods to rapidly assess a large number of chemicals, and thus cover a greater chemical space (OECD 2020a). The selection of data sources and NAMs in the IATA may be challenging and should be science-driven, a process in which the adverse outcome pathway (AOP) framework can play an important role (Bajard et al. 2023). Indeed, the AOP framework has already proven its usefulness by supporting the development of (conceptual) IATAs for skin sensitization and non-genotoxic carcinogens (Jacobs et al. 2020; "Guideline No. 497: Defined Approaches on Skin Sensitisation" 2023) and can facilitate the identification of the most suitable assays for measuring/informing on biological key events (Tollefsen et al. 2014; Willett 2019).

2 | Adverse Outcome Pathways

The conceptual framework of the Adverse Outcome Pathway (AOP) was designed to function as a knowledge compilation and communication instrument, enabling the transparent conversion of mechanistic data into outcomes that hold significance in the context of (chemical) safety assessment (Ankley and Edwards 2018). An AOP represents an analytical construct that describes a sequential chain of causally linked events at different biological levels leading to an adverse health or ecotoxicological effect of regulatory concern. The first biological event, called "Molecular Initiating Event (MIE)," describes the initial interaction between a stressor and a biomolecule within an organism causing a perturbation in its biology. This interaction is then followed by a cascade of intermediate Key Events (KEs) to finally culminate in an Adverse Outcome (AO) considered relevant to risk assessment or regulatory decision-making. All these KEs are linked by Key Event Relationships (KER), describing the causal and predictive relationship between the upstream and downstream KE with scientific knowledge (Villeneuve et al. 2014).

In this respect, AOPs define a sequence of biological changes expected to occur when the perturbation is sufficiently important to initiate the pathway and lead to the final AO. AOPs only focus on describing critical checkpoints along the path, which are both measurable and have potential predictive value. This focus on essentiality implies that each KE plays a causal role, meaning that if a KE fails to occur, the pathway cannot progress to the AO. In addition, AOPs are chemical agnostic. They can thus be initiated by any chemical or other agent capable of triggering the MIE (OECD 2018).

AOPs capture and organize what is known, and their development thus allows identifying current knowledge gaps, offering the opportunity to fill them and improve predictive utility. The objective underlying AOP development is to ultimately support extrapolations from one KE to another, which are consistent

with the proposed vision for regulatory toxicology in the 21st century. Indeed, extrapolations from KE measurements that are made efficiently and cost-effectively, typically at low levels of biological organization, to adverse effects at higher levels are relevant to regulatory protection goals and decision-making (OECD 2018; Krewski et al. 2010). In addition, AOPs sharing one or several common KEs can be combined into an AOP network, which is defined as an assembly of two or more AOPs (Knapen et al. 2018). AOP networks can capture broader knowledge concerning the range of possible AOs a perturbation may cause, or the range of ways in which an AO may occur. They are useful for addressing chemicals involved in multiple MIEs (OECD 2018) or evaluating the effects of combined chemicals. Several AOP networks have been presented and discussed in the literature (Veltman et al. 2023; Spinu et al. 2019; Cayley et al. 2023; Myden et al. 2023).

The AOP-Wiki (https://aopwiki.org) is a module of the AOP Knowledge base (AOP-KB), a central information and communication repository for AOPs. All AOPs included in the AOP-Wiki are monitored by members of the Society for the Advancement of AOPs (SAAOP) and thus described by a SAAOP status. AOPs relevant to regulatory applications can be proposed by scientists from OECD member countries for review by the Advisory Group on Emerging Science in Chemicals Assessment (ESCA), which oversees the essential elements of the OECD AOP Development Program. Proposals that receive support from ESCA will be recommended for inclusion in the workplan of the Working Party of the National Coordinators of the Test Guidelines Programme (WNT) or the Working Party on Hazard Assessment (WPHA), as appropriate. When an AOP project is accepted for inclusion in the work plan, it will also receive an "OECD status" (O.N.E, n.d.).

In the field of genotoxicity, AOPs can provide a framework to characterize relationships between the induction of DNA damage and adverse health outcomes, thereby supporting the organization of data generated with existing genotoxicity methods and providing insights into the information collected with new methods (Bajard et al. 2023; Sasaki et al. 2020). Moreover, the integration of AOPs linking different MIEs to either gene mutations, structural and/or numerical chromosome aberrations into a network could serve as a multi-entry structure for the induction of "permanent DNA damage." Such a network can provide an excellent basis for the integration of KE-specific NAMs into IATAs for genotoxicity aiming to address different regulatory questions.

3 | Draft AOP Network Leading to Permanent DNA Damage Based on Existing AOPs

3.1 | Compiling an Inventory of the AOPs Linked to DNA Damage Present in the AOP-Wiki

As a first step, an inventory of all AOPs present in the AOP-Wiki and with a link to DNA damage was compiled (Table 2).

Nineteen AOPs of interest were found on the AOP-wiki, 10 of which are included in the OECD work plan. At present, four of them are already endorsed by WPHA/WNT, the most advanced stage of AOP development. Two others are currently under review by ESCA, and four are under development. Next, a first draft AOP network combining the 10 AOPs included in the OECD work plan was built. Other AOPs (not included in the OECD work plan) were excluded from building this network, as further analysis showed that these AOPs did not bring supplementary information.

Although a draft AOP network could be designed based on the individual AOPs, several challenges were encountered, which are explained in the following paragraphs. These challenges have been discussed with the Society for Advancement of AOPs Knowledgebase Interest Group (SKIG) (Wittwehr et al. 2025).

3.2 | The Genotoxic AOs

From a regulatory point of view, the different genotoxic AOs that have to be covered are mutagenicity, that is, the ability to induce gene mutations, clastogenicity, that is, the ability to modify the structure of chromosomes, and aneugenicity, that is, the ability to alter the number of chromosomes. For each of the three AOs, existing KEs were directly found on the AOP-Wiki:

- "Increase, Mutations" (https://aopwiki.org/events/185): A mutation is a change in the DNA sequence that can affect the coding regions of genes, potentially resulting in malformed or truncated proteins. Mutations can also occur in promoter regions, splice sites, non-coding RNAs, or other functional genomic elements, potentially altering gene expression. Various types of mutations exist, including missense, nonsense, insertions, deletions, duplications, and frameshift mutations, and can uniquely impact the genome and its regulation (Loewe 2008).
- "Increase, Chromosomal aberrations" (https://aopwiki.org/events/1636): Structural chromosome aberrations refer to missing, extra, or altered segments of chromosomal DNA, often arising from errors in double-strand break (DSB) repair mechanisms. There are different types of chromosomal aberrations: deletions, duplications, translocations, and inversions. Moreover, structural chromosome aberrations can be categorized based on whether they affect the entire chromosome or a single chromatid. Chromosome-type aberrations include chromosome breaks, ring chromosomes, marker chromosomes, and dicentric chromosomes. In contrast, chromatid-type aberrations involve chromatid breaks and chromatid exchanges (Preston 2014). As this KE describes the different types of structural chromosome aberrations and thus refers to clastogenicity, we suggest renaming it as "Increase, Structural chromosome aberrations," which is more adequate to distinguish it from the third AO in our network, that is, the increase in numerical chromosome aberrations or aneugenicity.
- "Altered, Chromosome number" (https://aopwiki.org/events/723): Aneuploidy is an abnormality in the number of chromosomes in a cell due to loss or duplication. In humans, aneuploidy would be any number of chromosomes

TABLE 2 | Inventory of AOPs related to genotoxicity collected from www.AOP-Wiki.org in August 2024.

