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The Adverse Outcome Pathway approach in nanotoxicology

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ABSTRACT

An Adverse Outcome Pathway (AOP) is a conceptual construct that describes existing knowledge on the link between a molecular initiating event and an adverse outcome. A sequential chain of causally related events is portrayed at different levels of biological organisation. AOPs are considered to be useful mechanistic blueprints for the development of novel tools for human and environmental risk assessment. Following OECD guidance, an increasing number of AOPs for chemically-induced adverse effects in humans and environmental species are being proposed. Due to their unique properties, the toxicity of nanomaterials (NMs) and chemicals is often difficult to directly compare since their mechanisms usually differ. While there are still many knowledge gaps in our understanding of NM toxicity, an ever increasing number of mechanistic studies are shedding light on their toxicokinetic and toxicodynamic properties. In this paper, we introduce the concept of AOPs and analyse its possible implementation for nanotoxicology. We illustrate how the AOP framework can be used to rationally combine mechanistic knowledge relating to both NM- and chemically-induced liver toxicity to fill information gaps and guide the development of toxicity testing strategies. The differences between NM and chemically-induced adversity are proposed to be primarily related to differences in toxicokinetics and the nature of the initial Key Events in the AOP. Consequently, much of the mechanistic knowledge captured by AOPs that have been developed from consideration of chemically-induced toxicity is also relevant to describe AOPs applicable to NMs, at least in qualitative terms, and thus can be used to inform predictive modelling and risk assessment of NM toxicity.

Contents

Abbreviations: AO, adverse outcome; AOP, Adverse Outcome Pathway; CNTs, carbon nanotubes; H_2O_2, hydrogen peroxide; IATA, Integrated Approaches to Testing and Assessment; ILSI-RSI, International Life Sciences Risk Sciences Institute; IPCS, International Programme on Chemical Safety; ITS, Integrated Testing Strategies; KE, Key Event; KER, Key Event Relationship; MIE, molecular initiating event; MoA, Mode of Action; MWCNTs, multi-walled carbon nanotubes; NADPH, nicotinamide adenine dinucleotide phosphate; NM, nanomaterial; NLRP-3, NOD-like receptor family, pyrin domain containing 3; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, superoxide; OECD, Organisation for Economic Co-operation and Development; QSAR, quantitative structure–activity relationship; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; ROS, reactive oxygen species; SAS, synthetic amorphous SiO_2; SCCS, Scientific Committee on Consumer Safety; SiO_2, silicon dioxide; TGF-β1, transforming growth factor beta 1; TiO_2, titanium dioxide; WHO, World Health Organization.

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1. Introduction

For the regulatory assessment of chemicals, *in vivo* testing is still used extensively to fulfil information requirements, even though animal tests are typically very time-consuming, costly and questionable from an ethical perspective. Moreover, standard guideline tests offer sparse information on the mechanism of toxicity of a substance and thus provide little help in explaining why a substance might cause an adverse effect of regulatory concern. More than a decade ago, recommendations already emerged to focus on intelligent testing strategies [1] that move away from a "generalized, checklist approach" to cover data gaps by acquiring only essential information [2]. This has led to the development of Integrated Testing Strategies (ITS) to support the implementation of legislation such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) in the European Union [3,4], and to more recent efforts within the Organisation for Economic Co-operation and Development (OECD) to develop Integrated Approaches to Testing and Assessment (IATA) which optimally combine and exploit existing information, *in vitro* assay data and computational predictions to satisfy specific information requirements [5].

The International Programme on Chemical Safety (IPCS) of the World Health Organization (WHO) and the International Life Sciences Risk Sciences Institute (ILSI-RSI) initiated the Mode of Action (MoA) human relevance framework [6] for a better evaluation and harmonisation of the assessment of chemical risks. Following this, in 2012, a programme for the development of Adverse Outcome Pathways (AOPs) was launched by the OECD which has taken up many of the aspects of the WHO/IPCS work on MoA [7]. Initially described in the context of ecotoxicological risk assessment, an AOP was defined as "a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event (MIE) and an adverse outcome (AO)", by capturing the sequential chain of causally-linked Key Events (KEs) at different levels of biological organisation [8]. Subsequently, the AOP concept was extended to support the assessment of human health effects. AOPs aim to support regulatory decision-making by providing the knowledge base to support the development of novel test methods and (OECD) Test Guidelines, QSAR tools and IATA.

