N-Nitrosamine drug impurity research at FDA/NCTR

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N-Nitrosamine mutagenicity projects at NCTR

1. Optimize/enhance the Ames test for detecting the mutagenicity of *N*-nitrosamine drug impurities and *N*-nitrosamine drug-substance-related impurities (NDSRIs)
2. Developing mammalian cell mutagenicity assays using metabolically competent human cells to further study Ames test findings
3. Evaluating different in vivo genotoxicity endpoints, including TGR, *Pig-a* and ecNGS mutation, for detecting the genotoxicity of *N*-nitrosamine drug impurities and NDSRIs

Project 1: Optimizing the Ames test for detecting the mutagenicity of *N*-nitrosamine drug impurities and NDSRIs

- The Ames bacterial mutagenicity test is used to determine the mutagenicity of drug impurities and degradation products – mutagens are suspect carcinogens and controlled at low levels.
- Nitrosamine drug impurities are particularly troubling since many are known mutagenic carcinogens (one of the few chemical classes listed in ICH M7 cohort of concern).
- Conducting the standard **Ames test for nitrosamine impurities** as per OECD guideline has **produced inconsistent results**, including negative findings with otherwise potent mutagenic nitrosamines.
- Another issue is that **very little is known about** how these problems with the Ames test relate to **NDSRIs**, which are a recently recognized class of nitrosamine impurities formed from the drug substance itself. NDSRIs generally have more complex structures than the nitrosamines historically studied.
- **Thus, there is a need for a version of the Ames test 'optimized' or 'enhanced' for detecting nitrosamines that will increase FDA's confidence in the test's findings.**

Large literature on Ames testing nitrosamines… Going back 50 years (*e.g.*, Lijinsky, Guttenplan)

Ames protocol choices affecting nitrosamine mutagenicity

- Preincubation vs. plate incorporation assay
- Choice of species for S9: rat, hamster, mouse
- Concentration of S9
- Length of preincubation
- Choice of vehicle/solvent
- Tester strains employed
- Growth stage and concentration of the tester strains
- Slightly acid pH during preincubation

Approach to Ames study

- Using these historical observations, and our own experiences with assaying *N*-nitrosamines, we developed a strategy to test the most promising protocol choices on a series of nitrosamines, including NDSRIs.
  - Tester strain: TA1535, TA100, TA98, TA1537, WP2 uvrA (pKM101)
  - Metabolic action: No S9 and 10% and 30% S9; PB/BNF-induced rat and hamster liver S9 (5 conditions)
  - Preincubations of 30 and 60 min (plate incorporation used occasionally for comparison)
  - Solvent: limit concentration to $\leq$3.6%; priority: H_2O, acetone, methanol, DMSO
- Our initial goal is to perform Ames testing on 28 nitrosamines and NDSRIs (13 and 15) with different chemical structures to determine 'optimum' or 'enhanced' conditions for their assay.

Ames responses for 13 NDSRIs: Effect of tester strain and activation conditions

NDSRI	Overall call	Most sensitive strain	Most efficient activation
1	Negative	NA	NA
2	Negative	NA	NA
3	Negative	NA	NA
4	Positive	TA1535~ TA1537	30% hamster S9
5	Positive	TA1535~WP2 uvrA (pKM101)	30% hamster S9
6	Negative	NA	NA
7	Positive	TA1535~WP2 uvrA (pKM101)	30% hamster S9
8	Positive	TA1535~ TA98	10%~30% hamster S9
9	Positive	TA1535	30% hamster S9
10	Negative	NA	NA
11	Positive	TA1535	30% hamster S9
12	Negative	NA	NA
13	Positive	TA1535	30% hamster S9

NA = not applicable

Project 2: Developing human cell follow-up approaches

- 2021 CDER Workshop on nitrosamine drug impurities indicated that additional data to confirm or further study Ames mutagenicity findings may include human-cell assays with human metabolic capability.
- Concentrated our efforts on two systems:
  - Human lymphoblastoid **TK6 cells transduced with different human CYPs** (14 lines with different CYPs plus parent non-transduced line).
  - **HepaRG** cells expressing human metabolic enzymes.