AOP-Wiki ID	Title	SAAOP status	OECD status	Link
15	Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations	Included in OECD Work Plan	WPHA/WNT Endorsed	https://aopwiki.org/aops/15
46	AFB1: Mutagenic mode-of-action leading to hepatocellular carcinoma (HCC)	Included in OECD Work Plan	EAGMST under review	https://aopwiki.org/aops/46
106	Chemical binding to tubulin in oocytes leading to aneuploid offspring	Included in OECD Work Plan	EAGMST under review	https://aopwiki.org/aops/106
139	Alkylation of DNA leading to cancer 1	Under development	x	https://aopwiki.org/aops/139
202	Inhibitor binding to topoisomerase II leading to infant leukemia	Included in OECD Work Plan	WPHA/WNT Endorsed	https://aopwiki.org/aops/202
240	DNA Adducts Leading to liver hemangiosarcoma	Under development	x	https://aopwiki.org/aops/240
272	Direct deposition of ionizing energy onto DNA leading to lung cancer	Included in OECD Work Plan	WPHA/WNT Endorsed	https://aopwiki.org/aops/272
293	Increased DNA damage leading to increased risk of breast cancer	Included in OECD Work Plan	Under development	https://aopwiki.org/aops/293
294	Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer	Included in OECD Work Plan	Under development	https://aopwiki.org/aops/294
296	Oxidative DNA damage leading to chromosomal aberrations and mutations	Included in OECD Work Plan	WPHA/WNT Endorsed	https://aopwiki.org/aops/296
322	Alkylation of DNA leading to reduced sperm count	x	x	https://aopwiki.org/aops/322
331	Formation of DNA photoproducts leading to growth inhibition (1)	x	x	https://aopwiki.org/aops/331
332	Formation of DNA photoproducts leading to growth inhibition (2)	x	x	https://aopwiki.org/aops/332
333	Formation of DNA photoproducts leading to growth inhibition (3)	x	x	https://aopwiki.org/aops/333
397	Bulky DNA adducts leading to mutations	Included in OECD Work Plan	Under development	https://aopwiki.org/aops/397
441	Radiation-induced microcephaly	Under development	Under development	https://aopwiki.org/aops/441
443	Alcohol Induced DNA damage and mutations leading to metastatic breast cancer	Included in OECD Work Plan	Under development	https://aopwiki.org/aops/443
451	Interaction with lung resident cell membrane components leads to lung cancer	x	x	https://aopwiki.org/aops/451
472	DNA adduct formation leading to kidney failure	x	x	https://aopwiki.org/aops/472

other than the usual 46 (Aneuploidy, n.d.). This KE is included in one AOP (https://aopwiki.org/aops/106), which is under review (OECD status) by ESCA. However, to harmonize the terminology in the AOP network, we suggest renaming this KE as "Increase, Numerical chromosome aberrations."

Several of the AOPs included in the OECD workplan also contained KEs downstream to permanent DNA damage (e.g., infant leukemia or breast cancer). These downstream KEs were not included in the network as they were considered outside the scope of the current network. However, the AOP network could be connected to other AOPs/AOP networks at a later stage.

The selection of the KEs related to the three AOs also revealed an additional problem: the redundancy of certain KEs.

3.3 | Duplication of KEs

Although KE 1636 "Increase, Chromosomal aberrations" is part of an endorsed AOP, another KE describing this event was found in the AOP-Wiki, that is, KE 1554. Also for other KEs, "duplicates" were found. This phenomenon is due to the lack of reuse of existing KEs (Huliganga et al. 2022). During AOP development, many authors do not strictly follow the AOP development recommendations provided in the AOP Developers' Handbook and create their own KEs, leading to a pollution of the AOP-wiki. This conflicts with the originally anticipated strength of the AOP concept that by allowing the reuse of existing KEs, networks can be formed, capturing a broader space of events and allowing a complex understanding of the different pathways involved (Villeneuve et al. 2014).

To simplify our network, duplicate KEs (e.g., KE 1636 "Increase Chromosomal aberrations" and KE 1554 "Increase chromosomal aberrations," KE 1879 "Formation, Bulky DNA adducts" and KE 373 "Formation, Pro-mutagenic DNA") were identified and the most appropriate one was selected for inclusion. Preference was given to those KEs that are part of the AOPs with the most advanced OECD status and/or those for which the characterization was most in line with the AOP Developer's handbook's recommendations.

3.4 | Missing MIEs/Intermediate KEs

Several MIEs were identified through the AOP-Wiki. These MIEs can be divided into two subgroups; the ones leading to aneugenicity, that is, "Binding to Tubulin," and the others leading to clastogenicity and mutagenicity, that is, "Binding to topoisomerase II enzymes," "Formation, Bulky DNA adducts," "Alkylation of DNA," "Increase, RONS" and "Deposition of energy."

Reviewing the scientific literature revealed that some important MIEs and KEs leading to permanent DNA damage were still missing in the AOP network. The inventory was, therefore, complemented by two other AOPs found in the literature. More specifically, the AOPs "Chemical Binding to the Catalytic Domain of AURKs Leading to Aneuploidy Induction" and "DNA Synthesis Inhibition Leading to Chromosome Breaks and Rearrangements and Mutations" as reported in Sasaki et al. (2020) were integrated into the draft AOP network. The AOP "Chemical Binding to the Catalytic Domain of AURKs Leading to Aneuploidy Induction" was entirely new to the part of the network leading to "Increase, Numerical chromosome aberrations." The AOP related to the inhibition of DNA synthesis shared several KEs with the network, but brought also additional KEs, which could be named as follows:

- "Inhibition of DNA synthesis": This "umbrella" key event regroups several events that can all lead to the progression arrest of the replication fork on the DNA template (Sasaki et al. 2020).
- "Collapse, Stalled replication fork": Replication forks are susceptible to stalling and collapse when they encounter obstacles on the DNA template, such as unrepaired DNA damage, DNA-bound proteins, or secondary structures. Similarly, chemical agents like hydroxyurea and aphidicolin can inhibit replication elongation. A stalled replication fork is temporarily arrested but retains the ability to resume replication once the obstruction is resolved. In contrast, a collapsed fork becomes irreversibly inactivated due to the dissociation of the replication machinery or the formation of DNA double-strand breaks (DSBs) (Petermann and Helleday 2010).

Other MIEs that may lead to permanent DNA damage have not yet been described in the form of an AOP. Based on a rough literature search and expert knowledge, the following MIEs were added to the draft network (Figure 1):

- "Formation, DNA crosslinks": Several types of DNA crosslinks may occur in human cells. *Interstrand DNA crosslinks* (ICLs) are lesions characterized by covalent bonds forming between the opposite strands of double-stranded DNA (Luong et al. 2022; Hashimoto et al. 2016; Dronkert and Kanaar 2001). *DNA–protein crosslinks* (DPCs) are frequent lesions that occur when a protein, regardless of its size or type, becomes covalently attached to DNA following exposure to a physical or chemical crosslinking agent (Fielden et al. 2018; Duxin et al. 2014; Ruggiano and Ramadan 2021). Finally, *DNA intrastrand crosslinks* are covalent bonds formed between two bases on the same strand of DNA. These lesions often result from exposure to agents such as UV light, platinum-based chemotherapeutics (e.g., cisplatin), or reactive chemicals (Luong et al. 2022).
- "Binding to (Interferes with) topoisomerase enzymes": Topoisomerase I enzymes are important regulators of DNA topology. They catalyze changes in DNA topology through transient single-stranded DNA cleavage, strand passage, and relegation. Their involvement in DNA topology regulation potentially makes them critical targets of chemicals (Backer et al. 1990; Xu and Her 2015). As for the topoisomerase II enzyme causing TOP2-DNA complexes when inhibited (Pachva et al. 2020) (https://aopwiki.org/aops/202), inhibition of this enzyme is known to provoke DNA lesions, such as gene mutations and structural chromosome aberrations (Backer et al. 1990).

