Characterization of Murine Hepatocarcinogenesis Induced by the Nitrification Inhibitor Nitrapyrin: Mode of Action, Human Relevance Framework, and Risk Assessment Implications

RASS Webinar
February 10, 2016

Kerry Hastings, D.Phil — Risk Assessor, Dow AgroSciences
Jessica LaRocca, PhD — Toxicologist, Dow AgroSciences
Matthew LeBaron, PhD — Toxicologist, Dow Chemical

DOW® Dow AgroSciences

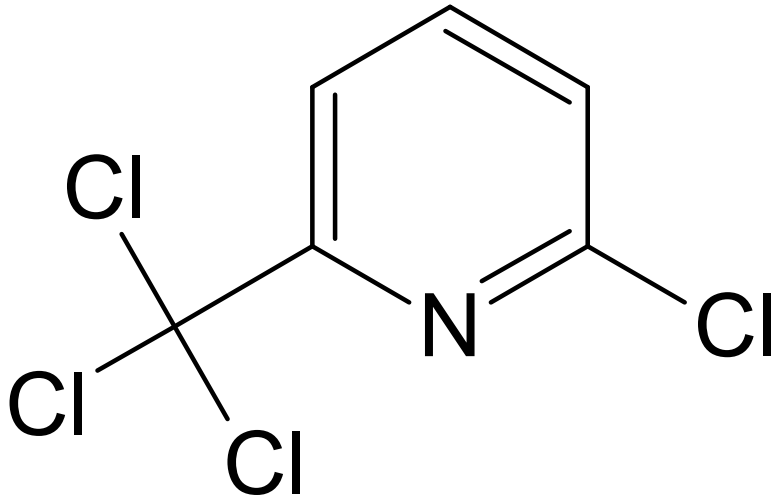
Solutions for the Growing World

Agenda

- Background/overview
- Liver MoA evaluation
- Application of the HRF
- Risk assessment overview

Nitrapyrin

- (2-chloro-6-trichloromethyl pyridine)
- Registered in the US since 1974
- Nitrification inhibitor
- Nitrogen stabilizer



Nitrate Loss

Ammonium

NH_4

(+) positive charge

Nitrosomonas bacteria →

Nitrite

NO_2

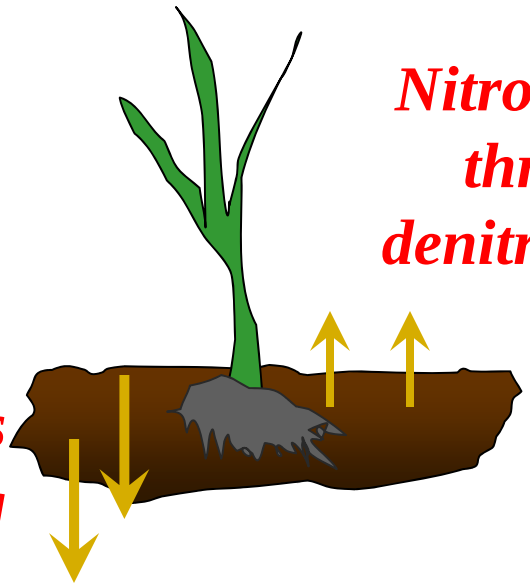
(-) soil negative charge

Nitrobacter bacteria →

Nitrate

NO_3

(-) negative charge



Nitrogen loss through denitrification

Nitrogen loss through nitrate leaching

Nitrapyrin Stabilizes Nitrogen

Ammonium

NH_4

(+) positive charge

Nitrosomonas bacteria

Nitrite

NO_2

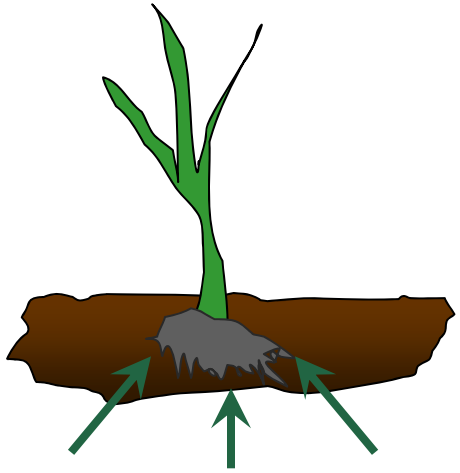
(-) soil negative charge

Nitrobacter bacteria

Nitrate

NO_3

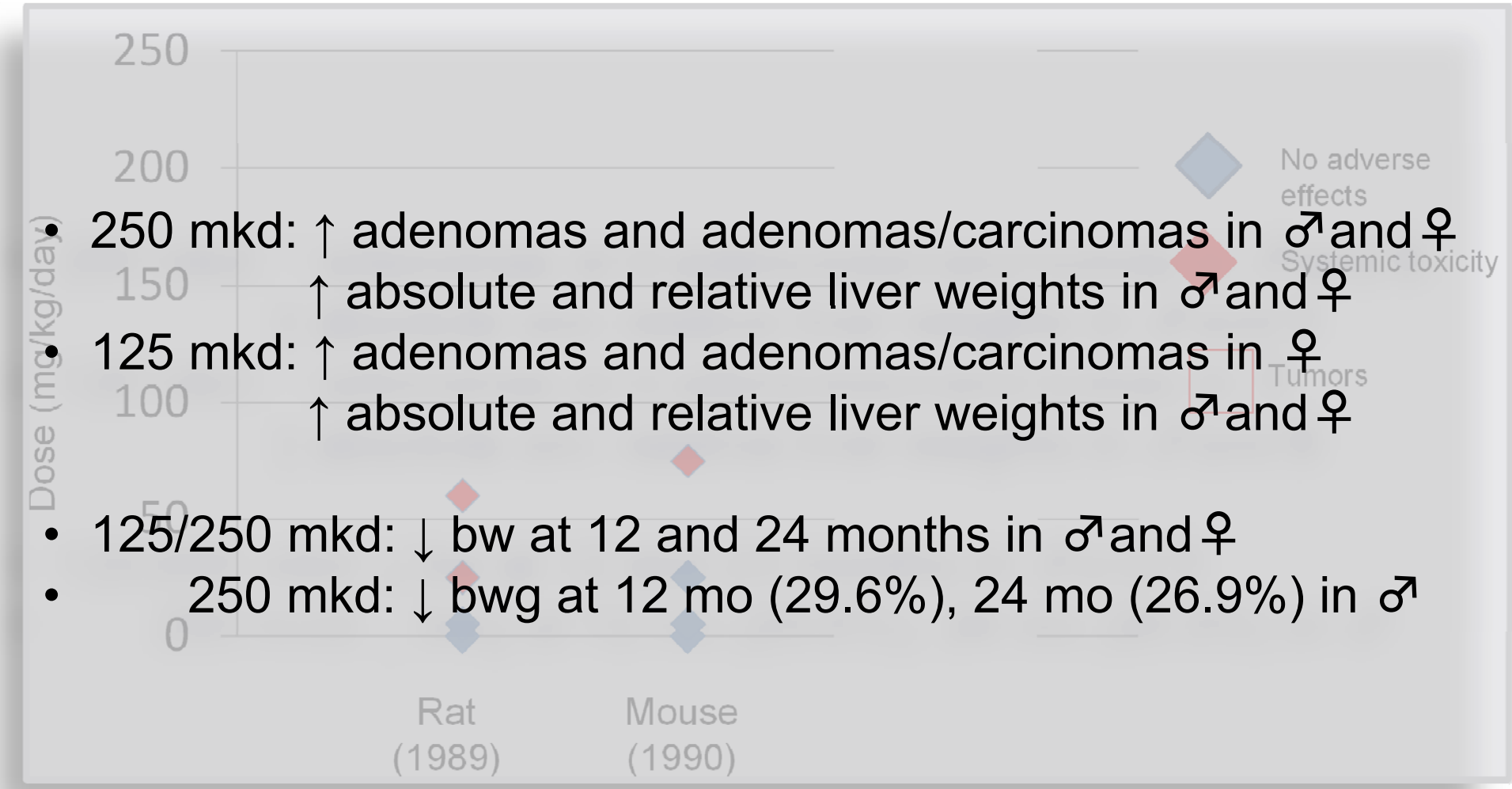
(-) negative charge



STABILIZED NITROGEN

Readily Available

Liver Tumors



- 250 mkd: ↑ adenomas and adenomas/carcinomas in ♂and♀
  ↑ absolute and relative liver weights in ♂and♀
- 125 mkd: ↑ adenomas and adenomas/carcinomas in ♀
  ↑ absolute and relative liver weights in ♂and♀

- 125/250 mkd: ↓ bw at 12 and 24 months in ♂and♀
- 250 mkd: ↓ bwg at 12 mo (29.6%), 24 mo (26.9%) in ♂

What is the MoA for nitrapyrin-mediated mouse liver tumors and is it relevant to humans?

Approach:

- Question 1: Can we assimilate/generate data to define an MoA for nitrapyrin-mediated mouse liver tumors?

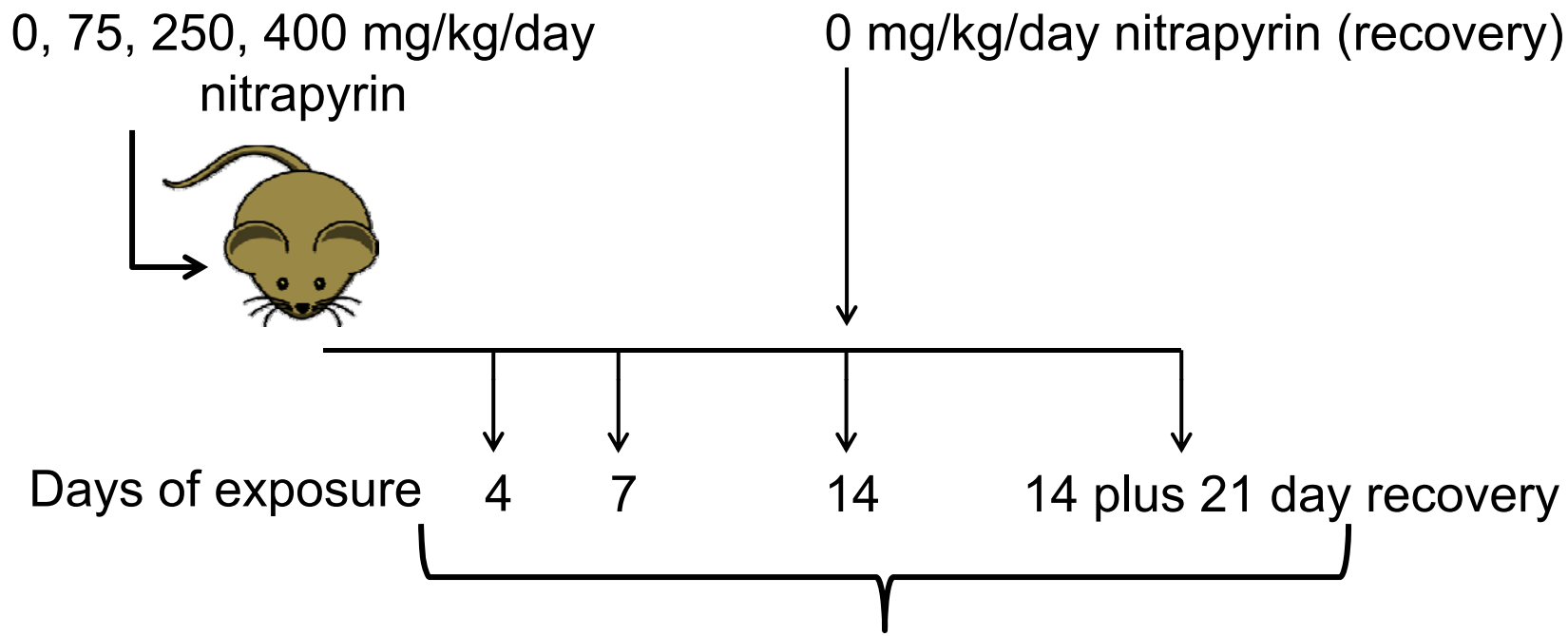
- Question 2: Can we exclude other MoAs?

- Question 3: Is the MoA relevant to humans?