In practical terms, the description of an AOP is highly structured and follows well-defined principles and conventions, as described in OECD guidance, a supplementary 'User handbook' [9], and in the scientific literature [10–12]. For example, KEs have to be both measurable and essential (but not necessarily sufficient) for the AO in question, and the evidence presented to support the causal linkages between individual KEs, termed Key Event Relationships (KERs), should be based on both biological plausibility and empirical data. Evidence can be derived from various sources including *in vivo* and *in vitro* studies, or from computational modelling [8]. An AO can be defined at various levels: for human health effects, an AO seldom relates to whole population level, but rather to individual organ damage (e.g. liver fibrosis), which has consequences on the individual, whereas in environmental toxicology the AO usually relates to growth inhibition, reduced survival or reproductive impairment of an individual (e.g. a fish) and the consequences on the whole population. The MIE describes the interaction of a material (e.g. chemical) with a biological target, and can be either specific, such as ligand–receptor interaction, or non-specific (e.g. a toxicant physically residing in a bio-membrane) [9]. By definition, an AOP consists of a single MIE and a single AO, but can have multiple causally-linked KEs (Fig. 1). This leads to a simplified and "linear" representation of an individual AOP, which may be an adequate basis for prediction in certain cases. However, since KEs can be shared by different AOPs, and one MIE can lead to multiple AOs and vice versa, AOP networks generally represent a more relevant basis for toxicity prediction [12]. To facilitate the development of AOPs within a network context and to provide a practical collaborative platform for AOP developers to systematically capture, share and integrate their AOP knowledge, the AOP Knowledge Base, including the AOP-Wiki, has been launched in 2014 as publicly accessible tool [13].

Building of networks can be further supported by the emerging concept of Aggregate Exposure Pathways (AEPs), which has been recently introduced to integrate also complex exposure scenarios

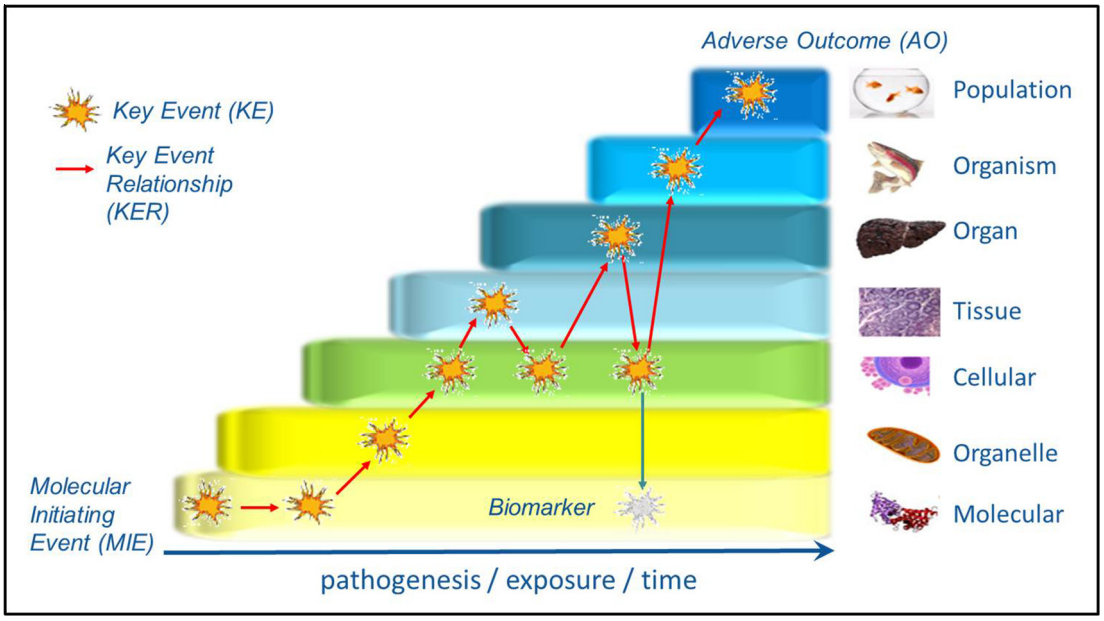

Fig. 1. Exemplary flow scheme of a typical AOP, starting from the molecular initiating event (MIE), inducing a variety of Key Events (KEs) connected by Key Event Relationships (KERs, red arrows) and resulting in a single specific Adverse Outcome (AO).

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and complement AOP development [14]. This would be a useful framework as so far, AOPs primarily focus on direct relationships between a toxicity endpoint resulting from the exposure to the trigger alone. In reality, human exposures are likely to occur as low dose co-exposures with a wide variety of materials, including bacterial agents, chemicals and pharmaceuticals. It is reasonable to suspect that toxicity, especially in the liver, may be driven by susceptibilities associated with such co-exposures.

To date, AOPs have been developed for chemically-induced AOs, although there is increasing awareness of this concept also in the nanotoxicology community. Recently, Vietti and colleagues published an overview of current knowledge and gaps on KEs involved in lung fibrosis development by carbon nanotubes (CNTs), with the intention to draft a respective AOP [15]. A large variety of KEs was described based exclusively on CNT-specific literature, leading to a complex pathway showing the various possibilities of CNT-induced lung fibrosis. Likewise, Labib and colleagues demonstrated the development of an AOP relevant for multi-walled carbon nanotubes (MWCNTs) based on *in vivo*-derived transcriptomic data. This exercise led to a simplified and linear AOP, as foreseen in the AOP guidelines, and demonstrates how transciptomic data can be used to derive pathway-based points of departure [16]. Again, however, this work is based solely on the literature on MWCNTs. In the present manuscript, we demonstrate how the AOP approach can be applied for nanotoxicology utilising not only NM-specific literature, but also existing knowledge on chemically-induced mechanistic toxicological processes. Following the initiator-agnostic AOP-philosophy, we show how the AOP approach can be used to describe the pathogenesis of NM-induced health effects of regulatory concern where NM-specific information is lacking. However, attention needs to be paid to distinguish NM-specific Key Events from generic ones. The case of liver toxicity induced by chemicals or metal oxide NMs is used as an illustration.

