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Adverse Outcome Pathways Mechanistically Describing Hepatotoxicity

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Chapter 12

Adverse Outcome Pathways Mechanistically Describing Hepatotoxicity

Ellen Callewaert, Jochem Louisse, Nynke Kramer, Julen Sanz-Serrano, and Mathieu Vinken

Abstract

Adverse outcome pathways (AOPs) describe toxicological processes from a dynamic perspective by linking a molecular initiating event to a specific adverse outcome via a series of key events and key event relationships. In the field of computational toxicology, AOPs can potentially facilitate the design and development of in silico prediction models for hazard identification. Various AOPs have been introduced for several types of hepatotoxicity, such as steatosis, cholestasis, fibrosis, and liver cancer. This chapter provides an overview of AOPs on hepatotoxicity, including their development, assessment, and applications in toxicology.

Key words AOP, Liver toxicity, Steatosis, Cholestasis, Fibrosis, Cancer

1 Introduction

To this day, it remains challenging to accurately assess the potential hazards of xenobiotics with limited toxicological data. Hazard and risk assessment traditionally relies upon apical toxicological outcome testing using laboratory animals. However, these animal-centered approaches have been challenged due to ethical, financial, and scientific concerns, including poor human predictivity and lack of mechanistic understanding [1, 2]. This has led to the emergence of alternative approaches, which provide the basis for next-generation risk assessment. This refers to an exposure-led and hypothesis-driven risk assessment approach that strives to replace and/or reduce animal testing by integrating in vitro, in chemico, and in silico methods [3–5]. In the field of next-generation risk assessment, the application of adverse outcome pathways (AOPs) is a promising method for hazard identification. AOPs provide a solid

Authors Julen Sanz-Serrano and Mathieu Vinken share equal seniorship to this chapter.

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mechanistic understanding of toxicological processes and, therefore, have the potential to enable more accurate predictions on chemical-induced toxicity [6–8]. The primary target for chemical-induced toxicity is the liver [9]. Currently, over 400 AOPs have been established for a plethora of human and ecotoxicological endpoints, including hepatotoxicity such as cholestasis, steatosis, fibrosis, and liver cancer [10]. This chapter will focus on the development, assessment, and applications of AOPs in the context of liver toxicity.

2 Structure

Linear AOPs visualize toxicological effects in a mechanistic way, starting from a molecular initiating event (MIE) resulting in an adverse outcome (AO) via a series of key events (KEs) linked by key event relationships (KERs) (*see* Fig. 1) [11, 12]. KEs are measurable and essential biological changes that capture relevant perturbations leading to a specific AO. The MIE and AO are two types of specialized KEs. The former is the primary anchor of an AOP and refers to the interaction of a chemical with a biological system at molecular level, such as covalent binding to proteins and nucleic acids or ligand–receptor interactions. The latter indicates the actual apical toxicological endpoint. The AO can be located at different levels of biological organization, ranging from the cellular to the population level, and can relate to either a chronic or systemic toxicological outcome or acute or local adverse effect [13, 14]. The connection between upstream and downstream KEs is described through KERs. They either represent direct links between KEs based on known mechanistic causality or indirect links where gaps persist in current mechanistic understanding [15]. Moreover, these KERs can be affected by extrinsic or intrinsic variables, designated modulating factors, such as genetic polymorphisms, disease states, and nutritional or environmental factors [16, 17]. They mediate the responses of KEs by changes in sensitivity, duration, and magnitude of the response, without directly

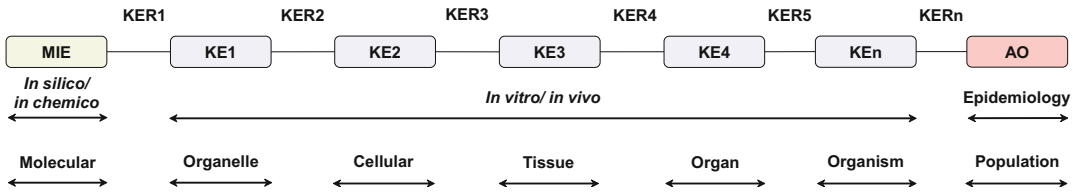

Fig. 1 Generic AOP structure starting with an MIE that is linked to KE by KER to an AO in the most simplistic and unidirectional manner. The AOP is substantiated by different types of information and covers different levels of biological organization. (Adapted from Arnesdotter et al. [15]). Abbreviations: adverse outcome (AO), adverse outcome pathway (AOP), key event (KE), key event relationship (KER), molecular initiating event (MIE)

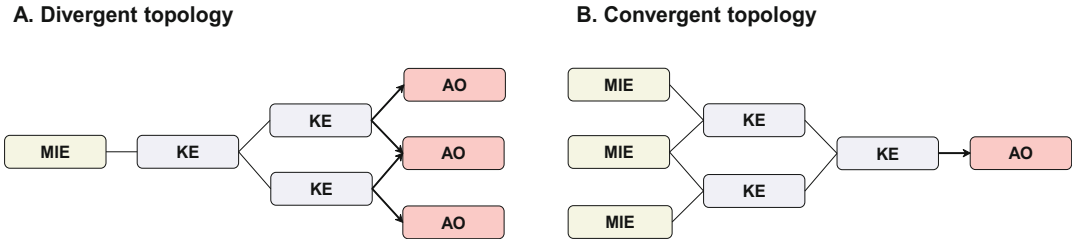

Fig. 2 Divergent topology (left) and convergent topology (right) for AOP networks in the most simplistic manner. (Adapted from Knapen et al. [18]). Abbreviations: adverse outcome (AO), adverse outcome pathway (AOP), key event (KE), molecular initiating event (MIE)

changing the causal response-relationship between KEs [18]. An individual linear AOP is thus anchored at one end by an MIE and at the other end by an AO, causally linked via KEs and KERs. However, in most real-life scenarios, toxicological responses are considerably more complex. Hence, interest is growing in developing AOP networks that better reflect the actual complexity of toxicity [19]. AOP networks are constructed by merging multiple AOPs sharing one or more KEs [18]. The topology of AOP networks can be classified in converging, diverging, or mixed patterns. In a converging topology network, the AOPs are directed toward a common KE or AO, while in a diverging topology network, the AOPs branch off from a common MIE or KE (*see* Fig. 2). In most cases, however, the AOP networks are mixed networks with both converging and diverging topologies [12]. Another way of visualizing an AOP network is by incorporating application-specific layers relevant for interpretation and application, such as layers related to quantitative data, taxonomic applicability, and modulating factors [18, 19]. Moreover, AOP networks have progressed to visualize features, such as the degree of prevalence and evidence for interconnected pathways [20]. The additional information included in layered AOP networks is intended to enhance their utility.