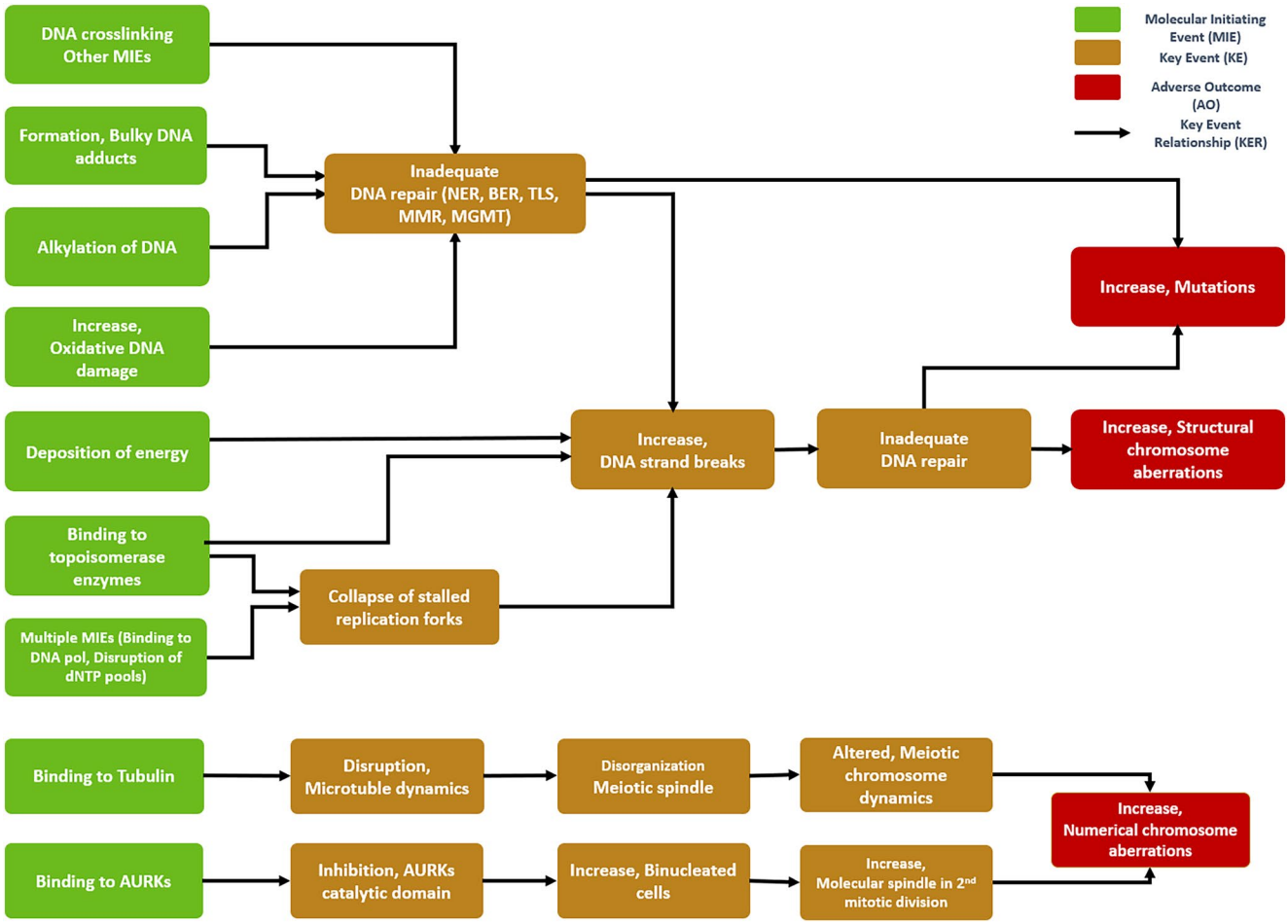

FIGURE 1 | Draft AOP network leading to permanent DNA damage (aneugenicity, clastogenicity, mutagenicity).

3.5 | Integration of Endorsed AOPs

As mentioned above, four endorsed AOPs were integrated into the AOP network. Although the scientific community has already thoroughly reviewed and discussed these AOPs, several challenges were encountered during their integration. For example, the endorsed AOP 15 "Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations" (https://aopwiki.org/aops/15) provides a very solid backbone for our AOP network. However, as the AO 336 "Increase, Heritable mutations in offspring" occurs downstream to the permanent DNA damage, this AO was not included in the network. Importantly, this AO determined the applicability domain of this AOP, that is, mature males and their pre-meiotic germ cells, as only permanent damage occurring in germ cells will lead to heritable mutations in the offspring. The MIE and other KEs in this AOP are not specific to germ cells and can also occur in somatic cells. In combination with other downstream KEs, these might lead to other AOs such as cancer. Except for AO 336, the evidence collected to characterize the KE(R)s in AOP 15 was collected in somatic cells. Consequently, these KE(R)s can be re-used in the AOPs 139 "Alkylation of DNA leading to cancer 1" (https://aopwiki.org/aops/139) and 141 "Alkylation of DNA leading to cancer 2" (https://aopwiki.org/aops/141), which are not yet included in the OECD work plan, as well as in our network which is not limited to germ cells. Also, for the other endorsed AOPs, the KEs downstream to permanent DNA damage were removed, except for the AOP 296 "Oxidative DNA damage leading to chromosomal aberrations and mutations" (https://aopwiki.org/aops/296) that could be fully included in the network (Cho et al. 2022). The inclusion of AOP 296 allows to link the network to other events leading to f reactive oxygen and nitrogen species (RONS) production. For the endorsed AOP 202 "Inhibitor binding to topoisomerase II leading to infant leukaemia," the specific KE 1253 "MLL chromosomal translocation" was not used as it is considered a sub-KE of KE 1636 "Increase, chromosome aberrations." Indeed, this specific KE focuses on a unique gene and the consequence of its translocation. Consequently, it is encompassed by the more general KE 1636.

3.6 | New KE Relationships

Compiling single AOPs into a network generates new relations between KEs of different pathways. For example, the KE "Increase, Structural chromosome aberrations" is now (indirectly) linked to the MIE "Alkylation of DNA." Similarly, the AOP "Formation, Bulky DNA adducts leading to Increase, Mutations" is now also connected to the AO "Increase, Structural chromosome aberrations". The AOP starting with "Binding to topoisomerase II enzyme" now has also a link with "Increase, Mutations." These new links are in line with the evidence that is already available in literature (Backer et al. 1990; Kaina 1998; Kopp et al. 2024; Boos and Stopper 2000).

Considering all the above, a draft network leading to permanent DNA damage emerged, consisting of nine MIEs converging to the three main genotoxic AOs. This network (Figure 1) has been submitted to the OECD WNT and was accepted for inclusion in the work plan.

4 | Refinement of the Draft AOP Network Leading to Permanent DNA Damage

Although the draft AOP network (Figure 1) looks less overwhelming and more comprehensive compared to the first version, some inconsistencies remain.

On one hand, the different steps that may occur between the MIEs and AOs are covered by only three intermediate KEs:

- "Inadequate DNA repair"
- "Increase, DNA strand breaks"
- "Collapse, stalled replication forks"

The KE "Increase, DNA strand breaks" is shown as a unique KE, creating the impression that both single and double-strand breaks arise at a unique moment in the cascade of genotoxic events and have exactly the same consequences. This is not correct as both types of strand breaks are repaired through different processes and/or can be induced through different MoAs. Furthermore, they can arise at different time points and in varying proportions depending on the MIE. Similarly, the KE "Inadequate, DNA repair" appears to encompass all types of DNA repair pathways. However, each DNA repair pathway is more or less specific to a type of DNA damage. The DNA repair processes can arise at different time points and at varying levels. These general genotoxic KEs complicate the accurate characterization and future quantification of the chemicals' MoA.

On the other hand, while some KEs, like "Increase, Oxidative DNA damage," are very general, encompassing several types of DNA damage, others are more specific, such as "Alkylation of DNA" focusing on one specific type of damage. This difference in the level of detail reduces the global harmony of the AOP network. Furthermore, some KERs are not correctly reflected in the network. Indeed, only two MIEs are linked to the KE "Collapse, Stalled replication fork." However, this KE seems to play a central role in the genotoxic cascade as several studies showed its implication after exposure to, among others, alkylating agents, following oxidative damage or even after deposition of energy (Vázquez et al. 2008; Torregrosa-Muñumer et al. 2015; Christensen et al. 2014).

