MutaTracker, a new approach method to measure gene mutations using error-corrected NGS to gain understanding of the genotoxic mode of action

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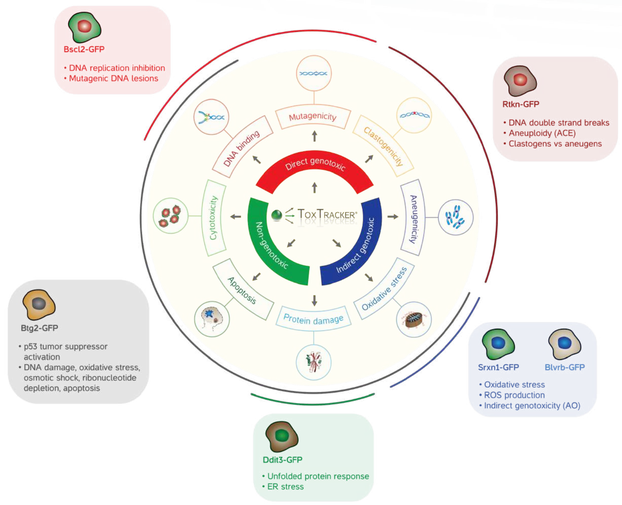
Introduction

Evaluating genotoxicity is a crucial part of chemical risk assessment and includes tests for the induction of gene mutations, chromosomal aberrations, and numerical chromosomal changes. Traditionally, mutagenicity is measured as a result of phenotypic changes but this underestimates the true mutation frequency (MF). A sequencing approach facilitates the detection of all mutations but historically suffered from poor precision due to inherent amplification errors when attempting to quantify < 1 x 10^{-7} mutations/kb.

However, with the introduction of error-corrected sequencing (ecSeq) methods, it is now possible to resolve ultra-low MFs. Single-molecule mutation sequencing (SMM-seq) is a highly sensitive technique for detecting single nucleotide variants (SNVs). SMM-seq utilizes Rolling Circle Amplification (RCA), which amplifies linked strands of each DNA fragment into a concatenated single-stranded DNA product. These ssDNA contigs act as a template for the sequencing library. This allows for the direct comparison of multiple copies of replicated strands, reliably distinguishing genuine mutations from sequencing artifacts.

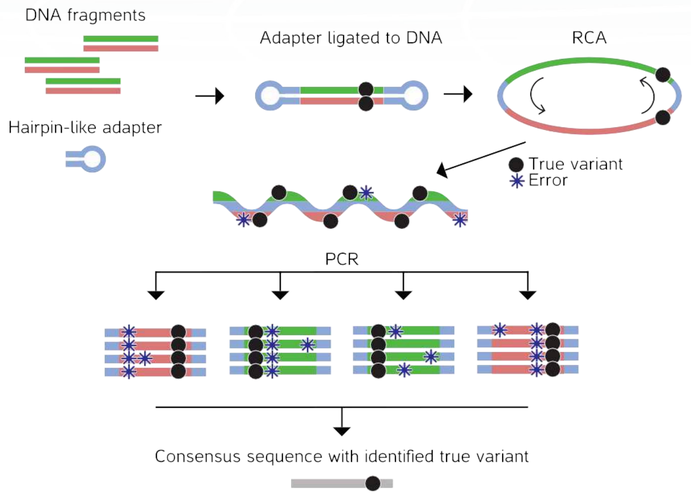
ToxTracker is a new approach methodology (NAM) that evaluates the induction of pathway-specific GFP-reporter genes to discriminate between primary and secondary genotoxicity, thereby distinguishing the likelihood of DNA reactivity. Specifically, the assay identifies secondary genotoxicity caused by oxidative stress or protein damage and can distinguish aneugens from clastogens. Here, we combined ToxTracker with SMM-seq to evaluate the genotoxic and mutagenic mode of action of nine substances.

