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# Integration of novel approaches demonstrates simultaneous metabolic inactivation and CAR-mediated hepatocarcinogenesis of a nitrification inhibitor

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ABSTRACT

Nitrapyrin, a nitrification inhibitor, produces liver tumors in mice at high doses. Several experiments were performed to investigate molecular, cellular, and apical endpoints to define the key events leading to the tumor formation. These data support a mode-of-action (MoA) characterized by constitutive androstane receptor (CAR) nuclear receptor activation, increased hepatocellular proliferation leading to hepatocellular foci and tumor formation. Specifically, nitrapyrin induced a dose-related increase in the Cyp2b10/CAR-associated transcript and protein. Interestingly, the corresponding enzyme activity (7-pentoxyresorufin-O-dealkylase (PROD) was not enhanced due to nitrapyrin-mediated suicide inhibition of PROD activity. Nitrapyrin exposure elicited a clear dose-responsive increase in hepatocellular proliferation in wild-type mice, but not in CAR knock-out mice, informing that CAR activation is an obligatory key event in this test material-induced hepatocarcinogenesis. Furthermore, nitrapyrin exposure induced a clear, concentration-responsive increase in cell proliferation in mouse, but not human, hepatocytes *in vitro*. Evaluation of the data from repeat dose and MoA studies by the Bradford Hill criteria and a Human Relevance Framework (HRF) suggested that nitrapyrin-induced mouse liver tumors are not relevant to human health risk assessment because of qualitative differences between these two species.

## 1. Introduction

Global plant protection product registration requires a comprehensive data package, which is designed to characterize the potential hazards to human health, the environment, and ecosystems, coupled with exposure assessments to perform a comprehensive risk assessment (for more information, please see Crop Life America (http://www.croplifeamerica.org/)). As a general practice, any treatment-related effects from the battery of toxicity tests should be evaluated, often by means of additional mechanistic data to characterize a mode-of-action (MoA). The MoA/Human Relevance Framework (HRF) developed by the International Programme on Chemical Safety (IPCS) of the World Health Organization [1,2] and International Life Sciences Institute (ILSI) [3] is typically the "gold standard" template used to assess the strength of the data in support of a MoA for a chemical, evaluate alternative or competing hypotheses for the MoA, and subsequently analyze the human relevance of the most plausible MoA for the animal effects in question.

Nitrapyrin (2-chloro-6-(trichloromethyl) pyridine; CAS Number 1929-82-4; Fig. 1) is the active ingredient in N-SERVE™[1]; nitrogen stabilizer. When applied to the soil nitrapyrin selectively inhibits the conversion of ammonium nitrogen to nitrite nitrogen by soil bacteria of the genus *Nitrosomonas* [33]. A complete database of toxicological studies has supported the registration of nitrapyrin in the United States for many years, including long term chronic/oncogenicity studies. Two separate chronic oncogenicity studies in mice were performed for nitrapyrin. In the first two-year oncogenicity study, B6C3F1 mice were administered 0, 5, 25, or 75 mg/kg body weight/day (mg/kg/day) nitrapyrin in the diet, and no increased incidence in hepatocellular tumors was observed. A subsequent two-year oncogenicity study was conducted with B6C3F1 mice that were administered 0, 125, or 250 mg/kg/day nitrapyrin. An increased incidence of mice with hepatocellular tumors was observed following exposure to 125 (females only) or 250 mg/kg/day (males and females) nitrapyrin in the diet.

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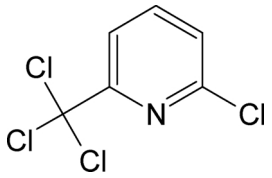


**Fig. 1.** Nitrapyrin Chemical Structure.

There are several well-characterized MoAs for hepatocarcinogenesis, and the analysis of robust mechanistic data can provide a reasonable basis for extrapolation of a rodent MoA to human relevance [4]. In order to elucidate the mechanism underlying nitrapyrin-mediated hepatocarcinogenesis in mice, targeted MoA experiments were conducted that included several apical, molecular, biochemical, and cellular end points. Additionally, the data from these experiments were evaluated against Bradford Hill criteria followed by subsequent application in a HRF.

## 2. Materials and methods

### 2.1. Animals and treatment

The carcinogenicity studies were conducted on approximately 6–8-week old B6C3F1 mice while the MoA study was performed on approximately 6–7-week old male B6C3F1 mice (Charles River Laboratories, Inc.; Portage, Michigan). The CAR KO mouse studies were performed on approximately 12-week old C57BL/6NTac mouse strain (WT and CAR KO; Taconic Inc, Hudson, NY). The purpose for using slightly older mice in the CAR KO study was to aid in reducing the background hepatocellular proliferation that may be observed with younger animals, which may confound the assessment and interpretation of the hepatic proliferative response to nitrapyrin. The studies were performed at The Dow Chemical Company, Toxicology & Environmental Research & Consulting (TERC), Midland, Michigan, 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. The MoA study included 7- and 14-day treatment periods (n = 6/group, except in the high dose group where n = 9), along with a recovery group that was treated for 14 days followed by a 21-day recovery period (n = 6/group). The 75 mg/kg/day dose level was selected because it was the high dose in the initial chronic/carcinogenicity study and was not carcinogenic. The 250 mg/kg/day dose level was selected because it was the high dose in the repeat mouse chronic/carcinogenicity study and resulted in increased incidence of liver tumors in both sexes. The 400 mg/kg/day dose level was selected because it was used in the previously conducted 2-week and 13-week subchronic studies and allowed for further insight into the dose-response of nitrapyrin administration.

A strain comparison study included 4- and 7-day treatment periods for B6C3F1 and C57BL/6NTac (background strain for the CAR KO animals) mice. Given the similarity of overall responsiveness to nitrapyrin-mediated hepatic effects between B6C3F1 and C57BL/6NTac mice at 4- and 7- days of exposure, the use of the C57BL/6NTac was validated for the CAR KO study. The CAR KO mouse studies included a 4-day treatment period in WT and CAR KO mice (n = 6/group).

### 2.2. Dietary test article preparation and analysis

Nitrapyrin was administered via the diet (LabDiet Certified Rodent Diet #5002, PMI Nutrition International, St. Louis, Missouri, USA) and analytical confirmation of dietary concentrations was performed. Feed consumption was determined for all mice by weighing feed containers at the beginning and end of the respective exposure period. Test material intake was calculated upon completion of the study using test material concentrations in the feed, body weights, and measured feed consumption.

### 2.3. Osmotic pumps

For the MoA study, on the first day of dosing, 7-day exposure group animals were implanted with miniosmotic pumps, model 2001 (Alzet Corporation, Palo Alto, California) for the duration of the treatment period. The 14-day exposure group animals were implanted with mini-osmotic pumps, model 2001, on day 7 of exposure which remained for the duration of the experiment. The 21-day recovery group animals were implanted with mini-osmotic pumps model 2001, beginning on day 14 of recovery (day 29 of the experiment) and remaining for the rest of the recovery period. For the CAR KO experiment, all animals were implanted with mini-osmotic pumps model 2001 (mice), (DURECT Corporation, Cupertino, California) 4 days prior to scheduled necropsy. Incorporation of 5-bromo-2′-deoxyuridine (BrdU; a structural analog of thymidine) into nuclear DNA was used as a surrogate marker of cell proliferation. Mice were continuously infused with BrdU via the implanted osmotic pumps filled with a 20 mg/ml solution of BrdU in phosphate buffered saline (pH 7.6), at a delivery rate of 1.0 µl/hour. The osmotic pumps were implanted subcutaneously into the lumbar or intrascapular region on the dorsum of each mouse. This implantation method assured a constant, uniform, systemic delivery of BrdU and this experimental design provided a labeling period of approximately 7 days (MoA experiment) or 4 days (CAR KO experiment) for each of the treatment groups. The 7-day treatment group allowed analysis of proliferation during the first week of treatment, the 14-day treatment group allowed analysis of the cell proliferation during the second week of treatment, and the recovery group provided proliferation data during the last 7 days of the recovery period. For the CAR KO experiment, the design provided a labeling period of approximately 5 days.

