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The role of ethyl acrylate induced GSH depletion in the rodent forestomach and its impact on MTD and in vivo genotoxicity in developing an adverse outcome pathway (AOP)

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ABSTRACT

Adverse outcome pathways (AOP) and mode of action (MOA) frameworks help evaluate the toxicity findings of animal studies and their relevance to humans. To effectively use these tools to improve hazard identification and risk assessments for ethyl acrylate (EA), knowledge gaps in metabolism and genotoxicity were identified and addressed. For EA, hypothesized early key events relate to its irritation potential: concentration dependent irritation and cytotoxicity, progressing to regenerative proliferation and forestomach carcinogenicity after repeated oral bolus application in rodents. The current research quantitated glutathione (GSH) depletion to assess a kinetically-derived maximum tolerated dose (MTD) in the target tissue and used this information to conduct an in vivo genotoxicity study using current methods. In the mouse forestomach, gavage doses of EA caused GSH depletion to 47% of control at 20 mg/kg and 28% at 100 mg/kg. Cellular redox changes and histopathology support saturation of metabolism and an MTD of ~50 mg/kg. No increases in point mutations or deletions occurred in the stomach or liver following a 28 day treatment of *gpt* delta transgenic mice at gavage doses up to 50 mg/kg/day. These results provide valuable information for evaluating AOP molecular initiating events or MOA key events for EA and other GSH depleting materials.

1. Introduction

Adverse outcome pathways (AOP) and mode of action (MOA) frameworks are being developed to help determine the in depth understanding of the findings of animal studies and their relevance to humans. An AOP is a conceptual framework that portrays existing knowledge concerning the linkage between a direct molecular initiating event (MIE) and an adverse outcome at a level of biological organization relevant to risk assessment (Ankley et al., 2010). Similarly, an MOA framework relies on the assumption that any health effect caused by exposure to a substance can be described by a series of causally linked biochemical or biological key events (KE) that result in an observed specified outcome (Meek et al., 2014). Both are tools to develop, organize, assess, and improve the understanding of the stressors leading to adverse outcomes in health and environmental risk assessments. The MIE in an AOP is a starting point, like the initial key event in an MOA. The MOA analysis begins with a specific scenario, identifying the substance, exposure, and effect. Listing the KE, arranging them in the likely sequence of occurrence and establishing a dose response pattern build the framework that can be analyzed. Guidance stresses the utility of applying modified Bradford-Hill criteria when considering KE and the data that support their position in the framework. An important consideration regarding the dose response relationship includes determination whether the KE are observed before, concurrent with, or after the toxic outcome is expressed. AOPs, on the other hand, are not intended to be chemical specific, but rather conceptually applied to any stressor that initiates a series of events, progressing from the molecular to cellular to organ to organism to population level. In this regard, AOPs are modular, and can describe a variety of pathways depending on the sequence of events occurring.

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Consideration of the next steps to improve the hazard and risk assessments for ethyl acrylate (EA, ethyl 2-propenoate, CAS# 140-88-5) included use of these tools to understand the relevance to humans of adverse outcomes identified in animal studies, including tumor and non-tumor findings. To that end, the existing animal database was evaluated against the available guidance in the MOA/species concordance analysis framework (Meek et al., 2014; Boobis et al., 2009). EA is a chemical substance that produced forestomach tumors in rodents via oral gavage, an adverse outcome for which an MOA framework (Proctor et al., 2007) was proposed and AOP analyses (AOP Wiki) are ongoing. In the case of EA, early KE are hypothesized to be related to the irritant characteristics of the substance which determine the dose response and the recovery relationship after acute and chronic exposure. These include concentration dependent irritation and cytotoxicity, with progression to more serious regenerative proliferation and even carcinogenicity under certain circumstances. The analyses are intended to focus on the contributing factors to each KE or MIE, and elucidation of circumstances that influence the outcome. Of particular interest for EA are the outcomes before and after exposure to irritating or cytotoxic doses, and reliably predicting the maximum tolerated dose of the substance under various testing regimens, including the chronic bioassays that resulted in forestomach tumors. An IARC technical report (2003) stated that the relevance of rodent forestomach tumors to humans is “probably limited for agents that have no demonstrable genotoxicity and that are solely carcinogenic for the forestomach squamous epithelium in rodents after oral administration”. The Proctor et al. (2007) review included EA among the examples, providing the assessment: “Tumor promulgation with cessation of exposure is another key consideration. Kagawa et al. (1993) demonstrated that forestomach lesions induced by genotoxic carcinogens did not regress with removal of exposure, while simple or papillary hyperplasia induced by non-genotoxic carcinogens did regress after cessation of exposure. Ghanayem et al. (1994) showed that forestomach epithelial hyperplasia continued as long as exposure to EA continued via gavage dosing, but cessation of exposure resulted in the regression of hyperplasia and lack of tumor development. Therefore, the effect of temporal dosing regimens on forestomach tumor development should be considered in assessing the MOA and relevance to human exposures.”