The ToxTracker® assay



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

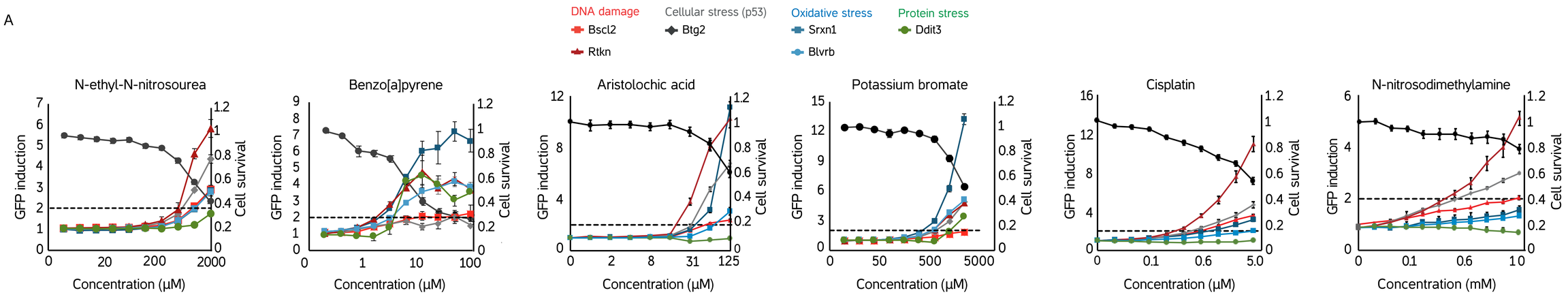
Single-molecule mutation Sequencing



Single-molecule mutation sequencing (SMM-seq) allows for the detection of gene mutations at an ultra-low frequency. SMM-seq utilizes 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.

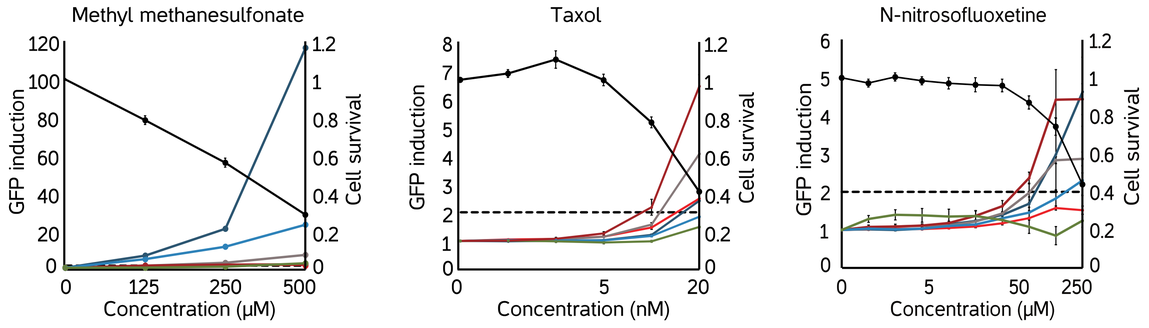
ToxTracker predicts the genotoxic mode of action

