Application of HepaRG Cells for Genetic Toxicity Testing as an In Vitro New Approach Methodology (NAM)

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Disclaimer

The information within this presentation is based on the presenter's experience and research, and does not necessarily represent the views of NCTR or U.S. FDA policy.

National Center for Toxicological Research - FDA

Mission

Address FDA's needs with high-quality research and serve as a global resource for collaboration, training, and innovative scientific solutions.

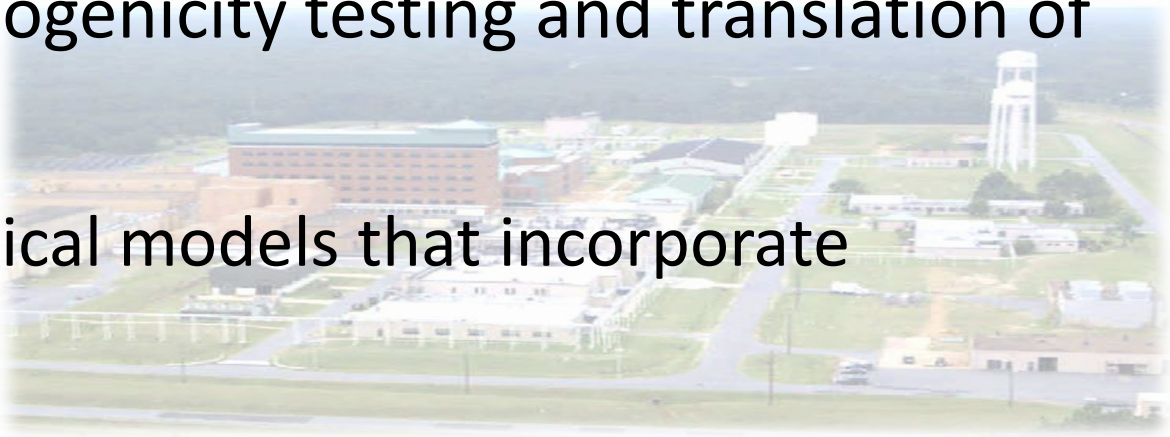
Vision

Conduct scientific research to provide reliable data for FDA's decision-making and develop innovative tools and approaches that support FDA's public health mission.

Division of Genetic and Molecular Toxicology (DGMT)

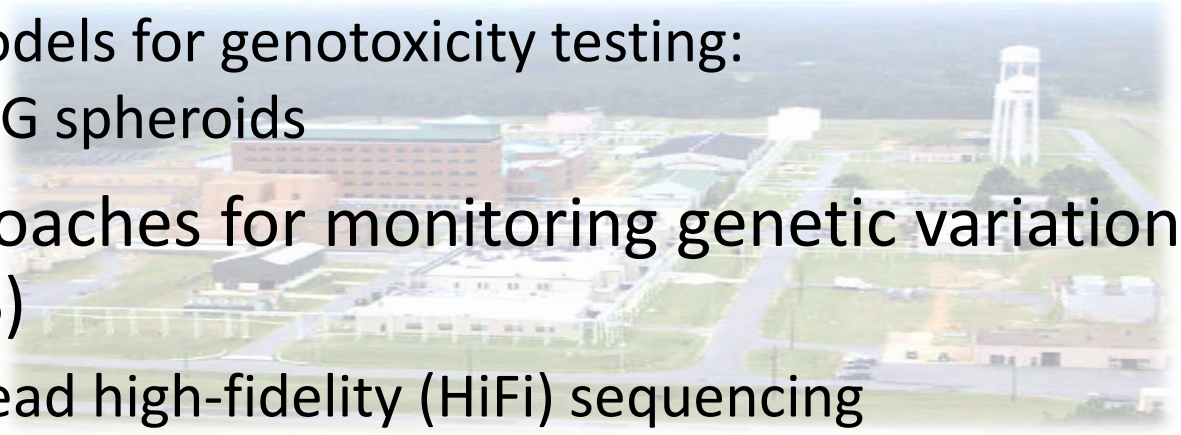
Goals

- Responding to Agency needs for genetic toxicology expertise and chemical-specific data (*e.g.*, nitrosamine impurities, CBD, and tobacco products).
- Maintaining DGMT's tradition of leadership in regulatory genetic toxicology assay development and validation (*e.g.*, Ames, TGR, MLA, TK/HPRT, Micronucleus, Comet, and *Pig-a* assays).
- Developing better methods for carcinogenicity testing and translation of rodent studies to human cancer risk.
- Developing advanced in vitro toxicological models that incorporate genotoxicity endpoints.

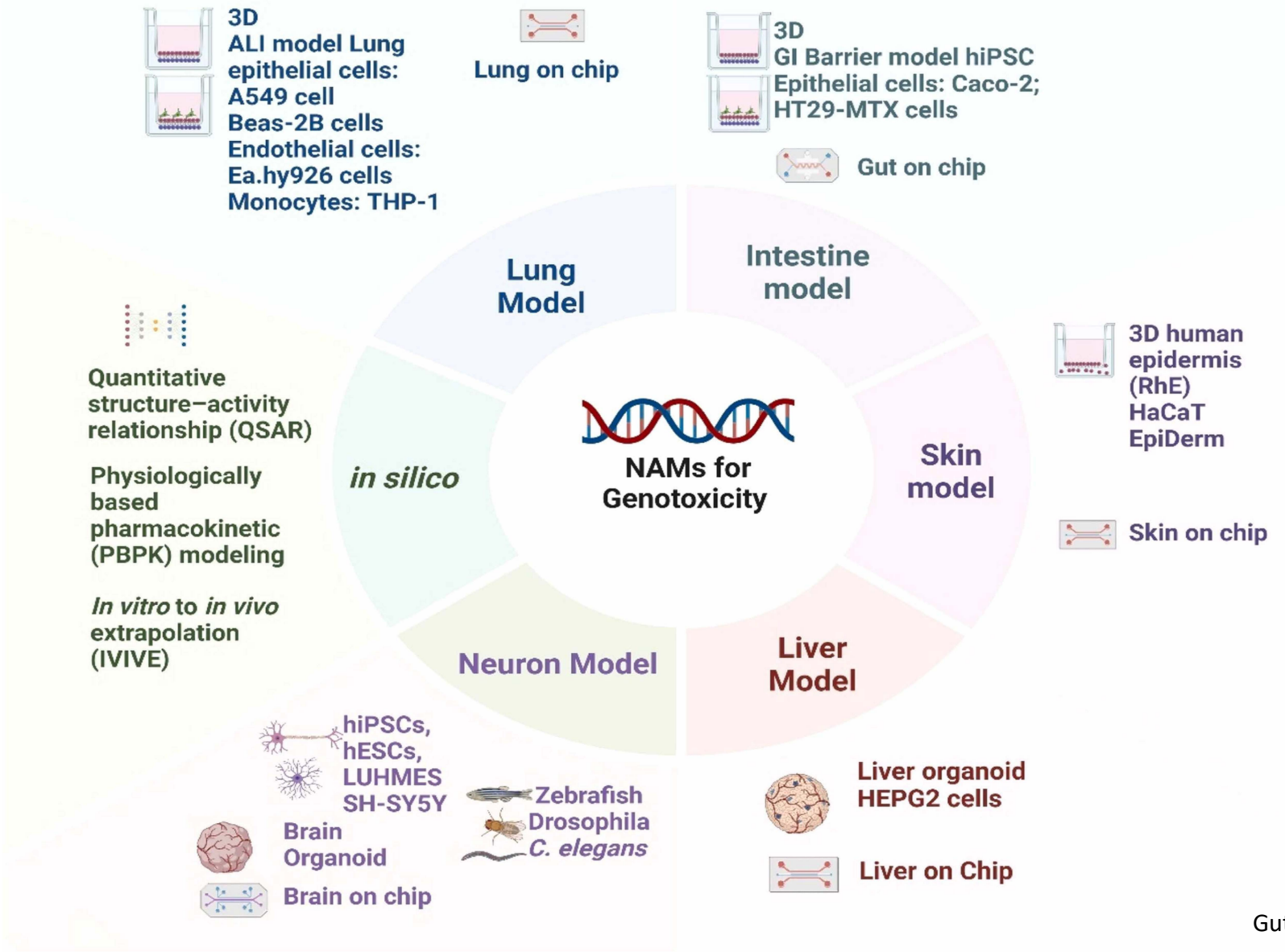


Division of Genetic and Molecular Toxicology (DGMT)

- Refining Regulatory Ames Assay (Enhanced Ames Test; EAT)
- Regulatory Acceptance of the *Pig-a* Gene Mutation Assay
- Developing Better Biological Models
  - Establishment of C. elegans for evaluating germ cell mutation
  - Rat in vitro testicular organoids as a model for Zika virus infection
  - Human Air-liquid-interface (ALI) airway tissue model for respiratory toxicology research
  - In vitro human metabolic competent models for genotoxicity testing: CYP450-transduced TK6 cells and HepaRG spheroids
- Developing more comprehensive approaches for monitoring genetic variation using Error-Corrected Sequencing (ECS)
  - CarcSeq, duplex sequencing, and long-read high-fidelity (HiFi) sequencing



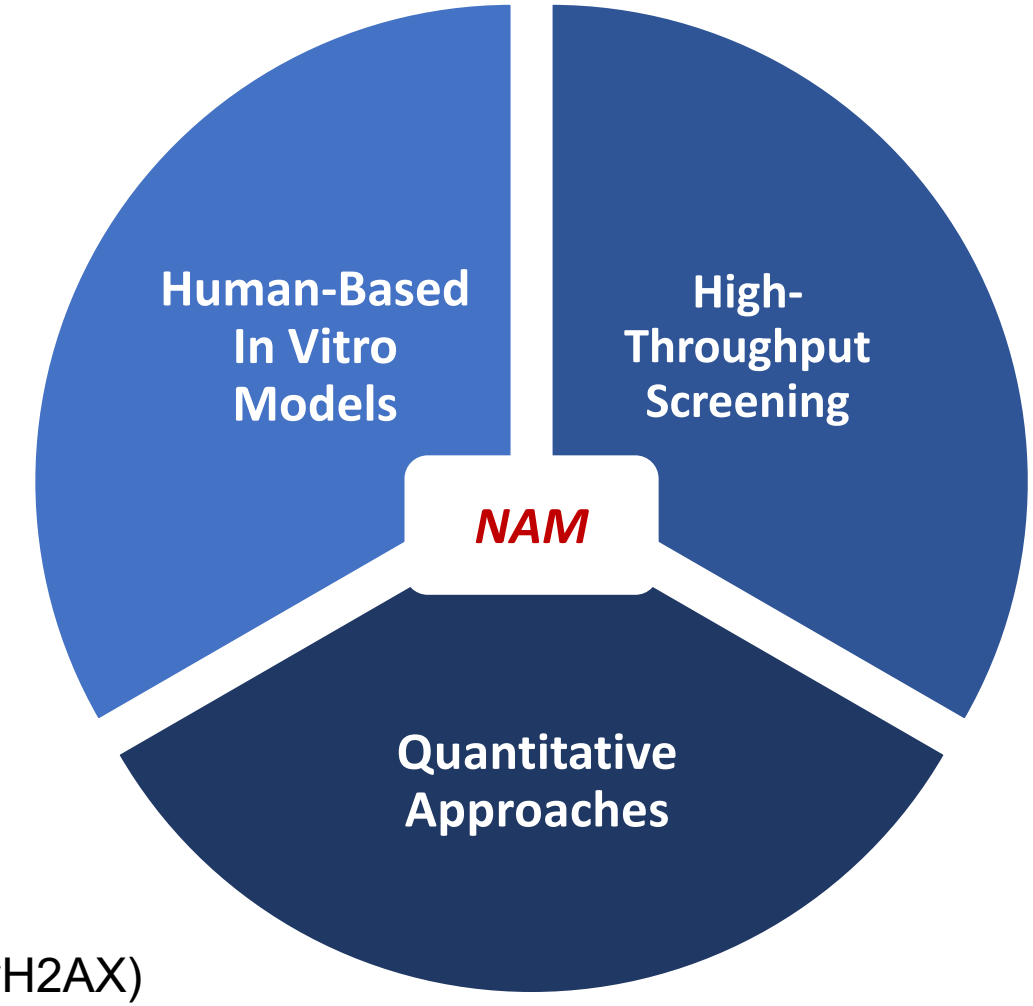
New Approach Methodologies (NAMs)



Gutleb *et al.*, *Mutation Research* **(2025)**

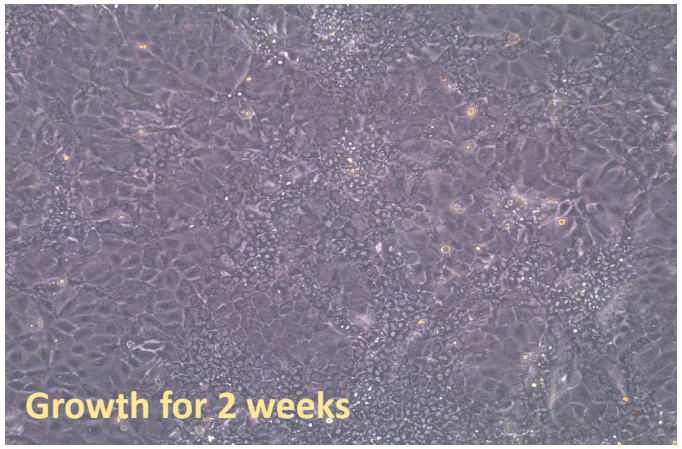
HepaRG cells

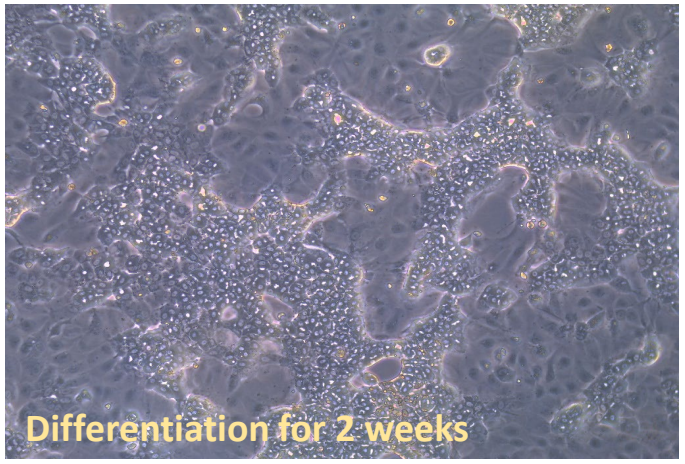
- **Origin:** From a female liver cancer patient (hepatocellular carcinoma).
- **Characteristics**
  - ✓ **p53-Proficient:** Suitable for genotoxicity studies.
  - ✓ **Metabolically Competent:**
    - Can differentiate into hepatocyte-like and biliary-like cells.
    - Express phase I and II enzymes similar to primary human hepatocytes.
- **2D & 3D culture conditions**
- **Endpoints:**
  - DNA damage assays (Comet assay and Multiflow assay; γH2AX)
  - Micronucleus assay [with human Epidermal Growth Factor (hEGF) stimulation]
  - ECS mutation assay



