**A global AOP network for genotoxicity to drive the integration of NAMs**

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

Keywords: Genotoxicity; s, AOP, IATA.

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 processes1. In humans, genetic damage in somatic cells is linked to severe health outcomes such as cancer, premature aging, and cardiovascular diseases. In contrast, damage to germ cells can result in infertility or inheritable genetic disorders2–4. 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, mutations, and/or chromosomal aberrations, all of which can significantly impact human health2.

Current genotoxicity testing of chemical compounds typically targets three main endpoints: gene mutations, structural chromosome aberrations (clastogenicity) and numerical chromosome aberrations (aneugenicity)5. There is no single test that covers all three genotoxic endpoints, and consequently, a battery of tests is required6. Although the composition of the test batteries varies between different regulatory domains (e.g. chemicals, cosmetics, pharmaceuticals, plant protection products) and jurisdictions, they follow the same principles. Genotoxicity testing of chemicals in Europe generally starts with a set of *in vitro* assays. If allowed, *in vivo* testing will be performed as a second step in case of a positive result or to fulfill the legal requirements. 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 test6–8. Based on the outcome of the *in vitro* tests and taking into account the legal requirements, an adequate *in vivo* follow-up test is then selected addressing either gene mutations or structural/numerical chromosome aberrations1,6–10.

Below, a brief overview of the different genotoxicity tests required per chemical domain within the EU is provided.

* **Registration, Evaluation, Authorisation and restriction of CHemicals (REACH)**: Information requirements for mutagenicity assessment, as well as for the other endpoints, increase with the volume of the substance to be registered. In the lowest tonnage band (1-10 tonnes per year), only the Ames test is requested. From 10 tonnes/year and above, *in vitro* evaluation of all the three genotoxic endpoints is needed, conducting preferentially, in addition to the Ames test, an *in vitro* mammalian cell micronucleus test. Only in case of positive results in one or more *in vitro* tests, an *in vivo* follow up is required, by appropriate *in vivo* mammalian somatic cell genotoxicity studies. Moreover, if the latter are positive, mutagenicity in germ cells should also be evaluated. Based on the results collected under REACH (and other regulations), it is possible to classify the substances according to the Regulation (EC) No 1272/2008 on **the classification, labelling and packaging (CLP) of chemical substances**. Germ cells mutagenicity is the hazard class under CLP that is relevant for the classification of genotoxic/mutagenic substances. Criteria to put harmful chemicals in one of the three categories (i.e., 1A, 1B or 2) are largely based on results of *in vivo* testing. Specifically, apart from category 1A which requires epidemiological evidence in humans, and which is in practice unpopulated, *in vivo* (germ cells) results are required to classify chemical substances as germ cell mutagens in categories 1B or 2.
* Genotoxicity testing of **plant protection products** under Regulation (EC) No. 1107/2009, involves an *in vitro* battery consisting of an Ames assay (OECD TG 471) and an *in vitro* micronucleus test (OECD TG 487) for the assessment of gene mutations and clastogenicity/aneugenicity, respectively. Additional *in vitro* testing may be needed for active substances bearing structural alerts, if the standard tests have not been optimised for these alerts. Although according to the *EFSA scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment*9, a substance is concluded to have no genotoxic potential in case of negative *in vitro* tests, Regulation (EC) No. 1107/2009 clearly states that at least one *in vivo* genotoxicity study in somatic cells shall always be conducted with demonstration of exposure to the test tissue, even if all the results of the *in vitro* studies are negative11. In the case of inconclusive, contradictory or equivocal results from *in vitro* testing, further *in vitro* testing is recommended, either by repeating a test already conducted under different conditions, or by conducting a different *in vitro* test9. . In case of positive *in vitro* data, *in vivo* genotoxicity testing in somatic cells is required to further investigate the *in vitro* positive results11. For active substances that are recognised as *in vivo* somatic cell mutagens no further genotoxicity testing is necessary since they will be considered to be potential genotoxic carcinogens and potential germ cell mutagens and ,therefore, *in vivo* studies in germ cells may only be triggered on a case-by-case basis and considering toxicokinetic data, use and expected human exposure.
* Under the **biocides** legislation, the first step of the genotoxicity assessment consists of evaluating all existing information, including physicochemical properties, grouping, [Q]SARs and expert systems, *in vitro* data, human data and animal data1. If existing information is not sufficient to conclude on the genotoxic potential of a compound, further *in vitro* testing is needed, including an Ames test (OECD TG 471), at least one *in vitro* mammalian cell test for gene mutation, i.e. either using the Thymidine Kinase gene (OECD TG 490) or using the Hprt and xprt genes (OECD TG 476) and an assay detecting clastogenicity/aneugenicity. The preferred method for examining *in vitro* chromosome damage in mammalian cells is the *in vitro* cell micronucleus test (OECD TG 487) in comparison to the *in vitro* chromosomal aberration assay (OECD TG 473) due to its higher sensitivity and ability to distinguish between clastogens and aneugens provided that appropriate centromere labelling is performed in case of positive results. Positive *in vitro* data trigger further *in vivo* testing that should be conducted depending on the endpoint of concern, i.e. mammalian erythrocyte micronucleus test (OECD TG 474) or mammalian bone marrow chromosome aberration test (OECD TG 475) in case of positive *in vitro* chromosome damage assays, transgenic rodent somatic and germ cell gene mutation assays (OECD TG 488) in case of positive *in vitro* gene mutation assay, and *in vivo* mammalian alkaline comet assay (OECD TG 489). Germ cell testing (i.e., OECD TG 488, OECD TG 483) is only needed when there is a positive result in an *in vivo* test for genotoxic effects in somatic cells and the toxicokinetic data on the capacity of the substance to reach the germ cells are inconclusive. As for plant protection products, in case of a positive result in the *in vivo* micronucleus assay, identification of an aneugenic and/or clastogenic potential (e.g., via appropriate staining procedure such as fluorescence in-situ hybridisation (FISH)) is necessary to conclude on a threshold or not in the mode of action11,12.
* **Cosmetics:** No *in vivo* genotoxicity tests are performed as the use of animal tests is prohibited for cosmetic ingredients (EC 2009). The testing strategy for genotoxicity assessment, recommended by the SCCS Note of Guidance (SCCS 2023 - SCCS/1647/22), implies initial consideration of available data (including *in silico* results) and subsequent application of the *in vitro* testing battery. Follow-up for inconclusive results entails the application of an *in vitro* testing toolbox, to gather additional evidence even from tests not routinely used in regulatory frameworks13.