N-Nitrosamine studies in mammalian cells

TK6 cell system

- 14 cell lines transduced with a single human CYP plus parent line
- Endogenous **human Phase I activation**; can combine parent TK6 with exogenous S9 activation
- Use to detect DNA damage (CometChip, Multiflow), and perform MN and phenotypic *TK* and *HPRT* mutation assays

HepaRG cells

- Human hepatic stem cell line that can be induced to differentiate into liver cells and then stimulated to divide
- Endogenous expression of large number of **human Phase I and Phase II** enzymes similar to primary human hepatocytes; spheroid cultures have higher Phase 1 activity (note that HepaRG cells have relatively low levels of CYP2D6, CYP2A6, and CYP3A7 compared to PHHs)
- Use to detect DNA damage (CometChip, Multiflow) and perform MN assay (following stimulation)

Applications for in vitro human cell genetox data

- Demonstrate relevance of Ames assay results (bacterial target; rodent activation) using human cells having endogenous human activation pathways
  - Confirm that positive results from Ames assays are positive in a human-based system
  - Confirm that a negative in Ames is also negative for a human-based system that is less selective for the type of genetic damage it detects than Ames
- Assay test substances not easily tested in bacteria (antibiotics)
- The TK6 assays can be viewed as **hazard ID** (since they use only Phase I activation-in that respect similar to the Ames test), while the HepaRG system may address **risk characterization** (since it has both Phase I and Phase II activities): assuming it can be validated, does doing HepaRG assays make more sense as a follow-up for Ames-positive findings than doing in vivo mutation assays in rodents?—some are advocating this.

Evaluating the genotoxicity of nitrosamine impurities and NDSRIs using human TK6 cells transduced with human cytochrome P450s

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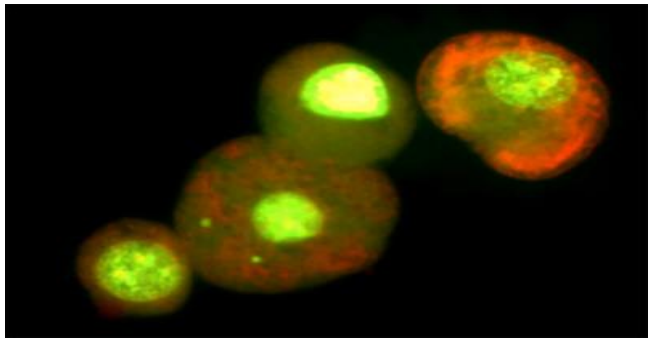
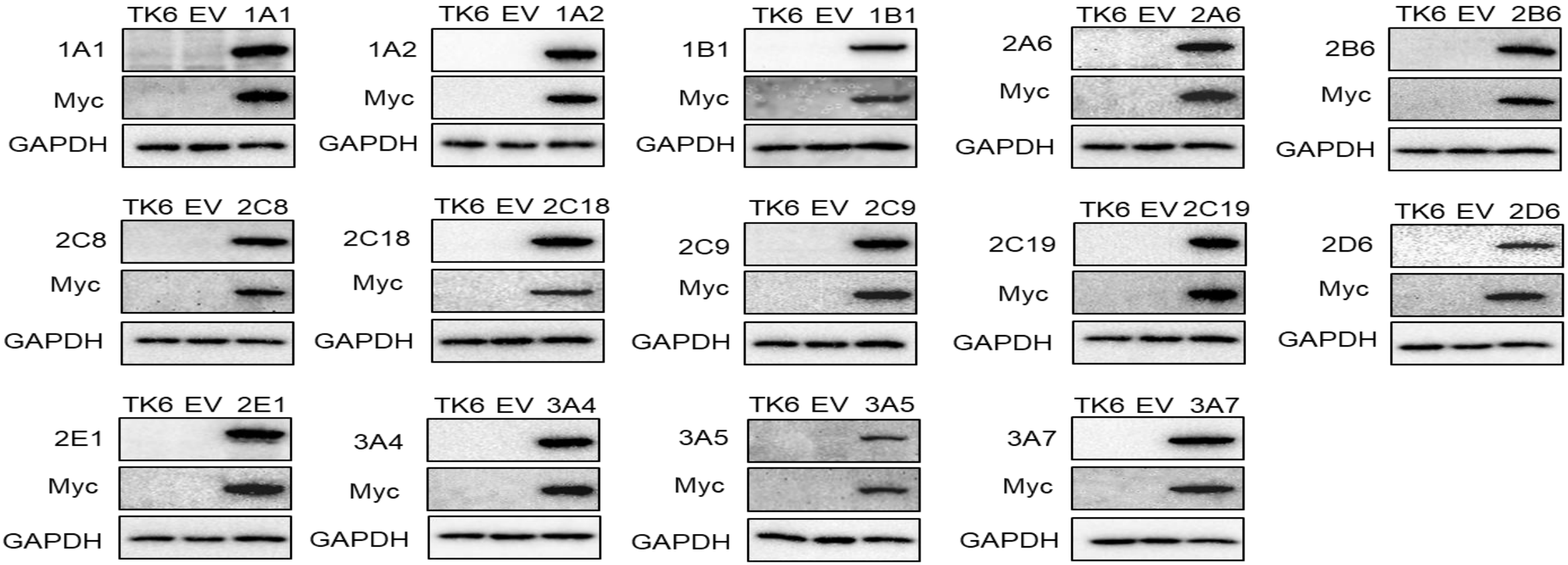