### 2.4. Clinical data and pathology

Mice submitted for necropsy were weighed (with the implanted osmotic pump *in situ*), anesthetized by inhalation of 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 reflected from the carcass, the abdominal cavity opened, and the liver 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 control for osmotic pump functionality and immunohistochemistry. The upper third of the left lateral liver lobe was processed in RNA*later*™ for targeted gene expression analysis. The middle third of the left lateral lobe was trimmed and preserved in neutral phosphate-buffered 10% formalin and was used for histological examination and BrdU proliferation analysis. A slice of the remaining left lateral lobe was diced into 1 mm cubes (~20) and placed in a phosphate-buffered solution of 2% glutaraldehyde-2% formaldehyde for at least two hours (maximum of 8 h) for rapid fixation. The samples were stored in 0.2 M phosphate buffer for possible ultrastructural analysis for peroxisomes by electron microscopy. However, this analysis was not performed based on the results of the gene expression analysis. The remaining liver was flash frozen and stored at −80 °C for enzyme activity analysis, contingencies, or additional analyses. Transponders were removed and placed in the jar with the tissues. Livers were processed by standard histologic procedures. Paraffin embedded tissues were sectioned approximately 6 µm thick, stained with hematoxylin and eosin, and examined by a veterinary pathologist using a light microscope.

### 2.5. Hepatic gene expression

Liver samples preserved in RNA*later* from all exposure groups were used for RNA isolation. Total RNA was extracted using the Qiagen RNeasy kit following the manufacturer's protocol. RNA quantity and quality was assessed by a NanoDrop ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer, respectively. Only samples with an OD 260/280 ratio greater than 1.8 and with clearly defined 28S and 18S bands were used for targeted gene expression experiments. Total RNA was treated with DNase enzyme to avoid DNA contamination. Targeted gene expression studies were conducted using an Applied Biosystems 7500 Real-Time Polymerase Chain Reaction system using Applied Biosystems TaqMan Gene Expression Assays. Due to the nature of the TaqMan system, dissociation curves were not required to verify the specificity of the PCR reactions.

The transcription levels of the following genes were examined to aid in understanding the MoA and other possible metabolic pathways. The following nuclear receptor pathways were analyzed: aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), and peroxisome proliferator activated receptor alpha (PPARα):

1. *Cyp1a1* "AhR response gene". Mouse- Mm00487218_m1
2. *Cyp2b10* "CAR response gene". Mouse-Mm00456591_m1
3. *Cyp3a11* "PXR response gene". Mouse-Mm00731567_m1
4. *Cyp4a10* "PPAR response gene". Mm01188913_g1
5. *β-actin* "Housekeeping gene". Mm00607939_s1

Data were analyzed by generation of the threshold cycle (Ct) and delta Ct (ΔCt) values for all genes. Each sample was analyzed in triplicate for the reference and target genes. Amplification curves were processed using Sequence Detection Software 1.4 (ABI). Gene expression was quantified using the comparative Ct method (ΔΔCt) (Schmittgen and Livak 2008). For this method, the amount of target mRNA for each treatment group was expressed relative to an endogenous reference or housekeeping gene (*β-actin*) and relative to vehicle control animals. The mean Ct of the housekeeping gene was subtracted from the mean Ct of the target genes (ΔCt). The mean ΔCt value from each treatment group was then subtracted from the mean ΔCt for the vehicle control group to generate the ΔΔCt value that was used to calculate the fold-induction for that treatment group, relative to controls.

### 2.6. Microsome isolation

Frozen liver samples were thawed on ice and homogenized using a Potter-Elvehjem apparatus. Microsomes were isolated using the method outlined by [5]. Total microsomal protein levels were determined for individual animals using the Pierce BCA method (Pierce Chemical Co., Rockford, Illinois, USA).

### 2.7. Western blot protein analysis

Immunoreactive Cyp2b10 protein levels were assessed by Western immunoblotting using microsomes prepared from mouse liver. Briefly, for each sample 30 μg of protein was applied for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred electrophoretically to nitrocellulose membranes for immunoblotting. Membranes were pre-blocked for 1 h using casein buffer followed by polyclonal rabbit anti-Cyp2b10 antibodies (Millipore, Billerica, Massachusetts, USA) for at least 2 h at room temperature with shaking. Membranes were rinsed with Tris-buffered saline with 0.1% Tween-20 buffer and probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat, anti-rabbit IgG, Millipore) for at least 45 min at room temperature with shaking. Immunoreactive bands were visualized on a Fluor-S multi-imager (BioRad, Hercules, CA) with SuperSignal detection systems (ThermoScientific, Waltham, MA). Commercially available control and phenobarbital-treated liver microsomes were included in each blot.

### 2.8. In vitro enzyme activity assays

Cyp2b10 enzyme activity (PROD) was determined using pentoxyresorufin as the substrate. Activities were measured using a microplate fluorometric method [6]. Briefly, this method involves the measurement of the *in vitro* O-dealkylation of 7-pentoxyresorufin to a highly fluorescent product, resorufin, by microsomal mixed function oxidases [7]. Samples analyzed included microsomes prepared from nitrapyrin-treated mouse liver samples from the 7-day treatment group, as well as DMSO control and commercially available phenobarbital-induced liver microsomes.

### 2.9. In vitro assay for mechanism-based (suicide) inhibition of p450 enzyme activity

Commercially available phenobarbital (PB)-induced rat liver microsomes (Celsis/*In Vitro* Technologies, Catalog# M70001, Lot# TVZ; 25 μg/well) along with reaction buffer and fluorometric substrate (7-pentoxyresorufin-O-dealkylase(PROD)) were prepared and pipetted into a 96-well plate for assay conduct. An appropriate concentration of one of the following test substances was added to this mixture: solvent (0.1% DMSO), negative control for inhibition (phenobarbital; 1-500 μM), positive control for inhibition (curcumin; 10–80 μM), or nitrapyrin (1–500 μM). The enzymatic activity was determined by the rate of PROD conversion to resorufin (pmol/min/mg protein) as detected by a fluorometric plate reader (fmax, Molecular Devices, Sunnyvale, California) in a 20-min kinetic assay [7,6]

### 2.10. Hepatocellular proliferation

Hepatic S-phase DNA synthesis was determined using 5-bromo-2′-deoxyuridine (BrdU) immunohistochemistry for identification of BrdU incorporation into nuclear DNA using the method outlined by Eldridge and Goldsworthy [8]. Upon euthanasia, a section of liver was recovered as noted above, processed by standard techniques, and mounted on glass slides. Tissue was stained for BrdU using the manufacturers' protocol (BD Biosciences, San Diego, California) and modified heat-induced antigen retrieval. A small section of duodenum from each animal was also processed to serve as a positive control for confirming systemic availability of the BrdU. Using light microscopy, a labeling index was calculated based on BrdU positive nuclei which were scored as percentages based on 1000 hepatocytes from each animal in each of three hepatolobular zones: centrilobular, midzonal, and periportal regions.