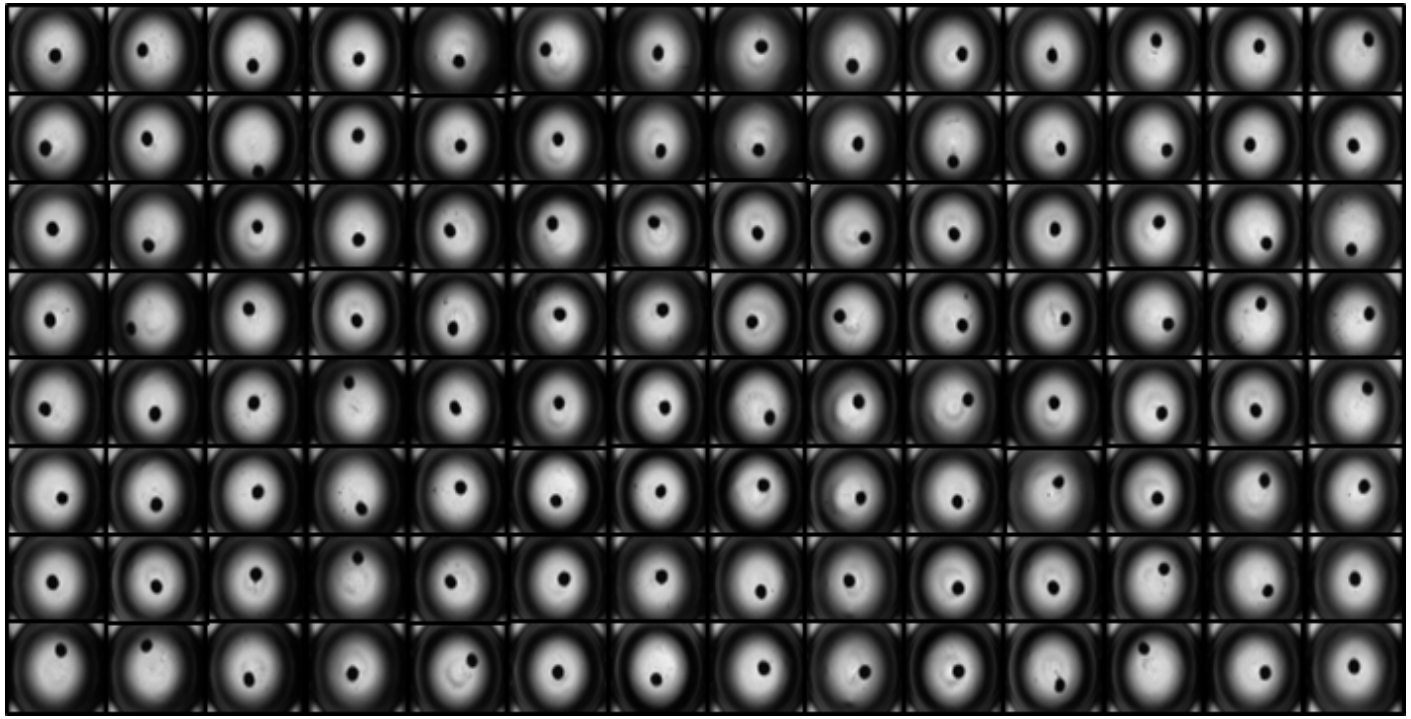
3D HepaRG spheroids

2D cells in 100-mm Petri dish



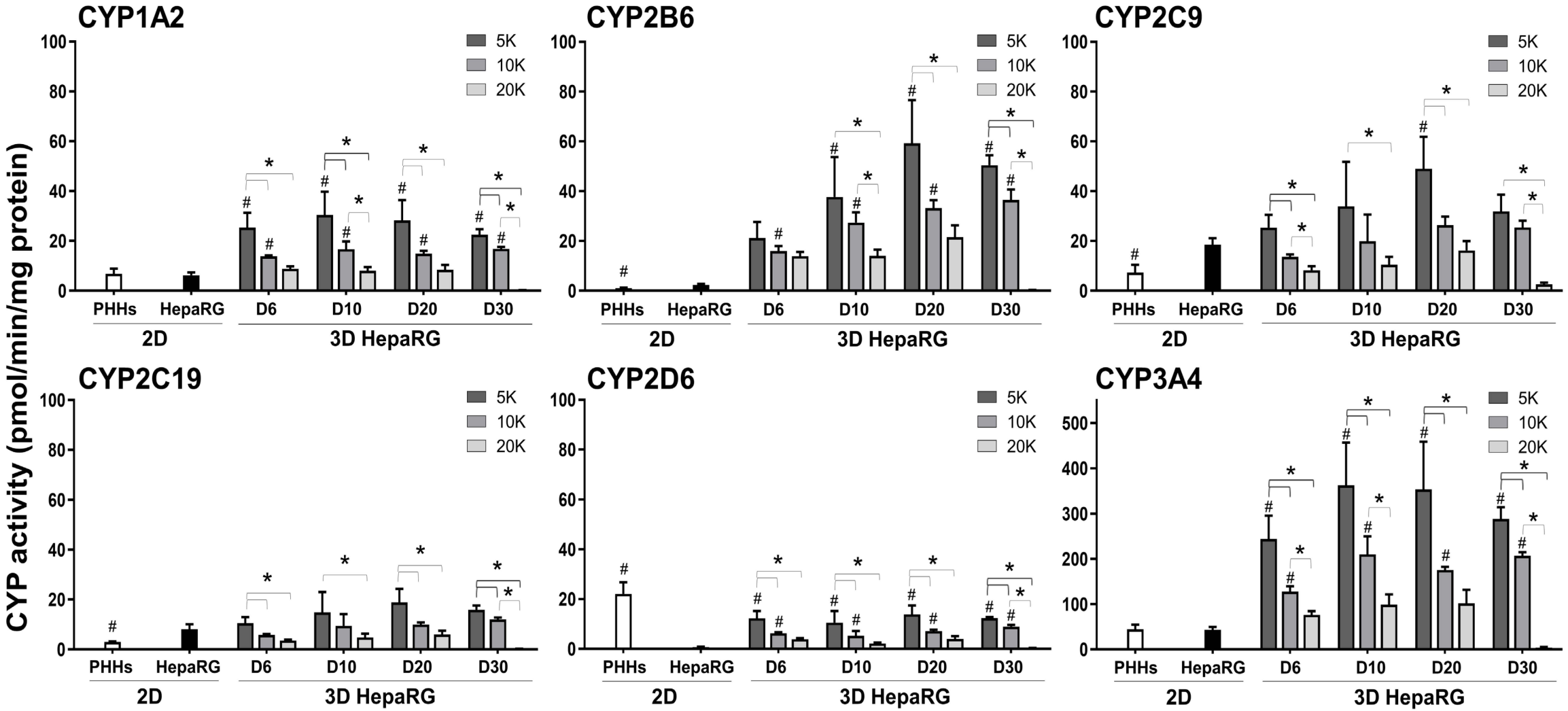


3D Spheroid in 384-well ultra-low attachment (ULA) plates



- 3D culture provides better cell-to-cell interactions and tissue-like structures compared to 2D culture.

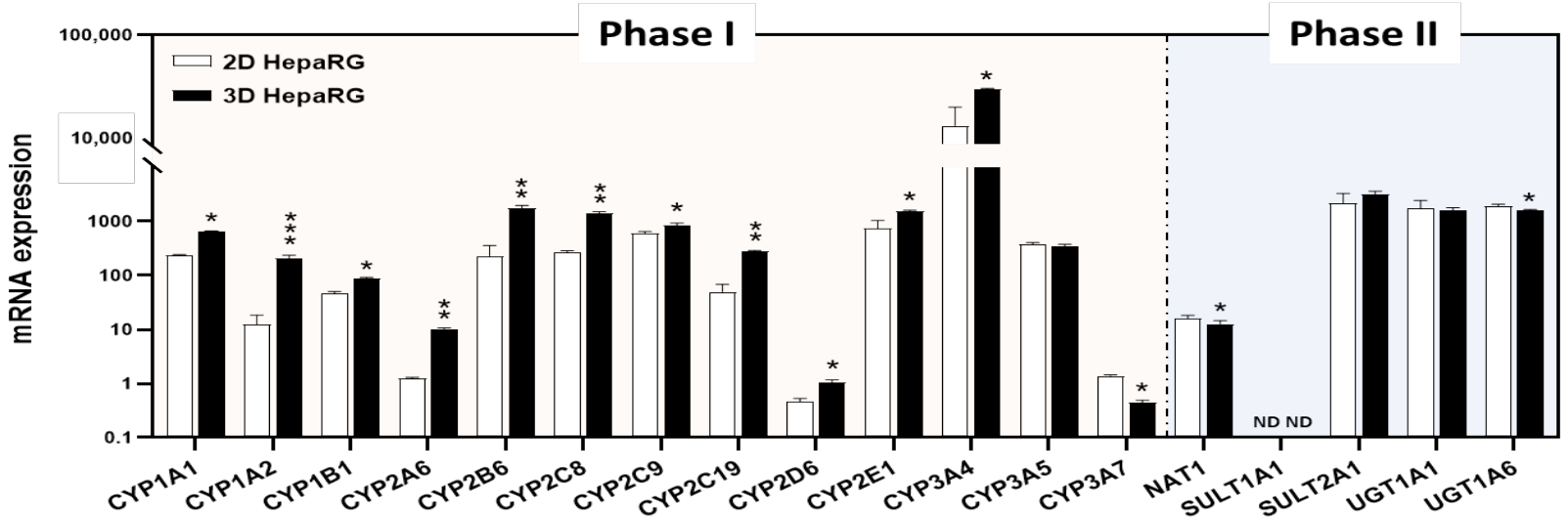
Metabolic capacity of 2D & 3D HepaRG models



Seo *et al., Alternatives to Animal Experimentation (ALTEX)* (2022)

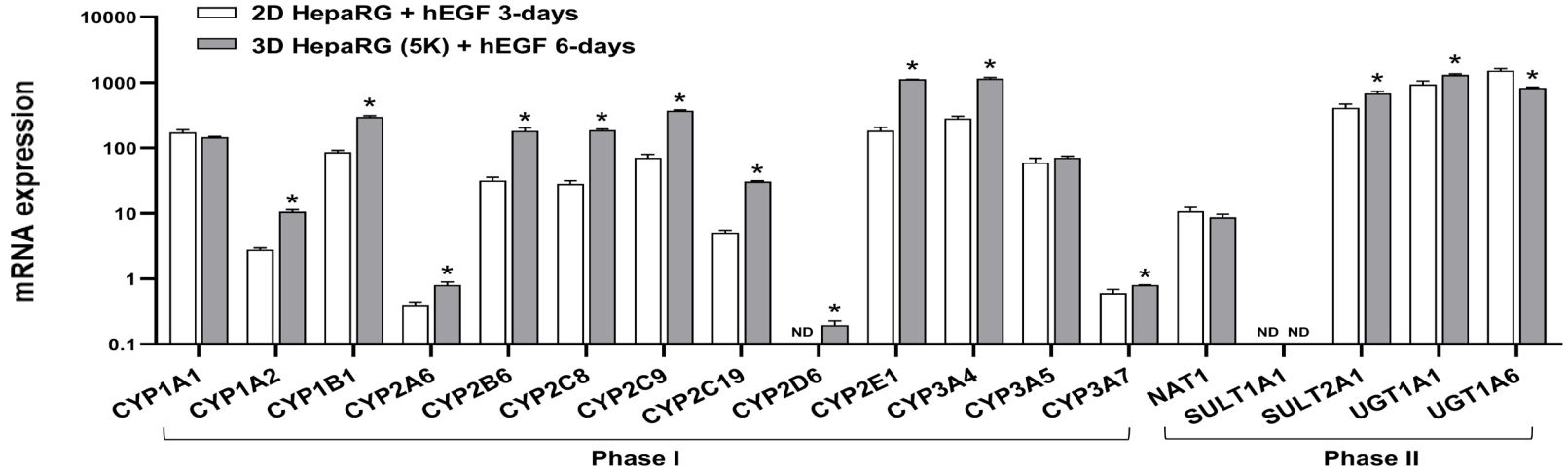
Metabolic capacity of 2D & 3D HepaRG models

A. Comet assay



Seo *et al.*, *Achieves of Toxicology* (Oct 2023)

B. Micronucleus assay

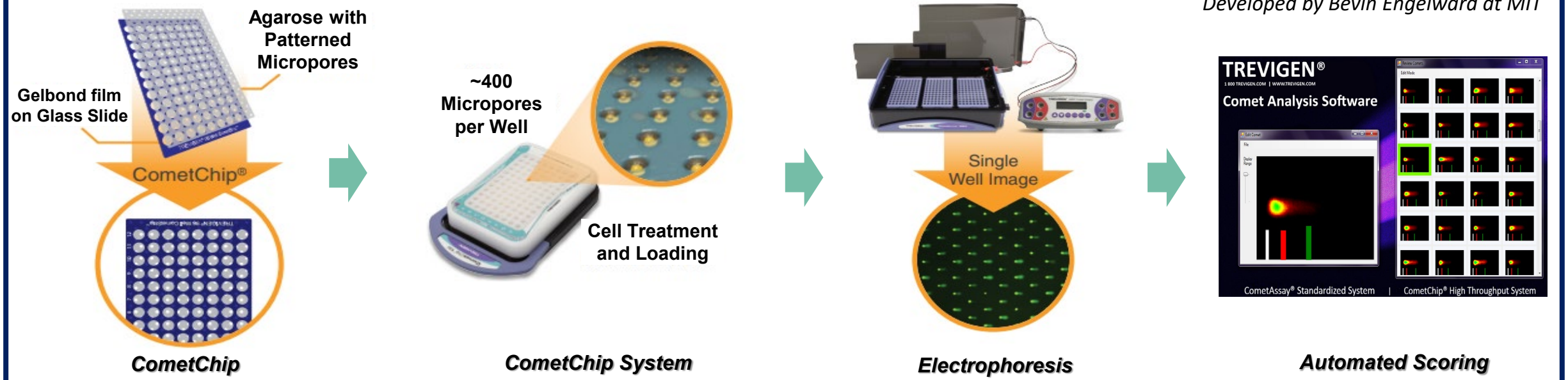


Seo *et al.*, *Achieves of Toxicology* (April 2023)