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), 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, also the REACH regulation requires that new testing of a substance involving vertebrate animals is only conducted as a last resort (EC 2006 ; EC 2009 ; SCCS 2022 ). 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 limitations14,15:

1. **The high rate of ‘misleading’ *in vitro* positive results:** the traditional *in vitro* genotoxicity tests have a rather low specificity, meaning that a positive result in the *in vitro* test is not confirmed in the adequate *in vivo* follow-up test5–7. For the *in vitro* micronucleus test, most false positive results have been related to cytotoxicity or the cell line used (e.g. TK6)8. This might trigger unnecessary animal testing or stop the further development of high-interest lead compounds.
2. **The lack of quantitative analysis of the collected genotoxicity data:** *in vitro* and *in vivo* genotoxicity assays have been mainly used for hazard identification and only to a very limited extent for hazard and risk assessment9. 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, 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 effects10,11.
3. **The limited mechanistic information provided:** the traditional *in vitro* tests provide no or only limited mechanistic information. By using different strains, the bacterial reverse gene mutation test (Ames test, OECD TG 471) allows to obtain some insights into the type of gene mutations induced (i.e. base pair substitutions versus frameshift mutations). The mammalian gene mutation tests based on the thymidine kinase gene (OECD TG 490), in turn, can distinguish between gene mutations and chromosome aberrations based on the type of colonies formed. The micronucleus test (OECD TG 487) detects both structural and numerical chromosome aberrations simultaneously, but when combined with a fluorescent *in situ* hybridization (FISH) or CREST technique, a distinction can be made between these two types of aberrations. Overall, the collected mechanistic information is very limited. Obtaining more insights into the underlying mode of action (MOA) of a chemical could help to identify if a threshold mechanism is involved and support quantitative analysis of the data10,11.
4. **The throughput of the assays:** current genotoxicity tests have a limited throughput and cannot address the high number of naturally occurring, newly developed or commercially available chemicals that have to be tested6,14. Indeed, the use of extensive, low-throughput, and resource-intensive animal testing is not adapted for addressing the wide range of chemicals of regulatory interest16. Even for the ‘traditional’ genotoxicity tests, the throughput is rather limited. This is an issue as the chemical space potentially encompasses billions of natural and synthetic molecules17 which are, for the most, uncharted in terms of toxicological characterization18,19.
5. **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 in vivo. Many chemicals require metabolic activation to become genotoxic (e.g. benzo[*a*]pyrene; acrylamide)20–23*. In vitro* test systems often 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 systems24, which contain enzymes like cytochrome P45025. However, these systems, especially those derived from non-human species, may not fully mimic human metabolism, causing inaccuracies in the safety assessment22,26–28.