3 Development and Assessment

3.1 Background

The conceptual basis for AOP frameworks has its origin in the late 1980s [8]. However, the first publication describing the AOP framework dates back from 2010, introduced by Ankley et al. [11] in the field of ecotoxicology and later in the area of human toxicology [11]. The AOP framework is a tool to collect and visualize mechanistic knowledge on toxicological effects of chemicals relevant to risk assessment [14]. It builds on the mode-of-action (MoA) concept, and although conceptually similar, the scope of an AOP is broader as it considers effects up to population level. Furthermore, MoAs tend to be chemical-specific and

consider kinetic aspects, whereas AOPs are chemical-agnostic and purely focus on the dynamic aspects [12, 17]. Hence, AOPs can be associated with any chemical, present at the site of action and able to activate the associated MIE [15]. This potential of AOPs for a range of applications has led to a rapid increase in AOP development. The substantial growth of AOPs called for harmonized guidelines and strategies regarding their development and assessment. Accordingly, the Organisation for Economic Co-operation and Development (OECD) published a *Guidance Document on Developing and Assessing Adverse Outcome Pathways* [14]. Additional recommendations are available in subsequent guideline updates, the OECD's users handbook, and in general scientific literature [12, 13, 17–19]. Furthermore, the OECD launched an international AOP development program that entails three main phases, namely, assembly, review, and endorsement. The first phase is the assembly of the data in the AOP Wiki, an internationally accessible and searchable knowledgebase archiving AOP information (https://aopwiki.org/) [10]. When the AOP is submitted in the OECD AOP development work plan within the AOP Wiki, the Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST) provides authors with coaching and feedback for the subsequent phases. The second phase involves the review of the AOP, which includes a compliance check and a scientific review. The third phase is the endorsement phase. This is an OECD-specific phase in which the AOP requires approval from the EAGMST, followed by the Working Group of the National Coordinators of the Test Guidelines Programme, the Working Party on Hazard Assessment, and the Chemicals and Biotechnology Committee. If the responsible OECD committees express confidence in the scientific review process, the AOP is labelled as "endorsed" and is published in the OECD dedicated Series on AOPs [21]. The purpose of publication is to provide a stable version over time (i.e., reviewed and revised version), as even endorsed AOPs can continue to evolve after their publication. On this note, endorsement of an AOP does not indicate that the AOP is ready or useful for direct regulatory applications.

3.2 Development

A number of different strategies can be adopted for AOP development. The seminal paper by Villeneuve et al. [12] describes strategies for AOP development, including case study, analogy, top-down, middle-out, bottom-up, and data-mining strategies [12]. Case study strategies use a well-studied pathway of a single chemical and generalize this pathway to other chemicals when enough supporting evidence is assembled. Analogy strategies use an AOP that is developed in a single organism and extrapolate this to other species. Top-down, middle-out, and bottom-up strategies start from an AO, a KE, or an MIE, respectively, and subsequently connect these to adjacent events. Data-mining strategies apply

high-throughput/high-content data (e.g., omics) and other types of database mining approaches to identify KEs and KERs.

The work of Knapen et al. [18] describes two strategies for AOP network development, namely, the network-guided and network derivation strategy [18]. The former involves the development of at least two individual AOPs containing one or more intentionally shared KEs, while the latter involves manual or programmatical extraction of relevant AOPs. Basically, any type of information can be fed into an AOP (network), including in vitro data (i.e., cell culture), in chemico data (i.e., abiotic chemical reactivity method), in vivo data (i.e., animal experimentation), in silico data (i.e., computational), and omics-based data [15]. Omics-based data, in particular transcriptomic data, is a major data source for detecting KEs [22]. All these types of data are assembled for AOP (network) development, often through a manually performed in-depth survey of relevant scientific literature. However, manual expert-driven data collection approaches are complex, time-consuming, and prone to data gaps. In this regard, new strategies have been proposed, such as deep text mining approaches and machine learning approaches [23]. Text mining, also known as text analytics or natural language processing, refers to the process of extracting knowledge from a large number of textual data [24]. Artificial intelligence-assisted data collection approaches can greatly facilitate the data extraction by automatically and systematically exploring available toxicological data [25, 26]. There is no universal strategy for AOP (network) development, and one or more strategies can be applied based on the availability of relevant data. Nevertheless, regardless of the adopted development strategy, five fundamental principles should be considered [12, 14]. Firstly, AOPs are not chemical specific. Secondly, AOPs are modular and composed of reusable components, notably KEs and KERs. Thirdly, an individual AOP, composed of a single sequence of KEs and KERs, is a pragmatic unit of AOP development and evaluation. Fourthly, AOP networks are composed of multiple AOPs that share common KEs and KERs and are likely to be the functional unit of prediction for most real-world scenarios, and fifthly, AOPs are living documents that evolve over time as new knowledge is generated [12].

3.3 Assessment

A major element in the *Guidance Document on Developing and Assessing Adverse Outcome Pathways* is the incorporation of a weight-of-evidence assessment [14]. The AOPs are hereby thoroughly assessed with a clear and transparent evaluation of reliability, robustness, and relevance. Assessment of AOPs relies on the so-called tailored Bradford-Hill criteria. Originally, the Bradford-Hill criteria were developed for causality evaluations in epidemiological studies [27]. Later on, the criteria were also applied for weight-of-evidence evaluations of MoAs with the aim of increasing

Table 1
Tailored Bradford-Hill criteria

Criteria	Driving questions
Biological plausibility of KERs	Is the mechanistic relationship between the upstream and downstream KE consistent with established biological knowledge?
Essentiality of KEs	What is the impact on downstream KEs and/or the AO if an upstream KE is modified or prevented?
Empirical support for KERs	Does the upstream KE occur at lower doses and earlier time points than the downstream KE, and at the same dose of prototypical stressor, is the incidence of the upstream KE more than that for the downstream KE? Are there inconsistencies in empirical support across taxa, species, and prototypical stressor that don't align with expected pattern for hypothesized AOP?

