AhR Activation in Pharmaceutical Development: Applying Liver Gene Expression Biomarker Thresholds to Identify Tumorigenic Dose Levels in Rats

European Centre for Ecotoxicology and Toxicology of Chemicals Workshop on Omics Threshold on Non-Adversity

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A set of 10* gene signatures have been optimized for inclusion in a routine early 5-day rat tolerability study for improving Safety Lead Optimization decision-making within Merck & Co., Inc.

IN A RECENT COMMENTARY FROM:
Gene Signatures Reduce the Stress of Preclinical Drug Hepatotoxicity Screening
Ian M. Copple Ph.D., B. Kevin Park Ph.D., Christopher E. Goldring Ph.D. *Hepatology* (2021) *74*: 513-515

"...the approach pioneered by Merck is likely to be seen as valuable by some companies in selected settings, which in itself would be regarded as a major achievement ***in an industry that has, to date, been largely reluctant to embrace gene expression technology within drug safety screening***.[7] [7]Vahle JL, Anderson U, Blomme EAG, Hoflack JC, Stiehl DP. Use of toxicogenomics in drug safety evaluation: Current status and an industry perspective. *Regul Toxicol Pharmacol* 2018; *96*: 18- 29

AND FROM VAHLE et al: ***"...these [EFPIA] survey data indicate that toxicogenomics is not widely used as a predictive tool in the pharmaceutical industry..."***

*Podtelezhnikov et al (2020) Toxicol. Sci. 175: 98-112

*#10 = Tissue Necrosis and Degeneration Gene Signature: Glaab et al (2021) Toxicol. Sci. 181: 148-159

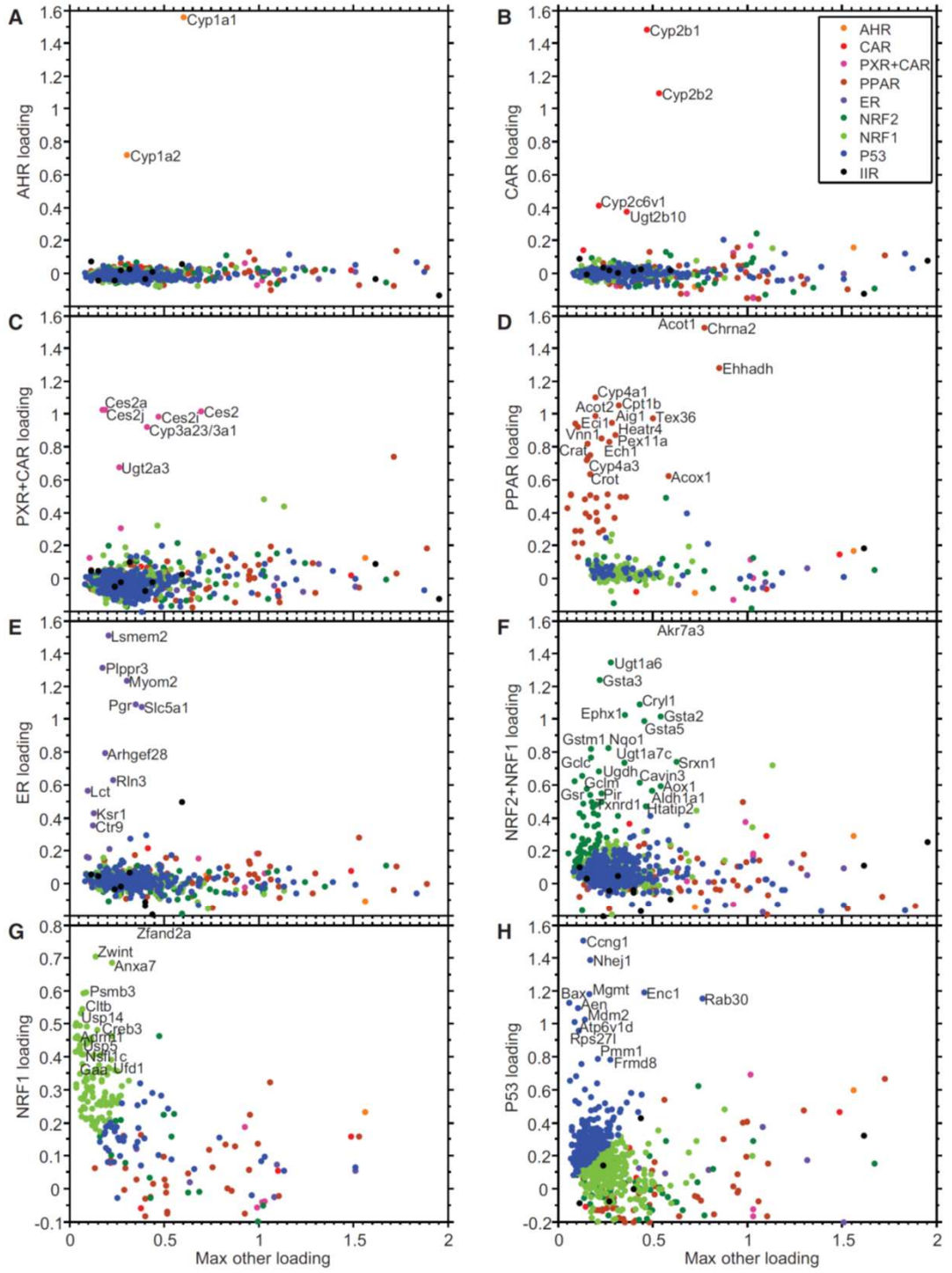


Figure 2. Loading coefficients from linear modeling. In panels A though H, the loading coefficients for AHR, CAR, PXR+CAR, PPAR, ER, NRF2+NRF1, NRF1, P53 are shown against another loading coefficient with maximum absolute value. The AHR, CAR, PXR+CAR, NRF2+NRF1, and P53 panels show genes with $R^2 > 0.7$, The PPAR and NRF1 panels use tighter $R^2 > 0.8$. Genes with highest loading coefficients are also labeled with their gene symbols. Colors indicate the factor with the highest loading for each gene.