Modern regulatory genotoxicology thus needs affordable tests with higher throughput and higher content, that are at the same time human-relevant and provide mechanistic information for effective chemical evaluation14. Over the last decades, efforts have been made to increase the throughput of standard tests, such as the *in vitro* micronucleus test using automated image analysis and Fluorescence-activated cell sorting (FACS)29–31. On the other hand, several new innovative animal-free methods have been developed32. Unfortunately, the integration of these new methods in the regulatory assessment of genotoxicants is still limited because validation, acceptance, and implementation of these approaches within regulatory decision-making are resource- and time-consuming32. However, several 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) and aim to help the transition towards next-generation genotoxicity assessment33–36. 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 continuedlearning37,38.

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* approaches28,29 and thus covers the ‘traditional’ *in vitro* genotoxicity tests as well as the new innovative methods. 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 space39. The selection of data sources and NAMs in the IATA may be challenging and should be science-driven, a process in which adverse outcome pathways (AOPs) could play an important role40. Indeed, the AOP framework has already proven its usefulness by supporting the development of (conceptual) IATAs for skin sensitization and non-genotoxic carcinogens41 and can facilitate the identification of the most suitable assays for measuring/informing on biological key events42,43.

1. **Adverse Outcome Pathways (AOPs)**

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 assessment44. 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 knowledge45.

In this respect, AOPs define a sequence of biological measurable 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 MIE46.

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-making38,46. 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 AOPs47. 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 MIEs46 or evaluating the effects of combined chemicals.

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’48.

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 methods40,49. 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. Additionally, the combination of IATA and AOP concepts could offer a better quantitative understanding of substances’ modes of action. Such molecular-level insight is expected to reduce the need for animal testing by increasing confidence. Finally, the use of AOPs can help identify knowledge gaps and prioritize new fields of research in genotoxicity.

1. **Draft AOP network leading to permanent DNA damage based on existing AOPs**

***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 1).

Nineteen AOPs of interest were found on the AOP-wiki, ten 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 at the OECD level. Two others are currently under review by ESCA and four are under development. Next, a first draft AOP network combining the ten AOPs included in the OECD work plan was built (Supplementary Figure 1). The AOPs that are 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 into the network.

Although a draft AOP network could be designed based on the individual AOPs, several challenges were encountered, which are explained in the following paragraphs.

