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Abstract

Introduction: The evaluation of the potential carcinogenicity is a key consideration in the risk assessment of chemicals. Predictive toxicology is currently switching towards non-animal approaches that rely on the mechanistic understanding of toxicity.

Areas covered: Adverse outcome pathways (AOPs) present toxicological processes, including chemical-induced carcinogenicity, in a visual and comprehensive manner, which serve as the conceptual backbone for the development of non-animal approaches eligible for hazard identification. The current review provides an overview of the available AOPs leading to liver cancer and discusses their use in advanced testing of liver carcinogenic chemicals. Moreover, the challenges related to their use in risk assessment are outlined, including the exploitation of available data, the need for semantic ontologies, and the development of quantitative AOPs.

Expert Opinion: To exploit the potential of liver cancer AOPs in the field of risk assessment, 3 immediate prerequisites need to be fulfilled. These include developing human relevant AOPs for chemical-induced liver cancer, increasing the number of AOPs integrating quantitative toxicodynamic and toxicokinetic data, and developing a liver cancer AOP network. As AOPs and other areas in the field continue to evolve, liver cancer AOPs will progress into a reliable and robust tool serving future risk assessment and management.

Keywords: Adverse outcome pathway, chemical, carcinogenicity prediction, liver cancer.

Abbreviations:

AFB_1	Aflatoxin B_1
AHR	Aryl hydrocarbon receptor
(q)AO(P)	(quantitative) Adverse outcome (pathway)
AOP-KB	Adverse outcome pathway knowledge base
AR	Androgen receptor
CAR	Constitutive androstane receptor
CYP(450)	Cytochrome P450
ER	Estrogen receptor
HCA	Hepatocellular adenoma

HCC	Hepatocellular carcinoma
IATA	Integrated approaches to testing and assessment
iNOS	Inducible nitric oxide synthase
KE(R)	Key event (relationship)
MIE	Molecular initiating event
MoA	Mode of action
NAM	New approach methodology
NGRA	Next generation risk assessment
OECD	Organization for economic co-operation and development
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PPAR	Peroxisome proliferator-activated receptor
WoE	Weight of evidence

1. Introduction

Assessing the potential adverse effects of chemicals on human health is crucial prior to marketing [1]. Amongst other toxicological endpoints, carcinogenicity is of great concern because of its impact and burden on society [2]. Evaluating the potential carcinogenicity of chemicals typically requires long-term rodent studies. Consequently, these assays use large numbers of animals, are time consuming, and entail a large financial investment [3,4]. In addition, animal use raises concerns regarding ethics and human relevance [5]. To address all these limitations, there is a need to focus on non-animal methods that are faster, more cost efficient, and better reflect the mechanisms of human chemical carcinogenesis [6].

Mechanistic information has become a critical aspect of the assessment of chemical-induced toxicity in the last decades [7]. A major milestone to set the basis for mechanistic reasoning behind cancer was the introduction of the hallmarks of cancer [8], which is a set of crucial functional capabilities acquired by human cells that ultimately lead to malignant tumor formation [9]. It is currently composed of 8 hallmark capabilities and 2 enabling characteristics (Figure 1). Recently, 2 hallmarks and 2 enabling characteristics were additionally proposed to refine the mechanistic understanding of cancer [9]. Moreover, the International Agency for Research on Cancer proposed 10 key characteristics of human carcinogens (Figure 1) [10]. Whereas the hallmarks are related to the cancer cells, the key characteristics are the properties of human carcinogens that can cause those hallmarks to become acquired [11].

Due to the increasing relevance of mechanistic toxicology, chemical toxicity testing is currently experiencing a fundamental change, leaning towards a reduction (or complete replacement) of animal approaches. Next generation risk assessment (NGRA) pursues human-relevant, exposure-led, and hypothesis-driven safety evaluation by integrating *in silico*, *in chemico* and *in vitro* approaches [12]. These approaches, named new approach methodologies (NAMs), include partial or full replacement alternatives to traditional animal-centered toxicity methods [13,14]. These alternatives are useful for chemical-induced toxicity prediction by providing mechanistic information for biologically complex endpoints [15,16]. In addition, NAMs raise less ethical concerns, are faster, and increasingly more relevant to humans [14].

Adverse outcome pathways (AOPs) have emerged as pragmatic frameworks to assist NAMs with a solid mechanistic basis [17,18]. AOPs are analytical constructs that visually describe the sequence of causally linked events leading to a toxic effect upon exposure to a chemical [17]. These events, named key events (KE), represent measurable biological changes that are essential for the progression towards the adverse effect. AOPs start with a molecular initiating event (MIE), which is a specialized

KE that represents the initial interaction and perturbation of the organism by a chemical. This is followed by one or more KEs at various biological levels (i.e., molecular, cellular, tissue and organ), which are connected by key event relationships (KERs) eventually leading to the adverse outcome (AO). Moreover, AOPs that share an MIE, KE and/or AO can be assembled into AOP networks [19].

Due to the increasing use of AOPs, the Organization for Economic Co-operation and Development (OECD) launched the OECD AOP Program for their development and dissemination, set on a web-based platform, the OECD AOP Knowledge Base (AOP-KB). The AOP-Wiki, the open-access AOP repository in AOP-KB, allows collaborative input and review of fellow experts [20].

Currently, the AOP-KB contains around 50 cancer-related AOPs, yet liver cancer has received limited attention. Primary liver cancer, mostly manifested as hepatocellular carcinoma (HCC), is the sixth most common cancer and the third most lethal cancer worldwide [2]. The development of chemical-induced HCC is a chronic multistep process driven by cytotoxicity and DNA damage, but with unknown etiology [21]. Mechanistic knowledge on chemical-induced cancer has been traditionally organized in mode-of-action (MoA) concept. MoAs are conceptually similar to AOPs in underlying the biological pathways associated with the development of an adverse effect. However, MoAs are commonly limited to individual chemicals and consider kinetic characteristics. AOPs, on the other hand, focus on the dynamic aspects and are chemical-agnostic [22]. To date, 9 AOPs leading to liver cancer have been included in the AOP-KB (Table 1). The current review provides an overview of these AOPs and highlights the scientific opportunities and principal challenges relevant to the future trajectory of AOPs on risk assessment of chemicals, focusing on liver carcinogenicity prediction.

