REVIEW

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Liver Adverse Outcome Pathways: What's in for the Hepatologist?

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ABSTRACT

Adverse outcome pathways are tools to capture and visualize mechanisms underlying adverse effects, and are currently emerging in the areas of toxicology and chemical risk assessment. Less attention has yet been paid to potential clinical applications of adverse outcome pathways, including in the hepatology field. Liver adverse outcome pathways can serve the development and optimization of the clinically relevant animal models of liver diseases for fundamental and translational research as well as for testing new liver therapeutics. They also can aid the characterization of novel and more specific diagnostic and prognostic biomarkers of liver disease. Full clinical exploitation in these directions requires further technical optimization of adverse outcome pathways as well as intensive interdisciplinary and intersectoral collaboration.

Key words: adverse outcome pathway – toxicology – liver – drug-induced liver injury.

Abbreviations: ALT: alanine aminotransferase; AOP: adverse outcome pathway; DILI: drug-induced liver injury; KE: key events; KER: key event relationship; MIE: molecular initiating event; OECD: Organization for Economic Co-operation and Development.

Setting the scene: hepatology meets toxicology

Acute and chronic liver toxicity as well as many types of liver disease are induced by chemicals. Most attention in this respect has yet been paid to pharmaceuticals. Drug-induced liver injury (DILI) is frequently misdiagnosed, but it has been estimated to develop in 1 in 100 patients during hospitalization [1]. In fact, DILI is responsible for more than half of all clinical cases of acute liver failure [2]. About 20-40% and 12-20% of DILI patients present a cholestatic and mixed hepatocellular/cholestatic injury pattern, respectively. Although more than one drug can be involved in DILI, single prescription medication underlies 73% of all DILI cases. More than 1,000 drugs have been associated with DILI, including anti-infectious, anti-diabetic, anti-inflammatory, psychotropic, cardiovascular drugs and steroids [3]. Drug-induced liver injury is a major reason of drug failure during pre-marketing and post-marketing phases, accounting for up to 29% of all drug withdrawals [4]. However, many other types of chemical compounds of various nature and applicability domains can also induce liver insults, including industrial chemicals, biocides, cosmetic ingredients, food additives and dietary/herbal supplements [5, 6] (Table I). Consequently, this is not a mere clinical and pharmaceutical issue, but has more general toxicological relevance.

Toxicology on the move: paradigm shift towards mechanistic approaches

The areas of toxicology and chemical risk assessment are presently undergoing tremendous changes. Historically, animal testing has formed the basis for toxicity testing and human safety evaluation of chemicals. The protocols for such studies have been introduced by the Organization for Economic Co-operation and Development (OECD) [7] and have been taken up in chemical legislations worldwide. These studies rely on the administration, mostly oral, of the chemical under investigation to groups of animals, typically rodents, for specific periods of time using well-defined dosing regimens. The testing as such is followed by the evaluation of a plethora of parameters, including histopathological and clinical chemistry endpoints. This allows to pinpoint the most relevant and sensitive adverse

Table I. Non-pharmaceutical chemicals associated with cholestatic liver injury [5, 6]

Industrial chemicals	
Alpha-naphthylisocyanate	Methylenedianiline
Diethylhexyl phthalate	Polyoxyethylene nonylphenol
Biocides	
Paraquat	Quizalofop-p-ethyl
Permethrin	Yellow phosphorus
N,N-diethyl-meta-toluamide	
Cosmetic ingredients	
Sunset Yellow FCF	2-Octynoic acid
Basic Red 51	2-Nonynoic acid
Triclosan	
Food additives	
Iron tartrate	Carmoisine
Tartrazine	Polysorbate 80
Neotame	Oxidized polyethylene wax
Trans-anethole	Propylene glycol
Brilliant blue FCF	
Dietary/herbal supplements	
Germander	*Polygonum multiflorum*
Artemisinin	*Fructus Psoraleae*
Celandine	Herbalife™
Kava extract	Hydroxycut®
Oleanolic acid	

effect, which is subsequently used to characterize the so-called point-of-departure in the dose-response curve for setting the safety limits for humans. The assumption is hereby made that the adverse effect identified in the laboratory animal will equally occur in humans. Nevertheless, an uncertainty factor is used to quantitatively extrapolate the point-of-departure to human. This uncertainty factor is usually set at 100, which counts for interspecies (animal-human) and intraspecies (human-human) differences.

