A framework for interpreting *in vitro* genotoxicity data: Using mechanistic data to interpret positive results

PETA SCIENCE CONSORTIUM INTERNATIONAL e.V.
Advancing 21st Century Toxicology

Paula Braun,[1] Paul Fowler,[2] Giel Hendriks,[3] Gilly Stoddart[1]

[1]PETA Science Consortium International e.V., Germany
[2]FStox consulting, England
[3]Toxys, The Netherlands

toxys®
FStox consulting

Introduction

The prediction of genotoxic hazard to humans usually follows a stepwise approach, beginning with an *in vitro* battery consisting of a gene mutation test in bacteria (OECD TG 471), and an *in vitro* test for chromosomal damage (OECD TG 487), depending on specific regulations this may also include a gene mutation test in cultured mammalian cells (OECD TGs 476, 490).[1] Depending on the results and regulatory requirements, the *in vitro* battery may be followed up by *in vivo* testing.

DNA or chromosome damage is often misidentified by *in vitro* tests and as positive responses can trigger *in vivo* follow-up tests, it is important to understand the mechanism driving any positive responses *in vitro*.[2,3]

To better interpret *in vitro* genotoxicity results, it has been suggested that all available information including *in silico* and *in vitro* data should be considered in a weight of evidence approach.[4] Clarification of the mechanism of action (MoA) of the test chemicals proves particularly valuable for decision-making.

This poster presents case studies for vincristine, curcumin and p-nitrophenol that demonstrate how MoA assessment using non-animal methods such as the ToxTracker system[5,6] can explain positive *in vitro* results, as well as mitigate those that are incorrectly identified as genotoxic, thereby potentially avoiding *in vivo* follow-up testing.

A framework for interpreting *in vitro* genotoxicity data

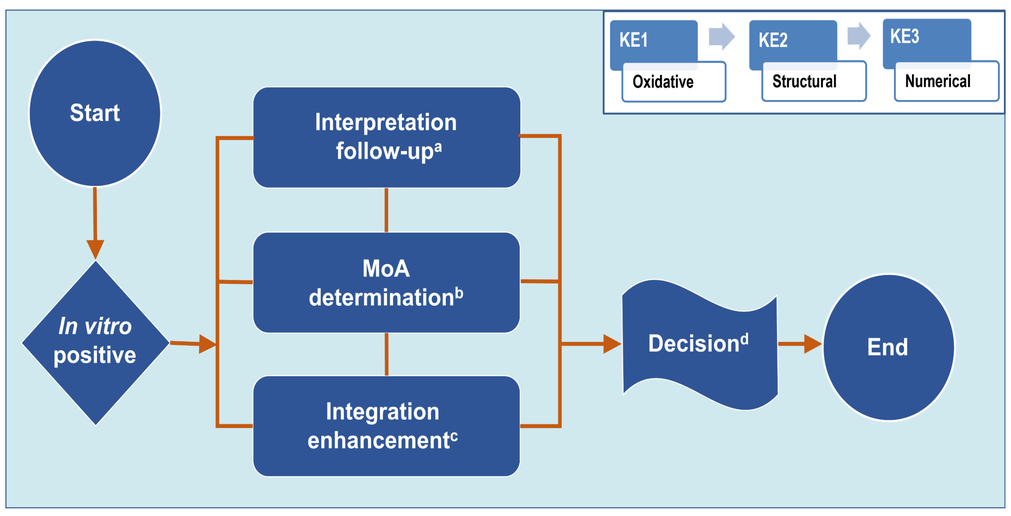


Figure 1. Framework to clarify *in vitro* positive genotoxicity results (modified after Dearfield et al, 2017).[4] [a]Analysis of all available data e.g., *in silico*, metabolic and/or kinetic modelling. [b]Use of fit-for-purpose non-animal methods (see Table 1). [c]Amplification of identified MoA by integrating data in AOPs and IATAs.[7] [d]Conclusion by combining all data on genotoxicity. In case of inconclusive result, repeat workflow to fill existing gaps. The insert depicts the key events addressed by the MoA determination by the ToxTracker system.

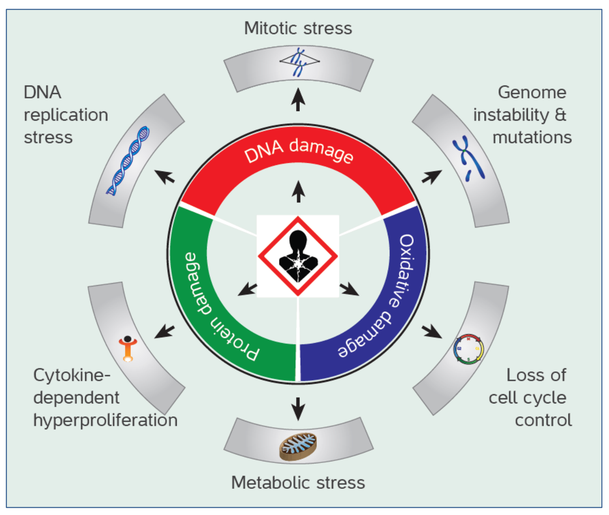


Figure 2. Insight into Mode-of-Action using ToxTracker. A panel of 6 reporter cell lines reveals activation of specific signalling pathways upon exposure to a test compound. ToxTracker discriminates between induction of direct DNA damage, oxidative stress and protein damage.

