Genotoxicity assessment of eight nitrosamine impurities using 2D and 3D in vitro human HepaRG models

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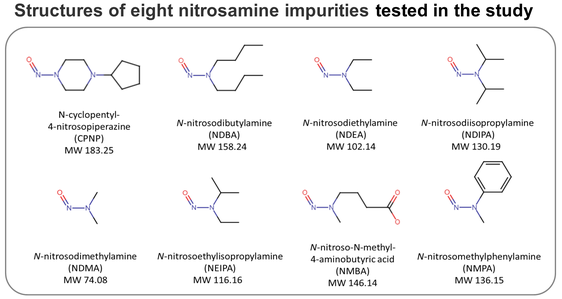
Abstract

N-nitrosamine impurities have been detected increasingly in human drugs. This is a safety concern as most nitrosamines are mutagenic in bacteria, genotoxic in mammalian cells and carcinogenic in rodent models. There are, however, only limited data on the genotoxic potency of nitrosamines in human cell systems. The mutagenic and carcinogenic activity of most nitrosamines require metabolic activation by CYP450 enzymes. In this study, we used metabolically competent human HepaRG cells, whose metabolic capability is comparable to that of primary human hepatocytes, to evaluate the genotoxicity of eight nitrosamine impurities [N-cyclopentyl-4-nitrosopiperazine (CPNP), N-nitrosodibutylamine (NDBA), N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), N-nitrosodiisopropylamine (NDIPA), N-nitrosoethylisopropylamine (NEIPA), N-nitroso-N-methyl-4-aminobutyric acid (NMBA), and N-nitrosomethylphenylamine (NMPA)]. Given that three-dimensional (3D) spheroids possess higher levels of CYP activity compared to 2D monolayer cells, the genotoxicity of the eight nitrosamines was investigated in both 2D and 3D HepaRG models using the high-throughput CometChip assay and the flow cytometry-based micronucleus (MN) assay. Following a 24-hr treatment, all the nitrosamines induced DNA damage in 3D spheroids, while only three nitrosamines, NDBA, NDEA, and NDMA produced positive responses in 2D HepaRG cells. In addition, these three nitrosamines also caused significant increases in MN frequency in both 2D and 3D HepaRG models, while NMBA and NMPA were positive only in the 3D HepaRG MN assay. Quantitative benchmark concentration analysis showed that under the current experimental conditions, NDMA produced the strongest response in 3D HepaRG models. Overall, benchmark concentration analysis indicated that 3D HepaRG spheroids were more sensitive at detecting the genotoxicity of the eight nitrosamines than 2D cultures. These results suggest that 3D HepaRG spheroids may be useful as a New Approach Method (NAM) for detecting the genotoxicity of nitrosamine impurities that results from human metabolic activation.

Introduction

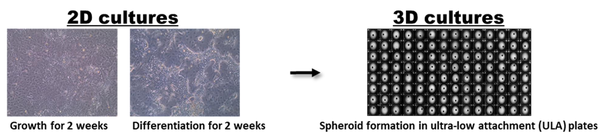
- Nitrosamine impurities, which are potent genotoxic agents, have been detected in several human drug products and some are classified as probable or possible human carcinogens based on animal studies. Despite the genotoxic/carcinogenic potency of nitrosamines in animal and bacteria-based assays, there are insufficient data on their genotoxicity in human systems.
- Most nitrosamines require metabolic activation by CYP450 enzymes to exert their mutagenic and carcinogenic properties
- The human hepatoma HepaRG cell line expresses both phase I and phase II enzymes. This study employed both 2D HepaRG cells and 3D HepaRG spheroids to evaluate the genotoxicity of eight nitrosamine drug impurities using high-throughput (HT) genotoxicity assays.

