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THE DEVELOPMENT AND USE OF ADVERSE OUTCOME PATHWAYS IN MECHANISTIC TOXICOLOGY

Bachelor's Thesis
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ABSTRACT

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The development and use of adverse outcome pathways in mechanistic toxicology
Haittavaikutusreittien kehitys ja käyttö mekanistisessa toksikologiassa
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Adverse Outcome Pathways (AOPs) offer a structured and mechanistic approach to understanding the toxicity of chemical substances, enabling a shift away from traditional animal-based testing methods. This thesis explores the historical development, conceptual structure, and practical applications of AOPs within the field of mechanistic toxicology. It outlines the emergence of AOPs in response to the ethical and scientific limitations of apical-endpoint testing and highlights the necessity of the paradigm shift in chemical safety assessment towards more rigorous, economical and sustainable methods such as new approach methodologies (NAMs) and integrated approaches to testing and assessment (IATAs).

The work also examines AOP development strategies, from manual curation to computational modelling, and the use of platforms like OECD-facilitated AOP-Wiki. Further, it considers the integration of omics data, artificial intelligence, and quantitative modelling (qAOPs) to enhance AOP utility in regulatory frameworks. Examples in related literature illustrate the use of AOPs in chemical prioritisation, biomarker discovery, nanomaterial assessment, and the development of predictive models. Ultimately, the thesis argues that AOPs are essential tools for enabling ethical, efficient, and scientifically robust chemical safety assessment, with significant potential to support the ongoing transformation of regulatory toxicology.

Keywords: Adverse outcome pathways, mechanism of action, mechanistic toxicology, new approach methodologies, integrated approaches to testing and assessment, AOP development

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PREFACE

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Jenni Leppänen

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

3R	Reduction, Refinement and Replacement
AI	Artificial Intelligence
AO	Adverse Outcome
AOP	Adverse Outcome Pathway
EC	European Commission
ECHA	European Chemicals Agency
EPAA	European Partnership for Alternative Approaches to Animal Testing
EU	European Union
HTP-seq	High-Throughput Sequencing
IATA	Integrated Approaches to Testing and Assessment
IOP	Impact Outcome Pathway
KE	Key Event
KER	Key Event Relationship
LD50	Median Lethal Dose (dose lethal to 50% of tested organisms)
LLM	Large Language Model
MIE	Molecular Initiating Event
MOA	Mechanism of Action
NAMs	New Approach Methodologies
NLP	Natural Language Processing
NM	Nanomaterial
OECD	Organisation for Economic Co-operation and Development
qAOP	Quantitative Adverse Outcome Pathway
QSAR	Quantitative Structure-Activity Relationship
REACH	Registration, Evaluation, Authorisation, and Restriction of Chemicals
TT21C	Toxicity Testing in the 21st Century
US EPA	United States Environmental Protection Agency
WHO	World Health Organisation
WoE	Weight-of-evidence

1. INTRODUCTION

Toxicology, as a scientific discipline, plays a crucial role in ensuring the safety of chemicals, pharmaceuticals, and environmental agents. Toxicology as a field is undergoing a structural change, and at the forefront of the change are Adverse Outcome Pathways (AOP) (Ankley et al., 2010), which provide a systematic framework for linking molecular-level events to adverse biological outcomes. These pathways are rooted in mechanistic toxicology, a branch of toxicology that focuses on determining the mechanism of action (MOA) behind toxic substances. The modular and reusable AOPs allow for the integration of mechanistically robust data sources to toxins, which have the potential to facilitate research and regulatory decision-making. They bring the necessary mechanistic background for New Approach Methodologies (NAMs) and Integrated Approaches to Testing and Assessment (IATAs), enabling more predictive and human-relevant models for chemical safety assessment. (Saarimäki et al., 2023b; Tollefsen et al., 2014)

This development is part of a broader paradigm shift in toxicology. Historically, the field has relied heavily on animal testing, which provided easily observable, apical-endpoint testing data and laid the groundwork for toxicity testing frameworks. (Hayes and Kobets, 2023) However, technological advancements have highlighted the limitations of these traditional approaches—including ethical concerns, inefficiencies, and poor predictive capacity for human outcomes. (Kiani et al., 2022; Krewski et al., 2010; Neuhaus et al., 2022) As a result, there is increasing demand for more mechanistically grounded, efficient, and ethically sound alternatives. (Parish et al., 2020)

This thesis explores the historical evolution, methodological development, and practical applications of AOPs in mechanistic toxicology. It examines the potential of AOPs to contribute to the transition away from animal-based methods, offering a hopeful vision of a future where ethical, efficient, and predictive chemical risk assessment is possible. With their structured, modular design and emphasis on mechanistic insight, AOPs have the potential to revolutionise toxicology. Their growing role in regulatory frameworks and research highlights their transformative potential in advancing the field toward a more sustainable future.

2. HISTORY AND BACKGROUND

Before we discuss AOPs further, it is important to examine the history of chemical safety assessment, and the paradigm shift that is currently shaping how we evaluate chemicals and shift away from current, apical-endpoint focused testing methods. It is also important to understand how and why the shift to more sustainable and alternative methods is necessary, what kind of organisations and legislation we must uphold to maintain the safety network, and what the rudimentary steps were to get to where we are now in chemical safety assessment.

2.1 History and development of chemical safety assessment

The modern use of chemicals, pharmaceuticals, and medical devices has always required safety and regulatory measures. This necessity led to the emergence of toxicology as a scientific discipline dedicated to studying the adverse effects of chemical, biological, and physical agents on living organisms. The roots of toxicology trace back to ancient civilisations, where early humans had to distinguish toxic plants and animals from those safe for consumption. As the field evolved, critical figures like Paracelsus in the 16th century shaped its foundational principles. Often regarded as one of toxicology's "Fathers," Paracelsus famously stated, "All substances are poisons; the right dose differentiates a poison from a remedy", which is modernly truncated to "the dose determines the poison". This concept of dose dependency remains central to modern toxicology. (Hayes and Kobets, 2023; Radenkova-Saeva, 2008)

As toxicology evolved through the centuries, animal testing became a cornerstone of chemical safety assessment due to its ability to provide observable outcomes, offering insights into whole-organism responses to acute toxicity. This was particularly crucial during the Industrial Revolution and the 20th century due to the rapidly increasing amounts of newly produced chemical substances and increased production, in many cases leading to chronic human exposure to certain chemicals, especially in occupational settings, where little mechanistic toxicological data was available (Hayes and Kobets, 2023).

