N-Nitrosamine Drug Impurity Research at FDA/NCTR: Assessing the Mutagenicity of *N*-Nitrosamines and NDSRIs

Xilin Li, Ph.D.
Division of Genetic and Molecular Toxicology
National Center for Toxicological Research
U.S. FDA

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Outlines

Ames: Optimizing the Ames test for detecting the mutagenicity of *N*-nitrosamine small-molecule drug impurities and nitrosamine drug substance-related impurities (NDSRIs)

- Rationale for optimizing the Ames test for *N*-nitrosamines
- Testing strategy
- General conclusions based on testing 29 *N*-nitrosamines

In vitro mammalian cells: Follow-up studies on the mutagenicity and genotoxicity of *N*-nitrosamines in human cells to further characterize their hazards beyond bacteria

- Addressing the relevance of NDSRI Ames mutagenicity findings in human cells with human metabolism
- Mutagenicity and genotoxicity of NDSRIs in human lymphoblastoid TK6 cells transduced with human CYPs
- Mutagenicity and genotoxicity of NDSRIs in HepaRG cells expressing human metabolic enzymes

HESI/GTTC/MGRA collaborations: From Ames testing to in vivo studies

- Ames: Phase I testing finished – preliminary results have been discussed, presented at GTA meeting in April
- Nitrosamine in vitro approaches working group: currently focusing on HepaRG cells
- Evaluating in vivo mutagenicity in TGR rodents dosed with small-molecule nitrosamines and NDSRIs—relationship to Ames and cancer findings, utility of error-corrected NGS

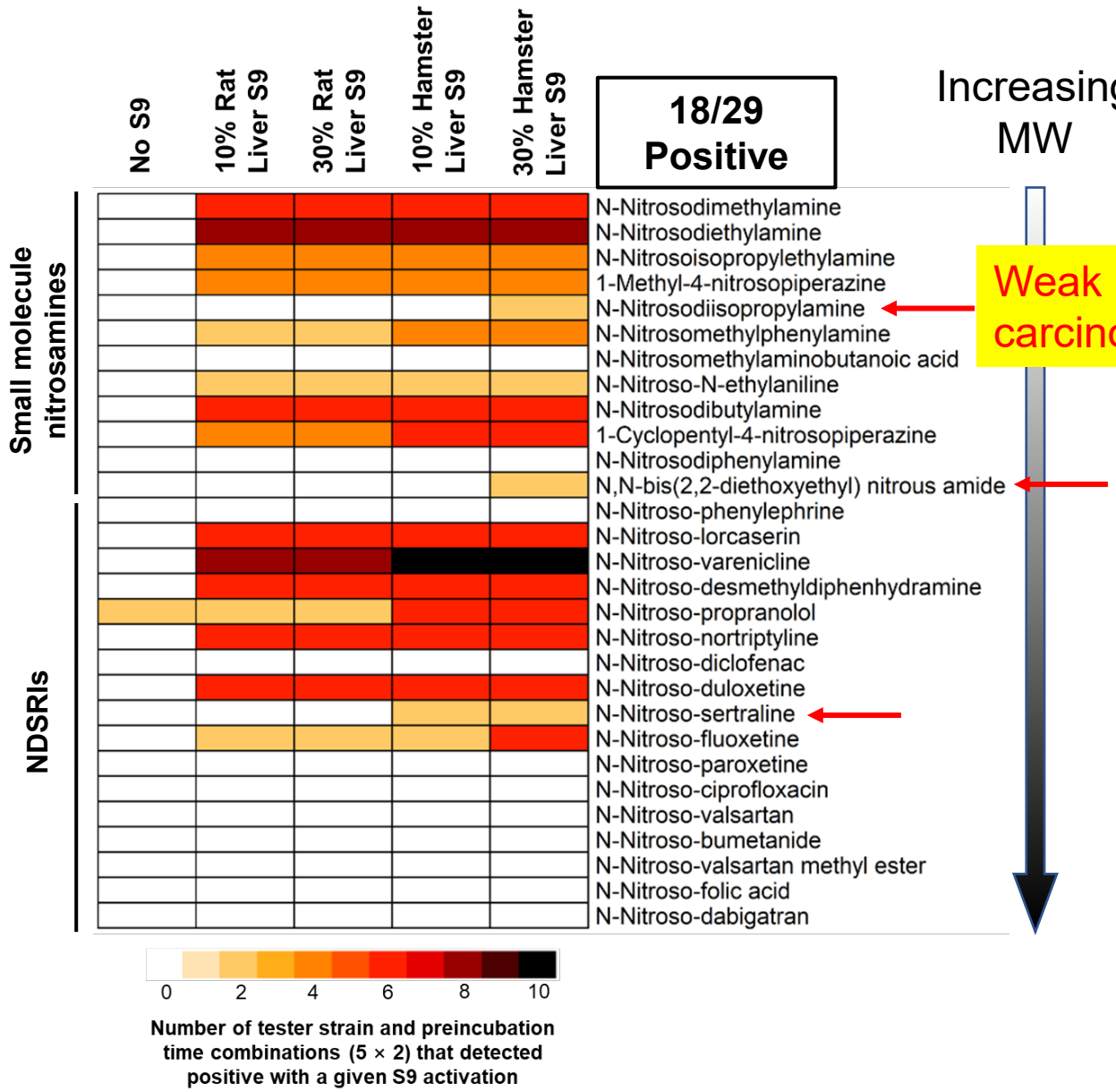
Optimizing the Ames test for *N*-nitrosamines

