**HepaRG™ Cells as a New Approach Methodology Follow Up to a Positive Response in Human TK6 Cell Micronucleus Assay: Naphthalene Case Study**

**Leslie Recio1,2, Jasmine Fowler2, Lincoln Martin2, Carol Swartz2**

1Corresponding Author Current Address:

Leslie Recio PhD DABT

ScitoVation

6 Davis Dr.

Durham, NC 27709

e-mail: [lrecio@scitovation.com](mailto:lrecio@scitovation.com)

Ph. No. (919) 670-8609

2Integrated Laboratory Systems, an Inotiv Company

Morrisville, NC

**Abstract**

We are evaluating the use of metabolically competent HepaRG™ cells combined with CometChip® for DNA damage and the micronucleus (MN) assay as a follow up for in vitro positive genotoxic response as alternatives to *in vivo* genotoxicity testing.. Naphthalene is genotoxic with rat liver S9 in human TK6 cells inducing a nonlinear dose-response for the induction of micronuclei in the presence of rat liver S9. To follow up this response, we used metabolically competent HepaRG™ cells as a New Approach Methodology (NAM) alternative to animals for genotoxicity assessment of naphthalene. In HepaRG™ cells, naphthalene genotoxicity was assessed using 12 concentrations of naphthalene with the top dose used for assessment of genotoxicity of 1.7 mM corresponding to 45% cell survival. In contrast to human TK6 cell with S9, Naphthalene was not genotoxic in either the HepaRG™ MN Assay or the Comet Assay using CometChip®. The lack of genotoxicity in both the MN and comet assays in HepaRG™ cells is likely due to Phase II enzymes removing phenols preventing further bioactivation to quinones and efficient detoxication of naphthalene quinones or epoxides by glutathione conjugation. In contrast to CYP450 mediated metabolism, these Phase II enzymes are inactive in rat liver S9 due to lack of appropriate cofactors causing a positive genotoxic response. This data indicates that rat liver S9-derived BMD10 over-predicts naphthalene genotoxicity BMD calculations when compared to hepatocytes. Metabolically competent hepatocyte models like HepaRG™ cells should be considered as human-relevant NAMs for use genotoxicity assessments to reduce reliance on rodents.

**INTRODUCTION**

Naphthalene is used as a chemical intermediate in the synthesis of dyes, plasticizers, insecticides, and is an important building block for synthesis of a broad-spectrum medicinal agents in drug discovery (International Agency for Research on Cancer [IARC], 2002; Makar et al., 2019). The toxicological properties of naphthalene have been the subject of numerous reviews (Griego et al., 2008; U.S. Environmental Protection Agency [U.S. EPA] 2004a, b). Systemic absorption of naphthalene after high acute exposure to naphthalene in humans, e.g. ingestion of moth balls, is associated with hemolytic anemia and reports of hepatotoxicity (jaundice, hepatomegaly, elevated serum enzyme levels) (see: <https://rais.ornl.gov/tox/profiles/naphthalene_f_V1.html>). The National Toxicology Program’s (NTP) cancer bioassays on inhaled naphthalene induced lung tumors in mice and nasal tumors in rats (Abdo et al., 1992, 2001). IARC classifies naphthalene as possibly carcinogenic to humans (Group 2B) (IARC, 2002) and the National Toxicology Program (NTP) Report on Carcinogens classifies naphthalene as “reasonably anticipated to be a human carcinogen” (NTP, 2016).

Naphthalene toxicity is dependent on biotransformation by cytochrome P450s (CYP450s) to reactive epoxide and quinone metabolites (Buckpitt et al., 2002; Cruzan et al., 2009; DeStefano-Shields et al., 2010; Bailey et al., 2016). Naphthalene inhalation at greater than 10 ppm induces cytotoxicity and cell proliferation in mouse lung and rat nasal tissue indicating site-of-contact bioactivation to reactive metabolites (Buckpitt et al., 2002; Dodd et al., 2010; Long et al., 2003; Phimister et al., 2004). In mice, Cyp2F2 is hypothesized to be a critical determinant in the species-specific and region-selective cytotoxicity and tumors in mouse lungs. Although, the human ortholog CYP2F1 of mouse Cyp2f2 shares 82% homology with mouse, the rate of naphthalene metabolism by human CYP2F1 is less than 0.1% that of mouse Cyp2F2. Rat nasal olfactory tissues, the primary site of nasal injury from inhaled naphthalene has high levels of Cyp450 enzymes and several rat CYP450s can biotransform naphthalene to cytotoxic metabolites in nasal tissues including Cyp2F4, the rat ortholog of mouse Cyp2F1, and Cyp2e1 with no single specific Cyp450 enzyme responsible for the bioactivation of naphthalene to reactive metabolites in nasal tissues (Cruzan et al., 2009; Li et al., 2011). In human liver microsomes, a spectrum of CYP450 enzymes can metabolize naphthalene to 1-, 2- naphthol, and dihydrodiol including CYP1A1/1A2, CYP2A6/2B6, CYP2E1 and CYP3A4 (Cho et al., 2006). CYP1A2 and 2D6\*1 can further biotransform naphthol to produce 1,4-naphthoquinone a known cytotoxic and genotoxic metabolite of naphthalene (Cho et al., 2006). The tissue-specific nature of individual P450 enzymes in mediating naphthalene toxicity in rodents and species differences in metabolic pathways to reactive metabolites for naphthalene has provided a basis for reliable assessments of the potential nasal toxicity and cancer risks of naphthalene inhalation in humans (Bailey et al., 2016).