One of the leading methods of modelling toxicity at the time was the LD50 test, also known as the median lethal dose, where the studied substance is lethal for 50% of animals tested (Hayes and Kobets, 2023; Hodgson and Roe, 2014), which at its core relies on the dose-dependency principle introduced by Paracelsus. It was introduced in

1927 and was widely used as a meaningful quantitative measure of acute toxicity to the degree that many regulatory authorities necessitated it (Hayes and Kobets, 2023).

Thus, the reliance on phenotype-based assessment—observing visible outcomes such as organ damage, lethality, or behavioural changes—became standard practice, as these endpoints were measurable and reproducible in controlled environments. However, for example, the previously mentioned LD50 tests were eventually, in numerous instances, deemed to be unethical use of animals due to the limited information they provided about the potential toxic actions of substances (Hodgson and Roe, 2014). So, although innovative for its time, the phenotype-based approach defined limitations that modern toxicology now seeks to overcome.

2.2 Transition from Animal Testing to Alternative Models

Toxicity testing has long relied on *in vivo* animal experiment-based methods, but around the 20th century, the idea of reducing animal testing became widespread. A broader recognition of the problems related to animal experimentation led to the need to move away from the apical end-point-based methodologies to more sustainable, alternative methods. The main driving forces of this change are the ethical, economic, regulatory, and logistical challenges animal testing poses (Kiani et al., 2022; Krewski et al., 2010; Neuhaus et al., 2022). As a result, this led to the coining of the 3Rs (Reduction, Refinement and Replacement) principle (Russell et al., 1959), which seeks to set the standards for the contraction of these challenges.

Additionally, animal experimentation is inefficient in producing robust biological data and results, both in predictive and transferable aspects, that can be paralleled in humans. In regulatory toxicology testing of inhaled substances, rats have commonly been used as test subjects for the potential inhaled toxic substances because of the similarities in the structure and function of the mammalian respiratory tract. However, a recent study found that further research in the anatomy and physiology of the rat respiratory tract revealed underlying issues in the use of rat data for predictive and translational effects in humans due to concerns about not understanding the mechanisms causing the adverse effects. (Stucki et al., 2024) Similarly, other examples highlight the challenges in translating animal-based evidence to human health, which proves that these concerns are not based on singular events but are a part of a wider problem with using animal-based data in safety assessment.

At present, the World Health Organisation (WHO) outlines (https://www.who.int/health-topics/chemical-safety#tab=tab_1, last visited 22.11.2024) the central aim of chemical

safety assessment to be the protection of human health and environment from the possible risks that different chemicals pose, along with the appropriate measures to deal with chemicals from their inception to their disposal from use. Thus, in the current day and time, many methods and legislation have been brought forth along with technological advancement to support these aims, described as the “paradigm shift” of animal experimentation and chemical safety assessment (Krewski et al., 2010; Parish et al., 2020).

In the European Union (EU), the current framework of chemical safety assessment is governed by the REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals) legislation set in place on the 1st of June 2007 and enforced by its overseeing political body, the European Chemicals Agency (ECHA). The REACH regulation shares the same aim, coinciding with the WHO’s, and it is outlined on ECHA’s website for this matter (https://echa.europa.eu/regulations/reach/understanding-reach, last visited 26.11.2024). It states its purpose to be in support of chemical safety assessment in the aspects of human health and environmental safety assessment, including promoting alternative hazard assessment methods, ensuring free circulation of substances, fostering innovation, and adhering to the precautionary principle. (European Parliament and Council, 2006)

As a procedure to initiate the structural transition from phenotype-based models to other alternatives in chemical safety assessment, many plans and strategies have been proposed. One of the most notable collections regarding the subject has been the introduction of the report *Toxicity Testing in the 21st Century* (TT21C) by the US National Academy of Sciences in 2007. It outlined the challenges of traditional animal testing and proposed a change in the focus of chemical safety assessment to centre on toxicity pathways and *in vitro* testing as the new toxicity testing paradigm, as detailed in Table 1. (Krewski et al., 2010)

The report outlined four options (I–IV) for conducting toxicity testing, ranging from *in vivo* phenotype-based assessments to more complex *in vitro* methods. It compared these options across various criteria, organised by topic in the rows, with each option's evaluation presented in the corresponding columns. Based on this analysis, it is evident that in vitro methods are something to aim for, but an overall integration of different methods to support chemical testing is a vital goal for the future of chemical assessment.

Table 1. *Table 1. Options explored for future chemical safety assessment. (Krewski et al., 2010, edited from Table 2.)*

Point of reference	*Option I, in vivo*	*Option II, tiered in vivo*	*Option III, in vitro and in vivo*	*Option IV, in vitro*
Biological basis	Animal biology	Animal biology	Primarily human biology	Primarily human biology
Dose amount	High doses	High doses	Broad range of doses	Broad range of doses
Testing and Screening	Low throughput	Improved throughput	High and medium throughput	High throughput
Cost	Expensive	Less expensive	Less expensive	Less expensive
Time	Time-consuming	Less time-consuming	Less time-consuming	Less time-consuming
Amount of test animals	Use of relatively large numbers of animals	Use of fewer animals	Use of substantially fewer animals	Use of virtually no animals
Response in subject	Based on apical endpoints	Based on apical endpoints	Based on perturbations of critical cellular responses	Based on perturbations of critical cellular responses
New approaches available		Some screening using computational and *in vitro* approaches; more flexibility than current methods	Screening using computational approaches possible; limited animal studies that focus on mechanism and metabolism	Screening using computational approaches

The report highlighted the importance of moving to new approaches, such as high-throughput sequencing (HTP-seq) techniques, human-cell-based toxicity pathways and understanding the mechanism of action (MOA) of dangerous substances (Krewski et al., 2010), — all of which now form the basis for a new era of chemical safety assessment.

In the scope of the development of AOPs and the future of chemical safety assessment, an important intergovernmental institution, the Organisation for Economic Cooperation and Development (OECD), is working with governments on economic, social, and environmental challenges. (Nikfar and Mozaffari, 2023). In 2012, the OECD initiated the AOP Development Program to improve and harmonize chemical assessment methods (Villeneuve et al., 2014).