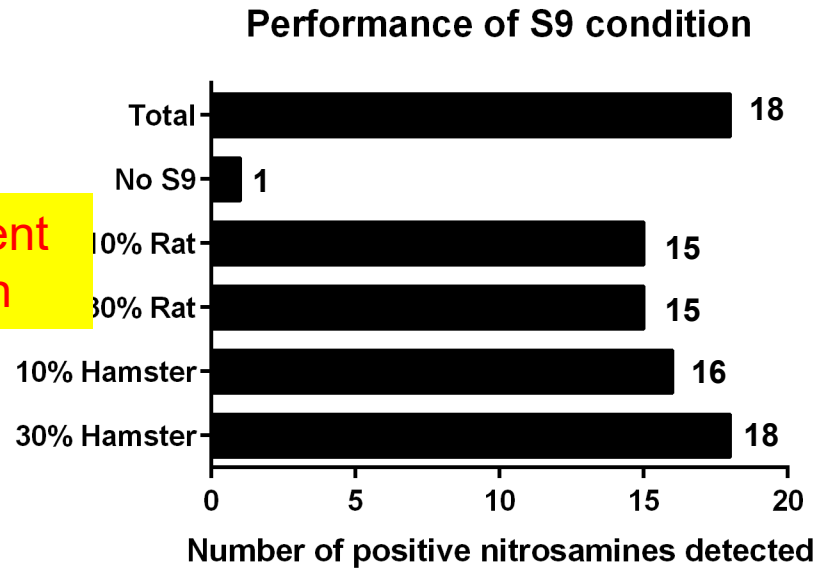
- Historically, conducting the **Ames test for nitrosamine impurities** has **produced inconsistent results** with otherwise potent mutagenic nitrosamines and a perception has developed (not held by all) that the standard Ames test is relatively insensitive to nitrosamine mutagenicity.
- Another issue is that **very little is known about the mutagenicity of NDSRIs in the Ames test**. NDSRIs generally have more complex structures than the small-molecule nitrosamines historically studied.
- **Thus, there is a need for an 'enhanced' version of the Ames test that detects mutagenic nitrosamines with the greatest possible sensitivity and that will increase FDA's confidence in the test's findings.**

Ames study strategies

- Using historical observations, and our own experiences, 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 activation: No S9, 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 ≤3.6%; priority: H_2O, acetone, methanol, DMSO
- Our initial trial involved testing 12 small-molecule nitrosamines and 17 NDSRIs with different chemical structures, using 50 different combinations of test conditions for each nitrosamine.

