Development of an Adverse Outcome Pathway From Drug-Mediated Bile Salt Export Pump Inhibition to Cholestatic Liver Injury

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Adverse outcome pathways (AOPs) have been recently introduced in human risk assessment as pragmatic tools with multiple applications. As such, AOPs intend to provide a clear-cut mechanistic representation of pertinent toxicological effects. AOPs are typically composed of a molecular initiating event, a series of intermediate steps and key events, and an adverse outcome. In this study, an AOP framework is proposed for cholestatic triggered by drug-mediated inhibition of the bile salt export pump transporter protein. For this purpose, an in-depth survey of relevant scientific literature was carried out in order to identify intermediate steps and key events. The latter include bile accumulation, the induction of oxidative stress and inflammation, and the activation of specific nuclear receptors. Collectively, these mechanisms drive both a deteriorative cellular response, which underlies directly caused cholestatic injury, and an adaptive cellular response, which is aimed at counteracting cholestatic insults. AOP development was performed according to Organisation for Economic Co-operation and Development (OECD) guidance, including critical consideration of the Bradford Hill criteria for weight of evidence assessment and the OECD key questions for evaluating AOP confidence. The postulated AOP is expected to serve as the basis for the development of new in vitro tests and the characterization of novel biomarkers of drug-induced cholestasis.

Key Words: drug-induced liver injury; cholestasis; adverse outcome pathway.

The fields of toxicology and risk assessment have witnessed a true paradigm shift in the last decade. Indeed, the tendency now is to rely on mechanistic data in the way forward to predictive toxicology. A first step in this direction came with the establishment of the mode of action concept, defined as a series of key events along a biological pathway from the initial chemical interaction to the adverse outcome (AO; OECD, 2012a). This mode of action concept was initially used by the U.S. Environmental Protection Agency in the cancer field (US EPA, 2005), but was also exploited for noncancer points as well (Bogdanfly et al., 2001; Julien et al., 2009; Meek et al., 2003; Seed et al., 2005). In 2007, the U.S. National Academy of Science issued their vision on toxicology in the 21st century, a report in which toxicity pathways play prominent roles (NRC, 2007). The latter, described as cellular pathways that, when disturbed, can lead to adverse health effects (OECD, 2012a), align with so-called adverse outcome pathways (AOPs), which originate from the area of ecotoxicology. Among the many proposed definitions, an AOP refers to a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event (MIE) and an AO at a biological level of organization relevant to risk assessment (Ankley et al., 2010; OECD, 2012a). In the last few years, the AOP concept has been successfully applied to a number of human-relevant toxicological endpoints, including phototoxicity, aryl hydrocarbon receptor-mediated toxicity (Ankley et al., 2010), estrogen receptor-mediated reproductive toxicity, voltage-gated sodium channel-mediated neural toxicity, hemolytic anemia induced by anilines, nephrotoxicity induced by 4-aminophenols (OECD, 2011), skin sensitization (OECD, 2012b), liver steatosis, and fibrosis (Landesmann et al., 2012). Recently, the OECD has anticipated the introduction of this tool into human risk assessment by publishing a draft guidance document for the development and assessment of the completeness of AOPs (OECD, 2012a).

Cholestatic insults are among the most severe clinical manifestations of drug-induced liver injury (DILI, Padda et al., 2011). From the epidemiologic point of view, cholestatic DILI may account for up to half of the cases of hepatic drug toxicity with
high mortality rates (Björnsson and Olsson, 2005). Cholestatic DILI can mimic either intrahepatic cholestasis or extrahepatic cholestasis and may be expressed as acute or chronic liver disease. Depending on the type (ie, occurring with or without hepatitis or bile duct injury), cholestatic DILI is clinically featured as jaundice, pruritus, hyperbilirubinemia, elevations in serum alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), 5’-nucleotidase (5’-NT), aspartate aminotransferase (AST), and alanine aminotransferase (ALT; Padda et al., 2011). At the molecular level, cholestatic DILI mainly results from the inhibition of the expression and function of transporter proteins located in the canalicular membrane area of hepatocytes, in particular the bile salt export pump (BSEP; Kis et al., 2012; Padda et al., 2011; Wagner et al., 2009; Zollner and Trauner, 2006, 2008). The resulting bile accumulation is paralleled by a series of transcriptionally regulated adaptive mechanisms that counteract the primary cholestatic insult (Wagner et al., 2009; Zollner and Trauner, 2006, 2008). The goal of this study was to set up an AOP framework for cholestatic DILI, in casu resulting from BSEP inhibition, which encompasses the most relevant molecular mechanisms. This initial framework includes qualitative information regarding key events in the pathway and, in keeping with the iterative and cumulative nature of AOP development, can be further supplemented by the addition of more rigorous causal and quantitative information.