Image source: ILS

Western blot analysis:

Expression of 14 CYP proteins in genetically modified TK6 cells



Li et al. (2020) *Tox Sci* 175:251-265; Li et al. (2020) *Food and Chem Toxicol* 145:111662

Applications of the TK6 cell system

Genotoxicity endpoints:

- Cytotoxicity (MTS, Cell viability, ATP,)
- Measure genotoxic metabolites by LCMS
- Comet assay
- Flow cytometer-based micronucleus assay
- Flow cytometer-based DNA damage assay
- Cell cycle analysis
- Western blotting measuring the expression of DNA damage/repair proteins
- *TK* mutation assay
- *HPRT* mutation assay

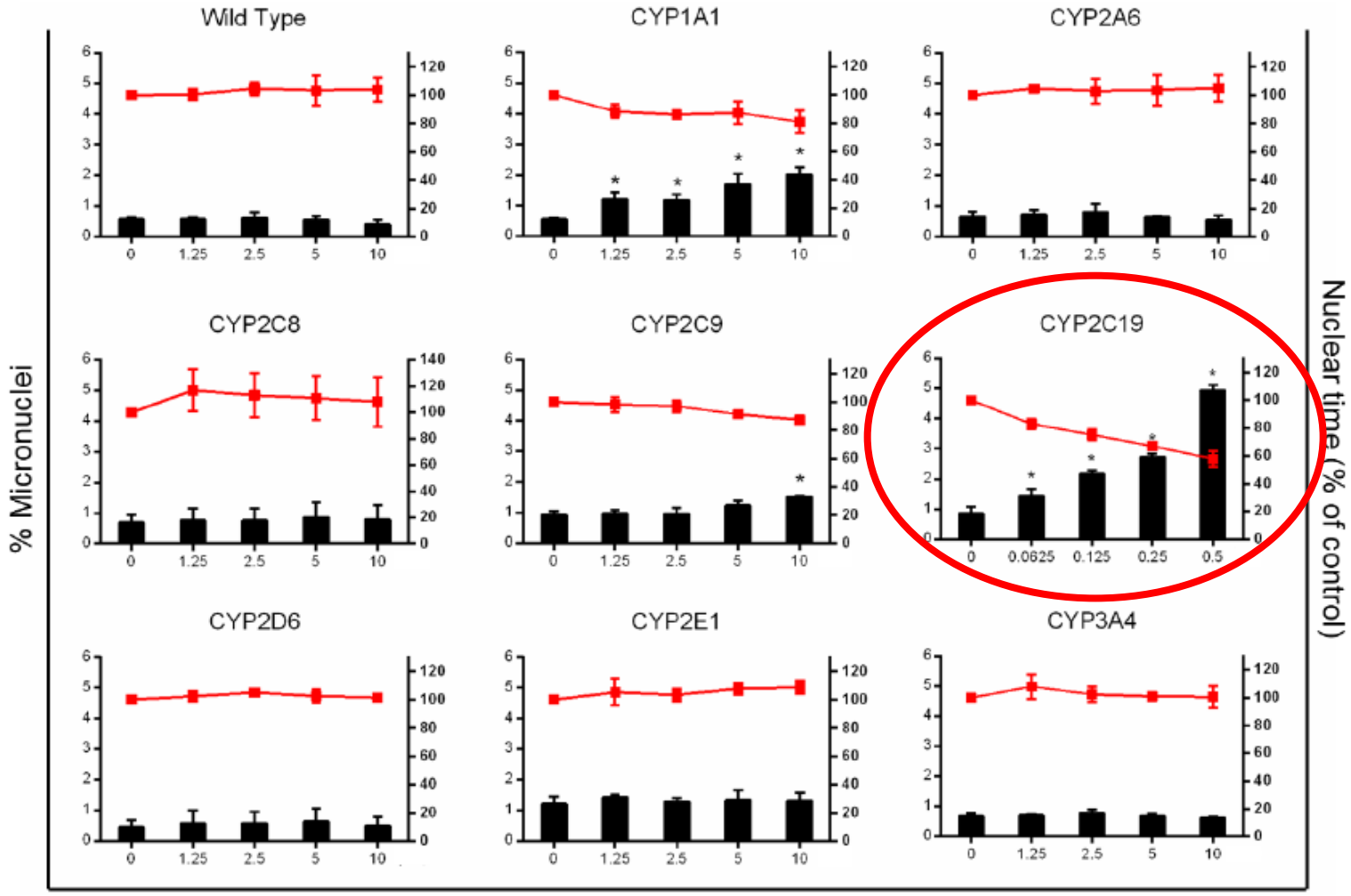
Chemicals (including some drugs) tested… before nitrosamines:

- Benzo[a]pyrene (CYP1A1,1B1,2C19)
- 7,12-Dimethylbenz[a]anthracene (CYP1A1,1B1,2C19)
- Acrylamide (CYP2E1)
- Cyclophosphamide (CYP2B6)
- Lasiocarpine (CYP3A4, 3A5, 3A7)
- Riddelliine (CYP3A4, 3A5, 3A7)
- Senkirkine (CYP3A4, 3A5, 3A7)
- Luteolin (CYP1A1, 1A2)
- Diosemetin (CYP1A1, 1A2)
- Chloroquine (CYP2C8, 3A4, 3A5)
- Hydroxychloroquine (CYP2C8, 3A4, 3A5)
- Methyl Methanesulfonate (Direct acting positive control)
- Mitomycin C (Direct acting positive control)

Li et al. (2020) *Tox Sci* 175:251-265; Li et al. (2020) *Food and Chem Toxicol* 145:111662; Li et al. (2021) *Toxicol Lett* 344:58-68

Screening for NDSRI genotoxic activity in TK6 cells transduced with different human CYPs

Genotoxicity screen conducted with high-throughput flow cytometric micronucleus assay



Xilin Li, Yuan Le, Nan Mei et al., Reg Toxicol Pharmacol 141 (2023) 105410

Genotoxicity of nitrosamines and NDSRIs using cultures of metabolically competent human HepaRG cells

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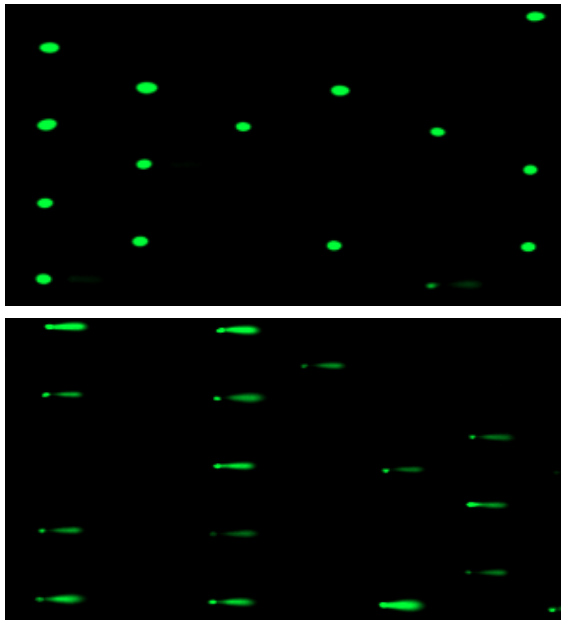

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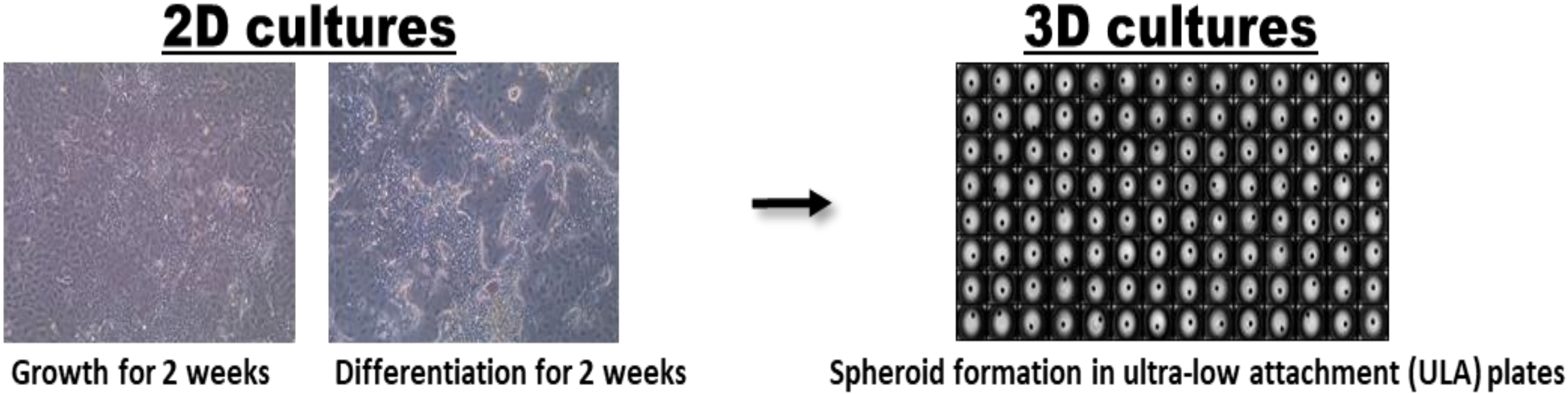
HepaRG cells grown as attached 2D cultures and as unattached spheroid cultures