### 2.11. In vitrohuman and mouse hepatocellular proliferation

Primary mouse and human hepatocytes were sourced from GIBCO-Life Technologies, a ThermoFisher Brand (Frederic, MD). For determination of replicative DNA synthesis, GIBCO fresh plated CD-1 mouse or human hepatocytes plated on collagen-coated glass coverslips in a 6-well format were used. For determination of cytotoxicity, the same culture system was used, except the cultures were in a 12-well format. Two human hepatocytes donors were used in two independent biological replicates. After acclimation, hepatocyte cultures were re-fed with 1 ml of pre-warmed culture medium and then an additional 1 ml of pre-warmed culture medium containing a 2X final concentration of the respective test material or vehicle. Following the addition of the test material, the plate was incubated for approximately 24 h at 37 °C with 5% $CO_2$. Following incubation, the culture medium was replaced with 1 ml of pre-warmed culture medium and 1 ml of pre-warmed culture medium containing a 2X final concentration of the respective test

material or vehicle and 10 μM final concentration of 5-ethynyl-2′-deoxyuridine (EdU) (Life Technologies, a ThermoFisher Brand; Frederic, MD). The hepatocyte culture plate was incubated at approximately 37 °C with 5% $CO_2$ for an additional 24 h. Cytotoxicity was assessed in primary mouse and human hepatocytes concurrently with the cell proliferation analysis. A cytotoxicity analysis of test material treatments was performed using group sizes of 2 − 3 technical replicates per biological replicate. Cytotoxicity was evaluated by monitoring mitochondrial metabolic activity (dehydrogenase activity) using a MTT reagent thiazolyl blue tetrazolium bromide. Only test material treatments showing ≤30% cytotoxicity (comparing mean values of treated groups to the control mean value) were used for analyzing cell proliferation. As a measure of cell proliferation, replicative DNA synthesis in mouse and human hepatocytes was measured by monitoring the incorporation of EdU into nuclear DNA. EdU is a nucleoside analog of thymidine that is incorporated into DNA during DNA synthesis. After exposing the cells to EdU for approximately 24 h as described above, the incorporation of EdU into hepatocyte nuclei was examined using a Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Life Technologies, a ThermoFisher Brand; Frederic, MD) according to the manufacturer's instructions. In brief, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS; pH 7.2–7.6), washed with PBS containing 3% bovine serum albumin, permeabilized with 0.5% Triton X-100 in PBS, incubated with Alexa Fluor® Click-iT® reagent, washed with PBS containing 3% bovine serum albumin, and counterstained with Hoescht 33342 to visualize all nuclei. Using this technique, cells incorporating EdU into nuclear DNA showed nuclear fluorescence of the Alexa Fluor® 488 dye (Fig. 1). One thousand nuclei (i.e. cells) per technical replicate were imaged with an epifluorescence microscope, and each nucleus was scored as being EdU positive or EdU negative. A single observer blinded to treatment scored each technical replicate within a biological replicate. For cell proliferation analysis, the procedure was performed twice with independent lots (i.e. biological replicates) of mouse or human hepatocyte isolates. For each biological replicate of mouse hepatocytes, group sizes (i.e. technical replicates) of 2–5 were used. Thus, a total of 5–7 technical replicates of mouse hepatocytes among two biological replicates were examined per group. For each biological replicate of human hepatocytes, group sizes (i.e. technical replicates) of 2–3 were used. Thus, a total of 5–6 technical replicates of human hepatocytes among two biological replicates were examined per group. For statistical purposes, the technical replicate was the statistical unit.

### 2.12. Statistics

Means and standard deviations were calculated for all continuous data. All parameters examined statistically (feed consumption and targeted gene expression are addressed separately) were first tested for equality of variance using Bartlett's test [9]. If the results from Bartlett's test were significant at alpha = 0.01, then the data for the parameter were subjected to a transformation to obtain equality of the variances. The transformations that were examined were the common log, the inverse, the square root, the arc sine square root, and rank. The data were then reviewed and an appropriate form of the data was selected. The selected form of the data was then subjected to the appropriate parametric analysis as described below.

In-life body weights, terminal body weight, liver weight, relative liver weight, cholesterol, ALT, AST, enzyme activity, and BrdU for proliferation were evaluated using a one-way ANOVA [10] with the factor of dose. Comparisons of individual dose groups to the control group were made with Dunnett's test (alpha = 0.05) only when a statistically significant dose effect existed. Targeted gene expression was quantified using the comparative Ct method (ΔΔCt); however, statistical analysis was not performed. Fold-change was calculated as the ratio of the treated/control values.

Determination of treatment-related effects was based on sound scientific principles such as biological plausibility, comparison to concurrent and historical control data, and dose-response relationships. For example, for a relative liver weight increase to be considered treatment-related, concomitant histopathological observations such as hepatocellular hypertrophy were required. In general, a treatment-related interpretation was based on the entirety of the data from a given experiment, or across multiple experiments, for consistency and robustness of a biological response.

## 3. Results

### 3.1. Nitrapyrin-exposure increased hepatocellular tumor formation and hepatocellular foci in mice

Two mouse oncogenicity studies were conducted for nitrapyrin. In the first mouse oncogenicity study, nitrapyrin was administered in feed to B6C3Fl mice (50/sex/dose) at 0, 5, 25, or 75 mg/kg/day for up to two years. While a slight increase in liver weight (absolute and relative) and histopathologic changes consisting of altered cytoplasmic staining/homogeneity of centrilobular hepatocytes occurred in high-dose males and females (data not shown), there was no treatment-related increase in the incidence of hepatic adenomas or carcinomas (Table 1) or any other types of tumors on this study (data not shown).

In the second mouse oncogenicity study, there were significant treatment-related depressions in body weight gains at 12 (decreased 29.6% compared to controls) and 24 (decreased 26.9%) months in males given 250 mg/kg/day nitrapyrin and statistically identified increases in liver weights (absolute and relative) in males and females given 125 or 250 mg/kg/day at 12 or 24 months (data not shown). The increased liver weights corresponded to the histopathologic observations of increased size hepatocytes in males and females given 125 or 250 mg/kg/day nitrapyrin at the 12- and 24-month sacrifices (data not shown). An increased incidence of mice with hepatocellular tumors (adenomas and/or carcinomas) were observed following exposure to 125 (females only) or 250 mg/kg/day (males and females) nitrapyrin in the diet (Table 1).

Nitrapyrin administration was also associated with an increase in the number of mice with one or more eosinophilic and/or basophilic

**Table 1**
Incidence of Hepatocellular Neoplasms in B6C3F1 Mice Treated with 0, 5, 25, or 75 or 0, 125 or 250 mg/kg/day Nitrapyrin for 2 Years.

| Sex | Males | | | | | | | Females | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dose (mg/kg/day) | 0 | 5 | 25 | 75 | 0 | 125 | 250 | 0 | 5 | 25 | 75 | 0 | 125 | 250 |
| # Examined | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| # Mice with one or more hepatocellular adenoma(s) | 20 | 12 | 18 | 20 | 12 | 19 | 45[a] | 11 | 6 | 7 | 14 | 6 | 27[a] | 32[a] |
| # Mice with one or more hepatocellular carcinoma(s) | 9 | 11 | 6 | 7 | 7 | 3 | 12 | 1 | 1 | 1 | 1 | 0 | 1 | 2 |
| # Mice with one or more hepatocellular adenoma(s) and/or carcinoma(s) | 23 | 20 | 23 | 23 | 17 | 20 | 46[a] | 12 | 7 | 7 | 15 | 6 | 28[a] | 33[a] |

Data are the number of animals with the specified observation.
[a] Statistically identified difference from control mean by Yate's Chi-square pair-wise test, alpha = 0.10, two sided.

**Table 2**
Incidence of Hepatocellular Foci in B6C3F1 Mice Treated with 125 or 250 mg/kg/day Nitrapyrin for 2 Years.

| Sex | Males | | | Females | | |
|---|---|---|---|---|---|---|
| Dose (mg/kg/day) | 0 | 125 | 250 | 0 | 125 | 250 |
| # Examined | 50 | 50 | 50 | 50 | 50 | 50 |
| # Mice with basophilic foci, focal or multifocal | 0 | 1 | 8[a] | 1 | 1 | 12[a] |
| # Mice with eosinophilic foci, focal or multifocal | 5 | 5 | 12 | 2 | 2 | 23[a] |
| # Mice with vacuolated or clear foci, focal or multifocal | 0 | 2 | 2 | 0 | 4 | 1 |

Data are the number of animals with the specified observation.
[a] Statistically identified difference from control mean by Yate's Chi-square pair-wise test, alpha = 0.10, two sided.

foci of altered hepatocytes in male and female mice administered 250 mg/kg/day (Table 2). The number of male and female mice with eosinophilic foci was greater than the number of mice with basophilic foci at 250 mg/kg/day. Also, the number of mice with multiple eosinophilic foci (0, 1, 9 males and 0, 0, 11 females at 0, 125, 250 mg/kg/day, respectively) was greater than the number of mice with multiple basophilic foci (0, 0, 6 males and 0, 0, 2 females at 0, 125, 250 mg/kg/day, respectively) (data not shown). The increased number of mice with foci and the increased number of foci per mouse (especially eosinophilic foci) correlated with the increased number of mice with hepatocellular adenomas and/or carcinomas attributed to nitrapyrin treatment at 250 mg/kg/day. The number of mice with foci of altered cells was not increased at 125 mg/kg/day, but this dose approximates a threshold dose for hepatocellular tumor formation as evidenced by the absence of an increase in male mice with hepatocellular adenomas and/or carcinomas at 125 mg/kg/day and no increases in hepatocellular tumors in either sex at ≤75 mg/kg/day.