MATERIALS AND METHODS

**AOP development.** By definition, an AOP shall have a single starting point, designated the MIE, and only one apical endpoint, called the AO (OECD, 2012a). In this study, BSEP inhibition was selected as the MIE, whereas the obvious AO is cholestasis. Identification of the response matrix between the MIE and the AO, composed of intermediate steps and key events, was achieved by an in-depth survey of appropriate scientific literature, using PubMed as the main resource. Experts in the area of fundamental and clinical cholestasis research were consulted in parallel. During this exercise, intermediate steps and key events were causally interlinked and analyzed according to the different levels of biological organization. Following OECD nomenclature, key events are a specific subset of intermediate steps that are toxicologically relevant to the AO and that are experimentally quantifiable (OECD, 2012a).

**AOP representation.** The most straightforward way of data summation is through graphic representation of the MIE, the intermediate steps, the key events, and the AO in a linear flow diagram (Ankley et al., 2010; OECD, 2012a). This graphical version of the AOP allows visualization of the sequence of events at the different biological levels of organization (OECD, 2012a).

**AOP evaluation.** According to the OECD guidelines (OECD, 2012a), the first step in the evaluation of newly established AOPs is the implementation of the Bradford Hill criteria for weight of evidence assessment (Hill, 1965; OECD, 2012a, Table 1). Furthermore, the OECD has defined a number of key questions to test overall confidence in de novo generated AOPs (Table 2).

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<tr>
<th>Table 1</th>
<th>Bradford Hill Criteria for AOP Weight of Evidence Assessment (Hill, 1965; OECD, 2012a)</th>
</tr>
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<tbody>
<tr>
<td>2.</td>
<td>Temporal concordance among the key events and adverse outcome.</td>
</tr>
<tr>
<td>3.</td>
<td>Strength, consistency, and specificity of association of the adverse outcome and the molecular initiating event.</td>
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<tr>
<td>4.</td>
<td>Biological plausibility, coherence, and consistency of the experimental evidence.</td>
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<tr>
<td>5.</td>
<td>Alternative mechanisms that logically present themselves and the extent to which they may distract from the postulated AOP.</td>
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<tr>
<td>6.</td>
<td>Uncertainties, inconsistencies, and data gaps.</td>
</tr>
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Abbreviation: AOP, adverse outcome pathway.

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<th>Table 2</th>
<th>Key Questions for Testing AOP Confidence (OECD, 2012a)</th>
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<tbody>
<tr>
<td>1.</td>
<td>How well characterized is the AOP?</td>
</tr>
<tr>
<td>2.</td>
<td>How well are the initiating and other key events causally linked to the outcome?</td>
</tr>
<tr>
<td>3.</td>
<td>What are the limitations in the evidence in support of the AOP?</td>
</tr>
<tr>
<td>4.</td>
<td>Is the AOP specific to certain tissues, life stages, or age classes?</td>
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<tr>
<td>5.</td>
<td>Are the initiating and key events expected to be conserved across taxa?</td>
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RESULTS

**AOP Development and Representation**

The established AOP is presented in a regular linear flow diagram (Fig. 1). The intermediate steps and key events between the MIE (ie, BSEP inhibition) and the AO (ie, cholestasis) are located at the cellular and organ levels. The scientific basis of the intermediate steps and key events, including their interrelationship, is outlined hereafter.