*from Podtelezhnikov et al (2020)

Safety Lead Optimization (SLO) Rat Study developed for use in Lead Op to guide Candidate Selection

- Cost sparing 5 day, control + 2 dose level, initial in vivo tolerability study using 12 male rats
  1. Drug exposure data provide perspective on margins and guide dose selection for future studies
  2. "Tissue Injury" gene signature[1] diagnostic of histopathology in primary organs of concern – liver, kidney, heart and skeletal muscle (9% positive 2011-14)
  3. "ADME" gene signatures[2] inform risks for certain tox endpoints, e.g., rodent carcinogenicity (and may inform poor exposures) (4% AhR positive 2011-14)
  4. "Liver Response Assay" genes[2,3] identify molecular response to reactive metabolite burden and predict potential for future DILI in rodents, non-rodents, & humans even when rat liver is not injured in 4 days (12% positive 2011-14)
  5. "Severe systemic inflammatory response" genes[2] identify molecular acute phase response (e.g., vascular inflammation, GI ulceration, etc.)
- Provides samples for same early views into other suspected tissue liabilities, e.g., testes, bladder, pancreas (& also bone marrow for early in vivo micronuclei genetox assessment)
- Offers future adoption opportunity for additional evolving low cost plasma and tissue endpoints on same quick turnaround platform

[1]Glaab et al (2021) Toxicol. Sci., 181: 148-159
[2]Podtelezhnikov et al (2020) Toxicol. Sci. 175: 98-112
[3]Monroe et al (2020) Toxicol. Sci. 177: 281-299

MRK Case Example: 1st in Class Molecule w AhR Activation (a Human Relevant Off Target Risk Factor)

- Gene expression of Cyp 1a1, Cyp 1a2 in rat liver shown below indicates potential for AhR induction by Compound X in an early 4 day rat tolerability study.
- After 7 days of dosing, potency and effectiveness of Compound X's induction of Cyp 1a1 and 1a2 further increase.

  [Data Not Shown: Cyp 1a1 and 1a2 induction by Compound X were also observed in vivo in monkey liver, and in an in vitro human cell assay]

Compound	Study (Days)	Dose (mkd)	AUC Multiple	mRNA Induction	
				Cyp 1a1	Cyp 1a2
X	Rat (4 Day)	300	113 X	242 X	6 X
		30	44 X	159 X	5 X
	Rat (7 Day)	400	140 X	2778 X	12 X
		10	5 X	163 X	4 X

2007 Iconix Publication: AhR Agonists that are non-persistent are not of toxicologic concern

Induction of Cyp1a1 Is a Nonspecific Biomarker of Aryl Hydrocarbon Receptor Activation: Results of Large Scale Screening of Pharmaceuticals and Toxicants in Vivo and in Vitro

Wenyue Hu, Claudio Sorrentino, Michael S. Denison, Kyle Kolaja, and Mark R. Fielden

Molecular Pharmacology June 2007, 71 (6) 1475-1486; DOI: http://dx.doi.org/10.1124/mol.106.032748

"…To evaluate the accuracy of in vivo Cyp1a1 induction as a biomarker of AhR agonist activity, we evaluated rat gene expression data in DrugMatrix, a large toxicogenomic database of gene expression profiles for 596 compounds (Ganter et al., 2005), and found that Cyp1a1 was induced by 239 compounds in a variety of tissues. The majority of the active compounds are marketed drugs with toxicity profiles unlike those produced by exposure to HAHs. To evaluate the sensitivity and specificity of in vivo Cyp1a1 induction to identify AhR agonists, a subset of 147 compounds was evaluated using a combination of in vitro assays to assess their ability to stimulate AhR transformation and DNA binding, dioxin response element (DRE)-driven reporter gene expression, and to compete with dioxin for binding to the AhR. The in vivo expression of other AhR-regulated genes, including Cyp1a2, Ugt1a1, and Nqo1, was also evaluated to determine whether the expression of these DRE-driven genes could improve the accuracy for identifying AhR agonists. Although all AhR agonists induce Cyp1a1 gene expression, the induction of Cyp1a1 expression in vivo does not necessarily implicate that a chemical is a direct AhR agonist. Furthermore, six marketed drugs that activate and bind to the rat AhR were identified and many treatments that induce Cyp1a1 in a tissue-specific manner and in a distinct pattern relative to other AhR-regulated genes. **These results lend support to the hypothesis that AhR activation is not synonymous with AhR agonist activity and HAH-like toxicity for nonpersistent compounds..."**