Driven by obvious ethical and scientific constraints, there is a clear tendency around the globe to increasingly address animal-free methods for toxicity testing of chemicals. A major milestone in this regard was the publication of the report "*Toxicity testing in the 21st century: a vision and a strategy*" by the US National Academy of Sciences in 2007. This landmark report reinforces the paradigm shift to move away from reliance on apical toxicological outcome testing in laboratory animals towards the use of human-based *in vitro* (cell culture) assays and *in silico* (computational) methods mainly designed to detect perturbations in toxicity pathways at the mechanistic level [8].

Adverse outcome pathways: tools to mechanistically map liver toxicity and disease

A major tool adopted in 21st century toxicology is the adverse outcome pathway (AOP) framework, first established in the area of ecotoxicology in 2010 [9] and introduced in the human toxicology domain over the past few years. An AOP is defined as a conceptual construct that represents existing knowledge concerning the linkage between a direct molecular initiating event (MIE) and an adverse outcome at a biological level of organization relevant to risk assessment. In practice, an AOP is a graphical scheme presenting the mechanisms driving a specific type of adverse effect. Each AOP consists of 2 critical elements, namely key events (KEs) and key event relationships (KERs) (Fig. 1). A KE represents a measurable change in a biological state that is essential, but not necessarily sufficient, for progression to the adverse outcome. The MIE and adverse outcome are 2 specialized KE types. The MIE occurs by definition at the molecular level and indicates the initial critical chemical-biological interaction within the organism. The adverse outcome, situated typically at the organ level or higher, indicates a change in morphology or physiology of an organism or system that results in the impairment of the functional capacity or the capacity to compensate for stress. A KER refers to a causal relationship between a pair of KEs, establishing one as upstream and one as downstream. It provides the scientifically plausible and evidence-based foundation for extrapolation from an upstream cause to a downstream effect, and thus for using KE information as indicators of adverse effects. Furthermore, a KER can reflect linkages between a pair of KEs that are either adjacent or non-adjacent in an AOP, allowing the possibility to capture parallel and interdependent processes within a single AOP [10, 11].

Although basically very similar, the scope of an AOP is broader compared to the mode-of-action concept, as it can go up to the population level. In addition, while the mode-of-action tends to be chemical-specific and takes into account kinetic aspects, such as metabolism, AOPs are chemical-agnostic and describe a toxicological process from a purely dynamic perspective. Different types of information can

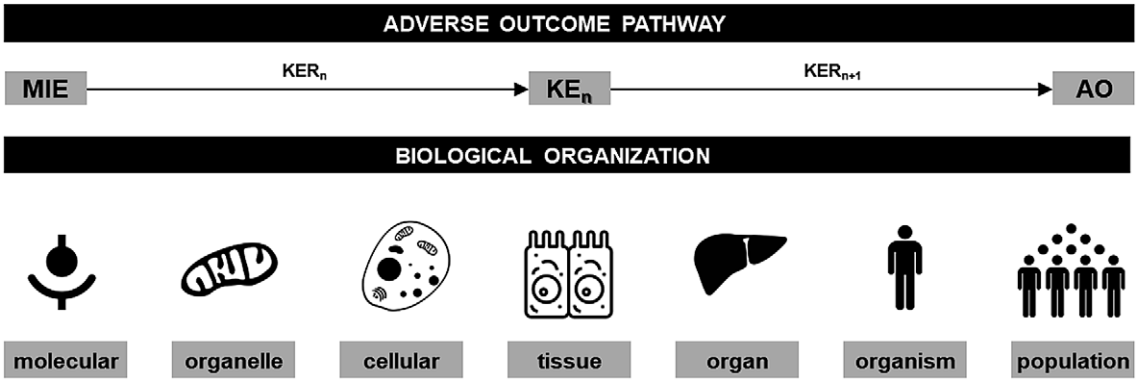

Fig. 1. Generic structure of an adverse outcome pathway. AO: adverse outcome; KE(R): key event (relationship); MIE: molecular initiating event.

be used during AOP development, including *in chemico, in silico, in vitro, in vivo*, clinical and epidemiological data [10-12]. Adverse outcome pathway development ideally complies with guidance from the OECD. In this context, the OECD, in collaboration with a number of other agencies, has established an electronic repository for AOPs, called the AOP Wiki. At present, the AOP Wiki contains more than 300 AOPs for a multitude of adverse effects [13]. Among those are several AOPs related to chemical-induced hepatotoxicity and liver pathology at different levels of development [14] (Table II).