The BSEP transporter protein is a prominent adenosine triphosphate-binding cassette transporter located at the canalicular pole of the hepatocyte membrane, which conveys bile acids from the hepatocyte cytosol into the bile canaliculi (Kis et al., 2012). BSEP can be directly cis-inhibited in a competitive way by several cholestasis-inducing drugs, including cyclosporine A, rifampicin, erythromycin estolate, 17α-ethinyl estradiol, bosentan, troglitazone, and glibenclamide (Dawson et al., 2011; Kis et al., 2012; Morgan et al., 2010; Padda et al., 2011; Wagner et al., 2009; Zollner and Trauner, 2006, 2008). As a result of BSEP inhibition, toxic bile acids, such as glychenodeoxycholic acid, accumulate into the cytosol of the hepatocytes or bile canaliculi into so-called bile plugs (Kuntz and Kuntz, 2008). Obviously, bile salt accumulation is a key event in the process of drug-induced cholestasis. These bile salts trigger the formation of the mitochondrial permeability pore, which leads to mitochondrial impairment and the production of reactive oxygen species (Botla et al., 1995; Gores et al., 1998; Sokol et al., 2005; Woolbright and Jaeschke, 2012). At the same time, hepatocytes start to express chemotactic factors, such as macrophage inflammatory protein-2, and consequently, neutrophils are recruited (Allen et al., 2011; Woolbright and Jaeschke, 2012; Zhang et al., 2012). The latter cause an inflammatory response and oxidative stress in hepatocytes (Gujral et al., 2003, 2004; Jaeschke and Hasegawa, 2006; Woolbright and Jaeschke,
AOP ON CHOLESTATIC DILI

which can be considered as key events that drive the direct deteriorative response to BSEP inhibition. Indeed, these events burgeon into the onset of cell death, which mainly is of necrotic nature (Woolbright and Jaeschke, 2012), although apoptotic mechanisms could also be involved (Botla et al., 1995; Gores et al., 1998; Schoemaker et al., 2004). This typically results from bile extravasating into liver parenchyma and pooling into highly concentrated areas referred to as bile lakes (Kuntz and Kuntz, 2008). As a result of membrane damage, hepatocytes release cytosolic enzymes, such as ALT and AST, which become increasingly measurable in the serum. A similar process takes place in cholangiocytes, whereby ALP, GGT, and 5’-NT as well as bilirubin leak into the serum (Hofmann, 2009; Kuntz and Kuntz, 2008; Padda et al., 2011).

A remarkable hallmark of cholestasis at the cellular level includes the induction of an adaptive response, which is aimed at counteracting bile accumulation and thus cholestatic liver injury. Accordingly, a complex machinery of transcriptionally coordinated mechanisms is activated by bile acids, which collectively decrease the uptake and increase the export of bile acids into and from hepatocytes, respectively. Simultaneously, detoxification of bile acids is enhanced, whereas their synthesis becomes downregulated. In addition, renal elimination of bile acids is strongly promoted (Boyer, 2009; Wagner et al., 2009;
Zollner and Trauner, 2006, 2008). These compensatory processes are mediated by a set of bile acid-activated nuclear receptors, in particular the farnesoid X receptor (FXR), the pregnane X receptor (PXR), the vitamin D receptor, and the constitutive androstane receptor (CAR, Wagner et al., 2009; Zollner and Trauner, 2006, 2008). Specifically, FXR directly activates the expression of the organic solute transporters α and β (OSTα/β), which are located at the basolateral membrane of hepatocytes and that provide an alternative route for the elimination of bile acids and bilirubin from cholestatic hepatocytes (Boyer et al., 2006; Boyer, 2009; Wagner et al., 2009; Zollner and Trauner, 2008). FXR also induces the production of the phase I biotransformation enzymes cytochrome P450 3A4 (CYP3A4, Boyer, 2009; Gnerre et al., 2004) and CYP2B10 (Boyer, 2009; Zollner et al., 2006b), the phase II bio-transformation enzyme uridine 5′-diphosphate-glucuronosyltransferase 2B4 (Barbier et al., 2003; Boyer, 2009), the multidrug resistance-associated protein 2 (MRP2, Boyer, 2009; Kast et al., 2002), as well as the expression of PXR (Boyer, 2009; Jung et al., 2006). Furthermore, FXR indirectly represses a number of genes through induction of a common gene silencer called small heterodimeric partner, including those encoding the sinusoidal bile acid uptake transporters sodium taurocholate cotransporting polypeptide (NTCP, Boyer, 2009; Denson et al., 2001; Wagner et al., 2009) and organic anion transporter 1B1 (Boyer, 2009; Jung et al., 2007; Wagner et al., 2009), and CYP7A1, which regulates the rate-limiting step of bile acid production (Boyer, 2009; Goodwin et al., 2000; Wagner et al., 2009). In addition to CYP3A4 (Boyer, 2009; Faucette et al., 2006), both PXR and CAR enhance the expression of dehydroepiandrosterone sulfo-transferase (SULT2A1), which catalyzes the sulfo-conjugation of bile acids (Boyer, 2009; Echchgadda et al., 2007). Also, PXR and CAR induce the production of MRP3 (Boyer, 2009; Maher et al., 2005). It is hence clear that activation of nuclear receptors, including FXR, PXR, and CAR, is indispensable for the induction of the adaptive response and therefore must be envisaged as a key event in the AOP. This key event also explains part of the clinical symptomatology of drug-induced cholestasis. Thus, the increased effort of cholestatic hepatocytes to remove bilirubin causes bilirubinuria and hyperbilirubinemia. As a result, a yellowish pigmentation of the skin and the conjunctival membranes over the sclera become visible, known as jaundice or icterus. Furthermore, although debated, the elevated presence of bile acids in the serum is thought to account for the typical skin itching (ie, pruritus) in cholestasis patients (Hofmann, 2009; Kuntz and Kuntz, 2008; Padda et al., 2011; Zollner and Trauner, 2008).