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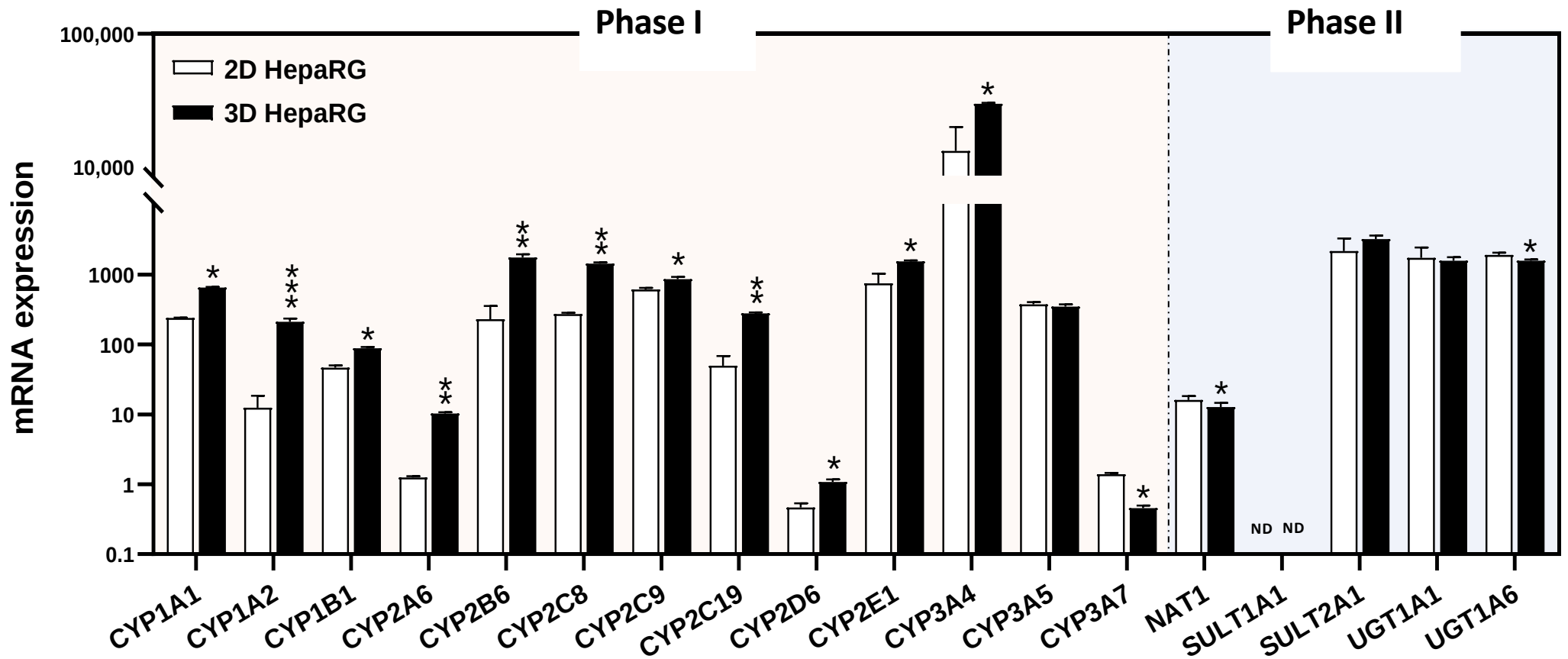
mRNA expression for Phase I and Phase II enzymes in HepaRG cells