### 3.2. Potential MoAs for nitrapyrin-mediated hepatocellular carcinogenesis

As listed in Table 3, the MoAs for hepatocellular carcinogenesis are broadly categorized as (1) increased cell proliferation or (2) DNA reactivity [4].The former can be subcategorized as (a) receptor mediated or (b) non-receptor mediated. The genotoxicity and apical endpoint data for nitrapyrin exclude some MoAs such as DNA reactivity, estrogenicity, and infection (further explained in Supplemental Data). However, other MoAs, including cytotoxicity and receptor-mediated, could not be excluded without additional supporting data. Considering that increases in liver weight, hypertrophy and an increase in hepatocellular foci are associated with a receptor mediated MoA, targeted experiments were conducted to explore if this was a plausible mechanism. B6C3F1 mice (6/dose/time period, except high-dose animals in the 14-day treatment groups, which were 9/dose) were exposed to 0, 75, 250, or 400 mg/kg/day nitrapyrin for 7 or 14 days. Recovery groups were given the same dosages of nitrapyrin for 14 days then switched to the control diet for 21 days to investigate recovery after treatment. In a second experiment, B6C3F1 male mice were fed diets targeting 0 and 250 mg/kg/day nitrapyrin for 4 or 7 days.

**Table 3**
Modes of Action for Hepatocellular Carcinogenesis [4].

| | |
|---|---|
| I | **DNA reactivity** |
| II. | Increased cell proliferation |
| | a. Receptor mediated |
| | i. PPAR (peroxisome proliferation) |
| | ii. Enzyme induction (CAR, PXR, AHR) |
| | iii. **Estrogen** |
| | iv. Statins |
| | v. **Cytotoxicity** |
| | vi. Other |
| | a. Non-receptor mediated |
| | i. **Cytotoxicity** |
| | ii. **Infectious** |
| | iii. **Iron (copper) overload** |
| | iv. Increased apoptosis (e.g., fumonisin B1) |
| | v. Other |

Modes of action in **bold** letters are likely to be relevant to humans.

### 3.3. Nitrapyrin-exposure increased liver weights and hepatocellular hypertrophy

After 4 days of 250 mg/kg/day nitrapyrin administration and after 7 days of 250 or 400 mg/kg/day nitrapyrin administration, increases in absolute (data not shown) and relative (Table 4) liver weights as compared to control were statistically identified. The mean liver weight changes were more pronounced in animals administered nitrapyrin for 14 days compared to 4- or 7-day duration. Animals treated with 250 mg/kg/day for 14 days exhibited a statistically significant increase of 20% and 24% for absolute and relative mean liver weights, respectively. Animals treated with 400 mg/kg/day nitrapyrin for 14 days had a statistically significant increase of 41% and 49% for absolute and relative mean liver weights, respectively. The liver weights for mice treated with 75 mg/kg/day were not statistically different from controls at 4, 7, or 14 days, supporting that there is a threshold for nitrapyrin-induced liver weight changes. Nitrapyrin-induced liver weight increases regressed such that there were no statistically identified liver weight changes (absolute or relative) for any of the treatment groups following 14 days of treatment with a subsequent 21-day recovery period on normal diets.

Nitrapyrin treatment-related alterations in the mouse were also reflected in histopathological evaluation of liver tissue. The data for microscopic hepatocellular hypertrophy for B6C3F1 mice treated with nitrapyrin from the MoA studies are presented in Table 4. A dose-responsive increase in hepatocellular hypertrophy was observed in nitrapyrin-treated animals, with a point of departure of 75 mg/kg/day. The hepatocellular hypertrophy data also correlated with the hepatocellular tumor results for the combined nitrapyrin mouse oncogenicity studies.

### 3.4. Nitrapyrin-exposure increased hepatocellular proliferation

Following 4 or 7 days of 250 mg/kg/day nitrapyrin exposure, B6C3F1 mice had a mild to moderate, statistically identified, treatment-related increase in hepatocellular proliferation (Table 4). Hepatocellular proliferation was similar at both timepoints for both the midzonal (2.3-fold vs. 2.5-fold) and periportal (5.9-fold vs. 6.2-fold) regions (Supplemental Table 1). Hepatocellular proliferation in the centrilobular zone was similar to controls following exposure to nitrapyrin at 4 days and was slightly increased at 7 days (up to 1.8-fold higher compared to controls) (Supplemental Table 1).

The trend of hepatocellular proliferation was similar, but more robust, in animals treated with 14 days of nitrapyrin by dietary administration when compared to 4 or 7 days of treatment. Specifically, in animals treated with 400 mg/kg/day there was a statistically identified increase in the proliferation index in midzonal, periportal, and total (panlobular) counts of 3.8-, 11.2-, and 4.9-fold, respectively, when compared to control animals (Table 4, Supplemental Table 1). In addition, animals treated with 250 mg/kg/day nitrapyrin had statistically identified increases in hepatocellular proliferation of 3.8-fold in the periportal region and 2.4-fold in the summed, panlobular count. Although not statistically significant, there was a 2.1- and 1.7-fold increase in labeled hepatocytes in the centrilobular and midzonal regions, respectively.

The induction of hepatocellular proliferation was completely resolved in animals fed control diet for 21 days following initial treatment with nitrapyrin for 14 days. There were no statistically significant

**Table 4**
Summary of Data Supporting Key Events #1 and #2 for Nitrapyrin-Mediated Liver Tumors.

| Treatment Period | Dose mg/kg/day (B6C3F1 strain) | Increased Relative Liver Weight (%) | Incidence Hepatocellular Hypertrophy | Panlobular Hepatocellular Proliferation (fold-change) | *Cyp1a1* – AhR (fold- change) | *Cyp2b10*–CAR (fold- change) | *Cyp3a11* – PXR (fold- change) | *Cyp4a10* – *PPAR* (fold- change) |
|---|---|---|---|---|---|---|---|---|
| 4 Days | 0 | – | 0/6 | – | 1 | 1 | 1 | 1 |
| | 75 | | | | | | | |
| | 250 | **27** | **6/6** | **3.5** | 2 | **370** | 2 | 3 |
| 7 Days | 0 | – | 0/6 | – | 1 | 1 | 1 | 1 |
| | 75 | – | 0/6 | −1.8 | 1 | 4 | 1 | 1 |
| | 250 | **20;34#** | **6/6; 6/6 #** | **2.0;3.9#** | 2 | **351;562#** | 1;2# | 7;4# |
| | 400 | **39** | **6/6** | **4.3** | 2 | **716** | 2 | 5 |
| 14 Days | 0 | – | 0/6 | – | 1 | 1 | 1 | 1 |
| | 75 | – | 0/6 | −1.1 | 1 | 4 | 1 | 1 |
| | 250 | **24** | **6/6** | **2.4** | 2 | **390** | 1 | 4 |
| | 400 | **49** | **9/9** | **4.9** | 2 | **1092** | 1 | 3 |
| 14 Days Plus 21-Days Recovery | 0 | – | 0/6 | – | 1 | 1 | 1 | 1 |
| | 75 | – | 0/6 | 1.0 | 1 | 2 | 1 | 1 |
| | 250 | – | 0/6 | 1.1 | 1 | 3 | 1 | 2 |
| | 400 | – | 0/9 | −2.9 | 1 | 3 | 1 | 1 |

#Combined results from two MoA experiments (7-day MoA experiment and strain bridging experiment). Minus "–" indicates not different than relevant control. Blank cell = No data. **Bolded** values were considered treatment-related effects

increases in hepatocellular proliferation at any dose level or in any of the hepatolobular regions analyzed. These data support a direct, causative effect of hepatocellular proliferation by nitrapyrin.