With respect to estimates of dioxin-like toxicity, a rich body of literature indicates that metabolically persistent halogenated ligands of the AhR cause sustained activation of the receptor and result in a wide spectrum of toxic responses similar to TCDD, whereas metabolically labile, nonhalogenated AhR ligands do not typically produce dioxin-like toxicities in animal studies. ….These results suggest that whereas binding and activation of the AhR are necessary prerequisite events for AhR-dependent dioxin-like toxicity, the actual occurrence of toxicity requires both continual presence of the AhR agonist and persistent activation of the AhR signaling pathway. In the current study, through a combination of in vivo and in vitro assays, a number of weak AhR ligands were identified, including nimodipine, leflunomide, flutamide, omeprazole, mexiletine, and atorvastatin. These compounds, which are approved for use by the U.S. FDA, do not produce dioxin-like toxicities in rats, and there is no evidence for chloracne, immunosuppression, or other adverse dioxin-like effects in exposed humans. This could be due to both their reduced potency relative to TCDD and/or their rapid rate of clearance from the body relative to persistent halogenated ligands. It would seem that **the toxicological consequences of transient or weak receptor activation are qualitatively and quantitatively distinct from persistent activation by metabolically stable and potent ligands.**

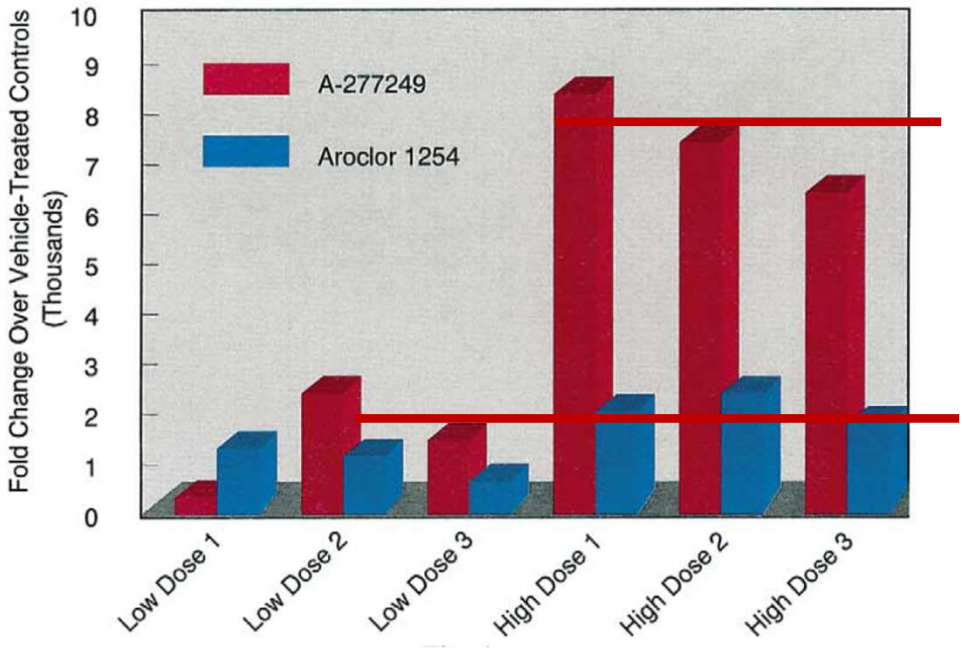
Hypotheses for AHR Association with Tumorigenesis

Threshold Magnitude

Sustained Activation

The Eventual Rat Carco study dose must be considered for proper perspective

Example 2 – Drug Discovery Investigators at Abbott Rapidly Identify A-277249 Toxic Liability in 2002: Candidate for Inflammatory Diseases found to be a Strong Activator of AhR at 10 and 100 mkd



8000-Fold Induction of CYP 1A1 mRNA @ 100 mkd A-2777249 x 3-D in SD Rats

2000-Fold Induction of CYP 1A1 mRNA @ 10 mkd A-2777249 x 3-D in SD Rats

Fig. 4. Analysis of CYP 1A1 mRNA by quantitative polymerase chain reaction shows a dose-dependent increase in expression by A-277249. At the high dose the expression was approximately 8000-fold, much higher that that seen for the positive control, Aroclor. Bars are values for individual animals.

Example 3 –De-Risking *proactively* for AhR Agonism carcinogenicity concern shared in Drug Discovery at GSK

Navigating CYP1A Induction and Arylhydrocarbon Receptor Agonism in Drug Discovery. A Case History with $S1P_1$ Agonists

Simon J. Taylor*†, Emmanuel H. Demont†, James Gray†, Nigel Deeks†, Aarti Patel‡, Dung Nguyen§, Maxine Taylor‡, Steve Hood‡, Robert J. Watson†, Rino A. Bit†, Fiona McClure∥, Holly Ashall∥, and Jason Witherington†