AOPs were initially introduced to support regulatory decision-making by making efficient use of mechanistic information, particularly novel data sets that can be generated rapidly and cost-effectively in an animal-free high-throughput format, rather than relying only on apical outcome data measured in animals. The specific application of an AOP is, however, dictated by its level of development [10-12]. In the toxicology field, AOPs can serve as the basis for setting up *in vitro* test batteries to predict hepatotoxic potential of chemicals. This has been nicely demonstrated for liver steatosis by using 4 *in vitro* assays based on lipid uptake, lipid efflux, fatty acid oxidation and lipid accumulation, being KEs in the AOP on liver steatosis [15]. Another toxicological AOP application is the development of chemical categories based on biological

Table II. Adverse outcome pathways (AOPs) related to liver toxicity and disease in the AOP Wiki [13, 14]

Number in AOP Wiki	Title in AOP Wiki	OECD status in AOP Wiki
1	Uncharacterized liver damage leading to hepatocellular carcinoma	Not under development
6	Antagonist binding to peroxisome proliferator-activated receptor alpha leading to body weight loss	Open for citation
27	Cholestatic liver injury induced by inhibition of the bile salt export pump	Under development
32	Inhibition of inducible nitric oxide synthase, hepatotoxicity and regenerative proliferation leading to liver tumors	Under development
34	Liver X receptor activation leading to hepatic steatosis	Under development
36	Peroxisomal fatty acid beta-oxidation inhibition leading to steatosis	Under development
37	Peroxisome proliferator-activated receptor alpha-dependent liver cancer	Under development
38	Protein alkylation leading to liver fibrosis	Open for citation
41	Sustained aryl hydrocarbon receptor activation leading to rodent liver tumors	Open for citation
46	Aflatoxin B1: mutagenic mode-of-action leading to hepatocellular carcinoma	Open for citation
57	Aryl hydrocarbon receptor activation leading to hepatic steatosis	Under development
58	Constitutive androstane receptor suppression leading to hepatic steatosis	Under development
59	Hepatocyte nuclear factor alpha suppression leading to hepatic steatosis	Under development
60	Pregnane X receptor activation leading to hepatic steatosis	Under development
61	Farnesoid X receptor activation leading to hepatic steatosis	Under development
62	Serine/threonine protein kinase 2 activation leading to hepatic steatosis	Under development
107	Constitutive androstane receptor activation leading to hepatocellular adenomas and carcinomas in the mouse and the rat	Under development
118	Chronic cytotoxicity leading to hepatocellular adenomas and carcinomas in mouse and rat	Under development
144	Endocytic lysosomal uptake leading to liver fibrosis	Under development
213	Inhibition of fatty acid beta-oxidation leading to non-alcoholic steatohepatitis	Under development
220	Cytochrome P450 2E1 activation leading to liver cancer	Open for citation
232	Nuclear erythroid 2-related factor repression to steatosis	Under development
240	Deoxyribonucleic acid adducts leading to liver hemangiosarcoma	Under development
273	Mitochondrial complex inhibition leading to liver injury	Under development
278	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor kinase complex inhibition leading to liver injury	Under development
285	Inhibition of N-linked glycosylation leads to liver injury	Under development

OECD: Organization for Economic Co-operation and Development

responses, as has been done for rat liver carcinogens using short-term assays [16].