Together, all these projects and legislation provide insight into the paradigm shift in chemical safety assessment. They outline a global phenomenon that will provide all the necessary tools for reducing animal testing without compromising chemical safety. Instead of focusing solely on animal-based methods, they will form a more informed and sustainable way of conducting toxicity testing in the future.

2.3 New Approach Methodologies

New Approach Methodologies (NAMs) are methods and technologies that are not necessarily recently established but new in the sense of innovation. They include methods that incorporate techniques highlighted in the TT21C report, such as *in vitro* testing that are novel in technique, and other procedures that focus on non-animal testing (Krewski et al., 2010; Westmoreland et al., 2022).

One of the main focuses of these methods is to move from animal-based assays to either human-cell-based assays or to methods such as *in silico,* entirely computational, and *in chemico* methods, which are not derived from living organisms but interact with chemical reactivity (Cronin et al., 2009; European Chemicals Agency (EU body or agency), 2016). Thus, NAMs work as tools for bringing mechanistic understanding and data to chemical safety assessment. These methods play an integral role in our understanding of the molecular initiating event (MIE), which is an essential part of the AOP model (Hayes and Kobets, 2023).

Thus far, the use of NAMs has mainly been industrial because of the narrow acceptance of their extensive use in regulatory settings in the EU and in the rest of the world. However, agencies such as the European Partnership for Alternative Approaches to Animal Testing (EPAA) also advocated for their use in these settings when

the aim is to support regulatory decisions addressing human health (Westmoreland et al., 2022). Other agencies worldwide have started to recognise NAMs as regulatorily robust methods of generating data in the testing of chemicals, following OECD Testing Guidelines and OECD Principles of Good Laboratory Practice, and with thoughtful consideration, agencies can include non-OECD guideline methods that they deem practicable and reliable for their needs (Stucki et al., 2022). This has also led to the increased development of non-OECD guideline methods alongside OECD's efforts, advancing regulatory practices, and bringing forth evaluation frameworks such as Integrated Approaches to Testing and Assessment (IATA) and the AOP framework (Stucki et al., 2022).

2.4 Integrated Approaches to Testing and Assessment

Due to the complexity of whole-organism models, using multiple testing methods to replace animal testing is generally necessary (Eskes, 2019). These testing methods, called partial testing methods, thus form an integrated approach to provide a holistic assessment and replace old, apical-endpoint-focused methods. Together, these systems of methods are referred to as IATAs.

One of the defining elements of IATAs are their capability to integrate and translate data from various sources and testing methods. They derive data from traditional *in vitro* and *in vivo* tests, in addition to using NAMs and computational methods to formulate a mode of action for the required system. The partial methods used may also be used to interpret and integrate the data. (European Commission, 2025; Organisation for Economic Co-operation and Development (OECD), 2025) While NAMs focus on developing and validating specific assays, IATA integrates these methods within a broader decision-making process, ensuring that data from various NAMs can be combined for a more robust safety evaluation (Eskes, 2019).

Both IATA and NAMs utilise the AOP framework, but IATAs incorporate it within a larger decision-making process, whereas NAMs focus on developing and validating specific test methods based on AOPs and singular assays, like singular KEs. Thus, IATA is a broader framework integrating various methods and data sources, including NAMs, to provide a holistic assessment.

One of the most valuable assets of integrating AOPs as a part of the IATA is the mechanical basis it provides for the development of said IATA (Tollefsen et al., 2014). The

AOP framework helps in organising and interpreting biological data relevant to toxicological endpoints, which then gives credibility for the use of IATAs in regulatory decision-making.

2.5 Mechanistic Toxicology

Mechanistic toxicology is a specialised branch of toxicology that focuses on understanding the MOA through which toxic substances exert their adverse effects. The focus of this field is to map out the sequence of events from the exposure to a harmful substance to the adverse outcome (AO), providing annotation and context to the pathway leading to the endpoint and providing a detailed explanation of the processes involved in the pathway.

On a molecular level, this means integrating chemical assessment with toxicogenomics, which involves the study of gene expression changes in response to toxicants. On higher levels of organisation, this can be perceived as systems toxicology, the description of all toxicological interactions in an organism computationally. Systems toxicology relies on the use of knowledge bases and integrated frameworks that compile data from multiple sources, along with computational modelling. (Waters and Fostel, 2004) For these purposes, the AOP framework is an essential tool for mechanistic toxicology to bring together pathways that span from molecular perturbations to toxicological endpoints. This also provides the means to model the network, or parts of it, depending on the requirements of the system.

3. ADVERSE OUTCOME PATHWAYS

This chapter explores the structure of AOPs, the most important structural building blocks, and how those blocks form the modular nature of this tool. Thus, this also introduces the network potential of AOPs, which is the starting point of the many important aspects of AOPs. AOPs can be developed in a multitude of ways, and the chapter also covers the development of these methods, from manual curation methods to mechanical and computational methods, encompassing how AOP development is regulated and shared within the scientific community.

3.1 Basic Structure

Ever since the introduction of the adverse outcome pathway (AOP) (Ankley et al., 2010), it has been a fundamental concept in toxicology and a tool for chemical safety assessment.

The AOP concept consists of multiple layers of information arranged to observable and measurable points throughout the relevant biological levels of relevance in the pathway termed key events (KE). KEs form a chain of events induced by a molecular stressor, coined as the molecular initiating event (MIE) with connecting key event relationships (KER) between the KEs that extend until a perceived AO (Ankley et al., 2010). The general structure is depicted in Figure 1, and examples of simple AOPs and how information can be formatted to different biological levels are depicted in Figure 2.