2. Liver cancer AOPs

2.1. Inhibition of inducible nitric oxide synthase, hepatotoxicity, and regenerative proliferation leading to liver tumors

The MoA of thiamethoxam metabolites eliciting liver tumors by the inhibition of the inducible nitric oxide synthase (iNOS) was published and partially transcribed to an AOP (Figure 2A) [23,24]. The mechanism hereby described is mouse liver specific and did not induce tumors in rats or in *in vitro* assays. Due to the intrinsic differences between MoAs and AOPs, this AOP lacks an MIE and an AO, and it is specific for thiamethoxam, thus not chemical-agnostic. The production of critical metabolites of thiamethoxam (KE 77) (i.e., GCA 330050 and GCA 265307), a liver non-carcinogenic toxicant (KE 164), and an inhibitor of iNOS (KE 147) respectively, serves as the first KE, since no MIE is presented. iNOS catalyzes arginine to citrulline, thereby producing endogenous nitric oxide, an important chronic inflammation mediator [25]. This leads to sustained hepatotoxicity (KE 270), which in turn induces sustained cell proliferation (KE 269), ultimately leading to liver tumor formation (KE 347). Liver tumors

induced through sustained liver cytotoxicity and subsequent regenerative hyperplasia in mice (KER 299 and KER 298) is a well-established MoA, which also relates to AOP 118 [26]. In this regard, liver cytotoxicity (i.e., cholesterol depletion, single cell necrosis, apoptosis) and cell proliferation leading to liver tumors (KER 299 and KER 298) follow a reproducible dose response and temporal relationship in mice fed on long-term diets containing thiamethoxan [23]. This has been confirmed in various human health risk assessments for thiamethoxan by the European Chemicals Agency [26], the U.S. National Toxicology Program [24], or the Health Canada Pest Management Regulatory Agency [27]. On the other hand, it is important to highlight that the role of iNOS in the AOP is not mentioned elsewhere. In addition, KER 159, inhibition of iNOS leading to induction of sustained hepatotoxicity, lacks systematic evidence. With one exception [23], no evidence on iNOS inhibition leading to liver cancer has been found. Inversely, an increase in iNOS activity is related to cell proliferation and genomic instability, which contributes to HCC progression [25,28,29]. Furthermore, iNOS upregulation is a poor prognostic and low survival marker for HCC [28,30]. Conversely, iNOS inhibition leads to a consistent decrease in HCC growth in mice and human HCC cell lines [25,28]. It is important to consider that thiamethoxan metabolites CGA265307 and CGA330050 are produced in substantially greater quantities by mice than by humans [23]. As a consequence, thiamethoxam would not pose a carcinogenic risk of developing liver tumors, as no sufficient level of these critical metabolites would be produced to initiate the progression of hepatic KEs [23,24,31].

2.2. Peroxisome proliferator-activated receptor alpha activation leading to hepatocellular adenomas and carcinomas in rodents

Peroxisome proliferator-activated receptors (PPARs) are a superfamily of nuclear receptors that function as transcription factors. PPARα subtype is known to be involved in fatty acid β-oxidation and energy homeostasis [32,33]. This AOP describes the development of liver adenomas and carcinomas in rodents through cell proliferation (KE 716) and clonal expansion of preneoplastic foci (KE 1171) as a consequence of chronic PPARα activation (KE 227) (Figure 2B). Chronic PPARα activation has been related to hepatocarcinogenesis in mice, as demonstrated by the resistance of PPARα-knockout mice to developing liver cancer in contrast to wild-type mice following a 1-year treatment with bezafibrate [34]. Hepatocyte-restricted PPARα activation shows hepatic proliferation after 1 week of treatment in transgenic mice [35] and chronic activation results in hepatocellular adenomas (HCA) and HCC [34]. The mechanism is solely restricted to rodents with a focus on adult mice and rats applicable to both genders. PPARα-mediated hepatocarcinogenesis is not established in humans [36,37], thereby limiting the extension of this AOP to species other than rodents. Conversely, a lower hepatic PPARα expression is correlated with a worse prognosis and higher tumor severity in humans suffering from HCC [38].

PPARα expression is also reduced in metabolic dysfunction-associated steatotic liver disease patients [39]. In contrast, transcriptional upregulation of PPARα is observed in lung cancer [40] and ampullary cancer [41], suggesting that PPARα might act differently in various cancers and metabolic diseases. It is also recognized that PPARα-mediated toxicants can also cause liver cancer via PPARα-independent pathways, as indicated by liver adenoma development in PPARα-knockout mice following perfluorooctanoic acid exposure [42].

2.3. Sustained aryl hydrocarbon receptor activation leading to rodent liver tumors

This AOP addresses the toxicological process of sustained activation of aryl hydrocarbon receptor (AHR) as an initiator of liver cancer in rodents mechanistically described for dioxin and dioxin-like compounds (Figure 2C) [43,44]. The MIE in this AOP is the long-term binding of a biologically persistent ligand to AHR (KE 165) [45]. AHR is a ligand-activated transcription factor that plays an important role in the regulation of cell growth and differentiation [46]. This is evidenced by the absence of downstream KEs in the case of impaired receptor binding, as shown by receptor mutations, polymorphisms, and in knockdown models [47]. The sustained AHR activation causes changes in cellular growth homeostasis likely associated with cell proliferation and inhibition of apoptosis within altered hepatic foci (KE 853). Although the exact mechanism is not known (KER 995), this often results in increased concentrations of reactive oxygen species (ROS), a potential trigger for cytotoxicity and hepatotoxicity (KE 139) [48]. In turn, the liver's regenerative capacity will create a highly proliferative environment to compensate for the extensive hepatopathy (KE 854) [48]. The proliferative response of the liver appears to be limited to rodents [49,50]. Dose-response temporality studies reveal an increase in KE severity with higher doses and when longer exposure duration is applied [43,51]. This chain reaction of KEs ultimately results in the development of HCC/HCA and cholangiocarcinomas (KE 856).

2.4. Aflatoxin B_1: mutagenic mode-of-action leading to hepatocellular carcinoma

HCC can arise from mutations in critical genes leading to uncontrolled cell proliferation and subsequent cancerous tumor formation. This AOP revolves around the MoA for the mycotoxin aflatoxin B1 (AFB_1), a natural food contaminant produced by *Aspergillus* fungi known for its strong carcinogenic potential that is often linked to HCC development (Figure 2D) [52,53]. Overall, the steps in the AOP encompass the metabolic activation of AFB_1 (KE 409), the formation of pro-mutagenic covalent DNA adducts (KE 373), inadequate DNA repair (KE 493) and the mutation of critical genes [54]. More specifically, the initial KE (KE 409) involves the hepatic biotransformation of AFB_1 into AFB_1-exo-8,9-epoxide by cytochrome P450 (CYP450), which is a fundamental event underlying AFB_1's strong

carcinogenic potential [55]. This results from the covalent binding of the instable and highly reactive AFB_1-exo-8,9-epoxide metabolite to genomic DNA, resulting in pro-mutagenic DNA adducts (KE 373). AFB_1 can indirectly induce intracellular ROS [56,57], which might contribute to oxidative DNA damage [58]. This indirect event is not included in the current AOP, but it is generally recognized by other AOPs, such as AOP 296 in the AOP-KB. In the presence of DNA adducts, inadequate DNA repair as a result of insufficient repair or mis-repair (KE 493) can induce mutations in critical genes required in physiological cell proliferation (KE 376). This ultimately induces uncontrolled cell division forming altered hepatic foci (KE 491), resulting in HCC (KE 378). CYP450-mediated metabolization of AFB_1 is required for its carcinogenic potential as demonstrated by an increased sensitivity of *in vitro* human liver cells with higher CYP450 activity [59]. AFB_1 has shown to induce altered hepatic foci in rat and mouse liver [60,61] with mutations that are similar to those observed in human HCC [61,62]. The P53 gene is considered a major target [63–65]. However, whole-genome analysis of rat liver recently showed that AFB_1 can induce mutations in various genes involved in cell proliferation, cell cycle, cell death and DNA repair pathways [66]. Additionally, retrospective data on human HCC samples has identified AFB_1-DNA adducts that are equivalent to those in AFB_1-induced HCC in mice [67]. In addition to the evidence in rodents, this AOP is therefore considered applicable to humans [68].