Question 1: MoA

- Assessed potential MoAs by evaluating previous toxicity data
- Generated additional MoA data to rule in or rule out nuclear receptor activation
  - Key events (NR activation, proliferation)
  - Recovery after removal of treatment

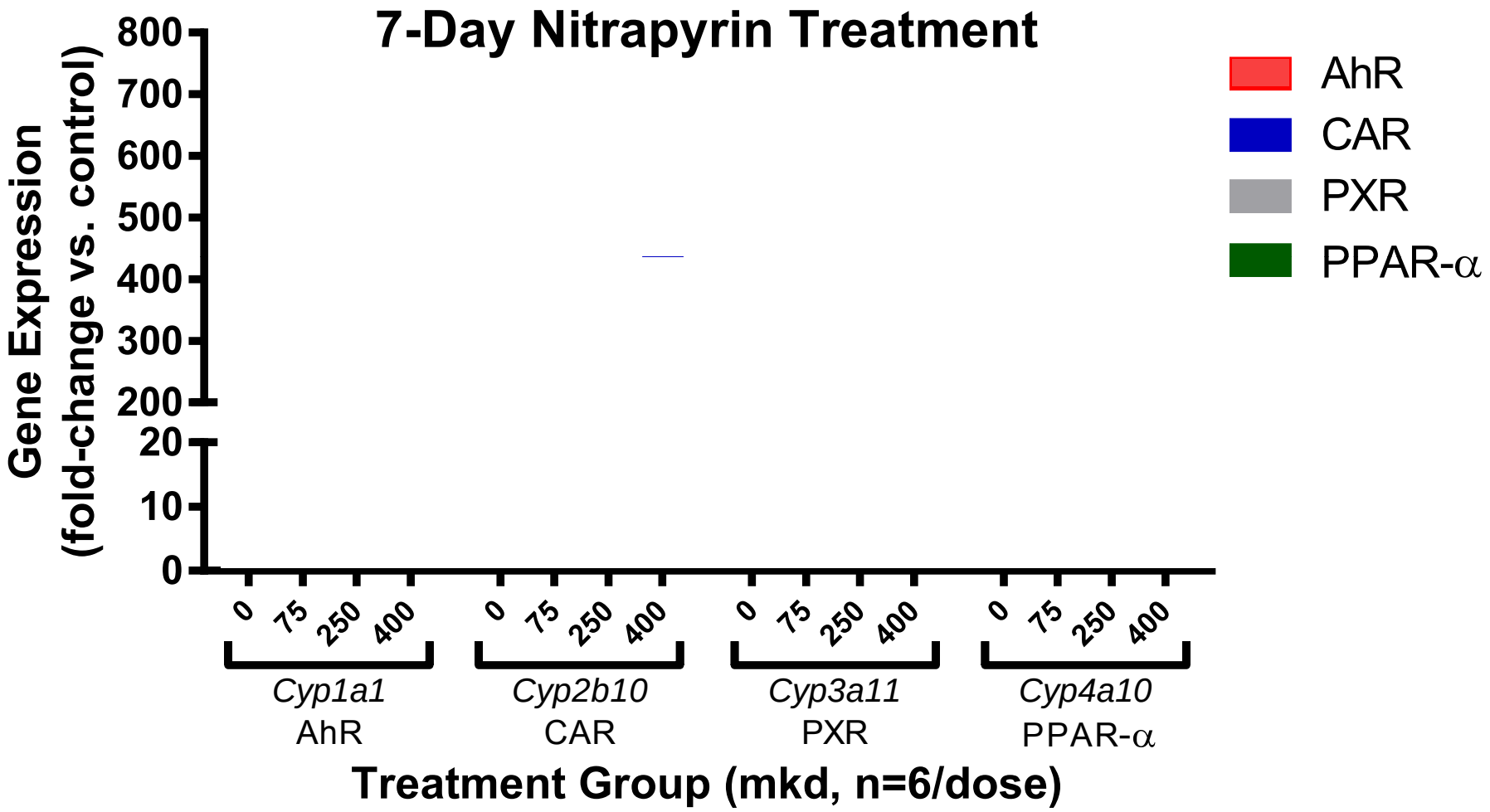
Nitrapyrin Liver MoA Study



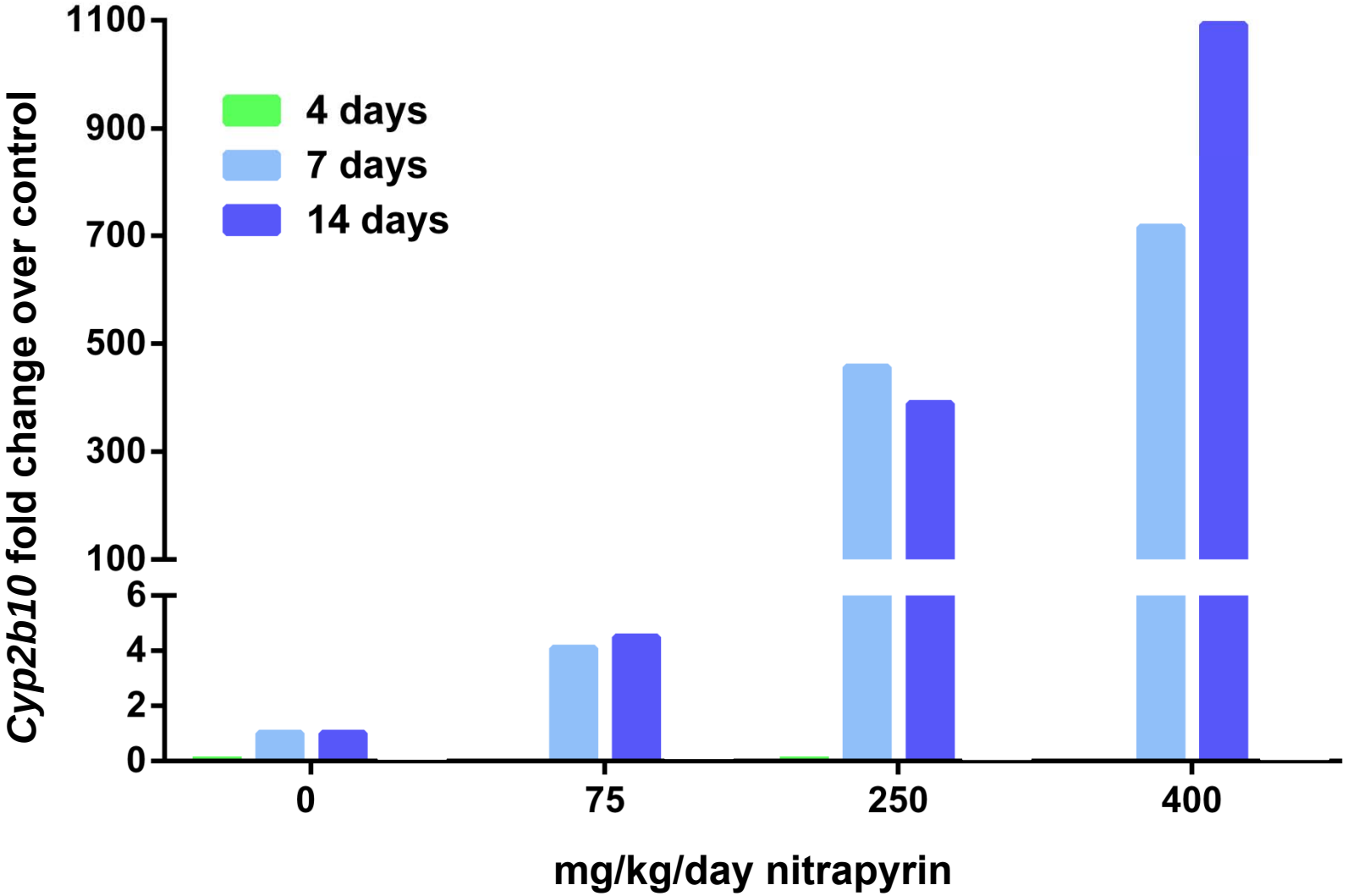
Endpoints:

- Gene expression of biomarkers of NR activation (AhR, CAR, PXR, PPAR-α)
  - Protein and enzyme activity
- Liver weight and histopathology
- Hepatocellular proliferation (via BrdU osmotic pumps)
- Assess recovery after treatment cessation

Key Event #1: NR Activation



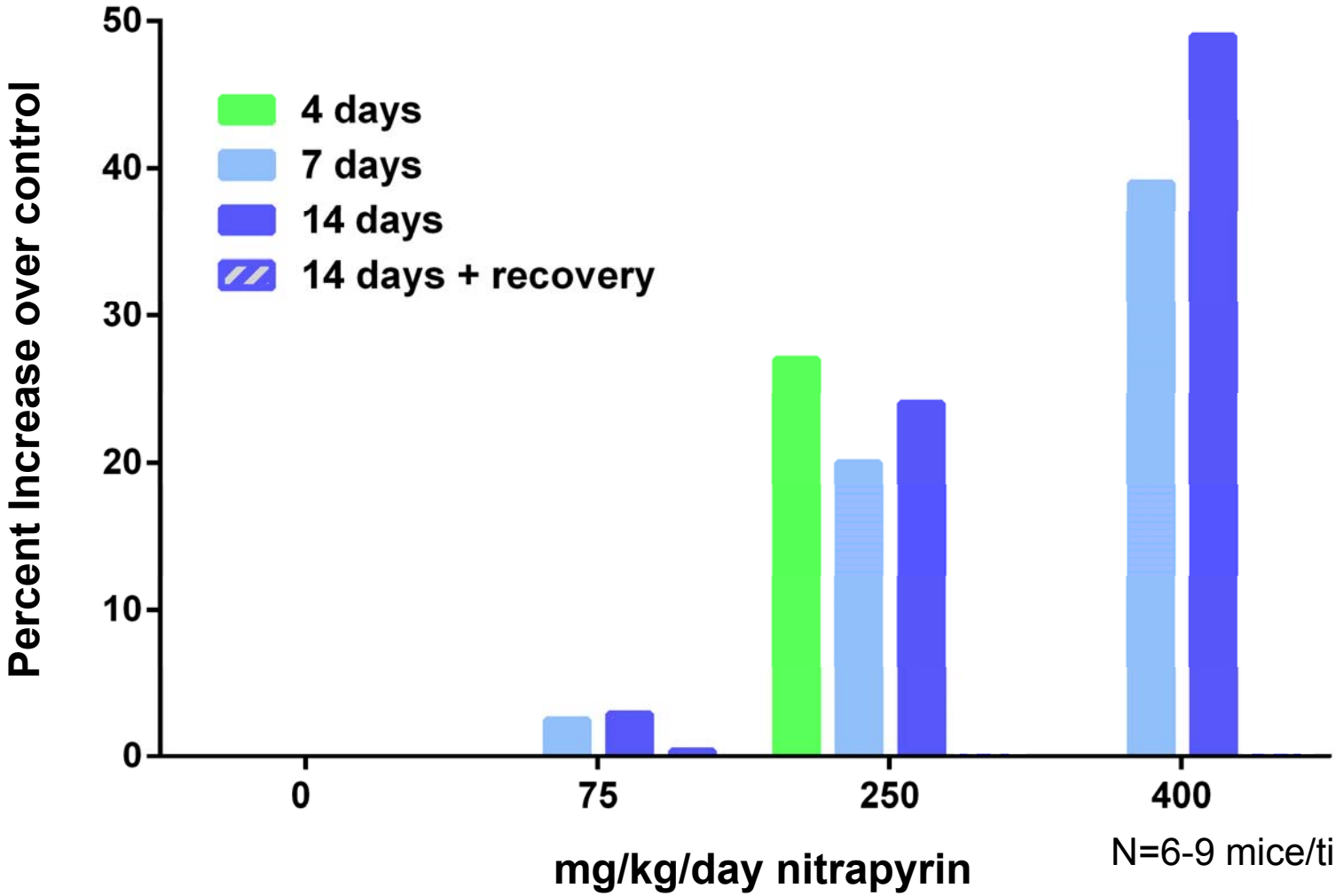
Key Event #1: CAR Activation (*Cyp2b10*)