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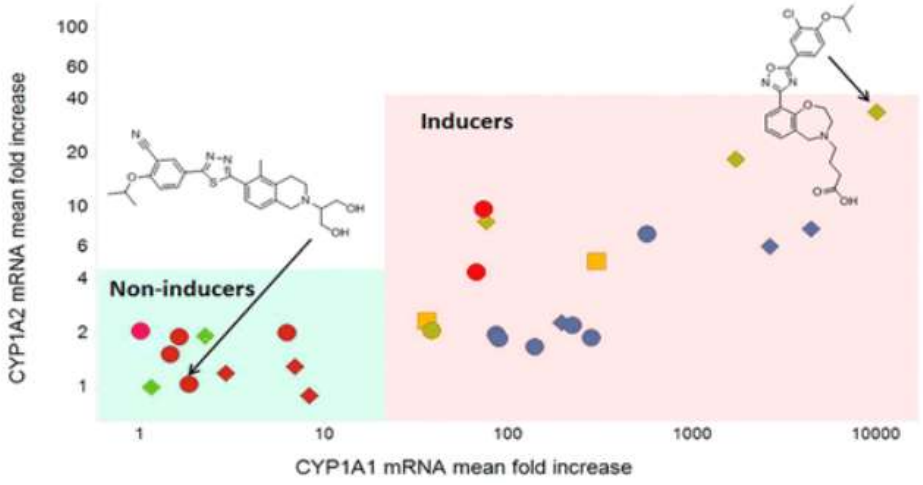
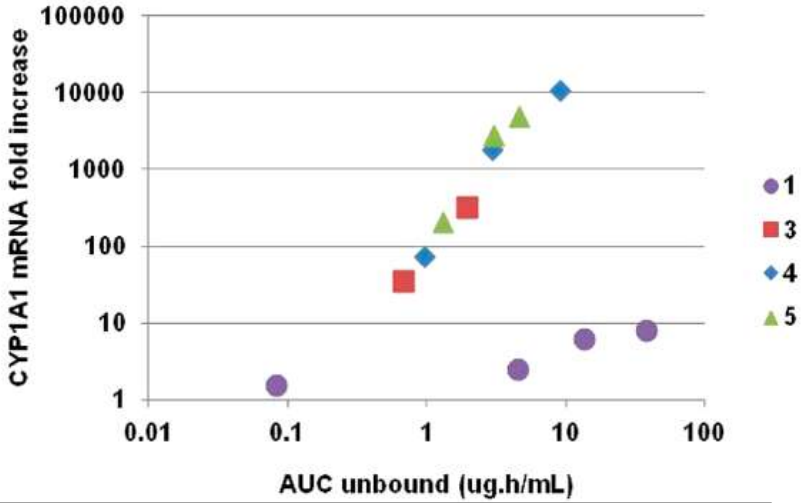
J. Med. Chem., **2015**, *58* (20), pp 8236–8256





This article describes the finding of substantial upregulation of mRNA and enzymes of the cytochrome P450 1A family **during a lead optimization campaign for small molecule $S1P_1$ agonists. Fold changes in mRNA up to 10 000-fold for CYP1A1 in vivo in rat and cynomolgus monkey** and up to 45-fold for CYP1A1 and CYP1A2 in vitro in rat and human hepatocytes were observed. Challenges observed with correlating induction in vitro and induction in vivo resulted in **the implementation of a short, 4 day in vivo screening study in the rat which successfully identified noninducers**.

MRK Safety Lead Optimization Rat Study Designed to Establish AhR Activation Gene Expression Thresholds for Rat Liver Tumorigenesis

