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Case study on the utility of hepatic global gene expression profiling in the risk assessment of the carcinogen furan ☆

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ABSTRACT

Furan is a chemical hepatocarcinogen in mice and rats. Its previously postulated cancer mode of action (MOA) is chronic cytotoxicity followed by sustained regenerative proliferation; however, its molecular basis is unknown. To this end, we conducted toxicogenomic analysis of B3C6F1 mouse livers following three week exposures to non-carcinogenic (0, 1, 2 mg/kg bw) or carcinogenic (4 and 8 mg/kg bw) doses of furan. We saw enrichment for pathways responsible for cytotoxicity: stress-activated protein kinase (SAPK) and death receptor (DR5 and TNF-alpha) signaling, and proliferation: extracellular signal-regulated kinases (ERKs) and TNF-alpha. We also noted the involvement of NF-kappaB and c-Jun in response to furan, which are genes that are known to be required for liver regeneration. Furan metabolism by CYP2E1 produces cis-2-butene-1,4-dial (BDA), which is required for ensuing cytotoxicity and oxidative stress. NRF2 is a master regulator of gene expression during oxidative stress and we suggest that chronic NFR2 activity and chronic inflammation may represent critical transition events between the adaptive (regeneration) and adverse (cancer) outcomes. Another objective of this study was to demonstrate the applicability of toxicogenomics data in quantitative risk assessment. We modeled benchmark doses for our transcriptional data and previously published cancer data, and observed consistency between the two. Margin of exposure values for both transcriptional and cancer endpoints were also similar. In conclusion, using furan as a case study we have demonstrated the value of toxicogenomics data in elucidating dose-dependent MOA transitions and in quantitative risk assessment.

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Introduction

Furan is a liver toxicant and rodent hepatocarcinogen that is classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC, 1995). Furan was first reported in foods over 30 years ago (Maga and Katz, 1979). It is formed during heat-treatment of food, probably through thermal decomposition of carbohydrates, and is commonly found in coffee and canned and jarred foods (including baby food) (Moro et al., 2012). It is also produced during combustion and is therefore found in engine exhaust, wood smoke and tobacco smoke (IARC, 1995). The National Toxicology Program's (NTP) two-year cancer bioassay showed that furan induces hepatocellular carcinoma (HCC) and adenoma (HCA) in a dose-dependent manner in B6C3F1 mice and HCC, HCA, cholangiocarcinoma, and mononuclear cell leukemia in F344 rats (NTP, 1993); however, due to mixed results in the standard battery of genotoxicity tests, furan's carcinogenic mode of action (MOA) was ambiguous. Briefly, at the time of the assessment the available data on furan indicated that it is negative in the Ames assay (±S9) but positive in the L5178Y/TK$^{+/-}$ mouse lymphoma assay

Abbreviations: APAP, Acetaminophen; AP-1, Activator protein 1; ASK1, Apoptosis signaling kinase; BMD, Benchmark dose; BMDL, Lower confidence limit of benchmark dose; BrdU, Bromodeoxyuridine; bw, Body weight; JNK1, c-Jun NH2-terminal kinase 1; CT, Carbon tetrachloride; BDA, cis-2-butene-1,4-dial; Cyp2E1, Cytochrome P450 2E1; EtOH, Ethanol; ERK, Extracellular signal-regulated kinase; GO, Gene ontology; GSH, Glutathione; HCA, Hepatocellular adenoma; HCC, Hepatocellular carcinoma; IPA, Ingenuity Pathway Analysis; IRIS, Integrated Risk Information System; IARC, International Agency for Research on Cancer; c-Jun, Jun proto-oncogene; MAPK, Mitogen-activated protein kinase; MPT, Mitochondrial permeability transition; MOA, Mode of action; NTP, National Toxicology Program; NF-κB, Nuclear factor kappa B; Nrf2/NFE2L2, Nuclear factor (erythroid-derived 2)-like 2; PH, Partial hepatectomy; POD, Point of departure; ROS, Reactive oxygen species; SAPK, Stress-activated protein kinase; TNF, Tumor necrosis factor; TNFR1, TNF receptor 1.