2. Toxicity mechanisms of metal oxide nanomaterials

The possible adverse effects of NMs on the human body are increasingly being discussed and investigated. In 2011, the European Commission proposed to define a NM as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm" [17]. However, this definition is under continuous discussion [18]. As the use and possible application areas of NMs for food and food-related products are increasing, it is not possible to test their toxicity on a case by case basis [19–22]. To tackle this, the ITS-NANO project has proposed an Intelligent Testing Strategy for NMs, similar to chemical testing. This strategy will help to identify priority research areas, ultimately limiting individual NM testing and supporting NM risk assessment [23,24]. The ITS-NANO report already acknowledged the usefulness of AOPs as a contributor to an ITS for NMs, and first AOP concepts, based on particle-specific literature and describing the species-specific outcomes of sustained particle overload in the lungs, have been described in an ECETOC technical report in 2013 [25] and by Morfeld and colleagues in 2015 [26].

Some of the major contributors to regular (nano)particle exposure are TiO_2 or amorphous SiO_2, all commonly used in cosmetics, pharmaceutical products or foods [20,22]. TiO_2 in its usual micron-size (inert) form is used as a whitener (E171), and also the synthetic amorphous SiO_2 (SAS) is accepted as a common food additive (E551), mainly in its micron-sized form, and used for example as an anticaking agent or thickener. A recent approach to estimate the content of nano-sized SiO_2 in food containing E551 revealed a likely worst case ingestion scenario of ~124 mg nano-SiO_2 per person per day [22], meaning that there might be continuous exposure and possible bioaccumulation. Peters and co-workers analysed the amounts of nano-sized SiO_2 in several of these E551 containing food products throughout a simulated gastro-intestinal digestion process. They report a disappearance of nano-sized particles in gastric pH conditions followed by their pronounced re-formation in an intestinal medium [27]. A recent approach to translate knowledge on SAS in food into risk assessment reported low gastrointestinal absorption rates of only 0.03, 0.06 and 0.2%, respectively, depending on the tested material and the treatment duration or reported study [28]. Interestingly, the absorption rate seems to decrease with an increase in administered SAS concentration, which was explained by possible gelation of this material in higher concentrations. This highlights once again that already low doses might result in accumulation and potentially in an adverse effect, since the uptake rate cannot necessarily be correlated to the administered dose. It also emphasises the necessity of appropriate kinetic models to accurately predict uptake rates [28,29].

The ability of NMs to directly induce the formation of reactive oxygen species (ROS) [19,30,31] is one of the most significant reasons for adverse NM effects. Likewise, oxidative stress is a known contributor to chemically-induced cell damage and toxicity [32]. A good correlation between cell free and cell-based *in vitro* ROS formation by NMs with their *in vivo* inflammation-generating potency has been described [33]. Low amounts of ROS can activate various signalling cascades within the cell, such as the phosphoinositide 3′-kinase/protein kinase B pathway which regulates cellular survival [34]. Excessive ROS formation however can induce genotoxicity, leading to DNA strand breakage, oxidative lesions, micronucleus formation or sister chromatid exchanges, and thus can be potentially carcinogenic [35].

In line with this, a classification model was developed to associate the reactivity of metal oxide NMs with the potential to generate oxidative stress [36]. The model is based on the ability of the NMs to exchange electrons with biological redox species in the cell (e.g. antioxidant molecules such as cytochrome C and glutathione). By using this simplified framework, it is possible to predict in a first ranking whether a given metal oxide has the potential to cause oxidative stress by checking whether its band energy levels (conduction and valence bands) overlap with the range of redox potentials of biological reactions occurring inside the cell. The model has been verified by independent experimental studies on 24 metal oxide NMs [37]. It should be kept in mind, however, that this model was only partially accurate in predicting the capacity of metal oxide NMs to induce oxidative stress, whereas other metal oxides induce similar effects through ion dissolution. This illustrates the importance of relating QSAR properties to proposed MIEs.

ROS formation is also an important player in the relationship between inflammation and carcinogenesis. The inflammatory phagocyte respiratory burst leads to the indirect formation of ROS, catalysed by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [38]. Indeed, activated neutrophils have been shown to cause oxidative DNA damage in rat lung epithelial cells [39,40]. Also macrophages are known to play a significant role in NM induced inflammatory processes. They are a key player in the uptake and elimination of inhaled NMs [41]. Upon the uptake of a NP by immune cells such as dendritic cells or macrophages, the NLRP-3 (NOD-like receptor family, pyrin domain containing 3) inflammasome can be activated. This process that has been described for both crystalline and amorphous SiO_2, but also for TiO_2 and can ultimately lead to oxidative stress, cell death and inflammation [42,43].