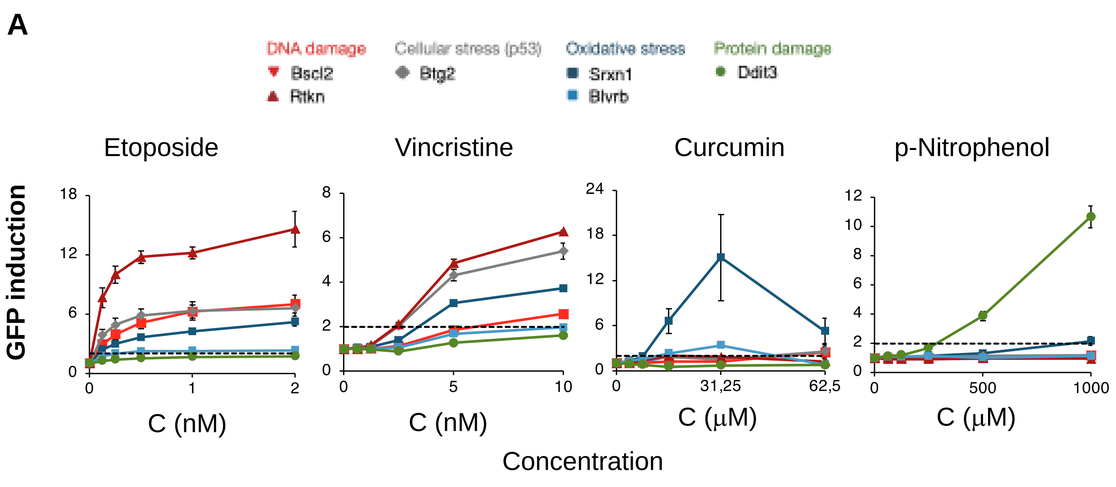
Non-animal method	Principle	Sensitivity/Specificity (%)	Indicator cell	Number & source compounds
RS skin comet	DNA strand break	77/88	Phenion (Henkel) FT	32; Cosmetic Master list
RS skin MN	MN formation	75/84	MatTek Epi-200™	47; Coded chemicals
ToxTracker	GFP-Biomarker	95/94	mouse ES	59; ECVAM list
TGxDDI	Transcription biomarker	83/100	TK6	45; FDA or literature
γH2AX	DNA strand break	95/88	HepG2 and TK6	329
In vitro comet	DNA strand break	88/64	Different cell lines	95; IARC, NTP, retrosp. Anal.
Transgenic reporter assay	Transgenic reporter	71/100	Lung epithel FE1	25; ECVAM list

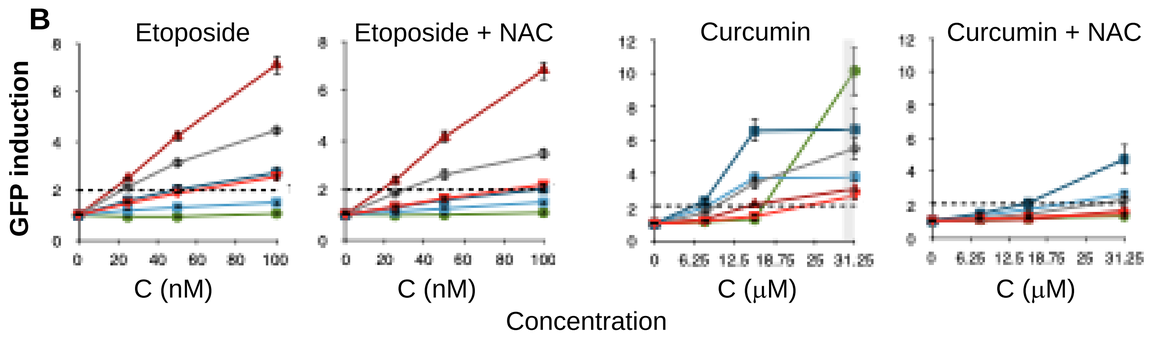
Table 1. Predictive value of non-animal methods for genotoxic MoA determination. Selected studies were chosen based on the best validation result (not shown).[8]

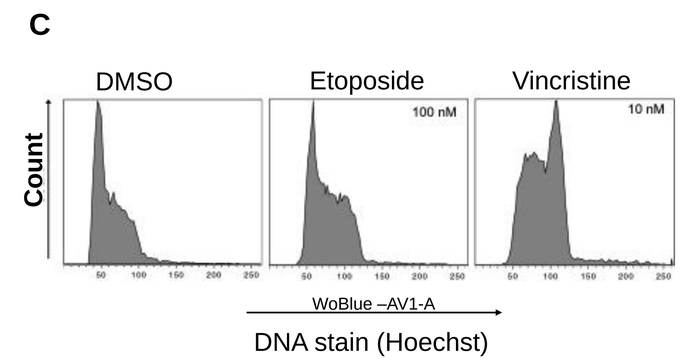
Case studies

Table 2 Genotoxicity assessment by the standard test battery of *in vitro* and *in vivo* genotoxicity assays for topoisomerase II poison etoposide, tubulin poison vincristine, antioxidant curcumin and agrochemical p-nitrophenol.