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(−S9). In addition, furan causes sister chromatid exchanges *in vitro* in Chinese hamster ovary (CHO) cells (±S9) but not *in vivo* in B6C3F1 mouse bone marrow cells. Finally, furan exposure induces chromosomal aberrations in both CHO cells (±S9) and B6C3F1 mouse bone marrow cells (NTP, 1993). Subsequent studies to clarify the genotoxicity of furan revealed that furan is not clastogenic, but that its metabolite (cis-2-butene-1,4-dial (BDA)) causes strand breaks in L5178Y $tk^{+/-}$ mouse lymphoma cells at high (often cytotoxic) doses (Kellert et al., 2008). The results of *in vivo* MN assays for furan in mouse and rat are typically negative (Durling et al., 2007; Leopardi et al., 2010; McDaniel et al., 2012). While some researchers caution against the dismissal of a genotoxic MOA for furan (Cordelli et al., 2010; Neuwirth et al., 2012), recent studies using male transgenic Big Blue® rats (McDaniel et al., 2012), and follow-up studies using the comet and micronucleus assays in male F344 rats (Ding et al., 2012) concluded that any genotoxic action by furan is likely to be a secondary consequence of oxidative stress produced during furan metabolism.

The MOA proposed for furan based on apical (phenotypic) data derived from female B6C3F1 mice (Fransson-Steen et al., 1997; Moser et al., 2009) (supported by a weight of evidence from furan studies conducted in other rodent models) is chronic cytotoxicity and inflammation followed by dysregulated regenerative proliferation, which is also the most common MOA for spontaneous HCC (Nakagawa and Maeda, 2012). Indeed, in the NTP's 13-week study, dose-dependent increases of histopathological markers for cytotoxicity, necrosis and cellular proliferation were observed in B6C3F1 mice and F344 rats (both genders) and, in the two-year study, there was evidence of chronic inflammation, hepatic fibrosis, hyperplasia, degeneration and necrosis in the liver. After two years the NTP researchers reported increased hepatic cancer rates in both B6C3F1 mice and F344 rats (both genders), with a higher sensitivity to furan in rats. Female mice had high hepatic cancer rates at both doses tested (34/50 and 50/50 at 8 and 15 mg/kg bw, respectively) and had a much lower spontaneous cancer rate than the corresponding male mice (26/50 and 7/50 in males and females, respectively). Moser et al. (2009) exposed female B6C3F1 mice to lower doses of furan (0, 0.5, 1, 2, 4 or 8 mg/kg bw) for three weeks or two years. In their three week study they observed a significant and dose-dependent increase in hepatic cytotoxicity beginning at 1 mg/kg bw (measured by serum alanine aminotransferase, ALT) and a significant increase in hepatocyte proliferation in the 8 mg/kg bw group (measured by bromodeoxyuridine, BrdU, incorporation). They observed tumorigenesis at 4 and 8 mg/kg bw after two years. This study suggests that furan's point of departure (POD) for carcinogenicity in female B6C3F1 mice lies between 2 and 4 mg/kg bw. Furan's no-observed adverse effect level (NOAEL) in B6C3F1 mice and F344 rats (males and females) has been reported as 0.12 mg/kg bw and 0.03 mg/kg bw, respectively (Gill et al., 2010, 2011). Ultimately, apical studies in both genders of mice and rats support the concept that furan causes cancer *via* a cytotoxicity and regenerative proliferation MOA.