Adapted from Ref. Becker et al. [29]
Abbreviations: *AO* adverse outcome, *AOP* adverse outcome pathway, *KE* key event, *KER* key event relationship

consistency and harmonization of evaluations [28]. In this respect, the Bradford-Hill criteria were considered to be a practical tool for weight-of-evidence assessment of AOPs; however, the criteria needed to be tailored for the AOP context (i.e., non-chemical-related elements) [29]. The tailored Bradford-Hill criteria relate to biological plausibility, essentiality, and empirical support [13]. Biological plausibility refers to the understanding of the fundamental biological processes and the consistency of the causal relationships. Essentiality considers the impact of modified/blocked upstream KEs on downstream KEs or on the AO. Empirical support is based on toxicological data derived by one or more prototypical stressors, such as dose–response, temporality, and incidence [14]. Each of these criteria consists of a number of defining questions, which are subjected to weight-of-evidence analysis and judged as high/strong, moderate, or low/-weak confidence for each of the KEs, KERs, and the overall AOP (*see* Table 1) [29]. Optionally, quantitative weight-of-evidence is added, which builds on the qualitative assessment by scoring the tailored Bradford-Hill considerations. Empirical support can be quantified by dose–incidence and/or temporal concordance [30]. Furthermore, the relevant biological domain of applicability (i.e., taxa, sex, life stage) should be reported when assessing AOPs [13]. While assessing AOPs, it is important to remember the fundamental principle stating that AOPs are living documents [12]. As such, development and assessment is a continuous and dynamic process, whereby AOPs gradually evolve by providing evidence for the KEs and KERs. Generally, three stages of maturity can be distinguished, namely, the putative, formal, and quantitative stage [18]. The putative stage refers to the assembly of general toxicological knowledge. In the formal stage, the AOP is refined with

additional evidence from literature. In the quantitative stage, the AOP is substantiated by more quantitative empirical evidence, usually at the level of KEs and KERs, along with a qualitative evaluation of the overall weight-of-evidence of the AOP [15].

3.4 Quantification

The use of quantitative AOPs (qAOPs) holds great potential for regulatory safety assessment [31]. A framework to guide development and assessment of qAOPs is currently lacking. Nevertheless, a paper by Spinu et al. [32] distinguishes three stages of qAOPs, namely, the semi-quantitative, probabilistic, and mechanistic qAOPs [32]. The semi-quantitative stage is an extension of a qualitative formal AOP with additional empirical data, whereby quantitative weighting and numerical assessments of KERs and KEs are included in the AOP description. Probabilistic qAOPs incorporate statistical or probabilistic approaches to build predictive relationships between KERs and KEs, whereas mechanistic qAOPs further include computational models in which mathematical functions predict the probability of a subsequent event given specified initial conditions, such as mathematical modelling by machine learning [32]. Overall, qAOPs quantify the magnitude of KEs along the pathway. The threshold required to progress from one KE to the next is integrated, but also exposure metrics such as dose–response interactions are often considered. The process of building qAOP models shares similarities with the construction of other computational models for decision support, and existing expertise can be leveraged. Generally, qAOP development can include a number of different modelling strategies that implement quantitative scoring systems according to the tailored Bradford-Hill considerations [29–32]. The Bayesian network model is a frequently adopted modelling strategy for directional and functional KERs, whereby a probabilistic model typically represented as a set of interconnected nodes (i.e., KEs) quantifies the links between the nodes by conditional probability tables based on the mathematical equation of Bayes [31, 33]. Regression modelling is often applied for (non-)linear KE relationships, including saturable response, while other types of mathematical models such as ordinary differential equation, individual-based modelling, and linear probability modelling are commonly applied for the predicting of temporal and time-resolved responses [31]. Overall, a qAOP provides quantitative, dose–response, and time-course predictions between the KEs that can support regulatory decision-making.

4 AOPs on Liver Toxicity

4.1 Cholestasis

Cholestasis is defined as disrupted bile formation, secretion, or excretion, resulting in accumulation of bile acids in the hepatic or systemic circulation. It can manifest as intrahepatic (i.e., functional

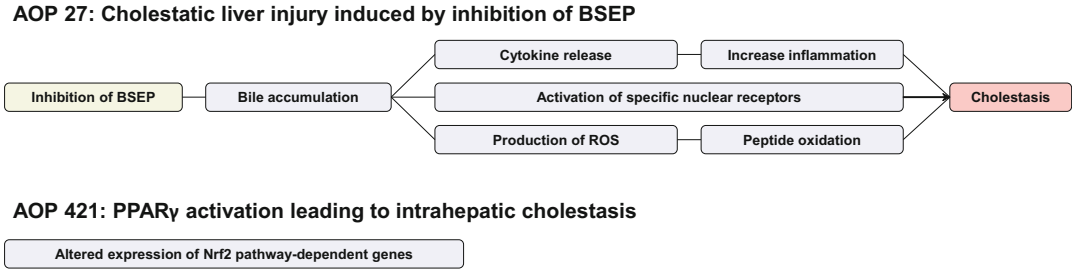

Fig. 3 Schematic representation of AOP (network)s that are related to cholestasis as described in the AOP Wiki [10]. Abbreviations: bile salt export pump (BSEP), nuclear factor-erythroid 2-related factor 2 (Nrf2), peroxisome proliferator-activated receptor gamma (PPARγ), reactive oxygen species (ROS)

defect in hepatocytes, bile canaliculi, or intrahepatic bile ducts) or extrahepatic cholestasis (i.e., blockages in extrahepatic ducts, the common hepatic duct, or the common bile duct) [34].

There are currently two AOPs related to intrahepatic cholestasis available in the AOP Wiki (*see* Fig. 3) [10]. The first AOP starts with the activation of peroxisome proliferator-activated receptor gamma (PPARγ). It is assumed that this activation alters the expression of nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway-dependent genes, ultimately resulting in cholestasis [10]. In the AOP Wiki, this AOP deviates from the guidelines as it only incorporates one KE, and no MIE/AO is described. The second AOP considers the inhibition of the bile salt export pump (BSEP) transporter as the MIE, which leads to bile accumulation, nuclear receptor activation (i.e., farnesoid X receptor (FXR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR)), oxidative stress, inflammation, and ultimately cholestatic injury [35]. Recently, this AOP has undergone revision and optimization through artificial intelligence-assisted data collection followed by quantitative confidence assessment according to the tailored Bradford-Hill criteria [36]. The optimized AOP network considers three types of MIEs, namely, hepatocellular changes, bile canalicular changes, and drug transporter changes [36]. Hepatocellular changes refer to cytoskeleton alterations and tight junction disruption, whereas bile canalicular changes refer to dilatation and constriction of bile canaliculi potentially due to rho kinase/myosin light chain kinase pathway interference [37, 38]. Transporter changes indicate alterations in activity and/or expression level of proteins mediating the transport of bile acids and drugs in hepatocytes [39]. The latter is reported as one of the most important MIEs [36]. In this regard, the role of BSEP, an important canalicular efflux transporter protein that regulates enterohepatic circulation of the bile acids, has been extensively studied. Impairment of BSEP function is closely linked to reduced secretion and subsequent accumulation of bile acids, resulting in severe forms of cholestasis [39–41]. Furthermore, transporters like sodium