We have previously examined the *in vitro* cytotoxicity of naphthalene in primary hepatocytes, nasal tissues and lung tissues from humans, mice, and rats. In human hepatocytes naphthalene induced dose dependent parallel decreases in ATP, glutathione (GSH) and cell viability at concentrations > 200 μM. In this study there were no significant species specificity in the cytotoxic responses to naphthalene exposures (Kedderis et al., 2014). However, there was significant differences in the spectrum of metabolites produced from human hepatocytes with naphthalene dihydrodiol as the primary metabolite detected compared to rat and mouse hepatocytes where glutathione-conjugates of naphthoquinone were the primary metabolites detected (Kedderis et al., 2014). This data supports known species differences in pathways of naphthalene metabolism and the biochemical reasons that underly tissue susceptibility and species-specific carcinogenic responses in rodents that are considered in naphthalene risk assessments.

Naphthalene CYP450 metabolites are substrates for Phase II conjugation enzymes that can eliminate the further bioactivation of phenols to reactive oxygen producing quinones, prevent covalent binding to macromolecules by naphthalene epoxide metabolites and eliminate reactive oxygen species. Phase II enzymes produce water soluble glutathione, sulfate, and glucuronide conjugates of naphthalene metabolites are recognized by transporter enzymes for export out of tissues and elimination from the body. Phase II enzymes participate in the detoxication and elimination of naphthalene with glutathione-derived metabolites accounting for 60-70% of the total measured metabolites excreted in urine with sulfate and glucuronide conjugates eliminated in equal amounts from mice exposed to inhaled naphthalene (Ayala et al., 2016). These Phase II metabolites are the primary metabolites excreted in urine from humans and can be used to biomonitor human exposure to naphthalene. Depletion of glutathione is a major determinant of naphthalene cytotoxicity that parallels loss of viability in human hepatocytes (Kedderis et al., 2014). Inhibition of sulfation and glucuronidation in hepatocytes causes 10-fold increases in naphthalene covalent binding (Schwarz et al. 1980), indicating that all three of the primary Phase II enzyme pathways operate in concert to eliminate naphthalene reactive metabolites. Phase II enzymes have a key role in determining nontarget/target tissue and species specificity of adverse outcomes from inhaled naphthalene.

Reviews on the genotoxicity of naphthalene have concluded that naphthalene is *not mutagenic* in bacterial or mammalian cell culture systems and is not genotoxic *in vivo* (Brusick, 2008; Brusick et al., 2008; IARC, 2002; Schreiner, 2003). However, naphthalene induces in vitro chromosomal damage at exposure levels that are cytotoxic, and the naphthalene metabolite 1,2-naphthoquinone is a direct mutagen in bacterial mutation tester strains that respond to oxidative stress (Brusick, 2008; Brusick et al., 2008; Bailey et al., 2016). The site specific tumors in mouse lung and rat nasal tissues are believed to be caused by high naphthalene exposures with tissue specific bioactivation to reactive metabolites that deplete Phase II enzyme detoxification. Depletion of GSH, sulfate and glucuronide conjugation will lead to cytotoxicity via free epoxide/quinone naphthalene metabolites, production of oxidative stress and indirect genotoxicity (Brusick, 2008; Brusick et al., 2008; U.S. EPA, 2004a,b; Bailey et al., 2016).

A positive response from the genetic toxicology test battery requires follow up in vivo genotoxicity testing with an appropriate endpoint that matches the in vitro response. In the case of an in vitro MN positive response the bone marrow micronucleus assay and the liver comet assay can be used to assess chromosomal damage and DNA damage in liver. In the case of Ames test positive the Pig-a or transgenic mouse mutation assay are appropriate follow ups (Robison et al., 2021). However, several industries are not supportive of animal testing yet still require a follow up from genetic toxicology hazard identification bioassays to human relevant bioassays that can be used to assess risk to humans. Secondly, there is an ongoing shift in the safety assessment of new drug candidates and environmental chemicals significantly impacting the practice of regulatory toxicology, including genetic toxicology. This shift includes reducing, and in some cases eliminating, traditional toxicity testing in animals with the development and implementation of human relevant New Alternative Methodologies (NAMs) using in vitro test systems that reflect human tissues (Stucki et al., 2022). However, replacing traditional animal toxicity tests requires the demonstration that NAMs can accurately predict both the responses known to occur in traditional regulatory testing and, more importantly, what can occur in humans.

For this study, we report the results of a positive response in the MN assay for naphthalene with rat liver S9 in human TK6 cells (Recio et al., 2012) and follow up these results with the comet assay and the MN assay using metabolically competent HepaRG™ cells. Rat liver S9 is devoid of Phase II enzyme activity that are key determinants of naphthalene tissue specific cytotoxicity and genotoxicity. Since Phase II enzymes play a key role in protecting cells from the effects of biologically reactive naphthalene metabolites, we have used naphthalene exposure in HepaRG™ to assess the effects of intact Phase I and Phase II metabolism on the genotoxicity of naphthalene,

**MATERIALS AND METHODS**

**Chemicals and No-Spin HepaRG™ cells**

Vehicle (methanol), positive controls, and naphthalene were purchased from Millipore Sigma (St. Louis, MO, USA) for exposures in fully differentiated, cryopreserved No-Spin HepaRG™ cells (Triangle Research Labs (TRL), Durham, NC, USA; acquired by Lonza Bioscience). Chemical exposures in HepaRG™ cells, cell viability studies, and the paired high-throughput CometChip® analysis were conducted at Integrated Laboratory Systems, Inc. (ILS; Research Triangle Park, Durham, NC, USA now Inotiv).