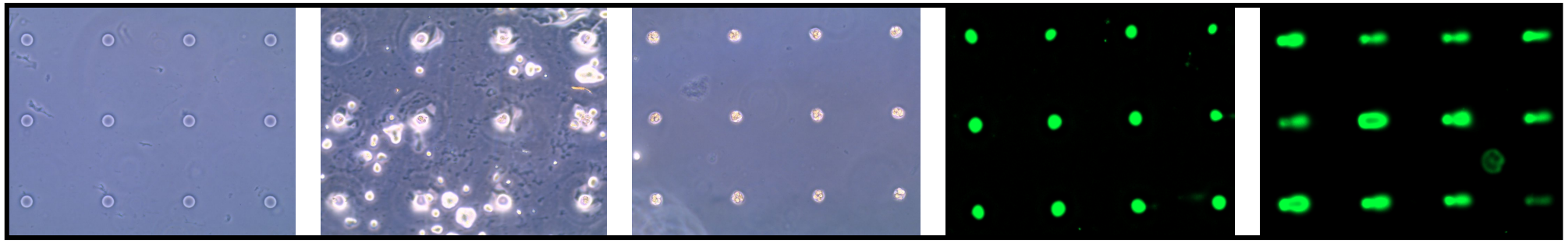
High-throughput CometChip assay

96-Well CometChip Platform

Developed by Bevin Engelward at MIT



NMP Agarose with micropores | Cell loading | Washing with PBS | Negative CON | Positive CON

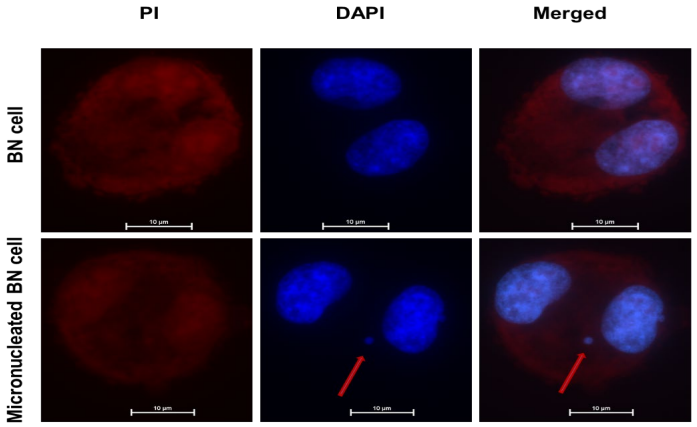


Using A polydimethylsiloxane (**PDMS**) stamp with an array of micropillars

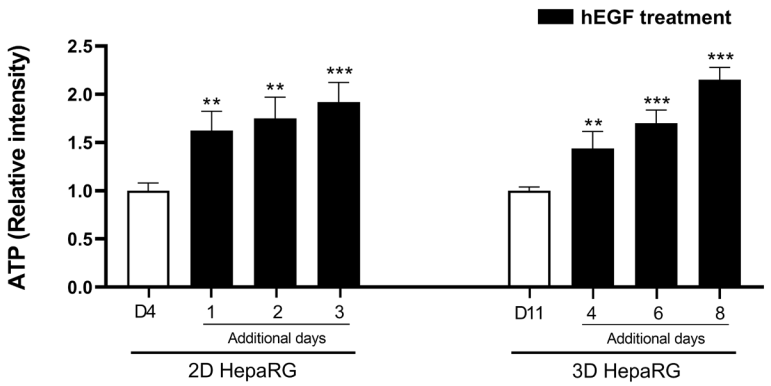
The Comet endpoints:
The fraction of migrated DNA = % Tail DNA

High-throughput micronucleus (MN) assay

Cytokinesis block micronucleus (CBMN) assay

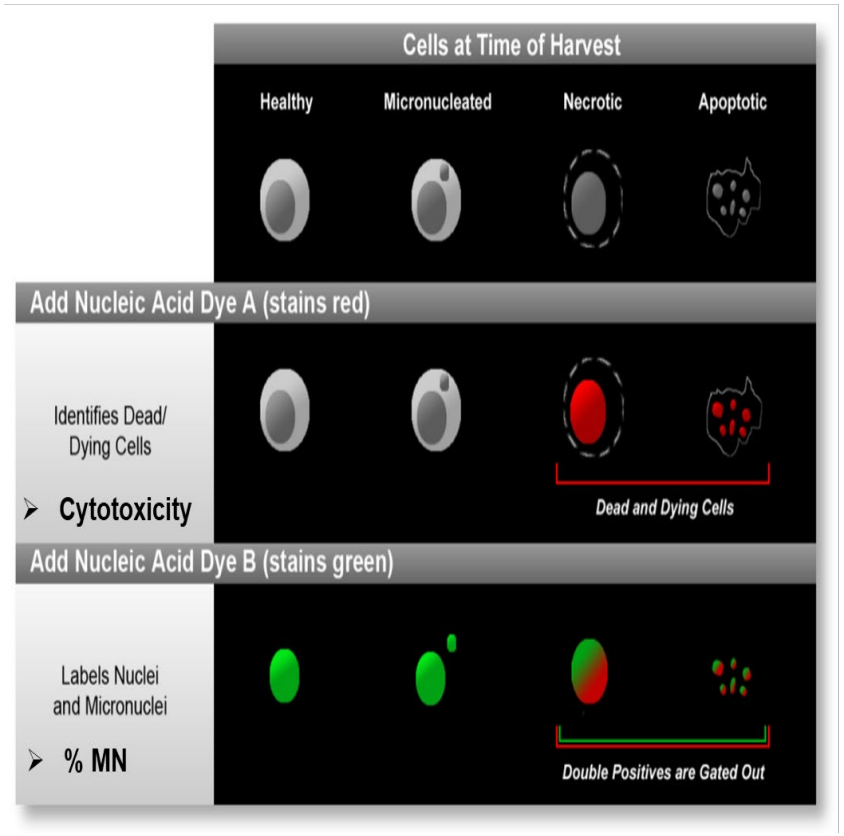


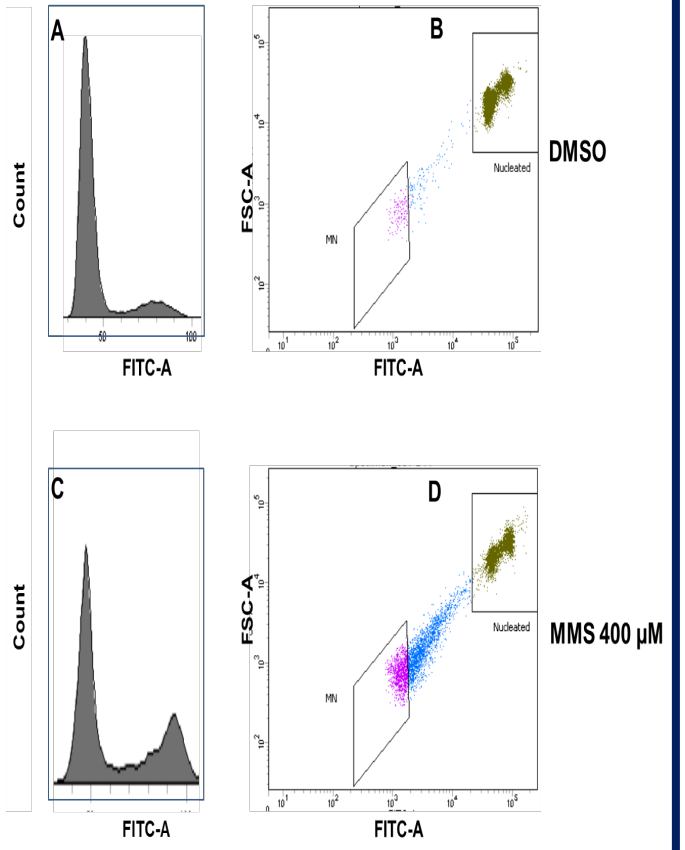
hEGF stimulation in HepaRG models (1.5 – 2 doubling time)



Flow cytometry-based MN assay

In Vitro MicroFlow kit (Litron Laboratories)





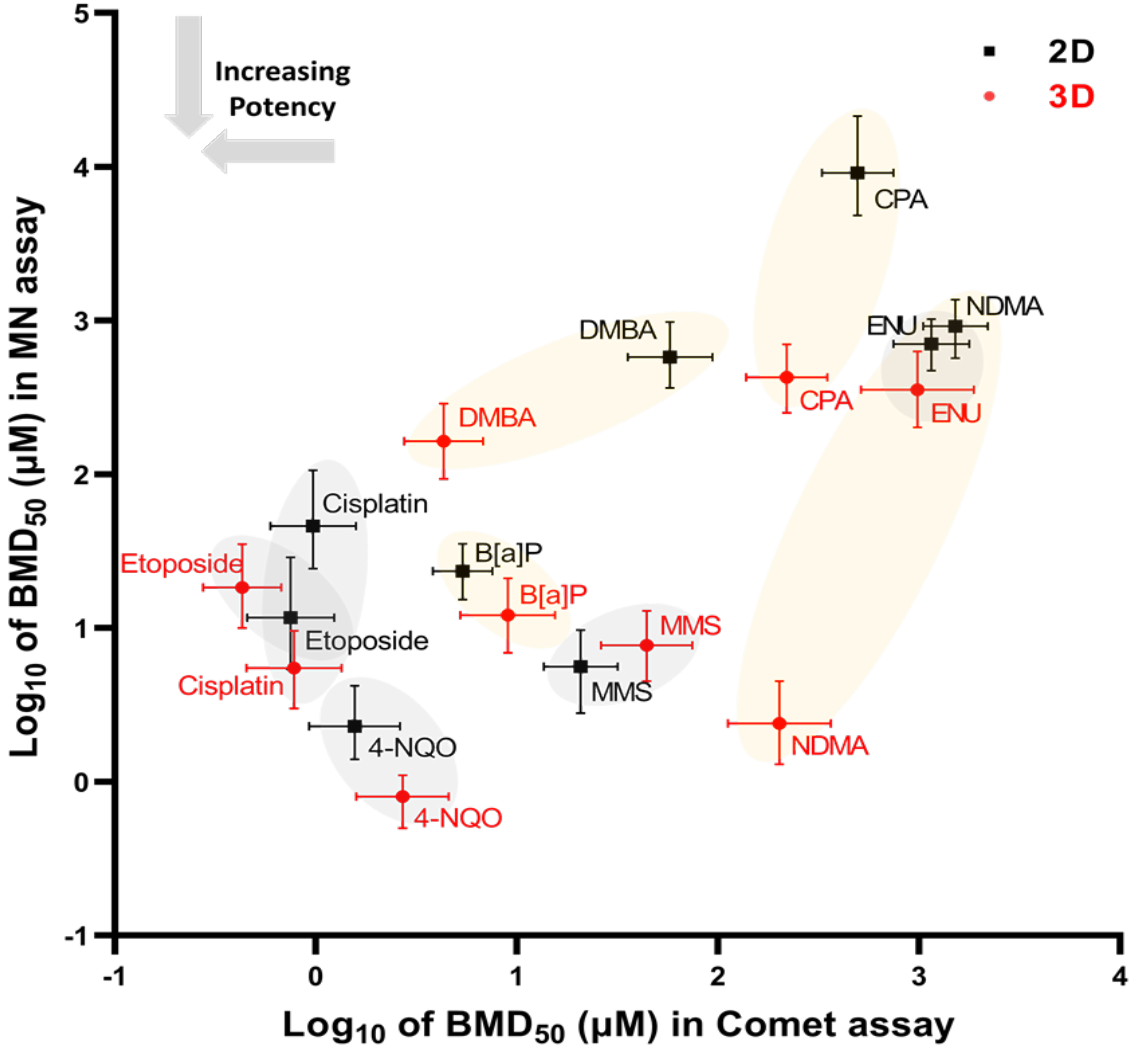
Developed by Litron Laboratories, Rochester, NY

Sensitivity of 2D and 3D HepaRG cultures in MN and Comet assays

Compound	MN assay		Comet assay		Overall	
	2D	3D	2D	3D	2D	3D
8 Direct-acting	88% (7/8)	**75% (6/8)**	63% (5/8)	**75% (6/8)**	88% (7/8)	**88% (7/8)**
11 Indirect-acting	55% (6/11)	**64% (7/11)**	45% (5/11)	**73% (8/11)**	64% (7/11)	**91% (10/11)**
19 Genotoxicants or carcinogens	68% (13/19)	**68% (13/19)**	53% (10/19)	**74% (14/19)**	74% (14/19)	**89% (17/19)**

Seo *et al.*, *Achieves of Toxicology* (April 2023)

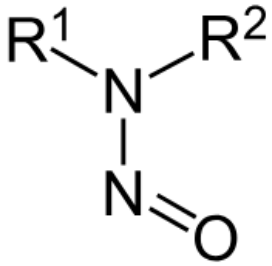
In Vitro Genotoxic Potency in HepaRG models (Benchmark dose analysis)



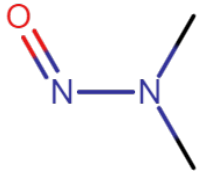
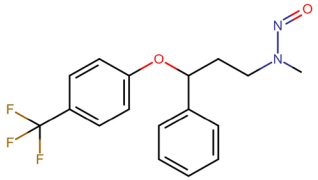
Seo *et al.*, *Achieves of Toxicology* (April 2023)

N-Nitrosamine risk

- *N*-Nitrosamines have been increasingly detected in human drugs.



- Due to their mutagenic and carcinogenic potential, *N*-nitrosamines are classified as a "cohort of concern" under the ICH M7(R2) guideline.
- *N*-Nitrosamines are classified into two categories:

	Small-molecule nitrosamines		Nitrosamine Drug Substance-Related Impurities (NDSRIs)	
Size	Low molecular weight		Larger, linked to drug substance structure	
Origin	Environmental contaminants, solvents, reagents		Formed from the Active Pharmaceutical Ingredient (API)	
Risk Concern	Lots of carcinogenicity data		Emerging concern, limited data	
Example		*N*-nitrosodimethylamine (NDMA, MW 74.08)		*N*-nitroso-fluoxetine (MW 338.32)

- NDSRIs present significant challenges for regulatory agencies due to their complex structures, unique nature to each drug, and the lack of existing safety data to set acceptable intake (AI) limits.