taurocholate co-transporting polypeptide, multidrug resistance-associated proteins (MRP2, MRP3, and MRP4), multidrug resistance protein 3, and organic anion transporting peptides are frequently associated with cholestasis [36, 42]. In fact, drug-induced cholestasis due to inhibition of BSEP is often accompanied with parallel inhibition of other hepatobiliary transporters. Either type of MIE can equally initiate intracellular bile accumulation, which in turn activates two types of responses, namely, the deteriorative response and the adaptive response [43]. The deteriorative response is typified by the opening of the mitochondrial membrane permeability pore, which subsequently results in the formation of reactive oxygen species (ROS), oxidative stress, endoplasmic reticulum stress, inflammation, cell death by both apoptosis and necrosis, and cholestatic injury [36, 44]. The adaptive response aims to counteract this deteriorative response and thus disturbed bile acid homeostasis [36, 43]. In this scenario, bile acids act as a signalling molecule and regulate specific nuclear receptors, such as FXR, PXR, CAR, and small heterodimer partner, through a number of transcriptionally regulated mechanisms [45, 46]. Simultaneously, bile acid synthesis and metabolism are altered. Overall, these changes result in decreased hepatocellular uptake and increased export of bile acids [36].

4.2 Liver Steatosis

Hepatic steatosis is characterized by excessive lipid accumulation within hepatocytes. A distinction can be made between macrovesicular and microvesicular steatosis depending on the size of the triglyceride droplets. The former is typified by single large lipid droplets or smaller well-defined fat droplets located in the cytoplasm of the hepatocytes and displacing the nucleus, while the latter is typified by smaller uniform lipid droplets dispersed throughout the hepatocytes [47].

Currently, the AOP Wiki contains ten AOPs related to hepatic steatosis (*see* Fig. 4) [10]. The first AOP regards the activation of PPARγ and liver X receptor (LXR) as the MIE. This can modulate expression of genes responsible for lipid homeostasis, such as carbohydrate response element binding protein, sterol response element binding protein 1c, free fatty acid uptake transporter FAT/CD36, fatty acid synthase, and stearoyl-CoA desaturase 1. These changes trigger a chain of KEs, including de novo synthesis of fatty acids, influx of fatty acids from the peripheral tissues to the liver, accumulation of triglycerides, cytoplasm displacement, nucleus distortion, mitochondrial toxicity, and eventually the onset of steatosis. The second AOP involves the decreased activation of PPARα/β/γ, leading to decreased mitochondrial fatty acid beta-oxidation, fatty acid accumulation, and steatosis [48]. Other AOPs describe the activation of the aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR), PXR, and FXR and the suppression of constitutive androstane receptor (CAR) as MIEs. The

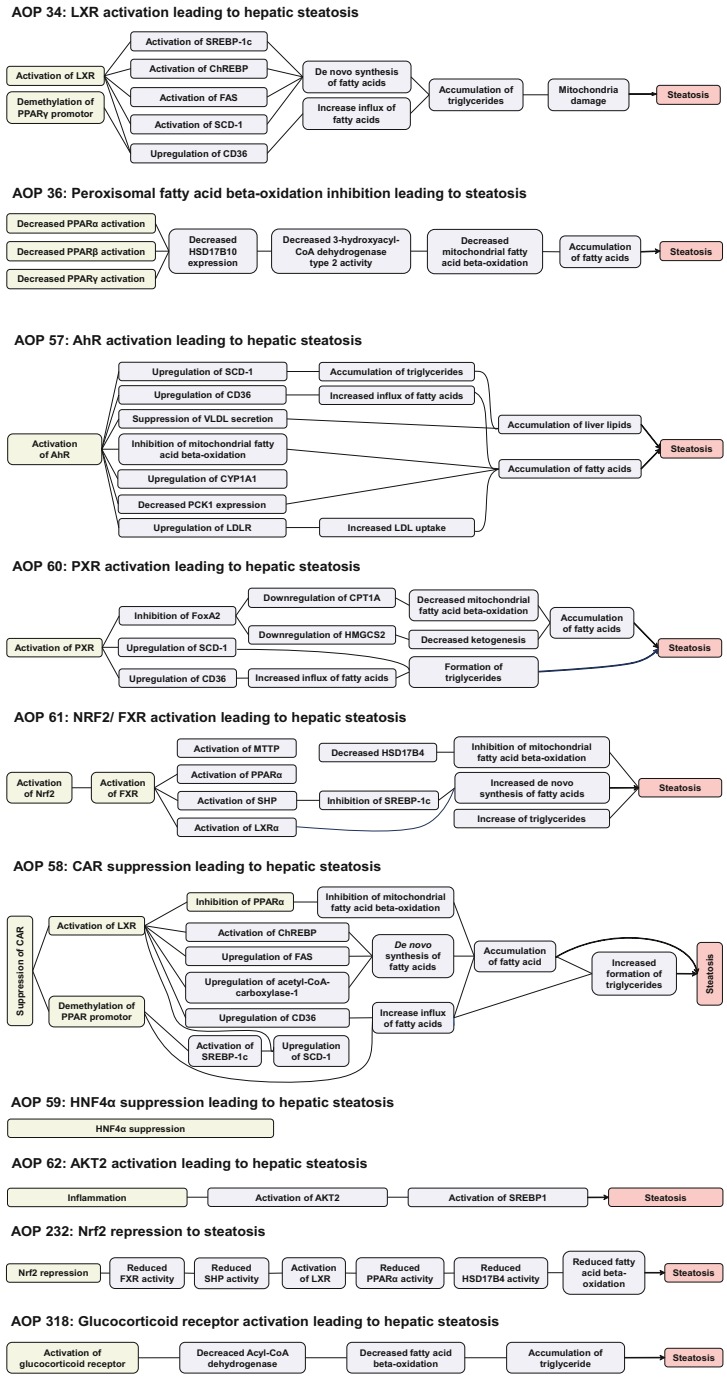

Fig. 4 Schematic representation of AOP (network)s that are related to steatosis as described in the AOP Wiki [10]. Abbreviations: aryl hydrocarbon receptor (AhR), carbohydrate-responsive element-binding protein (ChREBP), carnitine palmitoyltransferase IA (CPT1A), cluster of differentiation 36 (CD36), constitutive androstane receptor (CAR), cytochrome P450 (CYP), fatty acid synthase (FAS), farnesoid X receptor (FXR), forkhead box protein A2 (FoxA2), hepatocyte nuclear factor 4 alpha (HNF4α), hydroxysteroid 17-beta dehydrogenase 4/10 (HSD17B4/10), liver X receptor (alpha) (LXR(α)), microsomal triglyceride transfer protein (MTTP), nuclear factor-erythroid 2-related factor 2 (Nrf2), peroxisome proliferator-activated receptor alpha/beta/gamma (PPARα/β/γ), phosphoenolpyruvate carboxykinase 1 (PCK1), pregnane X receptor (PXR), protein tyrosine