***The genotoxic AOs***

From a regulatory point of view, the different genotoxic AOs that have to be covered are mutagenicity, i.e. the ability to induce gene mutations, clastogenicity, i.e. the ability to modify the structure of chromosomes, and aneugenicity, i.e. the ability to alter the number of chromosomes. These three types of permanent DNA damage were therefore selected as the AOs of our network. 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, each of which can uniquely impact the genome and its regulation50. This KE, which is related to mutagenicity, is included in three of the endorsed AOPs and thus already well described, documented and accepted.
* **“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. *Deletions* occur when a segment of genetic material is lost from a chromosome. Terminal deletions involve the loss of an end portion of the chromosome, while interstitial deletions arise when a chromosome breaks in two locations and rejoins incorrectly, omitting the middle segment. *Duplications* involve the addition or rearrangement of extra genetic material, which can take various forms, such as transpositions, tandem duplications, reverse duplications, or displaced duplications. *Translocations* occur when a segment of one chromosome is transferred to a non-homologous chromosome, with reciprocal translocations involving the exchange of segments between two non-homologous chromosomes. *Inversions* happen within a single chromosome, where both ends of a segment break and rejoin in reverse order, effectively inverting the DNA sequence. Finally, 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 exchanges51. This KE, which describes the different types of structural chromosome aberrations and thus refers to clastogenicity, is included in two of the endorsed AOPs and has also been characterized. However, for our AOP network, we suggest renaming this KE as **“Increase, Structural chromosome aberrations”** which is more adequate to distinguish it from the third AO in our network, i.e. 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 other than the usual 4652. 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 such as infant leukemia or breast cancer. As gene mutations and structural and numerical chromosome aberrations were selected as the AOs for our network, these downstream KEs were not taken into account. In a later stage, our AOP network can be integrated with other AOPs/AOP networks to make the link with other (downstream) AOs but this was considered outside the scope of the current network.

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

***Duplication of KEs***

Although KE 1636 “Increase, Chromosomal aberrations’ is part of an endorsed AOP, another KE describing the same event was found in the AOP-Wiki, i.e. KE 1554. Also for other KEs, ‘duplicates’ were found. The duplication of KEs is due to the lack of reuse of existing KEs53. 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 with redundant KEs. 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 after a specific compound exposure and allowing a complex understanding of the different pathways involved45.

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” or KE 1461 “DNA Double Strand Break” and KE 1635 “Increase, DNA strand breaks”

) 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

***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, i.e. “Binding to Tubulin”, and the others leading to clastogenicity and mutagenicity, i.e. “Binding to topoisomerase II enzymes”, “Formation, Bulky DNA adducts”, “Alkylation of DNA”, “Increase, RONS” and “Deposition of energy”. In this second group, MIEs can either represent structural DNA damage or can be an inhibitor of DNA replicator elements.

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 with 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 al49 were integrated into the draft AOP network bringing additional MIEs and KEs. 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 part of the network leading to mutagenicity and clastogenicity, 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 template49.
* **“Collapse, Stalled replication fork”:** Replication forks are susceptible to stalling or 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, causing fork stalling or collapse. A stalled replication fork is temporarily arrested but retains the ability to resume replication (replication fork restart) 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)54.

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 expertise knowledge, the following MIEs were added 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 DNA55–57. *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 agent58–60. 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 chemicals55.
* **“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 chemicals61,62. As for the topoisomerase II enzyme causing TOP2-DNA complexes when inhibited63 (<https://aopwiki.org/aops/202>), inhibition of this enzyme is known to provoke DNA lesions, such as gene mutations and structural chromosome aberrations61.

***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, i.e., 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 AOP 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 network64. The inclusion of AOP 296 will allow to link the network in the future to other events leading to the increase of reactive oxygen and nitrogen species (RONS). For the endorsed AOP 202 “Inhibitor binding to topoisomerase II leading to infant leukaemia”, the specific KE 1253: MLL chromosomal translocation was not included 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 “Increase, chromosome aberrations”, which has been renamed “Increase, Structural chromosome aberrations” to distinguish it from the genotoxic AO “Altered, Chromosome number”.

***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”. At the opposite, the AOP starting with “Binding to topoisomerase II enzyme” now has also an indirect link with “Increase, Mutations”. These new links are in line with the evidence that is already available in literature61,65–67.

By taking into account all the considerations described 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.

1. **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 (Supplementary Figure 1), 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, e.g. directly through deposition of energy (double-strand breaks) and indirectly through the accumulation of alkylated damages (single-strand breaks). 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, at varying levels, and sometimes, after the apparition of secondary DNA damage subsequent to the accumulation of primary damage. 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 energy68–70.