2.5. Constitutive androstane receptor activation leading to hepatocellular adenomas and carcinomas in the mouse and the rat

Constitutive androstane receptor (CAR) is a nuclear receptor primarily expressed in the liver and plays a crucial role in the regulation of genes involved in drug metabolism and detoxification [69]. Induced expression of xenobiotic-metabolizing enzymes belonging to the CYP2B and CYP3A subfamilies in rodents are associated with CAR activation. Other upregulated genes include biotransformation and transporter genes (i.e., UGT1A1, MRP2, SLC1A6, GSTA1, GSTA2 and sulfotransferase enzymes) and genes for cell proliferation markers (i.e., Ki67 and Gadd45b) [70,71]. This AOP is initiated by the activation of CAR (KE 715), for which transcriptional alterations (KE 1214) induce downstream KEs, namely cell proliferation (KE 716), preneoplastic foci (KE 774), and ultimately the AO (KE 719) (Figure 2E) [72]. In 2015, a gene expression biomarker to predict CAR activation upon exposure to a chemical was introduced [73]. While CAR activators induce cell proliferation responses in mice and rats, they do not induce these effects in humans and other species [74,75]. Epidemiological studies confirm that the effects in rodents might not be directly translatable to human risk [76,77]. Moreover, in contrast to the currently described AOP in rodents, recent literature suggests that CAR has a potential tumor-suppressive role in human HCC development [78]. As such, the applicability of this AOP is only relevant for both sexes of rodents.

2.6. Inhibition of pyruvate dehydrogenase kinase leading to hepatocellular adenomas and carcinomas in mouse and rat

Alterations in cell metabolism is a common feature of tumor cells. Metabolic reprogramming is frequently observed in cancerous cells under the form of the Warburg effect, in which cells shift from oxidative phosphorylation to the less efficient aerobic glycolysis [79,80]. The metabolic shift towards glycolysis offers a growth advantage and apoptosis inhibition in growing tumors [81]. Pyruvate dehydrogenase kinase (PDK) is a mitochondrial enzyme that regulates the activity of pyruvate dehydrogenase (PDH), a key enzyme involved in the dysregulation of Krebs cycle in tumors via phosphorylation [82,83]. In humans, 4 PDK isoforms have been characterized, namely PDK1-PDK4, each with different tissue expression profiles [84]. This AOP addresses the development of HCA and HCC through PDK inhibition (Figure 2F). The inhibition of mitochondrial PDK1 is the MIE (KE 724), which triggers a chain of downstream KEs (i.e., the induction of PDH (KE 726), the increase of oxidative metabolism (KE 769), peptide oxidation (KE 209) and cytotoxicity (KE 768)) resulting in HCA and HCC in mice and rats (KE 719). This is evidenced by an increased incidence in hepatocellular tumors in mice exposed to the PDK inhibitor dichloroacetate [85]. However, there are some discrepancies on the effect of PDK on tumor cells and its non-tissue-specificity or tumor-specificity [86,87].

2.7. Androgen receptor activation leading to hepatocellular adenomas and carcinomas in mouse and rat

As previously mentioned, nuclear receptors play a pivotal role in many biological processes, including proliferation and differentiation of cells, being associated with the development of HCA and HCC (Figure 2B, 2E, 2G) [88]. It has been described that HCC is a sexual dimorphism with a higher incidence in men compared to women [89]. A contributing factor for this gender disparity is the androgen receptor (AR), a transcription factor in the nuclear steroid receptor family that regulates genes playing a role in several oncogenic signaling pathways driving hepatocarcinogenesis [90]. Upon activation of AR (KE 785), sustained cell proliferation (KE 716) promotes the formation of preneoplastic cells (KE 774) by increasing the probability of cells to acquire DNA errors [91]. Preneoplastic cells exhibit higher replication rates, and therefore are more prone to HCA and HCC compared to normal cells (KE 719) [92]. Various studies using human liver cancer tissue and knock-out mice models indicate a causal link between AR modulations and the development of HCA and HCC [90,93]. These studies showed that increased AR expression and activity precede the formation of tumors. Additionally, the knock-out of AR decreases the tumor formation highlighting the existence of empirical evidence and essentiality for these KEs in the AOP. For KER 787, evidence indicates that the existence of diverse molecular mechanisms underpin this relationship [94,95]. In addition to different species and sex, this AOP is

applicable to endocrine disruptor chemicals other than hormones (i.e., androgen), which are of high interest to human risk assessment [96].

2.8. Chronic cytotoxicity leading to hepatocellular adenomas and carcinomas in mouse and rat

Chemicals inducing HCA and HCC via cytotoxic mechanisms accompanied with regenerative cell proliferation is a well-established MoA [97] (Figure 2H). An increase in hepatocytotoxicity (KE 788) is assigned as the MIE and is caused by a broad array of toxicological effects induced by non-DNA reactive chemicals [97]. A well-known inducer of cytotoxicity and hepatocarcinogenesis is chloroform. The initial KE for chloroform is the oxidative metabolic activation by CYP2E1 to phosgene. Biotransformation by CYP2E1 has previously been linked to liver cancer and is identified as an MIE in AOP 220 [98]. Since phosgene is an electrophilic metabolite, it can react with cellular proteins and macromolecules causing cytotoxicity [99,100]. Oxidative stress is another common KE causing cytotoxicity and has also previously been associated as a KE of liver cancer in AOP 220. Non-genotoxic carcinogens, such as epyrifenacil and arsenic trioxide, increase the cellular content of ROS resulting in cytotoxicity [101,102]. Prolonged cytotoxicity (KE 786) will result in cell death, both in the form of apoptosis and necrosis [97]. To counteract this continuous cell death, regenerative cell proliferation (KE 787) occurs. Sustained cell proliferation upon cytotoxicity is considered a plausible significant risk factor for the development of cancer, as the likelihood of spontaneous DNA errors increases with the number of cell divisions [103]. When these mutations are not fixed in functional sites of critical genes, preneoplastic foci are formed (KE 774) exhibiting a higher risk of malignant evolution than normal cells [104]. Further clonal expansion of preneoplastic foci can ultimately result in HCA and HCC (KE 719) [105]. Evidence can be retrieved from long-term and/or short-term *in vivo* rodent studies using histopathologic findings along with serum enzyme changes, to indicate cell death, necrosis or apoptosis, secondary to cytotoxicity, and following cell proliferation [97,103]. These studies indicated that cytotoxicity and cell proliferation always precede liver tumor formation in a dose-dependent manner regardless of the species [31,99,101]. Chemicals that induce tumors via cytotoxicity mechanisms are subjected to a non-linear dose threshold response, implying that the dose of the chemical must be sufficient to maintain a long-term stress environment for cancer development [104].