One important reason for these inconsistencies is that AOPs have been developed as "stand-alone" by different groups of scientists focusing on a specific AO and not as part of a larger network. In the next paragraph, we propose further modifications to harmonize the AOP network leading to permanent DNA damage, supported by evidence from relevant studies and reviews. Considering that efforts are ongoing at the level of HESI (Health and Environmental Sciences Institute)—Genetic Toxicology Technical Committee (HESI-GTTC) on the further development of the AOPs related to changes in the number of chromosomes, we will focus on the part of the AOP network leading to gene mutations and structural chromosome aberrations (Figure 2). It is important to note that the following description of our refined AOP network leading to gene mutations and structural chromosome aberrations is not an AOP report

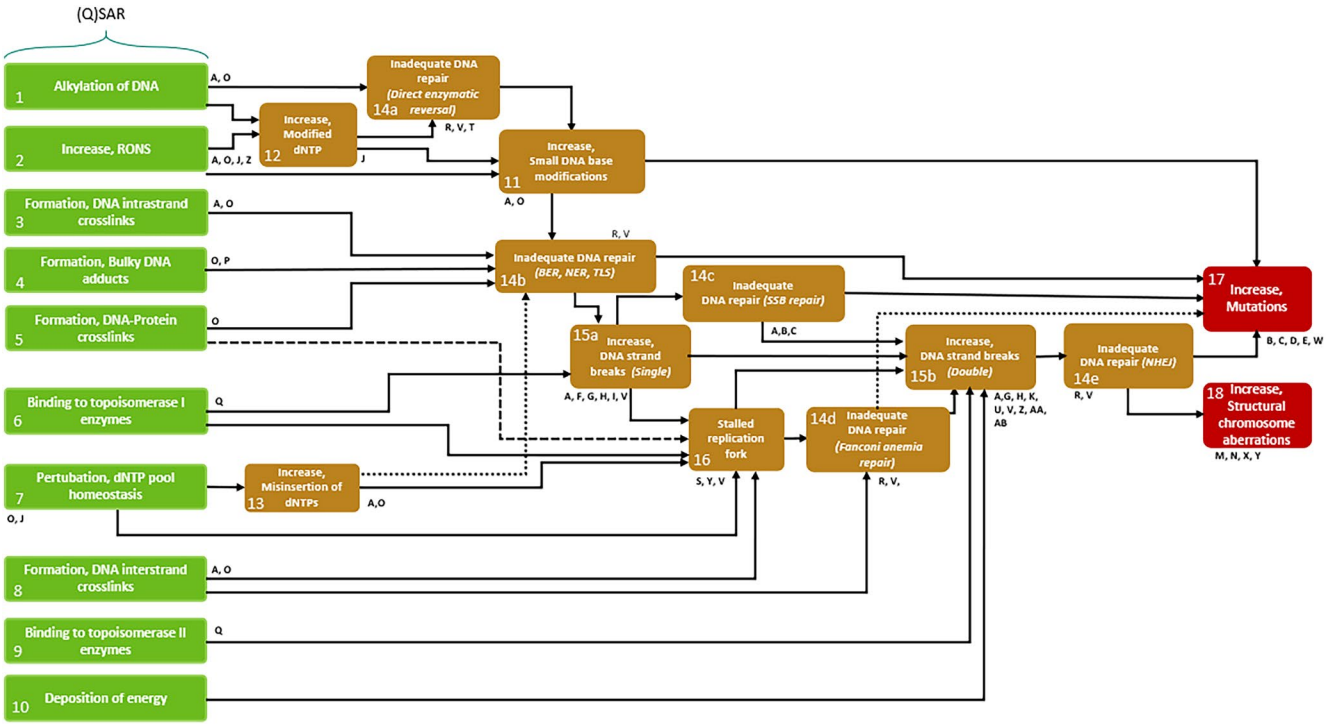

FIGURE 2 | AOP network leading to permanent DNA damage (mutagenicity and clastogenicity). Letters correspond to measurement methods presented in Table 3. Numbers allow to refer KEs in the manuscript.

TABLE 3 | Table linking measurement methods to keys events included in the AOP network leading to permanent DNA damage.

	Measurement method	Key event(s)	Reference(s)
A	Comet assays (CAs) and enzyme-modified CAs	Increase, DNA strand breaks (single)—alkaline CA Increase, DNA strand breaks (double)—neutral CA Alkylation of DNA—enzyme-modified CA Increase, small base modifications—enzyme-modified CA (alkylations, oxidized bases)	(Muruzabal, Collins, and Azqueta 2021; Muruzabal, Sanz-Serrano, et al. 2021; Muruzabal et al. 2020; Collins et al. 2001; Platel et al. 2011; Nikolova et al. 2017; Olive and Banáth 2006; Ge et al. 2014)
B	Mammalian gene mutation assays (HPRT and TK)	Increase, mutations	(Liber and Thilly 1982; Lloyd and Kidd 2012; Yamamoto et al. 2017; Ayres et al. 2006)
C	Bacterial reverse gene mutation assay (Ames test)	Increase, mutations	(Ames et al. 1973)
D	Pig-A/Pig-O assays	Increase, mutations	(Nakamura et al. 2012; Krüger et al. 2015; Chikura et al. 2019)
E	CRISPR-based mutation detection assays	Increase, mutations	(Malekshoar et al. 2023)
F	TUNEL assay	Increase, DNA strand breaks (single)	(Loo 2011)
G	STRIDE assay	Increase, DNA strand breaks (single)—sSTRIDE Increase, DNA strand breaks (double)—dSTRIDE	(Zilio and Ulrich 2021)
H	sBLISS assay	Increase, DNA strand breaks (double)	(Bouwman et al. 2020)
I	Unwiding assay	Increase, DNA strand breaks	(Nacci et al. 1992)
J	Immunological-based assays (ELISA)	Increase, Small DNA base modifications—8-oxodG Increase, DNA strand breaks (Double)—γ-H2AX Increase, modified dNTP	(Zhao et al. 2017)
K	γH2AX quantification	Increase, DNA strand breaks (double)	(Bryce et al. 2016; Rothkamm and Horn 2009; Burma et al. 2001; Revet et al. 2011; Garcia-Canton et al. 2013; Ji et al. 2017; Lorat et al. 2015; Khoury et al. 2016a, 2013)
L	pH 3 quantification	Increase, numerical chromosome aberrations	(Khoury et al. 2016a)
M	Micronucleus assays +FISH/Crest	Increase, structural chromosome aberrations Increase, numerical chromosome aberrations	(OECD, n.d.; Beaton et al. 2013)
N	Chromosomal aberration test	Increase, structural chromosome aberrations	(OECD 2014)

(Continues)

TABLE 3 | (Continued)

	Measurement method	Key event(s)	Reference(s)
O	Chromatography and mass spectrometry (MS) combined assays (HPLC, LC–MS/MS, HPLC-EC, LC-MRM/MS, GC–MS, MS-based proteomics)—DNA adductomics	Alkylation, DNA Increase, reactive oxygen and nitrogen species (RONS) Increase, small DNA base modifications Formation, bulky DNA adducts Formation, DNA crosslinks (intra-/inter-strand crosslinks, DNA-protein crosslinks) Perturbation, dNTP pool	(Groehler et al. 2017; Cao and Zhang 2024; Kanaly et al. 2006; de Groot et al. 1994; You and Wang 2016; Kang et al. 1992; Madugundu et al. 2014)
P	32P postlabeling	Formation, bulky DNA adducts	(Phillips and Arlt 2020)
Q	Cell-free decatenation assay	Binding to topoisomerase I and II enzymes	(Yu et al. 2021; Schroeter et al. 2015)
R	Fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR)	Inadequate DNA repair (direct enzymatic reversal)—MGMT reporter Inadequate DNA repair (NHEJ, HR)—NHEJ reporter, HR reporter	(Nagel et al. 2014)
S	Dual EdU-BrdU Pulse-Chase Labeling Flow Cytometry	Stalled, replication fork	(Bialic et al. 2022)
T	Repair synthesis measurement by 3H-thymine incorporation	Inadequate DNA repair (excision repair)	(Iyama and Wilson 2013)
U	Pusled Field Gel Electrophoresis (PFGE)	Increase, DNA strand breaks (double)	(Gardiner et al. 1986; Ager et al. 1990; Herschleb et al. 2007; Kawashima et al. 2017)
V	mRNA expression profiling (Transcriptomics)	Inadequate DNA repair Increase, DNA strand breaks Stalled, Replication fork	(Yu et al. 2022)
W	SMM-seq, ecNGS (NGS)	Increase, mutations	(Salk et al. 2018)
X	CNV detection (NGS)	Increase, structural chromosome aberrations	(Liu et al. 2013; Shen et al. 2016; Mukherjee et al. 2017)
Y	High content image mining	Stalled, Replication fork (S-Phase perturbation) Increase, structural chromosome aberrations	(Yin et al. 2021; Shahane et al. 2016)
Z	ToxTracker	Increase, reactive oxygen and nitrogen species Increase, DNA strand breaks (double) Increase, numerical chromosome aberrations	(Hendriks et al. 2012; Czekala et al. 2021)
AA	Prediscreen	Increase, DNA strand breaks (double) Increase, Numerical chromosome aberrations	(Khoury et al. 2013, 2016b)
AB	Multiflow	Increase, DNA strand breaks (double) Increase, numerical chromosome aberrations	(Bryce et al. 2016)