Performance of S9 conditions

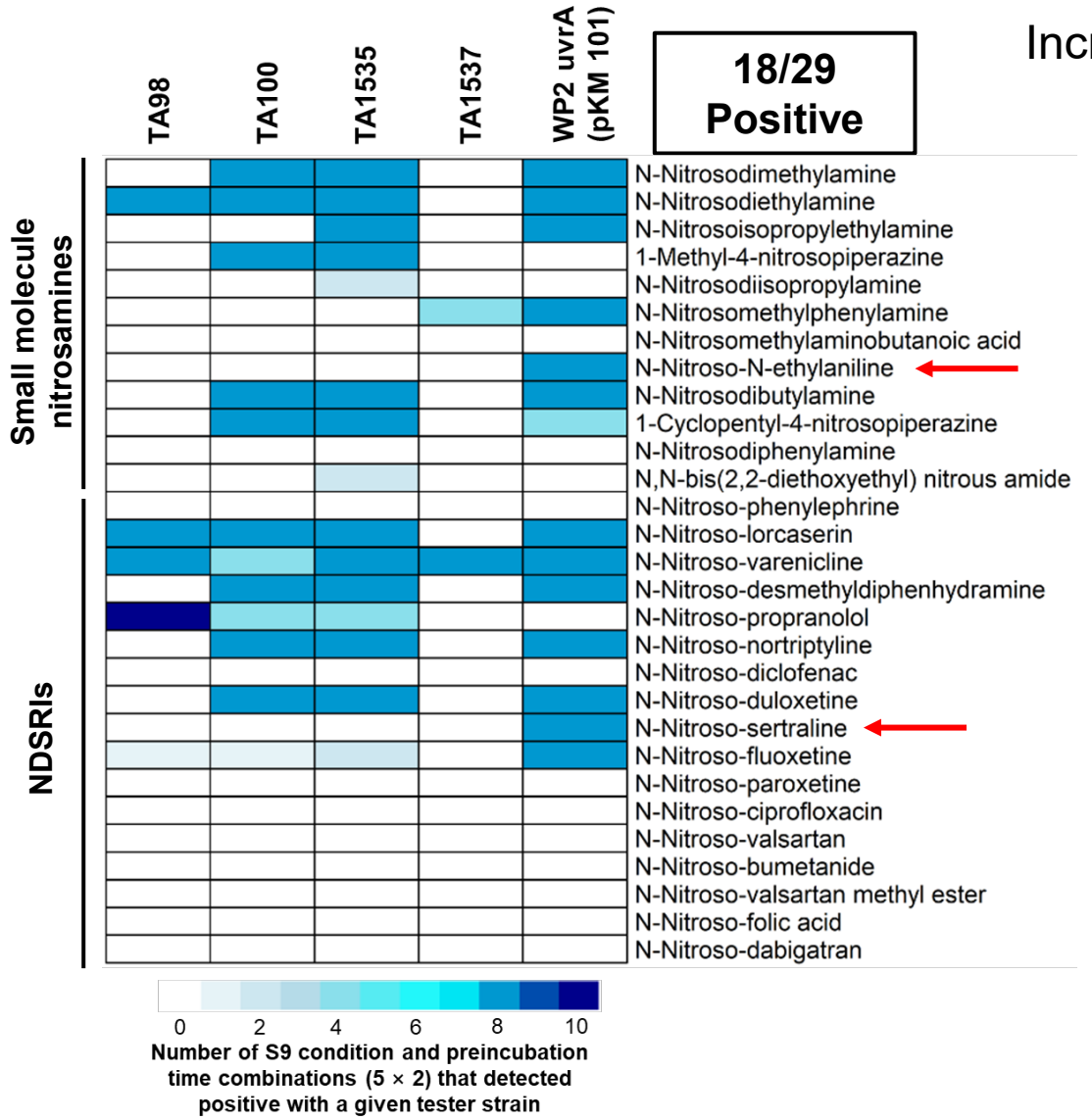




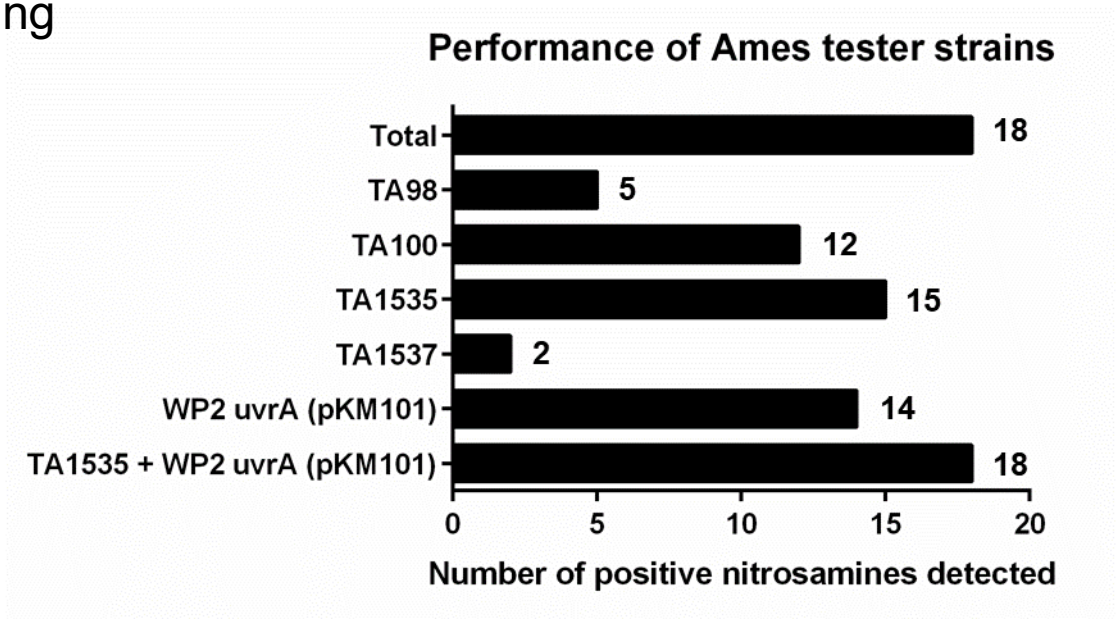
Observations:

- Preincubations with hamster S9 were generally more effective than rat S9
- Preincubations with 30% S9 were generally more effective than preincubations with 10% S9
- NDIPA and N,N-bis(2,2-diethyl) nitrous amide were only positive with 30% hamster S9
- *N*-nitroso-sertraline was only positive with hamster S9

Performance of Ames tester strains



Increasing MW



Observations:

- TA1535 and WP2 uvrA(pKM101) were the most useful tester strains
- No nitrosamine was uniquely mutagenic in TA100, TA98 or TA1537
- *N*-nitroso-N-ethylaniline and *N*-nitroso-sertraline were uniquely positive in WP2 uvrA(pKM101)

Follow-up studies: *in vitro* mammalian cells

TK6 cell system

- System consists of the parent TK6 line plus 14 cell lines transduced with a single human CYP providing endogenous **human Phase I activation**; can combine parent TK6 with exogenous S9 activation
- Endpoints: DNA damage (CometChip, Multiflow); MN and phenotypic *TK* and *HPRT* mutation assays