Assay		*In vitro*				*In vivo*		
Compound	CAS number	Ames	MLA	MN	CA	MN	CA	TGR
Etoposide	33419-42-0	P	P	P	P	P	P	N
Vincristine	57-22-7	N	P	P	P	P	P	
Curcumin	458-37-7	N		P		N		
p-Nitrophenol	100-02-7	N		E	P	N		







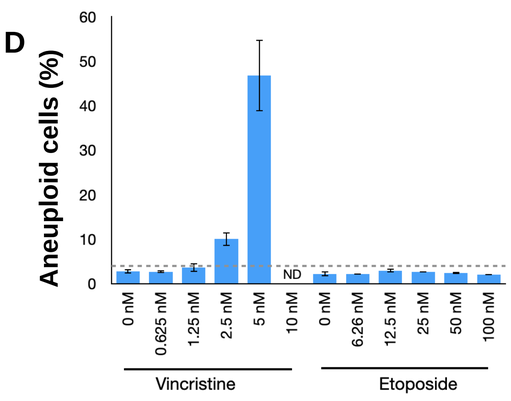


Figure 3. Mode of Action assessment using the ToxTracker
A. Activation of the GFP-biomarker upon exposure to the four test compounds (Table 2).
B. ToxTracker GFP reporter cells were exposed to increasing concentrations of etoposide or curcumin in absence and presence of the ROS scavenger N-Acetyl-L-cysteine (NAC, 10 mM). GFP induction levels in intact cells were determined by flow cytometry at 24 h after initiation of the exposure. The dashed line marks the threshold of positive induction.
C. Cell cycle analysis using Hoechst as a DNA stain after 4 h.
D. Quantification of the percentage of aneuploid cells after 24 h. A threshold of 4% aneuploid cells was selected based on control data (average + 2xSD).

Discussion and Conclusions

- ToxTracker GFP biomarkers are highly predictive of genotoxic, oxidative and protein damaging agents (Fig. 3A). ToxTracker AO identifies indirect genotoxic effects due to oxidative stress (Fig. 3B). ToxTracker ACE distinguishes between a clastogenic and an aneugenic MoA (Fig. 3C and D).
- The assignment of the compounds curcumin to the oxidative MoA and p-nitrophenol to the protein-damaging MoA eliminates the genotoxicty concerns raised by the positive and equivocal results of the *in vitro (*and *in vivo)* MN assay.

ToxTracker provides valuable MoA data that enables clarification of genotoxic potential for curcumin and p-nitrophenol. For curcumin, effects seen *in vitro* are from ROS which is better tolerated *in vivo* and in the case of p-nitrophenol a non genotoxic MoA may have led to the positive *in vitro*. Conversely, genotoxicity is predicted with very high sensitivity and specificity for materials with a well defined aneugenic (vincristine) and clastogenic (etoposide) MoA.

Investigation of MoA has great potential to clarify positive *in vitro* genotoxicity test results, thereby reducing the need for *in vivo* follow-up testing.

References

1. Chapter R.7a: Endpoint Specific Guidance Version 6.0-Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a: Endpoint Specific Guidance." 2017.
2. Kirkland, David et al. 2007. Mutation Research 628:31–55.
3. Fowler, Paul et al. 2012.. Mutation research 747:104–17.
4. Dearfield, Kerry L. et al. 2017. Environmental and molecular mutagenesis 58:264–83.
5. Brandsma, Inger et al. 2020. Toxicological Sciences, 177:202–213.
6. Hendriks, Giel., et al. 2016. Toxicological Sciences, 150:190–203.
7. Sasaki, Jennifer C. et al. 2020. Environmental and Molecular Mutagenesis 61:114–34.
8. Mišík, M., et al. 2022. Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 881.

The views, conclusions and recommendations are those of the authors and do not necessarily represent the policies or positions of the organisations to which the authors are affiliated.

Abbreviations

AOP	Adverse outcome pathway
AO	Adverse outcome
CA	Chromosome aberration test
EURL ECVAM	EU Reference Laboratory for alternatives to animal testing
FDA	Food and Drug Administration
GFP	Green Fluorescence Protein
IARC	International Agency for Research on Cancer
IATA	Integrated approach to testing and assessment
MLA	Mouse Lymphoma Assay
MoA	Mode of Action
MN	Micronucleus
NTP	National Toxicology Program
RS	Reconstructed Skin
ROS	Reactive Oxygen Species
TG	Test Guideline
TgR	Transgenic Rodent Assay
TGxDDI	Toxicogenomics x DNA damage inducing