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Recent studies underpinned the importance of lysosomal NM uptake for NM-induced toxicity. Once the material is taken up by a cell (such as macrophages or also human astrocytoma cells) and transported to the lysosome by autophagy, the acidic milieu therein can either enhance solubility of a NM, or the material remains in its initial nano-form. Both situations can induce toxicity, causing lysosomal swelling, followed by lysosomal disruption and the release of pro-apoptotic proteins and inflammation [44–46]. It is known that particles of low solubility and toxicity, such as TiO_2, may cause inflammation in proportion to their specific surface area [47,48] and, as more recently described, their zeta potential [46]. The zeta potential describes the electric potential between the surface of a NM (or associated groups thereon) and the suspension medium. It is known that the negatively charged cell membranes can interact more easily with positively charged NMs, making these potentially more toxic than neutral or negatively charged NMs [46]. Acute pulmonary inflammogenicity *in vivo* is highly correlated with the zeta potential in an acidic environment (as present in the lysosomes of a cell) for low-solubility NMs. Disruption of the lysosome can trigger an inflammation cascade in the target organ. The particle-driven inflammatory response is associated with tissue damage, remodelling and mutagenesis and is referred to as secondary particle toxicity following the exhaustion of antioxidant and DNA damage repair capacities, as has been described for the lung [30,35,49]. For highly soluble particles, however, the nature of the ion defines its toxic and/or inflammogenic potential (e.g. Zn^{2+} as an example of a highly toxic ion versus Mg^{2+} as an example of a low-toxicity ion) [46]. Thus it is obvious that solubility is an important aspect in nanotoxicology, and the question arises whether a dissolved material still acts as a "nanomaterial" in the target tissue. Therefore, here we focus on low-solubility NMs.

Once in a matrix (e.g. a food) or dispersed in biological fluids, such as mucins or the blood plasma, NMs come in contact with a large variety of proteins (e.g. approx. 3700 in the blood plasma), leading to the formation of a protein corona. This is the biomolecule coating that forms around NMs upon contact with biological molecules and depends on both the size and surface properties of the NM [50]. The corona consists of a hard and a soft corona: the hard corona is a tightly bound, near-monolayer of biomolecules around the NM, surrounded by a loose layer of biomolecules, the soft corona. The soft corona can easily be exchanged, whereas the hard corona often retains biomolecules from previous environments [51]. Therefore, its composition varies in time and depends on the environmental conditions [52]. The corona is stable for a longer time than the typical time scale of cellular uptake, thus acting as cell "mediator" in the interaction of the NM with cell receptors [53].

2.1. NM-induced liver toxicity

The liver is one of the main target organs for ingested NMs, but inhaled particles can also reach the liver upon clearance from the lung [54–57]. The mononuclear phagocyte system, also called reticuloendothelial system, is one of the important players in NM uptake and systemic distribution. It consists of the phagocytic monocytes and macrophages which are present in the body, especially in the liver but also in spleen, lymph nodes and bone marrow. Following recognition and phagocytosis by macrophages of both the mononuclear phagocyte system organs and in the blood, NMs are sequestered to those organs. NMs that are taken up by hepatocytes are potentially excreted into the bile, whereas NMs phagocytosed by Kupffer cells, the resident macrophages of the liver, generally remain in the cells for a long period of time if they can't be degraded intracellularly [58–61]. Once in the liver, TiO_2 nanoparticles may have the potential to induce DNA damage and mutagenesis [62,63], but this has been described mainly at high doses and not following inhalation and has been suggested to be due to induction of systemic inflammation [62]. *In vivo* experiments on gavaged or injected (intraperitoneal or intravenously) TiO_2 suggest a wide range of adverse effects on the liver: an increase in general serum markers for liver damage such as Alanine Aminotransferase or Aspartate Aminotransferase [64,65], an increase in inflammatory markers such as pro-inflammatory cytokines and/or infiltration of inflammatory cells [55,66,67], an increase of markers for oxidative stress [68,69], apoptosis, necrosis and also fibrosis [70,71]. It has to be noted, however, that many of these studies use relatively high particle doses, and the reported effects are usually seen at the highest treatment doses. When low doses of NMs induced an adverse effect, such as an influx of inflammatory cells, recovery to control levels on cessation of NM exposure was reported [67].

Liver damage and inflammation have also been reported for other metal oxide particles such as SiO_2 [29,72,73] via various application routes such as intraperitoneal injection or oral administration. Similarly, increased accumulation of the NMs in the liver is often reported. Oral NM administration appeared to induce overall milder adverse effects than systemic administration, most likely due to the typically limited absorption of NMs in the GI tract.