Structures of eight nitrosamine impurities tested in the study

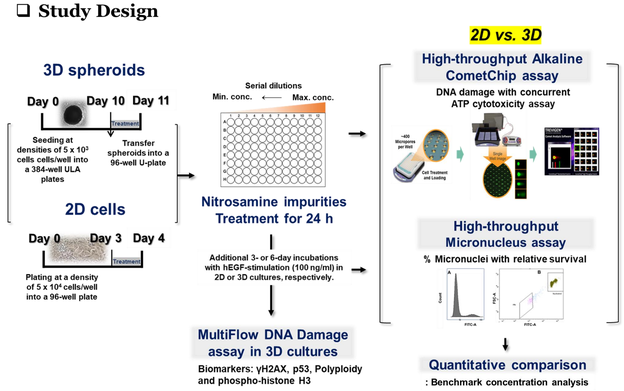


Methods

❑ HepaRG cultures



❑ Study Design



❑ Statistical analysis

Data are expressed as the mean ± standard deviation (SD). The statistical significance of Phase I and Phase II gene expression was evaluated by the two-tailed Student's t-test (*$p < 0.05$). One-way ANOVA followed by Dunnett's post hoc test was used for CometChip, MN, and Multiflow assays (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ *vs*. control).

Results

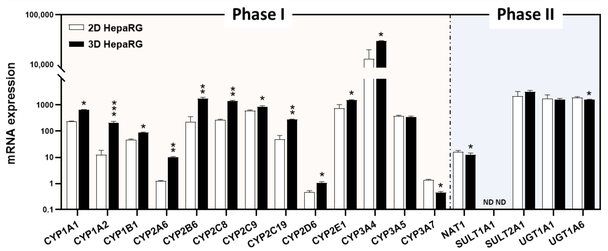


Figure 1. Gene expression of Phase I and Phase II enzymes in 2D and 3D HepaRG models. 3D spheroids showed significantly higher mRNA expression than 2D HepaRG cells for 11 phase I enzymes: CYP1A1 (2.7-fold), 1A2 (17.0-fold), 1B1 (1.9-fold), 2A6 (8.2-fold), 2B6 (7.7-fold), 2C8 (5.3-fold), 2C9 (1.4-fold), 2C19 (5.6-fold), 2D6 (2.3-fold), 2E1 (2.1-fold), and 3A4 (2.2-fold). Total RNA was extracted from 2D HepaRG cells at day 3 and 5K spheroids at day 10 for cDNA synthesis. mRNA expression was measured by quantitative real-time PCR (qPCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The expression value of each gene was defined by the equation: $E = 2^{-(Ct\ of\ test\ gene - Ct\ of\ reference\ gene)} \times 10{,}000$. *p < 0.05 and the fold change ≤ 5-fold; **p < 0.001 and the fold change ≤ 10; and ***p < 0.001 and the fold change > 10

Results

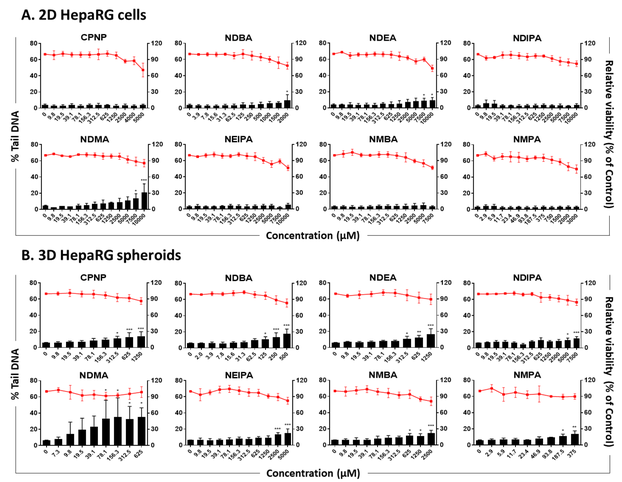


Figure 2. DNA damage and cytotoxicity in 2D (A) and 3D (B) HepaRG models. Three nitrosamines, NDBA, NDEA, and NDMA produced positive responses in 2D HepaRG cells, while all the nitrosamines induced DNA damage in 3D spheroids. DNA damage (% tail DNA; left y-axis and black bar) was detected using the CometChip assay. Cytotoxicity (% of control) was measured by ATP assay (right y-axis and red lines). The cell viability cutoff value was set as 70% for CometChip assay.