Liver adverse outcome pathways: applications for the clinical hepatologist

Although currently used in toxicology and chemical risk assessment, promising applications for AOPs in the clinical field, *in casu* in the hepatology area, remain to be explored. A first application includes the development and optimization of clinically relevant animal models of liver disease for fundamental and translational research purposes as well as for experimentally testing new liver therapeutics. A wide variety of animal models, mostly in rodents, is currently used for studying acute and chronic liver diseases, including acute liver failure, cholestatic disorders, non-alcoholic steatohepatitis, liver fibrosis and cirrhosis, and different types of liver cancer. They are typically based on the use of specific chemicals, well-defined diets, genetic modifications, surgical procedures or infection-based strategies [17, 18]. Although some of them appropriately reproduce the corresponding human pathology, all these animal models cope with specific flaws. Many scientific journals and agencies therefore request the use of 2 animal models of the same liver disorder in parallel to overcome such shortcomings. Nonetheless, the extrapolation of experimental findings from animal studies to the clinical situation still remains a challenge. Even for generic KEs, such as inflammation, the underlying mechanisms in mice and humans show poor correlation [19]. By scrutinizing the mechanistic level, AOPs allow to identify such critical discrepancies. This enables to leverage animal models of liver disease for translational research purposes and may even open perspectives for the development of more human-relevant models. Importantly, this is not restricted to interspecies differences, but equally applies to intraspecies differences. Many KEs and KERs can indeed undergo modulation by a diversity of endogenous and exogenous factors. In this respect, genetic predisposition, age, gender and gut microbiota composition, all are important endogenous modulating factors, while lifestyle, diet, medication and (occupational) environment are typical exogenous modulating factors of liver toxicity and disease. Recently, the environmental contributions to liver steatosis have been characterized in an AOP context [20]. AOPs allow to more accurately link such modulating factors both qualitatively and quantitatively to individual KEs or KERs, which paves the way to personalized toxicology and medicine.

Another promising application of AOPs lies with the identification of new diagnostic biomarkers of liver disease. Liver biopsy is currently the most reliable approach for diagnosis and staging of liver disease. Histopathological information can be used to indicate the type and the degree, but not to identify the actual etiology of the injury. The latter can be accomplished by transcriptomic analysis of the sampled liver tissue. A recent AOP-driven study identified a set of genes associated with liver steatogenic chemicals of which the response is conserved across species. Accordingly, this could be used as a transcriptomic signature of chemical-induced liver steatosis [21]. However, liver biopsy is limited by cost, sampling error and procedure-related morbidity and mortality. For this reason, diagnosis of liver disease heavily relies on non-invasive clinical chemistry parameters, yet they frequently lack specificity. The most commonly used clinical chemistry parameter in hepatology is alanine aminotransferase (ALT). Humans express 2 ALT iso-enzymes, of which ALT1 is produced by the kidney, the liver, fat and the heart, while ALT2 is detectable in muscle, fat, the brain and the kidney. Most assays presently used to detect ALT are not able to distinguish between the 2 ALT iso-enzymes. ALT is a relatively sensitive and fairly specific clinical biomarker of hepatotoxicity. This is much less the case for aspartate aminotransferase, which is expressed by the heart, the brain, skeletal muscle and the liver. For the diagnosis of cholestatic injury, alkaline phosphatase and gamma-glutamyltransferase are still among the most often assessed parameters, yet both are not exclusively produced by the liver. More recently, microRNAs have been identified as novel circulating biomarkers of liver disease, which in some cases can be detected before the onset of histopathological changes [22]. However, although the process of microRNA generation as such is well established, the precise link between specific microRNA species and defined liver diseases remains obscure. AOPs can assist in the elucidation of this mechanistic relationship, which in turn may lead to more accurate use and interpretation of microRNAs as diagnostic read-outs. Furthermore, elaboration of existing liver AOPs will unveil novel potential circulating diagnostic, and probably prognostic, biomarkers, which will support precision medicine. It seems likely to assume that the accuracy of diagnosis will greatly increase by combining the established with novel AOP-based biomarkers. This is reminiscent of the current mindset in the toxicology domain, where batteries of *in vitro* assays and associated read-outs, rather than stand-alone methods, are increasingly used to more meticulously predict the hepatotoxic potential of chemicals [10].