In addition to BSEP inhibition, other mechanisms by which drugs cause cholestatic DILI include disruption of tight junctions, deterioration of cytoarchitecture, alteration of membrane fluidity, and downregulation of vesicular targeting of transporter proteins to the membrane surface (Padda et al., 2011; Zollner and Trauner, 2008). In fact, these mechanisms can also be triggered by a number of intermediate steps, such as the oxidative stress and inflammatory processes, and may subsequently amplify bile accumulation induced by BSEP inhibition. Inflammatory stimuli indeed are known to negatively affect hepatic membrane fluidity (Salgia et al., 1993), tight junction expression and functionality (Mazzon and Cuzzocrea, 2003), cytoskeletal integrity (Pagani et al., 2003), and drug transporter localization (Sekine et al., 2010). Furthermore, pro-inflammatory cytokines are powerful downregulators of several cytochrome P450 enzymes and biliary transporters, which as such can be considered as a second critical hit that further aggravates the primary cholestatic injury (Padda et al., 2011). Thus, lipopolysaccharide was reported to suppress the expression of hepatic CYP3A4 (Gu et al., 2006), CYP2B10 (Li-Masters and Morgan, 2001), SULT2A1 (Kim et al., 2004), and MRP2 (Cherrington et al., 2004). On the other hand, lipopolysaccharide downregulates hepatic production of NTCP (Trauner et al., 1998a) and CYP7A1 (Feingold et al., 1996), whereas it promotes production of MRP3 (Cherrington et al., 2004), which may contribute to the adaptive response of the liver to the cholestatic insults.

**AOP Weight of Evidence Assessment According to the Bradford Hill Criteria**

**Concordance of dose-response relationships.** Morgan and colleagues investigated the potential of more than 200 benchmark drugs to inhibit BSEP. As much as 16% of the tested drugs displayed high potency of BSEP inhibition (IC₅₀ ≤ 25μM), the majority of which are associated with liver liabilities in humans (Morgan et al., 2010). Likewise, 17 of 85 pharmaceuticals tested by Dawson and coworkers inhibited BSEP (IC₅₀ ≤ 100μM), all of which are known to cause DILI (Dawson et al., 2011). Furthermore, several of the BSEP-inhibiting drugs cause cholestatic liver injury in a dose-dependent way, such as is the case for troglitazone and bosentan in rats (Funk et al., 2001) and humans (Fattinger et al., 2001), respectively. Thus, there is a clear relationship between the IC₅₀ of BSEP inhibition and the occurrence of (cholestatic) DILI.