Dow AgroSciences

N=6-9 mice/timepoint/dose

Key Event #1: Liver Weight Increases

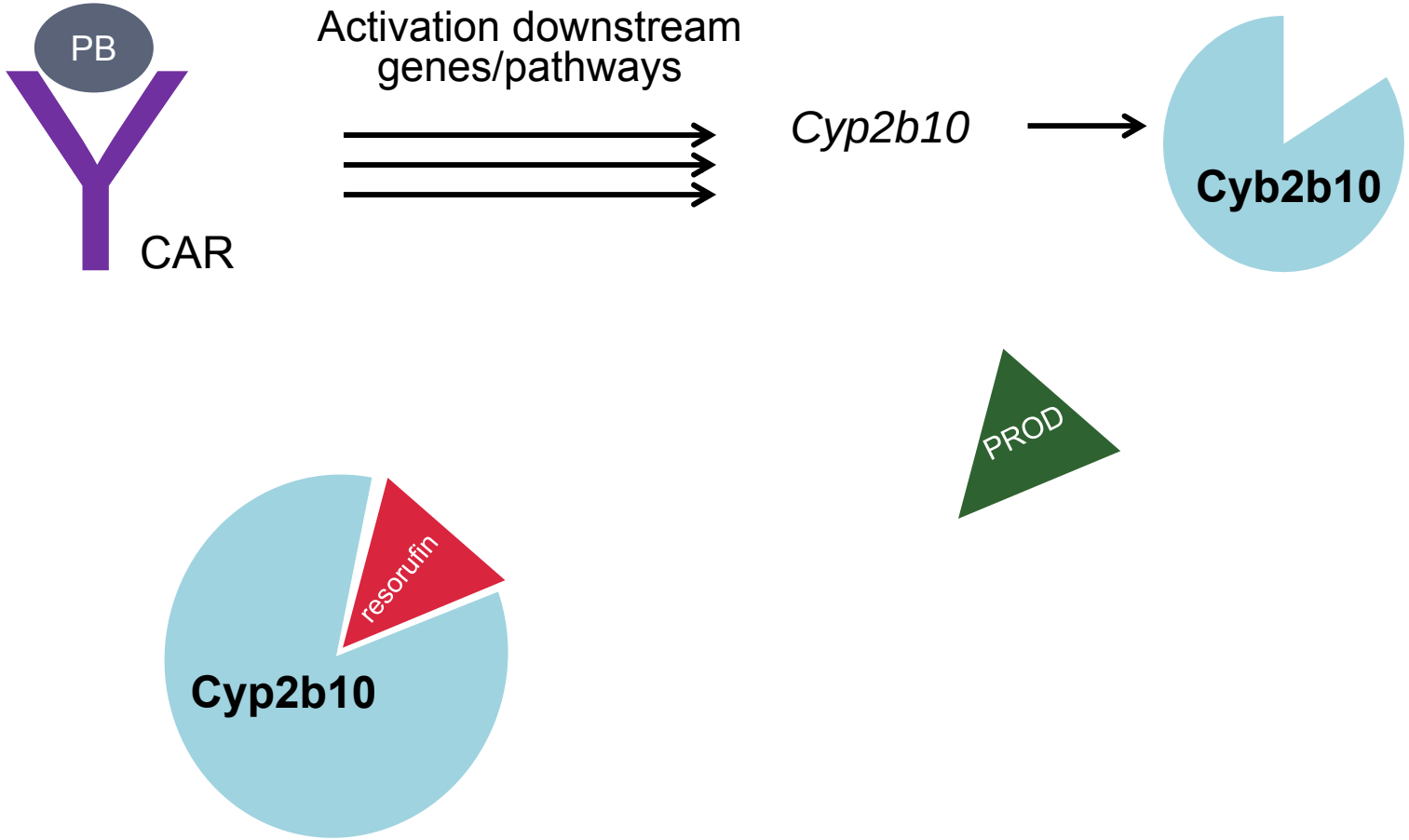


N=6-9 mice/timepoint/dose

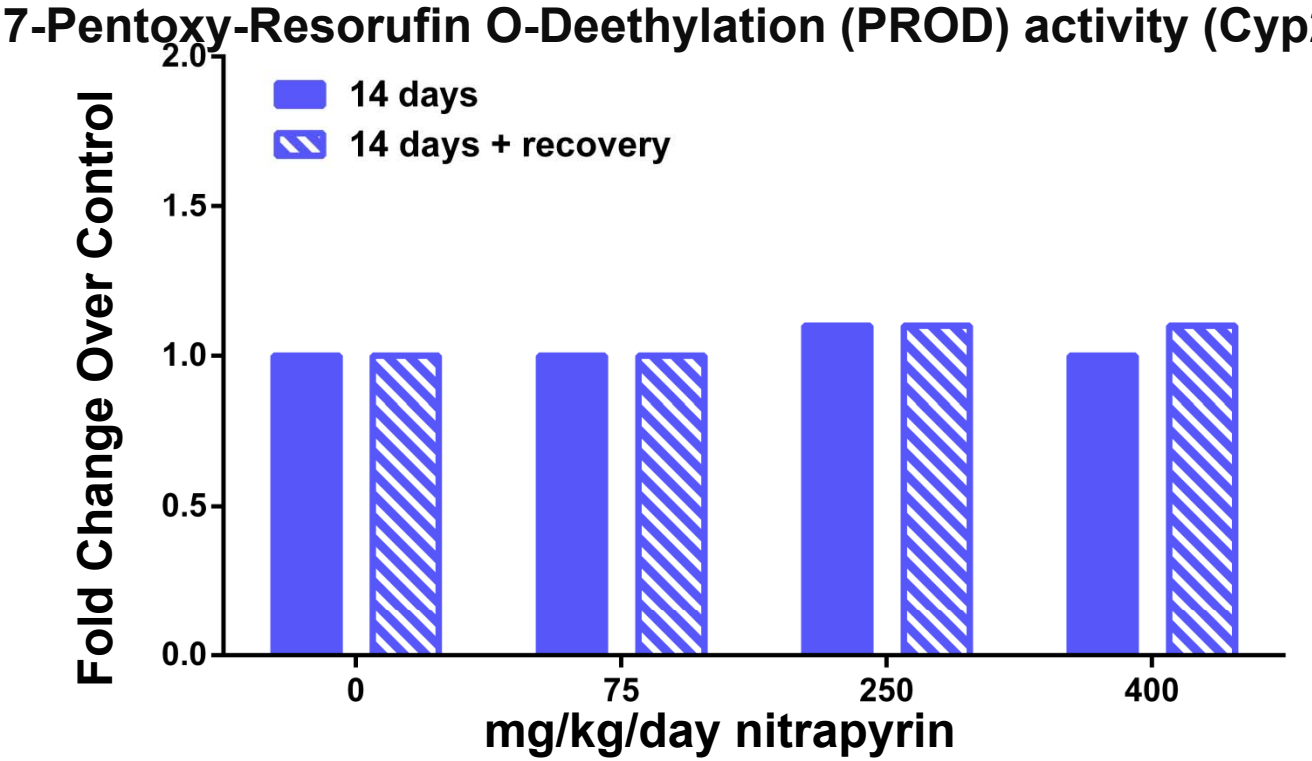
Similar responses for liver hypertrophy

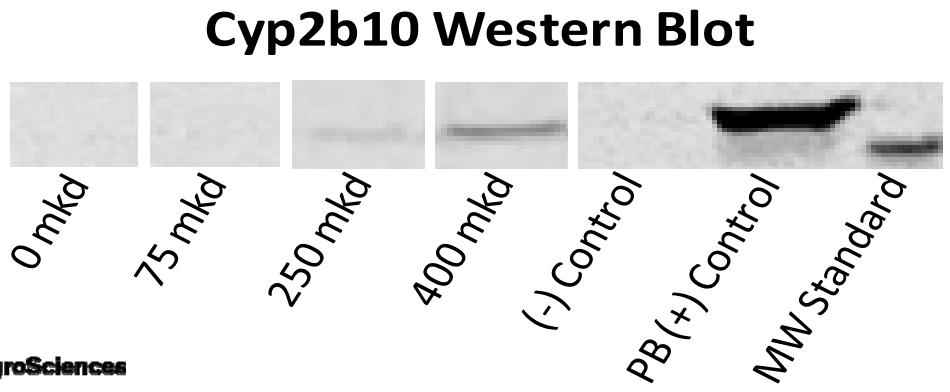
Cyp2b10 and Expected PROD Activity

7-Pentoxy-Resorufin O-Deethylation (PROD) activity (Cyp2b10-dependent)



Key Event #1: CYP Enzyme Induction





N=6 mice/timepoint/dose

Suicide Inhibition

- Inhibition of cytochrome activity (irreversible)
- **Phenobarbital (PB)-induced liver microsomes** used to investigate the role for suicide inhibition