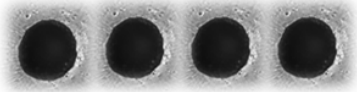
N-Nitrosamine risk

- Regulatory agencies like the FDA have adopted the Carcinogenic Potency Categorization Approach (CPCA) to establish AI limits for NDSRIs because of the limited data for these compounds
- In vitro and in vivo studies remain essential to validate CPCA predictions and to ensure the accuracy of CPCA-based strategies.
- Most *N*-nitrosamines require metabolic activation to form DNA adducts, leading to potential mutagenicity and carcinogenicity.
- The Enhanced Ames Test (EAT) improves Ames test sensitivity for NDSRI mutagenicity but is limited in predicting human risk, as it uses bacteria with metabolic activation from rodent liver S9.
- Conducting in vitro genotoxicity and mutagenicity testing in human-cell-based models for *N*-nitrosamines is essential, particularly in models that possess a human metabolic activation system.

Genotoxicity assessment of *N*-nitrosamines using HepaRG models

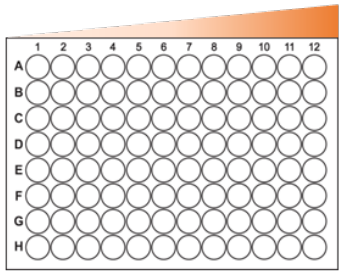
2D HepaRG at Day3

AND



3D spheroids at Day10

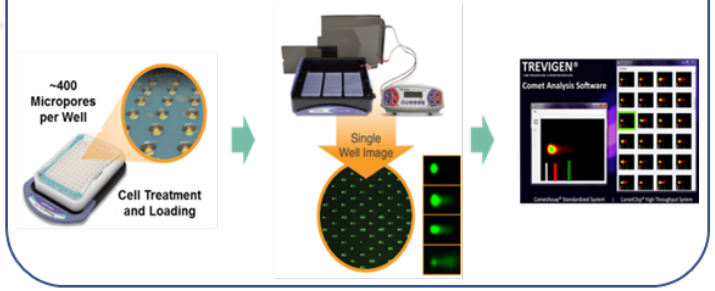
Min. conc. ← Max. conc.



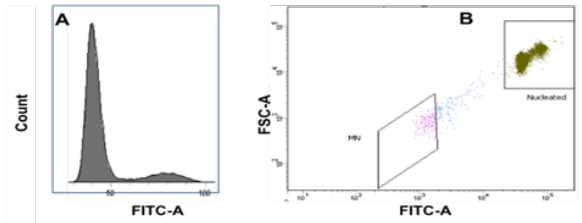
N-nitrosamine treatment for 24 h

Small-molecule nitrosamines & Nitrosamine drug substance-related impurities (NDSRIs)

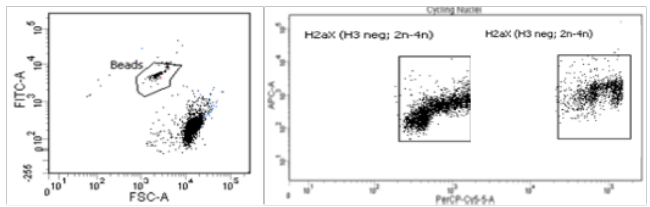
CometChip assay



Micronucleus assay

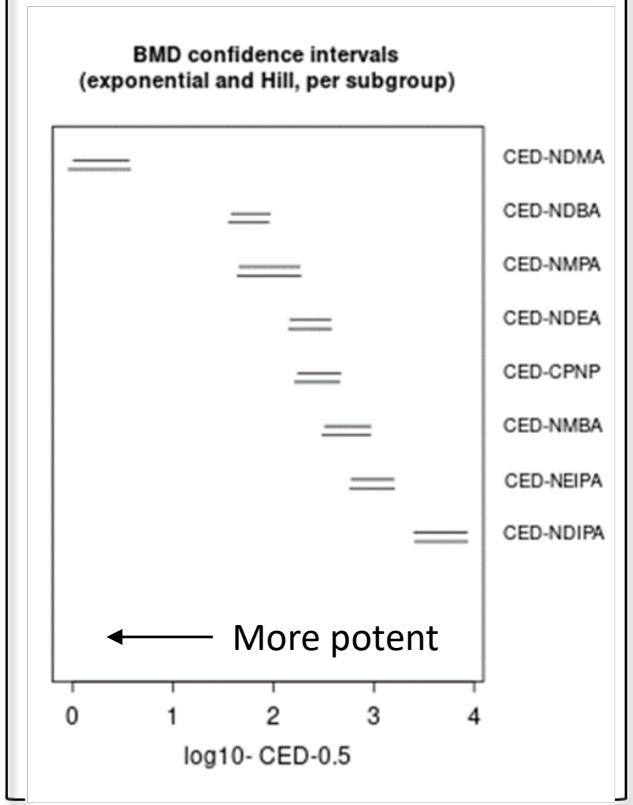


MultiFlow DNA damage assay



γH2A.X, P53, and phospho-histone H3

Quantitative comparison: Benchmark concentration modeling



Results of Ames test & in vitro mammalian cell assays

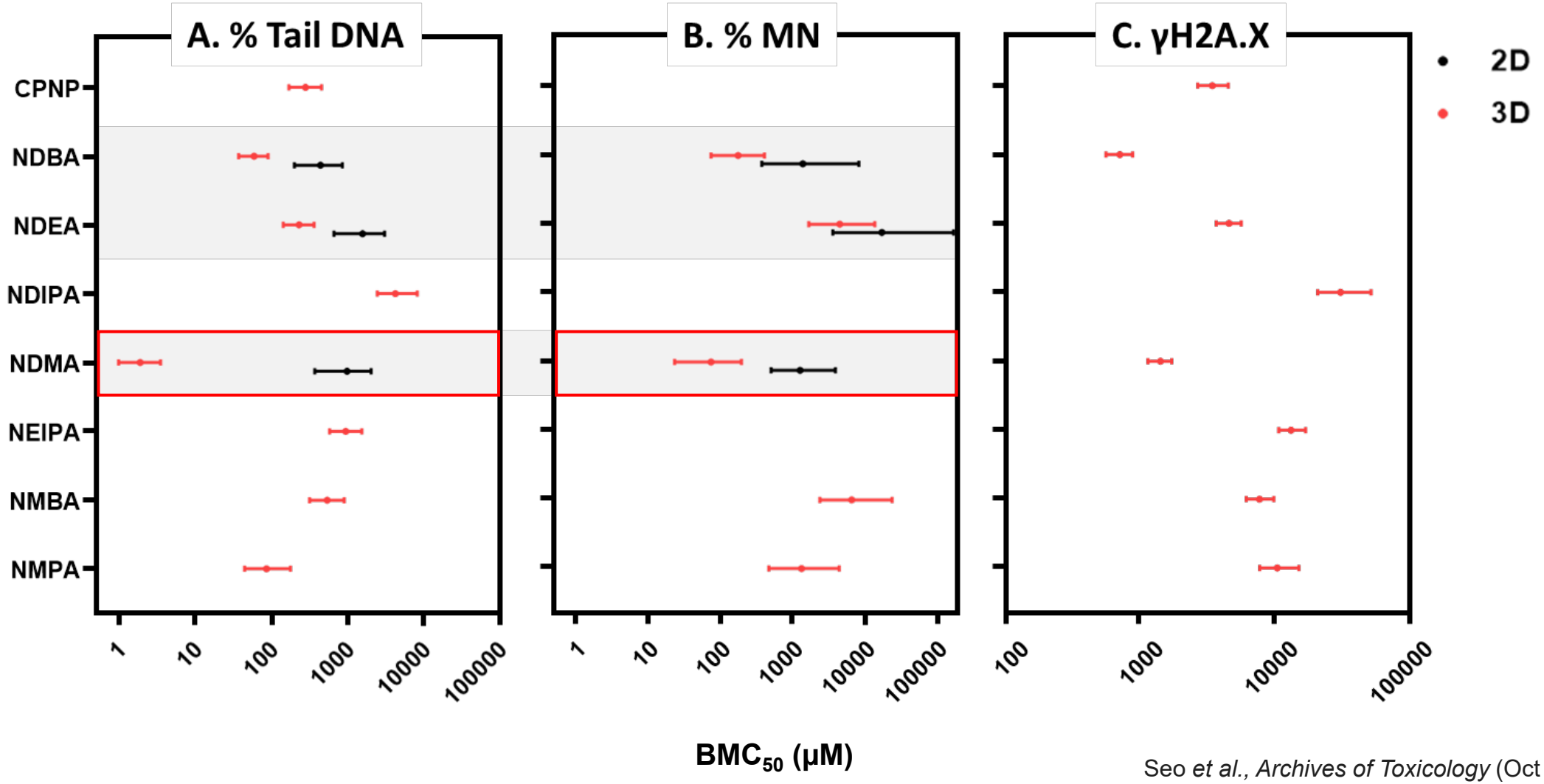
- **Small-molecule nitrosamines**

N-nitrosamines	Mutation		MN			Comet			γH2A.X	
	EAT[a]	CYP2A6-expressing TK6 cells[a]	2D HepaRG[b]	3D HepaRG[b]	2D HepaRG[b]	3D HepaRG[b]	PMHs (Monkey)[c]	PHHs (Human)[c]	CYP2A6-expressing TK6 cells[d]	3D HepaRG[b]
CPNP	+	N/A	-	-	-	+	+	+	N/A	+
NDBA	+	-	+	+	+	+	+	+	+	+
NDEA	+	+	+	+	+	+	+	+	+	+
NDIPA	+	-	-	-	-	+	+	+	+	+
NDMA	+	N/A	+	+	+	+	+	+	N/A	+
NEIPA	+	+	-	-	-	+	+	+	+	+
NMBA	-	+	-	+	-	+	+	+	+	+
NMPA	+	+	-	+	-	+	+	+	+	+

[a] Heflich *et al., Regulatory Toxicology Pharmacology* (2024)
[b] Seo *et al., Archives of Toxicology* (Oct 2023)
[c] Seo *et al., Chemico-Biological Interactions* (2025)
[d] Li *et al., Archives of Toxicology* (2022)

Genotoxic potency comparison (2D vs. 3D HepaRG)

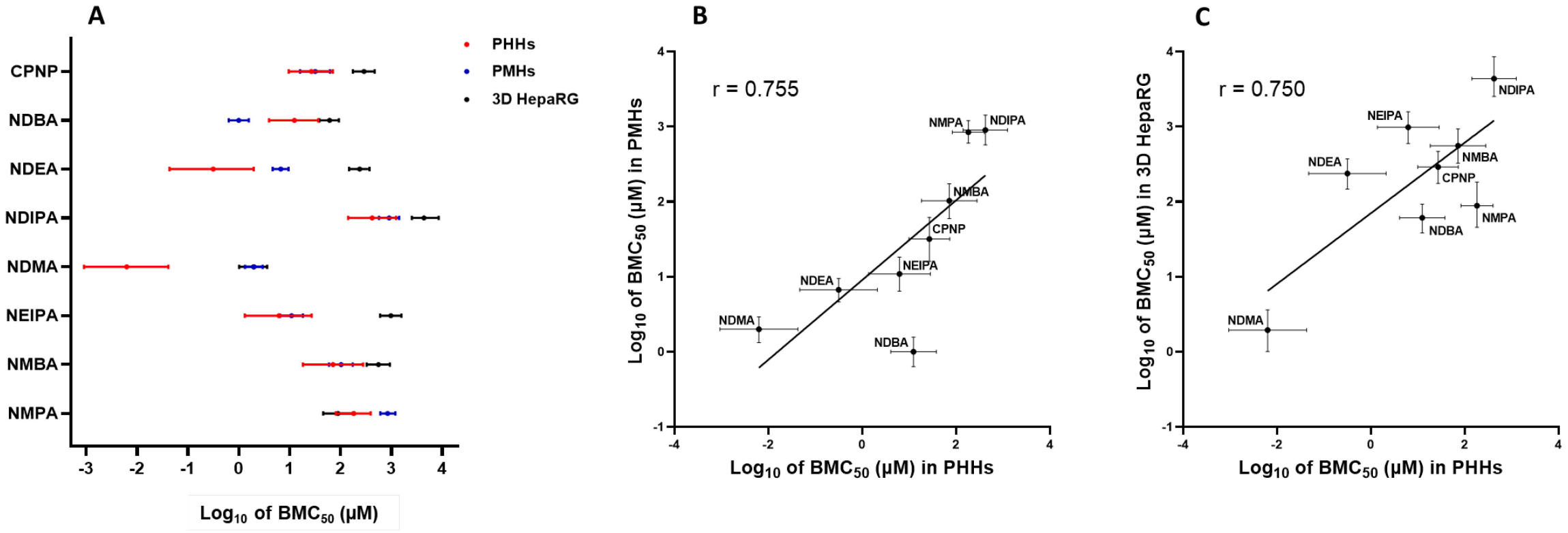
- **Small-molecule nitrosamines**



Seo *et al.*, *Archives of Toxicology* (Oct 2023)

Genotoxic potency comparison (PHHs vs. 3D HepaRG)

- **Small-molecule nitrosamines**



Seo *et al., Chemico-Biological Interactions* (2025)