(Continues)

TABLE 3 | (Continued)

	Measurement method	Key event(s)	Reference(s)
AC	GENOMARK, TGxDDI, MU2012 (transcriptomic biomarkers)	NA	(Thienpont et al. 2023, 2024; Li et al. 2019; Buick et al. 2021; Li et al., n.d.)
AD	(Quantitative) Structure Activity Relationship tools ((Q)SAR)	Molecular initiating events Increase, mutations Increase, structural chromosome aberrations Increase, numerical chromosome aberrations	(Bartsch 1996; Vogel and Nivard 1994; Pradeep et al. 2021)

nor an evidence assessment. The manuscript compiles sufficient, but not all, information to draft the network leading to gene mutations and structural chromosome aberrations. Thus, it does not follow the principles of documentation provided in the AOP Developers Handbook (O.N.E, n.d.) to generate individual AOPs on the AOP-wiki. Further characterization of certain KEs and KERs in the network using a systematic approach will thusbe needed in the future. Currently, such a systematic approach is being designed and applied to characterize KEs and KERs in the AOP linking the formation of bulky DNA adducts and the increase in gene mutations and structural chromosome aberrations.

4.1 | Refining the KEs Linking the MIEs to the Genotoxic AOs

To complete and harmonize our network, the seven MIEs leading to mutagenicity and/or clastogenicity from the first draft AOP network were rearranged, resulting in 10 MIEs:

- *Alkylation of DNA* (https://aopwiki.org/events/97, Figure 2—MIE 1): *DNA alkylation* is a chemical modification where *alkyl groups* (methyl (Me), ethyl (Et), or other small carbon-based groups) attach to various sites on *DNA bases* (Beranek 1990). Alkylation of DNA results in modified bases (KE 11, KE 14) via first or second-order nucleophilic substitutions (S_N1 or S_N2 reactions) on O- and N-atoms of bases. O^6-alkylguanines (O^6-MeG and O^6-EtG) are the most critical type of DNA alkylation (Fahrer and Christmann 2023).
- *Increase*, *reactive oxygen*, *and nitrogen species* (https://aopwiki.org/events/1632, Figure 2—MIE 2): Reactive oxygen and nitrogen species (RONS) are highly reactive oxygen- and nitrogen-based molecules that often contain or generate free radicals. RONS can provoke oxidation reactions with DNA bases (KE 11, KE 14), the most abundant lesion being the formation of 8-oxo-guanine (8-oxoG) (Kasai 1997; Kasai et al. 1986; Poetsch 2020). RONS are the result of upstream KEs triggered by the compound and, therefore, DNA damage induced by RONS is often referred to as secondary genotoxicity (Schins and Knaapen 2007).
- *Formation*, *DNA Intrastrand crosslinks* (Figure 2—MIE 3): DNA intrastrand crosslinks are DNA lesions where covalent bonds form between two adjacent bases on the same strand of the DNA. They are commonly caused by UV radiation or exposure to certain chemical agents such as cisplatin (McKay et al. 2001). Cyclobutane Pyrimidine Dimers (CPDs) are induced by UV radiation and result from covalent bonding between the C5 and C6 atoms of two adjacent pyrimidines (Mouret et al. 2006). 6–4 photoproducts are UV-induced lesions where covalent bonds form between the C6 atom of one pyrimidine and the C4 atom of an adjacent pyrimidine, causing significant distortion of the DNA helix (Hung et al. 2020). Chemotherapeutic agents like cisplatin and acetaldehyde form intrastrand crosslinks, primarily between two adjacent guanine bases (Sonohara et al. 2019).
- Formation, Bulky DNA adducts (https://aopwiki.org/events/1879, Figure 2—MIE 4): Bulky DNA adducts are generated when activated genotoxic compounds react with the nitrogenous bases of DNA at various sites. The most frequent reactive positions include C8, N7, N3, and N2 of guanine; N7, N6, N3, and N1 of adenine; N3, N4, and O2 of cytosine; and N3, O2, and O4 of thymine (Munnia et al. 2017; Hwa Yun et al. 2020). Bulky adducts are typically formed by exposure to environmental mutagens, chemicals, or certain metabolites (Munnia et al. 2017) such as benzo[a]pyrene and its metabolite BPDE, aflatoxin B1, and aristolochic acid.
- *Formation*, *DNA-protein crosslinks* (*DPCs*) (Figure 2—MIE 5): Chromosomes are associated with numerous structural and regulatory proteins that maintain genome stability, expression, and replication. These proteins can form covalent DPCs due to exposure to ionizing radiation, UV light, endogenous and exogenous reactive aldehydes, or chemotherapeutic agents such as nitrogen mustards and cisplatin (Fielden et al. 2018; Duxin et al. 2014; Ruggiano and Ramadan 2021; Ide et al. 2011; Stingele et al. 2017).
- *Binding to* (*interferes with*) *topoisomerase I* (*TOPO1*) *enzymes* (Figure 2—MIE 6): Topoisomerase I enzymes are important regulators of DNA topology. They catalyze changes in DNA topology through transient single-stranded DNA cleavage, strand passage, and relegation. Their involvement in DNA topology regulation potentially makes them critical targets of chemicals (Backer et al. 1990; Xu and Her 2015).
- *Disruption*, *dNTP pool homeostasis* (Figure 2—MIE 7): The four deoxyribonucleoside triphosphates (dNTPs), dATP, dTTP, dGTP, and dCTP, are the building blocks of DNA

and are then essential for the replication and repair of the nuclear and mitochondrial genome. The homeostasis of the dNTP pool is tightly regulated and is a key prerequisite to faithfully duplicate the human genome (Mathews 2015). Imbalances in their absolute and relative concentrations are then a critical genotoxic initiating event (Kumar et al. 2011). Although few or no studies on chemicals investigated this enhancing event for genotoxicity, the disruption of dNTP pool homeostasis is an important mode of action to monitor (Krawic et al. 2023).

- "Formation, DNA interstrand crosslinks" (Figure 2—MIE 8): Interstrand DNA crosslinks (ICLs) are lesions characterized by covalent bonds forming between the opposite strands of double-stranded DNA (Luong et al. 2022; Hashimoto et al. 2016; Dronkert and Kanaar 2001).
- *Binding to topoisomerase II* (*TOPO2*) *enzymes* (https://aopwiki.org/events/1252, Figure 2—MIE 9): TOPO2 enzymes are ubiquitous enzymes implicated in the maintenance of DNA integrity. Their function implies the transient formation of DNA double-strand breaks (Gasser et al. 1992), a process that becomes critical when disturbed (Backer et al. 1990; Boos and Stopper 2000; Gasser et al. 1992).
- "Deposition of energy" (https://aopwiki.org/events/1686, Figure 2—MIE 10): Ionizing radiation can cause the ejection of electrons from atoms and molecules, thereby resulting in their ionization and the breakage of chemical bonds. Ionizing energy can cause multiple ionization events targeting several structures in a cell including DNA. The breakage of chemical bonds can result in DNA double-strand breaks (Pajic and Rovcanin 2021).

All these MIEs will lead to changes in the DNA structure in the form of different lesion types or by disturbing transcription and replication processes. These critical situations for the cell are represented by the following intermediate KEs.