**TK6 Cell Micronucleus (MN) assay** Human TK6 cells were originally obtained in 2008 from the American Type Culture Collection: ATCC, 10801 University Boulevard, Manassas, VA 20110. The conduct of the MN assay in TK6 cells and corresponding details are in Recio et al., 2012. Some of those data are reproduced in this manuscript to permit a direct comparison for the readers to the data obtained with naphthalene exposures in metabolically competent HepaRG™ cells.

**HepaRG™ Cell Culture, Chemical Exposures and conduct of HepaRG™ CometChip® and MN Assay**

A detailed protocol for the cell culture of NoSpin® HepaRG™ cells (Lonza), chemical exposures, and conduct of the CometChip® assay has been published (Owiti et al., 2022; Buick et al., 2021). The details regarding the conduct of HepaRG™ MN assay have been published in detail (Buick et al., 2019). For the studies in this manuscript, we used the same 3-day repeat exposure regimen as done previously and described in detail (Buick et al., 2019, 2021, Owiti et al., 2022).

**Cell Viability Assay**

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was used to determine the number of viable HepaRG™ cells based on the quantification of ATP present following each chemical treatment. Cytotoxicity was evaluated 4 hrs after the last exposure following the manufacturer’s instructions in 96-well plates. Briefly, wells containing 100 µl cell samples were equilibrated at room temperature for 30 min prior to the addition of CellTiter-Glo® Reagent to each well in a volume equal to that of the cell culture medium (e.g., 100 µl). The contents were mixed for 2 min on an orbital shaker to induce cell lysis prior to incubation at room temperature for 10 min to stabilize the luminescent signal. Luminescence was measured on a SpectraMax® plate reader (Molecular Devices, San Jose, CA, USA). Luminescent signal is the result of the release of ATP from metabolically active cells and is directly proportional to the number of viable cells in the culture. The cytotoxicity cut-off was > 60% cytotoxic (equivalent to < 40% viable cells).

**Bio-techne® CometChip® Assay**

Exposed and control HepaRG™ cells were loaded into the CometChip® wells and were allowed to settle into microwells of a 96-well CometChip®. A 1% agarose overlay was then applied, and the cells were lysed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 with 1% Triton X-100 (Sigma, St. Louis, MO, USA) and 10% DMSO) overnight at 4°C. Following lysis, the CometChip® was equilibrated in an alkaline electrophoresis buffer (300 mM NaOH/1 mM EDTA) for 60 min and electrophoresed for 50 min under a 300 mA current at 4°C. These alkaline conditions are used to detect DNA single strand breaks. Following electrophoresis, the CometChip® was neutralized at 4°C for 2 × 15 min in 0.4 M Tris, pH 7.4 and equilibrated overnight at 4°C in 20 mM Tris, pH 7.4. Once equilibrated, the CometChip® was stained for 30 min at 4°C in 0.1X SYBR Gold and then de-stained for >1 hr. at 4°C in 20 mM Tris, pH 7.4. After de-staining, images were taken at 4X magnification of all 96 wells. The tiff images were captured and analyzed using Trevigen® Comet Analysis Software.

**Statistical Analysis of CometChip® Data**

The median % tail DNA for the CometChip® data was analyzed using one-way analysis of variance (ANOVA). The Anderson-Darling statistic was used to evaluate the normality assumption and the Fligner-Killeen test of homogeneity of variances was used to test the common variance assumption. If either assumption was not satisfied, the rank transformation was applied and a non-parametric one-way ANOVA was performed (Conover & Iman, 1981). Any pairwise comparisons to matched vehicle controls were conducted using the t-test. The resulting p-values were then family-wise error rate adjusted using Dunnett’s method.

**HepaRG™ Cell MN Assay Culture and Chemical Exposures**

Methods for the conduct of the in vitro MN assay in HepaRG cells have been published in detail (Buick et al., 2019). Briefly, HepaRG cells were exposed to six concentrations of naphthalene refreshed with media and test article daily for 3 days, i.e., 3-day repeat exposures (0 hr, 24 hr, and 48 hr). Twenty-four hrs after the day 3 exposure test articles were then removed, fresh media was added, and the cells were stimulated with human Epidermal Growth Factor-1 (hEGF) for an additional 72 hours to induce cell division (i.e., 144 hr total time following the last chemical exposure). The 3-day mitogen stimulation was found to increase the cell population by approximately 2-fold.

**In Vitro MicroFlow® MN Assay**

The flow cytometry-based cytotoxicity and MN assay was performed using the In Vitro MicroFlow® kit (Litron Laboratories, Rochester, New York, USA) (Bryce et al., 2008). Sample preparation, staining and other methods were performed according to the Instructional Manual provided with the kit. Data was collected using a Becton-Dickinson Faisalabad 2 laser 4-color instrument. Unless precluded by cytotoxicity, 5,000 (± 2,000) cells were analyzed to determine relative survival (% RS) and the MN frequency (% MN). A detailed description of the methods is outlined in Buick et al. (2019). In brief, % RS was determined using intact viable nuclei-to-bead ratios in exposed versus control cells by spiking in counting beads to the cell suspensions to function as the internal standards. MN induction was measured simultaneously using the double staining procedure. The RS and MN data were analyzed using generalized estimating equations (GEEs) as outlined in Yauk et al. (2016) (49). Briefly, a normal distribution for the RS data and a binomial distribution for MN data were assumed for the error terms. The geepack library in R was used for this analysis. GEEs only require specification of the first two moments, the mean and the variance. In the MN analysis, a log link function was used. The results were then back transformed to the original scale using the delta method. MN induction was positive if the MN frequency was statistically significant and there was at least a twofold increase above matched solvent vehicle controls.