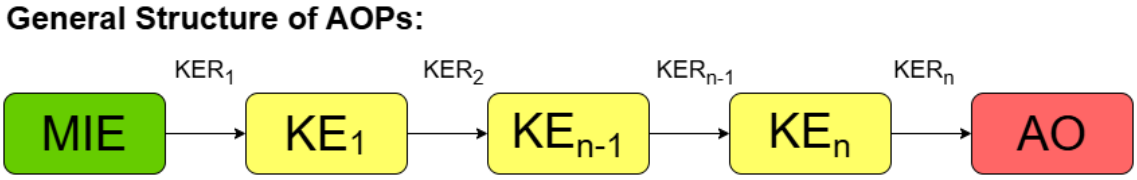

Figure 1. *The adverse outcome pathway (AOP) is a pathway of knowledge separated into different biological levels of interest. The molecular initiating event (MIE) is represented in the green box, and key events (KE) are represented in the yellow boxes. These lead to an adverse outcome (AO), which is represented in the red box. The connections between nodes, which represent key event relationships (KERs), are shown by the black arrows (OECD, 2018; edited from Figure 1).*

When multiple AOPs that share KEs or KERs are studied side-by-side, it is perceivable that the structure of AOPs enables the formation of networks. The general structure allows it to form networks naturally, offering a more comprehensive framework to model the complexity of biological systems. These networks are constructed by connecting

multiple AOPs that share KEs or KERs, creating a map of interconnected pathways influenced by a stressor or leading to a shared AO. (Knapen et al., 2018) This is demonstrated in Figure 2, where two original AOPs (AOP 1 and AOP 4) share KEs. This opens the possibility of two completely new AOPs (AOP 2 and AOP 3) being introduced to the network.

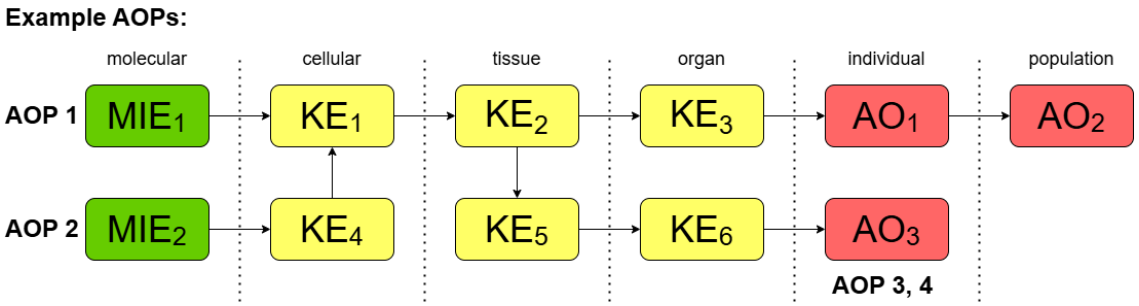

Figure 2. *The adverse outcome pathway (AOP) is a pathway of knowledge separated into different biological levels of interest. The AOPs 1–4 represent unique pathways from a MIE to an AO. AOPs are constructed by the following steps: AOP1 [MIE1, KE1, KE2, KE3, AO1, AO2]; AOP 2 [MIE2, KE4, KE1, KE2, KE3, AO1, AO2]; AOP 3 [MIE1, KE1, KE2, KE5, KE6, AO3]; AOP 4 [MIE2, KE4, KE1, KE2, KE5, KE6, AO3]. (Villeneuve et al., 2014, edited from Figure 1)*

The network concept acknowledges that single AOPs often simplify the interactions within biological systems, especially in scenarios involving chemical mixtures or stressors affecting multiple biological targets. As such, AOP networks provide a realistic representation of the cumulative and interactive effects of stressors on organisms, when multiple AOPs related to the same event are represented. (Knapen et al., 2018)

As models of toxicology, AOPs are toxicity pathways that can represent the MOA of multiple different chemical compounds in the studied organism. Generally, AOPs are derived from studies based on a single or few chemical compounds, but they are not chemical-specific, rather the MIE is the first step in the pathway caused by a certain substance or agent. The components of AOPs are also designed to be modular. This allows the reuse of the individual elements that make up the AOPs across multiple pathways. Therefore, by focusing on the systematic assembly of KEs and their connections, AOP development becomes a collaborative and iterative effort. This modular framework not only enhances efficiency but also supports the creation of AOP networks. (Villeneuve et al., 2014)

3.2 Early development strategies of AOPs

AOPs emerged as a tool of potential risk assessment at varying levels of biological organisation, spanning from the molecular level to the individual and eventually to greater

concepts such as the population level, based on the necessary relevance of the studied mechanism (Ankley et al., 2010). Its first use cases were based on ecotoxicology, but it was quickly adapted to human health assessment, organising mechanisms of disease progression and adverse health outcomes (Ankley et al., 2010; Clerbaux et al., 2022b; Nymark et al., 2021).

At first, the development of AOPs mainly focused on the manual assessment of already existing well-known toxicity pathways, such as singular case studies, where all the steps leading to the AO were not fully defined or understood (Ankley et al., 2010). However, it was soon realized that this method of working on a single case basis and generation of static documents was not enough to develop AOPs quickly and rigorously. Thus, multiple different methods of developing AOPs were outlined. (Villeneuve et al., 2014) The most common manual development methods and examples are presented in Table 2.

Table 2. *Manual development strategies of AOPs. (Villeneuve et al., 2014; based on Table 1)*

Strategy	Definition	Example
Top-down AOP development	Developer starts with an apical AO of interest and then delves down to progressively lower levels of biological organization in an effort to connect that outcome with a specific MIE (or multiple MIEs to construct a network of AOPs).	Investigators are interested in understanding the diversity of ways in which chemicals can adversely impact reproduction (Ankley et al., 2009). An important human health issue is strongly associated with chemical exposure, but the causes are poorly understood and predictive assays are lacking (Kimber et al., 2014).
Bottom-up AOP development	Developer starts with a well-defined MIE and begins linking to effects at higher levels of biological organization to develop the AOP.	A QSAR model or expert system for predicting chemical structures that will bind a receptor are available, but additional assays and endpoints are required to distinguish agonism from antagonism and link binding to hazard (Schmieder et al., 2004)
Middle-out AOP development	Developer starts with an observable phenotype or biological measurement (i.e., a KE) that is neither thought to be directly perturbed by exogenous chemicals or stressors nor considered to have regulatory relevance in and of itself. The developer then starts developing connections to the mechanisms underlying change in that KE and to the significance of that event as part of a causal chain leading to an AO.	Investigators are interested in developing alternatives to a widely used chronic toxicity test. Major morphological events during development are used as a set of observable KEs from which to initiate AOP development. (Villeneuve et al., 2013)
AOP development from case-study	Developer starts with a well defined sequence of biological events linking an MIE to AO for a single, well studied chemical, and then assembles evidence supporting generalization of that "motif of failure" for other chemicals/stressors that cause the same type of perturbation(s).	Detailed studies on the effects of 2,3,7,8-TCDD on cardiovascular development in fish was used as a basis for development of an AOP linking aryl hydrocarbon receptor agonism to early life stage mortality and/or impaired growth. (Volz et al., 2011)
AOP development by analogy	Developer starts with an AOP that has been well defined in a particular animal model or particular class of organisms. Development focuses on evaluation of which KEs and KERs in the extant AOP are conserved in another organism/organism class of interest and develops alternative KEs and KERs for those that are not.	While not originally intended for AOP development, the basic concepts behind frameworks for analyzing the human relevance of animal model-based modes of action provide a prominent example. (Boobis et al., 2008) Similarly, analogy between AOPs developed for fish and those that may occur in birds has been considered. (Lalone et al., 2013)
AOP development from data-mining	Developers utilize high content and/or highthroughput data sets (e.g., "omics," Toxcast screening data) and other types of automated literature and data-base mining approaches to infer (generally statistically) relationships between KEs. This strategy is most often used for early stages of AOP development.	(Knudsen and Kleinstreuer, 2011) (Perkins et al., 2011)