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 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 Handbook48 to generate individual AOPs on the AOP-wiki. Further characterization of certain KEs and KERs in the network using a systematic approach will thus still be 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.

***Refining the key events (KEs) linking the MIEs to the genotoxic AOs***

In the first draft of our network, seven MIE leading to mutagenicity and/or clastogenicity were included. To complete and harmonize our network, the MIEs were rearranged. As a result, the network includes now ten 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**71. Alkylation of DNA results in modified bases (KE 11, KE 14) via first or second-order nucleophilic substitutions (SN1 or SN2 reactions) on O- and N-atoms of bases. O6-alkylguanines (O6-MeG and O6-EtG) are the most critical type of DNA alkylation72.
* **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)73–75. RONS are the result of upstream KEs triggered by the compound and ,therefore, DNA damage induced by RONS is often referred to as secondary genotoxicity76.
* **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 cisplatin77. Cyclobutane Pyrimidine Dimers (CPDs) are induced by UV radiation, and result from covalent bonding between the C5 and C6 atoms of two adjacent pyrimidines78. 6-4 Photoproducts aire UV-induced lesion 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 helix79. Chemotherapeutic agents like cisplatin and acetaldehyde form intrastrand crosslinks, primarily between two adjacent guanine bases80.
* **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 thymine81,82. Bulky adducts are typically formed by exposure to environmental mutagens, chemicals, or certain metabolites81 such as benzo[a]pyrene and its metabolite BPDE, aflatoxin B1 and aristolochic acid.
* **Formation, DNA-protein crosslinks (DPCs**83**)** (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 cisplatin58–60,83,84.
* **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 chemicals61,62.
* **Disruption, dNTP pool homeostasis** (Figure 2 – MIE 7)**:** The four deoxyribonucleoside triphosphates (dNTPs), dATP, dTTP, dGTP and dCTP, are the building blocks of the 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 genome85. Imbalances in their absolute and relative concentrations are then a critical genotoxic initiating event86. 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 monitor87.
* **Binding to topoisomerase II (TOPO2) enzymes** (<https://aopwiki.org/events/1252>, Figure 2 – MIE 9): TOPO2 enzymes are ubiquitous enzymes implicated in maintenance of the DNA integrity. Their function implies the transient formation of DNA double-strand breaks88, a process that becomes critical when being disturbed61,67,88.
* **“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 DNA55–57.
* **“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 breaks89.

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 14): 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).
  + Oxidative damage (<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 mutations73.
  + 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 replication72.
* **Increase, Misinsertion of dNTPs** (Figure 2 – KE 12): When the homeostasis of the dNTP pool is disturbed, dNTP may be incorrectly inserted in the newly generated DNA strand86,90. Different models combining misinsertion, misalignment and mismatch extension mechanisms have been established to explain imbalanced dNTP-induced gene mutations86.
* **Increase, Modified dNTPs** (Figure 2, KE 11): As well as alkylating agents and RONS can modify DNA bases directly on the DNA helix, chemical modifications of DNA precursors, i.e. dNTPs, is possible91.
* **Increase, DNA strand breaks (Single)** (<https://aopwiki.org/events/1635>, Figure 2 – KE 16): 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)92, topoisomerase I-DNA complex stabilization (MIE 5)62, or misrepaired modified bases (KE 14). SSBs can also result from intermediate steps of DNA repair pathways (KE 17)93–97 which are discussed in more details 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 sites98.

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 18)**:** 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 lesions49,51, intrastrand crosslinks (MIE 3)55,80, bulky DNA adducts (MIE 5)99, AP sites, single strand breaks (KE 16)100, topo I-DNA cleavage complex stabilization (MIE 6)61,62, DPCs (MIE 4)84, mismatch (KE 12) or DNA interstrand crosslinks (MIE 8)101. 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 replication54. 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 20): 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)70,89,102. The inhibition of topoisomerase II enzymes can stabilize the TOPO2-DNA cleavage complex (MIE 9), leaving a “free” DNA DSB103 (<https://aopwiki.org/relationships/1634>). In addition, DNA SSBs can transform into DSBs104 via different mechanisms94, e.g. when two SSBs are close to each other or via the collapse of the replication fork105.