2.9. CYP2E1 activation leading to liver cancer

CYP2E1 is an enzyme involved in the biotransformation of xenobiotics. Its constitutive activation has been linked to the development of liver cancer (Figure 2I) [98]. The AOP is designed based on data from carbon tetrachloride, chloroform, ethanol and furan, which are considered non-genotoxic carcinogens in short-term testing that operate through cytotoxic and proliferation mechanisms to

establish cancer. The MIE involves the activation of CYP2E1 from substrate biotransformation (KE 1391), generating metabolites and ROS that can both result in oxidative stress (KE 1392) and hepatotoxicity (KE 1393). Counteractive liver regeneration is initiated by cell proliferation (KE 1394), which can potentially lead to tumor formation (KE 1395) under chronic conditions. This AOP is mainly supported by data obtained from rodents and human cells [106]. Inhibition studies revealed the involvement of CYP2E1 in the generation of oxidative stress and hepatotoxicity [107,108], and *in vitro* and *in vivo* antioxidant interventions have highlighted the direct effect of oxidative stress on hepatotoxicity [109].

3. Use of liver cancer AOPs for advanced testing of liver carcinogenic chemicals

In the last two decades, the European Union regulations have moved towards an animal-free regulatory system in chemical testing parallel to the paradigm shift in NGRA. As a cornerstone in chemical safety, the REACH Regulation emphasizes the importance of alternative testing methodologies and incorporates the 3Rs principle. Simultaneously, Directive 2010/63/EU provides a framework for the humane treatment of animals in chemical testing. NGRA builds upon the principles addressed in these regulations, encouraging the use of NAMs to animal testing. By providing an integrated biological mechanistic description, the AOP framework can help to predict the potential liver carcinogenicity of chemicals, identifying specific NAMs and testing strategies [18,110].

AOPs have been recently applied for the prediction and prioritization of potential liver carcinogens [111,112]. An AOP-driven short-term-exposure strategy was developed based on the 6 most common liver-cancer-related MIEs in rodents (i.e., genotoxicity, cytotoxicity and AHR, CAR, PPARα, and estrogen receptor (ER)) and other common KEs. An index integrating the ability of chemicals to activate these MIEs and KEs in short-term assays in rats was used to rank them for their carcinogenicity potential. The prediction derived from using this index was found to be equivalent to the gold-standard 2-year rodent bioassay for carcinogens. All these MIEs are currently represented in the AOP-KB except for the AOP related to ER activation (AOPs 37, 41, 46, 107, and 118 in Table 1). This approach was further refined by excluding KEs and only considering the 6 MIEs, achieving the same predictivity compared to the previous strategy (i.e., the combination of MIEs and KEs) [111]. This is a promising AOP-driven strategy facilitating hazard identification during early preclinical testing but also allowing prioritization of potential liver carcinogens. Nonetheless, this strategy relies on the fact that most chemicals cause rodent liver cancer through only 6 major pathways [111]. In this respect, it is known that PPARα and CAR pathways are rodent-specific [36,37,112]. Consequently, such AOPs alone still lack direct human relevance. Screening of human-relevant carcinogenic chemicals would therefore be dependent on considering additional mechanisms related to liver cancer development [103]. AOPs

also provide the opportunity to extrapolate effects to humans through the identification of conserved MIEs, KEs and KERs between species, consequently decreasing the numbers of different species, required in toxicity testing [22]. This is especially relevant in liver cancer AOPs, due to their strong reliance on rodent data.

The acceptance and application of AOPs in a regulatory and clinical context depends on the confidence in the quality of AOPs and therefore, the scientific evidence contained in the AOPs should be reviewed for its reliability, credibility and human relevance [18]. For this purpose, the OECD published a guidance document and users' handbook [18,113]. The assessment of AOPs is based on the evaluation of the tailored Bradford Hill criteria, including biological plausibility, essentiality, and empirical support, each subjected to weight-of-evidence (WoE) analysis. The evidence supporting both the AOP and each KEs and KERs is then determined qualitatively. Currently, 5 of 9 liver cancer AOPs (AOP 37, 41, 46, 107, 220 in Table 1) have been extensively assessed for WoE and are supported by a rich dataset of empirical data and experimental studies. These show strong essentiality for all KEs, strong concordance of dose-response understanding, and strong causality of KERs. This enhances the rigor, transparency and reproducibility of liver cancer AOPs, and plays an important role in the overall confidence of their application.

The full potential of AOPs in NGRA is also hampered by the intrinsic linearity of AOPs in liver cancer as most carcinogenic exposure conditions activate more than one MIE [111,114]. This could be overcome by an AOP network on liver cancer, which better represent the biological complexity of the toxicity in a real-life scenario [19]. Recently, an AOP network based on 14 linear AOPs related to human hepatotoxicity was characterized [115]. This approach described the linkages between several adverse effects, including liver cancer. Cell injury/death, increased ROS, mitochondrial dysfunction, and accumulation of fatty acids were identified as the most highly connected and central KEs. These KEs were then considered as the mechanistic basis to select and optimize *in vitro*/*in silico* assays for chemical-induced hepatotoxicity prediction in hazard identification [115]. AOP networks also serve as the cornerstone for integrated approaches to testing and assessment (IATA), providing the biological backbone as a platform for data integration, interpretation and decision-making. The AOP "Sustained AHR activation leading to rodent liver tumors" (AOP 41, in Table 1, Figure 2C) was used to frame an IATA in view of supporting the application of AOPs for regulatory purposes. This integrates the MIE/KEs as measurable endpoints in a stepwise decision tree approach and outlines what testing should be considered when a substance has the potential to bind to AHR or cause AHR-mediated transcription resulting in rat liver tumors [116]. The required assays in each step increase in complexity. A positive result pursues the next step, and a negative result in any step discards potential

AHR-mediated carcinogenicity. Fundamentally, IATA development and relevance depend greatly on the extent of completeness of an AOP.

Overcoming the challenges and applying the improvements available for AOPs will determine whether the AOP-based strategies and approaches can be used for prioritization, hazard identification or risk assessment [116].