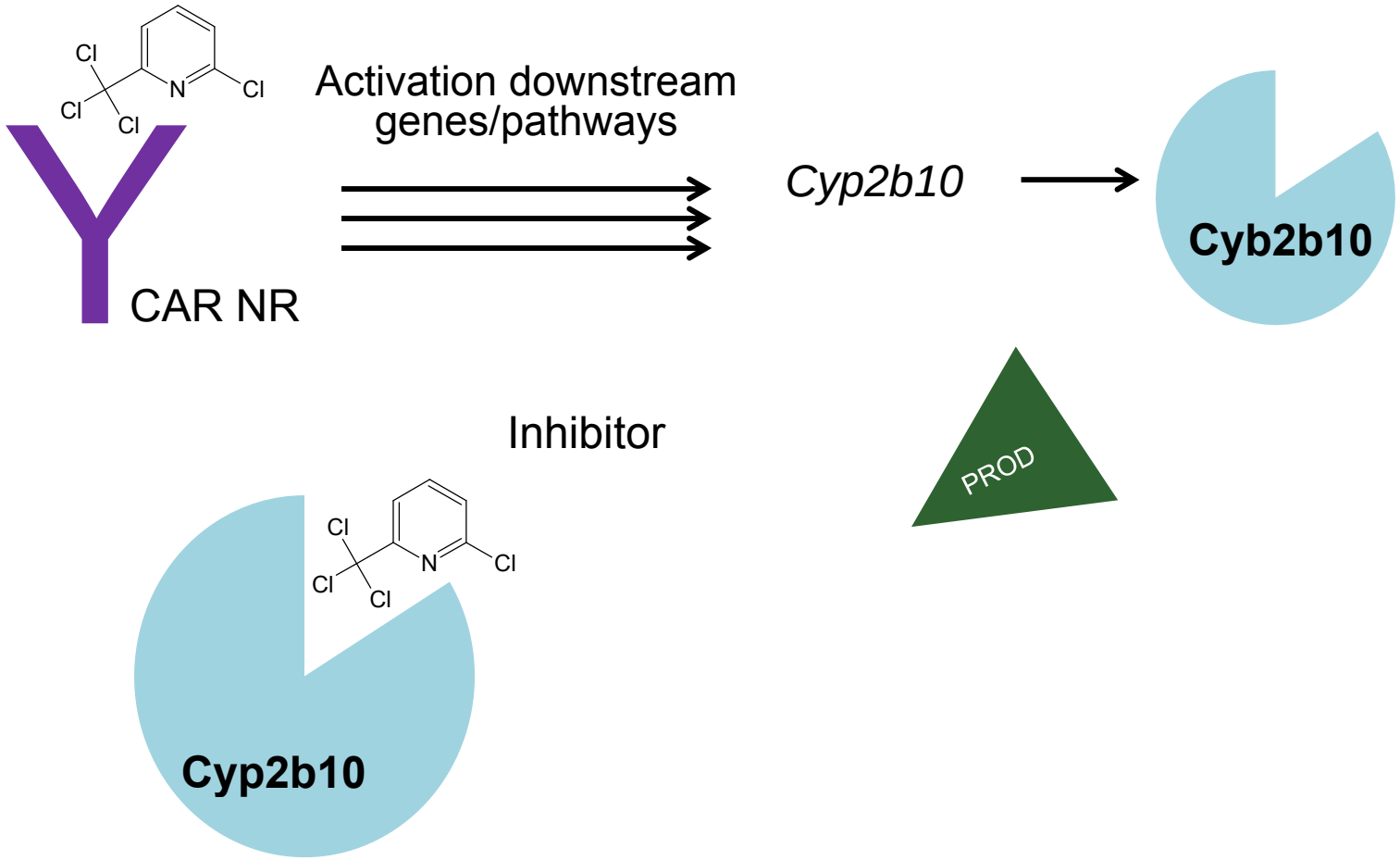
Inhibitor

Binds irreversibly, removes active enzyme from system

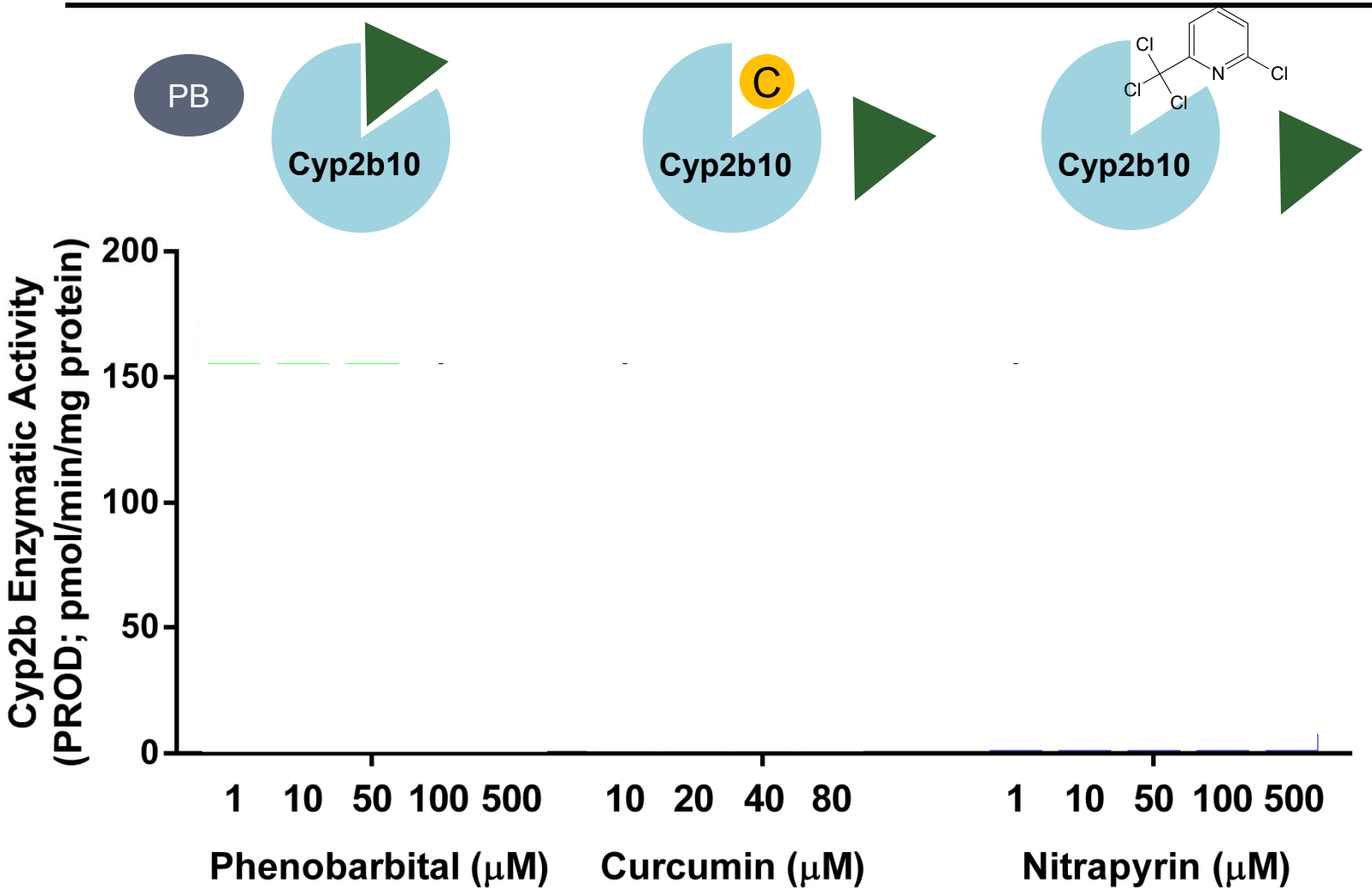
Enzyme

Suicide Inhibition

- Inhibition of cytochrome activity (irreversible)

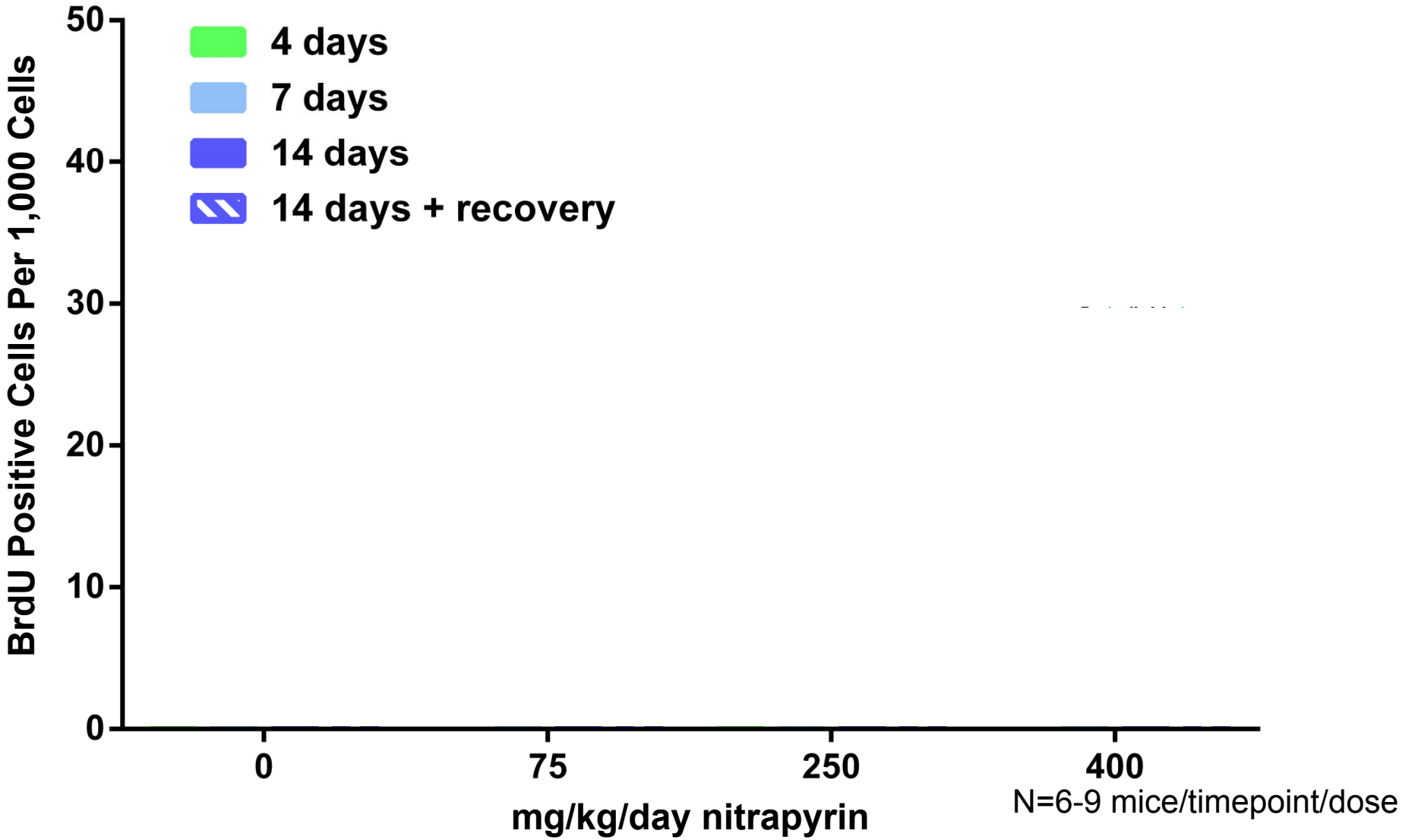


CYP Enzyme Induction/Suicide Inhibition



Cyp2b10 enzymatic inhibition similar to what was seen *in vivo*

Key Event #2: Increased Hepatocellular Proliferation



Summary Key Events #1 and 2

- Nitrapyrin exposure in mice causes:
  - Key Event #1 – CAR activation
    - –Cyp2b10 gene and protein expression
    - –Liver weight increases
    - –Liver hypertrophy
    - –Suicide inhibition of PROD
  - Key Event #2 – Hepatocellular proliferation
    - –BrdU Labeling Index

Question 1: Conclusion

Can we assimilate/generate data to define an MoA for nitrapyrin-mediated mouse liver tumors?

YES

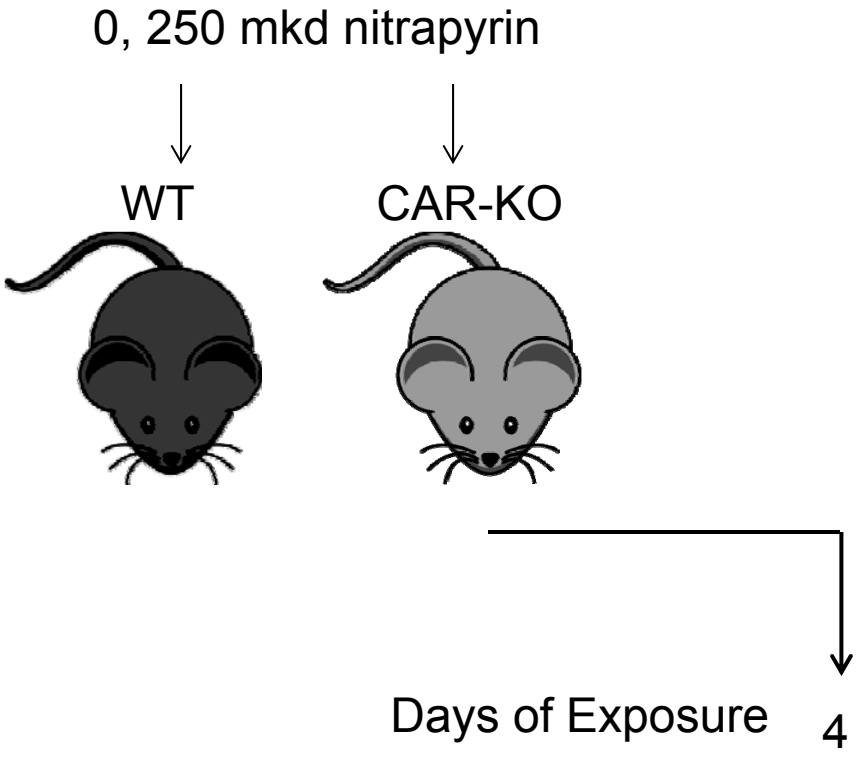
Key events #1 (CAR) and #2 (Proliferation)

Question 2

- Can we exclude other MoAs?
  - Is CAR necessary for nitrapyrin-mediated liver effects (proliferation)?