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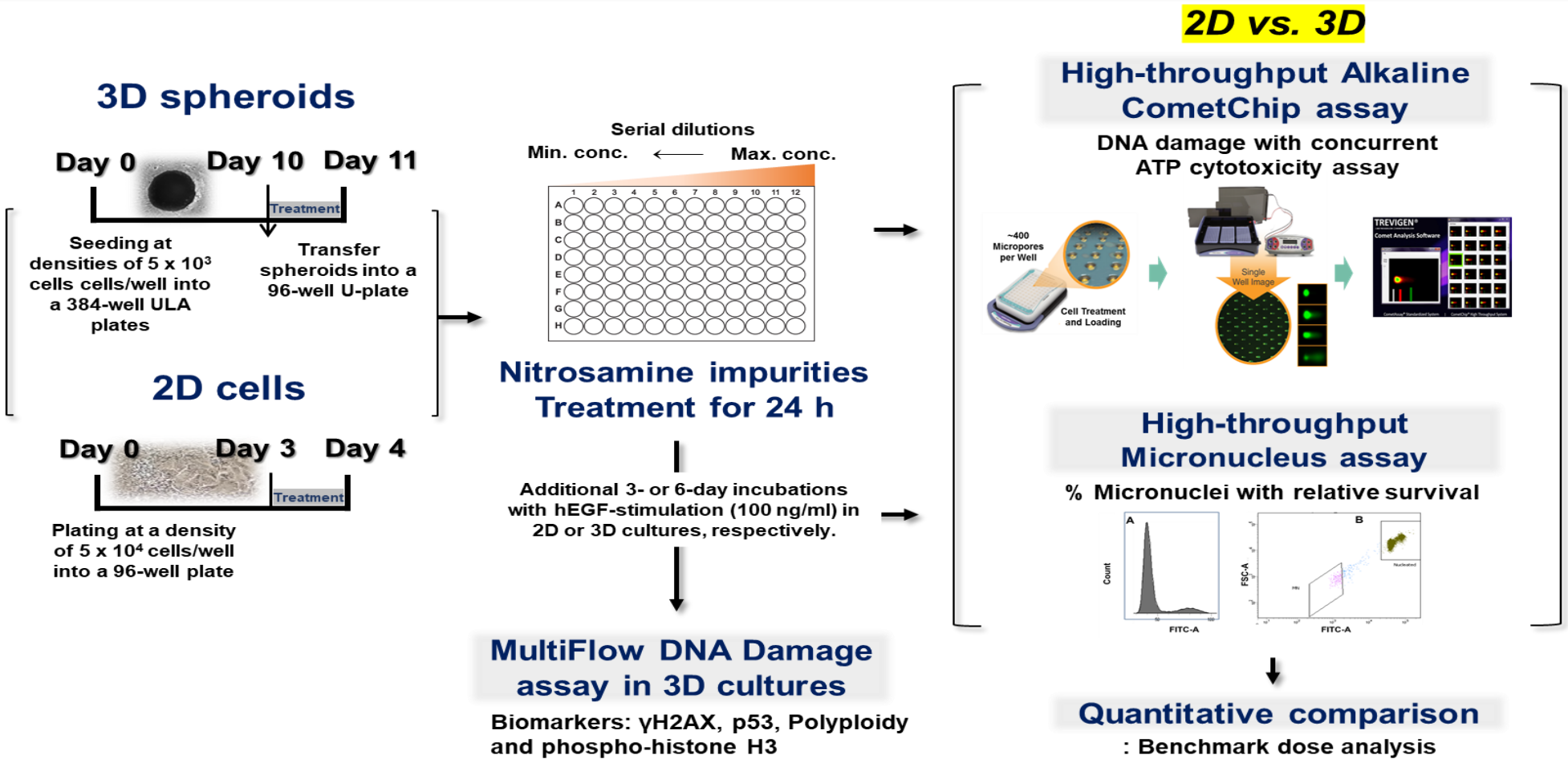
HepaRG protocols for assessing DNA damage and MN endpoints