A



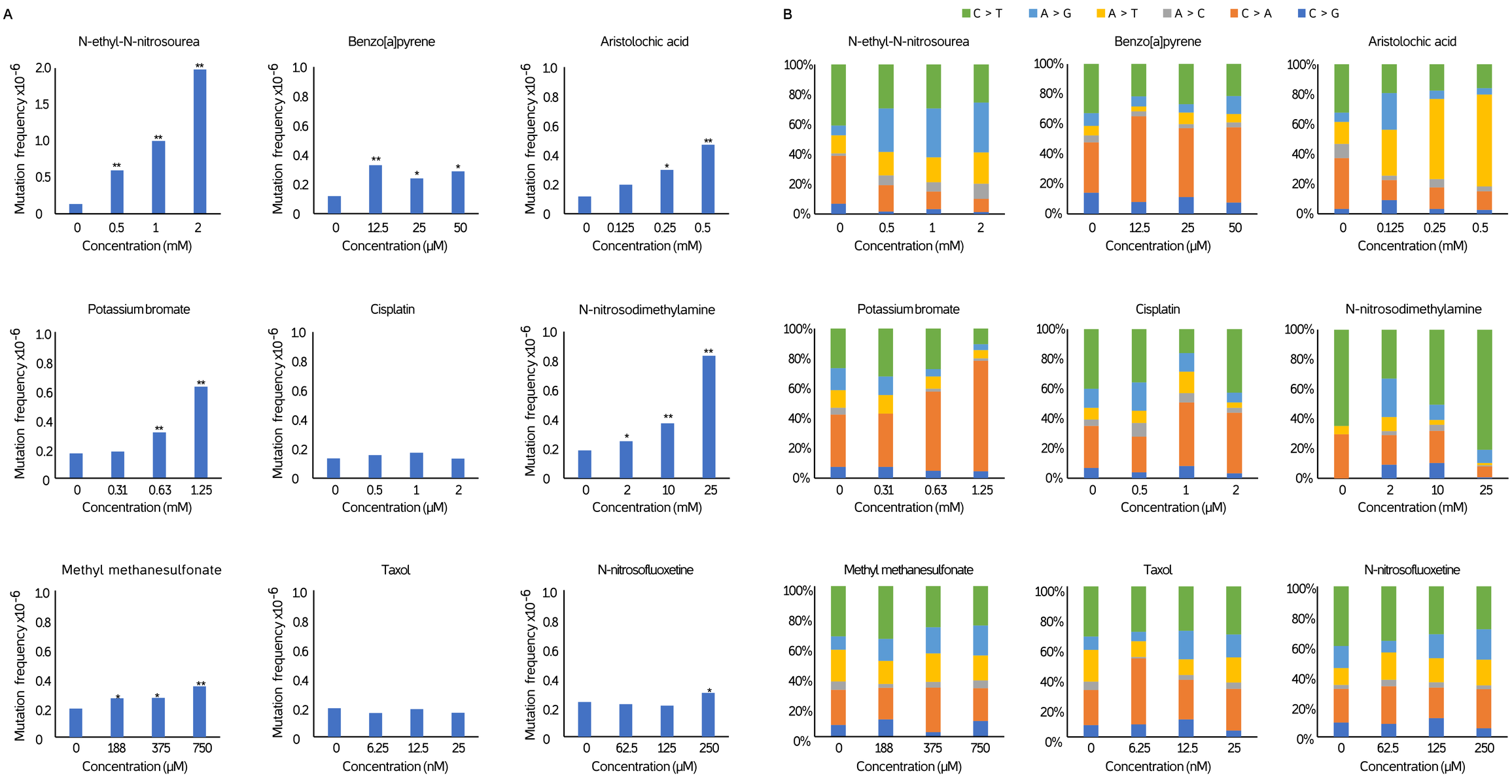
B

Compound		CAS number	*In vitro* Ames	MLA/HPRT	MN-vit	CA-vit	*In vivo* MN	CA	TgR
N-ethyl-N-nitrosourea	ENU	759-73-9	P	P	P	P	P	P	P
Benzo[a]pyrene	B[a]P	50-32-8	P	P	P	P	P	P	P
Aristolochic acid	AA	313-67-7	P	P	P	P	P	P	P
Potassium bromate	$KBrO_3$	7758-01-0	P	P	P	P	P		
Cisplatin	CisPt	624-18-0	P	P	P	P	P	P	P
N-nitrosodimethylamine	NDMA	62-75-9	P	P	P	P	P		P
Taxol	Taxol	33069-62-4	N	P	P	P	P	P	
N-nitrosofluoxetine	NF	150494-06-7	P						
Methyl methanesulfonate	MMS	66-27-3	P	P	P	P	P	P	P



ENU, B[a]P, AA, CisPt, NDMA, and MMS are directly genotoxic, $KBrO_3$ is indirectly genotoxic due to oxidative stress, taxol is an aneugen, and NF is genotoxic. (A) GFP-reporter cells were exposed to increasing concentrations of ENU, B[a]P (in the presence of rat S9), AA, $KBrO_3$, CisPt, MMS, and taxol for 24h or to NDMA and NF (in the presence of hamster S9 and enhanced cofactor mix) for 4h. GFP induction in intact cells was determined by flow cytometry and plotted on the primary y-axis. The relative cell survival was determined by cell counts using flow cytometry and plotted on the secondary y-axis. Dashed lines indicate the threshold for a positive response (2-fold). (B) An overview of public genotoxicity data for the tested substances in various *in vitro* and *in vivo* assays.

Mutational signatures reveal the mode of action of genotoxic compounds



Induction of gene mutations following exposure to various (genotoxic) compounds detected by SMM-seq. ToxTracker cells were exposed to increasing concentrations of ENU, B[a]P (+S9), AA, $KBrO_3$, CisPt, NDMA (+hamster S9 and enhanced cofactor mix), MMS, taxol, and NF (+hamster S9 and enhanced cofactor mix) for 24h or 4h. Exposed cells were maintained for two passages to fix mutations before isolating DNA, library preparation, and single-molecule mutation sequencing. (A) 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 MF increased after exposure to ENU, B[a]P, AA, $KBrO_3$, NDMA, MMS, and NF but not CisPt and taxol. (B) The base substitution spectra show the substitution type for each treatment. For ENU, predominantly A>G and A>T substitutions were observed. B[a]P caused C>A mutations. AA mainly induced A>T transversions. Exposure to $KBrO_3$ mainly resulted in an increase in C>A mutations, often observed for oxidative stress-related 8-Oxoguanine DNA damage. Exposure to NDMA mainly resulted in an increase in C>T mutations due to O^6-methylguanine. Exposure to MMS and NF mostly induced A>G mutations. Exposure to CisPt and taxol did not induce gene mutations.