Many AOPs have a basis in the manual curation methods, and these methods are still used to develop AOPs to this day. The prevalence of manual curation methods is highlighted in studies where the study focus is limited or based on only a few determinants, like in case studies, or when relevant and well-defined AOPs can be mirrored to study

new stressors. (Clerbaux et al., 2022a; Sandhu et al., 2024) However, as stated before, even in the early stages of development, there was already a need for more effective development methods. This led to the recognition that more flexible means of handling AOP-related data computationally and more mechanistic approaches are indispensable for processing the great amounts of data related to the generation and development of AOPs, as well as to the models derived from them (Wittwehr et al., 2017).

Out of the development methods presented in Table 2, the development method presented in the last row and column, “AOP development from data mining”, stands as an example for the need for computational methods in early development strategies. Even though better than working on a single case basis, this revealed the main problem concerning the manual development methods; although these methods often produce accurate and rigorous end-products and are broader than single case studies, the methods are too slow and arduous compared to the possible mechanistic development strategies.

One general problem affecting the development of AOPs in is the fact that it is often not academically well recognised for the researchers to conduct these studies. Although beneficial for their great potential, it typically does not grant academic recognition to the author (Bajard et al., 2023). Thus, publishing and updating of AOPs is currently a group effort for the betterment of toxicology development. Still, it is generally not motivating for the creators to work on tasks, and there is a need to move to more pragmatic approaches in AOP development (Svingen et al., 2021).

3.3 AOP Development Platforms

The urgent need for computational platforms for handling AOP-related documents led to the establishment of the OECD-facilitated AOP-Wiki, which is an open-source interface for collaborative AOP development and a platform for sharing AOPs, which is a part of the broader AOP knowledgebase (AOP-KB), which is a collection of individually developed platforms (Villeneuve et al., 2014). AOP-KB is still under development and will contain many modules to support AOP development. Currently, out of the modules present, the most through one and furthest in the development progress is AOP-Wiki.

AOP-Wiki is a group information gathering platform, where individual researchers may upload their related AOP data, with reusable modularity for mapping out the AOP itself, thus adding onto already existing information in the wiki, whilst contributing to the AOP network as a whole. The wiki is an effective method of gathering and distributing AOP

information, but it does suffer from the common problems of group information sourcing. There are many instances where the efficacy of the language used in the AOP-wiki would need revisions and often the biological annotations only partially cover the KE (Saarimäki et al., 2023b). There is also a need for the homogenisation of terms for the nodes. In addition, although KEs are meant to be modular and completely reusable, there are still instances where some KEs are limited in reusability due to being tied to the context of the AOP where the KE was first introduced (Saarimäki et al., 2023b), thus further hindering the AOP network through duplication of some KEs and creating unlinked sections in the AOP network.

These aspects need to be taken into consideration when using the AOP-Wiki as a resource, and the AOP-KB is currently under development and working on improving these matters as they become acknowledged (OECD, 2018). However, as of now, only a small portion of the AOPs available in AOP-Wiki are even considered for the OECD AOP development work plan and are endorsed by the OECD or its working committees, and the progress is slow. Currently, out of 501 AOPs available on AOP-Wiki, none of which are fully approved, and only 35 are endorsed by the working committees of OECD. However, a total of 103 AOPs are either under development or under review by the OECD. Thus, in total, only about 28% of the AOPs in AOP-Wiki are reviewed by OECD to some degree, and the rest are completely supported by individual researchers and scientists contributing to the AOP-Wiki project. (https://aopwiki.org/, last accessed 22.6.2025)

Once the development of AOPs began, there was no standardisation or guidance on the format of the AOPs, apart from the few model examples explored by previous researchers, which were limited in availability and scope. To address this, the OECD launched an international AOP development program in 2012 to unify AOP development efforts and provide a structured approach to including the necessary information. This initiative aimed to ensure that AOP descriptions adhered to a standardised format, enabling rigorous assessment of the measurements, the weight of evidence (WoE), and the overall suitability of AOPs for various regulatory applications. (Villeneuve et al., 2014)

The OECD, soon after the release of this program, introduced a new document to support the AOP development efforts of individuals and organisations; the first edition of *Users' Handbook Supplement to the Guidance Document for Developing and Assessing Adverse Outcome Pathways* became one of the first major projects aimed at unifying and systematically collecting AOP-based information (Villeneuve et al., 2014). The second edition, released in 2017, was improved as the experience gained over the

years in developing and assessing AOPs expanded along with the supporting material (*Users' Handbook supplement to the Guidance Document for developing and assessing Adverse Outcome Pathways*, 2018). The second edition of the handbook is currently available as an online material on AOP-Wiki for all users and is meant to be used as a supporting supplement for AOP developers and wiki-users alike.

3.4 Mechanistic and Computational Creation of AOPs

As shown in Table 2, computational tools were essential even in the early stages of AOP development. While initially applied primarily during the early phases, advancements in these technologies have enabled their integration across all stages of AOP development. Some tools, such as those used for quantitative structure–activity relationship (QSAR) analysis, were already used earlier, during more manually driven approaches. However, the range and application of computational tools have since expanded significantly and now play a role throughout the entire development process.