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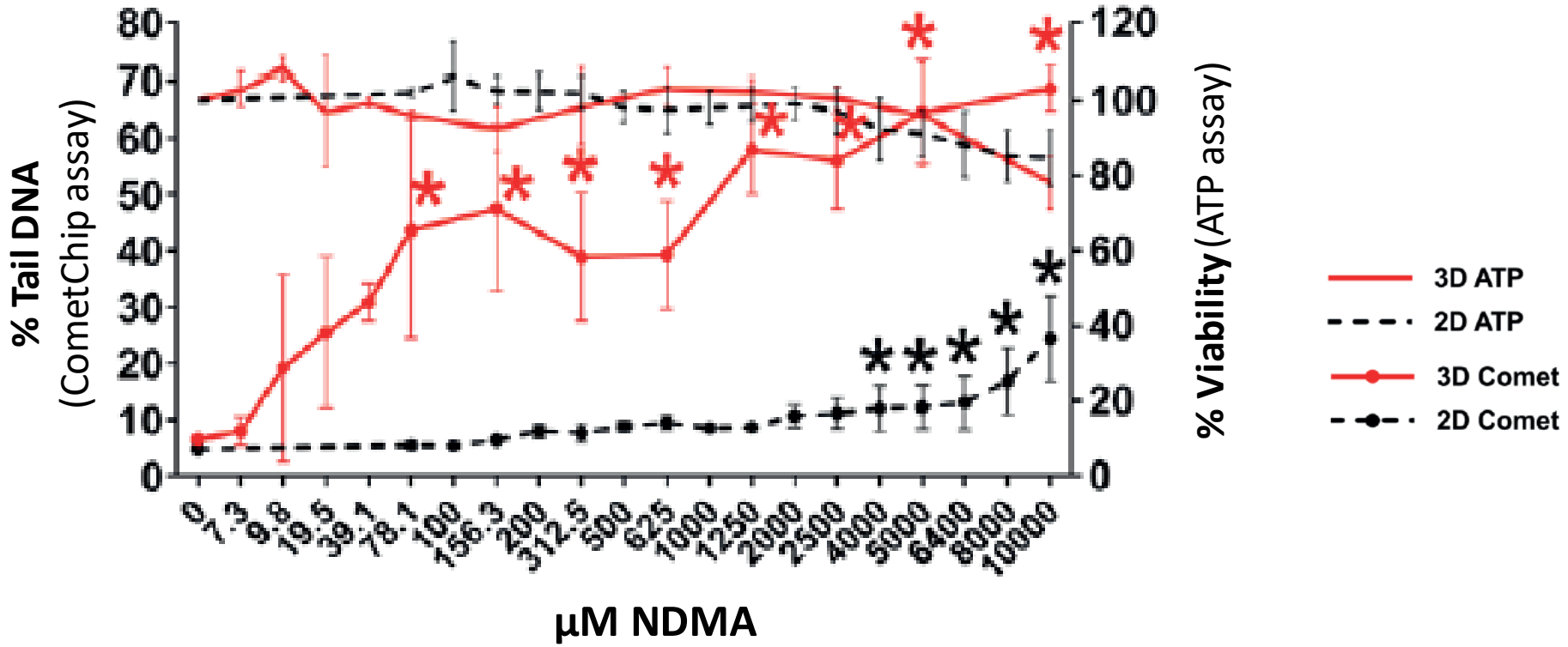
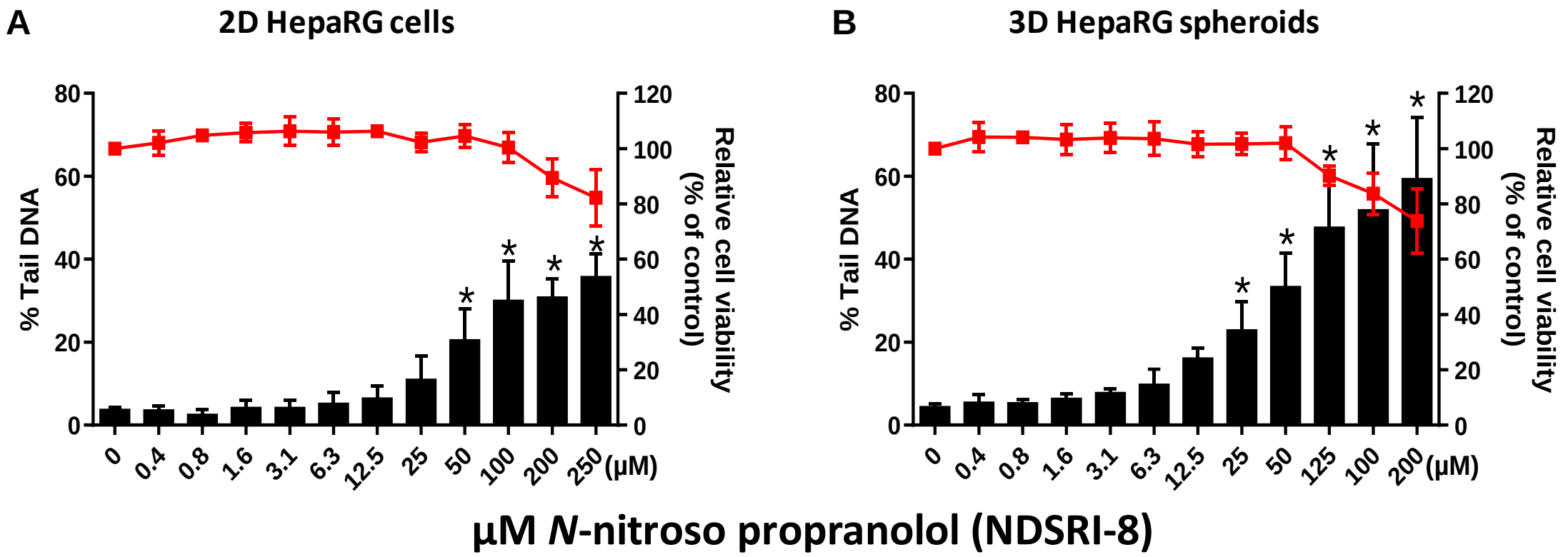
DNA damage induction by *N*-nitroso dimethylamine in 2D and spheroid cultures of HepaRG cells