Results of Ames test & in vitro mammalian cell assays

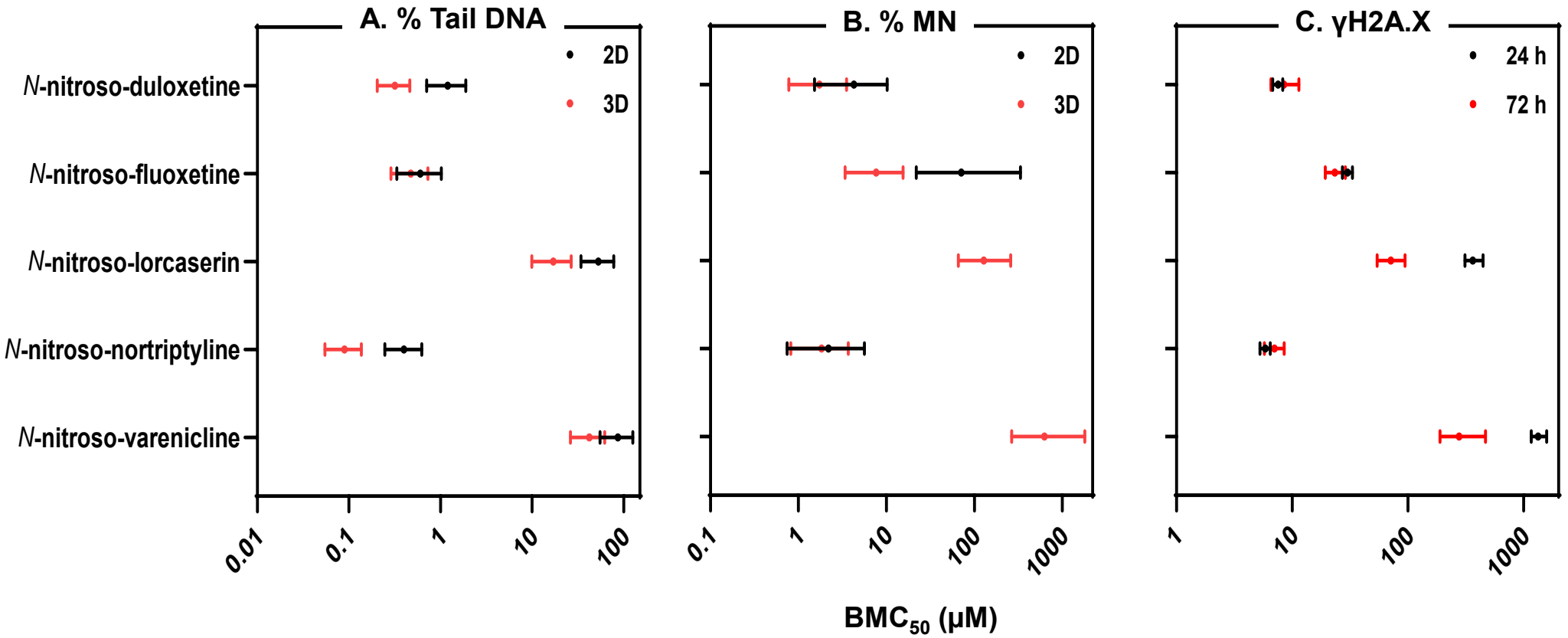
- **Nitrosamine drug substance-related impurities (NDSRIs)**

NDSRIs	Mutation		MN				Comet		γH2A.X
	EAT[a]	TK/ HPRT[b]	TK6 no S9[b]	TK6 + 2% hamster S9[b]	2D HepaRG[c]	3D HepaRG[c]	2D HepaRG[c]	3D HepaRG[c]	3D HepaRG[c]
N-nitroso-duloxetine	+	+	+	+	+	+	+	+	+
N-nitroso-fluoxetine	+	+	+	+	+	+	+	+	+
N-nitroso-lorcaserin	+	+	-	+	-	+	+	+	+
N-nitroso-nortriptyline	+	+	+	+	+	+	+	+	+
N-nitroso-varenicline	+	+	-	+	-	+	+	+	+
N-nitroso-diclofenac	-	-	+	-	-	-	-	-	-
N-nitroso-folic acid	-	N/A	N/A	N/A	-	-	-	-	-
N-nitroso-paroxetine	-	-	-	-	-	-	-	-	-
N-nitroso-desvaleryl-valsartan	-	-	-	-	-	-	-	-	-
N-nitroso-desvaleryl-valsartan methyl ester	-	-	+	-	-	-	-	-	-

[a] Heflich et al., *Regulatory Toxicology Pharmacology* (2024)
[b] Li et al., *Regulatory Toxicology Pharmacology* (2024)
[C] Seo et al., *Regulatory Toxicology Pharmacology* (2025)

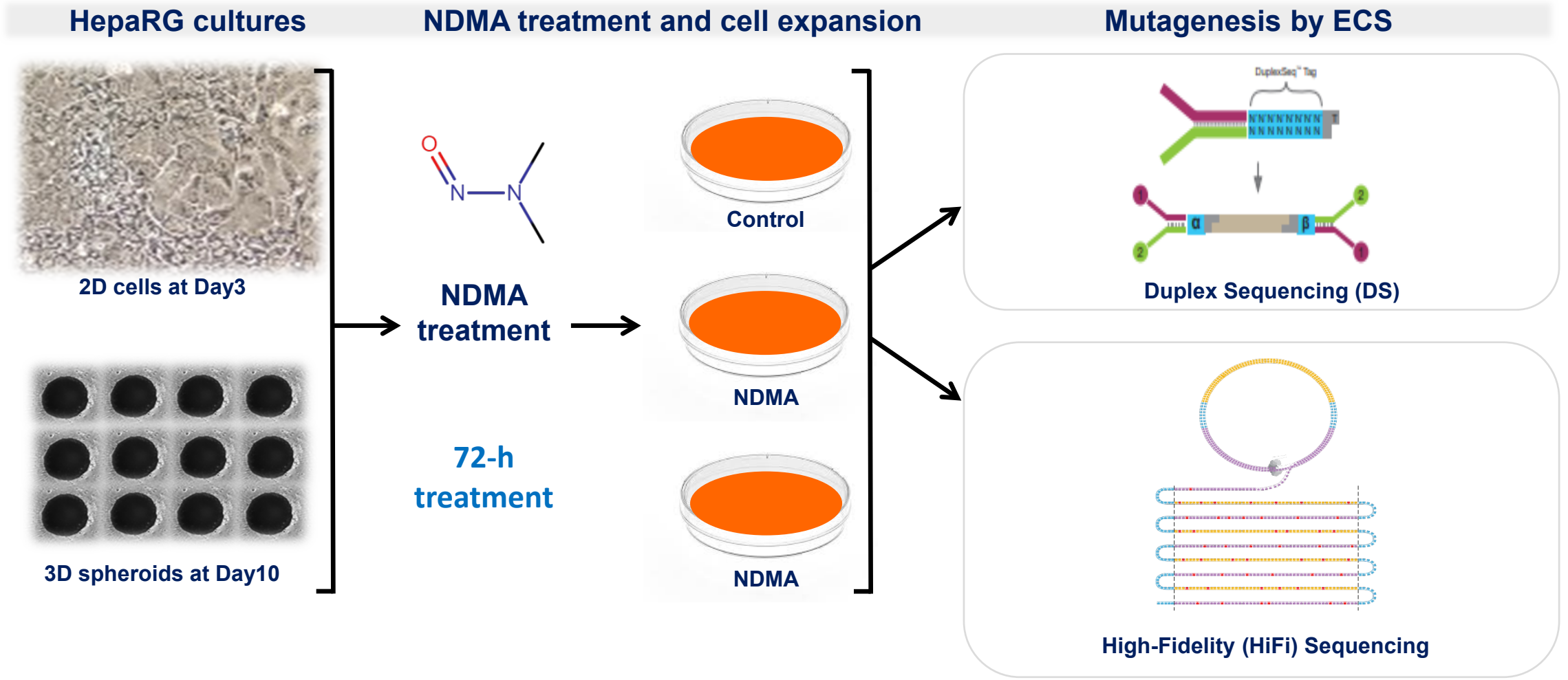
Genotoxic potency comparison (2D vs. 3D HepaRG)

- **Nitrosamine drug substance-related impurities (NDSRIs)**



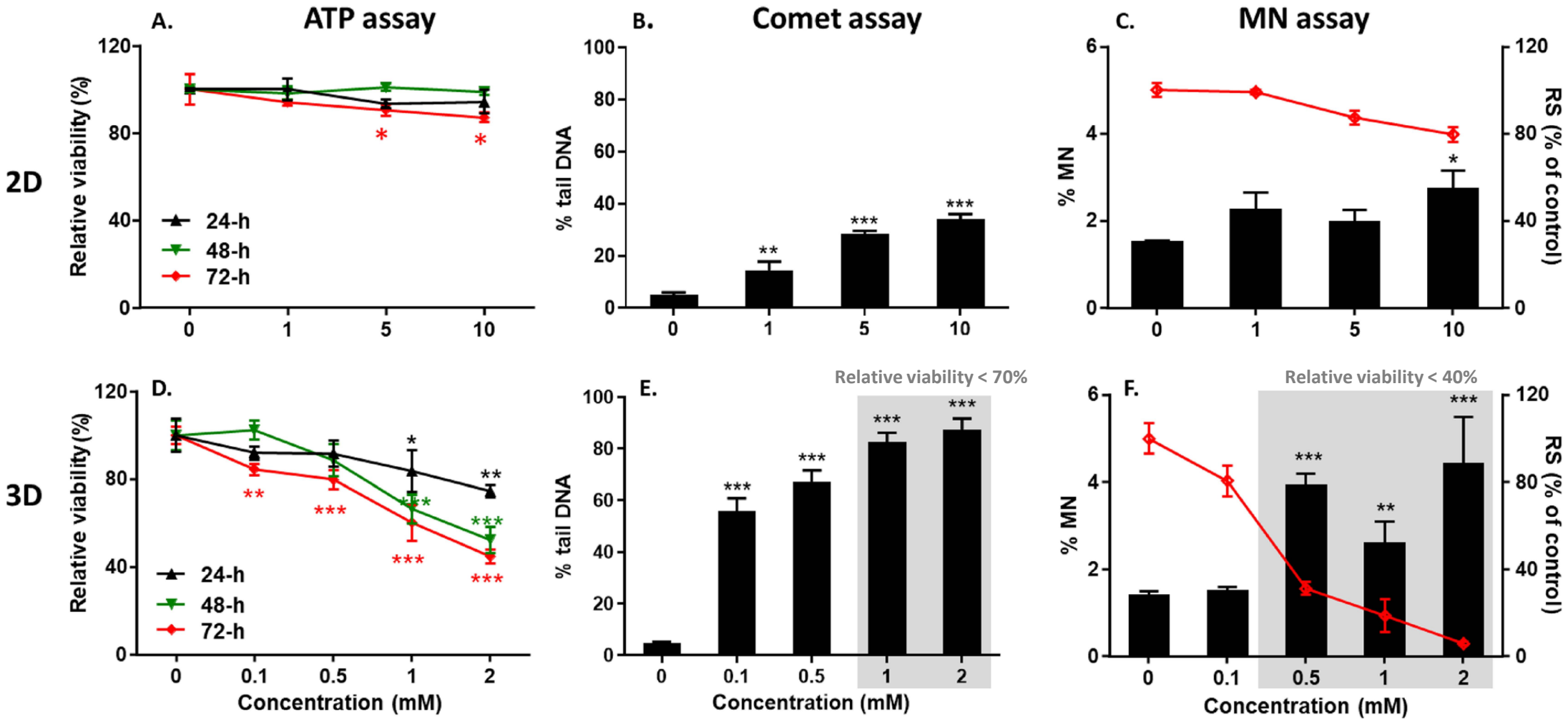
Seo et al., *Regulatory Toxicology Pharmacology* (2025)

Mutagenicity assessment of *N*-nitrosamines using HepaRG models

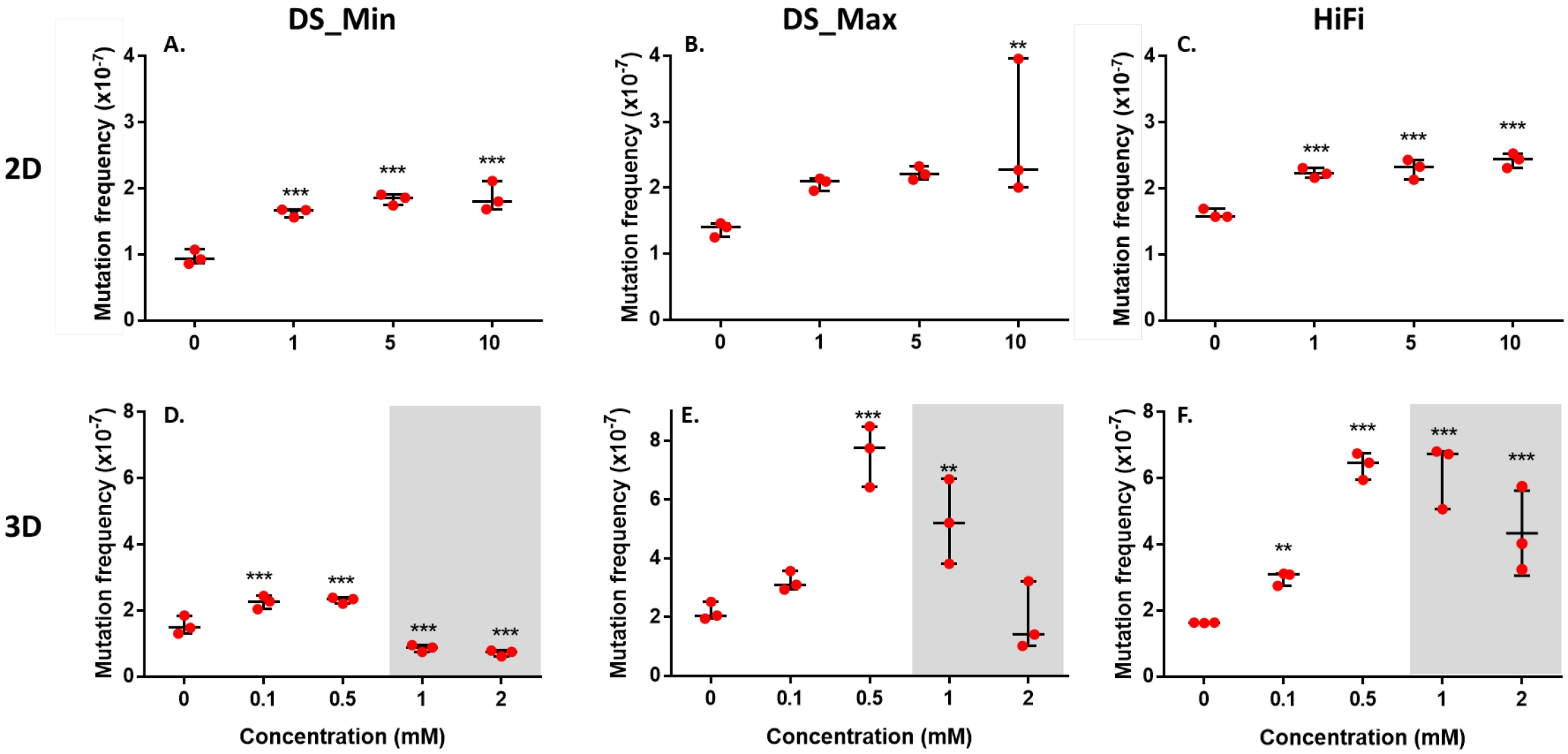


Seo *et al.*, *Archives of Toxicology* (2024)