4. Challenges for liver cancer AOPs

4.1. Exploitation of available data for liver cancer AOPs

Available AOPs greatly depend on the granularity of the mechanistic insights in the onset and progression of liver cancer. In the last few years, efforts have been made to integrate the toxicity pathways of liver carcinogens into AOPs with an increase in the number of liver cancer AOPs from 4 to 9 [20,117]. However, liver cancer AOPs currently included in the AOP-KB only represent a part of the complex etiology of liver cancer triggered by chemicals (Table 1). This multi-step process, sometimes secondary to other liver diseases, hampers the identification of MIEs [118]. Moreover, although many new theories have been proposed, research on liver cancer mechanisms is relatively slow, and practical studies that fully examine the interplay between these mechanisms are scarce [119]. These gaps in knowledge should be filled through fundamental research.

The elaboration of AOPs is time-consuming, such effort lacks incentives, and is poorly recognized by the scientific community [5]. In fact, manual data extraction of abundant evidence in the form of text results in a tedious process. The field of artificial intelligence (AI), and specifically machine learning approaches such as natural language processing, provide a solution to this challenge [120]. AI assisted data collection has risen as a useful and rapidly evolving tool directly applicable to AOP development. When building AOPs, automated machine learning approaches can be used to aid in the selection of relevant articles and directly perform data mining. In liver AOPs, a novel approach using automated machine learning assisted article selection has been used to develop an AOP network on cholestasis [121]. Moreover, genes mechanistically linked to this AOP network were identified using machine-learning-assisted data mining from human *in vitro* data sets [122]. In liver cancer, a wealth of data is already available in databases and peer-reviewed literature that awaits extraction and embedment into an AOP construct. Potential MIEs specific to chemicals inducing HCC have been recently identified using an adverse drug event database and quantitative structure-activity relationship predictions [118]. This leads to the identification of 6 MIEs specific to HCC-inducing drugs that are currently not described in the AOP-KB: estrogen receptor-β antagonist, estrogen-related receptor agonist and antagonist, ATAD5 inducer, vitamin D receptor agonist, and TRHR agonist [123]. The hallmarks of cancer and the 10 key specific characteristics of human carcinogens also provide the basis for

identifying and organizing results from mechanistic studies, and an opportunity to guide the development of AOPs (Figure 1) [11]. As illustrated in Figure 1, current liver cancer AOPs can be related to the hallmarks “sustaining proliferative signaling”, “deregulating cellular metabolism”, “genome instability and mutation”, and “tumor-promoting inflammation”. In addition, not all the key characteristics of carcinogens are present in liver cancer AOPs, which represent “genotoxicity”, “oxidative stress induction”, “induction of inflammation”, “cell proliferation alteration” and “cell death induction”. Although progression of HCC is modulated by the immune system, no AOP on liver cancer is yet related to immune-specific pathways [124]. Recently, prognostic gene-set signatures to predict HCC risk linked to “cell death resistance” hallmark and immortalization-related key characteristics, such as ferroptosis-related genes or hypoxia-related genes have been developed [125]. Using the hallmarks and key characteristics as a basis, the collected information can provide guidance to discover gaps in AOPs, identify molecular mechanisms, and develop AOPs and their networks (Figure 1). Moreover, MoAs in liver carcinogenesis provide a direct way to develop AOPs [3]. The mechanistic information contained in form of MoAs could be therefore integrated into AOPs, which represents a more useful framework for hazard and risk assessment purposes [126]. Some liver cancer AOPs are described by specific stressors due to their MoA-related origin. Except for the inhibition of iNOS induced by the metabolites of thiamethoxam leading to liver tumors (AOP32), all liver cancer AOPs are not chemical-specific. Nonetheless, this aspect should be carefully addressed, and future efforts should aim at refining this information, as the advantages of AOPs towards chemical toxicity prediction partially lie in their chemical-independent nature (Table 1, Figure 2).

4.2. Semantic ontologies in liver cancer AOPs

The lack of consensus on the extent and detail of AOPs presents a challenge for their inter-applicability. Terminology should be subjected to standards, and the minimum level of information and detail required for their application in routine practice should be harmonized [22]. KE/KER information currently included in the AOP-KB is of variable evaluation and the descriptions of KE/KERs are not consistent. This results in the duplication of KEs and KERs, being added to AOP-KB with different descriptors, resulting in the loss of interrelation. Controlled vocabularies will therefore allow a more pragmatic use of AOPs, the interconnection of different AOPs, the unification into a single AO, and ultimately the development of an AOP network. In this respect, Table 2 shows highly similar MIEs/KEs/AOs grouped under a single common concept; 5 of the 9 uploaded AOPs referring to “liver cancer” used “Increase of HCA and HCC” (KE 719) as the AO, while others used similar terms such as “Promotion of HCC” (KE 334) or “Formation of hepatocellular and bile duct tumors” (KE 856). However, unifying such events into one is debatable. A balance between the harmonization of the KE denominator and the specificity of the biological event described should be achieved. In other words,

it should be discussed to what extent event title specificity is advantageous for the applicability of AOPs, and likewise, to what extent normalized event titles do not impair the accuracy on the represented biological event. Regardless, it should be cautiously performed on a case-by-case basis, not only by the pertinent organizations, but also by the scientific community. The AOP-KB has been upgraded to facilitate the use of a defined set of ontologies as consistent structured descriptions of KEs [114]. Marginal differences in event titles are highly relevant for the applicability of AOPs, especially when applying computer-based approaches. With the improved use of semantic ontologies, the integration of data could be performed with automated AI-based approaches to combine and interrelate AOPs, therefore accelerating their development [114].

4.3. Quantification of liver cancer AOPs

Qualitative AOPs help to assess hazard identification, and therefore can be used for prioritization of testing strategies, screening of chemicals, and development of assays to guide chemical decision-making during the development of novel chemicals [127]. The application of AOPs in risk assessment yet requires quantitative time- and dose-response information, underlying the need to develop quantitative AOPs (qAOPs) [3,127]. However, a framework to guide such development and assessment of qAOPs is currently lacking [20].