A multitude of AOP development methods are already in use, and they apply foundational mechanistic understanding and computational approaches to leverage the arduous generation process. These methods include, for instance, QSAR (Ankley et al., 2010; Villeneuve et al., 2014), Bayesian network modelling and regression modelling (Jeong et al., 2025), and automated workflows (Wiklund et al., 2023). This development has added quantitative, statistical and mathematical information to AOP development, providing a more precise understanding at all stages of development. By extending existing quantitative knowledge through the assessment of the quantitative plausibility of an AOP's KERs, the pathway can be characterized as quantitative in nature, referred to as a quantitative AOP (qAOP) (Conolly et al., 2017; Jeong et al., 2025)

HTP-seq, data mining and rigorous curation of molecular events associated with AOPs could facilitate the implementation of omics-based evidence. Saarimäki et al. outlined that AOPs, when combined with omics evidence, can "1) guide the interpretation of omics data readout, 2) support the development of new AOPs, 3) identify and fill gaps in knowledge, and 4) transfer AOP-based knowledge into robust assays to support chemical safety assessment" (Saarimäki et al., 2023b). Omics-based evidence is thus beneficial and provides further opportunities to link the mechanistic information needed for AOP development (Q. Vuong et al., 2025), serving as a valuable tool in the mechanistic creation of AOPs. Additionally, the insight into underlying mechanisms that omics data provides can inform chemical read-across by strengthening the evidence base for identifying MOAs, enhancing our understanding of biological pathways, and supporting more accurate predictions of species-specific sensitivities (Brockmeier et al., 2017).

The introduction of omics data associations between different data sources further strengthens the development of AOPs.

Due to the previously noted network capabilities of AOPs and the common problems with crowd-sourced information gathering, by rigorous annotation and refinement, it is possible to computationally discover novel AOPs in the already existing AOP network. These methods often integrate tools of systems biology (Jaylet et al., 2023; Saarimäki et al., 2023b), such as introducing supplementary annotation data to find important new links between KEs and AOs (Chai et al., 2021) and expanding the annotation for the pre-existing nodes in AOP-wiki with natural language processing (NLP) (Saarimäki et al., 2023b, 2023a). It is also evident that by providing supplementary annotation, the efficacy of the already existing network connections of biological system representations of AOPs can be improved. With the help of supplementary annotation, Saarimäki et al., were able to reduce the redundancy of a modelled AOP network by fixing duplicated KEs due to semantics, combining system-specific KEs to their general counterparts, distinguishing consecutive KEs from each other due to the lack of specificity and combining the up- and downregulation of the same biological event. An example in the article related to the AOP network on pulmonary fibrosis led to a 23% decrease in the total amount of KEs in the network, from 30 KEs to 23 KEs. Also, after refinement, one of the six AOPs in the network was entirely encompassed by the other five AOPs. (Saarimäki et al., 2023b) A development which shows that by curating the AOP data, we can achieve more efficient and definite results.

3.5 Future Approaches

There are still many different outlooks for the future growth of AOP development. One of the most prominent currently is using artificial intelligence (AI), especially large language models (LLMs), to enhance the mechanistic development of AOPs.

LLMs have already been recognised as a way to increase clinical efficiency and ease many practices in health care, although they have been scrutinised for their tendency to propagate bias and they have faced concerns regarding the limitations, transparency, ethics, and legality of these practices (Arighi et al., 2024; Kunze et al., 2025; Rao et al., 2023; Sallam, 2023). However, it is undeniable that the potential of AI and LLMs is similar to other significant technological developments like calculators and computers (Arighi et al., 2024), and will be crucial tools to biomedicine and toxicology, along with other fields. Many insights into using LLMs in AOP development have already been utilised, mainly employing the language processing and text-mining abilities of these mod-

els. Their use can improve the predictability and interpretability of chemical and material impacts, significantly reducing the time and effort needed for data identification and synthesis and minimising the risk of missing critical information. (Serra et al., 2025; Shi and Zhao, 2024).

Other methods explore the potential of using automated and harmonised approaches to generate AOP networks. For example, Wiklund et al. (2023) present a case study on endocrine disruptor-related AOPs, proposing a data-driven workflow that automatically formats and filters AOP-Wiki data for seamless import into network visualisation software. Their method demonstrates how automated workflows can streamline data extraction and facilitate AOP network generation (Wiklund et al., 2023). Thus, this and many of the methods mentioned in section 3.4 can also be considered future methods. Although partially in use, mechanistic development methods are, in many aspects, still under development and can be considered the future of AOPs and chemical safety assessment.

Even though generative tools have already been used in AOP development, the same limitations and concerns discussed broadly in healthcare applications similarly apply to the use of generative AI in AOP development. In the end, it is always the responsibility of the researchers to verify that the AI-assisted results are up to standards and void of errors. Thus, even though these tools are helpful, we always need to ensure the correctness of data and material processed by non-human tools to validate the results.

4. APPLICATIONS OF AOPS

As we have now explored why AOPs are a great and flexible framework to support chemical safety assessment, this chapter outlines some examples of applications for AOPs, especially from the perspective of research and regulation. AOPs are the strongest in their capability to provide and organise mechanistic reasoning, which can then be further utilised to find solutions to several questions related to the structurally linked and derivative aspects of AOPs. Quantitative and qualitative understanding of chemicals and toxicological events is required to solidify a rigorous reasoning to support chemical safety assessment. Evaluating how we can extrapolate AOPs to widen their application breadth is essential, which this thesis will explore in the last part of this chapter.

4.1 Contextualising methodology and facilitating regulation

The AOP framework is increasingly used in research to uncover toxicological mechanisms, predict biological responses, and support the development of new tools for chemical safety assessment. These research applications are foundational for scientific understanding and serve as a bridge toward regulatory use.

As the main aim of AOPs is broadening the understanding of toxicological mechanisms, their power lies in exploring causal pathways and predicting biological responses. They can be used to support their WoE evaluation, chemical prioritisation, and hypothesis creation (Bajard et al., 2023; Willett, 2019). There are already multiple instances where AOPs have been used for qualitative research and hazard recognition, both in human health and environment-related matters(e.g. He et al., 2024; Knapen et al., 2020; Lautan et al., 2025), and have been used as a tool for disease prediction on contemporary issues as well, such as for COVID-19 (Clerbaux et al., 2022a). Many studies also focus on only one or some of the KEs in the AOP, but more rarely on the whole AOP, and knowledge caps along the toxicity pathway are common. This hinders the applicability of the AOP, but developing AOPs still helps identify these gaps and helps guide future research. (Leist et al., 2017) Once entire AOP framework is rigorous enough, AOPs and the networks they form can be used as tools for multichemical risk assessment as well (Bajard et al., 2023).