### 3.5. Nitrapyrin-induced nuclear receptor activation

Representative transcripts were selected to query several distinct pathways for nuclear receptor activation that may be involved in nitrapyrin-related pathologic changes in the murine liver, specifically AhR signaling (*Cyp1a1*), CAR signaling (*Cyp2b10)*, PXR signaling (*Cyp3a11)*, and PPAR-α signaling (*Cyp4a10)*. Following 4 or 7 days of nitrapyrin treatment, there was a clear, threshold-based biological signature of CAR activation when measured at the gene expression level based on *Cyp2b10* gene expression levels (Table 4). For instance, after 7 days of exposure *Cyp2b10* showed a clear, dose-related increase in transcript level compared to control of 4-, 351-, or 716-fold at 75, 250, or 400 mg/kg/day, respectively. After 4 days of exposure, *Cyp2b10* was similarly increased compared to control by 370-fold at 250 mg/kg/day. The results of the targeted gene expression analysis revealed similar CAR-related changes after 14 days of nitrapyrin administration when compared to those seen with 4 and 7 days of treatment. The AhR-responsive *Cyp1a1* and PXR-responsive *Cyp3a11* transcripts were essentially unchanged at all dose levels, indicating that nitrapyrin is not a direct activator for AhR or PXR (Table 4). The PPAR-α-inducible transcript *Cyp4a10* was essentially unchanged at the 75 mg/kg/day dose level and was increased 6.8-fold at 250 mg/kg/day and 5.2-fold at 400 mg/kg/day. This minor, non-dose-responsive increase of *Cyp4a10* at the highest doses suggests a potential non-specific or indirect result of general increased cytochrome induction. Prototypical PPAR-α activators induce *Cyp4a10* in the range of 500- to 1000-fold [11].

Data from the 21-day recovery groups indicated recovery from the gene expression changes that were induced following 14 days of nitrapyrin administration. Although the *Cyp2b10* levels in the recovery groups were elevated slightly when compared to controls, the values were minimal when compared to the significant increase after 14 days of treatment and before removal of the test material.

The dose response and temporality of *Cyp2b10* gene expression in male mice support a CAR- mediated MoA. CAR activation, as evidenced by *Cyp2b10* gene expression, as well as reversibility upon discontinuance of treatment, are considered essential data to support this MoA [4].

### 3.6. Hepatic cytochrome P450 protein content

To further assess the observed increase in *Cyp2b10* transcript following nitrapyrin treatment, analyses of the total cytochrome P450 content and Cyp2b10 protein content were performed. The analyses were performed for all samples in the 14-day treatment group, all samples in the 14-day treatment group and 21-day recovery samples. No significant alterations in total P450 levels were identified in any of the treatment groups compared to controls (Supplemental Data Table 2). Specific Cyp2b10 protein measurement was undertaken by western immunoblotting. After 14 days of nitrapyrin treatment there was a clear, dose-responsive increase in Cyp2b10 protein in mice treated with 250 or 400 mg/kg/day nitrapyrin (Supplemental Data Fig. 1). There was no apparent expression of Cyp2b10 protein in any of the animals fed control diet or 75 mg/kg/day nitrapyrin. These data are consistent with the gene expression analysis of *Cyp2b10*, where a notable increase in transcript was identified at 250 or 400 mg/kg/day nitrapyrin exposure.

### 3.7. Hepatic metabolic enzyme activity

Increased PROD enzyme activity is one of the primary alterations observed following CAR activation [12]. Therefore, a functional analysis of the Cyp2b10 enzyme was undertaken using 7-Pentoxy-Resorufin O-Deethylation (PROD) activity as the metric. The analysis was performed for all samples in the 14-day treatment group as well as all samples in the 14-day treatment group plus recovery. Surprisingly, there were no significant alterations in the PROD activities for any of the treatment groups compared to control after 14 days of nitrapyrin treatment or after 14 days of treatment and 21 days of recovery (Table 5).

### 3.8. Suicide inhibition of P450 enzyme activity

Given the paradoxical findings of increased *Cyp2b10* expression without a concomitant increase in PROD enzyme activity, experiments were conducted to understand the underlying mechanism of these results. A number of studies using structurally diverse compounds have previously identified inhibitors of specific cytochrome activity while increasing the expression of the relevant gene as well as the protein levels. Inhibition of cytochrome activity may occur as a result of substrate competition, metabolic intermediate complexation, or

**Table 5**
Summary of Liver Relative PROD Enzymatic Activity in Male Mice.

| Dose (mg/kg/day) | Mean PROD |
|---|---|
| 14-day Treatment | |
| 0 | 1.0 |
| 75 | 1.0 |
| 250 | 1.1 |
| 400 | 1.0 |
| 14-day Treatment and 21-day Recovery | |
| 0 | 1.0 |
| 75 | 1.0 |
| 250 | 1.1 |
| 400 | 1.1 |

Data are relative fold-change versus control activity (N = 6).

mechanism-based (suicide) inhibition [13,14]. For example, in the rat and dog the antibiotic chloramphenicol has been shown to be a mechanism-based inactivator of PB-induced (*i.e.,* Cyp2b family) cytochrome activity [15,16].

To investigate the role of suicide inhibition as a potential mechanism for the observed absence of PROD activity, *in vitro* experiments were conducted with phenobarbital (PB)-induced liver microsomes. In this system, PB induces PROD activity in microsomes *in vivo*, therefore, as PB is known not to be a suicide inhibitor of Cyp2b activity, exposure to additional PB to these isolated microsomes *in vitro* should not affect PROD activity in either direction. PROD activity should only be reduced following *in vitro* exposure to a suicide inhibitor of PROD, such as curcumin [17]. In this experiment, PB-induced microsomes were analyzed for PROD activity following treatment with PB (negative control), curcumin (positive control), or nitrapyrin (Fig. 2). As expected, PB had no effect on PROD activity. Curcumin had a dose-related inhibition of PROD activity of up to 63% at 40 μM, also consistent as a positive control. Finally, nitrapyrin inhibited PROD activity in a dose-related manner and up to 96% at 500 μM. These results indicated that nitrapyrin and/or its metabolites irreversibly inhibited Cyp2b10-mediated PROD activity of PB-induced microsomes. This elucidates the apparent inconsistency between protein levels and enzyme activity of nitrapyrin-treated livers. Hence, the lack of PROD activity in nitrapyrin-treated liver does not indicate a lack of CAR-mediated activity; in fact, nitrapyrin-mediated CAR-activation is supported by gene expression and protein levels of Cyp2b10.

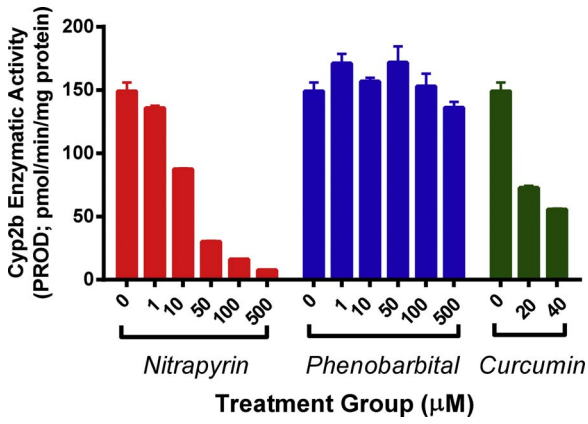


**Fig. 2.** Nitrapyrin Inhibition Cyp2b-Mediated Metabolic Activity of PB-Induced Rodent Liver Microsomes. PROD enzymatic activity was measured in PB-induced liver microsomes following addition of PB (negative control), curcumin (positive control), or nitrapyrin. All test materials were administered in 0.1% DMSO, which served as the solvent control. Data presented are from a representative experiment. Error bars represent standard deviation of technical replicates.

### 3.9. CAR is necessary for nitrapyrin-mediated hepatocyte proliferation

Nitrapyrin-mediated increases in *Cyp2b10* gene expression, liver weights and hypertrophy, and hepatocellular proliferation support a CAR-mediated MoA. In order to confirm this MoA, and eliminate alternative MoAs, CAR KO and WT mice were compared for their hepatic response to nitrapyrin.