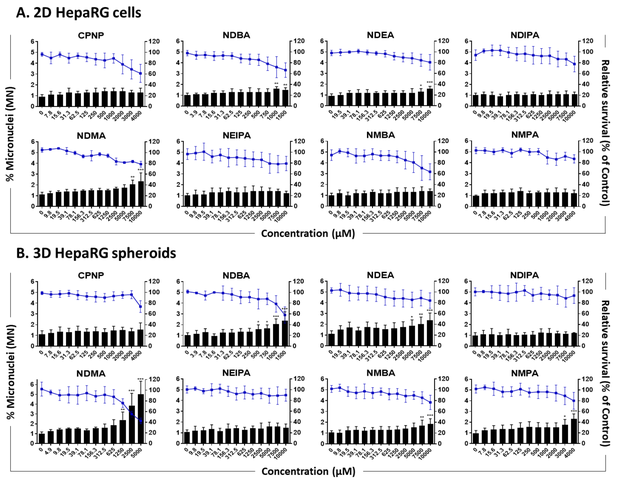


Figure 3. MN induction and relative survival in 2D (A) and 3D (B) HepaRG models. NDBA, NDEA, and NDMA caused significant increases in MN frequency in both 2D and 3D HepaRG models, while NMBA and NMPA were positive only in the 3D MN assay. MN frequency is presented as the percentage of micronuclei relative to intact nuclei (% MN; left y-axis and black bar) and cytotoxicity is presented as the percentage of relative survival (%RS; right y-axis and blue line). The RS cutoff value was set as 40% for the MN assay.

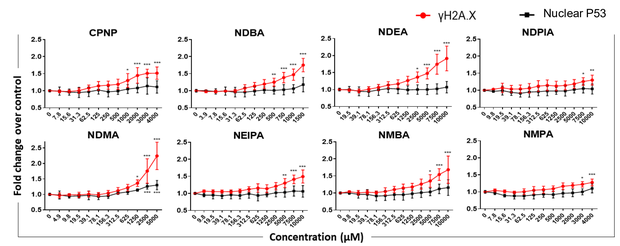


Figure 4. The MultiFlow® DNA damage assay in 3D HepaRG models. All the nitrosamines significantly increased γH2A.X formation in 3D spheroids, while only NDMA caused a statistically significant fold-increase in nuclear p53. γH2A.X shift (red filled circles and lines) and nuclear p53 shift (black filled squares and lines) were determined as indicators of DNA double strand breaks and p53 activation in response to genotoxic stress, respectively, using in vitro MultiFlow DNA damage kits.

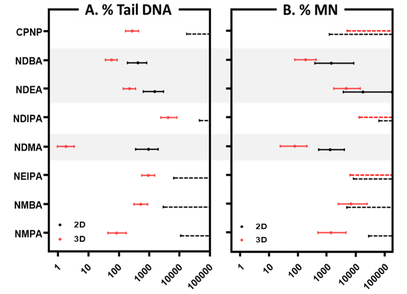


Figure 5. Genotoxic potency of nitrosamine impurities. NDMA produced the strongest response among the nitrosamine impurities. Benchmark concentration$_{50}$ (BMC_{50}) values with the 90% confidence intervals were calculated from 2D and 3D comet and MN data using PROAST software. The bars represent the uncertainty of BMD_{50} estimates. Negative responses in the assays are presented as the dotted line. Black, 2D; Red, 3D models.

Summary & Conclusion

Table 1. Summary of eight nitrosamine impurities-induced genotoxic responses.

Compound	Comet		MN		3D MultiFlow	
	2D	3D	2D	3D	γH2A.X	p53
CPNP	-	++	-	-	±	-
NDBA	++	++	+	++	+	-
NDEA	++	++	+	++	+	-
NDIPA	-	+	-	-	±	-
NDMA	++	+++	++	++	++	+
NEIPA	-	++	-	-	±	-
NMBA	-	++	-	+	+	-
NMPA	-	++	-	++	±	-
Positivity	37.5%	100%	37.5%	62.5%	100%	12.5%

−, The ratio ≤ 1.5-fold and $p \geq 0.05$ (green color);
±, The ratio ≤ 1.5-fold but $p < 0.05$; + 1.5 < ratio ≤ 2; ++, 2 < ratio ≤ 5; +++, and the ratio > 5 ($p < 0.05$, red color).

Following a 24-h treatment, NDBA, NDEA, and NDMA showed positive genotoxic responses in both 2D and 3D HepaRG cultures (relative potency: NDMA > NDBA > NDEA). Overall, 3D HepaRG spheroids were more sensitive than 2D cultures at detecting the genotoxicity of the eight nitrosamines. Our results demonstrate that 3D HepaRG spheroids may be useful as a New Approach Method (NAM) for detecting the genotoxicity of nitrosamine impurities using a HT human-based, metabolism-competent cell system.