3. Considerations for the development of AOPs for nanomaterials

As for most risk assessment approaches, the lack of human data is a major difficulty in evaluating the human liver toxicity potential of metal oxide NMs. Risk assessment is based on data from animal studies, mainly conducted in mice and rats, and from *in vitro* experiments with human or rodent cell lines. Moreover, the properties of the nanomaterials tested in various studies vary greatly. The most apparent differences lie within the primary particle size distribution of the materials. NMs of an average primary particle size of 5 nm may lead to a different AO or altered severity of the AO than materials of 100 nm size on average. Furthermore, the tested NMs can vary in their crystalline structure (such as rutile versus anatase in the case of TiO_2) and the ratio of these crystalline forms, their surface charge, shape or specific surface area. The toxicokinetics of the NM, including its solubility, is obviously a key factor in describing a material's toxicity, but are often unknown or only partially described. Another major issue is the lack of sufficient physicochemical characterisation especially in early publications. The problem and its consequences have been described extensively [74] and has led to an increased awareness of researchers [75,76]. Scientific journals imposed minimal requirements for adequate NM characterisation and an internationally recognised guidance has been published by OECD [77]. But even if NMs are well-described in their pristine form many publications still lack a thorough characterisation *in situ*, i.e. the biological fluids the NM interacts with. Furthermore, tested doses in different studies can differ immensely and are therefore not comparable; sometimes the high concentrations used are not relevant for real life exposure conditions. Further, exposure scenarios vary in terms of duration (days versus weeks or months), the route of exposure (for example oral gavage vs injection), and the investigated toxicological endpoint.

Understanding the relevant physico-chemical properties of NMs in biological systems is vital when it comes to defining the characteristics of the NM that initiates an event which could potentially result in an AO. Knowledge about the initial fate and biotransformation of the NM *in vivo* prior to reaching the biological target is often sparse, although the route of uptake plays a major role. Ingested NMs pass through different pH environments prior

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to their intestinal uptake, affecting the net surface charge [78] and solubility. The physicochemical properties of the NM and its transformation products have a considerable influence on the absorption, distribution and excretion processes that ultimately determine the fate of the NM in the body. Moreover, a NM might not only act directly at its target organ, but might also cause toxicity via second messengers. E.g. inhaled particles are shown to increase the risk of cardiovascular disease indirectly via the induction of a pulmonary acute phase response and enhancing atherosclerotic changes. However, no or only low hepatic acute phase response could be found following inhalation or instillation [79].

Information on both toxicokinetics and toxicodynamics needs to be combined for hazard and risk assessment purposes. By definition [9], an AOP is limited to the description of toxicodynamics, but the kinetics can influence the occurrence of the initiating event.

Another issue in using the AOP framework to describe NM toxicity is the nature of the MIE. It is plausible, and probably likely, that not all interactions of NMs with cells or cellular components involve a specific molecular interaction or reaction as seen for many chemicals (e.g. pharmaceuticals or pesticides). NMs could in fact induce mechanical/physical damage, e.g. to the cell membrane or to the lysosome, which would not be best described as a "Molecular" Initiating Event. However, AOP principles and guidance make provision for describing non-specific interactions with a biological target [9] and for describing an AOP with unknown MIE. Therefore, when describing NM-relevant AOPs, it is probably often more appropriate to assign the first KE as initiating event for the respective AOP that could be termed the initial KE.

A large body of knowledge on NM-induced toxicity and underlying mechanisms exists but for the most part this is fragmented and dispersed across the literature. Different NMs can exhibit toxicity through different mechanisms, but at least qualitatively common mechanisms are shared by many NMs. In fact, there is also evidence for the comparable toxicological behaviour of nano- and bulk particles [80]. Developing an AOP is about looking across different material-specific studies and extracting relevant information, reduced and discretised into a series of causally linked KEs, that is applicable to any NM that has the potential to trigger the (M)IE or initial KE. Restricting this knowledge mining and curation exercise to NM-specific literature makes knowledge gaps evident. Considering that downstream toxicological processes are biology-related rather than substance-specific, the huge chemical-based mechanistic knowledge base can be used to elucidate toxicological processes of NMs. Major differences lie in the initial events that reflect how the chemical or NM perturb the biological system. Using liver toxicity as a case study, we describe how evidence on chemically-induced toxicity can be used to develop an AOP for NM-induced adverse effects.

4. Development of a liver-specific AOP applicable to NMs

A number of AOPs for adverse liver outcomes are being elaborated within the OECD AOP development programme [13]. These AOPs are based on MIEs that are induced by chemicals, but nevertheless their value for developing NM-specific AOPs should not be underestimated. As described in Section 3, NM-induced (M)IEs might differ from chemically-induced ones, as in specific cases they are caused by physical damage rather than molecular interactions. However, certain chemicals can also induce similar (M)IEs as NMs. Moreover, the downstream biological effects are essentially the same. To demonstrate this, a well-advanced AOP for (chemically-induced) liver-fibrosis is presented here and used to describe similarities and differences to mechanisms known for NM-derived toxicity.