**RESULTS**

**Micronucleus assay in human TK6 cells with rat liver S9**

In parallel with naphthalene studies in hepatocytes (Kedderis et al., 2014), naphthalene cytotoxicity and genotoxicity were determined in the micronucleus (MN) assay using human TK6 cells with Phenobarbital/β-naphthoflavone rat liver S9 supplemented with CYP450 cofactors to provide CYP450 mediated biotransformation (OECD 487) (Recio et al., 2012). In this study, naphthalene induced a dose-dependent increase in cytotoxicity and a nonlinear dose-response for the induction of MN (P < 0.05 at ≥ 5.0 µM by ANOVA Dunnett’s), with a pronounced break in the dose-response curve at exposure concentrations > 20 µM corresponding to approximately 80% survival and a twofold increase MN. The dose-response curves for MN and fraction of apoptotic/necrotic cells increased in parallel with increased exposure to naphthalene. The BMD10 for naphthalene induced MN was 6.2 µM (3.3 – 9.2 µM 95% confidence interval). Since the primary pathway for detoxication of naphthalene reactive metabolites is glutathione conjugation addition of GSH at physiological concentrations of 5 mM to the S9 eliminated the cytotoxicity and genotoxicity of naphthalene in human TK6 cells when evaluated up to 500 µM (Table I) (Recio et al., 2012). This data in TK6 cells with GSH supplemented S9 and data in human hepatocytes (Kedderis et al., 2014) indicates that GSH conjugation is a critical determinant of naphthalene cytotoxicity and genotoxicity.

**HepaRG™ CometChip® Assay**

Naphthalene was tested for DNA damage in the HepaRG™ CometChip® assay using a 3-day repeat dosing protocol as detailed in Owiti et al., 2022. After the conduct of a dose range finder study using eight concentrations of naphthalene, 6 exposure concentrations of naphthalene were tested based on cytotoxicity for DNA damage ranging from 39 µM to 1.25 mM (**Fig. 2**). There was no significant cytotoxicity based on levels of cellular ATP until an exposure level of 1.25 mM naphthalene that induced 50% cytotoxicity. Despite significant cytotoxicity induced by naphthalene at the top exposure concentration, there was no increase in naphthalene-induced DNA damage across all the exposure levels of naphthalene tested at up to 1.25 mM that caused ~50% cytotoxicity.

**Micronucleus Assay in HepaRG cells**

Naphthalene was tested for the induction of MN in HepaRG™ cells using a 3-day repeat dosing protocol as detailed in Buick et al., 2019 and Owiti et al., 2022. After the conduct of a doserange finder study, twelve exposure concentrations of naphthalene were tested for MN induction ranging from 340 µM to 2.5 mM (**Fig. 3**). There was no dose dependent decline in cell survival based on nuclei:bead ratio by flow cytometry until an exposure level of ≥ 1.00 mM naphthalene followed by a dose dependent decrease in cell survival to 30% survival at 2.08 mM, exceeding cytotoxicity criteria for the MN assay in OECD 487. There was no increase in naphthalene-induced MN across all the exposure levels of naphthalene tested, until the top level of cytotoxicity (≥ 55 ± 5%) was exceeded at 2.08 and 2.5 mM. The increases in MN observed at ≥ 2.08 mM are not relevant since they exceed the criteria for cytotoxicity. However, this demonstrates that highly cytotoxic irrelevant exposures of hepatocytes to naphthalene can produce “false positive” MN responses likely due to depletion glutathione by reactive naphthalene metabolites.

**DISCUSSION**

A number of laboratories are developing NAMs that recapitulate the biology and responses of human tissues to xenobiotic exposures as in vitro toxicology alternatives to animal testing. Relying primarily or exclusively on animals for safety and risk assessments may not adequately characterize the spectrum of potential effects in humans limiting the ability to accurately assess human health hazards and potential risk. (National Academy of Sciences, 2023). Secondly, these human-relevant NAMs are being employed in the pharmaceutical and chemical industries to identify human-relevant potential hazards early development prior to going forward for regulatory safety assessment testing (Avila et al., 2020). Integrating data from these test systems with computational tools for in vitro to in vivo extrapolation (IVIVE) modeling are beginning to impact risk assessments to humans (Ramanarayanan et al., 2022; Moreau et al., 2022). Our laboratory is focused on developing multiple genetic toxicology endpoints in human hepatocytes since liver is the primary target organ affected in regulatory toxicology testing in rodents, drug induced liver injury in humans remains a significant issue in drug development exposed during clinical trials, and liver is the primary tissues for the in vivo comet assay used for regulatory genetic toxicology testing. The current study examines the use HepaRG™ as a genetic toxicology NAM alternative to the in vivo comet and MN assays using naphthalene as a prototype in vitro genotoxic compound requiring metabolic activation.

To provide xenobiotic metabolizing capability to cells devoid of CYP450, genetic toxicology employs a highly induced liver homogenate preparation referred to as S9 (McGregor et al., 1991). For the preparation of rat liver S9 for commercial sale, rats are dosed with the highly potent CYP450 enzyme inducers, phenobarbital and β-naphthoflavone causing proliferation of the endoplasmic reticulum, hepatocellular hypertrophy, and increased liver weight. The liver homogenate as used in regulatory genetic toxicology testing is the supernatant of a 9000xg spin (S9) of homogenized livers from phenobarbital/β-naphthoflavone induced rats. For regulatory testing, S9 is supplemented with CYP450 cofactors only (NADPH) referred to as S9 mix. The induction regimen used to prepare rat liver S9 induces Cyp450s greater than 100-fold enabling the enhanced biotransformation of a broad spectrum of xenobiotics. This high level of induction can provide enhanced CYP450 mediated bioactivation without detoxication since rat liver S9 is supplemented with CYP450 cofactors (NADPH) only and Phase II biotransformation enzymes are inactive due to lack of cofactors required by glucuronyl transferases, sulfotransferases, and glutathione-S-transferases. This high level of CYP450 bioactivation without Phase II detoxication represents a significant imbalance in xenobiotic biotransformation. In liver and in hepatocytes Phase I and Phase II metabolism are intact and linked in contrast to rat liver S9 supplemented with Cyp450 cofactors only supporting Cyp450 mediated bioactivation. This imbalance of bioactivation with detoxication can lead to false positive responses in genetic toxicology teting.