modulation of these receptors triggers downstream connections that via upregulation of fatty acid transport induce steatosis [49–51]. Furthermore, suppression of hepatocyte nuclear factor 4 alpha (HNF4α) and Nrf2 expression are recognized as the MIEs leading to hepatic steatosis. Both proteins play important roles in the prevention of liver injury [52, 53]. Another AOP indicates systemic inflammation as initial trigger to activate serine/threonine protein kinase 2, which ultimately leads to hepatic steatosis [10]. The exact mechanisms by which some MIEs contribute to hepatic steatosis involve multiple pathways related to lipid metabolism and often remain to be further elucidated. There are many other AOPs related to hepatic steatosis that are not included in the AOP Wiki. Recently, Escher et al. [54] published an AOP network for microvesicular hepatic steatosis, which includes nine AOPs from the AOP Wiki and MoAs on valproic acid (i.e., prototypical inducer of hepatic steatosis) [54]. This AOP network closely resembles the previously proposed networks by Mellor et al. [55] and van Breda et al. [56], but with recent updates integrated [55, 56]. The network considers different MIEs, namely, modulation of nuclear receptors (i.e., PPARα, PPARδ, PPARγ, FXR, CAR, PXR, AhR, GR, and LXR), suppression of transcription factors (i.e., HNF4α and Nrf2), and activation of serine/threonine kinase 2 [54]. These MIEs trigger a downstream cascade of KEs, including enhanced transcription of genes encoding mediators of cholesterol and lipid metabolism. Subsequently, de novo synthesis and influx of fatty acids increase, and triglycerides accumulate within the hepatocytes. At the organelle level, hepatocellular lipid accumulation can cause cytoplasm displacement, nucleus distortion, endoplasmic reticulum stress, and mitochondrial disruption. Furthermore, these effects contribute to an altered influx/efflux and metabolism of fatty acids, leading to a net increase in cellular fatty acids and the development of the typical fatty liver cell phenotype, known as steatosis [54].

Steatosis is a progressive disease that may evolve to more severe forms of liver diseases, such as steatohepatitis, cirrhosis, hepatocellular carcinoma, and ultimately liver failure [57]. Metabolic dysfunction-associated steatohepatitis (MASH), formerly referred to as non-alcoholic steatohepatitis, is presented as steatosis (i.e., lipid accumulation) combined with hepatitis (i.e., inflammation) [58]. The only AOP focused on MASH considers the inhibition of fatty acid beta-oxidation as MIE [10]. This results in an overall increase of cytosolic fatty acids, which undergo lipid peroxidation

Fig. 4 (continued) phosphatase (SHP), RAC-beta serine/threonine-protein kinase (AKT2), sterol regulatory element-binding protein 1c (SREBP-1c), stearoyl-CoA desaturase 1 (SCD-1), (very-)low-density lipoprotein (receptor) ((V)LDL(R)), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2)

induced by ROS and thereby form free radicals [59–61]. Subsequently, these free radicals trigger a cascade of protein and membrane damage, resulting in oncotic necrosis. The cell damage causes leakage of cytoplasmic content, which stimulates the inflammatory response associated with steatohepatitis [62]. If injury continues, MASH may further lead to cirrhosis, hepatocellular carcinoma, and liver failure [63].

4.3 Liver Fibrosis

Liver fibrosis denotes the reversible wound-healing response to acute or chronic cellular damage and reflects a balance between liver regeneration and scar formation [64]. The development of liver fibrosis is primarily attributed to the activation of hepatic stellate cells (HSCs) and occurs in two major phases, namely, initiation and perpetuation, followed by a resolution phase if the injury regresses [65]. The initiation phase involves early triggers, including ROS and apoptotic bodies originating from dying hepatocytes, which make quiescent HSCs responsive to growth factors. The perpetuation phase covers changes in cell behavior of the previously primed HSCs, like proliferation, contractility, fibrogenesis, chemotaxis, extracellular matrix degradation, and retinoid loss. Subsequently, the HSCs adopt a myofibroblast-like phenotype [66]. The resolution phase refers to pathways that counteract the activation of HSCs through apoptosis, senescence, or quiescence [67]. The liver has a remarkable regenerative capacity; however, due to chronic injury, fibrosis may progress in cirrhosis, which, unlike fibrosis, is considered an irreversible event [65].

The AOP Wiki contains four AOPs related to liver fibrosis (*see* Fig. 5) [10]. The first AOP includes protein alkylation as the MIE. Subsequent KEs at the cellular and tissue level have been defined, including hepatocyte injury and cell death, Kupffer cell activation, expression of transforming growth factor beta 1, HSC activation, oxidative stress, chronic inflammation, collagen accumulation, and changes in hepatic extracellular matrix composition [10, 68]. The second AOP describes endocytic lysosomal uptake as the MIE. The KEs leading to liver fibrosis include lysosomal disruption, mitochondrial dysfunction, cell death, inflammation, leukocyte recruitment, activation of HSCs, and changes in hepatic extracellular matrix composition [10]. The third AOP proposes angiotensin-converting enzyme 2 inhibition as MIE. This triggers a cascade of KEs, including an increase of angiotensin II type 1 receptor, ROS, and extracellular matrix deposition [10]. In the fourth AOP, the activation of the AhR is considered as the MIE. Subsequently, this activation leads to liver steatosis. However, there are some gaps in the AOP as it does not mention the downstream KEs responsible for the onset of this KE. In this regard, the previously described AOP "AhR activation leading to hepatic steatosis" could potentially help elucidate the underlying mechanisms. Steatosis triggers a downstream cascade of KEs, including cell injury, HSC activation,