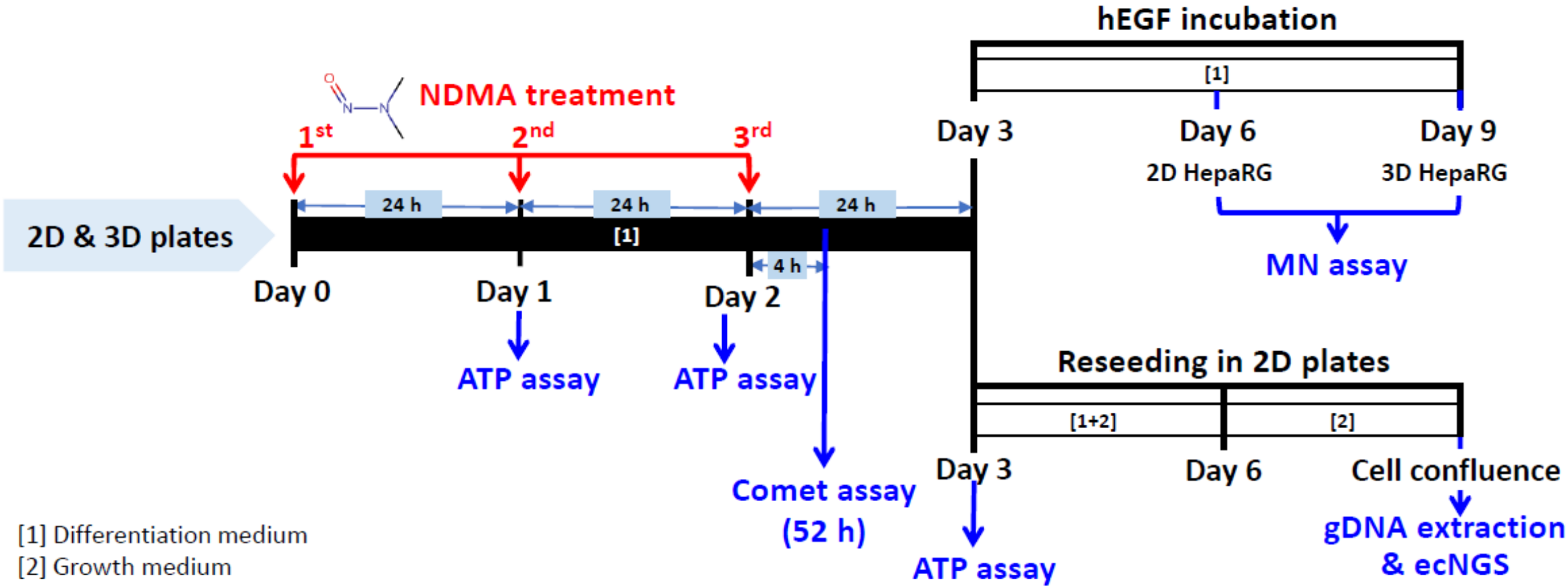
HepaRG cells

- 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 (much more robust than HepG2 cells); spheroid cultures have higher Phase 1 activity
- Endpoints: DNA damage (CometChip, Multiflow), MN assay and ecNGS mutation assay

Mutagenicity of NDSRIs in TK6 cells

NDSRIs *N*-nitroso-	Enhanced Ames test	In vitro mammalian gene mutation	Micronucleus with S9	Micronucleus without S9	Human CYP activation
Diphenhydramine	+	+	+	+	CYP2C19, 2B6
Duloxetine	+	+	+	+	CYP2C19, 2B6
Fluoxetine	+	+	+	+	CYP2C19, 2B6
Nortriptyline	+	+	+	+	CYP2C19, 2B6
Propranolol	+	+	+	-	CYP2C19, 1A1
Varenicline	+	+	+	-	CYP3A4, 2B6
Bumetanide	-	-	-	-	N/A
Dabigatran	-	-	-	-	N/A
Diclofenac	-	-	-	+	N/A
Phenylephrine	-	-	-	-	N/A
Valsartan	-	-	-	-	N/A
Valsartan methyl ester	-	-	-	+	N/A