Considering the CAR KO mouse is only available in the C57BL/6NTac strain, an experiment was first conducted to investigate potential differences in nitrapyrin responsiveness between B6C3F1 and C57BL/6NTac mice. B6C3F1 and C57BL/6NTac mice were fed diets targeting 0 and 250 mg/kg/day nitrapyrin for 4 or 7 days and liver weights, hypertrophy, hepatocellular proliferation, and gene expression were analyzed. B6C3F1 and C57BL/6NTac mice had similar effects for all endpoints analyzed, justifying the use of the C57BL/6NTac strain for these experiments (Summarized in Supplemental Table 3).

In the CAR KO experiment, C57BL/6NTac (wild type; WT) and CAR knockout (CAR KO) mice were fed diets targeting 0 or 250 mg/kg/day nitrapyin for 4 days to elicit the role of CAR in the hepatic response to nitrapyrin. Consistent with previous MoA studies, administration of the carcinogenic dose level of 250 mg/kg/day nitrapyrin to WT mice resulted in treatment-related increases in relative (20%) and absolute (24%) liver weights compared to untreated control WT animals. These liver weight changes were consistent with treatment-related histopathological alterations of: 1) a very slight increase in mitotic figures (hepatocytes in mitoses) (data not shown), 2) slight centrilobular/midzonal hepatocellular hypertrophy with increased cytoplasmic eosinophilia (Table 6), and 3) a very slight vacuolization of centrilobular/midzonal hepatocytes consistent with a minimal fatty change (Supplemental Table 4). Gene expression analysis of the liver indicated a robust increase in the *Cyp2b10*/CAR-associated transcript (494-fold higher than unexposed WT animals) consistent with direct activation of this nuclear receptor, but no significant increase in the transcripts associated with AhR, PXR, or PPAR-α (Table 6).

Unexpectedly, liver weights were increased in CAR KO mice following exposure to 250 mg/kg/day nitrapyrin (Table 6). However, while the liver weight changes in nitrapyrin-exposed CAR KO mice were similar to treated WT mice, the histopathological and molecular responses were markedly different. In CAR KO mice given 250 mg/kg/day nitrapyrin, the histopathological findings were limited to very slight hepatocellular hypertrophy with increased eosinophilia and very slight vacuolization, consistent with fatty change in centrilobular/midzonal hepatocytes. In contrast to nitrapyrin-treated WT mice, there was no indication of a proliferative response in CAR KO mice, as indicated by the absence of mitotic figures (Supplemental Table 4).

Furthermore, the gene expression response of the liver following nitrapyrin exposure in CAR KO mice was distinctly different than treated WT mice. Specifically, there was no biologically significant induction of the *Cyp2b10*/CAR-associated transcript (2-fold vs. 494-fold in WT mice). Similar to the response to nitrapyrin in WT mice, the *Cyp3a11*/PXR- and *Cyp4a10*/PPAR-α-associated transcripts were unchanged, indicating no clear activation of those pathways. However, the *Cyp1a1*/AhR-associated transcript was induced to a greater degree in treated CAR KO mice compared to treated WT mice. It has been established that there is extensive crosstalk between nuclear receptors and transcription factors [18–20]. The presence of various nuclear receptors that have different yet overlapping specificity for ligands allows for an organism to respond to exposure to xenobiotics more effectively. Competition for cofactors and other cellular transcription resources may account for differences in CYP induction following exposure to xenobiotics. In fact, for AhR specifically, reduction in the amount of cofactor and increasing the amount of competing non-AhR binding proteins reduces *Cyp1a1* gene expression [21]. For nitrapyrin, in the absence of a functional CAR signaling pathway, the AhR pathway is weakly activated presumably as an adaptive response due to the lack of competition for cofactors and co-activators. Importantly, the magnitude

**Table 6**
Comparison of WT and CAR KO Mice Response Following 4 Days of Nitrapyrin Exposure.

| Dose mg/kg/day (strain) | Increased Relative Liver Weight (%) | Incidence Hepatocellular Hypertrophy | Panlobular Hepatocellular Proliferation (fold-change) | *Cyp1a1* – AhR (fold change) | *Cyp2b10*–CAR (fold change) | *Cyp3a11* – PXR (fold change) | *Cyp4a10* – *PPAR* (fold change) |
|---|---|---|---|---|---|---|---|
| 0 (C57BL/6) | – | 0/6 | – | 1 | 1 | 1 | 1 |
| 250 (C57BL/6) | **24** | **6/6** | **1.5** | 3 | **494** | 2 | 2 |
| 250 (CAR-KO) | **23** | **6/6** | 0.8 | **104** | 2 | 3 | 3 |

Minus "–" indicates not different than relevant control. Blank cell = No data. **Bolded** values were considered treatment-related effects

of induction (104-fold) in the CAR KO mouse was substantially less than that seen with a prototypical AhR activator (up to 5000-fold for a similar exposure period) [22]. This supports that AhR activation observed in the CAR KO animals was likely an adaptive liver enzyme response due to the absence of a functional CAR signaling pathway. The observed changes in liver histopathology in CAR KO mice also likely reflect activation of an adaptive enzyme response.

Finally, hepatocellular proliferation was evaluated in WT and CAR KO mice given it is the critical event in nitrapyrin-induced liver tumor formation (Table 6, Supplemental Table 5). Similar to what was observed in the strain comparison experiment, there was a clear increase in hepatocellular proliferation as measured by BrdU incorporation in WT mice following nitrapyrin administration. However, no increase in proliferation was observed in CAR KO animals. This is consistent with the previously discussed differences (increase in mitotic figures) between CAR KO and WT responses to nitrapyrin exposure. These results, combined with the observed liver weight and histopathologic changes in CAR KO mice, suggest that while nitrapyrin exposure still confers some form of hypertrophic response in the absence of CAR, it does not confer the critical hyperplastic response (proliferation) that is necessary for tumor formation in this MoA.

### *3.10. Nitrapyrin elicited increased proliferation in mouse but not human hepatocytes*

Considering CAR activation causes liver tumors and hepatocyte proliferation in mice but not in humans [23], and our data support that CAR is necessary for nitrapyrin-mediated liver proliferation in mice, we tested the hypothesis that nitrapyrin increases proliferation of primary mouse hepatocytes but not primary human hepatocytes. A primary hepatocyte culture system was used to evaluate proliferation of mouse and human hepatocytes in response to nitrapyrin. The adequacy of the experimental conditions for induction of cell proliferation in mouse and human hepatocytes was evaluated by employing a positive control chemical (25 ng/μl epidermal growth factor (EGF)) that is known to increase primary hepatocyte proliferation [24].

Primary CD-1 mouse hepatocytes and primary human hepatocytes were first assessed for cytotoxicity following exposure to nitrapyrin. Hepatocyte proliferation was scored at test material concentrations causing less than 30% cytotoxicity. EGF did not alter the viability of either mouse or human primary hepatocytes. Cytotoxicity above the 30% threshold level for examination of cell proliferation was observed in mouse primary hepatocytes exposed to nitrapyrin concentrations ≥30 μM. Nitrapyrin did not cause cytotoxicity of human hepatocytes above the 30% threshold level for examination of cell proliferation at concentrations up to and including 100 μM.

Nitrapyrin caused a treatment-related increase in proliferation of mouse primary hepatocytes but not human primary hepatocytes (Fig. 3). EGF increased cell proliferation approximately four- to five-fold in mouse and human primary hepatocytes, demonstrating the similar responsiveness of mouse and human primary hepatocyte cultures to a proliferative stimulus. A treatment-related three-fold increase in proliferation of mouse hepatocytes was observed after exposure to 10 μM nitrapyrin. Nitrapyrin did not increase the proliferation of human hepatocytes at concentrations up to and including 100 μM (10x the concentration necessary to induce proliferation in mouse hepatocytes). Therefore, under the conditions of this experiment, nitrapyrin-induced mouse hepatocyte proliferation was consistent with the *in vivo* response in mice; however, human hepatocytes did not proliferate in response to nitrapyrin administration. This is consistent with the PB-like (CAR activation) hepatocarcinogenic response in rodents and humans, where upon exposure to a CAR activator different enzymes are induced in humans compared to rodents and, more importantly, there is no evidence that this results in an increase in cell proliferation in the human liver.