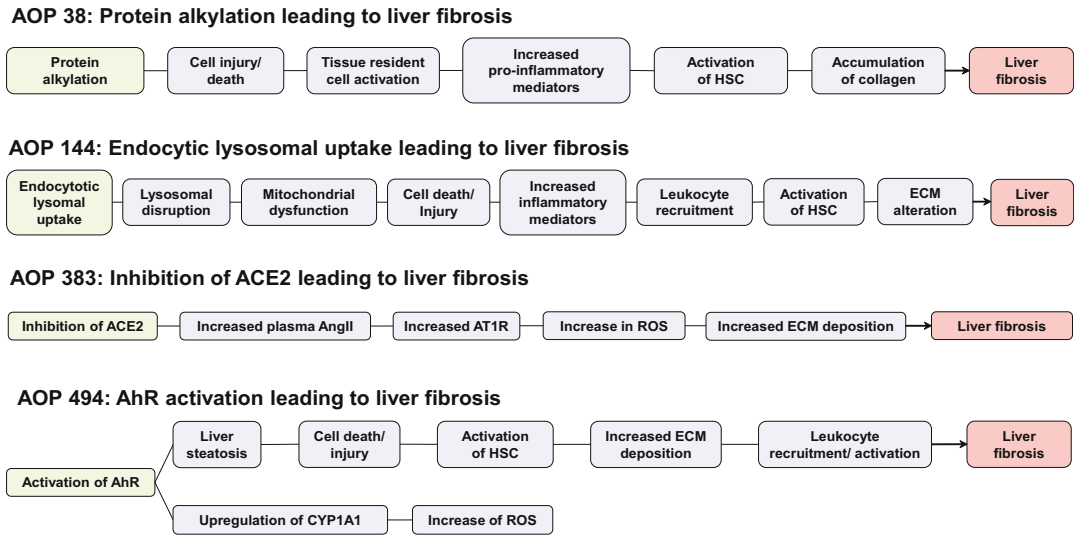

Fig. 5 Schematic representation of AOP (network)s that are related to liver fibrosis as described in the AOP Wiki [10]. Abbreviations: angiotensin-converting enzyme 2 (ACE2), angiotensin II (AngII), angiotensin II receptor type 1 (AT1R), aryl hydrocarbon receptor (AhR), cytochrome P450 (CYP), extracellular matrix (ECM), hepatic stellate cell (HSC), reactive oxygen species (ROS)

increase in extracellular matrix deposition, leukocyte activation, and ultimately liver fibrosis [10, 15]. Furthermore, AhR activation also causes an upregulation of CYP1A1 and an increase in ROS, but unlike the AOP guidelines, these KEs are not linked via KERs to other KEs or AO in the AOP Wiki.

4.4 Liver Cancer

Liver cancer is characterized by the presence of malignant cells localized in the liver. The shift from normal to malignant cells is designated tumorigenesis [69]. In the latter process, cells acquire functional capabilities, known as the hallmarks of cancer, which promote tumor development by allowing survival, proliferation, and dissemination of the cells. There are eight hallmarks of cancer, namely, sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing/accessing vasculature, activating invasion and metastasis, reprogramming cellular metabolism, and avoiding immune destruction. Furthermore, two enabling characteristics, including genome instability and tumor-promoting inflammation, are involved in cancer development [70]. Liver cancer can be classified as primary liver cancer (i.e., originates in the liver) or secondary liver cancer (i.e., spread from another location in the body). Primary liver cancer can manifest in a number of different ways, such as hepatocellular carcinoma, intrahepatic cholangiocarcinoma, and other rare tumors, notably fibrolamellar carcinoma and hepatoblastoma [71].

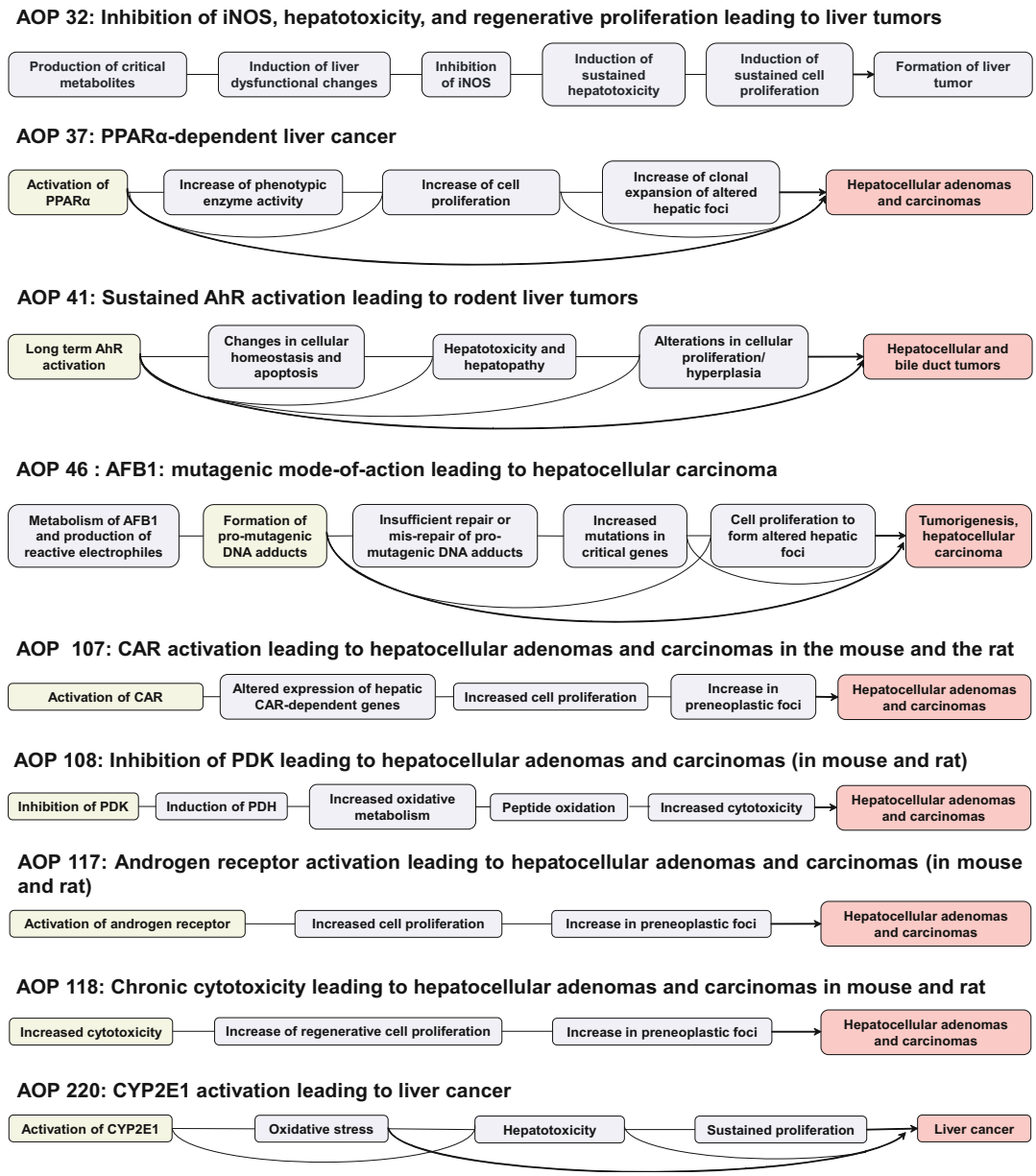

Fig. 6 Schematic representation of AOP (network)s that are related to liver cancer as described in the AOP Wiki [10]. Abbreviations: aflatoxin B1 (AFB1), aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), cytochrome P450 (CYP), inducible nitric oxide synthase (iNOS), peroxisome proliferator-activated receptor alpha (PPARα), pyruvate dehydrogenase (PDH), pyruvate dehydrogenase kinase (PDK)