Proof-of-principle study measuring the mutagenicity of NDMA in HepaRG cells



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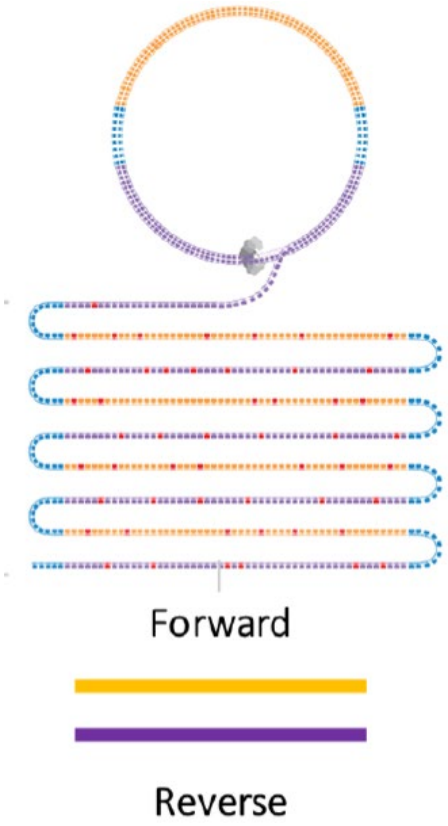
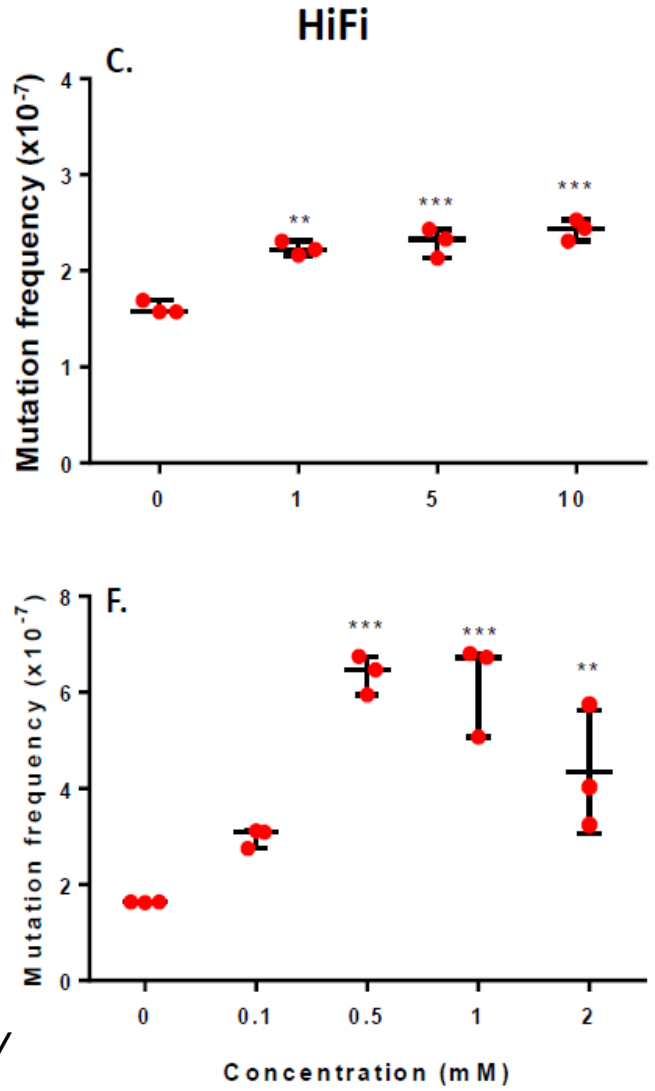
Mutation analysis in NDMA-treated HepaRG cells by PacBio HiFi ecNGS





2D cultures

3D spheroids

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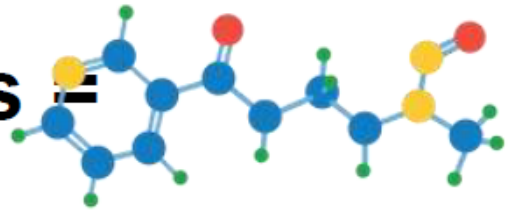
MGRA Nitrosamines



