A novel approach method combining GFP reporter cell lines with error-corrected next generation sequencing for in vitro genotoxicity assessment of N-nitrosamines

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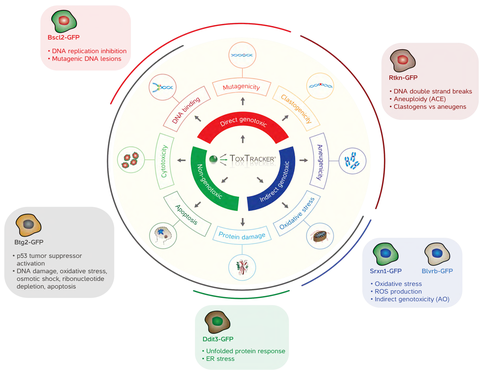
Introduction

N-nitrosamines (NAs) are considered probable human carcinogens and were recently detected as impurities in pharmaceuticals leading to a concern for human health. NAs require metabolic activation before they become mutagenic. NAs vary largely in their molecular weight and not all NAs are mutagenic. Understanding which NAs are genotoxic and their mode of action (MoA) will improve understanding of how the NA structure relates to mutagenic potential.

While NAs are potent *in vivo* mutagens, metabolism *in vitro* is generally less efficient. Hence, we first optimized a hamster S9-based protocol for *in vitro* use that used a short exposure period with enhanced cofactor mix. Next, we assessed the genotoxic potential of 8 different NAs to which humans are commonly exposed using the ToxTracker assay. Seven of the tested NAs were classified as genotoxic by ToxTracker in the presence of hamster S9 with a clastogenic MoA. Benchmark dose (BMD) analysis showed that larger NAs were more potent than smaller NAs.

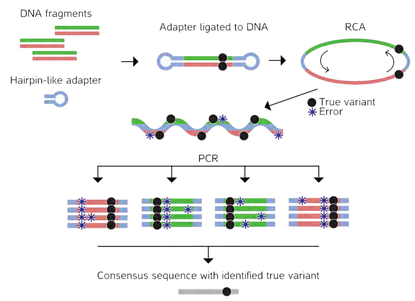
Next, we investigated the mutagenic potential of NAs by subjecting N-nitrosodimethylamine (NDMA) and N-nitrosofluoxetine (NF), a nitrosamine-derived substance related impurity (NDSRI), to single-molecule mutation sequencing (SMM-seq). NDMA induced the mutation frequency 4-fold with mostly C>T mutations and NF showed a minor increase in mutations. N-ethyl-n-nitrosourea (ENU), was subjected to SMM-seq to determine the mutagenic potential and mutation spectrum and show functionality of this new form of error-corrected sequencing (ecSeq).

The ToxTracker® assay



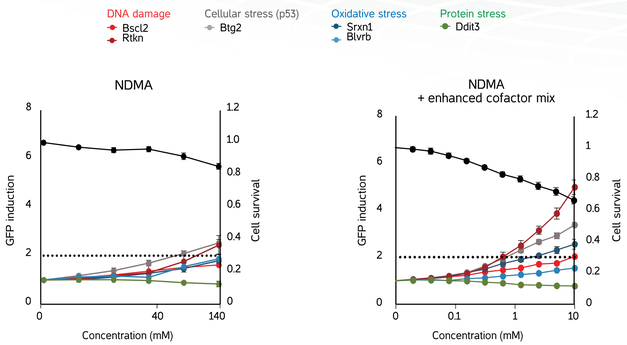
Genotoxic and non-genotoxic endpoints in the ToxTracker assay. ToxTracker combines six fluorescent reporter genes that are specifically activated by different cellular signaling responses associated with genotoxicity. ToxTracker uses mouse embryonic stem cells and activation of the different GFP-reporters is determined by flow cytometry 24 hours after exposure initiation.

Single-molecule mutation sequencing



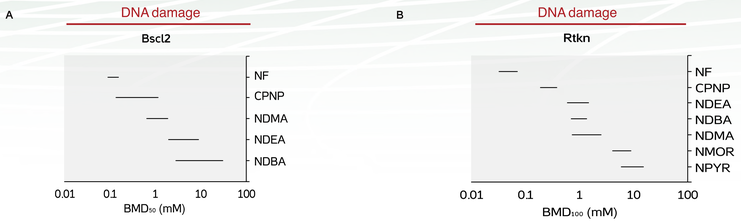
Single-molecule mutation sequencing (SMM-seq) allows for the detection of gene mutations at an ulta-low frequency. SMM-seq makes use of rolling circle amplification (RCA). DNA strands are linked together and RCA amplifies one long strand. This enables the comparison of two strands and distinguishes amplification artifacts from true mutations. Thereby we can rule out false positives with extremely high confidence.

The enhanced metabolization protocol improved prediction of genotoxicity for NDMA



The efficiency of *in vitro* metabolic activation of N-nitrosodimethylamine (NDMA) and subsequent genotoxicity was strongly enhanced when using hamster S9 liver fraction and enhanced cofactor mix. GFP reporter cells were incubated for 30 min in 10% hamster S9, cofactor mix, and NDMA followed by a 4h exposure to 3% hamster S9, cofactor mix and NDMA. Compound was removed and fresh medium was added. GFP reporter induction in intact cells was determined by flow cytometry 24 hours after exposure initiation. Dashed lines indicate threshold for a positive response (2-fold). The relative cell survival was determined by cell count using flow cytometry.