## 4. Discussion

An essential factor in establishing a MoA is an evaluation of the evidence using the Bradford Hill criteria, indicating the strength, consistency, and specificity of the data supporting the key events, along with biological plausibility and coherence. The relevant molecular and pathological endpoints for nitrapyrin-induced liver effects in mice are summarized in Table 7. The table is organized such that the metrics are consistent with the established key events of nuclear receptor-mediated rodent hepatocarcinogenesis [25,26,23]. Nuclear receptor- (i.e. CAR-) mediated induction of cytochrome P450 enzyme activity is a well-known and characterized MoA for rodent hepatocarcinogens and PB is a standard example [27,28,4,23]. The mechanistic and apical data points clearly demonstrate Key Event #1 (activation of CAR), followed by Key Event #2 (increased hepatocellular proliferation), ultimately leading to the apical endpoint (increased hepatocellular tumors).

### *4.1. Strength, consistency and specificity of association of effects with key events*

Key Event #1 for the nitrapyrin liver tumor MoA is defined as activation of the nuclear receptor (i.e., CAR), which was measured by induction of the Cyp2b10 transcript as well as the protein. Experimental evidence support the hypothesis that the lack of elevated PROD metabolic activity typically observed with CAR activation was attributed to mechanism-based (suicide) inhibition of the enzyme. Supportive, associative key events to #1 include increased liver weight and microscopic hepatocellular hypertrophy, which were increased in a dose-dependent manner following nitrapyrin exposure. Key Event #2 is characterized as an increase in hepatocellular proliferation. Exposure at or above the tumorigenic dose of nitrapyrin for males (250 mg/kg/day) consistently produced characteristic causal and associative events for this MoA in several different experiments and in multiple strains of mice. The key events demonstrate reversibility upon discontinuance of treatment, a fundamental element typical for the nuclear receptor mediated liver tumor MoA. Studies utilizing CAR KO and WT mice corroborated that increased hepatocellular proliferation, a key event for nitrapyrin-induced liver tumors, was dependent on CAR activation, thereby confirming the proposed MoA. Furthermore, *in vitro* experiments conducted with mouse and human primary hepatocytes demonstrated that nitrapyrin exposure induced cellular proliferation (Key Event #2) in rodent cells but not humans, consistent with the fact that this MoA is not relevant for human health risk assessment given qualitative differences between species.

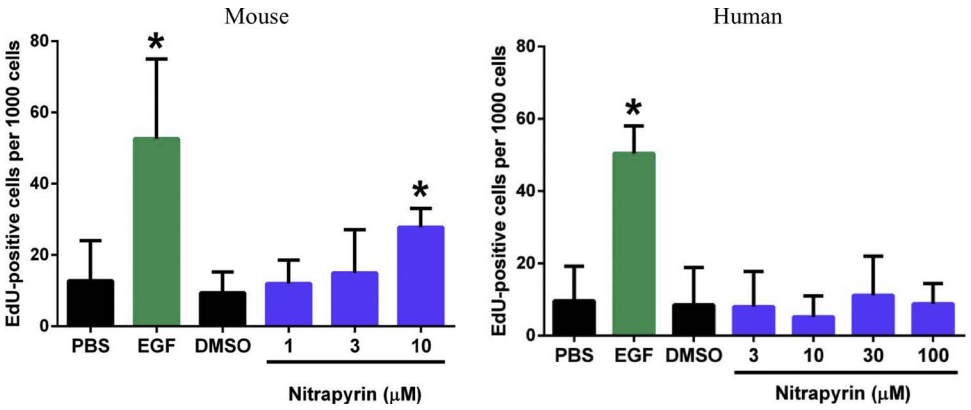


**Fig. 3.** Mouse and Human Primary Hepatocyte Response to EGF or Nitrapyrin Exposure.
Compared to vehicle control (PBS), 25 ng/ml EGF exposure caused a statistically-identified approximately five-fold increase in human hepatocyte proliferation and approximately four-fold increase in mouse hepatocyte proliferation. Compared to vehicle control (DMSO), nitrapyrin exposure increased mouse hepatocyte proliferation, which was approximately three-fold and statistically-identified at 10 μM. Compared to vehicle control (DMSO), nitrapyrin exposure did not alter human hepatocyte proliferation at any concentration tested. Data shown are the mean ± standard deviation. *p-value < 0.05. n = 2 biological replicates/treatment group.

The key events for nitrapyrin show clear, threshold, dose-responsive alterations and provide informative, temporal-specific characterization of nitrapyrin-induced liver effects, which are consistent with a CAR-mediated MoA (Table 7).

Taking into consideration both nitrapyrin mouse cancer studies as well as the shorter-term MoA data, it is clear that the lower doses of nitrapyrin (5, 25, or 75 mg/kg/day) were not associated with CAR activation, hypertrophy, hyperplasia, or hepatocellular tumors while higher dose levels (≥125 mg/kg/day for females, ≥250 mg/kg/day for males) resulted in CAR activation, hypertrophy, hyperplasia, and hepatocellular tumors. Notably, the data generated by the CAR KO and WT mice experiment clearly reinforce that CAR activation is necessary for the critical key event of hepatocellular proliferation in the pathogenesis of nitrapyrin-induced rodent hepatocellular tumors. Overall, the experiments conducted with nitrapyrin provide a strong and consistent association that links the nitrapyrin-induced key events to a specific MoA that results in mouse hepatocellular tumors.

### 4.2. Biological plausibility and coherence

Dietary administration of nitrapyrin to mice results in early key events (CAR activation and hepatocellular proliferation) that can lead to hepatocellular tumors after prolonged exposure to relatively high dose levels (125 mg/kg/day for females or 250 mg/kg/day for males). The early key events are reversible upon cessation of treatment with nitrapyrin, which is consistent with the defined P450 enzyme induction MoA. The MoA described for nitrapyrin is consistent with both the well-known MoA for PB in rodents [27,28,4,23] and the current understanding of cancer biology. The observed lack of PROD activity in nitrapyrin-treated livers was attributable to nitrapyrin irreversibly inhibiting Cyp2b10-mediated PROD activity via suicide inhibition, and elucidates the apparent inconsistency between protein levels and enzyme activity. Indeed, given the evident, dose-dependent key events that are consistently observed across multiple studies and mouse strains, in conjunction with the absence of these key events in CAR KO mice, the data for nitrapyrin are strongly compatible with this non-genotoxic liver tumor MoA. Critically, human primary hepatoctyes did

**Table 7**
Relevant Molecular and Pathological Endpoints for Nitrapyrin-Induced Liver Effects in Male Mice.

Temporal →

Dose ↓

| Dose for 2-Yr Studies (mg/kg/day) | *Key Event 1* | | *Key Event 2* | Key Events After Recovery | *Apical Endpoints*: Increased Hepatocellular Tumors and Altered Foci |
|---|---|---|---|---|---|
| | Causal: CAR Activation (Cyp2b10 Transcript & Protein) | Associated: Increased Liver Weights/ Hypertrophy | Hepatocellular Proliferation | | |
| | 4-14 Days | 4-14 Days | 4-14 Days | 14 Days Plus 21 Days Recovery | 2 Yrs |
| 5 | | - | | | - |
| 25 | | - | | | - |
| 75 | - | - | - | - | - |
| 125 | | +,+[#] | +,+[#] | | - |
| 250 | + | + | + | - | + |
| | | | | | |
| 250 CAR KO | - | +[@] | - | | |

+ Indicates effect present, − indicates effect absent at indicated duration of treatment. Blank cell = No data. [#]Data only from 1-year interim sacrifice. [@]Suggestive of compensatory changes in CAR KO mice.

not exhibit a proliferative response (Key Event #2) following exposure to levels of nitrapyrin that significantly increased proliferation in mouse hepatocytes. One weakness in the *in vitro* experiment was the low number of replicates as the use of fresh primary hepatocytes from human donors was limited by the hepatocytes available for the experiment at the time. Another weakness is the lack of gene expression data from the primary hepatocytes. Prior scientific evidence supports that exposure to CAR-activators in both humans and mice results in activation of the CAR/PXR receptors. However, exposure results in induction of different enzymes in humans compared to rodents, and an increase in hepatocellular proliferation is only observed in rodents and not humans. While a data gap remains that it is still unknown what enzymes and genes are necessary to induce hepatocellular proliferation *in vitro* following exposure to nitrapyrin, our experiment was designed to focus on the critical key event, an increase in hepatocellular proliferation. Given the current state of the science suggests that a hepatocarcinogenic response in rodents for compounds which have data to support a CAR-mediated (i.e., PB-like) MoA is not relevant to humans, the primary hepatocyte experiment acts as supportive evidence by providing nitrapyrin-specific data for the critical key event, an increase in hepatocellular proliferation.