- Addressed this question with a CAR-KO mouse study

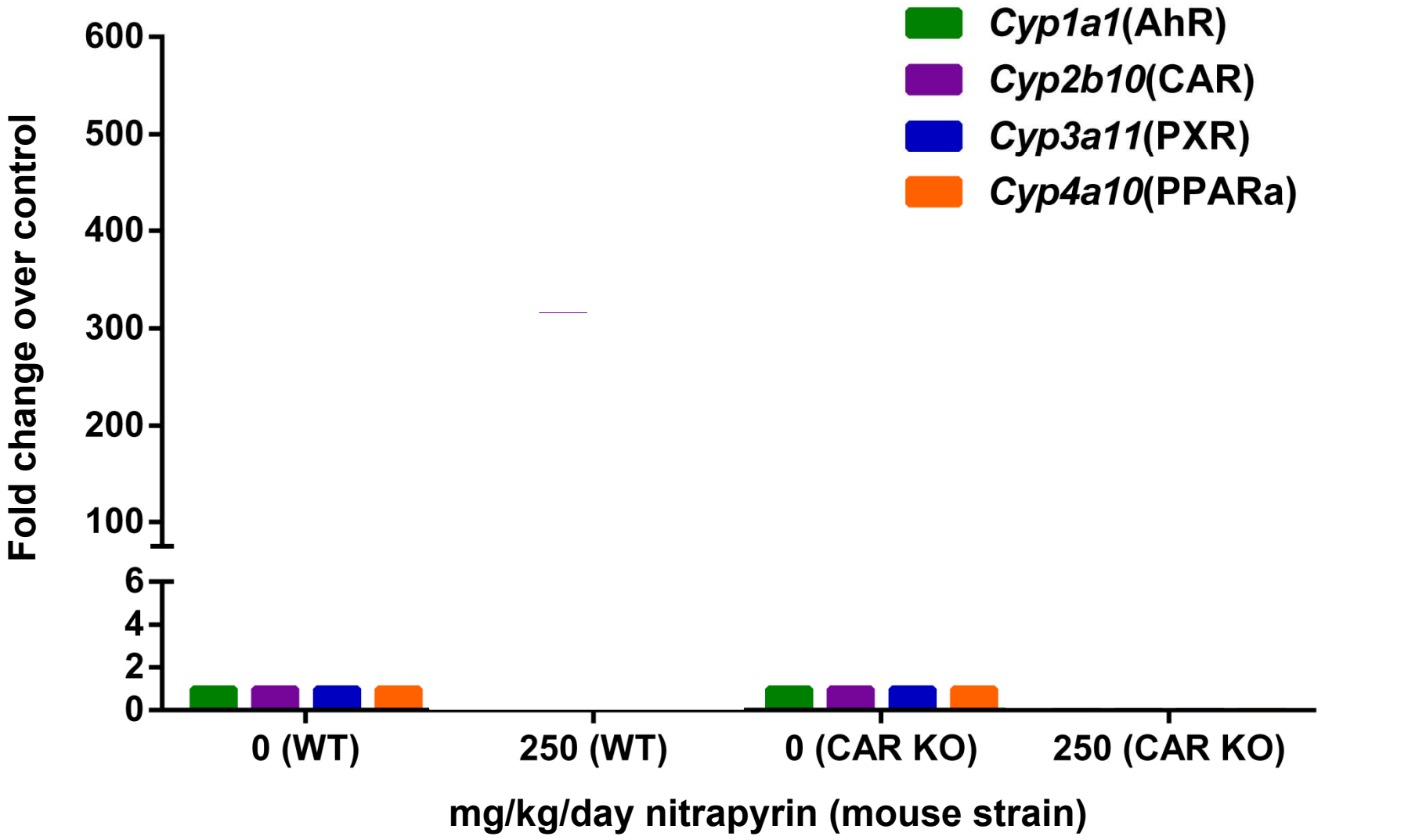
CAR-KO Mouse Study Design



Endpoints:

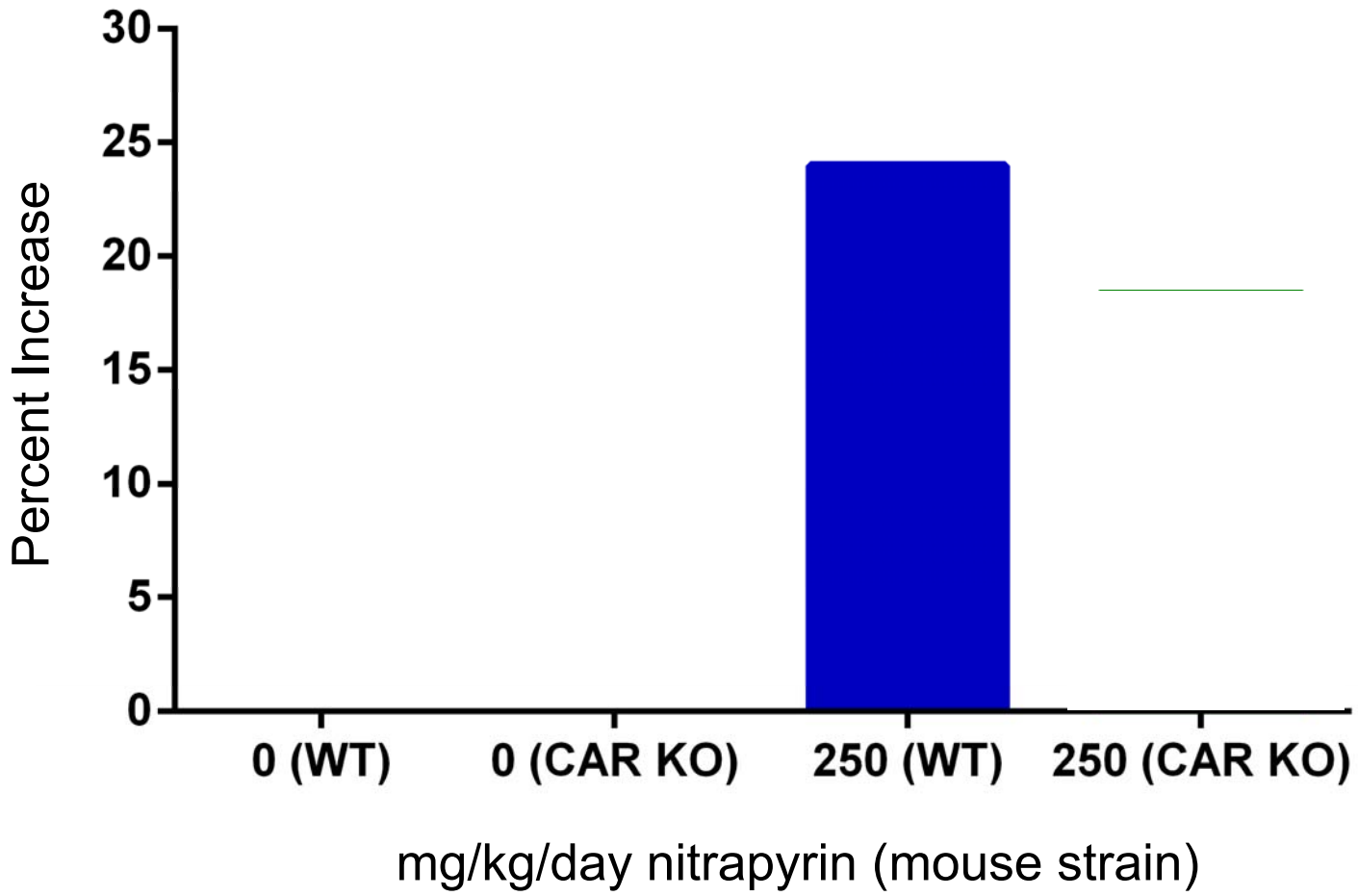
- *Cyp2b10*
- Liver weight increases, histopathology
- Hepatocellular proliferation