OECD Project 1.14 "The Adverse Outcome Pathways from Protein Alkylation to Liver Fibrosis", AOP number 38 [81] (Fig. 2) describes the relevant KEs in the development of liver fibrosis in humans. With chemically-induced protein alkylation being the MIE, hepatocyte injury/apoptosis is described as the subsequent KE. This leads to the activation of Kupffer cells, which account for approximately 15% of the total liver cell population and are involved in the pathogenesis of chemical- or toxin-induced liver injury through the release of inflammatory mediators such as cytokines, chemokines and lysosomal or proteolytic enzymes. Kupffer cells are a main source of the most important profibrogenic cytokine in this process, TGF-β1, and this is described as KE-3. KE-4 describes the subsequent activation of hepatic stellate cells, which ultimately results in progressive collagen accumulation, the onset of fibrosis. This process is accompanied throughout by oxidative stress, which further promotes the development of fibrosis. Moreover, the inflammatory response is a continuous driver in the development of the AO. As a matter of convention, the oxidative stress and inflammatory responses are therefore not described as specific KEs in their own right, but are captured in the KERs. The presented AOP illustrates how a complex biological process can be simplified and described in a linear manner.

To describe adverse effects of NMs on the liver, we have focused on metal oxide NMs such as TiO_2 or SiO_2. A large number of studies on general hepatotoxicity endpoints (such as inflammation, liver damage) have been performed *in vivo,* whereas *in vitro* studies have mainly focused on unveiling the underlying mechanisms. This has led to the development of the AOP "Lysosomal damage leading to liver inflammation" under the OECD framework (AOP number 144) [82]. As described in Section 2, insoluble NMs as well as toxic

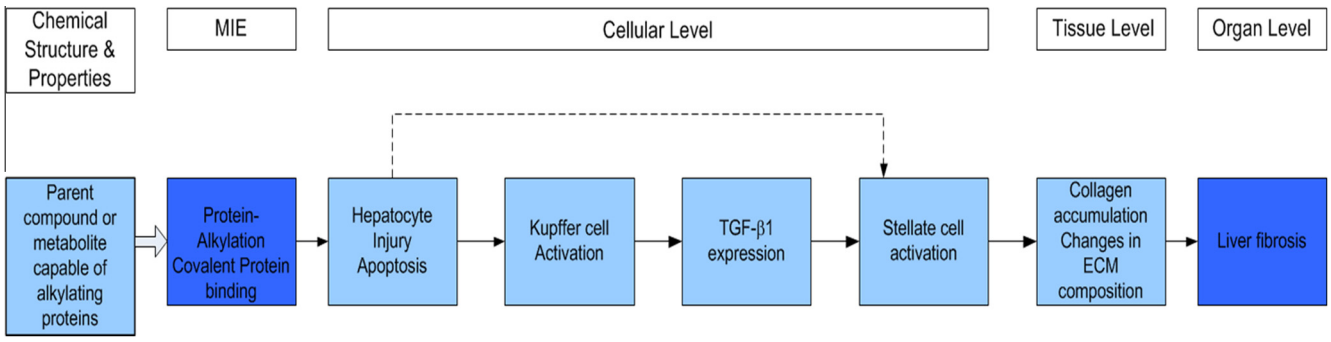

Fig. 2. AOP "The Adverse Outcome Pathways from protein alkylation to liver fibrosis". AOP under development in the AOP wiki under the OECD framework titled "The Adverse Outcome Pathways from Protein Alkylation to Liver Fibrosis". It illustrates a good example of the simplification and linearisation of a complex biological process by narrowing the representation down to the most essential and measureable events.

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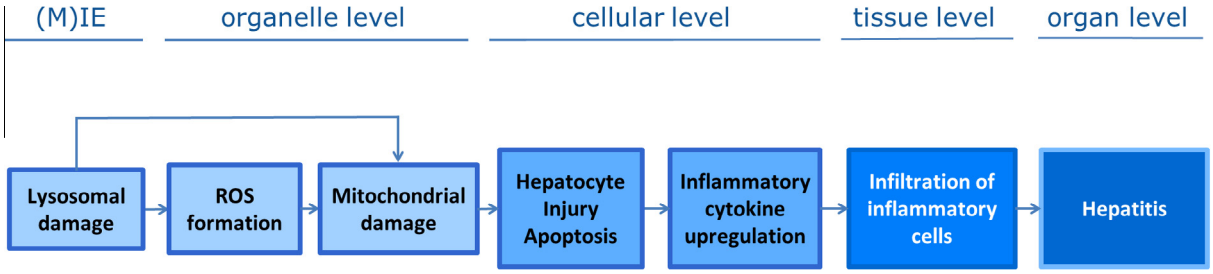

Fig. 3. Putative AOP applicable to metal oxide NMs leading to hepatitis. AOP under development in the AOP wiki under the OECD framework titled "Lysosomal damage leading to liver inflammation". Metal oxide NMs can induce the AO via lysosomal damage as one relevant (M)IE or initial KE, initiated in hepatocytes. The described AOP leads to hepatitis as the AO, as described in the literature. The formation of ROS can lead to hepatocyte injury/apoptosis induced by mitochondrial damage, ultimately resulting in the infiltration of inflammatory cells, inducing the AO.