The ability of furan to induce levels of cytotoxicity sufficient to promote regenerative proliferation is related to the chemical's metabolism. Furan is metabolized into an electrophilic metabolite (BDA) by cytochrome P450 2E1 (CYP2E1) (Kedderis and Held, 1996), which is the only cytochrome P450 whose expression is not receptor-mediated. Instead, CYP2E1 is constitutively expressed and its activity is induced post-transcriptionally by stabilization in the presence of its substrate. Low molecular weight ligands, including ethanol (EtOH), acetaminophen (APAP), carbon tetrachloride (CT), chloroform and furan, increase Cyp2E1's half-life from seven to 32 h (Gonzalez, 2007). *In vitro* experiments have demonstrated that metabolic activation to BDA is necessary for furan-induced cytotoxicity (Kellert et al., 2008), and inhibition of CYP2E1 is sufficient to prevent cytotoxicity in female B6C3F1 mice and in mouse, rat or human microsomes (Fransson-Steen et al., 1997; Gates et al., 2012). The CYP2E1 catalytic cycle produces reactive oxygen species (ROS) (Gonzalez, 2005; Lu and Cederbaum, 2008) and BDA has been shown to deplete cellular glutathione (GSH) levels in F344 rat hepatocytes (Carfagna et al., 1993). There is evidence of oxidative stress-induced genomic damage in response to furan exposure including 8-oxo-dG adducts (Hickling et al., 2010) and oxidized purine and pyrimidine bases (Ding et al., 2012) in rat liver. However, it is unclear whether the indirect consequences of genotoxicity mediated *via* ROS contribute to the MOA of furan.

Established assays for the identification of non-genotoxic carcinogens are a current gap in the standard battery of short-term carcinogenicity tests. Toxicogenomics is the study of changes in gene expression following chemical exposure; induced perturbations in gene expression are related to the MOA of carcinogens. Thus, toxicogenomics is expected to provide an effective tool for the identification of diverse MOAs and may fill an important testing gap for non-genotoxic carcinogens (Waters et al., 2010). MOA information gleaned from toxicogenomic studies of carcinogens has the added benefit of providing mechanistic information, thereby facilitating inter-species comparisons for inferring human risk. In this study we characterize global gene expression profiles in liver tissue taken from female B6C3F1 mice that were sub-chronically exposed to non-carcinogenic (0, 1, 2 mg/kg bw) and carcinogenic (4 and 8 mg/kg bw) doses of furan. We perform extensive bioinformatics analyses that are anchored in previously published apical (phenotypic) endpoint data (Gill et al., 2011; Moser et al., 2009; NTP, 1993) in order to elucidate the molecular mechanism by which furan causes liver cancer. More broadly, our goal is to use furan as a case study to champion the concept of using sub-chronic exposures and genomic tools to inform risk assessment of non-genotoxic carcinogens.

Methods

Chemical

Furan (CAS no. 110-00-9) (>99% pure) (Sigma-Aldrich Chemical Co., Milwaukee, WI) was mixed with Mazola corn oil to the appropriate concentration. Doses were prepared separately on a volume-to-weight ratio (v:w), were de-aerated with inert gas, and were stored in 8 mL brown glass vials (sealed with plastic closures and modified silicon septa) in the refrigerator for up to 14 days (based on previous reports of furan stability (NTP, 1993)).

Animals

5–6 week old female specific pathogen free B6C3F1 mice were purchased from Charles River Breeding Laboratories (Portage, ME) and were allowed to acclimatize for at least 7 days prior to the start of the study. Feed (NIH-07; Zeigler Brothers, Inc., Gardners, PA) and tap water were available *ad libitum* up until the time of necropsy. Mice were housed five per cage in polycarbonate cages in a specific pathogen free (SPF) and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility. All procedures were conducted in compliance with the Animal Welfare Act Regulations (9CFR1–4). Mice were handled and treated according to the guidelines provided in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (ILAR, 1996; http://dels.nas.edu/ilar/).

Female mice were dosed with furan in corn oil at 0, 1, 2, 4, or 8 mg/kg bw per day by oral gavage for three weeks ($n = 10$ per dose). We chose to use female mice because they have a lower spontaneous tumor rate than males (Haseman et al., 1998). $n = 4, 5, 3, 4,$ or 5 per dose group were treated with 0.02% Bromodeoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO, USA) in drinking water for 5 days just prior to sacrifice. Upon necropsy, there remained $n = 5$ mice in each non-BrdU group. Some mice were lost due to early (pre-BrdU treatment) mis-dosing or esophageal puncture. Four hours after their final dosing, mice were anesthetized by CO_2 inhalation prior to euthanasia by exsanguination achieved by cutting the caudal vena cava after blood collection. One animal per group was killed and this continued until all mice had been sacrificed;

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