As a high volume material, EA has a comprehensive traditional toxicology database of in vivo and in vitro studies (ECHA, 2016; McLaughlin et al., 1993; OECD, 2005). The predominant adverse findings include skin sensitization and strong irritation to all tissues upon immediate contact. Systemic toxicity was only observed at lethal doses. Chronic drinking water (Borzelleca et al., 1964), inhalation (Miller et al., 1985), and dermal (DePass et al., 1984) studies did not produce increases in tumor incidence. EA produced forestomach tumors in rats and mice after chronic administration by oral gavage (NTP, 1986). No other tumors were observed. The formation of the forestomach tumors was preceded by dose related chronic irritation, inflammation, hyperkeratosis and hyperplasia of the forestomach in both sexes of both species tested. The dermal and inhalation routes of administration are most relevant to workplace exposures, and notably EA was not carcinogenic by these exposure routes.

After the bioassays revealed forestomach tumors, mechanistic data for EA were developed primarily in the rat, with emphasis on metabolism, genotoxicity, and physiologically based pharmacokinetic (PBPK) modeling. Metabolism of EA occurs in two major pathways: carboxylesterase mediated hydrolysis and conjugation with glutathione (GSH). Radiolabeled EA was rapidly eliminated after oral administration to rats (deBethizy et al., 1987). Most was hydrolyzed, and the remainder was conjugated with nonprotein sulfhydryls (NPSH) such as GSH with mercapturic-acid derivatives excreted in the urine. After gavage administration, dose related depletion of NPSH in forestomach and glandular stomach was observed, with maximum efficiency of conjugation to be at less than 20 mg/kg, citing a precipitous drop in NPSH content at the dosing site between 2 and 20 mg/kg. At > 100 mg/kg the NPSH content did not change with dose, suggesting that reactive thiols were depleted. In vitro studies using cell free systems have shown that Michael addition of EA with GSH can occur in the absence of glutathione S-transferase enzyme (McCarthy et al., 1994) while enzyme-catalyzed GSH conjugation has been shown in a variety of tissue homogenates (Potter and Tran, 1992). Importantly, following GSH adduct formation no reactive chemical bonds will remain in the conjugate due to the lack of a bond available for further Michael additions.

Frederick et al. (1992) described that rapid detoxification of EA prevents toxic responses occurring in tissues remote from the dosing site. Significant GSH depletion was associated with the toxic response only at the site of gavage dosing. In time course studies forestomach GSH levels recovered from acute insult and returned to normal within several days, suggesting that when recovery is possible, rapid detoxification can be expected. Stimulation of S-phase activity in the forestomach and glandular stomach correlated with the replenishment and overshoot of tissue NPS levels with differences in the responses of the two tissues (Gillette and Frederick, 1993). After two weeks of administration the forestomach tissue appeared to have significantly increased detoxification potential (NPS level) at doses up to 50 mg/kg, but at 200 mg/kg dramatic proliferative activity was noted. The glandular stomach, a non-target tissue, showed only a marginal S phase increase at 200 mg/kg, reflecting more effective detoxification processes.

Comprehensive reviews of the genotoxicity data related to mechanisms of carcinogenesis for EA are available in the literature (Heine and Schneider, 2012; Johannsen et al., 2008; WHO, 2006; Williams and Iatropoulos, 2009). The weight of evidence indicates that EA does not present a genotoxic hazard to humans.

Electrophiles such as EA (Freidig et al., 2001) can form GSH conjugates irreversibly consuming a substantial portion of cellular GSH. Below a certain critical threshold, depletion of GSH impairs cellular protective mechanisms resulting in cytotoxicity (Lushchak, 2012; Lu, 2009). Mechanistic studies using mouse lymphoma cells indicated that the EA-induced mutagenic response in vitro correlated best with cellular cytotoxicity mediated by NPSH depletion and mitochondrial membrane impairment, rather than a direct DNA effect (Ciaccio et al., 1998). This aligns with the report by Morimoto et al. (1990) that no DNA damage was noted in the forestomach of F344 rats after single gavage doses of EA. This also supports GSH depletion as evidence of saturation of metabolism, as an MIE or initial KE that adequately defines the maximum tolerated dose (MTD) for tissues in which this pathway is crucial.

Critical review of each hypothesized MOA and the data that support the KE often identifies gaps in the data, particularly if there are multiple plausible pathways to a given endpoint. Despite the extensive data base for EA, data gaps existed in the areas of in vivo mutagenicity and dose related quantitation of GSH depletion in the mouse forestomach. The following studies were undertaken to determine the extent of GSH depletion as an indicator of cellular toxicity in the forestomach of rats and mice after exposure to EA and to understand the role of GSH in determining the MTD in this and other tissues and then to generate contemporary guideline (OECD 488) compliant mutation data with doses not exceeding the MTD.

2. Materials and methods

2.1. GSH and GSSG studies

2.1.1 Test Materials: 2-Propenoic acid ethyl ester, ethyl acrylate (EA), $C_5H_8O_2$, MW 100.1, CAS# 140-88-5, purity 99.9% (The Dow Chemical Company, Midland, MI) and Corn oil (Sigma-Aldrich, St. Louis, MO) were used for this study.

2.1.2 Dose preparation: Corn oil suspensions were prepared to achieve the targeted dose levels for mice at a dose volume of 10 mL/kg