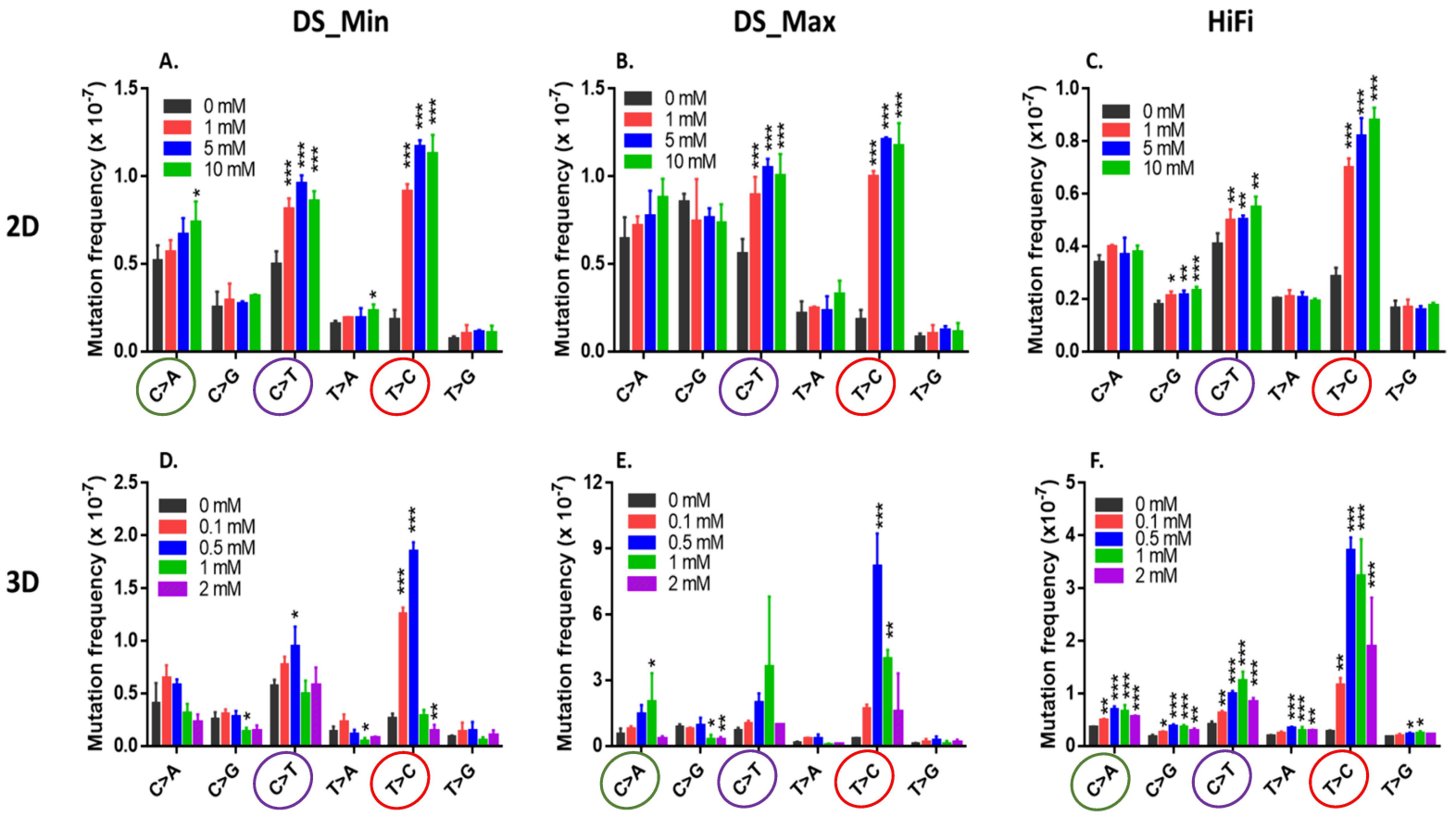
NDMA-induced cytotoxicity and genotoxicity (72-h)

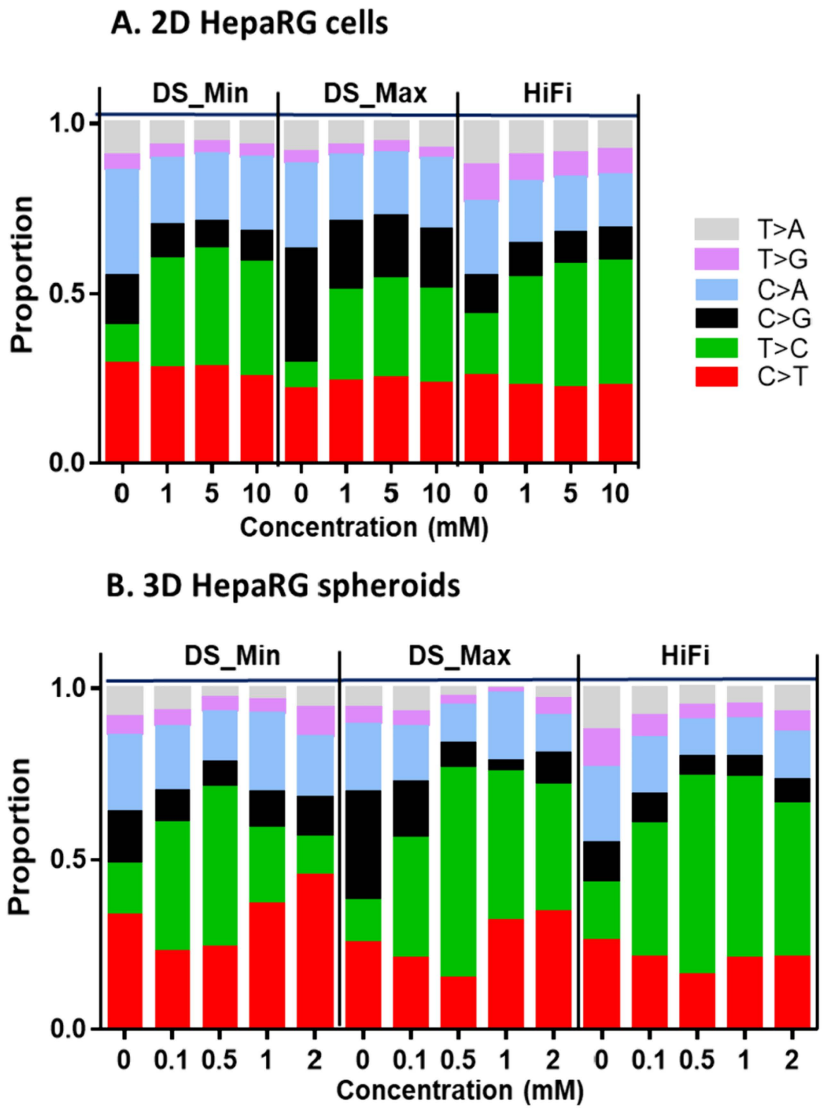


NDMA-induced mutation frequency (72-h)

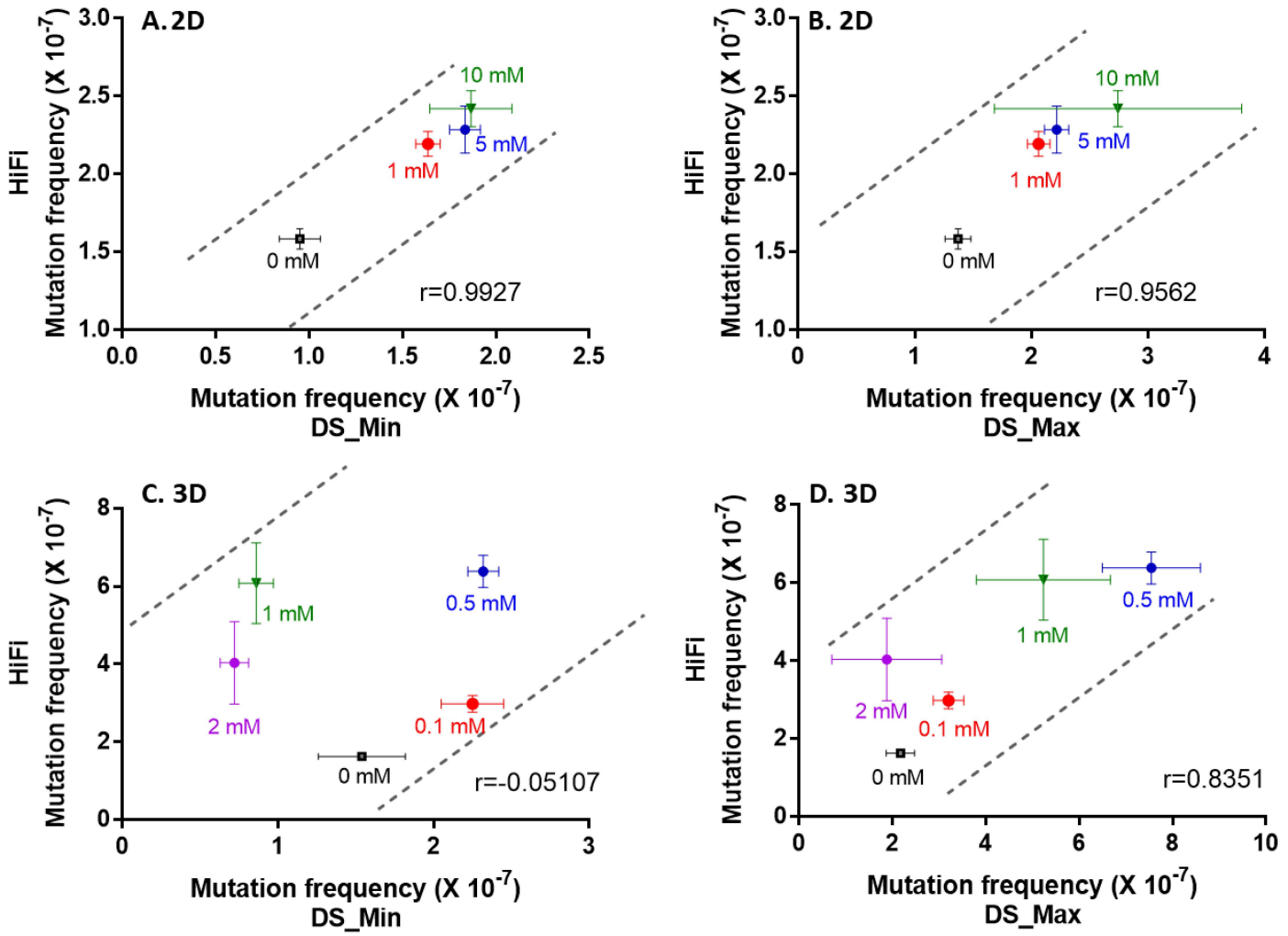


NDMA-induced individual base substitution (72-h)

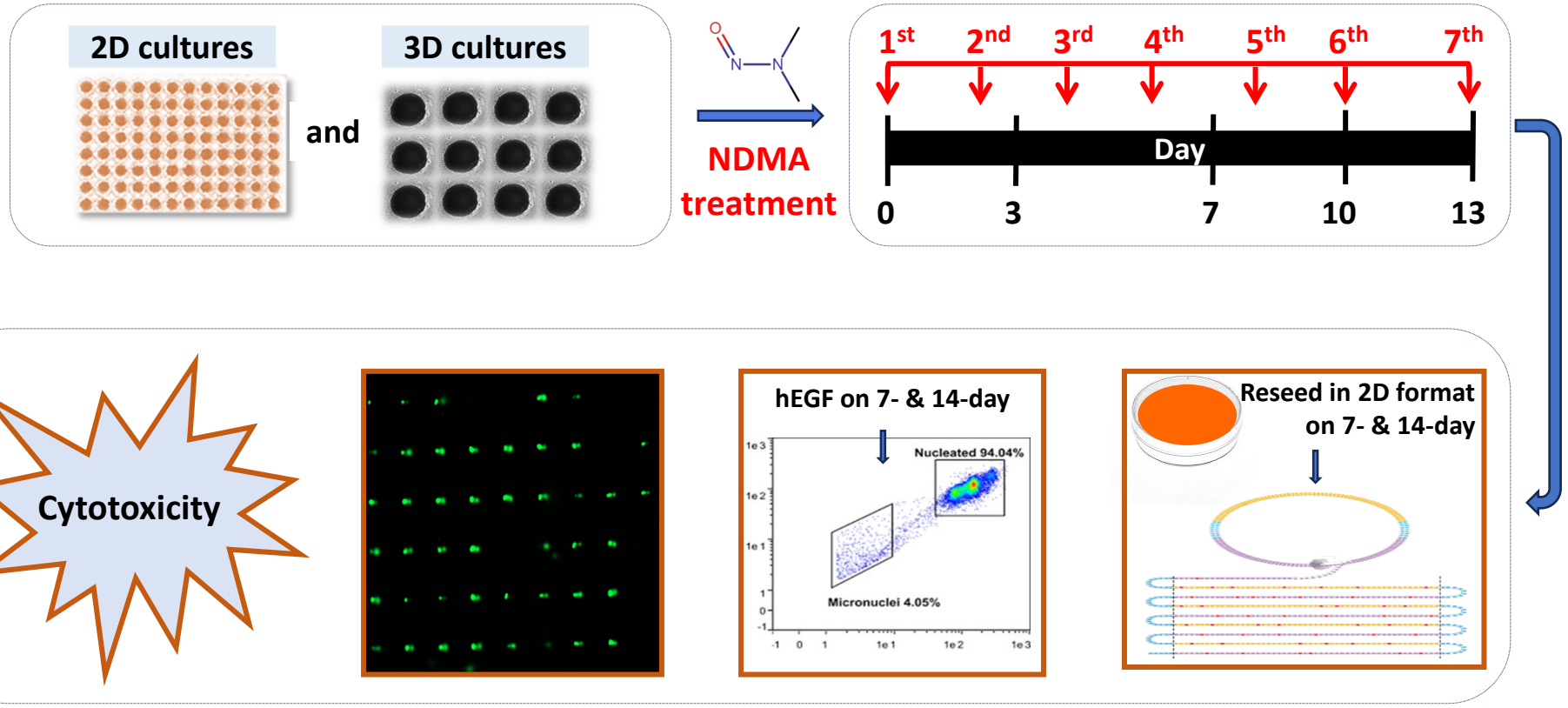




Correlation of mutation frequency obtained from Duplex and HiFi Sequencing



Mutation accumulation following extended exposure of HepaRG cells to NDMA



ATP assay on
3-, 7-, 10- & 14-day
(2D & 3D)