Gene expression in WT and CAR-KO Livers



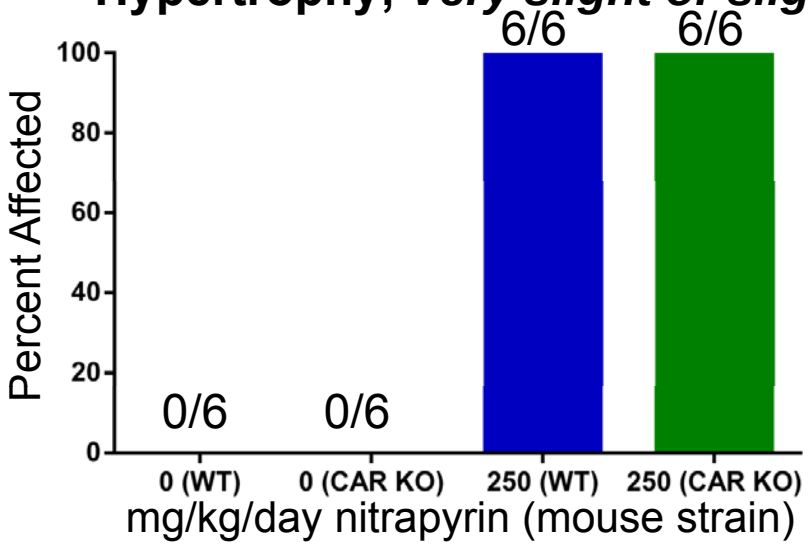
N=6 mice/strain/dose

Relative liver weight increases

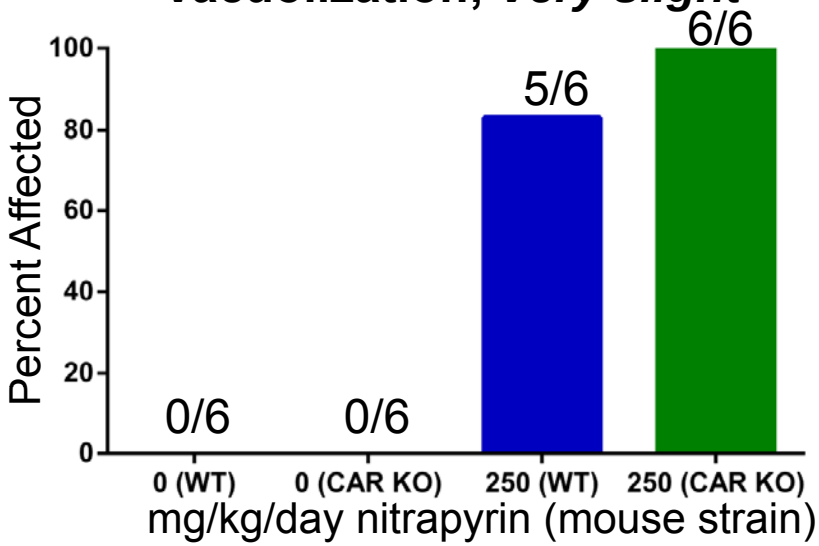


Histopathologic changes in WT and CAR-KO Mice

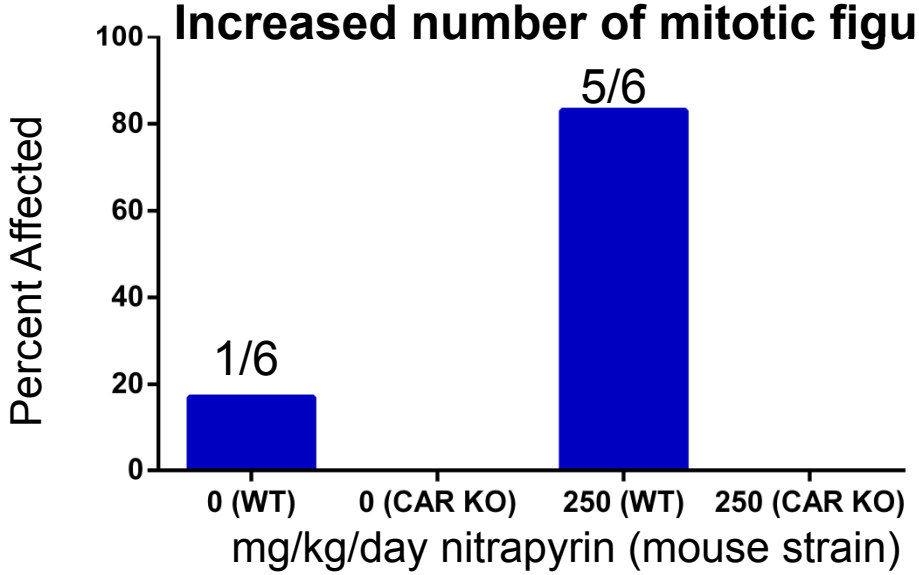
Hypertrophy; *Very slight or slight*



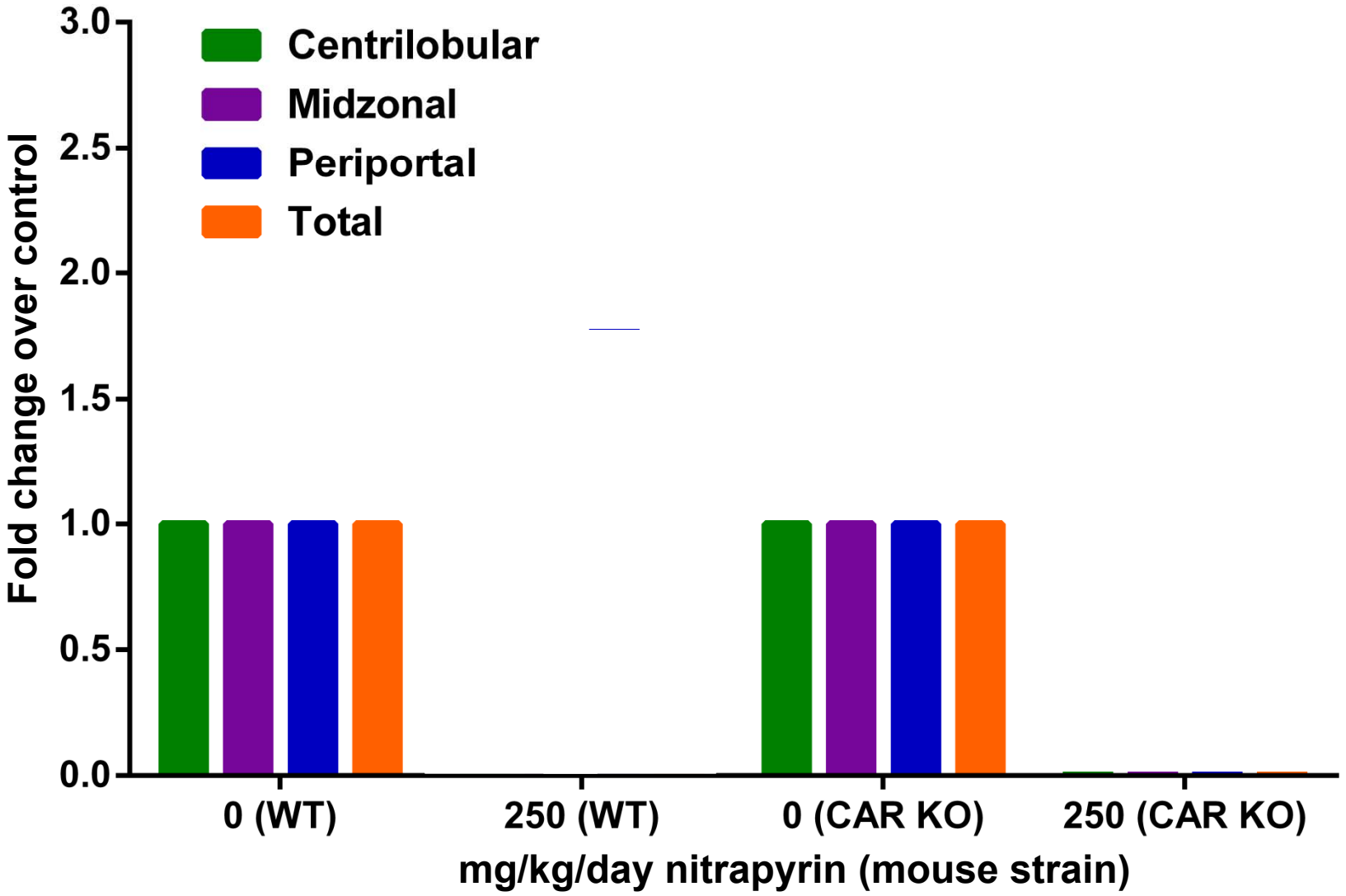
Vacuolization; *Very slight*



Increased number of mitotic figures



Hepatocellular Proliferation in WT and CAR-KO Mice

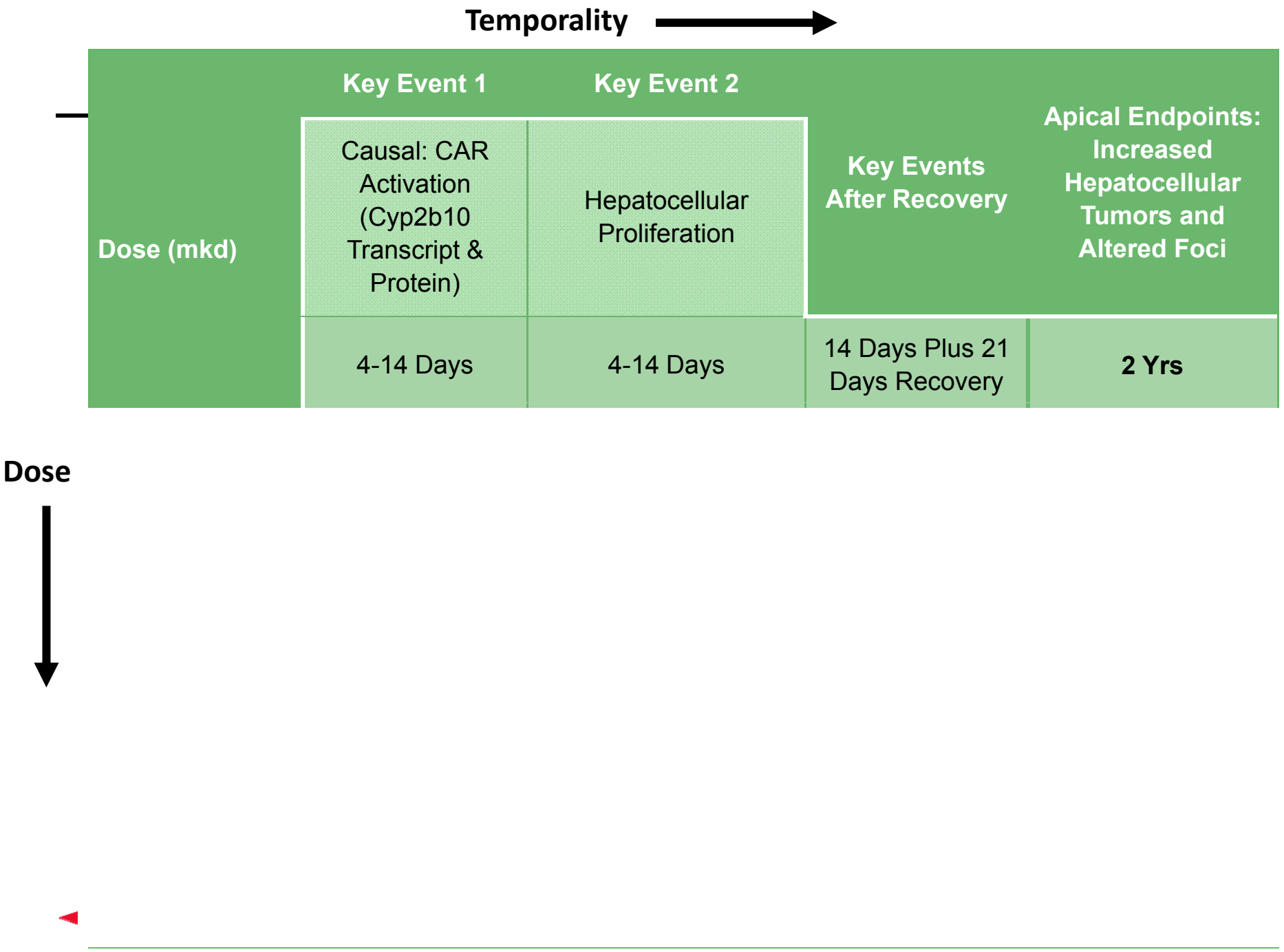


Nitrapyrin-induced proliferation requires CAR activation

Alternative MoAs

Alternative MoAs were evaluated for plausibility and coherence by Bradford Hill Criteria:

- **DNA Reactivity**
  - Not plausible
  - No coherence
- **AhR, PXR, PPARα Activation**
  - Not plausible
  - No coherence
- **Cytotoxicity (1 Wk – 12 Mo)**
  - Plausible
  - No coherence: based on magnitude of effect, entirety of data
- **Increased Apoptosis**
  - Not plausible
  - No coherence
- **Estrogens, Statins, Metals, Infectious**
  - Not plausible
  - No coherence


Temporality
Dose (mkd)
Key Event 1
Key Event 2
Key Events After Recovery
Apical Endpoints: Increased Hepatocellular Tumors and Altered Foci
Causal: CAR Activation (Cyp2b10 Transcript & Protein)
Hepatocellular Proliferation
4-14 Days
4-14 Days
14 Days Plus 21 Days Recovery
2 Yrs
Dose

Question 2: Conclusion

Can we exclude other MoAs?

YES

CAR is necessary for nitrapyrin-induced hepatocellular proliferation

Question 3: Relevance to Humans?

- CAR activation has been shown to be not relevant to humans:

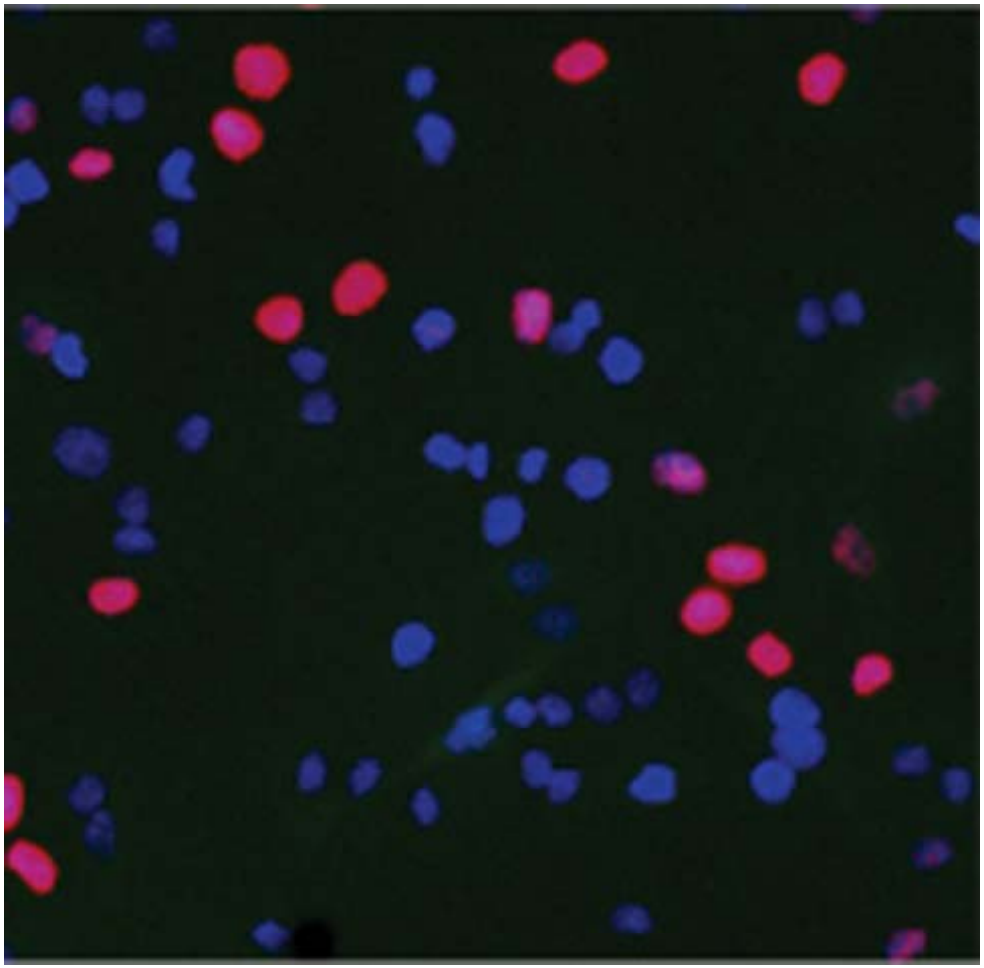
Relevance to Humans for Nitrapyrin?