Larger N-nitrosamines are more potent than smaller N-nitrosamines

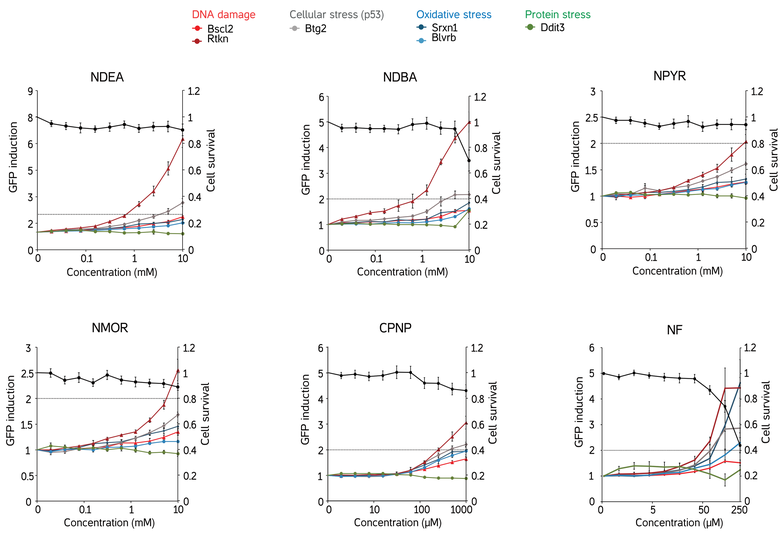


	Bscl2	
Compound	BMDL (mM)	BMDU (mM)
NF	0.0915	0.154
CPNP	0.136	1.16
NDMA	0.642	1.84
NDEA	1.98	8.9
NDBA	2.87	30.2

	Rtkn	
Compound	BMDL (mM)	BMDU (mM)
NF	0.0325	0.0715
CPNP	0.188	0.378
NDEA	0.598	1.48
NDBA	0.709	1.37
NDMA	0.738	2.5
NMOR	4.08	8.95
NPYR	5.86	15

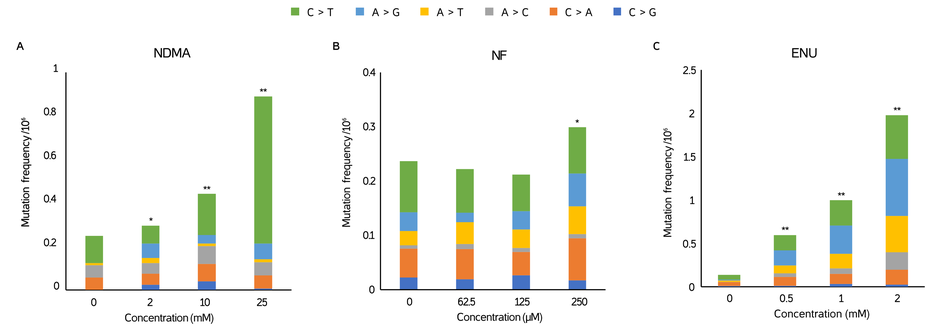
Overview of potency ranking for NAs that induced the DNA damage reporters Bscl2 and Rtkn. (A) Quantitative confidence limits of benchmark doses (BMDs) are presented for the NAs that induced Bscl2 at least 1.5-fold compared to vehicle. Three experimental replicates per compound were analysed using PROAST software. A BMR/CES of 0.5 was used. The figure (top) shows potency ranking of N-nitrosamines with the most potent NA at the top. The table (bottom) shows the lower (BMDL) and upper (BMDU) limits of the BMD confidence interval in mM with the most potent NA at the top. (B) As A but for activation of the Rtkn reporter. A BMR/CES of 1 was used to represent the 2-fold positive response criteria for GFP induction.

ToxTracker predicts the genotoxic mode of action of N-nitrosamines



Exposure to NDEA, NDBA, NPYR, NMOR, CPNP, and NF in the presence of S9 results in DNA damage reporter activation indicating genotoxicity. GFP-reporter cells were exposed for 4h to increasing concentrations of each NA in the presence of hamster S9 and enhanced cofactor mix. GFP reporter induction in intact cells was determined by flow cytometry 24 hours after exposure initiation. Dashed lines indicate threshold for a positive response (2-fold). The relative cell survival was determined by cell counts using flow cytometry.

Clastogens increased the mutation frequency with distinct mutation spectra



Induction of gene mutations and mutation spectra following exposure to NDMA, NF, and ENU detected by SMM-seq. ToxTracker cells were exposed to increasing concentrations of NDMA (+ hamster S9 and enhanced cofactor mix), NF (+ hamster S9 and enhanced cofactor mix), and ENU for 4h or 24h. Exposed cells were maintained for two passages to fix mutations before isolating DNA, library preparation, and SMM-seq. The mutation frequency for each substance was calculated as the single nucleotide variant (SNV) count divided by the total number of duplex bases analyzed. Mutation frequencies are represented as SNVs per 1M bases. A threshold for increased MF was determined based on the average MF for all vehicles + 1xSD (*) or 2xSD (**). The frequency per mutation type is shown for each concentration. The MF increased after exposure to both NAs and ENU. Exposure to NDMA mainly resulted in an increase in C>T mutations due to O^6-methylguanine. Exposure to NF mostly induced A>G mutations. For ENU, predominantly C>T, A>G, and A>T substitutions were observed.

Summary

- Using the enhanced cofactor mix for metabolization strongly improved detection of NDMA-induced genotoxicity by ToxTracker.
- NDMA, NDEA, NDBA, NPYR, NMOR, CPNP, and NF were classified as genotoxic using ToxTracker.
- Larger NAs appear more potent than smaller NAs when inducing DNA damage reporters Bscl2 and Rtkn.
- NDMA and NF increased mutation frequency in mES cells. NDMA mostly induced C>T mutations, which fits with its known MoA (O6-meG adducts).
- Overall, the enhanced metabolization protocol facilitates the use of ToxTracker, a mammalian cell assay, and SMM-seq to evaluate genotoxic and mutagenic potential of N-nitrosamines.