- *Increase*, *small DNA base modifications* (Figure 2—KE 11): Chemical alterations to individual nucleobases may disrupt base pairing and compromise genomic integrity. These modifications are typically caused by endogenous metabolic processes or environmental factors such as RONS (MIE 2) and alkylating agents (MIE 1).
  - Oxidativedamage(https://aopwiki.org/events/1634):RONS can oxidize guanine to form 8-oxo-7,8-dihydroguanine (8-oxoG), which can mispair with adenine, leading to transversion mutations (Kasai 1997).
  - Alkylation: Alkylating agents can add alkyl groups to bases, such as forming 7-methylguanine or O6-methylguanine, which can disrupt normal base pairing and result in miscoding during replication (Fahrer and Christmann 2023).
- Increase, misinsertion of dNTPs (Figure 2—KE 13): When the homeostasis of the dNTP pool is disturbed, dNTPs may be incorrectly inserted in the newly generated DNA strand (Kumar et al. 2011; D et al. 2010). Different models combining misinsertion, misalignment, and mismatch extension mechanisms have been established to explain imbalanced dNTP-induced gene mutations (Kumar et al. 2011).
- *Increase, Modified dNTPs* (Figure 2, KE 12): As well as alkylating agents and RONS can modify DNA bases directly on the DNA helix, chemical modifications of DNA precursors, that is, dNTPs, are possible (Topal et al. 1982).
- Increase, DNA strand breaks (single) (https://aopwiki.org/events/1635, Figure 2—KE 15a): Single-strand breaks (SSBs) occur when the sugar-phosphate backbone of DNA is hydrolyzed, disrupting the structure to the extent that the hydrogen bonds between complementary bases can no longer maintain the integrity of the two strands. DNA SSBs are caused by various conditions: oxidative stress (MIE 2) (Kay et al. 2019), topoisomerase I-DNA complex stabilization (MIE 5) (Xu and Her 2015), or misrepaired modified bases (KE 11). SSBs can also result from intermediate steps of DNA repair pathways (KE 14b) (Chatterjee and Walker 2017; Yang et al. 2004; Popov et al. 2023; Hegde et al. 2008; Fousteri and Mullenders 2008), which are discussed in more detail below. Furthermore, thymineless episodes (MIE 7), caused by folate deficiency or dTMP synthase inhibitors, have been associated with DNA strand breakage, notably at specific chromosomal locations, the so-called fragile sites (Kunz et al. 1994).

If not or incorrectly repaired, all the above-mentioned damage can interfere with DNA transcription and replication. If complexes involved in these processes are likely to be stalled for a prolonged period, the latter can collapse and transform into DNA double-strand breaks (DSBs), a critical situation for the cell.

- Stalled replication fork (Inhibition of DNA synthesis) (Figure 2—KE 16): A stalled replication fork occurs when the progression of the DNA replication machinery is impeded, typically due to obstacles on the DNA helix such as alkylated lesions (Torregrosa-Muñumer et al. 2015; Fahrer and Christmann 2023), intrastrand crosslinks (MIE 3) (Luong et al. 2022; Sonohara et al. 2019), bulky DNA adducts (MIE 5) (Piberger et al. 2020), AP sites, single strand breaks (KE 15a) (Ensminger et al. 2014), topo I-DNA cleavage complex stabilization (MIE) (Backer et al. 1990; Xu and Her 2015), DPCs (MIE 4) (Stingele et al. 2017), mismatch (KE 13) or DNA interstrand crosslinks (MIE 8) (Carusillo and Mussolino 2020). Blocking of the DNA replication machinery disrupts the normal synthesis of new DNA strands. Cells employ various mechanisms, such as fork stabilization, translesion synthesis, or homologous recombination, to address the issue and restart replication (Petermann and Helleday 2010). However, a prolonged period in a stalled situation can lead to the collapse of the replication fork, generating a DNA DSBs.
- *Increase, DNA strand breaks* (*double*) (Figure 2—KE 15b): Double-strand breaks (DSBs) occur when both DNA strands are broken close enough that base-pairing and chromatin structure cannot maintain the alignment of the two ends. As a result, the DNA ends can physically separate. Direct breakage of DNA double strands is also possible through energy deposition (https://aopwiki.org/relationships/1977, Figure 2—MIE 10) (Christensen et al. 2014; Pajic and Rovcanin 2021; Georgakilas

et al. 2010). The inhibition of topoisomerase II enzymes can stabilize the TOPO2-DNA cleavage complex (MIE 9), leaving a "free" DNA DSB (Lynch et al. 2003) (https://aopwiki.org/relationships/1634). In addition, DNA SSBs can transform into DSBs (Ho et al. 2007) via different mechanisms (Yang et al. 2004), for example, when two SSBs are close to each other or via the collapse of the replication fork (Caldecott 2024).

Fortunately, our complex biological system is provided with repair mechanisms that can eventually limit exogenous-induced DNA damage (Ciccia and Elledge 2010). Multiple DNA repair processes exist to face these critical situations. The type of DNA repair process involved will depend on the type of lesions. An overview of these processes is provided below, starting with processes targeting simple damage and ending with those aiming to repair more complex damage:

- *Direct reversal of DNA damage*: Several enzymes are able to directly reverse small DNA lesions (Fahrer and Christmann 2023; Chatterjee and Walker 2017), for example, alkylguanine alkyltransferase (AGT/MGMT) and AlkB-related alpha-ketoglutarate-dependent dioxygenases (AlkB), which are able to address certain alkylated lesions (Fahrer et al. 2015), allowing the cell to rapidly recover from the damage in an error-free manner. However, this type of repair, in addition to being specific to a few types of DNA lesions, showed a threshold of maximal activity above which other repair pathways, notably the base excision repair pathway, are necessary to limit apoptotic and carcinogenic effects (Fahrer et al. 2015).

The saturation of these enzymes is therefore an important biological KE. Indeed, the KE "Inadequate DNA repair (Direct enzymatic reversal)" (Figure 2, KE 14a) will lead to the KE "Increase, small DNA base modifications" (Figure 2, KE 11), which mainly encompasses DNA lesions caused by RONS and alkylating agents (https://aopwiki.org/relationships/24).

The base excision repair (*BER*) deals with minor damages affecting individual bases (KE 11) without distorting the overall structure of the DNA double helix (Fahrer and Christmann 2023). These damages include oxidized bases (MIE 2) (Bjørås et al. 1997), methylated and alkylated bases (MIE 1), deaminated bases, minor adducts that do not require nucleotide excision repair (NER), and abasic (AP) sites and SSBs. The last two can be either primary damage triggered by certain MIE or formed as intermediates of the BER (Caldecott 2008; Hegde et al. 2008). Five steps have been identified in this process: (i) base removal by specific DNA glycosylase, (ii) incision of the resulting abasic site, (iii) processing of the generated termini at the strand break, (iv) DNA synthesis to fill in the gap, and (v) ligation of the damaged DNA strand. Gap filling and ligation are carried out by two alternative pathways, that is, short-patch (SP) or long-patch (LP) repair, whose distinct feature is the size of the repair patch: one nucleotide in the case of SP repair and two or more nucleotides (2–12 nt) in the case of LP repair (Fortini and Dogliotti 2007). The most established BER pathway model is then subdivided into two subtypes: the short-patch (or single-nucleotide) BER (SP-BER) and the long-patch BER (LP-BER) (Hegde et al. 2008). If DNA damage accumulates, the BER will be overwhelmed, which will lead to the accumulation of abasic sites and SSBs, ultimately resulting in DSBs.

The nucleotide excision repair (*NER*) deals with lesions that distort the DNA double helix and interfere with replication and transcription. These damages include bulky adducts (MIE 4) (Popov et al. 2023), pyrimidine dimers and intrastrand crosslinks (MIE 3), large DPCs (MIE 5), and oxidative damage (MIE 2). NER consists of two sub-pathways: (i) global genome repair (GGR), which localizes lesions anywhere in the genome sensing damage-induced DNA helix distortions, and (ii) transcription-coupled repair (TCR) initiated by stalling RNA polymerase II at transcription-blocking lesions (Fousteri and Mullenders 2008; Marteijn et al. 2014).

In case DNA damage persists in the cell during replication, a special pathway allows cells to tolerate certain types of DNA damage by bypassing lesions and incorporating a base in front of the damaged template, albeit with lower fidelity than replicative DNA polymerase.

The translesion synthesis (*TLS*): The TLS occurs during DNA replication (S phase of the cell cycle) and bypasses DNA lesions by incorporating nucleotides in front of damaged bases (KE 11). TLS is also known to address DPCs (MIE 5). DPCs are particularly challenging to repair because they involve both nucleic acid and protein components, requiring a proteolysis step followed by an excision of the remaining fragment and the bypass of the peptide adduct requiring DNA pol ζ (Ide et al. 2011; Fielden et al. 2018; Duxin et al. 2014; Klages-Mundt and Li 2017; Stingele et al. 2017). However, TLS is error-prone, directly increasing the gene mutation rate (Figure 2—AO 17) (Sale 2013; Shilkin et al. 2020; Póti et al. 2022).