As indicated previously, when naphthalene was tested according to OECD 487 in TK6 cells with S9, naphthalene induced a dose-dependent increase in MN at exposure concentrations > 20 µM corresponding to approximately 80% survival and a twofold increase in MN as a second criterion for positive test concentrations (Recio et al., 2012). The benchmark dose in TK6 cells with 95% confidence interval in human TK6 cells was 3.4- 9.8 µM, well below the concentration in liver (96 µM) of rodents exposed to inhaled naphthalene (Willems et al., 2001). Addition of glutathione at liver physiological concentrations of 5 mM to the S9 mix eliminated naphthalene induced cytotoxicity and genotoxicity. Although naphthalene is genotoxic by inducing chromosome aberrations in mammalian cell systems (Galloway et al., 1987), and the induction of MN in human TK6 cells with rat liver S9, naphthalene is not genotoxic in rodent liver or bone marrow (Brusick 2008; Brusick et al., 2008).

The previous results testing naphthalene with rat liver S9 in TK6 cells is in direct contrast to the results observed with metabolically competent HepaRG™ (Fig. 3 and 4). These data indicate that in HepaRG™ cells naphthalene reactive epoxide metabolites are efficiently conjugated and detoxified by glutathione conjugation and phenols/dihydrodiols are conjugated by glucuronidation or sulfation blocking further metabolism to quinones and the formation or cytotoxic/genotoxic reactive oxygen species. These data indicate that the positive response for naphthalene in TK6 cells with S9 tested as per OECD 487 was a false positive response due to bioactivation by CYP450 without Phase II detoxication that is absent in rat liver S9. The lack of a genotoxic effect for naphthalene in HepaRG™ cells is consistent with the lack of naphthalene genotoxicity in rat hepatocytes using the alkaline elution assay (Sina et al., 1983) and the *in vivo* MN assay.

Current genetic toxicology requirements for the pharmaceutical industry dictates that a positive result from a single bacterial mutation assay or *in vitro* mammalian cell genotoxicity assay used in the regulatory testing battery can trigger costly, time-consuming animal studies (Robison et al., 2022). The liver homogenate preparations incorporated into the regulatory genetic toxicology test battery used to provide biotransformation/bioactivation of xenobiotics integrated with cells devoid of endogenous metabolism does not include Phase II conjugation enzymes, and false positives due to CYP450 bioactivation without Phase II detoxication can cause needless animal testing. The use of S9 mix in short-term genetic toxicology results in a bias toward the production of toxic metabolites formed that can differ significantly from *in vivo* pathways of biotransformation or those that occur in intact hepatocytes. In many cases but not all, *in vitro* positives are not positive *in vivo* and frequently in vitro positive results are overruled by the conduct of an in vivo genotoxicity assay. However, as indicated previously, certain industries are reducing reliance on animals while for others animal testing cannot be employed to follow up in vitro positive genetic toxicology tests, therefore in vitro alternatives need to be developed as human relevant alternatives to animals to assess the genotoxic potential of xenobiotics.

We suggest that S9 mediated positives from the *in vitro* MN assay can be followed in human relevant NAMs employing metabolic competent HepaRG™ cells with CometChip for DNA damage, the MN assay for chromosomal aberrations and TGx-DDI (Buick et al., 2019; 2021; Owiti et al., 2022), rather than the conduct of *in vivo* Comet and MN assays in rodents using up to 90 animals when conducted independently. Use of human relevant metabolically competent models from various tissues such as liver, lung, skin or GI tract that measure the identical genetic toxicology endpoints as measured *in vivo* can be used to replace, reduce, and refine the use of animals in the practice of investigative and regulatory genetic toxicology.

**Funding**

This work was supported by a grant 4R44ES024698-02 funded by the NIEHS.

**Role of each Author**

**LR –** Principal Investigator, Experimental design, wrote manuscript.

**JF** – Conduct of MN assay.

**LM** – Conduct of CometChip®.

**CS** – Laboratory Manager, review data

**REFERENCES**

Abdo, KM, Eustis, SL, McDonald, M, Jokinen, MP, Adkins Jr, B, Haseman,

JK (1992). Naphthalene: a respiratory tract toxicant and carcinogen for mice. *Inhal.Toxicol.* **4**, 393-409.

Abdo, KM, Grumbein, S, Chou, BJ and Herbert, R. (2001). Toxicity and carcinogenicity study in F344 rats following 2 years of whole-body exposure to naphthalene vapors. *Inhal Toxicol.* **13**, 931-950.

Avila AM, Bebenek I, Bonzo JA, Bourcier T, Davis Bruno KL, Carlson DB, Dubinion J, Elayan I, Harrouk W, Lee SL, Mendrick DL, Merrill JC, Peretz J, Place E, Saulnier M, Wange RL, Yao J, Zhao D, Brown PC. (2020). An FDA/CDER perspective on nonclinical testing strategies: Classical toxicology approaches and new approach methodologies (NAMs). *Regul Toxicol Pharmacol.* 2020 Jul;114:

Bailey LA, Nascarella MA, Kerper LE, Rhomberg LR. (2016) Hypothesis-based weight-of-evidence evaluation and risk assessment for naphthalene carcinogenesis. *Crit Rev Toxicol*. 46(1):1-42.