Fortunately, our complex biological system is provided with repair mechanisms, that can eventually limit exogenous-induced DNA damage106. 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 lesions72,93, e.g. alkylguanine alkyltransferase (AGT/MGMT) and AlkB-related alpha-ketoglutarate-dependent dioxygenases (AlkB) which are able to address certain alkylated lesions107, 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 effects107.

The saturation of these enzymes is therefore an important biological KE. Indeed, the KE “**Inadequate DNA repair (Direct enzymatic reversal)”** (Figure 2, KE 13) will lead to the KE **“Increase, small DNA base modifications”** (Figure 2, KE 14), which mainly encompasses DNA lesions caused by RONS and alkylating agents (<https://aopwiki.org/relationships/24>).

* **The base excision repair (BER):** The base excision repair process deals with minor damages affecting individual bases (KE 16) without distorting the overall structure of the DNA double helix72. These damages include oxidized bases (MIE 2)108, 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 be formed as intermediates of the BER96,109. 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, i.e., 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-repair110. 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). With the discovery of the concept of protein interactome, the complexity of the BER processes increases and new hypotheses are emerging, such as the replication-associated (RA-)BER model96. 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):** The NER process deals with lesions that distort the DNA double helix and interfere with replication and transcription. These damages include bulky adducts (MIE 4)95, pyrimidine dimers and intrastrand crosslinks (MIE 3), large DPCs, 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. Following the detection of the lesion, the damaged strand is incised on both sides of the lesion, marking a point of no return after which the reaction must be efficiently concluded to avoid leaving potentially dangerous intermediates. The damage is excised with short flanking sequences. Then, DNA synthesis occurs to fill in the gap using the undamaged complementary strand as template97,111.

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 the DNA replication (S phase of the cell cycle) and bypasses DNA lesions by incorporating nucleotides in front of damaged bases (KE 16)112–115. TLS is also known to address DPCs. 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 ζ58,59,83,84,116. However, this process is error-prone and direclty increases the gene mutation rate (Figure 2 - AO 24)113–115.

This set of DNA repair pathways (BER, NER and TLS) can be summarized in one KE, which is already described in the AOP-Wiki, i.e. **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 257 “Oxidative DNA damage leading to chromosomal aberrations and mutations” (<https://aopwiki.org/aops/296>). Indeed, other repair pathways address different types of DNA damage. The KE was therefore named **“Inadequate, DNA repair (BER, NER, TLS)”** (Figure 2, KE 15), focusing only on the BER, NER and TLS DNA repair pathways.

* **Inadequate, DNA repair (BER, NER, TLS)** (Figure 2 – KE 15): This KE regroups DNA repair pathways addressing alkylated lesions117 (<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 14). These pathways also address DNA intrastrand crosslinks (MIE 3)118 and bulky DNA adducts (MIE 4)119. 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 sites75 and DNA SSBs in the cell (Figure 2 – KE 16)100,120.

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 DSB109. To avoid such dramatic consequences, the cell will implement single-strand break repair processes.

* **The SSB repair (SSBR) pathways:** SSBR is predicted to act through different enzymatic complexes depending 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. PARP1 accelerates chromosomal SSBR by stabilizing repair protein complexes, such as XRCC1, and regulating chromatin structure. Additionally, it may facilitate DNA gap filling, energy generation for ligation, and suppression of illegitimate recombination. 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), which removes TOP1 from the 3′-termini at such breaks, resulting in a 3′-phosphate terminus, which is subsequently repaired by PNKP. PNKP also repairs the 5′-hydroxy termini present at TOP1 breaks.
  + DNA gap filling: At most SSBs, Pol β inserts the missing nucleotide, this is termed short-patch repair (common with SP-BER). Under some circumstances (for example, an oxidized deoxyribose phosphate that cannot be repaired by Pol β) 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 long-patch repair involves the removal of the damaged 5′-terminus as a flap of two or more displaced nucleotides by flap endonuclease 1 (FEN1), in a reaction stimulated by PARP1 and proliferating cell nuclear antigen (PCNA). Also 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)109.
* **“Inadequate DNA repair (SSBR)”** (Figure 2 – KE 17): 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 22), i.e. point mutations or indels depending the length of the naked patch, or could also block the replication fork (Figure 2 – KE 18). Additionally, clusters of  SSBs on opposite DNA strands (KE 16), 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>, Figure2 – KE 20)105,109.