This set of DNA repair pathways (BER, NER, and TLS) can be summarized in one KE, which is already described in the AOP-Wiki, that is, KE 155 "Inadequate DNA repair" (https://aopwiki.org/events/155). The KE represents the incapacity of the cell to deal with induced DNA damage and encompasses all types of DNA repair pathways. In our network, the KE is subdivided into specific sub-KE, similar to the approach used in the AOP 296 "Oxidative DNA damage leading to chromosomal aberrations and mutations" (https://aopwiki.org/aops/296).

- *Inadequate, DNA repair* (*BER*, *NER*, *TLS*) (Figure 2—KE 14b): This KE regroups DNA repair pathways addressing alkylated lesions (Soll et al. 2017) (https://aopwiki.org/relationships/24, MIE 1) and oxidative damage (https://aopwiki.org/relationships/1909, MIE 2) merged in the KE "Increase, small DNA base modifications" (KE 11), DNA intrastrand crosslinks (MIE 3) (Douki et al. 2017) and bulky DNA adducts (MIE 4) (Skosareva et al. 2013). Moreover, TLS is implicated in the late stage of DPCs (MIE 5) repair, as mentioned above. In case of a high level of damage, while TLS increases the gene mutation rate, intermediates from BER and NER pathways can accumulate, increasing the rate of AP sites (Poetsch 2020) and DNA SSBs in the cell (Figure 2—KE 15a) (Ensminger et al. 2014; Horton et al. 2008).

The accumulation of DNA SSBs generates a stressful situation for the cell and has different consequences, leading either to

a gene mutation if incorrectly repaired or, ultimately through the stall and collapse of the replication fork, to a DNA DSB (Caldecott 2008). To avoid such dramatic consequences, the cell will implement single-strand break repair processes.

The SSB repair (SSBR) pathway is predicted to act through different enzymatic complexes depending on the origin of the SSB (BER-intermediate SSB (indirect), NER-intermediate SSB (indirect), Sugar damage-caused SSB (direct) or TOP1-SSB (TOP1-DNA crosslink)), and the type of damaged termini, but similar steps are undergone in SSBR processes.

- SSB detection: The SSB sensor Protein Poly(ADP-Ribose) Polymerase 1 (PARP1) plays a crucial role in detecting and repairing single-strand breaks (SSBs) in DNA, primarily through its rapid binding and activation at breaks. The exact roles of PARP1 in certain contexts, such as base excision repair (BER) and abortive TOP1–SSBs, remain unclear.
- DNA end processing: Once a SSB has been detected, it undergoes end processing. Damaged termini that are present at BER-induced SSBs are repaired by APE1, DNA polymerase (Pol) β, polynucleotide kinase 3′-phosphatase (PNKP) and aprataxin (APTX). Direct sugar-damage induced SSBs are repaired by APE1, PNKP, and APTX. TOP1–SSBs are repaired by tyrosyl-DNA phosphodiesterase 1 (TDP1) and PNKP.
- DNA gap filling: At most SSBs, Pol β inserts the missing nucleotide, known as short-patch repair (common with SP-BER). Under some circumstances gap filling might be extended for ~2–12 nucleotides (nt) by Pol β, Pol δ and/or Pol ε (Pol δ/ε) during long-patch repair (common with LP-BER). Note that TOP1–SSBs are DNA nicks and therefore might not require a gap-filling step.
- DNA Ligation: Short-patch repair is primarily completed by DNA ligase 3 (LIG3), while long-patch repair is predominantly carried out by DNA ligase 1 (LIG1) (Caldecott 2008).

- "Inadequate DNA repair (SSBR)" (Figure 2—KE 14c): The incorrect repair of SSBs, a sub-KE of KE 155 "Inadequate, DNA repair" (https://aopwiki.org/events/155), may lead to gene mutations (Figure 2—AO 17), that is, point mutations or indels depending the length of the naked patch, or could also block the replication fork (Figure 2—KE 16). Additionally, clusters of SSBs on opposite DNA strands (KE 15a), the collapse of a stalled replication fork or direct strand breakage by deposition of energy (MIE 10) may lead to the formation of DNA DSBs (https://aopwiki.org/relationships/1911, Figure 2—KE 25b) (Caldecott 2024, 2008).

DNA DSBs are repaired by the two most prominent processes. *The homologous recombination* (HR) process deals with DNA DSBs during the S and G2 phases. This process is highly accurate and error-free (Chapman et al. 2012; Schipler and Iliakis 2013; Karanam et al. 2012). In *the non-homologous end-joining* (*NHEJ*), the DSB ends are blocked from 5′ end resection and held in close proximity by the double-stranded DNA end-binding protein complex Ku70-Ku80 heterodimer. This complex allows the direct ligation of DSB ends, but this process is error-prone, often leading to small insertions, deletions, or substitutions at the break site, and potentially causing structural aberrations by joining ends from different genomic regions (Chapman et al. 2012; Schipler and Iliakis 2013; Lieber 2010). Globally, studies show that the NHEJ pathway is predominant compared to HR, repairing up to ~80% of all DSBs in human cells (Karanam et al. 2012; Stinson and Loparo 2021; Mao et al. 2008).

- "Inadequate DNA repair (NHEJ)" (Figure 2—KE 14e): The inadequate repair of DNA DSBs by NHEJ provokes genotoxic AOs, that is, "Increase, Structural chromosome aberrations" (*AO 18*) *and KE 185* "*Increase*, *Mutations*" (*AO 17*).

Finally, more complex damages, that is, DNA interstrand crosslinks (MIE 8), are repaired through a combination of repair pathways grouped under the *Fanconi Anemia* (*FA*) *repair pathway* (Kratz et al. 2010; Moldovan and D'Andrea 2009; Fu et al. 2012). This damage blocks both replication and transcription processes by impeding the strands' disjunction. The FA pathway is believed to orchestrate a complex repair mechanism that integrates components from three major DNA repair pathways: homologous recombination, NER, and TLS (Moldovan and D'Andrea 2009). Although primordial for cell survival, the FA pathway involves mutagenic polymerases (TLS) to bypass lesions at the cost of potentially introducing mutations (AO 17) in the genome (Ceccaldi et al. 2016). However, the FA pathway seems to limit the use of NHEJ for the repair of transient DSBs step, reducing the probability of chromosomal rearrangements. FA proteins also have a crucial role in replication fork protection and are implicated in replication fork restart (KE 16) (Ceccaldi et al. 2016).

- *Inadequate DNA repair* (*Fanconi anemia repair*) (Figure 2—KE 14d): This pathway thus represents the last sub-KE of KE 155 "Inadequate DNA repair" (http://aopwiki.org/events/155). Overall, a high rate of interstrand crosslinks implies a high rate of transient DSBs (KE 15b), a critical situation for a cell to deal with in case of interrupted repair processes.

All types of DNA lesions presented above, if not or incorrectly addressed by repair processes, will converge through the different KEs to form gene mutations and structural chromosome aberrations.

- *Increase, Mutations* (https://aopwiki.org/events/185, Figure 2—AO 17):

  Mutations can have different origins and appear through different MoAs following the different MIEs. Alkylation of DNA can directly enhance gene mutations (https://aopwiki.org/relationships/25). Structurally mimicking thymine, O^6-alkylguanines (KE 11) are mispaired with adenine (Fahrer et al. 2015), leading to GC → AT transitions after two replication cycles (Loechler et al. 1984). Similarly, 8-oxoG (KE 11) generated through oxidation of guanines by RONS (MIE 2) are structurally similar to natural nucleotides and might escape lesion detection mechanisms, also leading to GC → AT transitions (Suzuki et al. 2010; Kamiya et al. 2010) (https://aopwiki.org/relationships/1914). All DNA damage susceptible to being addressed by TLS repair processes (KE 14b—Inadequate DNA repair [BER, NER, TLS]), that is,

modified DNA bases (KE 11), DNA intrastrand crosslinks (MIE 3), DPCs (MIE 4) and bulky DNA adducts (MIE 5), as well as incorrectly repaired DNA SSBs (KE 15a), may lead to an increase in the gene mutation rate (Sale 2013; Shilkin et al. 2020). Imbalances in the dNTP pool (MIE 7), both in the overall concentration and in proportions of individual dNTPs or their precursors, are also known to provoke enhanced mutagenesis in yeast, bacterial, or mammalian cells (D et al. 2010; Kunz et al. 1994). Different models combining misinsertion, misalignment, and mismatch extension mechanisms have been established to explain imbalanced dNTP-induced gene mutations (Kumar et al. 2011). Moreover, DNA DSBs (KE 15b) have been shown to cause gene mutations (https://aopwiki.org/relationships/1931) through inadequate repair (KE 14e) (McMahon et al. 2016; Bétermier et al. 2014). Topo-II inhibitors induce more DNA DSBs than alkylating agents and are less likely to generate gene mutations (Nicolette et al. 2021). This reduced probability could be explained by several reasons, including the fact that DNA DSBs cause much more lethality than modified bases, reducing the probability of observing a point mutation event, and that point mutations arising from DSBs require a mis-repaired-inside-gene DSB, a rare event compared to multiple-point gene alterations provoked by alkylating agents. Consequently, all MIEs/KEs causing DNA DSBs, that is, inhibition of topoisomerase II (MIE 9), stalled replication fork (KE 16) and deposition of energy (https://aopwiki.org/relationships/1981, MIE 10), DNA SSBs accumulation (KE 15a) and their inadequate repair (KE 14e), are also able to increase the gene mutation rate through NHEJ (Lieber 2010; Stinson and Loparo 2021; Mao et al. 2008; Bétermier et al. 2014; Takata et al. 1998).

