Food and Chemical Toxicology xxx (2017) 1–13

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Dose-response analysis of epigenetic, metabolic, and apical endpoints after short-term exposure to experimental hepatotoxicants☆

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ARTICLE INFO

Article history:
Received 31 March 2017
Received in revised form
5 May 2017
Accepted 7 May 2017
Available online xxx

Keywords:
Benchmark dose modeling
DNA methylation
LINE-1
miRNA
Metabolomics
Hepatotoxicants
Dose-response

ABSTRACT

Identification of sensitive and novel biomarkers or endpoints associated with toxicity and carcinogenesis is of a high priority. There is increasing interest in the incorporation of epigenetic and metabolic biomarkers to complement apical data; however, a number of questions, including the tissue specificity, dose-response patterns, early detection of those endpoints, and the added value need to be addressed. In this study, we investigated the dose-response relationship between apical, epigenetic, and metabolomics endpoints following short-term exposure to experimental hepatotoxicants, clofibrate (CF) and phenobarbital (PB). Male F344 rats were exposed to PB (0, 5, 25, and 100 mg/kg/day) or CF (0, 10, 50, and 250 mg/kg/day) for seven days. Exposure to PB or CF resulted in dose-dependent increases in relative liver weights, hepatocellular hypertrophy and proliferation, and increases in Cyp2b1 and Cyp4a1 transcripts. These changes were associated with altered histone modifications within the regulatory units of cytochrome genes, LINE-1 DNA hypomethylation, and altered microRNA profiles. Metabolomics data indicated alterations in the metabolism of bile acids. This study provides the first comprehensive analysis of the apical, epigenetic and metabolic alterations, and suggests that the latter two occur within or near the dose response curve of apical endpoint alterations following exposure to experimental hepatotoxicants.

Abbreviations: Ahr, Aryl Hydrocarbon receptor; BA, Bile Acid; BMD, Benchmark Dose; BrdU, Bromodeoxyuridine; CAR, Constitutive Androgen Receptor; CF, Clofibrate; ChIP, Chromatin Immunoprecipitation; DNA, Deoxyribonucleic Acid; Dnmt, DNA methyltransferase; LC/MS, Liquid Chromatography/Mass Spectrometry; LI, Labeling Indices; LINE-1, Long Interspersed Nuclear Element 1; MBD, Methyl-binding Domain; miRNA, microRNA; MS-PCR, Methylation-Sensitive quantitative Polymerase Chain Reaction; NMR, Nuclear Magnetic Resonance; NOAEL, No-Observed-Adverse-Effect Level; ORF, Open Reading Frame; PB, Phenobarbital; PBRE, Phenobarbital Response Element; PPARα, Peroxisome Proliferator-Activated Receptor alpha; PPRE, Peroxisome Proliferative Response Element; PXR, Pregnane X receptor; qRT-PCR, quantitative Real-Time Polymerase Chain Reaction; RNA, Ribonucleic Acid; SAH, S-adenosyl-homocysteine; TCA, Taurocholic Acid; TCDCA, Taurochenodeoxycholic Acid; TDCA, Taurodeoxycholic Acid; TSS, Transcription Start Site; TUCA, Tauroursocholic Acid; TUDCA, Tauroursodeoxycholic Acid.

☆ #The views expressed in this manuscript do not necessarily represent those of the US FDA.

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http://dx.doi.org/10.1016/j.fct.2017.05.013
0278-6915/

1. Introduction

The current product safety assessment paradigm identifies adverse apical effects to establish a dose-response relationship between exposure and health-related outcomes and has been used for many years in human risk assessment. The molecular mechanisms leading to adverse apical endpoints are not typically evaluated in the risk assessment process, although molecular changes underlying the apical effect may be considered. Accumulating evidence indicates that exposure to various toxicants and chemical carcinogens can affect the cellular epigenome (Hou et al., 2012; Koturbash et al., 2011c; Thomson et al., 2014); however, the lack of causal links between epigenetic and apical effects is an obstacle to the incorporation of epigenetic endpoints into the human risk assessment process (Alyea et al., 2014; Miousse et al., 2015b; Priestley et al., 2012). Furthermore, specific epigenetic endpoints, target tissue specificity, dose-response, and approaches for their evaluation need to be identified. This is particularly relevant in the case of hepatotoxicants and carcinogens with the non-genotoxic mode of action, where DNA damage cannot be utilized as an endpoint, and the reliance on apical endpoints is therefore greater. In these regards, experimental non-genotoxic rodent hepatocarcinogens, such as phenobarbital (PB) and clofibrate (CF), serve as suitable chemicals to investigate the relationship between the apical and epigenetic endpoints.

Epigenetics is defined as changes in gene expression and chromatin organization that occur without alterations in DNA sequence and are heritable through cell division. Disruption of the balanced cellular epigenetic status may result in the development of a variety of pathological states, including cancer (Baylin and Jones, 2011; Waldmann and Schneider, 2013). Exposure to numerous liver toxicants, including the rodent experimental hepatocarcinogen PB, has been associated with a variety of epigenetic alterations, such as changes in DNA methylation, histone modifications and non-coding RNAs (Koturbash et al., 2011b; Koufaris et al., 2013; Phillips et al., 2009; Phillips and Goodman, 2009; Thomson et al., 2012, 2014; Watson and Goodman, 2002). Therefore, DNA methylation status of the Long Interspersed Nuclear Element-1 (LINE-1) and expression of microRNAs (miRNAs), were proposed to be utilized as early biomarkers of exposure to hepatotoxicants and carcinogens (Herceg et al., 2013; Koturbash et al., 2012, 2015; Lambert et al., 2015; Vliegenthart et al., 2015).