From Seo et al. (2022) ALTEX 39:583-604

DNA damage induction by an NDSRI in 2D and spheroid cultures of HepaRG cells



μM *N*-nitroso propranolol (NDSRI-8)

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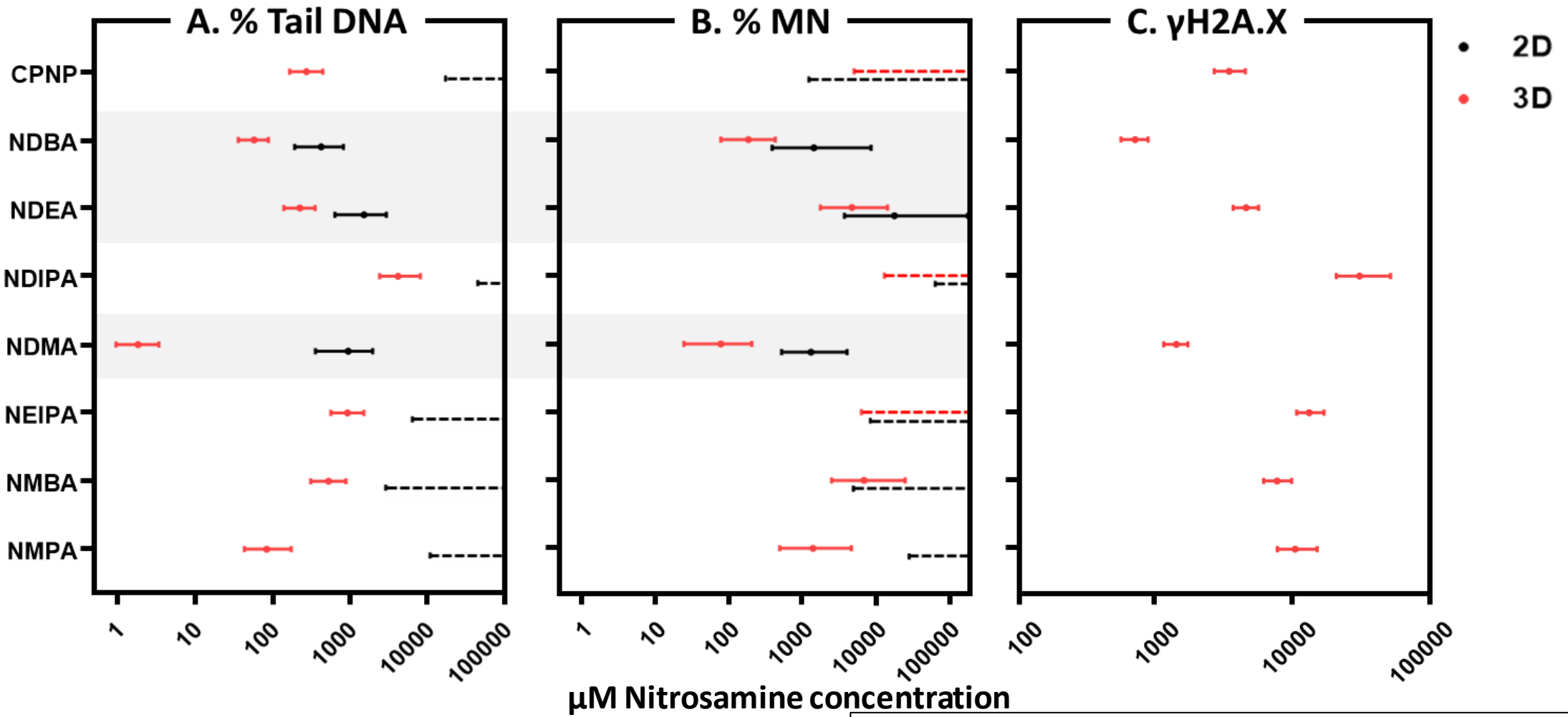
Genetox results for 8 *N*-nitrosamines in 2D and spheroid HepaRG cultures: BMD dose response analysis



From Seo et al, 2023 SOT meeting poster

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Thank you: questions?