Bryce SM, Avlasevich SL, Bemis JC, Lukamowicz M, Elhajouji A, Van Goethem F, De Boeck M, Beerens D, Aerts H, Van Gompel J, Collins JE, Ellis PC, White AT, Lynch AM, Dertinger SD. (2008). Interlaboratory evaluation of a flow cytometric high content *in vitro* micronucleus assay. *Mutat Res*. 650, 181-195.

Brusick, D (2008). Critical assessment of the genetic toxicity of naphthalene.

*Regul Toxicol Pharmacol*. **51(2 Suppl)**, S37-42.

Brusick, D, Small, MS, Cavalieri, EL, Chakravarti, D, Ding, X, Longfellow, DG, Nakamura, J, Rogan, EC, Swenberg, JA (2008). Possible genotoxic modes of action for naphthalene. *Regul Toxicol Pharmacol*. **51(2 Suppl)**, S43-50.

Buckpitt, A, Boland, B, Isbell, M, Morin, D, Shultz, M, Baldwin, R, Chan, K, Karlsson, A, Lin, C, Taff, A, West, J, Fanucchi, M, Van Winkle, L, Plopper, C (2002). Naphthalene-induced respiratory tract toxicity: metabolic mechanisms of toxicity. *Drug Metab Rev*. **34**, 791-820

Buckpitt, A R, Bahnson, LS, Franklin, RB (1984) [Hepatic and pulmonary microsomal metabolism of naphthalene to glutathione adducts: factors affecting the relative rates of conjugate formation.](http://www.ncbi.nlm.nih.gov/pubmed/6491983) *J Pharmacol Exp Ther.* **231**, 291-300.

Buick JK, Williams A, Gagné R, Swartz CD, Recio L, Ferguson SS, Yauk CL. (2020). Flow cytometric micronucleus assay and TGx-DDI transcriptomic biomarker analysis of ten genotoxic and non-genotoxic chemicals in human HepaRG™ cells. *Genes Environ.* 4;42:5.

Buick JK, Williams A, Meier MJ, Swartz CD, Recio L, Gagné R, Ferguson SS, Engelward BP, Yauk CL. (2021). A Modern Genotoxicity Testing Paradigm: Integration of the High-Throughput CometChip® and the TGx-DDI Transcriptomic Biomarker in Human HepaRG™ Cell Cultures. *Front Public Health*. Aug 18;9:694834.

Callander, RD, Mackay, JM, Clay, P, Elcombe, CR, Elliott, BM (1995). Evaluation of phenobarbital/beta-naphthoflavone as an alternative S9-induction regime to Aroclor 1254 in the rat for use in in vitro genotoxicity assays. *Mutagenesis* **10**, 517-522.

Cruzan, G, Bus, J, Banton, M, Gingell, R, Carlson, G (2009). Mouse specific lung tumors from CYP2F2-mediated cytotoxic metabolism: an endpoint/toxic response where data from multiple chemicals converge to support a mode of action. *Regul Toxicol Pharmacol.* **55(2)**, 205-218.

Dodd, DE, Gross, EA, Miller, RA, Wong, BA (2010) [Nasal olfactory epithelial lesions in F344 and SD rats following 1- and 5-day inhalation exposure to naphthalene vapor.](http://www.ncbi.nlm.nih.gov/pubmed/20086191) *Int J Toxicol*. 29, 175-184.

Galloway, SM, *et al*. (1987) Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. Environ Mol Mutagen. **10** **Suppl 10**, 1-175.

Griego, FY, Bogen, KT, Price, PS, Weed, DL (2008). Exposure, epidemiology and human cancer incidence of naphthalene. *Regul Toxicol Pharmacol*. **51(2 Suppl)**,S22-26.

IARC Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene. (2002). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. IARC, Lyon, France, 367-435.

Kelty, J, Kovalchuk, N, Uwimana, E, Yin, L, Ding, X, Van Winkle L (2022) In vitro airway models from mice, rhesus macaques, and humans maintain species differences in xenobiotic metabolism and cellular responses to naphthalene. *Am J Physiol Lung Cell Mol Physiol* 323(3):L308-L328.

Long, P H, Herbert, RA, Peckham, JC, Grumbein, SL, Shackelford, CC, Abdo, K (2003). Morphology of nasal lesions in F344/N rats following chronic inhalation exposure to naphthalene vapors. *Toxicol Pathol*. **31**, 655-664.

McGregor, DB, Edwards, I, Wolf CR, Forrester, LM, Caspary, WJ (1991). [Endogenous xenobiotic enzyme levels in mammalian cells.](http://www.ncbi.nlm.nih.gov/pubmed/1715512) *Mutat. Res*. **261**, 29-39.

Moreau M, Fisher J, Andersen ME, Barnwell A, Corzine S, Ranade A, McMullen PD, Slattery SD (2022) NAM-based prediction of point-of-contact toxicity in the lung: A case example with 1,3-dichloropropene. *Toxicology* 481:153340.

National Academies of Sciences, Engineering, and Medicine. 2023. *Building Confidence in New Evidence Streams for Human Health Risk Assessment: Lessons Learned from Laboratory Mammalian Toxicity Tests*. Washington, DC: The National Academies Press. https://doi.org/10.17226/26906

Organisation for Economic Co-operation and Development (OECD) 487 (2010). OECD Guideline for the Testing of Chemicals, *In Vitro* mammalian cell micronucleus test.