**Temporal concordance among the key events and AO.** Inhibition of BSEP activity and the resulting accumulation of bile acids primarily trigger a direct cellular response, which is associated with deteriorative processes, such as inflammation, oxidative stress, and cell death. It also causes a secondary and rather indirect cellular response, which is adaptive in nature (Fig. 1). Indeed, a well-orchestrated network of mechanisms is activated, all of which are targeted toward the elimination of bile from the liver (Boyer, 2009; Wagner et al., 2009; Zollner and Trauner, 2006, 2008). The temporal concordance between these cellular responses is not clear. It is, however, conceivable to assume that the adaptive response becomes manifested at a somewhat later stage when compared with the primary events, especially because this secondary response highly depends on transcriptional regulation. Nonetheless, it is clear that the graphical linear representation of cholestatic DILI
resulting from BSEP inhibition as a sequence of events (Fig. 1) is an oversimplification of a probably very complex network of entangled consecutive and parallel reactions.

**Strength, consistency, and specificity of association of the AO and the MIE.** BSEP is considered as the major apical transporter protein that pumps bile salts from hepatocytes into bile canaliculi. As a part of this pivotal task, BSEP has a very narrow substrate specificity with only a few known nonbile substrates (Dawson et al., 2011; Kis et al., 2012; Morgan et al., 2010). Defects in BSEP expression or function, therefore, can be anticipated to have drastic consequences with respect to bile homeostasis. Indeed, a plethora of studies has demonstrated that BSEP inhibition or impairment is probably causally linked to the induction of cholestasis in a dose-dependent way, both in experimental animals and in humans (Wagner et al., 2009; Zollner and Trauner, 2006, 2008). Thus, it is very likely that there is a direct and quantitative association between BSEP inhibition and the onset of cholestatic DILI. Additional evidence in this direction comes from progressive familial intrahepatic cholestasis type 2 patients who have been causally linked to BSEP deficiency (Morotti et al., 2011). Furthermore, autoantibodies against BSEP have been observed in progressive familial intrahepatic cholestasis type 2 patients who underwent liver transplantation (Jara et al., 2009; Keitel et al., 2009). These examples show causality due to pharmacological inhibition of BSEP, albeit not via a drug, but by means of antibodies.

**Biological plausibility, coherence, and consistency of the experimental evidence.** The rationale, coherence, and consistency along with the experimental data that support the proposed AOP are discussed in detail under AOP Development and Representation. In essence, BSEP inhibition (ie, the MIE) activates a number of mechanisms that drive a deteriorative cellular response, which underlies directly caused cholestatic injury, as well as an adaptive cellular response, which is aimed at countering cholestatic insults. Both these responses contribute to the clinical manifestation of cholestasis (ie, the AO). Serum concentrations of ALT, AST, ALP, GGT, and 5'-NT indeed increase because of bile acid-induced membrane damage of hepatocytes and cholangiocytes (Hofmann, 2009; Kuntz and Kuntz, 2008; Padda et al., 2011). At the same time, elevated concentrations of bilirubin in serum and urine are observed, reflecting the compensatory response of the organism to counteract bile acid accumulation. Hyperbilirubinemia causes jaundice, whereas the increased presence of bile acids in the serum is thought to induce pruritus (Hofmann, 2009; Kuntz and Kuntz, 2008; Padda et al., 2011; Zollner and Trauner, 2008).

**Alternative mechanisms that logically present themselves and the extent to which they may distract from the postulated AOP.** Although predominant, BSEP inhibition is not the sole MIE in cholestatic DILI, as depicted in the established AOP (Fig. 1). In this regard, cyclosporine A not only inhibits BSEP (Dawson et al., 2011; Kis et al., 2012; Morgan et al., 2010) but also induces cholestasis by inhibition of intrahepatic vesicle transport (Román et al., 1990) and by affecting canalicular membrane fluidity (Yasumiba et al., 2001). Several of these events, in particular the cytoskeletal changes, might be considered as secondary and nonspecific phenomena (Trauner et al., 1998b). Other drugs clearly evoke cholestasis through non-BSEP-related mechanisms. This is the case for zonisamide, which causes destruction and disappearance of bile ducts (Vuppalanchi et al., 2006), and itraconazole, which inhibits multidrug resistance P-glycoprotein 3 (Yoshikado et al., 2011). Furthermore, MRP4 inhibition has also been linked to cholestatic injury (Yang et al., 2013). Separate AOPs could be drafted for each of these alternative mechanisms in cholestatic DILI.

