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# The adverse outcome pathway for rodent liver tumor promotion by sustained activation of the aryl hydrocarbon receptor

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ABSTRACT

An Adverse Outcome Pathway (AOP) represents the existing knowledge of a biological pathway leading from initial molecular interactions of a toxicant and progressing through a series of key events (KEs), culminating with an apical adverse outcome (AO) that has to be of regulatory relevance. An AOP based on the mode of action (MOA) of rodent liver tumor promotion by dioxin-like compounds (DLCs) has been developed and the weight of evidence (WoE) of key event relationships (KERs) evaluated using evolved Bradford Hill considerations. Dioxins and DLCs are potent aryl hydrocarbon receptor (AHR) ligands that cause a range of species-specific adverse outcomes. The occurrence of KEs is necessary for inducing downstream biological responses and KEs may occur at the molecular, cellular, tissue and organ levels. The common convention is that an AOP begins with the toxicant interaction with a biological response element; for this AOP, this initial event is binding of a DLC ligand to the AHR. Data from mechanistic studies, lifetime bioassays and approximately thirty initiation-promotion studies have established dioxin and DLCs as rat liver tumor promoters. Such studies clearly show that sustained AHR activation, weeks or months in duration, is necessary to induce rodent liver tumor promotion – hence, sustained AHR activation is deemed the molecular initiating event (MIE). After this MIE, subsequent KEs are 1) changes in cellular growth homeostasis likely associated with expression changes in a number of genes and observed as development of hepatic foci and decreases in apoptosis within foci; 2) extensive liver toxicity observed as the constellation of effects called toxic hepatopathy; 3) cellular proliferation and hyperplasia in several hepatic cell types. This progression of KEs culminates in the AO, the development of hepatocellular adenomas and carcinomas and cholangiolar carcinomas. A rich data set provides both qualitative and quantitative knowledge of the progression of this AOP through KEs and the KERs. Thus, the WoE for this AOP is judged to be strong. Species-specific effects of dioxins and DLCs are well known – humans are less responsive than rodents and rodent species differ in sensitivity between strains. Consequently, application of this AOP to evaluate potential human health risks must take these differences into account.



## 1. Introduction

The source-to-outcome pathway sequence consists of release, transport, contact, absorption, dose, molecular interactions, cellular responses, tissue and organ changes, culminating in an adverse effect (Sobus et al., 2011). An adverse outcome pathway (AOP) (Ankley et al., 2010) is a subset of the source to outcome sequence. An AOP, by convention, starts at the molecular interaction step, in which a xenobiotic chemical moiety interacts with biological molecule and proceeds to an adverse outcome (AO) via a series of Key Events (KEs). The sequence of the hierarchical AOP framework (see SF1 in Supplemental Material), and guidance on the development and evaluation of AOPs has been developed (OECD, 2013; OECD, 2014). Ideally, an AOP will be applicable to a broad

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spectrum of chemicals that have the potential to operate via a common pathway or to have KEs in common. However, the OECD AOP program (OECD, 2013) permits submission of an AOP case study applicable to a single chemical, or a very limited number of chemicals, recognizing that, data permitting, such an AOP may be expanded in the future to cover a category of chemicals.

The molecular initiating event (MIE) is a fundamental concept of all AOPs (Ankley et al., 2010). The MIE has been operationally defined as the first key event (KE) and necessary but not sufficient to produce the AO. Other KEs within a Mode of Action (MOA) are defined similarly—necessary but not sufficient for the AO to occur (Julien et al., 2009; Simon et al., 2014; USEPA, 2005). Although it can be argued that initial molecular interactions such as absorption or parent compound interaction with an enzyme that leads to production of a toxicologically-active moiety meet the definition of an MIE, these events are considered to be upstream of an MIE. The MIE, by convention (OECD, 2014), "involves a chemical interaction (e.g., a reaction, covalent binding, hydrogen bonding, electrostatic interaction, etc.) between a chemical stressor and chemically defined biomolecules within an organism." Identifying the MIE as the first KE within the AOP and thus distinguishing it from early upstream events within the source-to-outcome pathway is important, especially when adverse outcomes require chronic exposure and dose-dependent transitions (Patlewicz et al., 2013). Hence, Patlewicz et al. (2013) introduced the idea of the "Initial Molecular Event" (IME) to capture necessary biological responses that are chemically induced early on in the pathway but may not be sufficient to "initiate" an adverse outcome. Drewe et al. (2014) used the term pre-MIE to make the same distinction. These alternative constructs reflect concern that some would infer certainty of an adverse outcome based solely on responses from assays associated with an MIE. In this AOP, we use the term pre-MIE.