DNA DSBs are repaired by two most prominent processes described below:

* **The homologous recombination (HR):** The HR process deals with DNA DSBs during S and G2 phases of the cell cycle when sister chromatid sequences are available and can be used as template to mediate faithful repair. This process is highly accurate and error-free121–123.
* **The non-homologous end-joining (NHEJ):** In NHEJ, the DSB ends are blocked from 5′ end resection and held in close proximity by the double-stranded DNA (dsDNA) end-binding protein complex, the Ku70-Ku80 heterodimer (Ku). This pathway facilitates the direct ligation of DSB ends but is error-prone, often leading to small insertions, deletions, or substitutions at the break site, and potentially causing translocations when DSBs from different genomic regions are joined121,122,124. Globally, studies show that the NHEJ pathway is predominant compared to HR, repairing up to ∼80% of all DSBs in human cells123,125,126.

Consequently, this DNA DSB repair pathway is an important genotoxic parameter to monitor and is included in the AOP network through the following KE:

* **“Inadequate DNA repair (NHEJ)”** (Figure 2 – KE 21): The inadequate repair of DNA DSBs by NHEJ, notably due to its intrinsic reduced accuracy, provokes genotoxic AOs, i.e. **“Increase, Structural chromosome aberrations” (AO 23)**  and  **KE 185 “Increase, Mutations” (AO 22)**.

Finally, more complex damages, i.e. DNA interstrand crosslinks (MIE 8), are repaired through a combination of repair pathways112,127,128.

* **The Fanconi Anemia (FA) repair pathway**: The FA repair pathway is primarily dedicated to dealing with the most cytotoxic primary DNA damage, the DNA interstrand crosslink. This damage, blocking both replication and transcription processes by impeding the strands' disjunction. The Fanconi Anemia pathway is believed to orchestrate a complex repair mechanism that integrates components from three major DNA repair pathways: homologous recombination, nucleotide excision repair, and mutagenic translesion synthesis. This process relies on a specialized nuclear protein complex that ubiquitinates FANCD2 and FANCI, facilitating the assembly of DNA repair structures128. The FA pathway, although primordial for cell survival, involves mutagenic polymerases (TLS) to bypass lesions at the cost of potentially introducing mutations (AO 22) in the genome129. 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 have also a crucial role in replication fork protection and implicated in replication fork restart (KE 18)129.
* **Inadequate DNA repair (Fanconi anemia repair)** (Figure 2 – KE 19: This last pathway thus represents the last sub-KE of KE 155 “Inadequate DNA repair” (<http://aopwiki.org/events/155>) which implies the creation of transient DNA DSBs (KE 20)**.** Overall, a high rate of interstrand crosslinks implies a high rate of transient DSBs, 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 and reversion processes, will converge through the different KEs to form gene mutations and sutrucural chromosome aberrations.

* **Increase, Mutations** (<https://aopwiki.org/events/185>, Figure 2 – AO 22):