- Wanted to generate nitrapyrin-specific data

- How?
  - Mouse vs. human hepatocyte proliferation study

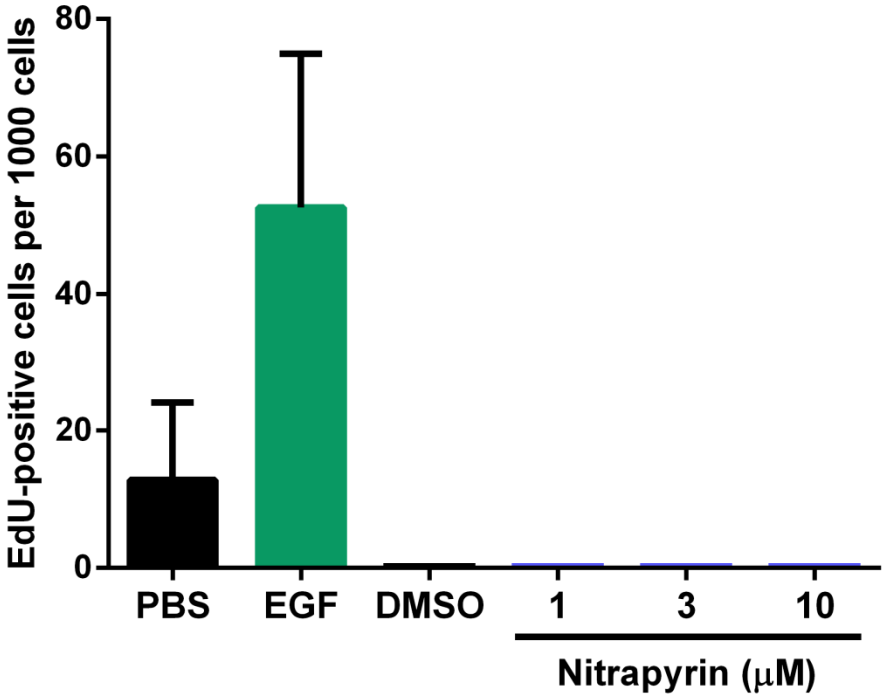
Study Design

- Mouse hepatocytes treated with 0, 1, 3, 10 µM nitrapyrin
- Human hepatocytes treated with 0, 3, 10, 30, 100 µM nitrapyrin
- Positive control EGF
- DNA synthesis analyzed via EdU staining (fluorescent alternative to BrdU)



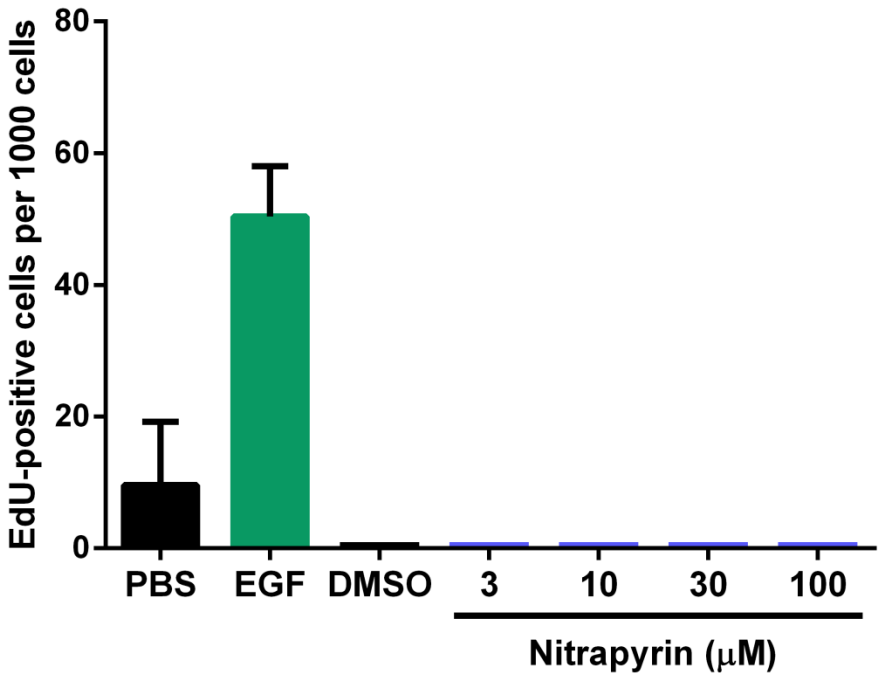
Hepatocyte Proliferation in Mice and Humans