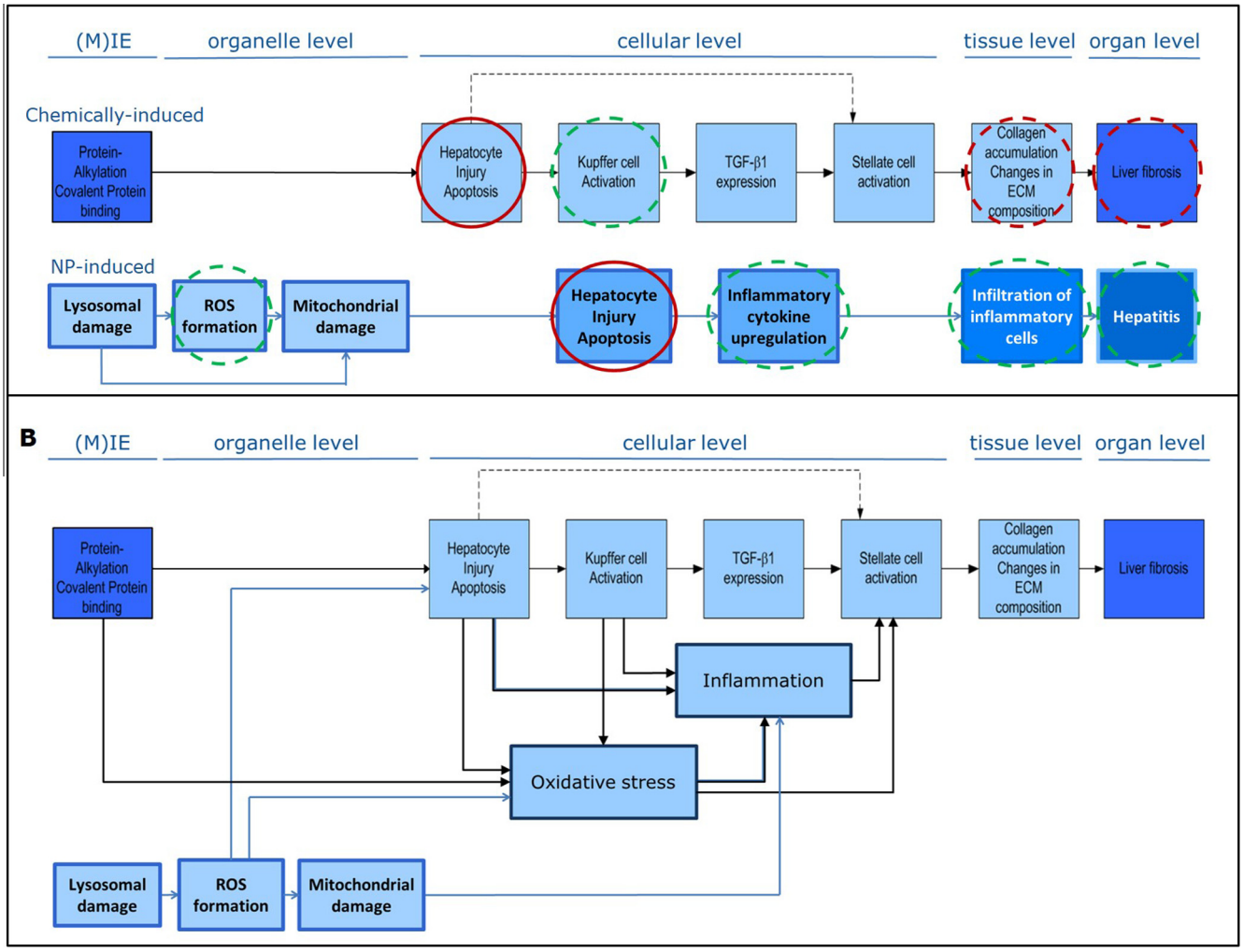

Fig. 4. Merging of the existing chemically-induced fibrosis-AOP and the putative AOP for metal oxide NMs leading to hepatitis. (A) The major differences lie within the (M)IE and early KEs. A major downstream KE, "Hepatic Injury, Apoptosis", is the same for both AOPs (red circle). Also fibrosis induced by NMs has been reported, but the direct links are not fully described yet (dotted red circle). KEs in the putative AOP for metal oxide NMs such as ROS formation or inflammation are described in the KERs of the fibrosis-AOP as they continuously occur throughout the whole process and are not listed here separately (dotted green circle), and similarly, Kupffer cell activation has been described following metal oxide exposure, but is not part of the actual AOP relevant to NMs. A merging of the two AOPs unveils the direct integration and overlap of mechanisms (B). The black arrows show the described KERs in the AOP for alkylating agent-induced fibrosis, whereas the blue arrows show the proven links in the putative AOP for metal oxide NM-induced hepatitis; further proof of the specific KEs leading to fibrosis is still lacking. Combined black and blue arrows show the KERs proven for both approaches. Oxidative stress and inflammation are included here and linked to the various KEs of the fibrosis-AOP for clarity, and both together with Hepatocyte Injury/Apoptosis are the KEs that directly merge both AOPs. This suggests that incubation with metal oxide NMs is ultimately expected to induce liver fibrosis development via the KEs known for chemically-induced fibrosis. However, this has not been confirmed experimentally yet.