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 close to the thymine, O6-alkylguanines (KE 14) are mispaired with adenine107, leading to GC → AT transitions after two replication cycles130. Similarly, 8-oxoG (KE 14) 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 transitions131,132 (<https://aopwiki.org/relationships/1914>). All DNA damage susceptible to be addressed by TLS repair processes (KE 15 – Inadequate DNA repair [BER, NER, TLS]), i.e. modified DNA bases (KE 14), DNA intrastrand crosslinks (MIE 3), DPCs (MIE 4) and bulky DNA adducts (MIE 5), as well as incorrectly repaired DNA SSBs (KE 17), may lead to an increase in the gene mutation rate113,115. Imbalances in dNTP pool, 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 cells90,98. Different models combining misinsertion, misalignment and mismatch extension mechanisms have been established to explain imbalanced dNTP-induced gene mutations86. Moreover, DNA DSBs (KE 20) have shown to cause gene mutations (<https://aopwiki.org/relationships/1931>) through inadequate repair (KE 21)133. Indeed, the NHEJ process is known to be error-prone, while its ability to provoke gene mutations depends on the types of ends created during the generation of the DSB134. Large deletions, more than point mutations, seem to be the cause of mutagenicity in this case. Moreover, these mutations are typically rare events133 compared to the mutation rate after exposure to base-modifying agents such as alkylating agents. Thus, topo-II inhibitors, which induce more DNA DSBs than alkylating agents, are less likely to generate gene mutations34. 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 DSB require a mis-repaired-inside-gene DSB, which could also be a rare event compared to multiple-points-gene small alterations provoked by alkylating agents. Consequently, all MIEs/KEs causing DNA DSBs, i.e. inhibition of topoisomerase II (MIE 9), stalled replication fork (KE 18) and deposition of energy (<https://aopwiki.org/relationships/1981>, MIE 10), DNA SSBs accumulation (KE 16) and their inadequate repair (KE 17), are also able to increase the gene mutation rate through NHEJ124–126,134,135.

* **Increase, Structural chromosome aberrations** (<https://aopwiki.org/events/1635>, Figure 2 – AO 23):

Uncontrolled DNA DSBs (KE 20), that can directly appear after topo-II enzyme inhibition (MIE 9)34,67, deposition of energy (<https://aopwiki.org/relationships/1982>) and direct strand breakage (MIE 10)70, collapse of stalled replication forks (KE 18)62 or through opposed-strand SSBs proximity (KE 16)105,109 (<https://aopwiki.org/relationships/1939>), will be mainly repaired through the NHEJ process (KE 23)124–126,134,135. However, this error-prone repair process is known to provoke structural chromosomal rearrangements (<https://aopwiki.org/relationships/1912>)124–126,134.

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

1. **Towards AOP-based IATAs for genotoxicity**

The AOP network provides a structured and scientifically robust framework for advancing IATA development in genotoxicity assessment. As indicated in the introduction, the aim is to build IATAs that do not rely on animal testing and thus solely combine traditional and new innovative *in vitro* methods with existing knowledge and *in silico* models. 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 understanding2. For example, methods collecting data related to the AO ‘Increase, Structural chromosome aberrations’ do not provide insights into whether this permanent DNA damage results from a direct interaction with DNA (e.g. DNA alkylation) or rather indirectly via the generation of RONS. Such information is important for regulatory decision-making. 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 to 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. Such a mapping exercise has previously been done for the KEs and KERs in the AOP Oxidative DNA damage leading to mutations and chromosomal aberrations64 and forms a good starting point for mapping methods to KEs within the bigger network. However, to be useful in a regulatory context, it is important to characterize the “regulatory readiness" of the different methods. NAM development is indeed a long, timely and resource-demanding process that can roughly be subdivided into four major phases before regulatory implementation namely136:

**i. 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.

**ii. 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.

**iii. 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.

**iv. International harmonisation**: Several routes for international harmonisation 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 over 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-Price137. 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 studies136.

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 modelling.

1. **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 human cells. Moreover, this AOP network provides a solid basis for the science-driven development of IATAs for genotoxicity.

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**AUTHOR CONTRIBUTIONS**

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

**CONFLICT OF INTEREST**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Table 1. Inventory of AOPs related to genotoxicity collected from www.AOP-Wiki.org in August 2024**

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| --- | --- | --- | --- | --- |
| **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 leukaemia | 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> |

**FIGURES**

**Figure 1. Draft AOP network leading to permanent DNA damage.**

**Figure 2. AOP network leading to permanent DNA damage (mutagenicity and clastogenicity)**