Different approaches can be followed to develop qAOPs, ranging from Bayesian network and regression modeling to ordinary differential equation and individual based models [128]. Bayesian networks are used to predict the probability that a downstream KE occurs, based on the change of an upstream KE. Regression models predict how different levels of perturbation in an upstream KE would affect a downstream KE. Other types of mathematical models, such as ordinary differential equation or individual-based modeling, are commonly applied for predicting of temporal and time-resolved responses [128]. To feed the models, information between KEs can be derived from experimental data indicating causality, including KE expression knock-down or activity inhibition, and/or response-response dependence, such as dose-response measurement of upstream and downstream KE activities. All liver cancer AOPs remain devoid of quantification today [113,114]. In this regard, Bayesian models are of special interest in qAOP development, and have already been applied to quantify an AOP network on liver steatosis [124,125]. In any case, the choice of the quantification approach for liver cancer AOPs will depend on how the KER is described and the data available [128]. It is necessary to bear in mind that the conditions that propagate an AOP may differ depending on specific circumstances, such as type of exposure, cell type, or species. Therefore, it is essential that these conditions are clearly defined and accurately recorded. It is worthwhile to highlight the importance of chronic or sustained activation of MIEs observed in liver cancer AOPs (AOP 37, 41, 118,

in Table 1). This might require the use of models that are suited to incorporate the effect of time-dependent perturbations, such as ordinary differential equation models. Moreover, AOPs in liver cancer are currently not fully representative for humans. Upon the development of a rodent-based qAOP, the modulation of a downstream KE by an upstream KE observed in rodents occurs to the same extent in humans.

The development of liver cancer involves interactions between multiple pathways and cell types. Due to its inherent complex etiology, additional critical aspects of liver cancer should be considered when developing a qAOP network. This includes the non-linear nature of cancer, and the dichotomy of non-genotoxic and genotoxic carcinogens. Cancer is considered a non-linear pathogenic process with described feedback loops, affecting several signaling pathways and cell cycle dysregulation [129–131]. In addition, from the mechanistic point of view, carcinogens can be regarded as genotoxic and non-genotoxic (Figure 1). Although widely debated in the past years, it is historically assumed that a single molecule of a genotoxic chemical may cause a mutation, and thus result in an increased cancer incidence at any dose [132]. This has a direct impact on the development of qAOPs and therefore in risk evaluation. Despite the small increase in risk, genotoxic chemicals are considered not to have a safe exposure threshold. This is the case of AOP 46 (see 2.5), and KEs like DNA adducts (MIE), mutations (KE) and tumors (AO). It should therefore be assumed that in the presence of activated MIEs, all other steps (KEs) are also active, leading to an increased probability of AO. Moreover, the link between genotoxicity and chemical carcinogenesis is well established and standardized testing batteries have been used for decades [133]. On the other hand, for non-genotoxic carcinogens that work under threshold mechanisms, if an MIE occurs at levels that are too low to induce a KE, no AO would be expected.

In a human real-life scenario, the concentration at which a chemical triggers a KE might not be relevant at the target site. For this reason, AOPs also need to be integrated with exposure assessment to translate the external exposure levels into internal doses at the site of action. In practice, physiologically based pharmacokinetic modelling can perform these estimations [5]. The Aggregate Exposure Pathway (AEP) framework has been proposed to organize these exposure data, from the source of a chemical to its concentration at a site of action [134,135]. AEPs share several key features with AOPs, such as the structure and terminology, to provide a smooth integration between these two frameworks [135]. As risk assessment is moving towards a better mechanistic understanding on how chemicals interact with biological pathways that lead to an apical outcome, the integration of AOPs with quantitative toxicodynamic and toxicokinetic data can address risk queries in a comprehensive and efficient manner [5,134,135]. Moreover, the application of AOPs in a regulatory context relies on standardized evaluation and acceptance [126]. Their use for hazard identification and risk assessment

ultimately depends on the confidence in the quality of AOPs, and thus the scientific evidence contained in the AOPs should be reviewed for its reliability and credibility [18]. As abovementioned, WoE evaluation is included as a key element in the OECD guidance document and users' handbook for developing and assessing AOPs [18,113]. This provides a useful approach for evaluating the extent of support for the hypothesized mechanisms, ultimately improving the overall confidence of the AOP [113,126].

5. **Expert opinion**

Over the past three decades, liver cancer incidence has tripled globally [136]. In 2020 alone, liver cancer approached 1 million diagnoses and caused over 800,000 fatalities. These trends are continuously increasing and anticipate 1.4 and 1.3 million diagnoses and deaths from liver cancer by 2040, respectively [136]. The etiology of this rise is linked to exposure to various carcinogens that contribute significantly to the onset and progression of liver cancer. Chemical toxicity testing of new compounds requires long-term rodent studies, with a 2-year rodent carcinogenicity test currently estimated to cost over $1 million and utilizing more than 880 rodents [137]. The application of AOPs favors the use of NAMs as alternative test methods in toxicity assessment, thereby reducing and ultimately replacing the reliance on animal testing. This transition marks a milestone in chemical assessment, moving towards NGRA, prioritizing apical endpoints, and combining *in vitro* and *in silico* data.

In light of these developments, this review identifies 3 critical prerequisites essential for leveraging the potential of liver cancer AOPs in NGRA (Figure 3):

1. Developing human-relevant AOPs for chemical-induced liver cancer. Formulating human-centered AOPs for chemical-induced liver cancer involves systematically gathering data on the pathways and mechanisms triggered by chemical exposures, translating this knowledge into AOP frameworks. Applying machine learning techniques facilitates this process, aiding in tasks such as article selection, text analysis, and data mining. The use of improved semantic ontologies allows for computational integration of the collected data, automating the generation and accelerating the development of AOPs.
2. Increasing the number of qAOPs. Incorporating quantitative measurements and exposure data into AOPs is crucial for effective risk assessment. The models employed in qAOPs should be sufficiently sophisticated to replicate the inherent complexities in liver cancer, including exposure metrics and toxicokinetic considerations. Integration of AOPs with dose-response data and AEPs enables a more comprehensive assessment of the health impacts associated with real-life scenarios.

3. Developing a liver cancer AOP network. The biochemical and biological pathways contributing to chemical-induced liver cancer are inherently complex, thus isolated and linear AOPs are of limited relevance. An AOP network emerges as a robust tool for risk assessment, capable of describing the intricate interplay of factors contributing to chemical-induced liver cancer with greater fidelity to biological reality.

Ultimately, regulatory acceptance of AOPs stands as a pivotal factor for their integration into risk assessment practices (Figure 3) and continued efforts are needed to demonstrate the reliability of AOPs to regulatory agencies. Demonstrating the practical applications and tangible benefits of AOPs in human-relevant scenarios provides the confidence required for regulatory bodies to integrate these frameworks into their decision-making processes.

The development of AOPs aligns with a broader movement towards more predictive, mechanistically informed, and humane approaches in NGRA. Along with AOPs, various areas hold promise for advancing our understanding of toxicological mechanisms and refining risk assessment methodologies (Figure 3). The integration of *in vitro* testing, high-throughput screening, and omics data with AOPs enriches the understanding of key events and molecular mechanisms associated with AOs, aiding in the identification of pertinent biomarkers for predicting toxicity. Furthermore, AOPs can guide the incorporation of alternative models, such as 3D cell culture models and organ-on-a-chip systems, aligning with mechanistic insights into toxicity pathways and providing more physiologically relevant platforms for toxicity testing.