**Uncertainties, inconsistencies, and data gaps.** Although a clear causal and dose-dependent relationship has been established between BSEP inhibition and the clinical onset of cholestasis (Wagner et al., 2009; Zollner and Trauner, 2006, 2008), several mechanisms of the intermediate steps and key events as well as their linkage are not fully understood. A prominent discussion in this respect relates to the nature of the cell death mode, namely apoptosis or necrosis, associated with cholestasis (Woolbright and Jaeschke, 2012). High concentrations of hydrophobic bile acids induce apoptotic cell death in cultures of primary hepatocytes (Botla et al., 1995; Gores et al., 1998; Schoemaker et al., 2004), yet such concentrations are not achieved in vivo (Zhang et al., 2012). It has, therefore, been suggested that the main mechanism of cell death in cholestasis in vivo is necrosis (Woolbright and Jaeschke, 2012). In fact, this seems to be a general consideration of the AOP, as several other constituting data also have been derived from in vitro experimentation and need to be substantiated in vivo. On the other hand, a number of data are still lacking, including the full identification of FXR, PXR, and CAR target genes, which may additionally contribute to the adaptive response to BSEP inhibition. Along the same line, alternative nuclear receptors, such as peroxisome proliferator-activated receptor α (Li et al., 2012; Zollnet et al., 2006a, 2010), involved in bile acid homeostasis will need to be included in the AOP. Furthermore, ongoing research regarding the regulation of these nuclear receptors in cholestatic DILI might add complexity to the AOP. It is known that they act, at least in part, by recruiting coactivators and corepressors (Gollamudi et al., 2008; Wagner et al., 2009). Moreover, compelling evidence suggests that nuclear receptors are regulated epigenetically, which might necessitate inclusion in the AOP (Eloranta and Kullak-Ublick, 2005; Wagner et al., 2009). Additional uncertainties, inconsistencies, and data gaps associated with the established AOP relate to the temporal concordance of the intermediate steps and key events, the consistency of the available experimental data, alternative mechanisms involved, interspecies and intraspecies differences. These issues are addressed in the respective sections.
under AOP Weight of Evidence Assessment According to the Bradford Hill Criteria and AOP Confidence Testing According to the OECD Key Questions.

AOP Confidence Testing According to the OECD Key Questions

How well characterized is the AOP? Drug-induced cholestasis is a well understood AO that is likely to be causally and dose-dependently linked to BSEP inhibition (Dawson et al., 2011; Kis et al., 2012; Morgan et al., 2010). Furthermore, the critical role of the key events, namely the accumulation of bile, the induction of inflammation and oxidative stress, and the activation of specific nuclear receptors, as well as the different intermediate steps in the AOP (Fig. 1), is supported by a wealth of experimental data, as described in detail under AOP Development and Representation. Thus, despite a number of limitations in scientific evidence, as will be discussed further, the established general structure and components of the AOP can be considered as being well characterized.

How well are the initiating and other key events causally linked to the outcome? It has been demonstrated on numerous occasions that BSEP inhibition is probably causally linked to the induction of cholestasis in a dose-dependent way, both in experimental animals and in humans (Wagner et al., 2009; Zollner and Trauner, 2006, 2008). BSEP inhibition directly leads to bile accumulation, which subsequently activates an inflammatory reaction as well as the occurrence of oxidative stress (Gujral et al., 2003, 2004; Jaeschke and Hasegawa, 2006; Woolbright and Jaeschke, 2012). The resulting cell death and associated bile acid-induced membrane damage of hepatocytes and cholangiocytes underlie the increased serum concentrations of ALT, AST, ALP, GGT, and 5'-NT, being a prominent clinical hallmark of cholestasis (Hofmann, 2009; Kuntz and Kuntz, 2008; Padda et al., 2011). To compensate for the cholestatic insults, an adaptive response is induced, which is initiated by nuclear receptor activation and that is targeted toward the elimination of bile from the organism (Boyer, 2009; Wagner et al., 2009; Zollner and Trauner, 2006, 2008). Consequently, elevated concentrations of bilirubin in the serum and urine are observed. The former causes jaundice, whereas the increased presence of bile acids in serum is thought to induce pruritus (Hofmann, 2009; Kuntz and Kuntz, 2008; Padda et al., 2011; Zollner and Trauner, 2008). Thus, it is likely that there is a direct, and, at least in some cases, quantitative association between the MIE, the key events, and the AO in the established AOP. Additional evidence in this direction comes from progressive familial intrahepatic cholestasis type 2 patients who have been causally linked to BSEP deficiency (Morotti et al., 2011). Furthermore, autoantibodies against BSEP have been observed in progressive familial intrahepatic cholestasis type 2 patients who underwent liver transplantation (Jara et al., 2009; Keitel et al., 2009). These examples show causality due to pharmacological inhibition of BSEP, albeit not via a drug, but by means of antibodies.