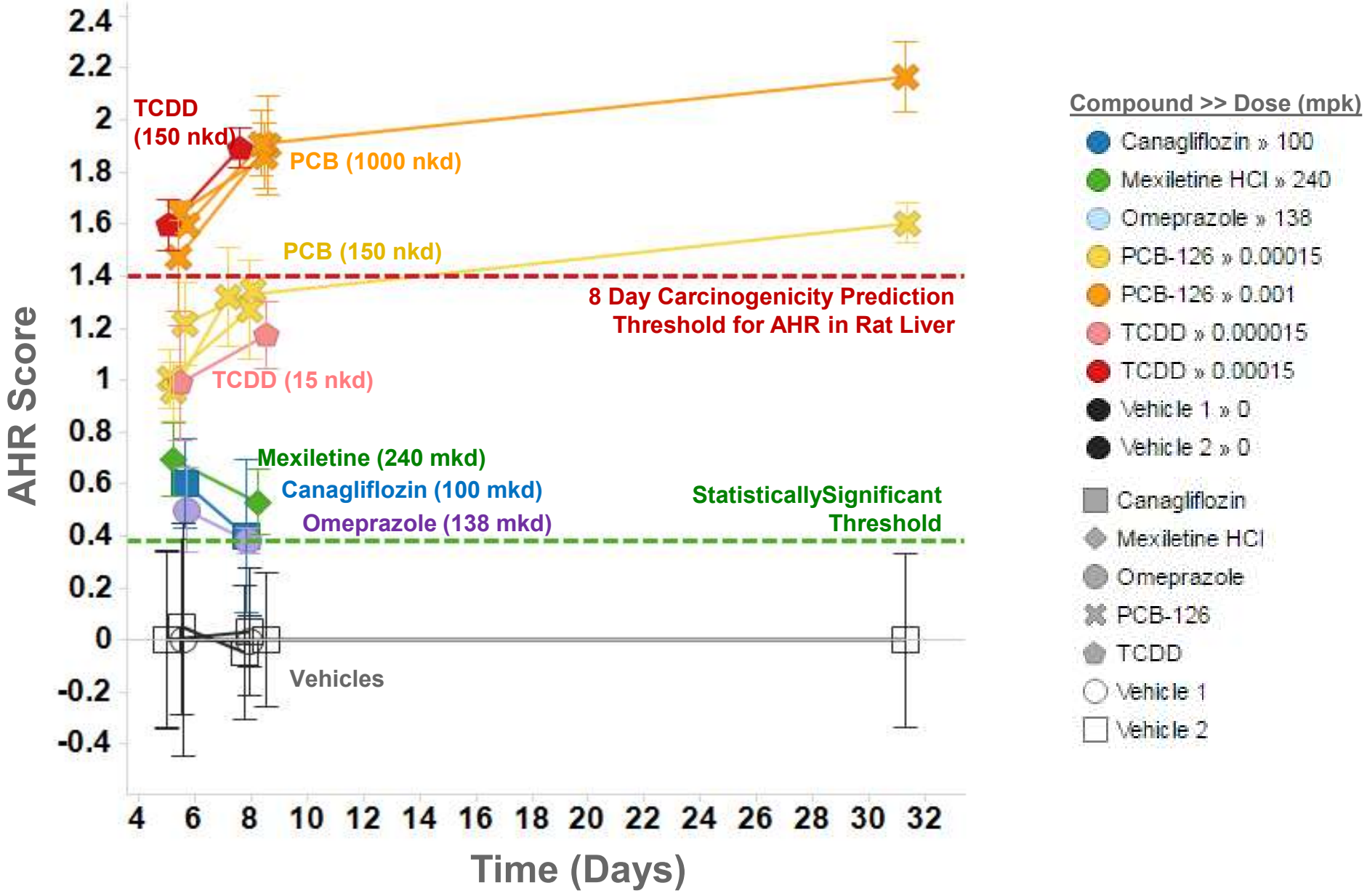
Table 1. Rat Study Design

Group	Compound Class	Test Material	Abbreviation	Vehicle	Dose (mg/kg)	Dose Class
1	Vehicle	—		1	0	
2	Vehicle	—		3	0	
3	Carcinogen	Hexachlorobenzene	HCB	1	0.5	Noncarcinogenic
4	Carcinogen	Hexachlorobenzene	HCB	1	5	Carcinogenic
5	Carcinogen	PCB126	PCB126	1	0.00015	Noncarcinogenic
6	Carcinogen	PCB126	PCB126	1	0.001	Carcinogenic
7	Carcinogen	TCDD	TCDD	1	0.000015	Noncarcinogenic
8	Carcinogen	TCDD	TCDD	1	0.00015	Carcinogenic
9	Noncarcinogen	Omeprazole	OMEP	2	138	Noncarcinogenic
10	Noncarcinogen	Omeprazole	OMEP	2	600	2-week MTD
11	Noncarcinogen	Mexiletine	MEXI	2	240	Noncarcinogenic
12	Noncarcinogen	Mexiletine	MEXI	2	400	2-week MTD
13	Noncarcinogen	Canagliflozin	CANA	3	100	Noncarcinogenic
14	Noncarcinogen	Canagliflozin	CANA	3	600	2-week MTD

Vehicle 1: 99% corn oil, 1% acetone; Vehicle 2: 0.5% (w/v) methylcellulose in deionized water; Vehicle 3: 10% (w/w) Polysorbate 80, 0.5% (w/v) methylcellulose in deionized water.

From: Qin, et al (2019) "AhR Activation in Pharmaceutical Development: Applying Liver Gene Expression Biomarker Thresholds to Identify Doses Associated with Tumorigenic Risks in Rats," Toxicol Sci 171: 46-55

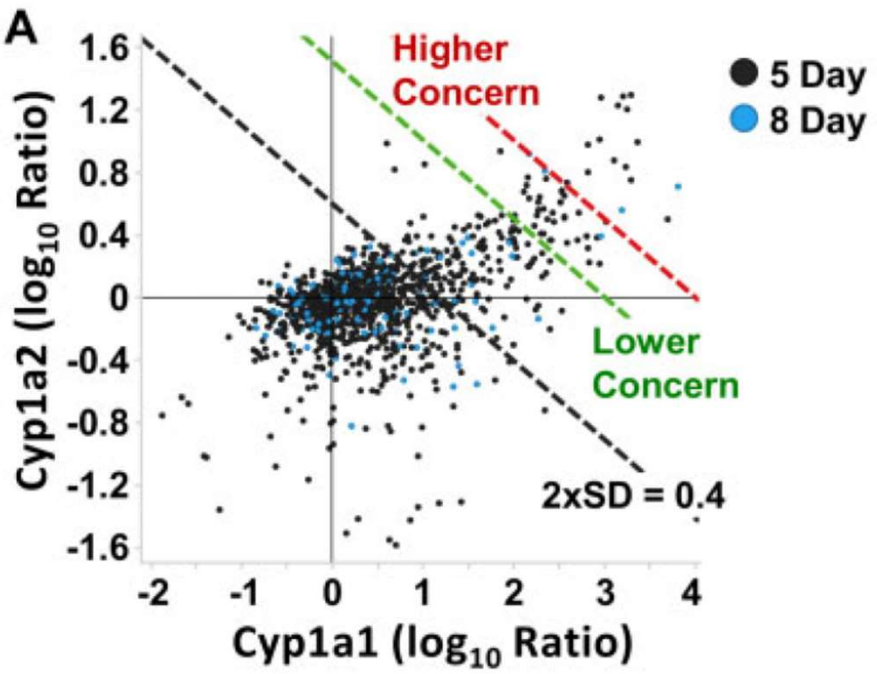
TCDD and PCB126 discriminate the same tumorigenic thresholds and they sustain AhR activation. 2-yr rat study doses of Omeprazole, Mexiletine and Canagliflozin fall well below threshold & do not sustain