As AOPs continue to evolve, their assimilation into regulatory decision-making processes holds the potential to revolutionize NGRA. The progression of liver cancer AOPs into a robust regulatory tool will lead to a paradigm shift towards more informed, efficient, and ethical approaches in assessing and managing the hepatotoxic risks associated with chemical exposure.

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Figures and tables

Figure 1. Hallmarks of cancer and key characteristics of carcinogens (adapted from [9,11]) focused on liver cancer AOPs. The nomenclature of liver cancer AOPs has been adapted from AOP-Wiki.

Figure 2. Representation of the liver cancer AOPs. Their nomenclature has been adapted from AOP-Wiki. Abbreviations: AFB1, aflatoxin B1; AHR, sustained aryl hydrocarbon receptor; AOP, adverse outcome pathway; AR, androgen receptor; CAR, constitutive androstane receptor; CYP, cytochrome P450; iNOS, inducible nitric oxide synthase; KE, key event; KER, key event relationship; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase.

Figure 3. Future perspectives in liver cancer AOPs. Abbreviations: AOP, adverse outcome pathways; qAOP, quantitative adverse outcome pathway.

Table 1. Liver cancer AOPs in the AOP-KB. Abbreviations: AOP, adverse outcome pathway; AOP-KB, adverse outcome pathway-knowledge base.

Table 2. Semantic ontologies in MIE/KE/AOs from different liver cancer AOPs. Abbreviations: AO, adverse outcome; AOP, adverse outcome pathway; KE, key event; MIE, molecular key event.

Figure 1

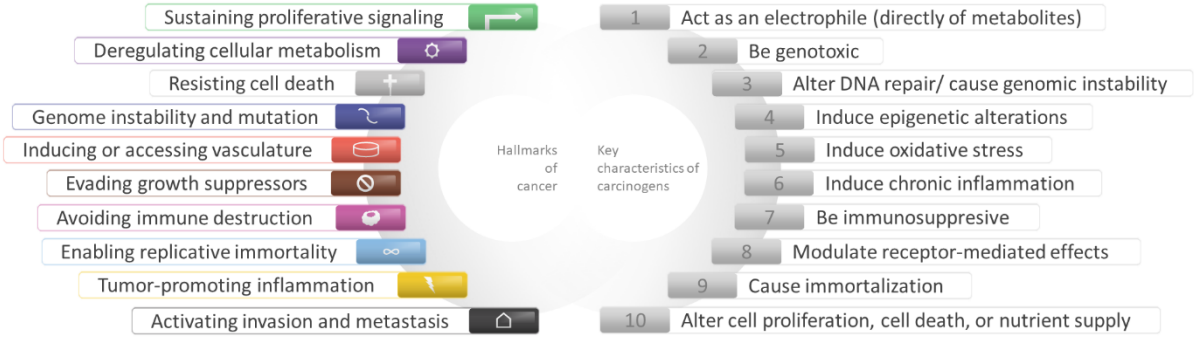

Figure 2

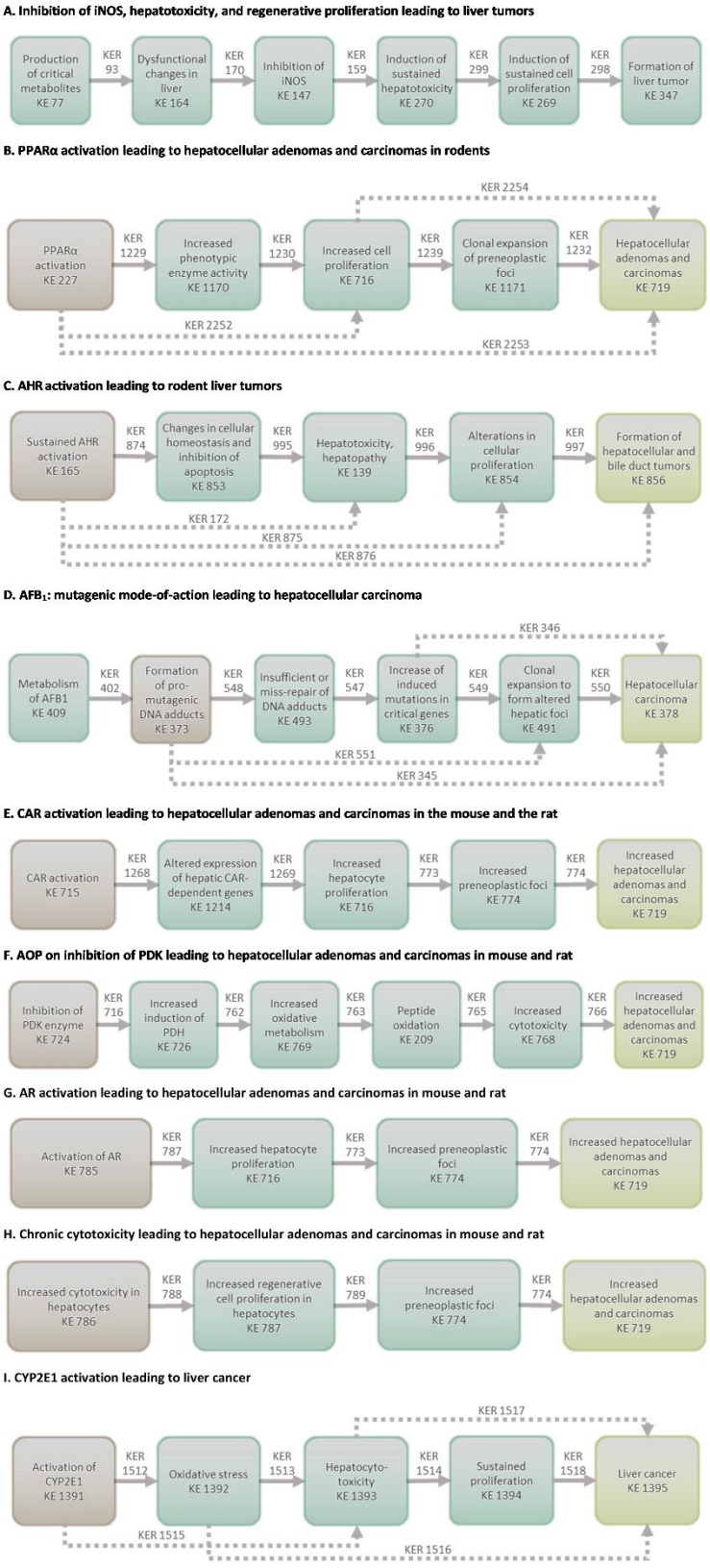
A. Inhibition of iNOS, hepatotoxicity, and regenerative proliferation leading to liver tumors
Production of critical metabolites KE 77
KER 93
Dysfunctional changes in liver KE 164
KER 170
Inhibition of iNOS KE 147
KER 159
Induction of sustained hepatotoxicity KE 270
KER 299
Induction of sustained cell proliferation KE 269
KER 298
Formation of liver tumor KE 347
B. PPARα activation leading to hepatocellular adenomas and carcinomas in rodents
KER 2254
PPARα activation KE 227
KER 1229
Increased phenotypic enzyme activity KE 1170
KER 1230
Increased cell proliferation KE 716
KER 1239
Clonal expansion of preneoplastic foci KE 1171
KER 1232
Hepatocellular adenomas and carcinomas KE 719
KER 2252
KER 2253
C. AHR activation leading to rodent liver tumors
Sustained AHR activation KE 165
KER 874
Changes in cellular homeostasis and inhibition of apoptosis KE 853
KER 995
Hepatotoxicity, hepatopathy KE 139
KER 996
Alterations in cellular proliferation KE 854
KER 997
Formation of hepatocellular and bile duct tumors KE 856
KER 172
KER 875
KER 876
D. AFB1: mutagenic mode-of-action leading to hepatocellular carcinoma
KER 346
Metabolism of AFB1 KE 409
KER 402
Formation of pro-mutagenic DNA adducts KE 373
KER 548
Insufficient or miss-repair of DNA adducts KE 493
KER 547
Increase of induced mutations in critical genes KE 376
KER 549
Clonal expansion to form altered hepatic foci KE 491
KER 550
Hepatocellular carcinoma KE 378
KER 551
KER 345
E. CAR activation leading to hepatocellular adenomas and carcinomas in the mouse and the rat
CAR activation KE 715
KER 1268
Altered expression of hepatic CAR-dependent genes KE 1214
KER 1269
Increased hepatocyte proliferation KE 716
KER 773
Increased preneoplastic foci KE 774
KER 774
Increased hepatocellular adenomas and carcinomas KE 719
F. AOP on inhibition of PDK leading to hepatocellular adenomas and carcinomas in mouse and rat
Inhibition of PDK enzyme KE 724
KER 716
Increased induction of PDH KE 726
KER 762
Increased oxidative metabolism KE 769
KER 763
Peptide oxidation KE 209
KER 765
Increased cytotoxicity KE 768
KER 766
Increased hepatocellular adenomas and carcinomas KE 719
G. AR activation leading to hepatocellular adenomas and carcinomas in mouse and rat
Activation of AR KE 785
KER 787
Increased hepatocyte proliferation KE 716
KER 773
Increased preneoplastic foci KE 774
KER 774
Increased hepatocellular adenomas and carcinomas KE 719
H. Chronic cytotoxicity leading to hepatocellular adenomas and carcinomas in mouse and rat
Increased cytotoxicity in hepatocytes KE 786
KER 788
Increased regenerative cell proliferation in hepatocytes KE 787
KER 789
Increased preneoplastic foci KE 774
KER 774
Increased hepatocellular adenomas and carcinomas KE 719
I. CYP2E1 activation leading to liver cancer
KER 1517
Activation of CYP2E1 KE 1391
KER 1512
Oxidative stress KE 1392
KER 1513
Hepatocyto-toxicity KE 1393
KER 1514
Sustained proliferation KE 1394
KER 1518
Liver cancer KE 1395
KER 1515
KER 1516