What are the limitations in the evidence in support of the AOP? There are a number of limitations in the scientific evidence in support of the AOP in relation to temporal concordance of the intermediate steps and key events, the consistency of the available experimental data, alternative mechanisms involved, interspecies and intraindividual differences, uncertainties, inconsistencies, and data gaps. These shortcomings are addressed in the respective sections under AOP Weight of Evidence Assessment According to the Bradford Hill Criteria and AOP Confidence Testing According to the OECD Key Questions.

Is the AOP specific to certain tissues, life stages, or age classes? Although the entire process of drug-induced cholestasis mainly takes place in the liver, more specifically in hepatocytes, the adaptive changes to this insult also occur in other tissues, including the kidney and the intestine. In this context, expression of MRP2 is induced in renal tubular cells in experimental models of cholestasis (Lee et al., 2001). Alterations in transporter protein expression during cholestasis also occur in other tissues, such as the ileum (Mennone et al., 2010). Like in the liver, the overall goal of these alterations is to increase elimination of bile salts via the urine and feces (Wagner et al., 2009; Zollner and Trauner, 2006, 2008). Regarding age specificity, no significant quantitative differences in BSEP expression between fetal and human liver have been detected (Chen et al., 2005). Hepatocellular accumulation of bile acids causes giant cell hepatitis and progressive liver damage in children (Oude Elferink et al., 2006), which may burgeon into hepatocellular carcinoma (Bernstein et al., 2005; Knisely et al., 2006). On the other hand, it is well established that age over 50 years poses an increased risk to develop drug-induced hepatic damage (Pauli-Magnus and Meier, 2006) and that DILI in elder people is of cholestatic rather than of hepatocellular nature (Lucena et al., 2009). In general, women are more susceptible for developing DILI than men (Pauli-Magnus and Meier, 2006), yet no gender differences exist in liver BSEP expression in humans (Cheng et al., 2007). In contrast, male rats are more prone to troglitazone-induced cholestasis than female rats because of higher rates of troglitazone sulfate formation (Funk et al., 2001; Kostrubsky et al., 2001). At the population level, genetic variability in the BSEP gene, leading to its decreased expression, may predispose different ethnic populations to drug-induced cholestasis (Lang et al., 2006, 2007; Meier et al., 2006).

Are the initiating and key events expected to be conserved across taxa? Standard animal studies conducted during drug development, using mainly rodents, usually pick up about half of all human hepatotoxic compounds because of interspecies differences (Blomme et al., 2009; Ozer et al., 2008). In the case of BSEP inhibition, however, interspecies differences are mostly of quantitative nature. This could be due to the fact that there is a high amino acid similarity between human BSEP and its rodent counterparts, namely 80% in mouse and 82% in rat (Green et al., 2000; LeCureur et al., 2000). Accordingly, although IC50 values...
for BSEP inhibition differ only minimally between human and mouse for troglitazone, they differ by almost an order of magnitude for glibenclamide (Kis et al., 2012). Other reasons for the absence of hepatotoxicity induced by human-relevant cholestatic drugs in rats include higher rates of basolateral bile salt efflux, which could represent an additional protective mechanism against cholestasis (Jemmnez et al., 2010). Furthermore, the human bile acid pool is more hydrophobic than that of rat (Heuman, 1989). In addition to BSEP inhibition, the key events of the proposed AOP are expected to be generally well conserved among taxa. Nevertheless, a recent report showed that considerable differences exist in inflammatory responses between human and mouse (Seok et al., 2013). Despite the occurrence of interspecies differences in their expression or ligand binding, such as shown for PXR (Krasowski et al., 2005), activation of nuclear receptors is a critical event in different animal models of cholestasis (Wagner et al., 2009; Zollner and Trauner, 2006, 2008). It remains to be established whether data included in the AOP can be extrapolated from animals to humans and vice versa.