ions of soluble NMs can trigger an inflammation cascade by disruption of the lysosomal membrane, which is considered as the initial KE in the present AOP (Fig. 3). Alternative NM-induced (M)IEs or initial KEs can also be envisaged; for example direct ROS formation, leading to the induction of oxidative stress and inflammation in the target organ. However, the lysosomal damage in hepatocytes is known to be a relevant starting point for NM-induced toxicity, and thus we describe it here as the initial KE. Once the lysosome is damaged, ROS are formed as a consequence and mitochondrial damage is initiated, both events further amplifying each other, followed by hepatocyte apoptosis and the inflammatory cascade (Fig. 3).

There are several similarities between KEs of the chemically-induced fibrosis-AOP and NM-induced toxic effects: liver fibrosis has been described as the AO resulting from repeated treatment with TiO_2 or SiO_2 NMs, following administration via oral treatment [29,83], i.p. [70,73] or i.v. injection [84]. Exposure of Kupffer cells to SiO_2 NMs leads to their activation via inflammasome activation [85], causing oxidative stress and inflammation [85,86]. Also, the appearance of collagen fibres around silicotic nodular like lesions has been described for NMs [73,84]. However, the downstream KEs leading to fibrosis development, such as TGF-β1 expression or hepatic stellate cell activation, are still to be investigated for NMs. Recent findings on renal cells and mouse kidneys describe an increase of TGF-β expression following nano-TiO_2 treatment [87], further supporting the assumption that excessive exposure to metal oxide NMs will ultimately lead to liver fibrosis. However, it is currently not possible to describe a full NM-relevant AOP leading to liver fibrosis based on NM toxicology literature alone. Since the development of liver inflammation due to systemic exposure to metal oxides is a well-documented phenomenon, this led us to define hepatitis as the AO, which is in fact a known intermediate event leading to the development of fibrosis (assuming sustained exposure).

In an attempt to learn from the well-developed chemically-induced fibrosis AOP and use this knowledge for the nanotoxicology domain, the two AOPs were directly compared and merged (Fig. 4). Fig. 4A displays both AOPs in parallel, which highlights similarities and differences. As mentioned above in this section, “Collagen accumulation” and the AO “Liver fibrosis” have been described in the literature for NMs, but no direct link to preceding KEs has been established yet.

Based on the KER descriptions and an earlier version of the chemically-induced fibrosis-AOP graph [88], which includes oxidative stress and inflammation (black arrows, Fig. 4B), it is possible to directly link the KEs of the AOP relevant to NM toxicity (blue arrows) to the existing fibrosis-AOP. The combined black and blue arrows display KERs common to both AOPs. This merging clearly shows that there are major overlaps of KEs, and that the main differences between these AOPs lie within the (M)IE or initial KE, and subsequent (early) KEs. It is very likely that TGF-β1 expression and hepatic stellate cell activation can be identified as KEs following NM-treatment, leading to liver fibrosis. Based on this assumption, research strategies to confirm or refute this hypothesis can be designed. This also implies that it might be more efficient to base the predictive modelling of NM toxicity on common KEs, rather than on widely varying and poorly characterised (M)IEs.

5. Conclusions

We demonstrate that the mechanistic knowledge captured in AOPs that have been developed to describe chemically-induced toxicity can be utilised to fill knowledge gaps related to the toxicity of poorly soluble NMs. The major differences in the toxicodynamics of NMs and chemicals lie in the initial upstream events, and in particular the (M)IE, although here also overlaps exist. However, chemically- and NM-induced toxicological processes share downstream events that lead to a particular AO. Of course, NMs and chemicals differ considerably in their *in vivo* biokinetics and in fact this is the primary factor that sets them apart from a toxicological perspective, at least when considering the current generation of engineered NMs. Considering only the toxicodynamic processes that are described in AOPs, apart from the non-specific nature of NM induced (M)IEs, much of the mechanistic knowledge that is required to describe AOPs relevant to NM toxicity can be found in the chemicals domain. Thus, as AOP networks evolve, the mechanistic knowledge captured in KEs and KERs can be also used to inform on downstream events related to NM toxicity, and ultimately for NM hazard assessment. This provides a tool for a more effective and efficient targeted testing to fill data and knowledge gaps that clearly remain in nanotoxicology.

Competing interests

The authors declare no competing interests.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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