Mouse Hepatocytes



N=5-7 technical replicates

Human Hepatocytes

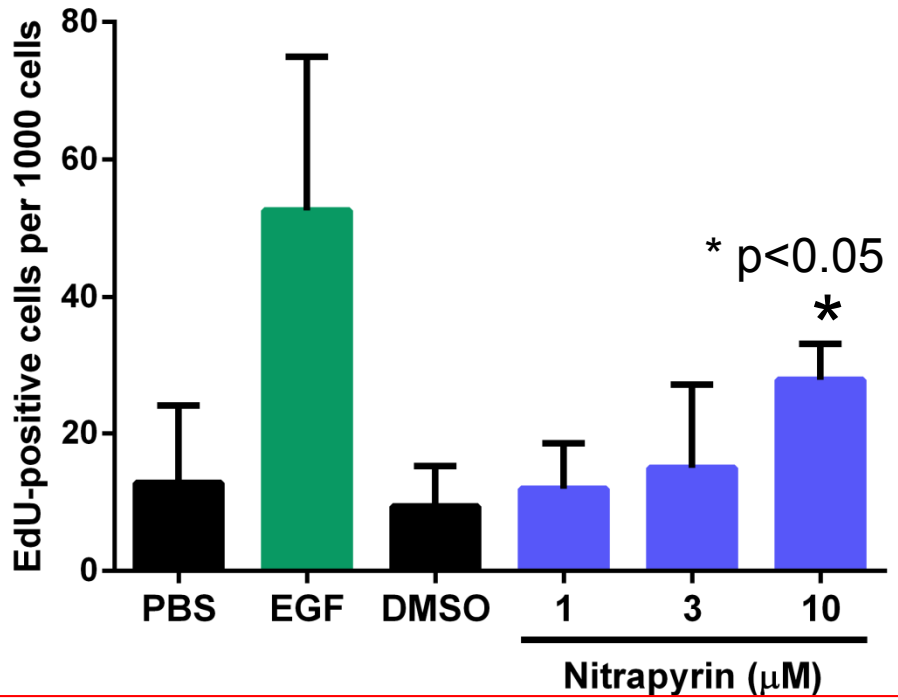


N=2 human donors,
2-3 technical replicates/donor/dose

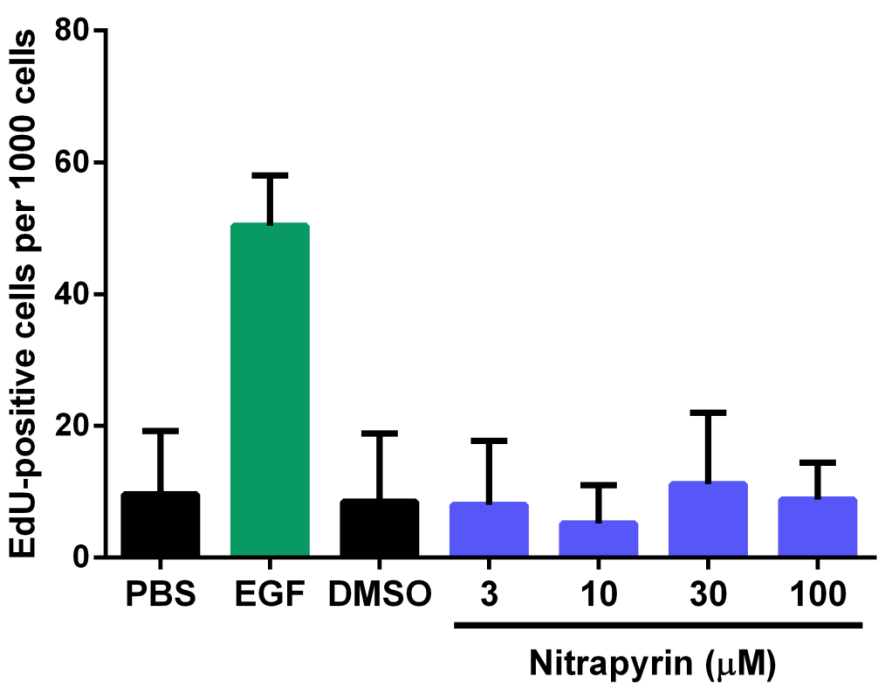
Dow AgroSciences

Hepatocyte Proliferation in Mice and Humans

Mouse Hepatocytes

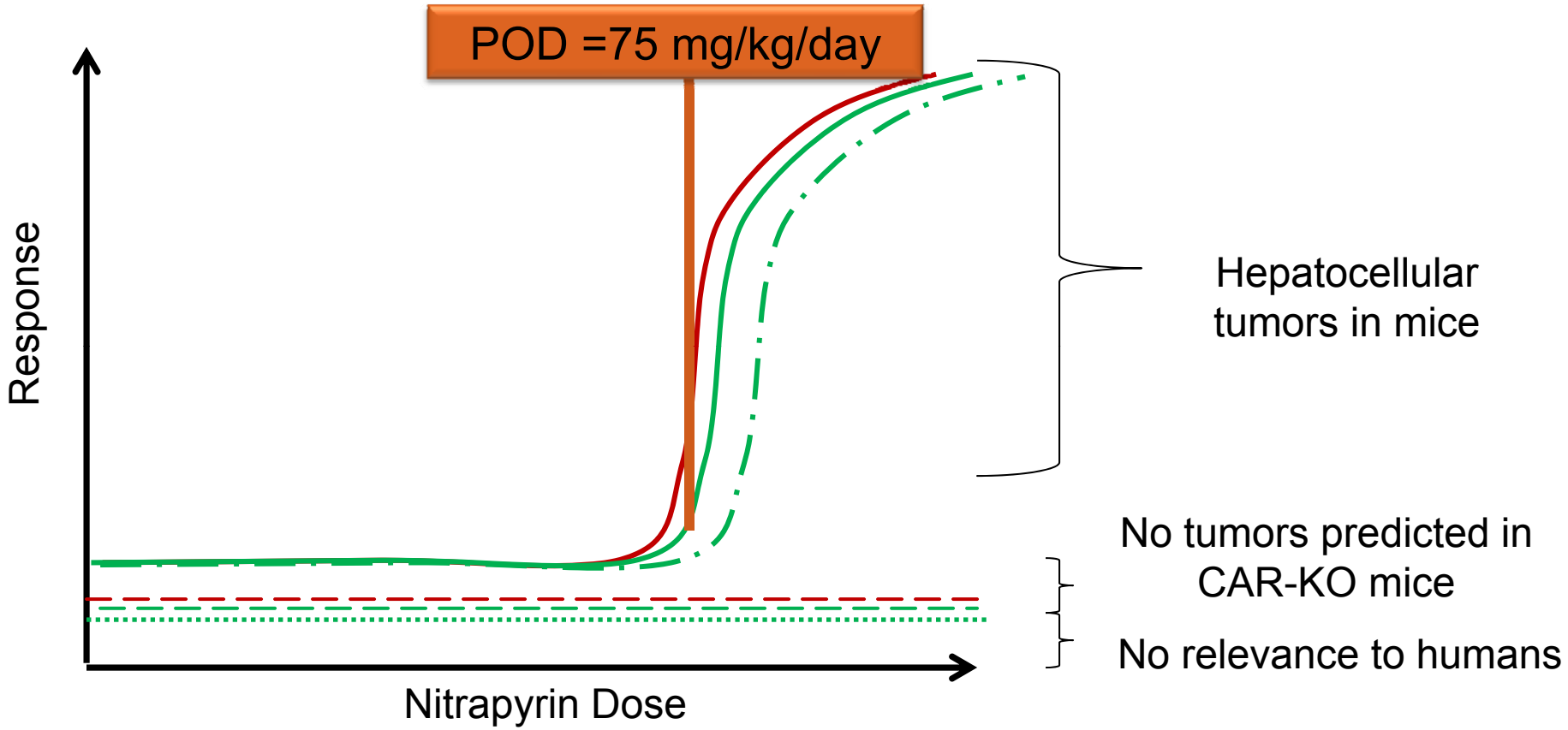


Human Hepatocytes

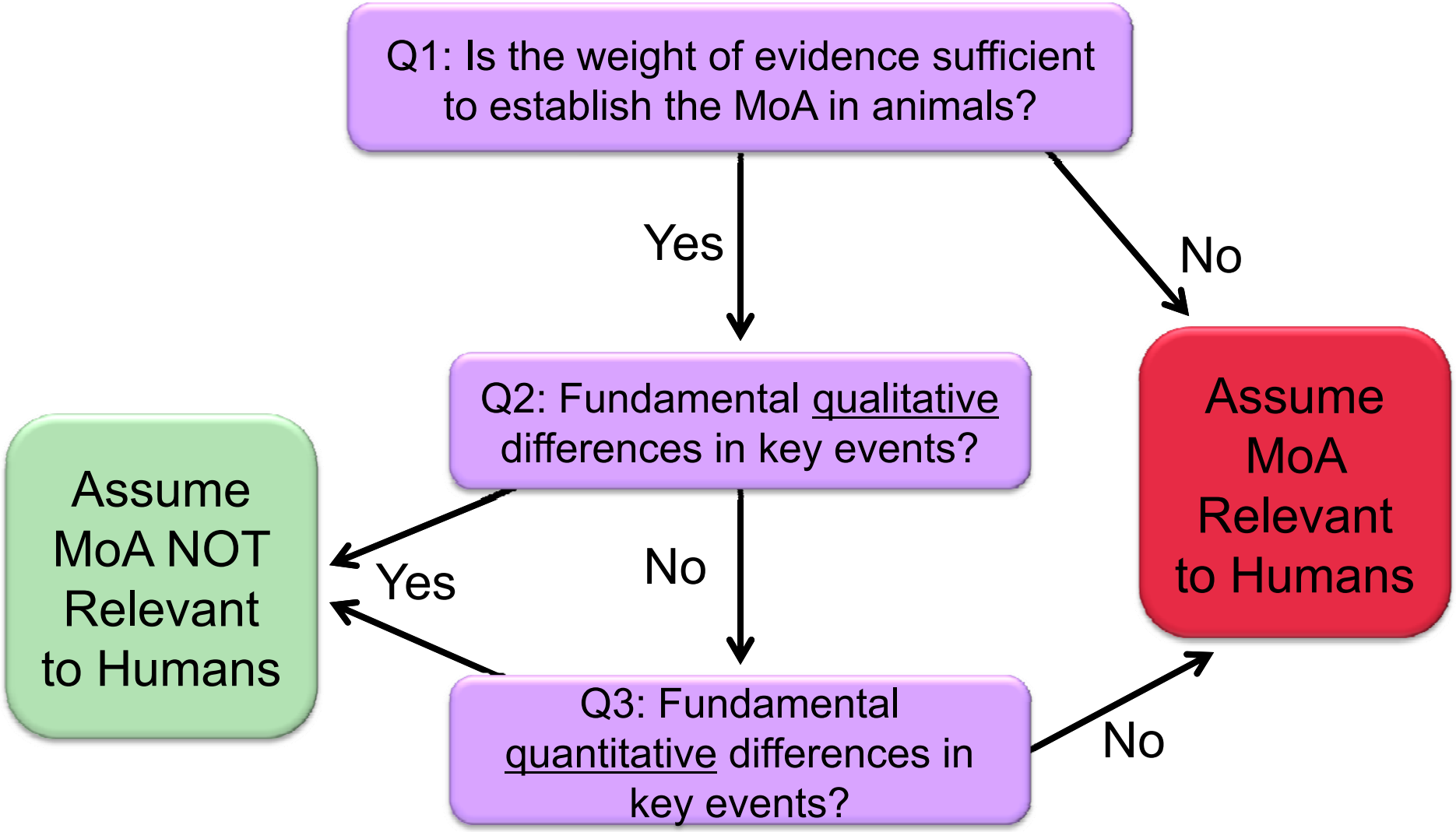


Conclusion: Nitrapyrin does not increase hepatocellular proliferation in human hepatocytes

Conclusions: Nitrapyrin MOA and Relevance to Humans



ILSI/IPCS Mode-of-Action/Human Relevance Framework



As described by Meek et al. (2003) and revised by Seed et al.(2005).

Nitrapyin MoA/HRF

- Data support CAR activation as MoA
  - Key Event #1 – CAR Activation
  - Key Event #2 – Hepatocellular Proliferation

- Alternative MoAs can be excluded

- Due to qualitative differences, MoA for nitrapyrin is not relevant to humans

Conclusion – Part #1

Questions on the MoA/HRF evaluation?

Regulatory Reviews

- EPA
  - CPRC (1992) - Not classifiable
  - CARC (2000) - Likely
  - CARC (2005) - Likely
  - CARC (2012) - **Suggestive Evidence**
  - CARC (2017) – Under Review

Dietary Risk Assessment

- Dietary exposure is estimated using
  - Food consumption data (from NHANES surveys)
  - Potential values for pesticide residues in different foods (tolerances)

§180.350 Nitrapyrin; tolerances for residues.

(a) *General.* Tolerances are established for the combined residues of the soil microbiocide nitrapyrin [2-chloro-6-(trichloromethyl) pyridine] and its metabolite, 6-chloropicolinic acid in or on the following raw agricultural commodities:

Commodity	Parts per million
Corn, field, forage	1.0
Corn, field, grain	0.1
Corn, field, milled byproducts	0.2
Corn, field, stover	1.0
Corn, pop, grain	0.1
Corn, pop, stover	1.0
Corn, sweet, forage	1.0
Corn, sweet, kernel plus cob with husks removed	0.1
Corn, sweet, stover	1.0
Sorghum, forage, forage	0.5
Sorghum, grain, forage	0.5
Sorghum, grain, grain	0.1
Sorghum, grain, stover	0.5
Wheat, bran	3.0
Wheat, forage	2.0
Wheat, grain	0.5
Wheat, milled byproducts, except flour	2.0
Wheat, straw	6.0

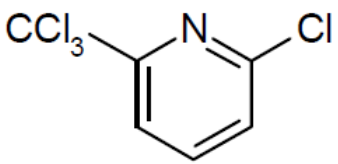
NHANES = National Health and Nutrition Examination Survey

nitrapyrin tolerances

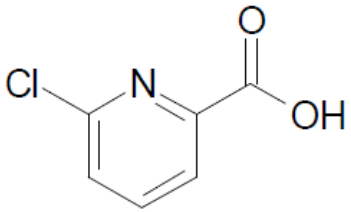
Dietary Risk Assessment

- Diet
  - Residues of concern in food:

nitrapyrin



6-chloropicolinic acid



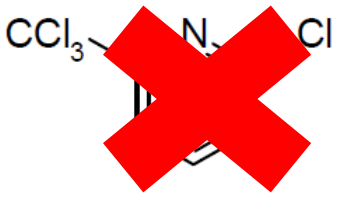
§180.350 Nitrapyrin; tolerances for residues.

(a) *General.* Tolerances are established for the combined residues of the soil microbiocide nitrapyrin [2-chloro-6-(trichloromethyl) pyridine] and its metabolite, 6-chloropicolinic acid in or on the following raw agricultural commodities:

Dietary Risk Assessment

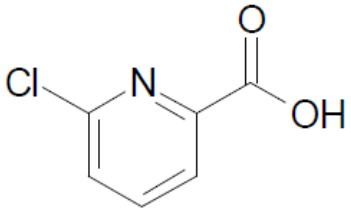
- Diet
  - Dietary cancer risk assessment:

nitrapyrin



No residues ever detected in food commodities

6-chloropicolinic acid



  - [Former] Cancer endpoint only relevant to nitrapyrin; not 6-CPA

Dietary Risk Assessment

- Chronic RfD = 0.03mg/kg bw/day (based on NOAEL of 3mg/kg/day (chronic feeding – dog) and uncertainty factor = 100; FQPA = 1)
- cPAD = RfD÷FQPA = 0.03mg/kg bw/day
- Exposure ≤1% cPAD for

  US population and all subgroups

Table 3. Summary of Dietary Exposure and Risk for Nitrapyrin

Population Subgroup	Chronic Dietary Exposure and Risk			
	DEEM		Lifeline	
	Dietary Exposure (mg/kg/day)	% cPAD	Dietary Exposure (mg/kg/day)	% cPAD
General U.S. Population	0.000013	<1	0.000012	<1
All Infants (< 1 year old)	0.000015	<1	0.000012	<1
Children 1-2 years old	0.000027	<1	0.000026	<1
Children 3-5 years old	0.000031	<1	0.000029	<1
Children 6-12 years old	0.000023	<1	0.000021	<1
Youth 13-19 years old	0.000017	<1	0.000015	<1
Adults 20-49 years old	0.000011	<1	0.000011	<1
Adults 50+ years old	0.000007	<1	0.000009	<1
Females 13-49 years old	0.000011	<1	0.000013	<1

Table 3 extracted from Nitrapyrin Chronic Dietary Exposure Assessment for the Registration Eligibility Decision. D. Soderburg, EPA 2004. D299299

Dietary Risk Assessment

Occupational Risk Assessment

- Occupational
  - Potential exposure through mixing, loading and application:



  - Exposure is calculated using unit exposure values from specific studies (such as those conducted by the Agricultural Handlers Exposure Task Force).

Occupational Risk Assessment

$$\text{Exposure} = \frac{\text{AR (lb ai/acre)} \times \text{AT(acre/day)} \times \text{unit exposure (mg/lb ai)}}{\text{body weight (kg)}}$$

AR = application rate

AT = area treated

- Exposure (in mg/kg/day) is used to assess risk in two ways:

- Cancer and non-cancer

Occupational Risk Assessment

- Cancer: Lifetime Average Daily Dose (LADD) calculated from daily exposure. LADD used to calculate risk:

$$\text{LADD} = \text{total exposure} \times \frac{\text{no. of days exposed per year}^{*}}{\text{365 days per year}} \times \frac{\text{35 working years}}{\text{70 year lifetime}}$$

*3 day per year for private applicators; 30 day per year for commercial applicators

$$\text{Risk} = Q_1^{*} \left[4.25 \times 10^{-2} \text{ (mg/kg/day)}^{-1} \text{ human equivalents}\right] \times \text{LADD}.$$

Occupational Risk Assessment

- Cancer risks marginally higher than LOC (LOC = 1 x 10^{-6}) but risk vs. benefit recommends no additional mitigation beyond long pants, long sleeves and gloves for mixing/loading

Table 6: Occupational Handler, Summary of Cancer (Q*) Risk for Nitrapyrin

Exposure Scenario #	Application Rate lb ai/A	Acres treated A/day	Crop Type	Baseline Risk 3/30	PPE1 Risk 3/30	PPE2 Risk 3/30	PPE 3 Risk 3/30	E. control Risk 3/30
Mixer/Loader Exposure								
Mixing/Loading Liquids for Groundboom application (1)	1.00	200	Wheat, Corn, Sorghum	6.66e-4 /6.66e-3	5.88e-6 /5.88e-5	4.5e-6 /4.5e-5	3.96e-6 /3.96e-5	2.02e-6 /2.02e-5
Applicator								
Sprays for Groundboom application (2)	1.00	200	Wheat, Corn, Sorghum	3.6e-6 /3.6e-5	3.6e-6 /3.6e-5	2.9e-6 /2.9e-5	2.6e-6 /2.6e-5	1.2e-6 /1.2e-5

Baseline dermal unit exposure scenarios includes long pants, long sleeved shirts and no gloves.
PPE1 long pants, long sleeved shirts and gloves (no respirator)
PPE 2 cancer risk includes long pants, long sleeved shirts, double layer, gloves and no respirator.
PPE 3 cancer risk includes long pants, long sleeved shirts, double layer, gloves and organic vapor respirator.
Engineering Control dermal unit exposure scenarios includes long pants, long sleeved shirts, gloves.
Engineering inhalation unit exposure represents no respirator.
Two exposure frequencies were used for cancer, the first represented the maximum number of applications per site per season to represent private use (3), and the second frequen mmercial handlers making multiple applications per site per seaso

(extracted from occupational exposure assessment, 2005)

- Cancer-based risk assessment not required since 2012 (re-classification)

Occupational Risk Assessment

- Non-cancer: the MOE is calculated:

$$MOE = \frac{NOAEL \ (mg/kg/day)}{Exposure \ (mg/kg/day)}$$

Need MOE ≥ 100 for pass

Table 5: Summary of Occupational Handler Risk for Nitrapyrin

Exposure Scenario (Scenario #)	Crop	Application Rate lb ai/A	Daily Area Treated A/day	Total Baseline Short-Term MOE	Total Baseline Intermediate-Term MOE	Total PPE Short-Term MOE	Total PPE Intermediate-Term MOE
Mixer/Loader							
Mixing/Loading Liquids for Groundboom application (1)	Wheat, Corn, Sorghum	1	200	3	1	250 (gloves)	100 (gloves)
Applicator							
Sprays for Groundboom application (2)	Wheat, Corn, Sorghum	1	200	420	150	420	150

Baseline dermal unit exposure scenarios includes long pants, long sleeved shirts and no gloves.
Baseline inhalation unit exposure represents no respirator
PPE dermal unit exposure includes long pants, long sleeved shirts and gloves for mixer/loaders only.

(extracted from occupational exposure assessment, 2005)

Occupational Risk Assessment

- All MOEs ≥ 100 when long pants, long sleeves and gloves worn for mixing/loading

Acknowledgements

Dow AgroSciences Human Health Assessment

Dow Toxicology & Environmental Research & Consulting (TERC) Laboratories

- Reza Rasoulpour
- B. Bhaskar Gollapudi
- Dave Eisenbrandt
- H. Lynn Kan
- Melissa Schisler
- Val Marshall
- Johnson Thomas
- Lynea Murphy
- Kamin Johnson
- Nico Visconti
- Lindsay Sosinski
- Dave DeLine