Figure 3

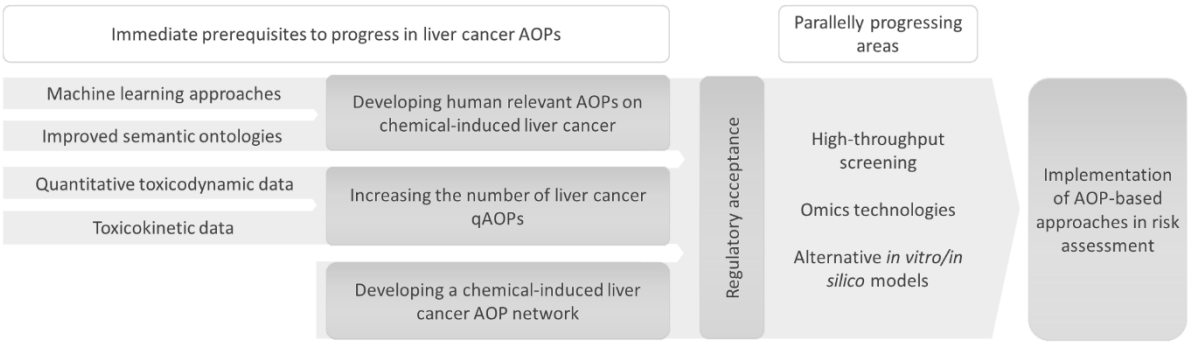

Table 1

AOP no.	AOP title
32	Inhibition of inducible nitric oxide synthase, hepatotoxicity, and regenerative proliferation leading to liver tumors
37	Peroxisome proliferator-activated receptor alpha activation leading to hepatocellular adenomas and carcinomas in rodents
41	Sustained aryl hydrocarbon receptor activation leading to rodent liver tumors
46	Aflatoxin B_1: mutagenic mode-of-action leading to hepatocellular carcinoma
107	Constitutive androstane receptor activation leading to hepatocellular adenomas and carcinomas in the mouse and the rat
108	Inhibition of pyruvate dehydrogenase kinase leading to hepatocellular adenomas and carcinomas (in mouse and rat)
117	Androgen receptor activation leading to hepatocellular adenomas and carcinomas (in mouse and rat)
118	Chronic cytotoxicity leading to hepatocellular adenomas and carcinomas (in mouse and rat)
220	Cytochrome P450 2E1 activation leading to liver cancer

Abbreviations: AOP, adverse outcome pathway; AOP-KB, adverse outcome pathway-knowledge base.

Table 2

Type	ID no.	Title	AOP no.
KE	768	Increase, cytotoxicity	108
MIE	786	Increase, cytotoxicity (hepatocytes)	118
KE	139	N/A, hepatotoxicity, hepatopathy, including a constellation of observable effects	41
KE	270	Induction, Sustained Hepatotoxicity	32
KE	1393	Hepatotoxicity	220
KE	269	Induction, sustained cell proliferation	32
KE	716	Increase, cell proliferation (hepatocytes)	37, 107, 117
KE	787	Increase, Regenerative cell proliferation (hepatocytes)	118
KE	854	Alterations, cellular proliferation / hyperplasia	41
KE	1394	Induction, persistent proliferation/sustained proliferation	220
KE	347	Formation, liver tumor	32
AO	378	Tumorigenesis, hepatocellular carcinoma	46
AO	719	Increase, hepatocellular adenomas and carcinomas	37, 107, 108, 117, 118
AO	856	Formation, hepatocellular and bile duct tumors	41
AO	1395	Liver cancer	220

Abbreviations: AO, adverse outcome; AOP, adverse outcome pathway; KE, key event; MIE, molecular key event.