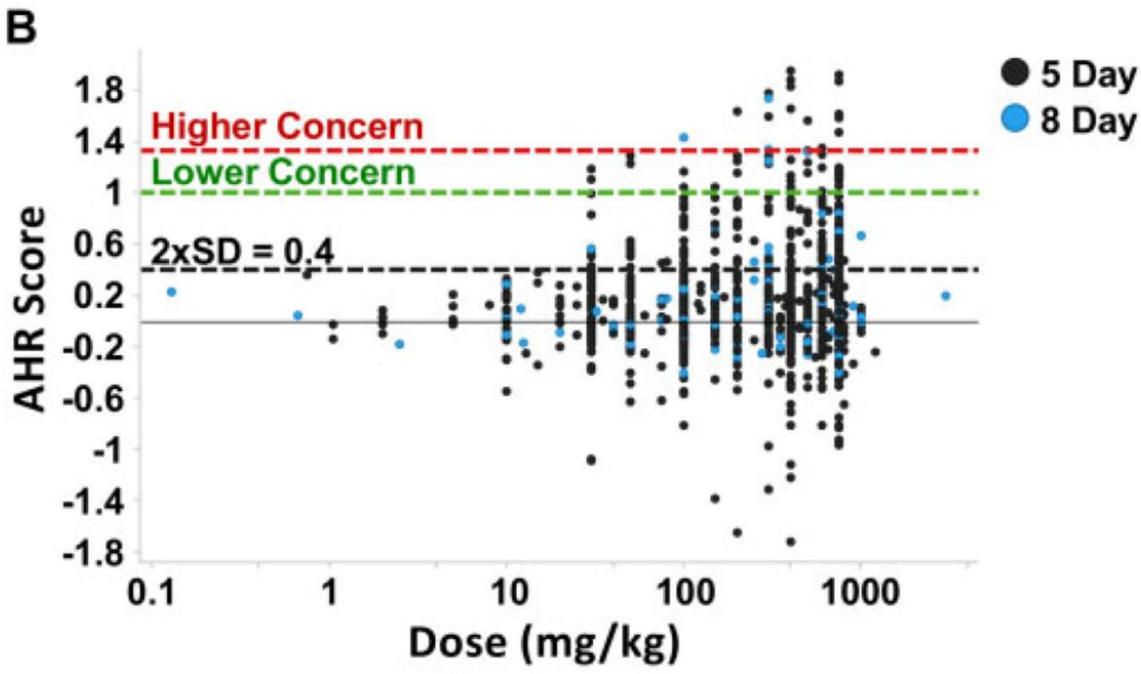


AHR Score = (0.33 x Cyp1a1*) + (0.66 x Cyp1a2*)
*Log10 Fold-Induction Ratio

From: Qin, et al (2019) Toxicol Sci 171: 46-55

Internal Merck & Co., Inc. Historical Experience with (A) Cyp 1A1 and 1A2 Rat Liver Gene Expression/ (B) AhR Receptor Activation Scores across 713 Drug Candidates





AHR Score = (0.33 x Cyp1a1*) + (0.66 x Cyp1a2*)

***Log10 Fold-Induction Ratio**

Table 2. Thresholds for Cyp1a1, Cyp1a2, and AhR Scores at Different Significance Levels

Significance Level	Cyp1a1[a]	Cyp1a2[a]	AhR Score
Statistical cutoff	0.84	0.26	0.40
Lower concern	2.00	0.48	1.00
Higher concern	2.60	0.70	1.33

[a]Log_{10} fold-induction ratio.

Rule of Thumb Fold-Inductions		
Threshold	Cyp 1A1	Cyp 1A2
Statistical	7 X	1.8 X
Lower Concern	< 100 X	< 3X
Higher Concern	> 400 X	> 20 X

From: Qin, et al (2019) Toxicol Sci 171: 46-55

MRK Case Example #1: 1st in Class Molecule w AhR Activation (a Human Relevant Off Target Risk Factor)

- Compound X de-selected due to **POTENT** and **SUSTAINED** rat liver AhR activation (… actually increased with longer duration dosing).
- Compound Y yielded far less Cyp 1a1 which decreased with continued dosing, and no apparent 1a2 induction in rat liver at greater human exposure multiples than Compound X.