One of the more practical applications of AOPs is the identification of biomarkers. Different biomarkers can be set for different KEs along with the AOP, and certain measurable KEs may be used as biomarkers of exposure and effect by the KERs. These may include alterations in gene expression, enzyme function, metabolite levels, hormone concentrations, or tissue structure. While such markers are commonly used in research, their use in regulatory decision-making has been limited. One key challenge is the lack of a clear connection between these molecular or cellular changes and the regulatory endpoints typically used in policy. AOPs help bridge this gap by contextualising biomarkers within a defined sequence of biological events, increasing their relevance and potential for application in research and risk assessment. (dos Santos et al., 2020; Saarimäki et al., 2023b; Villeneuve, 2017)

Due to the aforementioned reasons, AOPs have plenty of regulatory applications. As briefly introduced in sections 2.3 and 2.4, there is also a call for better use of NAMs and IATA in regulatory settings. AOPs stand to bridge the gap between these methods, mechanistic understanding, and predictive toxicology. They provide the qualitative reasoning necessary behind chemical risk assessments and offer a transparent, scientifically grounded framework that supports the integration of NAMs into regulatory decision-making. In this way, AOPs contribute significantly to the acceptance and validation of NAMs by illustrating how mechanistic data can be linked to adverse outcomes of regulatory concern (https://www.oecd.org/en/topics/sub-issues/testing-of-chemicals/adverse-outcome-pathways.html, last accessed 5.8.2025). Thus, AOPs can enable the contextualization of individual NAMs. For example, the Endocrine Disruptor Screening Program of the United States Environmental Protection Agency (US EPA) used AOPs to screen potential endocrine disruptors, mapping NAMs to multiple AOPs to connect and evaluate mechanistic information (Browne et al., 2017). As detailed, AOPs can be used to outline biomarkers, and AOPs have been used to validate some human effect biomarkers of phthalates and reproductive effects in 2019, too (Baken et al., 2019).

Moreover, AOPs support the development and application of IATA by structuring evidence across different levels of biological organisation. This enhances the reliability of predictions and facilitates weight-of-evidence considerations in risk assessments. Their contribution to structured decision-making is increasingly recognized, especially as regulators seek to replace or reduce animal testing while maintaining robust safety standards (Willett, 2019). As a result, this would strengthen the evidence-based rigour of AOPs and support their acceptance in regulatory settings.

Despite these successful inclusions of AOPs in regulation, the relative use of AOPs in validation is scarce. One fundamental limitation is the incomplete coverage of the biological landscape within current AOP repositories. While some topics—such as those related to oxidative stress, thyroid hormone disruption, and reproductive toxicity—are well represented, others like immunotoxicity and metabolic disorders remain underdeveloped. This limited scope reflects the relatively recent adoption of the AOP framework and the significant effort required to construct and maintain high-quality AOPs. (Bajard et al., 2023)

A key barrier to broader regulatory adoption of AOPs is the limited regulatory confidence, stemming from the slow and resource-intensive endorsement process, reliance on volunteer reviewers, and problems in data homogenisation. The lack of standardised and validated methods for measuring KEs also reduces confidence in their use for risk assessment. However, ongoing efforts to align test methods with AOP components and implement harmonised ontologies show promise. Additionally, the disconnect between AOPs and chemical-specific data remains a significant limitation. Because AOPs are designed to be chemically agnostic, integrating them with real-world exposure information, like with biomonitoring data, toxicokinetics, or QSARs, is essential but remains fragmented. Lastly, the absence of quantitative data describing relationships between MIEs and downstream events limits the predictive value of AOPs. Although initial steps toward qAOPs are underway, their development remains complex. (Bajard et al., 2023)

4.2 Evaluation of novel substances

AOPs can also help in evaluating emerging substances. Most commonly, the MIE of an AOP is a chemical or biological perpetrator, but AOPs are not limited to only those groups. For example, AOPs have been utilised in nanomaterial (NM) safety assessment, as the safety of many NMs cannot be fully characterised by conventional toxicity testing (Halappanavar et al., 2021). AOPs are regarded as a future consequential factor for NM safety assessment, and there are already applications for utilising AOPs for NM safety characterisation in environmental and health safety research, but there is a call for more (Khosrovyan et al., 2025; Li et al., 2025). An important strategy in advancing NM safety assessment involves drawing parallels between NMs and well-characterised chemicals (Halappanavar et al., 2020). If a NM can be compared with a well-characterized chemical perpetrator that is known to be the molecular perpetrator of a MIE in an AOP, there is a basis to suspect that the NM might share the same toxicological pathway, and this parallel can be drawn to other substances as well. These analogies are typically based on shared physiological, chemical, or biological properties, and can

have basis methods like exposure testing or QSARs. For instance, multi-walled carbon nanotubes have been associated with pathogenic outcomes similar to those caused by asbestos exposure (Poland et al., 2008). Likewise, metal oxide nanoparticles have been shown to induce toxicological effects resembling those observed in occupational settings involving exposure to silica, coal dust, or welding fumes (Donaldson and Seaton, 2012; Murugadoss et al., 2017; Napierska et al., 2010). These observations underscore the need for a more comprehensive understanding of NM-induced toxicity. They also support the hypothesis that substance-induced KEs and AOs elicited by conventional chemicals should be translatable to NMs (Halappanavar et al., 2020). Consequently, this further establishes that AOPs offer a valuable framework for predicting the toxicological behaviour of novel materials.