Future perspectives: technical optimization and interdisciplinary collaboration

More than 25 different liver AOPs have been introduced today, most of which are in various degrees of maturity [13, 14]. The latter is critical in view of appropriate application. In this context, a recent study showed that the only available AOP on cholestatic liver injury seems fit to model the intrahepatic type of cholestasis, but less for the extrahepatic counterpart [23]. This suggests that more than one AOP is required to mechanistically describe a liver disorder with different etiology. A way to pragmatically tackle this is to generate so-called AOP networks by merging individual AOPs. Such an AOP network has already been proposed for chemical-induced liver steatosis [24]. Another feature that deserves more attention is the inclusion of feedback and feedforward loops as well as of homeostatic adaptation mechanisms in AOPs. This has been initiated for the existing AOP on cholestatic liver injury [13, 14], but is largely lacking for other liver AOPs. Furthermore, efforts should be focused on the quantification of AOP constructs, as the vast majority of current AOPs are merely qualitatively describing adverse effects. Quantification of AOPs typically occurs at the KER level by using dose/response relationships, Bayesian methods or systems biology approaches [10, 25]. Such well-defined and quantified AOP networks are

of paramount importance for reliable use by toxicologists as well as by clinicians.

AOPs offer great opportunities for the hepatology field. This is, however, still in its infancy. A *conditio sine qua non* for further exploration in this regard includes close interaction between (fundamental) toxicologists and (clinical) hepatologists. This has been challenging so far, but could be easily triggered by setting up targeted workshops, which could be supported by organizations such as the European Association for the Study of the Liver (EASL), the American Association for the Study of Liver Diseases (AASLD), the Federation of European Toxicologists and European Societies of Toxicology (EUROTOX) and the Society of Toxicology (SOT). The AOP field could greatly benefit from clinical input in order to optimize overall translational value of AOPs. Besides expert and conceptual advice, clinicians could majorly contribute by providing human liver and serum samples of specific etiologies as well as epidemiological data in order to verify clinical relevance of the liver AOPs. As a matter of fact, this desirable collaboration between toxicologists and hepatologists should ideally be part of a larger interdisciplinary endeavor, including experts from other fields pertinent to AOPs, such as bio-informaticians and bio-engineers. In view of exploiting AOPs to the maximum extent, an intersectoral dialogue should be set-up as well. As holds for the toxicology domain, AOPs have the potential to support future regulatory decision-making in the pharmaceutical field. This will necessitate awareness and proper training of regulators, such as at the European Medicines Agency and the US Food and Drug Administration, to correctly interpret and apply AOP-based information when evaluating dossiers of candidate liver therapeutics. Finally, collaboration between different industries should be strongly encouraged. This is because it has become increasingly clear that chemicals from various sectors can cause hepatotoxic effects and frequently act by similar mechanisms. This certainly is the case for cholestatic liver injury, which can be induced by chemicals from the pharmaceutical, biocide, cosmetics and food industries (Table I). As a consequence, these sectors face identical challenges regarding the early prediction of such adverse effects in the liver. AOPs offer a means to exchange such expertise among disciplines and sectors, which will directly save resources, money, time, efforts and ultimately lives.

Conflicts of interest: None to declare.