Compound	Study (Days)	Dose (mkd)	AUC Multiple	mRNA Induction	
				Cyp 1a1	Cyp 1a2
X	Rat (4 Day)	300	113 X	242 X	6 X
		30	44 X	159 X	5 X
	Rat (7 Day)	400	140 X	2778 X	12 X
		10	5 X	163 X	4 X
Y	Rat (4 Day)	200	356 X	111 X	1 X
		50	57 X	9 X	1 X
	Rat (7 Day)	270	> 400 X	43 X	1 X
		90	91 X	13 X	1 X

Liver ADME gene expression scores from >400 early rat high dose tolerability studies at Merck show that potentially confounding nuclear receptor activation is VERY common

- **AhR Gene Signatures**
  - Overall **4%** of the compounds tested, presented with a Significantly Positive Score (seen in 3 programs)
  - An additional **20%** of compounds tested, presented with weaker positive AhR Scores of less concern

- **CAR Gene Signatures**
  - Overall **20%** of compounds tested had positive CAR dominant signatures
- **PXR Gene Signatures**
  - Overall **30%** of compounds tested had positive PXR dominant signatures
- **PPARα Gene Signatures**
  - Overall **16%** of compounds tested had positive PPARα signatures

NOTE that these are initial HIGH DOSE tolerability studies, with an unclear relationship to doses appropriate for 2 yr rat carc studies

Rooney et al (2018): Transcriptomics for 6 Molecular Initiating Events to Assess 77 Chemicals shows >90% Sensitivity for Predicting Chemical / Pharmaceutical Off-target Mechanisms of Rat Liver Carcinogenicity

Toxicology and Applied Pharmacology 356 (2018) 99–113

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Adverse outcome pathway-driven identification of rat liver tumorigens in short-term assays

John Rooney[a,b,1], Thomas Hill III[a,b,1], Chunhua Qin[c], Frank D. Sistare[c], J. Christopher Corton[b,*]