Owiti NA, Kaushal S, Martin L, Sly J, Swartz CD, Fowler J, Corrigan JJ, Recio L, Engelward BP (2022). Using the HepaCometChip® Assay for Broad-Spectrum DNA Damage Analysis. *Curr Protoc*. Sep;2(9):e563

Phimister, AJ, Lee, MG, Morin, D, Buckpitt, AR and Plopper, CG (2004). Glutathione depletion is a major determinant of inhaled naphthalene respiratory toxicity and naphthalene metabolism in mice, *Toxicological Sciences* **82**, 268-278.

Ramanarayanan T, Szarka A, Flack S, Hinderliter P, Corley R, Charlton A, Pyles S, Wolf D. (2022) Application of a new approach method (NAM) for inhalation risk assessment. *Regul Toxicol Pharmacol.* 133:105216.

Robison TW, Heflich RH, Manjanatha MG, Elespuru R, Atrakchi A, Mei N, Ding W. (2021) Appropriate in vivo follow-up assays to an in vitro bacterial reverse mutation (Ames) test positive investigational drug candidate (active pharmaceutical ingredient), drug-related metabolite, or drug-related impurity. *Mutat Res Genet Toxicol Environ Mutagen.* Aug-Sep;868-869

Saeed, M, Higginbotham, S, Rogan, E and Cavalieri, E (2007). Formation of depurinating N3adenine and N7guanine adducts after reaction of 1,2-naphthoquinone or enzyme-activated 1,2-dihydroxynaphthalene with DNA. Implications for the mechanism of tumor initiation by naphthalene*, Chem Biol Interact* **165**, 175-188.

Schreiner, CA (2003). Genetic toxicity of naphthalene: a review. *J Toxicol Environ Health B Crit Rev.* **6**,161-83.

Schwarz LR, Mezger M, Hesse S (1980) Effect of decreased glucuronidation and sulfation on covalent binding of naphthalene in isolated rat hepatocytes. *Toxicology* 17(2):119-22.

Sina JF, Bean CL, Dysart GR, Taylor VI, M.O. Bradley MO (1983). Evaluation of the alkaline elution/rat hepatocyte assay as a predictor of carcinogenic/mutagenic potential. *Mutat. Res.*, 113 (1983) 357-391.

Stucki AO, Barton-Maclaren TS, Bhuller Y, Henriquez JE, Henry TR, Hirn C, Miller-Holt J, Nagy EG, Perron MM, Ratzlaff DE, Stedeford TJ, Clippinger AJ.(2022) Use of new approach methodologies (NAMs) to meet regulatory requirements for the assessment of industrial chemicals and pesticides for effects on human health. *Front Toxicol.* 4:964553.

US EPA Toxicological Review of Naphthalene, in Support of Summary Information

on the Integrated Risk Information System (IRIS). US Environmental Protection

Agency, Office of Research and Development, NCEA-S-1707, External Review

Draft. (2004a). Available at:

http://oaspub.epa.gov/eims/eimscomm.getfile?p\_download\_id=434727.

US EPA Environmental Protection Agency (EPA). (2004b). Summary Review of

Health Effects Associated with Naphthalene: Health Issue Assessment.,

EPA/600/8-87/055F (NTIS PB88172374). Available at:

http://oaspub.epa.gov/eims/eimscomm.getfile?p\_download\_id=434728.

Wilson, AS, Davis, CD, Williams, DP, Buckpitt, AR, Pirmohamed, M and Park, BK (1996). Characterisation of the toxic metabolite(s) of naphthalene, *Toxicology* **114**, 233-242.

**Table 1. Relative Percent Survival Based on Bead:Nuclei Ratio in Unexposed and Naphthalene-Exposed Human TK6 Cells (From Recio et al., 2012).**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Naphthalene**  **Concentration (μM)** | **(–) L-Glutathione** | | | **(+) L-Glutathione (5.0 mM)** | | |
| **Mean Bead:Nuclei** | **Standard Deviation** | **Relative Percent Survival*a*** | **Mean Bead:Nuclei** | **Standard Deviation** | **Relative Percent Survival*a*** |
| **0.0** | 3.6 | 0.19 | 100 | 3.9 | 0.01 | 100 |
| **25.0** | 3.0 | 0.18 | 83.1 | 4.0 | 0.05 | 103.6 |
| **37.5** | 2.4 | 0.02 | 66.8 | 3.9 | 0.01 | 101.4 |
| **50.0** | 1.8 | 0.01 | 48.1 | 3.9 | 0.03 | 101.2 |
| **100.0** |  |  |  | 3.7 | 0.12 | 95.9 |
| **250.0** |  |  |  | 3.8 | 0.1 | 98.9 |
| **500.0** |  |  |  | 4.1 | 0.05 | 106.2 |
|  |  |  |  |  |  |  |
| **Cyclophosphamide 12.5 μg/ml** | 2.3 | 0.7 | 64.1 | 3.7 | 0.2 | 94 |
|  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |

*a*Relative percent survival for naphthalene exposures – L-Glutathione and + L-Glutathione calculated independently.

Unexposed controls used as 100

**Figure Legends**

**Fig. 1. Figure 1.** Assessment of naphthalene induced cytotoxicity (bead:nuclei ratio) and genotoxicity in human TK6 cells with rat liver S9. The dashed line indicates a 2-fold increase over the background MN frequency. The **\*** indicates *P* < 0.05 using ANOVA by Kruskal-Wallis with Bonferroni correction. Error bars indicate one standard deviation. (Figure from Recio et al., 2012)

**Fig. 2.** Cytotoxicity andDNA damageassessmentof naphthalene in HepaRG™ CometChip® assay. The %Tail methanol vehicle control was 4.2 ± 0.7, the positive control Ethyl Methane Sulfonate 6 mM was 57.1 ± 9.1 and the positive control cyclophosphamide 10 mM was 12.4 ± 3.6.