- *Increase, Structural chromosome aberrations* (https://aopwiki.org/events/1635, Figure 2—AO 18):

  Uncontrolled DNA DSBs (KE 15b), that can directly appear after topo-II enzyme inhibition (MIE 9) (Nicolette et al. 2021; Boos and Stopper 2000), deposition of energy (https://aopwiki.org/relationships/1982) and direct strand breakage (MIE 10) (Christensen et al. 2014), collapse of stalled replication forks (KE 16) (Xu and Her 2015) or through opposed-strand SSBs proximity (KE 15a) (Caldecott 2024, 2008) (https://aopwiki.org/relationships/1939), will be mainly repaired through the NHEJ process (KE 14e) (Lieber 2010; Stinson and Loparo 2021; Mao et al. 2008; Bétermier et al. 2014; Takata et al. 1998). However, this process is known to provoke structural chromosomal rearrangements (https://aopwiki.org/relationships/1912) (Bétermier et al. 2014; Lieber 2010; Stinson and Loparo 2021; Mao et al. 2008).

Considering all the above-described biological events, a refined AOP network leading to "Increase, Mutations" and "Increase, Structural chromosome aberrations" has emerged (Figure 2).

5 | Towards AOP-Based IATAs for Genotoxicity

The AOP network provides a structured and scientifically robust framework to advance genotoxicity assessment. First, linking *in silico* and in vitro methods to the KEs within the AOP network ensures that the data generated with these methods is biologically meaningful and mechanistically interpretable, offering sufficient context to understand the mode of action (MoA) of chemicals. Especially, the methods addressing the earlier KEs could improve our mechanistic understanding (Phillips and Arlt 2009). For example, the current in vitro genotoxicity testing battery collects data related to the AOs without providing insights into whether this permanent DNA damage results from a direct interaction with DNA (e.g., DNA alkylation) or rather indirectly (e.g., via the generation of RONS). Such information is important for regulatory decision-making (Muruzabal, Collins, and Azqueta 2021). Several NAMs, such as the enzyme-modified comet assay, ToxTracker, Prediscreen, or MultiFlow, already allow the collection of MoA information and can thus increase the confidence in results from AO's measurement methods. Furthermore, the AOP network also allows for more critical reflection on the genotoxicity test systems. For example, the AOP network shows an important role for MGMT. However, this repair process is lacking in TK6 cells, the cell system that is now most frequently used in genotoxicity testing. Moreover, modeling relationships between results obtained with various KE-associated methods can increase the quantitative understanding of these MoAs. On the other hand, this modeling can also provide more insights into the performance of different methods to measure a specific KE. For example, if methods addressing the same KE detect the effects of a chemical in the same cell system at very different concentrations, the method detecting the effect only at higher concentrations could be considered less appropriate. Similarly, if despite the existence of strong evidence that a certain KEx occurs upstream of another KEy in the AOP, the method for KEx only detects this event at higher concentrations compared to the method addressing KEy, this also might indicate that the method is less suitable, at least when using the data for quantitative analysis. Additionally, as the network integrates several individual AOPs, it offers the opportunity to address the complexity of chemicals with multiple modes of action, allowing for a more precise and comprehensive analysis. Finally, it also provides a framework to evaluate the combined effects of genotoxicants. This improved qualitative and quantitative insight is expected to increase the sensitivity and specificity of genotoxicity assessment strategies, aligning with regulatory expectations and supporting the reduction and eventual replacement of animal testing.

One important next step in building AOP-based IATAs for genotoxicity consists of inventorizing the available in silico and in vitro genotoxicity methods and mapping them to the KEs of the AOP network that leads to permanent DNA damage (Table 3). Such a mapping exercise has previously been done for the KEs and KERs in the AOP Oxidative DNA damage leading to mutations and chromosomal aberrations (Cho et al. 2022) and was used as a starting point for mapping the methods to KEs within our AOP network (Figure 2). By carefully selecting the most appropriate methods to address the regulatory question of interest, new genotoxicity testing strategies integrating NAMs can be designed. Interestingly, several high-content information methods (e.g., MultiFlow approaches and the MutaTracker with ACE extension within the ToxTracker suite) have recently been developed that allow the simultaneous collection of information on different KEs. These developments suggest that, moving forward, the reliance on multiple assays may be

reduced, facilitating streamlined testing strategies. Moreover, omics methods, such as transcriptomics, could have a key role in these testing strategies, as when combined with quantitative and time-scaled study designs, they allow the evaluation of the dynamics of genotoxic responses by evaluating key drivers of genotoxicity (e.g., MGMT, P53 pathway, H2AX, Excision repair gene sets, NHEJ-related gene sets) and identifying biomarkers for toxicity endpoints (such as GENOMARK and TGxDDI in case of genotoxicity).

However, to be useful in a regulatory context, it is important to characterize the "regulatory readiness" of the different methods to be integrated in the IATA. NAM development is indeed a long, timely, and resource-demanding process that can roughly be subdivided into four major phases before regulatory implementation, namely (Haase et al. 2024):

- *Research*: This phase is mainly driven by novelty and ends with a "scientifically plausible" method. A method description is generally available as well as selected performance parameters.
- *Optimisation*: During this phase, the method is further optimized including a refinement of the method description to ensure reproducibility. The optimization phase ends with a "harmonized" or "standardized" method description often released as standard operating procedure (SOP). The specific regulatory application of the method is often still unclear at this stage.
- *Validation*: Results obtained with the method are used to test one or several hypotheses. If the hypothesis is not valid, the test method development returns to the research phase.
- *International harmonization*: Several routes for international harmonization exist, among which the endorsement of an OECD test guideline is the most common one for chemical risk assessment.

As explained before, the available NAMs for genotoxicity cover the four stages, ranging from methods that just come out of the research phase to methods undergoing validation to the traditional in vitro tests described in OECD TGs. In order to have more insights into the regulatory readiness of the non-guideline genotoxicity methods, it is important to clearly describe and assess the characteristics of these assays. One way to do this is by describing the method using ToxTemp, which allows the evaluation of the methods according to the readiness criteria defined by Bal-Price (Bal-Price et al. 2019). Although these criteria were established initially for evaluating developmental neurotoxicity assays, they are now more broadly accepted as criteria for the assessment of non-guideline studies (Haase et al. 2024).

NAMs will then be integrated into an IATA based on the type of information they provide (thus on which KE) and their regulatory readiness. Evaluation of the IATAs will be done through the design of case studies addressing specific regulatory questions. For each case study, the performance of different NAM combinations to predict genotoxicity will be assessed and compared, thereby also integrating QIVIVE and PBK modeling.

6 | Conclusion

Combining different AOPs into a network leading to permanent DNA damage more correctly reflects the current state of knowledge in genetic toxicology compared to individual AOPs. Moreover, this network could also be seen as the intersect between on the one hand other upstream effects such as MIEs and KEs triggering oxidative stress leading to RO(N)S and on the other hand, downstream AOs such as genetic diseases or cancer. Its mechanistic representation of genotoxic biological pathways should be applicable to any type of mammalian cells. Moreover, this AOP network provides a solid foundation for the science-driven development of IATAs for genotoxicity.

Author Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflicts of Interest

The authors declare no conflicts of interest.

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