Nitrosamines Research Program

Leadership
- Tetyana Cheairs (New York Medical College)
- Andreas Czich (Sanofi)

Goal 1: Ames Optimization

Develop an Ames protocol predictive for carcinogenic potential of Nitrosamines

Leadership
Anthony Lynch (GSK)
Joel Bercu (Gilead)

- FDA grant provided for purchase of 35 compounds to be tested under varying assay conditions and external statistical analysis
- 16 participating organization providing in-kind work

Goal 2: In vitro test strategy

Identification and verification of in vitro assays with alternative cell models supporting Nitrosamines risk identification

Leadership
Xiaoqing Guo (US FDA/NCTR)
Wen Sun (Pfizer)

- Landscape analysis to identify and prioritize and key in vitro test systems for testing
- Experimental comparison of assay systems

Goal 3: In vivo Ames follow-up

Develop in vivo Strategy to verify Ames Data within the frame of ICH M7

Leadership
Bob Jolly (Lilly)
Joel Bercu (Gilead)
Sheroy Minocherhomji (Lilly)
Timothy McGovern (US FDA)

- Comparison of in vitro/in vivo testing for NDSRIS for hazard ID
- Comparison of in vivo mutagenicity and carcinogenicity of NAs
- LTL literature analysis

Goal 4: (Q)SAR/QM

Refine and extend the CPCA using (Q)SAR and QM for more predictive and accurate predictions

Leadership
David Ponting (Lhasa)
Jakub Kostal (GW University)
Kevin Cross (Instem)

https://hesiglobal.org/gttc-nitrosamines/.

HESI

Our collaborations with HESI - Ames

Mixture of carcinogenic and non-carcinogenic

- 32 Nitrosamines (2 labs per compounds)
- 11 negatives and 21 positives for carcinogenicity

CPCA Category	# of NAs
1	6
2	6
3	6
4	9
5	2
NA	3

FDA/NCTR was assigned 6 HESI compounds:

- *N*-nitroso-ephedrine (positive)
- 4-Benzoyl-3,5-dimethyl N-nitrosopiperazine (positive)
- *N*-nitroso-methylphenidate (equivocal)
- *N*-nitroso-chlorodiazepoxide (direct-acting positive)
- 2-methyl-1-nitrosopiperidine (positive)
- N-methyl-N-nitroso-1H-purin-6-amine (*N*-nitroso- methyladenine) (direct-acting positive)

Our collaborations with HESI - Ames

Indications from preliminary results

- The EAT protocol is highly sensitive (~95%) for predicting carcinogenicity of nitrosamines
- Hamster S9 was more sensitive than rat, and 30% S9 improved sensitivity; 30% hamster liver S9 did not significantly decrease specificity
- Accuracy rate around 77%
- The low specificity (~45%) may be linked with the false positive compounds that are direct-acting (potential follow-up with in vitro mammalian cells to identify those direct-acting bacterial mutagens?)

Our collaborations with HESI – in vitro and in vivo

- In vitro mammalian cell assays
  - Goals: identify alternative in-vitro approaches as follow-up assay to Ames test findings
    - Informative for in vivo study design;
    - Potency ranking;
    - WoE;
  - Currently focusing on HepaRG cells: Protocol harmonization, endpoint development
- In vivo
  - Evaluating in vivo mutagenicity in TGR gene mutation assay: utility of error-corrected NGS (PacBio Hi-Fi sequencing) as an alternative to transgene assays in rats dosed with NDSRIs

Future directions

- EAT: test whether pH will change the assay sensitivity
- Characterize the mutagenicity of NDSRIs in human cells using ecNGS
- Transgenic rodent gene mutation assays (*cII*) on selected NDSRIs
- Compare in vivo mutagenicity results between TGR and ecNGS
- Carcinogenicity study?

Project contributors (2021-present)

U.S. FOOD & DRUG ADMINISTRATION

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