Metabolomics evaluates “the metabolite pool that exists within a cell under a particular set of conditions” (Fiehn, 2002). Metabolite profiling, typically using nuclear magnetic resonance (NMR) spectroscopy- or liquid chromatography/mass spectrometry (LC/MS)-based methods, has been utilized to evaluate biomarkers of hepatocarcinogenesis (Ohta et al., 2009; Tan et al., 2012; Unterberger et al., 2014). Metabolomics methods have been applied previously to evaluate the toxicity of CF in rats (Ishihara et al., 2006; Strauss et al., 2012) and mice (Wheelock et al., 2007) and the effects of exposure to PB in rats (Rubtsov et al., 2010; Waterman et al., 2010). These approaches have the potential to identify metabolite biomarkers related to hepatotoxicity and hepatocarcinogenesis.

Currently, there are limited data available to establish the toxicological significance of epigenetic changes and their causal relationship to apical endpoints and alterations in metabolism in response to exposure to hepatotoxicants and non-genotoxic liver carcinogens, as assessed in regulatory guideline toxicology studies. Toxicological studies incorporating epigenetic endpoints are typically conducted at a single high-dose level of exposure and do not contain appropriate concurrent assessment of apical toxicity endpoints such as histopathology and clinical chemistry to correlate epigenetic changes to apical endpoints (Alyea et al., 2012; Miousse et al., 2015b; Rasoulpour et al., 2011). In addition, few studies have utilized multiple doses to investigate a dose-response relationship between apical effects, specific genes associated with alteration in epigenetic regulation, and metabolism and metabolite profiling. Currently, employing benchmark dose (BMD) response analysis has become a useful tool to estimate dose levels corresponding to specific response by selecting a suitable dose-response modeling in risk assessment. Therefore, this study was designed to explore the similarities and differences between the dose-response relationship and associated benchmark doses of apical and metabolomics endpoints, as well as transcriptional changes in the genes involved in epigenetic regulation following short-term exposure to experimental hepatotoxicants, CF and PB, and provide useful information on early toxicity at the multiple levels of biomarkers underpinning quantitative risk assessment.

2. Materials and methods

2.1. Animals and treatment

Ten-week old male F344/DuCrl rats were purchased from Charles River Laboratories, Inc. (Kingston, New York). This strain was selected as it is commonly used in repeat dose toxicology studies and previous toxicity studies on CF and PB have used this strain. Rats were randomized according to body weight, identified via subcutaneously implanted transponders, housed one per cage in stainless steel cages, and provided LabDiet Certified Rodent Diet (PMI Nutrition International, St. Louis, MO) and water *ad libitum*. CF and PB were obtained from TOCRIS (BioScience, Bristol, United Kingdom) and Sigma-Aldrich (Saint Louis, MO), respectively. After acclimation, rats were administered PB or CF by oral gavage at dose levels of 0, 5, 25 or 100 mg/kg/day (PB) or 0, 10, 50, or 250 mg/kg/day (CF) in propylene glycol for seven days ($n = 5$ per dose). Those doses are generally considered as NOAEL, LOAEL, and lowest carcinogenic doses, respectively. Animals were terminated 24 h after the last dosing. The animal studies were performed at The Dow Chemical Company, Toxicology and Environmental Research & Consulting (TERC), Midland, MI, which is accredited by the American Association for Accreditation of Laboratory Animal Care. All animal care and use activities were reviewed and approved by the Institutional Animal Care and Use Committee.

2.2. Osmotic pumps

Rats were implanted with mini-osmotic pumps (model 2ML1; Alzet Corporation, Palo Alto, CA) to allow for continuous administration of bromodeoxyuridine (BrdU; a structural analog of thymidine that incorporates into nuclear DNA and is used as a surrogate marker of cell proliferation) during the seven days of treatment (Eldridge et al., 1990). The pumps contained 20 mg/ml of BrdU in phosphate buffered saline (pH 7.6) and delivered at a rate of 10 μl/h.

2.3. Clinical data and pathology

Rats submitted for necropsy were weighed (with the implanted osmotic pump *in situ*), anesthetized by inhalation of CO_2/O_2 or isoflurane/O_2, and blood samples were obtained from the orbital sinus. The animals were then euthanized by decapitation. The osmotic pumps were removed and weighed. The weights of the osmotic pumps were subtracted from the recorded body weights to determine the final body weight. The skin was removed from the carcass, the abdominal cavity opened, and the liver and kidneys excised and weighed. In addition, a 2–3 cm segment of the proximal duodenum was excised, flushed with fixative, and placed in the same fixative with the liver tissue, which served as a positive

Please cite this article in press as: Miousse, I.R., et al., Dose-response analysis of epigenetic, metabolic, and apical endpoints after short-term exposure to experimental hepatotoxicants, Food and Chemical Toxicology (2017), http://dx.doi.org/10.1016/j.fct.2017.05.013