Within an AOP, the MIE is followed by one or more intermediate KEs, which are connected in a sequential and integrated manner, culminating in the AO (Andersen et al., 2014). A Key Event Relationship (KER) "connects one key event to another, defines a directed relationship between the two (i.e., identifies one as upstream and the other as downstream), and facilitates inference or extrapolation of the state of the downstream key event from the known, measured, or the predicted state of the upstream key event" (OECD, 2014). In other words, a KER captures the knowledge of the toxicodynamic relationship between $KE_n$ and $KE_{n+1}$, and this knowledge may be sufficient to develop a prediction model. KERs necessarily include homeostatic mechanisms, and therefore, understanding dose-dependent transitions and tipping points along the sequence of KEs is important when developing, evaluating and applying AOPs. Further, responses can be influenced by co-exposure to other substances, and these components are referred to as modulating factors when considered within a MOA (Andersen et al., 2014); modulating factors affect the nature of KE dose–response relationships. Associative events serve as reliable biomarkers or indicators of KE(s) but may not be causal themselves. Thus, one needs to consider exposure pathways, chemical properties, ADME, modulating factors, and associative events when developing and applying AOPs (Carmichael et al., 2011; Dellarco and Fenner-Crisp, 2012; Fenner-Crisp, 2012; Julien et al., 2009). Benefiting from experience gained from over the last 2–3 years, largely through the OECD AOP program (OECD, 2014) and the AOP Wiki (USEPA, 2014), a component of the OECD-sponsored AOP Knowledgebase, recent publications provide greater clarity with respect to the concepts of KEs, KERs, (Villeneuve et al. (2014a, 2014b), strategies and practices to use when developing AOPs. Scientific confidence in the identification of KEs and in KERs may be judged using the evolved Bradford Hill (BH) considerations (Hill, 1965; Meek et al., 2013, 2014a, 2014b; OECD, 2014; Patlewicz et al., 2015; Becker et al., 2015).

In constructing AOPs, OECD recognizes the importance of initially drawing upon case examples applicable to a single chemical or a limited number of chemicals (OECD, 2015). The goal of these case examples is to expand the AOP concept to a broader chemical domain as experience and knowledge is gained. Consistent with this goal, we have chosen to draw from the MOA of aryl hydrocarbon receptor (AHR) activation causing liver tumors in rodents, including the extensive of knowledge of the biological process of rodent liver tumorigenesis (Budinsky et al., 2014). In the following case study, we describe the AOP of sustained AHR activation leading to rodent liver tumor promotion. This AOP case study forms a project within the OECD AOP work program and is also being summarized for inclusion into the AOP Wiki. The sustained AHR activation rodent liver tumor promotion AOP is shown in Fig. 1, organized according to the OECD AOP template.

The MIE is identified as sustained activation of the AHR produced by biologically persistent dioxin-like chemicals (DLCs). DLCs are potent AHR ligands that cause a range of species-specific adverse outcomes (Okey, 2007). The role of the AHR in carcinogenicity has been extensively studied and reviewed (Beebe et al., 1995; Bock and Kohle, 2005; Gasiewicz et al., 2008; Goodman and Sauer, 1992; Hailey et al., 2005; Knerr et al., 2006; Kociba et al., 1978; Stinchcombe et al., 1995). A number of cancer bioassays demonstrate that such compounds can induce hepatocellular adenomas/carcinomas and cholangiomas/carcinomas in various test species (Table 1) (Della Porta et al., 1987; Kociba et al., 1978; NTP, 1980; NTP, 1982a; NTP, 1982b; NTP, 2006a; NTP, 2006b; NTP, 2006c; NTP, 2006d; NTP, 2006e; NTP, 2006f; NTP, 2010). Approximately thirty initiation-promotion studies provide a complement to these cancer bioassays and, together have established the mechanism by which dioxin and DLCs act as rodent liver tumor promoters. Initiation-promotion studies rely on an initiating agent, generally diethylnitrosamine, to increase the population of initiated liver cells. Following initiation, treatment with DLCs or other tumor promoters, with or without partial hepatectomy, promotes the development and growth of pre-neoplastic altered hepatic foci. When treatment with promoters is continued for a sufficient time and dose, tumor formation occurs (Buchmann et al., 1994; Dragan et al., 1992; Maronpot et al., 1993; Teeguarden et al., 1999).

The mechanistic causal link between earlier histopathological KEs in liver and tumor development likely resides in the proliferative potential of the liver and the ability of this organ to regenerate following cytotoxicity (e.g., Cohen and Ellwein, 1990; Cohen and Arnold, 2011; Tomasseti and Vogelstein, 2015). Replacement of damaged hepatocytes may occur through replication of neighboring hepatocytes or, if damage is extensive, through recruitment of liver stem cells (Alison, 2005; Pintilie et al., 2010; Tanaka et al., 2011; Wang et al., 2003; Wolfle et al., 1993). Although DLCs have also been reported to cause tumors of the lung and oral mucosa in rodents, these tumor types are not considered further since they are outside the scope of this AOP (NTP, 2006a; NTP, 2006b; NTP, 2006c; NTP, 2006d; NTP, 2006e; NTP, 2006f; NTP, 2010; Wang et al., 2011).

Each component and KE of the AOP is discussed below in greater detail and in temporal sequence. As is the case for all AOPs, as further research increases knowledge of the mechanism of action (detailed molecular events and cellular processes), the KEs and KERs for sustained AHR activation leading to rodent liver tumor promotion are likely to evolve; the online AOP Wiki is well suited to accommodate such an evolution.

## 2. Toxicant (chemical properties)

Co-planar halogenated polyaromatic hydrocarbon structures with halogens in specific locations bind to and activate the AHR,