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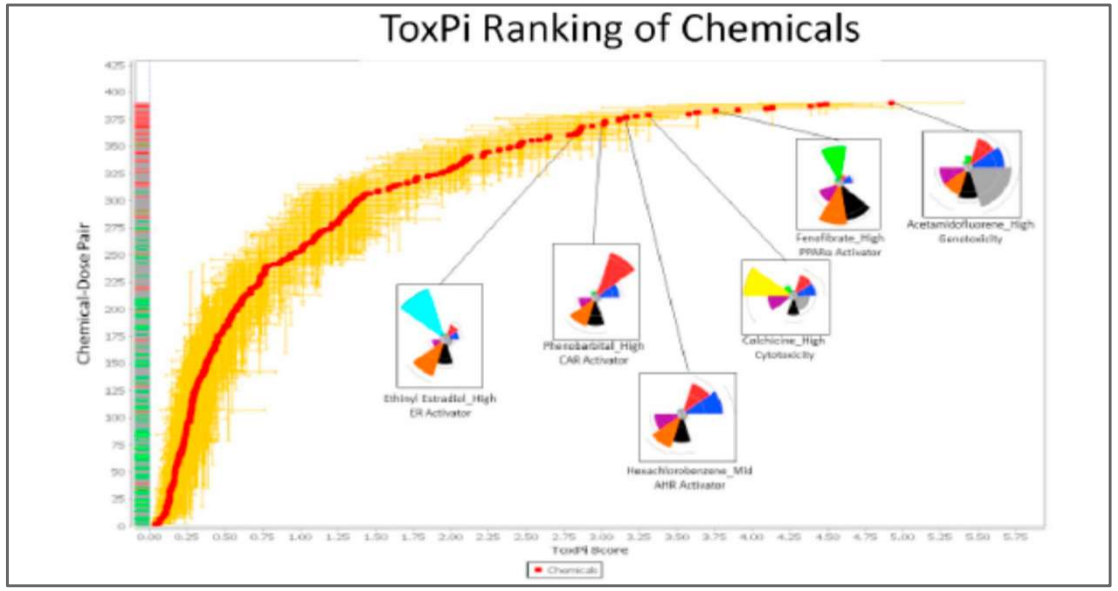
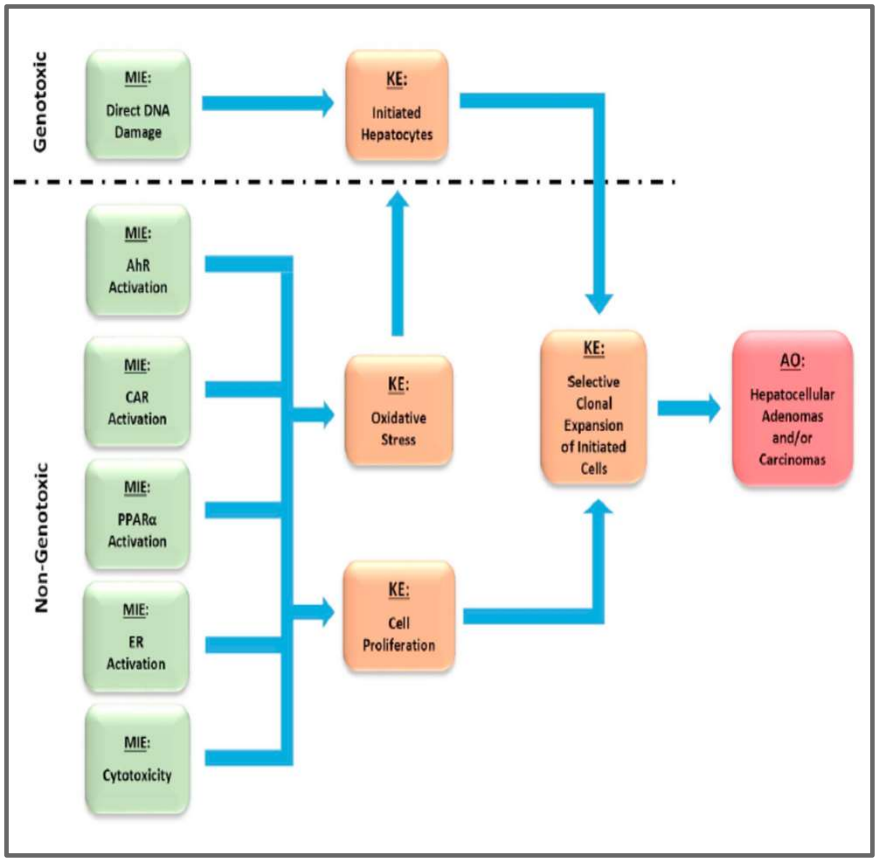
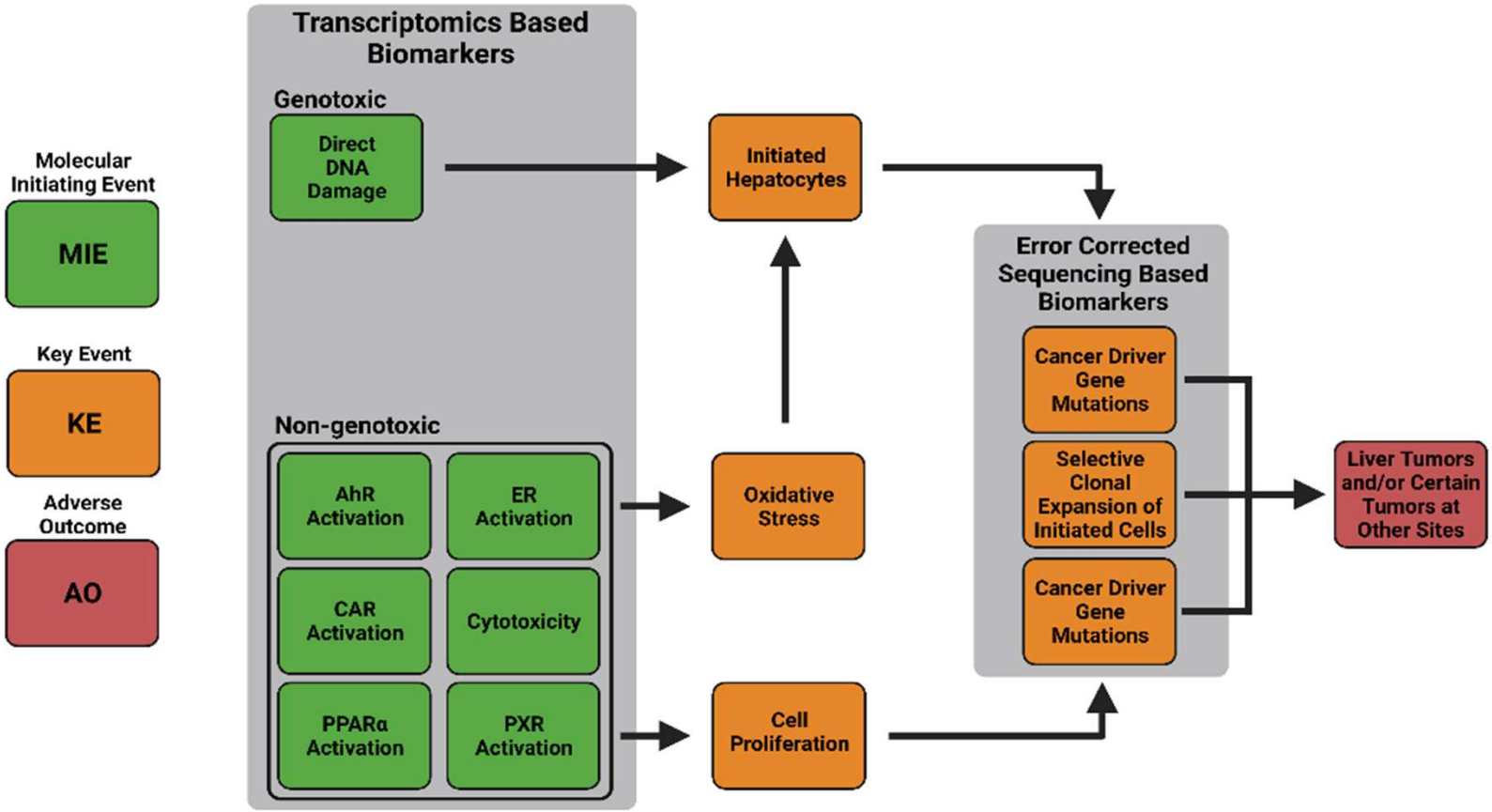




HESI eSTAR Carcinogenomics Project Launched to Leverage Emerging ICH S1 Guidance Modifications (Weight-of-Evidence Based Approach) and Molecular Biomarkers toward Reducing Reliance on the 2-year Rodent Bioassay



Early Whole Tissue "Field Effect"

Use Transcriptomics Based Biomarkers to query 7 commonly observed molecular initating events in sub-chronic/chronic rat studies with histologic risk factors of neoplasia to provide explanations for chemical carcinogenic mechanims and inform human relevance.

Late Rare Clonal Cellular Expanisons

Error Corrected Next Generation Sequencing technology to identify DNA Cancer Driver Gene Mutation based biomarkers for selective clonal expansion that could address earlier hypothetical concerns of carcinogenic risk.

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