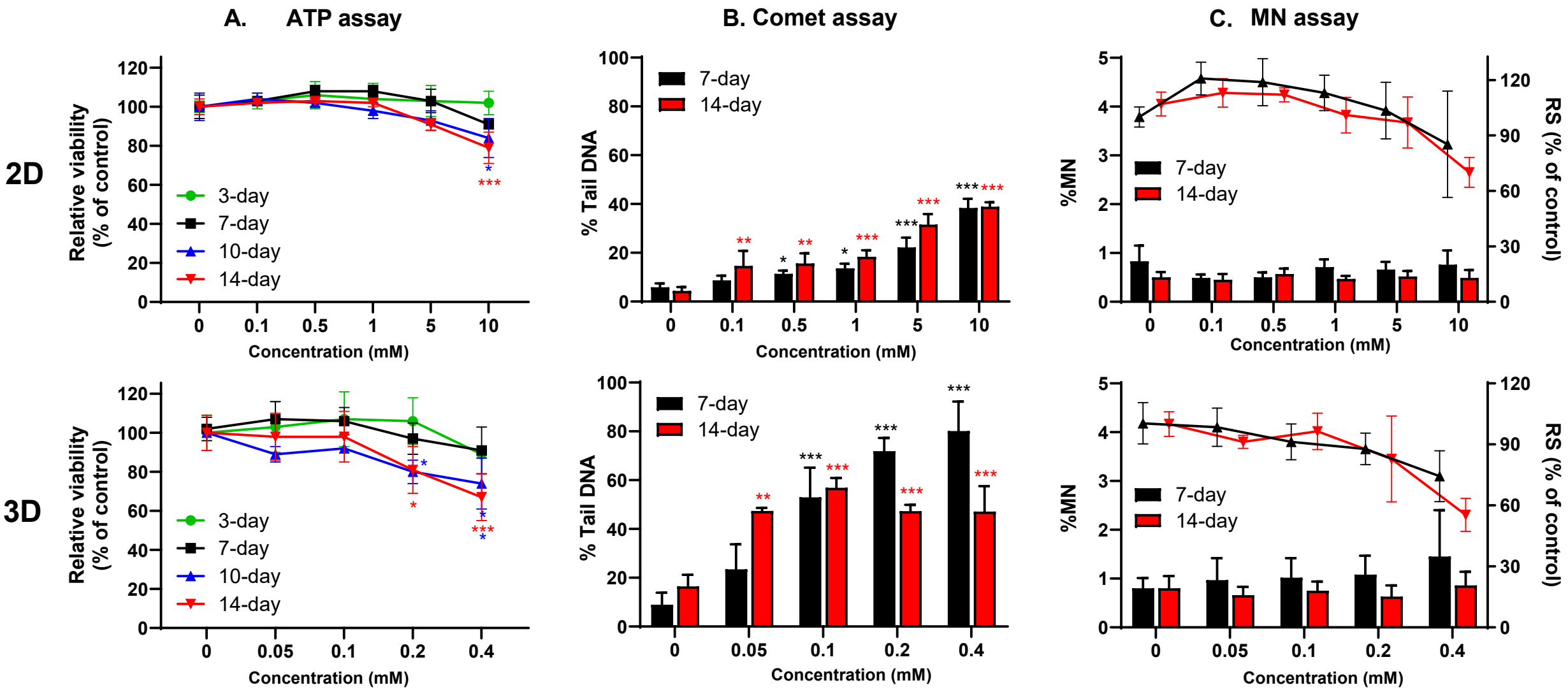
CometChip assay
on 7- & 14-day
(2D & 3D)

MN assay on
10- & 17-day (2D)
13- & 20-day (3D)

Extract gDNA
HiFi Sequencing
at cell confluence

Guo *et al., Journal of Environmental Science and Health, Part C* (2025)

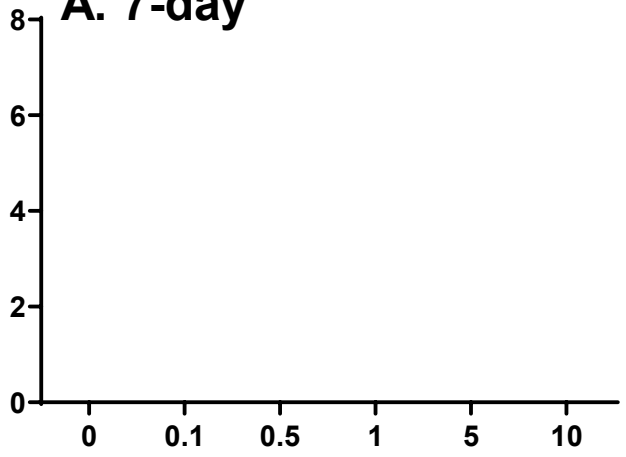
NDMA-induced cytotoxicity and genotoxicity (7- & 14-days)



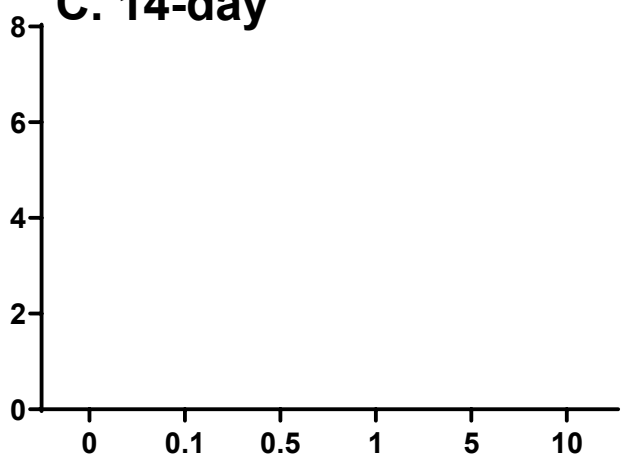
NDMA-induced mutation frequency (7- & 14-days)

2D

A. 7-day

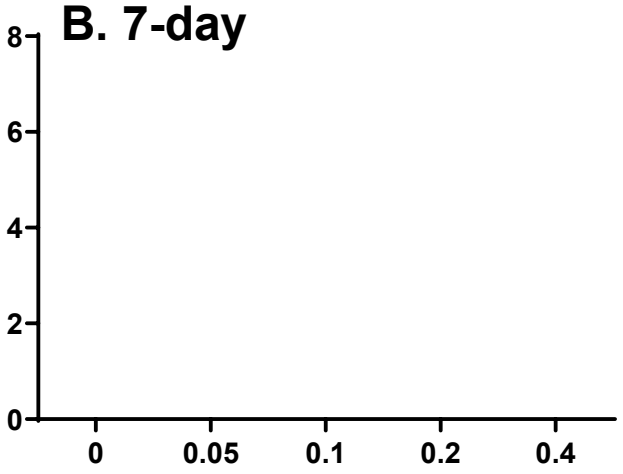


C. 14-day

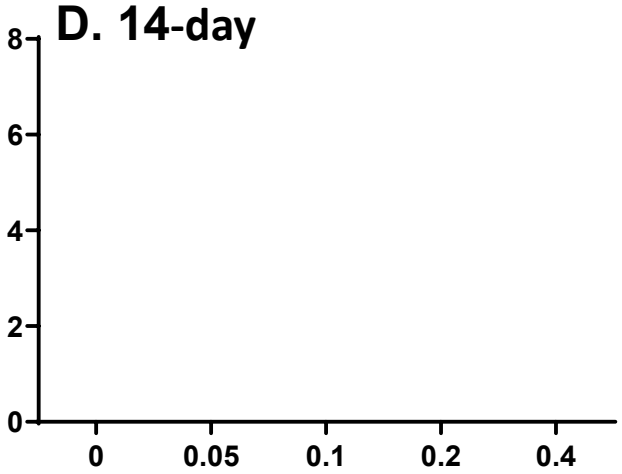


3D

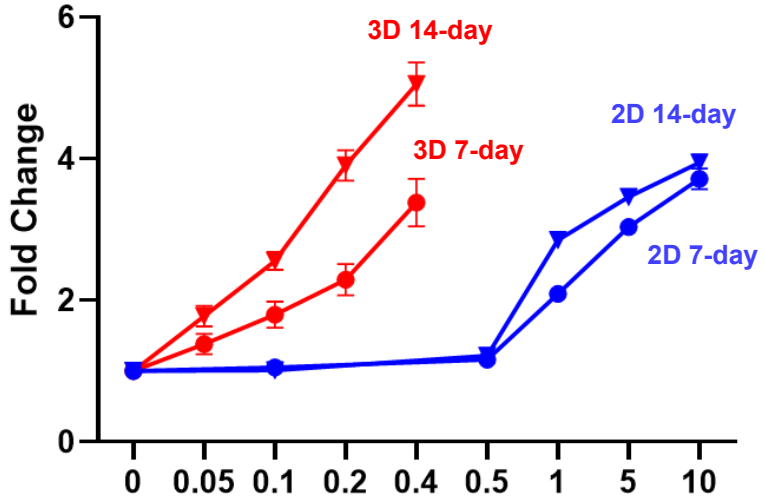
B. 7-day



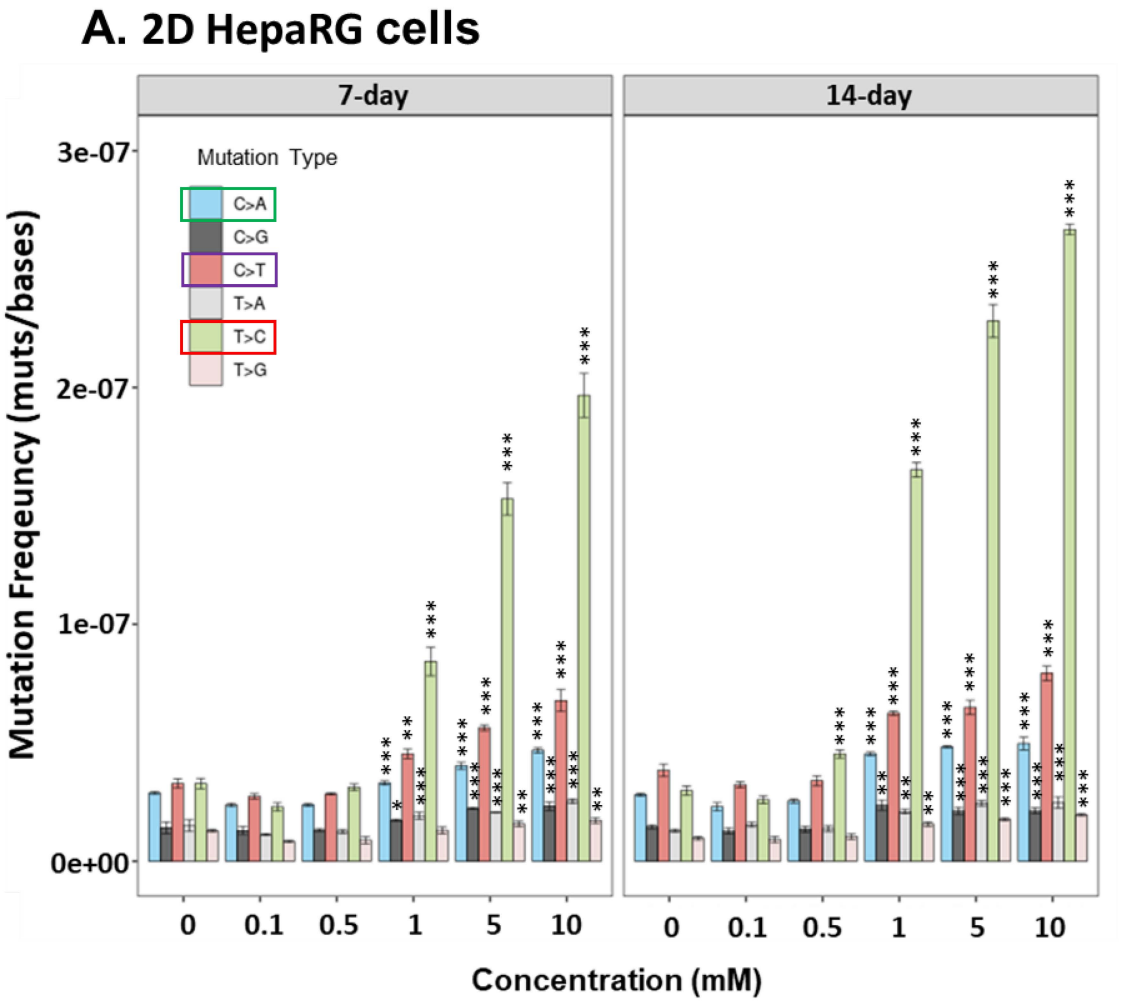
D. 14-day

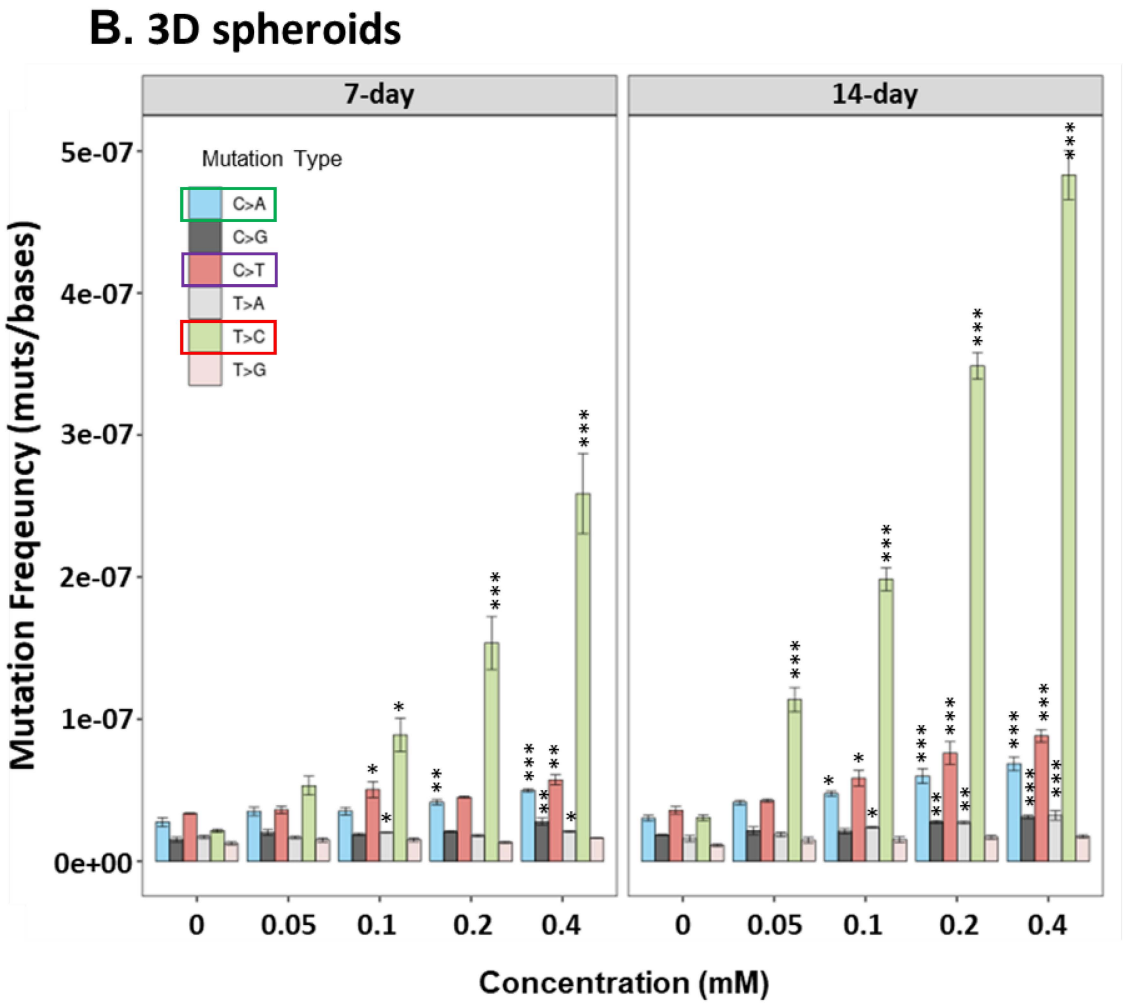


E.