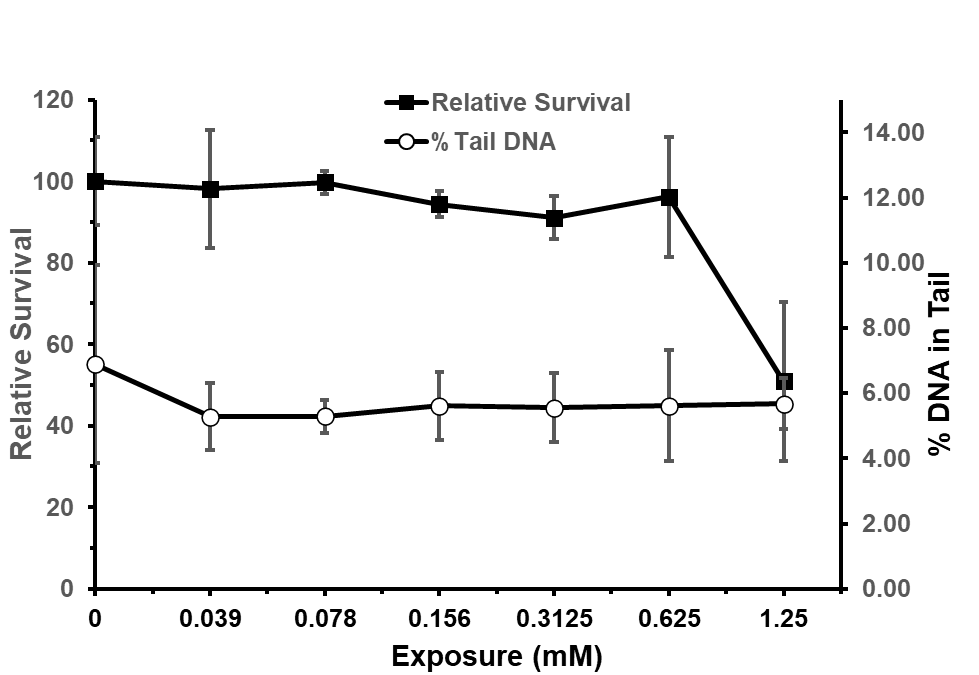
**Fig. 3.** Cytotoxicity andMN assessmentof naphthalene in HepaRG™ using the MN assay. The MN frequency in methanol vehicle control was 1.9 ± 0.2 , the MN frequency positive control vinblastine (6.2 nM) was 38.1 ± 2.7 and for the positive control Benzo(a)pyrene (50.0 µM) was 10.6 ± 1.3.

**Fig. 4.** The difference between rat liver S9CYP450 bioactivation to produce epoxide and quinone reactive metabolites of naphthalene that induce cytotoxicity and MN response in TK6 cells contrasted to the limited cytotoxicity and no genotoxicity observed in Phase I and Phase IImetabolically competent HepaRG™ cells exposed to naphthalene.

Fig. 1.

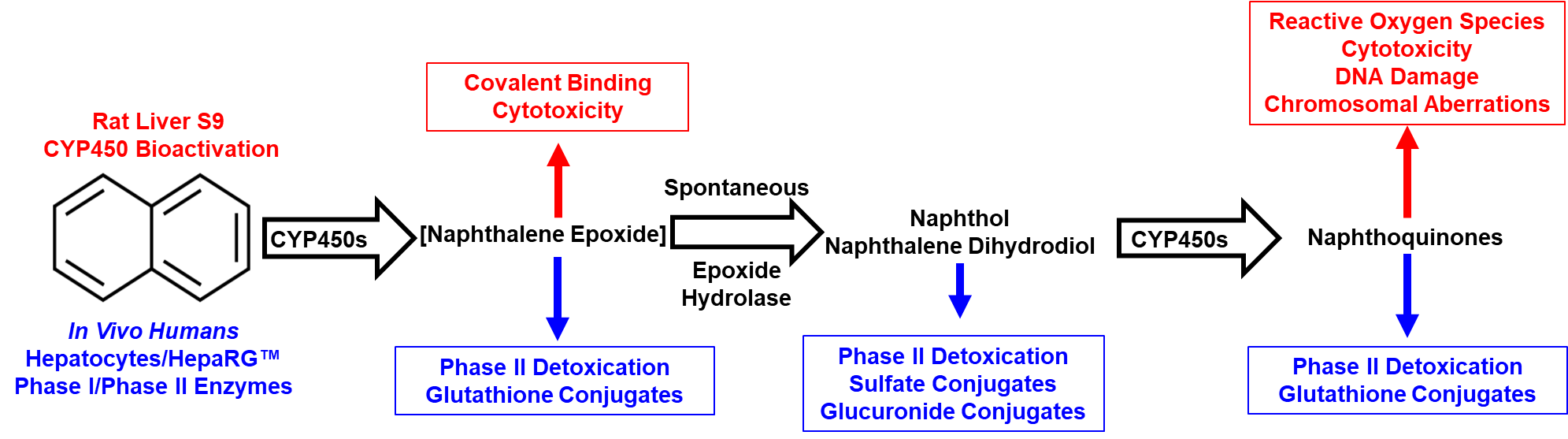
****

**Fig. 2**



**Fig. 3**

***Fig. 4***

******