REFERENCES

1. Meier Y, Cavallaro M, Roos M, et al. Incidence of drug-induced liver injury in medical inpatients. Eur J Clin. Pharmacol 2005;61:135-143. doi:10.1007/s00228-004-0888-z
2. Goldberg DS, Forde KA, Carbonari DM, et al. Population-representative incidence of drug-induced acute liver failure based on an analysis of an integrated health care system. Gastroenterology 2015;148:1353-1361. e3. doi:10.1053/j.gastro.2015.02.050
3. Bhamidimarri KR, Schiff E. Drug-induced cholestasis. Clin Liver Dis 2013;17:519-531. doi:10.1016/j.cld.2013.07.015
4. Lee WM. Drug-induced acute liver failure. Clin Liver Dis 2013;17:575-586. doi:10.1016/j.cld.2013.07.001
5. Vilas-Boas V, Gijbels E, Cooreman A, et al. Industrial, biocide and cosmetic chemical inducers of cholestasis. Chem Res Toxicol 2019;32:1327-1334. doi:10.1021/acs.chemrestox.9b00148
6. Vilas-Boas V, Gijbels E, Jonckheer J, De Waele E, Vinken M. Cholestatic liver injury induced by food additives, dietary supplements and parenteral nutrition. Environ Int 2020;136:105422. doi:10.1016/j.envint.2019.105422
7. OECD. Available at:https://www.oecd.org/. Accessed: August 2020.
8. National Research Council. Toxicity testing in the 21st century: a vision and a strategy. The National Academies Press (Washington DC): 2007:1-196. doi:10.17226/11970
9. Ankley GT, Bennett RS, Erickson RJ, et al. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem 2010;29:730-741. doi:10.1002/etc.34
10. Vinken M, Knapen D, Vergauwen L, Hengstler JG, Angrish M, Whelan M. Adverse outcome pathways: a concise introduction for toxicologists. Arch Toxicol 2017;91:3697-3707. doi:10.1007/s00204-017-2020-z
11. Vinken M. The adverse outcome pathway concept: a pragmatic tool in toxicology. Toxicology 2013;312:158-165. doi:10.1016/j.tox.2013.08.011
12. Vinken M. Adverse outcome pathways and drug-induced liver injury testing. Chem Res Toxicol 2015;28:1391-1397. doi:10.1021/acs.chemrestox.5b00208
13. Collaborative Adverse Outcome Pathway Wiki (AOP-Wiki). Available at: https://aopwiki.org/. Accessed: August 2020.
14. Gijbels E, Vinken M. An update on adverse outcome pathways leading to liver injury. Appl In Vitro Toxicol 2017;3:283-285. doi:10.1089/aivt.2017.0027
15. Angrish MM, McQueen CA, Cohen-Hubal E, Bruno M, Ge Y, Chorley BN. Editor's highlight: mechanistic toxicity tests based on an adverse outcome pathway network for hepatic steatosis. Toxicol Sci 2017;159:159-169. doi:10.1093/toxsci/kfx121
16. Rooney J, Hill T 3rd, Qin C, Sistare FD, Corton JC. Adverse outcome pathway-driven identification of rat liver tumorigens in short-term assays. Toxicol Appl Pharmacol 2018;356:99-113. doi:10.1016/j.taap.2018.07.023
17. Willebrords J, Pereira IV, Maes M, et al. Strategies, models and biomarkers in experimental non-alcoholic fatty liver disease research. Prog Lipid Res 2015;59:106-125. doi:10.1016/j.plipres.2015.05.002
18. Yanguas SC, Cogliati B, Willebrords J, et al. Experimental models of liver fibrosis. Arch Toxicol 2016;90:1025-1048. doi:10.1007/s00204-015-1543-4
19. Seok J, Warren HS, Cuenca AG, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A 2013;110:3507-3512. doi:10.1073/pnas.1222878110
20. Wahlang B, Jin J, Beier JI, Hardesty JE, et al. Mechanisms of environmental contributions to fatty liver disease. Curr Environ Health Rep 2019;6:80-94. doi:10.1007/s40572-019-00232-w
21. AbdulHameed MDM, Pannala VR, Wallqvist A. Mining public toxicogenomic data reveals insights and challenges in delineating liver steatosis adverse outcome pathways. Front Genet 2019;10:1007. doi:10.3389/fgene.2019.01007
22. Vinken M, Maes M, Vanhaecke T, Rogiers V. Drug-induced liver injury: mechanisms types and biomarkers. Curr Med Chem 2013;20:3011-3021. doi:10.2174/0929867311320240006

23. Gijbels E, Vilas-Boas V, Annaert P, Vanhaecke T, Devisscher L, Vinken M. Robustness testing and optimization of an adverse outcome pathway on cholestatic liver injury. Arch Toxicol 2020;94:1151-1172. doi:10.1007/s00204-020-02691-9
24. Mellor CL, Steinmetz FP, Cronin MT. The identification of nuclear receptors associated with hepatic steatosis to develop and extend adverse outcome pathways. Crit Rev Toxicol 2016;46:138-152. doi:10.3109/10408444.2015.1089471
25. Knapen D, Angrish MM, Fortin MC, et al. Adverse outcome pathway networks I: development and applications. Environ. Toxicol Chem 2018;37:1723-1733. doi:10.1002/etc.4125