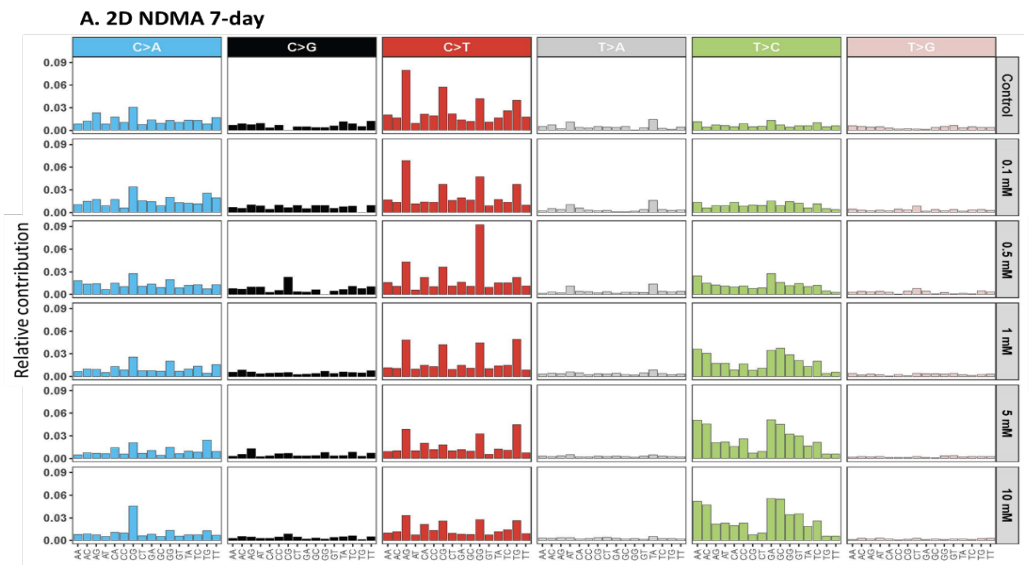


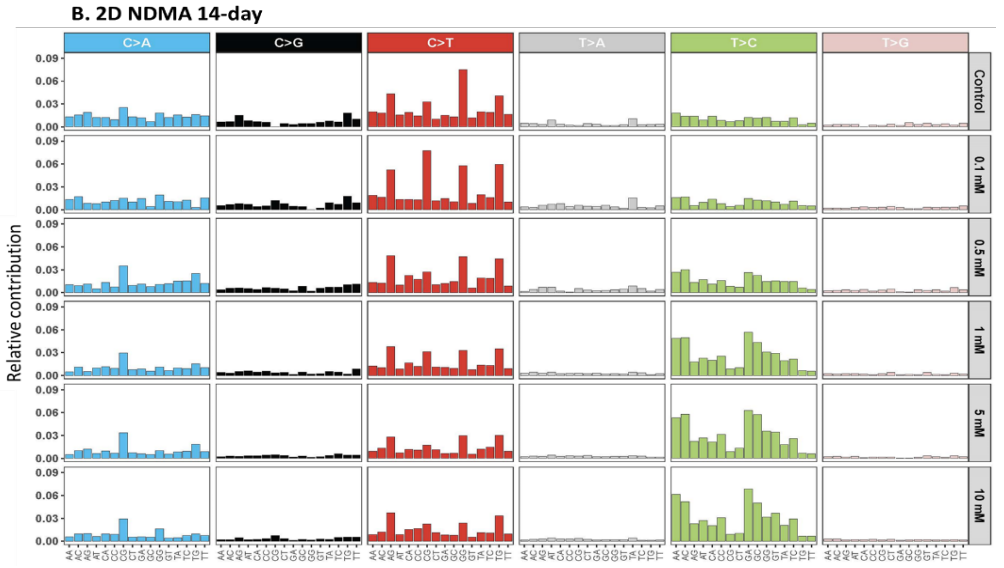
NDMA-induced individual base substitution (7- & 14-days)

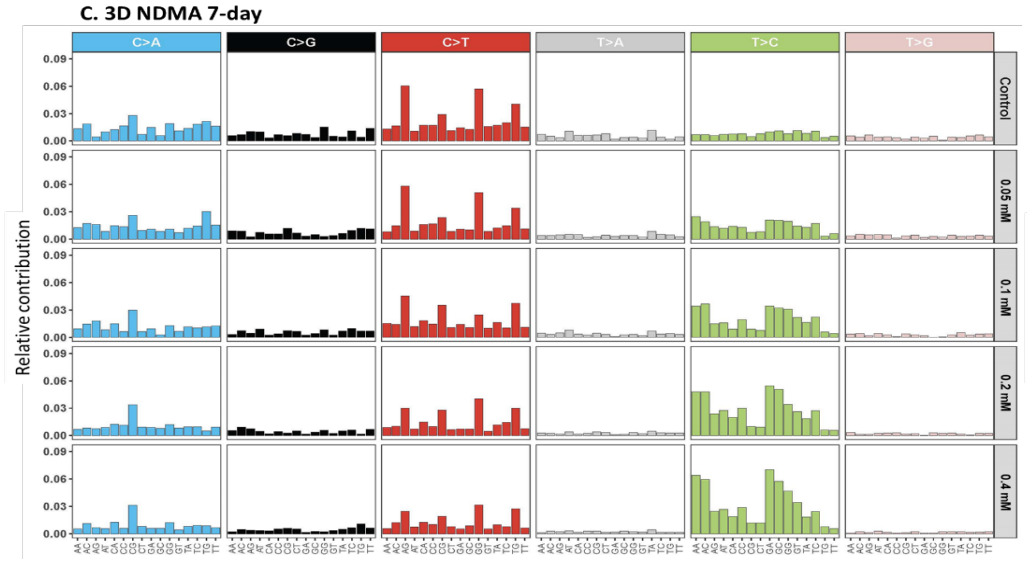


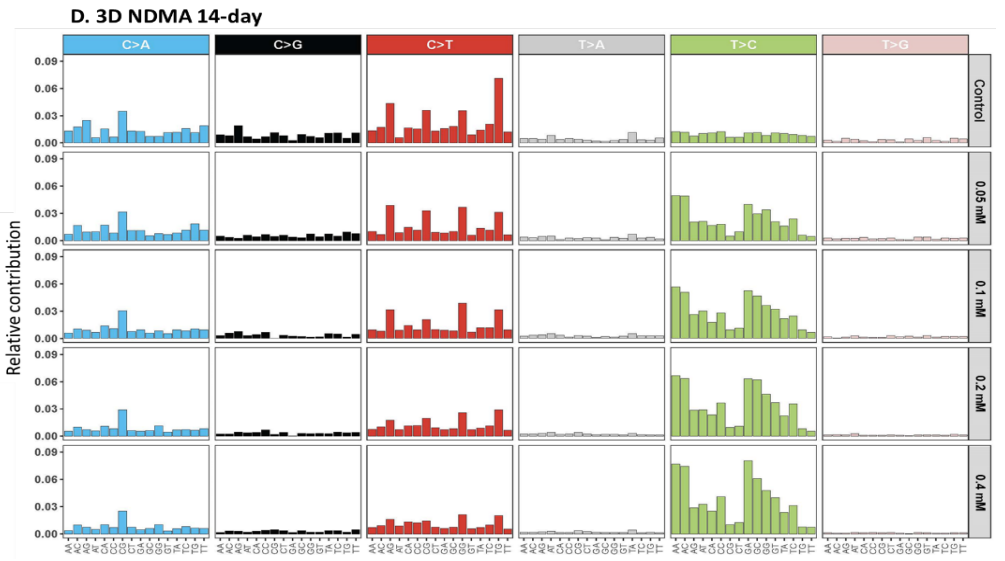


The 96-trinucleotides spectrum of NDMA

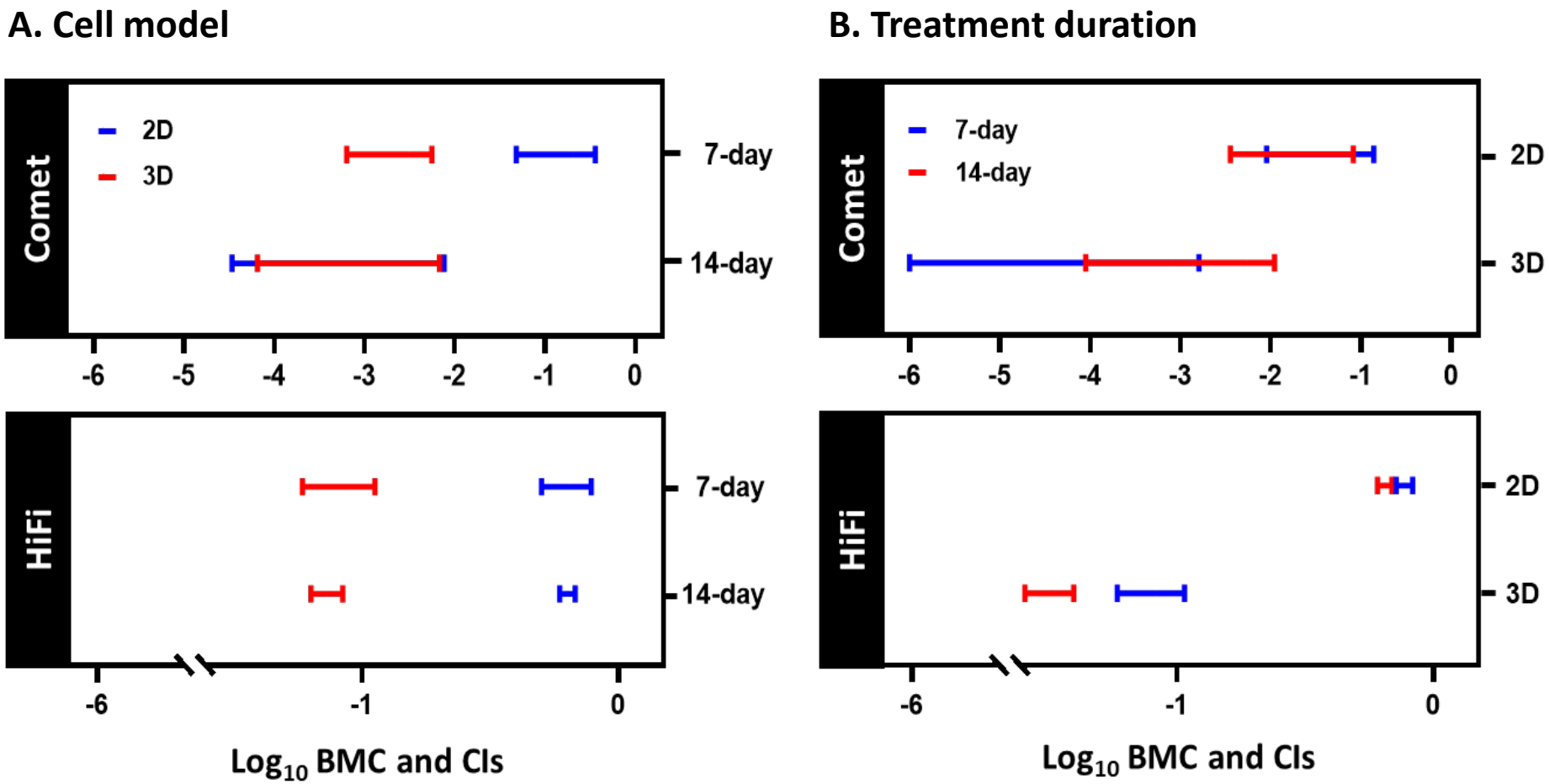








Quantitative analysis of comet and HiFi sequencing data



Summary – HepaRG genotoxicity

- 2D and 3D HepaRG models were successfully employed in high-throughput CometChip, micronucleus, and Multiflow assays for genotoxicity assessment.
- 3D HepaRG spheroid model can serve as a sensitive, human-based cell system expressing Phase I and Phase II enzymes for detecting the genotoxicity of small-molecule nitrosamines and nitrosamine drug substance-related impurities (NDSRIs).
- 3D HepaRG spheroids produced greater genotoxic responses to *N*-nitrosamines (related to higher levels of metabolic activity) than 2D cells, and the results were generally consistent with EAT results.
- Quantitative analysis using high-throughput genotoxicity data from 3D HepaRG spheroids can enhance the accuracy of benchmark dose in risk assessments.

Summary – HepaRG mutagenicity

- A method for detecting mutations in differentiated HepaRG cells was established using error-corrected sequencing (ECS).
- Both Duplex Sequencing (DS) and High-Fidelity (HiFi) Sequencing detected NDMA-induced mutations in both 2D and 3D HepaRG models.
- Severely cytotoxic concentrations should be avoided for ECS.
- NDMA treatments resulted in concentration- and time-dependent increases in mutation frequency in both 2D and 3D HepaRG models, with 3D spheroids showing higher mutation responses than 2D cells.
- NDMA predominantly induced T→C transitions, along with a lower frequency of C→T transitions in HepaRG models.

Conclusion

- **Although utilizing 3D HepaRG spheroids requires more time and labor, it provides higher sensitivity in detecting the genotoxicity and mutagenicity of *N*-nitrosamines.**

- **We are currently conducting the genotoxicity and mutagenicity evaluations of additional small-molecule nitrosamines and NDSRIs using 3D HepaRG spheroids.**

- **HepaRG cell system can potentially serve as a human-relevant in vitro new approach methodology (NAM) for studying the mutagenicity of NDSRIs.**

  - HepaRG cell system can be used for In Vitro to In Vivo Extrapolation (IVIVE).

  - With improved prediction of in vivo genotoxicity responses, 3D HepaRG spheroid model will generate more human-relevant data and minimize the use of animal models.

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Thank you for your attention!

감사합니다.

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