**DISCUSSION**

In recent years, AOPs have found their way to human risk assessment as pragmatic tools with multiple uses. AOPs can indeed serve variable purposes, including the establishment of chemical categories, the advancement of integrated testing strategies, the development of new ex vivo and in vitro assays, as well as the identification of novel biomarkers (OECD, 2012a). With respect to the latter, a Safety Evaluation Ultimately Replacing Animal Testing (SEURAT) research initiative was set up in 2011 between the European Commission and Cosmetics Europe, representing the European cosmetics industry, which intends to develop an in vitro strategy to replace repeated dose toxicity testing in experimental animals. Regarding the hepatotoxicity research in SEURAT, focus is put on liver steatosis, liver fibrosis, and cholestasis (http://www.seurat-1.eu/; Vinken et al., 2012). AOP proposals for the former two types of DILI have been established (Landesmann et al., 2012) and will be used as the basis for in vitro test development and biomarker characterization within the SEURAT consortium. The aim of this study was to repeat this exercise for drug-induced cholestasis, a prominent form of DILI (Björnsson and Olsson, 2005; Padda et al., 2011). It should be stressed, however, that the relevance and potential of this newly established AOP is much broader, as several efforts are currently ongoing worldwide in the field of AOP development, including at the OECD level (OECD, 2012a), the U.S. Hamner Institutes of Health (Andersen et al., 2012, 2013), and the U.S. Center for Alternatives to Animal Testing (http://caat.jhsph.edu/).

Because inhibition of BSEP has been shown to underlie cholestatic injury induced by a plethora of drugs (Dawson et al., 2011; Kis et al., 2012; Morgan et al., 2010), it was considered as the most prominent MIE in the current AOP. The matrix of events between the MIE and the AO consisted of a series of processes that are part of a primary response, the suite of directly caused deteriorative processes, and a secondary response, the indirectly triggered cellular adaptation and counteraction to BSEP inhibition. Among the various mechanisms involved, and as supported by a substantial body of scientific evidence (Boyer, 2009; Gujral et al., 2003, 2004; Jaeschke and Hasegawa, 2006; Kuntz and Kuntz, 2008; Padda et al., 2011; Wagner et al., 2009; Woolbright and Jaeschke, 2012; Zollner and Trauner, 2006, 2008), the accumulation of bile, the induction of oxidative stress and inflammation, and the activation of the nuclear receptors PXR, FXR, and CAR can be envisaged as key events in the AOP. In line with the OECD definition (OECD, 2012a), these key events indeed are indispensable for achieving the AO and all are experimentally measurable through a number of established methods, including the testing of nuclear receptor activation in reporter genes constructs (Kocarek et al., 2002). Likewise, elegant in vitro assays for measuring BSEP inhibition have been developed and successfully applied to demonstrate the cholestasis-inducing potential of drugs (Dawson et al., 2011; Kis et al., 2012; Morgan et al., 2010).

Although useful and based upon currently available scientific data, the postulated AOP should be interpreted and applied with caution, part of which became evident while meeting the Bradford Hill criteria for weight of evidence assessment as well as upon answering the OECD key questions for evaluating AOP confidence. Thus, it might only be applicable to cholestatic DILI resulting from BSEP inhibition and not from other causes whether or not induced by drugs. Furthermore, it is realized that the AOP in its current form is an oversimplification of a complex pathological process, which is incomplete from a number of angles. It must be considered as an open and flexible framework that will need continuous refinement. Research efforts, such as those of the SEURAT project cluster (http://www.seurat-1.eu/; Vinken et al., 2012), are expected to produce results that can be fed into the AOP in an iterative way, which can either reinforce the relevance of existing intermediate steps and key events or that enable identification of additional and yet unexplored ones. The latter is anticipated to open new perspectives for in vitro test development and biomarker characterization. In turn, this will contribute to the reduction and replacement of animal testing in the safety evaluation of chemical compounds, which complies with current European legislation, especially in the area of cosmetic products and their ingredients (EU, 2003; Pauwels and Rogiers, 2010).

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