The AOP Wiki contains nine AOPs related to liver cancer (*see* Fig. 6) [10]. The first AOP begins with the inhibition of inducible nitric oxide synthase. This enzyme catalyzes the conversion of L-arginine to citrulline, resulting in the production of endogenous nitric oxide, an important chronic inflammation mediator

[72]. This leads to sustained hepatotoxicity, which in turn induces sustained cell proliferation, ultimately resulting in liver tumor formation. In the second AOP, the activation of PPARα serves as MIE. This triggers an array of downstream KEs, including cell proliferation, clonal expansion of preneoplastic foci, and development of liver adenomas and carcinomas in rodents [73]. The third AOP describes the development of liver tumors in rodents as a consequence of sustained AhR activation through changes in cellular growth homeostasis likely associated with cell proliferation and inhibition of apoptosis within altered hepatic foci. This results in the formation of hepatocellular and bile duct tumors. A fourth AOP addresses the metabolic activation of mycotoxin aflatoxin B1. The covalent binding of the reactive metabolite of mycotoxin aflatoxin B1 to the DNA and the consequent formation of pro-mutagenic covalent DNA adducts are considered the MIE. The involved downstream KEs encompass the inadequate DNA repair and the mutation of critical genes, which in turn leads to the formation of hepatocellular carcinoma [74]. There are some incongruences related to this AOP, as an AOP is normally considered chemical agnostic. The fifth AOP considers the activation of CAR as the MIE, whereby transcriptional alterations induce cell proliferation and preneoplastic foci and ultimately lead to hepatocellular adenomas and carcinomas in rodents [75]. A sixth AOP describes the alteration of glucose metabolism, through inhibition of pyruvate dehydrogenase kinase. Inhibition of pyruvate dehydrogenase kinase leads to increased pyruvate dehydrogenase activity, oxidative metabolism, peptide oxidation and cytotoxicity, and ultimately the development of hepatocellular adenomas and carcinomas in mouse and rat. The seventh AOP addresses the activation of androgen receptor as MIE, followed by increased cell proliferation, preneoplastic foci, and development of hepatocellular adenomas and carcinomas in rodents [76]. Chronic cytotoxicity in hepatocytes is also characterized as an MIE in an eighth AOP. Sustained hepatocytotoxicity, caused by a wide range of toxicological effects, such as oxidative metabolic activation, oxidative stress, or increase of ROS, causes cell death through apoptosis and necrosis. To counteract this cell death, regenerative cell proliferation occurs. However, sustained cell proliferation poses a significant risk for cancer development due to increased chances of errors in the DNA replication process, potentially leading to the formation of preneoplastic foci and liver cancer. The ninth AOP regards CYP2E1 activation from substrate biotransformation as MIE. Metabolites and ROS are formed, leading to oxidative stress and hepatotoxicity. Consequently, counteractive liver regeneration initiates cell proliferation, which can potentially lead to tumor formation under prolonged conditions [77]. In conclusion, several of these AOPs are based on sustained hepatotoxicity, which can eventually promote hepatocarcinogenesis via a number of KEs. These KEs are

frequently associated with alteration of the homeostatic balance in favor of cell growth, including reduction of apoptotic activity, increase in cell proliferation, hyperplasia in various liver cell types, and clonal expansion of preneoplastic foci cells.

5 Applications

Risk assessment is a process that typically consists of four steps, namely, hazard identification, hazard characterization, exposure assessment, and risk characterization [78]. AOPs can be applied in each of these steps, mainly in hazard assessment steps, and can support the development of integrated approaches to testing and assessment (IATA) [79]. An IATA can be developed for different fit-for-purpose applications [80]. The specific application is usually determined by the available data and the degree of maturity of the AOP but is constrained by the taxonomic, sex, and life-stage applicability domain [81]. In general, qualitative AOPs are valuable tools for hazard identification purposes, whereas qAOPs with sufficient quantitative information on dose–response and/or response–response relationships are useful for hazard characterization [31, 32]. Currently, most applications of AOPs are indicated for regulatory purposes within the area of risk assessment.

5.1 Development of Quantitative Structure–Activity Relationships

The MIE of an AOP reflects a specific interaction of a chemical with a biological target. It can be used as the basis for generating mechanistically based structure–activity relationships (SARs), whether or not quantifiable. These SARs can predict if a chemical can trigger an AOP but can also be utilized for chemical grouping and read-across strategies [80]. In this context, the OECD provides free computer software (https://qsartoolbox.org/), also known as the quantitative SAR (QSAR) Toolbox. This software can help identify potential chemical hazards by assessing structural similarity to known substances with available toxicity data and thereby enables pragmatic QSAR method-based toxicity predictions [14, 82]. QSAR approaches have proven useful in many cases. This is illustrated by QSAR approaches for cholestasis that demonstrated that chemicals with an ester or thioester group attached to a carbon atom of a heterocyclic group cause BSEP inhibition [83]. Likewise, a carbocyclic system with at least one aromatic ring and mononuclear heterocycles contributes to inhibition of BSEP [84]. In contrast, hydroxyl groups bound to aliphatic carbon atoms result in increased BSEP activity [83]. Another QSAR model described several methotrexate analogues with MRP2 inhibition potency caused by similar structural features, such as lipophilicity and aromaticity [85]. Furthermore, halogen substitution and widened angle of biphenyl-substituted heterocyclic compounds with bulky ortho-substituents increase MRP2 inhibition

[86, 87]. QSAR studies have been performed on LXR ligands, which is considered a type of MIE for liver steatosis. In this respect, phenyl rings, chloro-groups, and methyl substituents have been identified as determinants of LXR binding and activation [88]. Moreover, QSAR models are also available for other nuclear receptors associated with steatosis, such as PXR, AhR, and PPARγ [89–91].

5.2 Grouping of Chemicals into Chemical Categories

The grouping of chemicals is not always solely based on structural similarities, but also biological activity at different levels of biological organization [80]. Chemicals that activate the same AOP based on in vitro and in silico assays or predictions of the KEs can be grouped together in a chemical category. As such, AOPs provide an opportunity to group chemicals. Most grouping approaches start from a toxicological mechanism and then search for chemical structures that can trigger it, thereby applying in silico tools like QSAR strategies. However, some strategies take the opposite approach [92]. In a study, 16 structural alerts for hepatotoxicity were established based on a dataset of 951 compounds [93]. Once a chemical category is established, it can be used for data gap filling strategies, such as with read-across approaches [92]. Read-across refers to the process of reading information from a set of toxicologically well-characterized chemicals (i.e., source chemicals) to a chemical for which limited information is available (i.e., target chemical), with the aim to predict the hazard (s) of the target chemical [93].