4.3 Quantitative Risk Assessment and Predictive Models

AOPs can be used for quantitative risk assessment when combined with quantitative data and an understanding of the needed perturbations driving the activation of KERs , such as time-course and dose-response predictions. In a report by Conolly et al., administered by the US EPA, they introduce a key application-oriented extension of traditional AOPs called qAOPs. qAOP is a vital tool for predicting the severity and probability of AOs, thus proving to be a critical part of targeted assays for different substances and chemical safety assessment. (Conolly et al., 2017)

In a qualitative AOP, the underlying assumption is often that once the MIE is reached in terms of dose and duration of that effect, it is sufficient to achieve the AO at the end of the toxicity pathway. There are no descriptive factors for the progress, regardless of the dose and time needed, that describe the KER activation that leads to advancement in the string of events. The qAOP takes the probability and severity of the AO into consideration (Conolly et al., 2017; Patlewicz et al., 2015) and the qAOPs can incorporate many types of quantitative data to be considered quantitative, from the quantitative WoE-based AOPs to qAOPs that utilise mathematical modelling to support mechanistic and probabilistic evaluation (Spinu et al., 2020). Similarly, AOP-informed predictive models vary in design, from agent-based to probabilistic methods (Wittwehr et al., 2017). At this stage, however, only a few resources exist to support the creation and regulatory evaluation of qAOP models (Spinu et al., 2020).

Due to the modular nature and network boundness, AOPs are well-versed for predictive modelling. KEs and KERs provide biologically defined anchor points that can guide QSARs, chemical categorisation, and model scaling. The biological description of these units also provides a basis of information for where the modeller can refer if needed,

since it is not granted that the modeller is as well-equipped to analyse the information with the AOP as the creators of the AOP are. This bridges gaps between fields of study and provides a starting point for multidisciplinary collaboration. (Wittwehr et al., 2017)

A practical example is the use of three linked models integrated into an AOP describing aromatase inhibition in female fish (https://aopwiki.org/wiki/index.php/Aop:25, last accessed 3.8.2025; Wittwehr et al.,2017). The AOP is linear with eight KEs in total, including the MIE and AOs. The series includes different predictive models that are utilised in different parts of the AOP, and where the downstream models use the output of the previous models. First, a biologically based dose–response model predicts plasma 17-beta-estradiol and vitellogenin levels (Breen et al., 2016). Next, a statistical model translates vitellogenin concentrations into effects on oocyte growth and ovulation (Li et al., 2011; Watanabe et al., 2016). Finally, a Leslie matrix population estimates density-dependent population changes (Miller et al., 2015; Miller and Ankley, 2004). This system of predictive models demonstrates how AOP-informed predictive models can be used in predictive toxicology, further expanding our understanding of the topic with concrete, quantitative mechanistic reasoning, which can then be applied in regulatory consideration of endocrine-disrupting substances.

4.4 Expanding on the AOP framework

There are numerous ways that the AOP framework can be expanded and combined with different data to encompass other applications and cover a wide range of outcomes. The qAOP and AOP-informed predictive models are great quantitative examples of this, but plenty more demonstrate the utility of AOPs in other dimensions.

The AOP fingerprint is an innovative way to present all the enriched AOPs and KEs of a given chemical. To build it, AOPs and KEs are first linked to specific gene sets through NLP and manual assessment to assess relevant matches. This step provides a bridge between molecular-level data and the AOP framework. Once this mapping is established, toxicogenomic data, for example, differentially expressed genes from experimental exposures, can be tested for enrichment against the mapped AOPs and KEs. The result is a distinctive “fingerprint” showing which AOPs and KEs are most strongly associated with a given chemical. By doing this, it is possible to examine, sort and curate chemicals by their enriched AOPs, to for example, find new AOs for said chemicals and better chemical grouping with precise and mechanism-based evidence. (Saarimäki et al., 2023b)

By centralising metainformation and annotation, the US EPA's project, the AOP-DB allows the integration of various data sources in a structured and accessible way. It is the first effort of its kind to automatically relate AOP mechanistic information to functional biological entities. The database supports exploration across multiple levels of biological organisation and includes information on species-specific variation. This enables researchers to link molecular-level data to adverse outcomes in a systematic and transparent manner, improving interoperability between datasets and enhancing the practical use of AOPs in both research and regulatory contexts. (Pittman et al., 2018)

To also account for sustainability, the INSIGHT project introduced the concept of impact outcome pathways (IOPs). These extend AOPs by incorporating environmental and socio-economic dimensions, offering a unified framework for assessing the broader risks of chemicals. IOPs promote the safe and sustainable development and use of chemicals and exemplify how AOPs can be extrapolated beyond their initial use cases. (Serra et al., 2025)

These developments illustrate the adaptability of the AOP framework and its potential for integration with diverse data sources, predictive tools, and broader impact assessments. As demonstrated by the examples above, AOPs can support more refined, transparent, and forward-looking approaches to chemical safety. Together, they highlight the framework's capacity to evolve alongside scientific and regulatory needs. This is an aspect that is central to the discussions and conclusions that follow chemical safety assessment.

5. CONCLUSIONS

AOPs represent a pivotal advancement in mechanistic toxicology, offering a structured, modular, and increasingly data-driven framework for linking molecular-level perturbations to adverse biological outcomes. This thesis has examined the evolution of AOPs from manually curated pathways to sophisticated, computationally enhanced networks that integrate diverse data sources, support predictive toxicology and possess a multitude of key applications for research and regulation.

The development of AOPs has been propelled by collaborative platforms such as the OECD's AOP-KB, alongside innovations that have facilitated the advancement of AOPs, such as integrating omics technologies, mechanistic methods, and AI improvements. These tools have expanded the scope and precision of AOPs, improving their application in regulatory decision-making, enabling their use in applications like biomarker identification, chemical prioritisation, and the evaluation of emerging substances such as nanomaterials.

AOPs are not merely theoretical constructs — they are practical instruments that can transform chemical safety assessment. Their ability to contextualise mechanistic data within a reproducible and transparent framework makes them essential for advancing non-animal testing strategies. AOPs stand to bridge the necessary mechanistic information for NAMs and IATA to facilitate their use in regulation as well. Moreover, their modularity and network potential position them as foundational tools for building predictive models and supporting both qualitative and quantitative risk assessment.

To fully realise the transformative potential of AOPs, a concerted effort is needed across academia, industry, and regulatory bodies. Researchers must continue to innovate and refine AOP development methods; regulators must work toward harmonised frameworks that embrace mechanistic evidence, and collaborative platforms must evolve to support the growing complexity of toxicological data. By investing in these areas, we can move toward a future where chemical safety assessment is not only more predictive and efficient, but also ethically responsible and scientifically robust.

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