### 4.3. Assessment of postulated nitrapyrin mouse liver tumor MoA

The data for nitrapyrin support a threshold-based, dose responsive MoA for hepatocellular adenomas and carcinomas in mice. The MoA demonstrated for nitrapyrin is consistent with the CAR nuclear receptor-mediated induction of cytochrome P450 enzyme activity, of which PB is a classic example [27,28,4,23]. The data for nitrapyrin are consistent with the current understanding of biology with this non-genotoxic MoA in the rodent liver. *In vitro* experiments support that the nitrapyrin MoA is not relevant to humans, given that Key Event #2 occurs in a clear, dose-responsive manner in primary mouse hepatocytes but is absent in primary human hepatocytes following nitrapyrin administration. This supports the conclusion that the proposed MoA for nitrapyrin is not relevant for human health risk assessment given qualitative differences between rodents and humans that are archetypal for this well-established MoA. The weight of evidence gathered from *in vitro* and *in vivo* studies collectively indicates that the nitrapyrin MoA is via CAR nuclear receptor activation and that all other MoAs can be reasonably and confidently excluded (see supplemental material for additional discussion on exclusion of alternative MoAs).

### 4.4. Nitrapyrin mouse liver tumor human relevance framework

#### 4.4.1. Question 1. Is the weight of evidence sufficient to establish the MoA in animals?

The answer is yes. The data presented herein support that the MoA for nitrapyrin-induced mouse liver tumors is congruent with the well-established MoA of CAR nuclear receptor activation, similar to the PB-like MoA [27,28,26,4,23]. The relevant molecular and pathological endpoints for nitrapyrin-induced liver effects in mice via this MoA are consistently supported across several repeat-dose studies and carcinogenicity studies and there is clear correlation of the dose responses between the MoA data and the hepatocellular tumors. Key Event #1 for the nitrapyrin liver tumor MoA is defined as activation of the CAR nuclear receptor which is surrogately measured by induction of the Cyp2b10 transcript as well as the protein. Key Event #1 is accompanied by mechanism-based (suicide) inhibition of the enzyme PROD, which elucidates the unexpected lack of elevated of PROD metabolic activity. Supportive, associative key events to Key Event #1 include increased liver weight and microscopic hepatocellular hypertrophy. Key Event #2 is an increase in hepatocellular proliferation at tumorigenic dose levels. Importantly, both of these key events demonstrate reversibility upon discontinuance of treatment and show consistency across multiple studies. The key events for nitrapyrin show clear, thresholded, dose-responsive alterations and are consistent with a CAR-mediated MoA. The absence of effects associated with these key events in CAR KO mice exposed to a tumorigenic dose of nitrapyrin confirmed that CAR is necessary for nitrapyrin-mediated liver effects that lead to tumorigenesis. Furthermore, other possible MoAs are considered unlikely based on an analysis of all relevant data for nitrapyrin.

#### 4.4.2. Question 2. Can human relevance of the MoA be reasonably excluded based on fundamental qualitative differences in key events between experimental animals and humans?

The answer is yes. The key events for this MoA in rodents include activation of CAR and leads to an increase in hepatocellular proliferation and subsequent induction of proliferative lesions in the liver including foci, adenomas, and carcinomas. Activation of CAR in rodents leads to activation of genes that leads to hepatocellular proliferation, which is critical for development of liver tumors [27,4,23]. While exposure to PB, a prototypical CAR activator, in humans results in activation of the CAR/PXR receptors and induction of CYP enzymes, different enzymes are induced in humans compared to rodents and, more importantly, there is no evidence that this results in an increase in cell proliferation in the human liver [29,23]. Consistent with this prior evidence, while nitrapyrin exposure to primary mouse hepatocytes led to increased proliferation, this effect was not observed in primary human hepatocytes, thereby substantiating that the MoA for nitrapyrin-mediated liver tumors in mice is not relevant for human health risk assessment [23]. Regarding cancer risk for humans, extensive epidemiologic studies in humans with PB at exposure levels that are comparable to those in rodent bioassays conclude that PB exposure at these levels does not result in increased cancer risks [27,30]. Based on the MoA assessment, PB is not a hepatocarcinogen in humans. Furthermore, a hepatocarcinogenic response in rodents for compounds which have data to support a CAR-mediated (i.e., PB-like) MoA, in this case nitrapyrin, is not likely to be carcinogenic to humans [28,23]. On this basis, the mouse liver tumors associated with administration of higher dose levels of nitrapyrin would not pose a cancer hazard to humans.

A concordance analysis for the response in mice (rodents) and humans of the key events for the nitrapyrin CAR-mediated MoA is presented in Table 8.

**Table 8**
Evidence of Key Events for the Nitrapyrin (CAR nuclear receptor activation) MoA in Rodents and Humans.

| Key Events | | Evidence in Rodents | Evidence in Humans |
|---|---|---|---|
| **#1** | Activation of CAR | Yes | Yes |
| | CYP Enzyme Induction Associated Liver Hypertrophy | Yes; unclear if critical step or indicator of activity secondary to CAR activation | Yes; different enzymes induced compared to rodents |
| **#2** | Hepatocellular Proliferation | Yes | No evidence of increased cell proliferation in the human liver (limited *in vitro* and *in vivo* data). |
| **Apical Endpoints** | Selective Clonal Expansion (Foci) | Yes | No; none reported |
| | Occurrence of Hepatocellular Tumors | Yes | No; based on epidemiological data |

*4.4.3. Question 3. Can human relevance of the MoA be reasonably excluded based on quantitative differences in either kinetic or dynamic factors between experimental animals and humans?*

Given that human relevance of the experimental animal MoA can be reasonably excluded on the basis of qualitative differences in key events (Question 2), a quantitative assessment of kinetic or dynamic factors is not necessary.

## 5. Conclusions

The investigation for the nitrapyrin-induced mouse liver tumor MoA demonstrated dose, temporal, apical, and molecular concordance with a CAR-mediated effect. Specifically, the MoA key events are threshold-based and hepatocellular tumor formation in mice does not occur at or below 75 mg/kg/day. Alternative MoAs were excluded based on plausibility and concordance, along with a CAR KO mouse experiment which demonstrated that CAR was necessary for a nitrapyrin-mediated increase in hepatocellular proliferation. Furthermore, the PB-like CAR-mediated MoA for hepatocarcinogenesis has been well established as not being relevant to humans [23]. The nitrapyrin MoA may provide an interesting and applicable case study for next-generation chemical risk assessment, such as the ILSI Health and Environmental Sciences Institute's (HESI's) RISK21 approach. Understanding the MoA of a chemical is important for cumulative risk assessment, as chemicals that share the same MoA belong to the same cumulative assessment group [31]. Furthermore, as described in a publication by Simon et al., MOA/HRF in conjunction with Quantitative Key Events/Dose-Response Framework is suggested to reduce uncertainty in risk assessments [32]. Indeed, for nitrapyrin the key events are threshold based, as expected for nuclear receptor mediated MoAs, which suggests nonlinear extrapolation would be appropriate for quantitative risk assessment. Regarding human relevance for hazard identification, as anticipated for a CAR-mediated MoA, hepatocellular proliferation was established both *in vivo* and *in vitro* in mice, but was not observed in primary human hepatocytes following nitrapyrin exposure *in vitro*. The results of the studies presented in this manuscript support the conclusion that nitrapyrin-induced mouse liver tumors are mediated by CAR activation and are therefore not relevant to humans.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2017.10.007.

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