5.3 Elaboration of Prioritization Strategies

Prioritization of chemicals refers to the process in which less complex, cheaper, and faster assays are used to select chemicals to be subjected to more elaborate, expensive, and time-consuming testing. Chemicals are screened and ranked according to their potency, whereby the most potent chemical receives the highest priority to undergo more detailed testing and/or evaluation [7, 92]. AOPs have great potential with respect to prioritization strategies, as they can increase confidence in the integration of information, such as obtained from in vitro assays. An example in this context is the case study integrating molecular docking, QSAR, and structure knowledge approaches for ranking potential LXR binders according to potency for development of liver steatosis [94]. Recently, AOP-derived approaches have also been adopted for the prediction and prioritization of potential liver carcinogens. Chemicals were tested using short-term assays for an array of MIEs and KEs associated with liver cancer. Then, the Toxicological Priority Index was then used to rank chemicals based on their ability to activate the KEs [95]. Furthermore, mechanistic toxicological information from AOPs has been used to screen 62 flame retardants and highlight priority compounds that critically need more toxicological studies regarding liver hepatotoxicity. Hereby, the flame retardants

were first grouped into five prioritization categories. Afterward, AOPs were used to identify plausible toxicity mechanisms for high-priority compounds [96].

5.4 Development of Testing Strategies

AOPs have emerged as versatile tools to support development of new testing strategies [6]. Proposals for the development of new ex vivo, but especially in vitro, toxicity screening assays can be established by linking assays to toxicological endpoints and KEs anchored in AOPs (networks) [13]. MIEs and KEs hereby serve as the basis for the characterization of biomarkers. The testing strategy aims to collect information from a combination of assays that cover different KEs along the AOP in a tiered approach. The latter refers to a systematic and structured strategy, whereby information from one tier determines the subsequent test for the next tier in order to generate the most relevant information [80]. The purpose of a tiered approach is to efficiently allocate resources, reduce unnecessary testing, and focus on obtaining the most relevant information. The level of confidence in an AOP can be used when deciding how many and which of the assays or prediction models developed for particular KEs need to be included in the testing strategy. Furthermore, it is of utmost importance that quality criteria of the AOP are sufficiently substantiated, such as strong KE (R)s and relevant chemicals, to ensure confidence in the application of the developed testing strategy [80]. Especially in the field of hepatotoxicity, these new testing strategies can greatly improve the general detection and prediction of drug-induced hepatotoxicity. In this respect, the use of AOP-based approaches has been demonstrated in the in vitro assessment of steatotic mixture effects of hepatotoxic compounds [97–101]. Furthermore, Bayesian AOP networks have been used to identify the most informative KEs for predicting steatosis and developing a model to predict the occurrence of steatosis under different chemical exposure conditions [102]. Recently, a tiered testing strategy has been generated based on the AOP steatosis network of Escher et al. [54]. This testing strategy integrates transcriptome data and AOP-specific human in vitro and in silico data to test a read-across hypothesis based on the most critical in vivo effects [54]. An in vitro test battery to screen for the potential of chemicals to induce liver triglyceride accumulation, a hallmark of liver steatosis, has also been proposed. These in vitro assays cover different MIEs and KEs of the respective AOPs by using reporter gene assays at MIE level (i.e., nuclear receptor transactivation), gene expression analysis, and triglyceride accumulation assays at KE level [103]. Moreover, AOP-derived approaches have also been adopted for the identification of potential liver carcinogens [96]. The six most common liver cancer-related MIEs in rodents (i.e., genotoxicity, cytotoxicity, and AhR, CAR, PPARα, and estrogen receptor activation) and other common KEs have been integrated as biomarkers in a novel short-term

exposure assay to predict potential liver carcinogens [104]. As regards cholestasis, a recent publication based on the AOP network identified a transcriptomic signature of 13 genes involved in drug-induced cholestasis by applying machine learning algorithms [105]. This signature can be implemented for the development of future tiered testing strategies.

6 Conclusions and Perspectives

The AOP framework has evolved from a largely conceptual construct into a powerful and versatile tool with regulatory and clinical applications in the hepatology field. The first applications are emerging; however, there is still a long way to go until AOP-based hazard assessment based on in vitro testing will replace the current toxicological approaches. Indeed, there are still some limitations concerning the utility of AOP networks, such as guidance gaps for AOP development, the variety in biological applicability of AOPs, and the limited quantitative data [106]. In view of the fragmented guidance landscape, overarching advice on the different guidance documents and associated tools, harmonization of AOP development and assessment concepts, and general definitions for some high-level principles concerning the design and applications of IATA would be beneficial [107]. Furthermore, future efforts should be focused on harmonization and reassessment of available AOPs with regard to continuous updating. In this context, the use of artificial intelligence and machine learning techniques has opened new avenues in the AOP field. Furthermore, the establishment of qAOPs, which are structurally complex and parameter-rich computational models, can improve quantitative understanding of the KERs [108]. Currently, the vast majority of AOPs in the AOP Wiki do not yet reach the quantitative stage. It is recommended to further evolve these AOPs to this quantitative stage. In the future, AOPs should also more accurately capture toxicodynamic processes and include exposure aspects for risk assessment. Additional actions are required to bridge the gap between kinetic profiles of chemicals and the initiation of MIEs, allowing chemical-specific kinetic data to be included in chemical-agnostic AOPs for hazard identification and risk assessment purposes [106]. The application of a combined aggregate exposure pathway and adverse outcome pathway approach can help to inform a cumulative risk assessment [109]. In the field of clinical and translational hepatology, AOPs also offer great opportunities, although this is still in its early stages of development. These applications include identification of novel diagnostic and probably prognostic biomarkers of liver disease, which can ultimately support precision medicine [110]. Furthermore, AOPs can assist in better understanding the pathophysiology of liver diseases and in

identifying druggable targets [111]. Another application might be the development and optimization of clinically relevant animal models of liver disease for fundamental and translational research purposes as well as for experimentally testing of new liver therapeutics [110]. To further explore these opportunities, it is crucial to establish close collaborations between fundamental toxicologists and clinical hepatologists, a connection that has posed challenges, but can be facilitated through targeted interdisciplinary workshops. The first steps have been taken for AOPs; however, the future potential holds exciting perspectives in the field of toxicology, such as personalized toxicology and prediction of idiosyncratic reactions, and also in the clinical area, including disease modelling and personalized medicine.

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