Mechanistically informed *in vitro* genotoxicity model for complex molecules: *NAMs* to prevent unnecessary *in vivo* follow-up tests in Regulatory field

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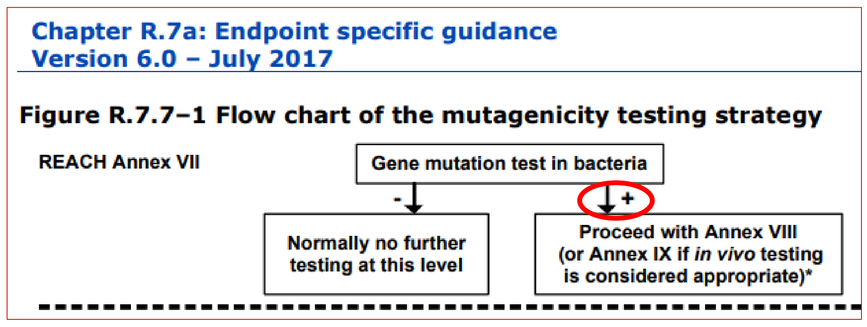
SCAN TO HAVE ME IN DIGITAL FORM

INTRODUCTION

Currently, under **REACH (EC 1907/2006)**, a positive result in a bacteria *in vitro* genotoxicity test triggers mandatory *in vivo* follow-up studies



Synthetic organic dyes frequently test **positive in the Ames test (OECD TG 471)**, yet the underlying mechanisms remain unclear

356	156	≈92%
dyes in the reference database	positive in the Ames test	**NOT genotoxic / carcinogenic in vivo**

follow-up in vivo tests show ≈92% of these dyes are not genotoxic

AIM: Gather *in vitro* evidence before *in vivo* testing

It is necessary to include **mechanistically informative, human-relevant *in vitro* approaches** that elucidate genotoxicity pathways, optimise *in vivo* follow-up decisions, and advance non-animal methods

KEY POINTS OF THE PROPOSED ALTERNATIVE STRATEGY

- **ToxTracker assay:** High-throughput *in vitro* reporter-gene assay (mES cells, 6 GFP biomarkers) providing **qualitative genotox data & Mode of Action (MoA)** information.
- **Pilot study:** 18 substances with *in vivo* data for assay performance evaluation and generation of an internal reference dataset
- **MoA dataset:** correlation of reporter-gene activation patterns with positive/negative *in vitro* and *in vivo* results + biological data for a scientifically valid Read-Across approach.
- *In vivo* tests: **reserved as a last resort confirmatory studies (Article 13 (1) of the EU REACH Reg. No. 1907/2006)**

1. Ames test positive → *true or false?* (OECD TG 471)
2. ToxTracker assay used to reliably evaluate the genotoxicity of compounds before conducting *in vivo* tests
3. ToxTracker assay to obtain MoA data and perform mechanistic mapping of complex molecules
4. Grouping & Read-Across with biological data from ToxTracker
5. Reduce & Replace *in vivo* testing

RESULTS

≈78%	100%	MoA
concordance with the *in vivo* Comet assay	sensitivity (no false negatives)	oxidative stress identified as main driver

	in vitro bacteria	*in vitro* mammalian			*in vitro* mammalian	*in vivo*		
Ref Comp.	AMES	OECD 476/490	OECD 487	OECD 473	Tox Tracker	OECD 489	OECD 474	OECD 486
Substance 1	+	NA	NA	NA	+	+	-	NA
Substance 2	+	NA	NA	+	+	+	-	NA
Substance 3	+	NA	NA	+	+	-	+	NA
Substance 4	+	-	NA	+	+	-	NA	NA
Substance 5	+	NA	-	NA	+	-	NA	NA
Substance 6	+	-	NA	+	-	-	-	NA
Substance 7	+	-	-	NA	-	-	-	NA
Substance 8	+	+	-	NA	-	-	NA	NA
Substance 9	+	NA	NA	NA	-	-	NA	NA
Substance 10	+	NA	-	NA	-	-	-	NA
Substance 11	+	ambiguos	NA	NA	-	-	-	NA
Substance 12	+	-	NA	NA	-	NA	NA	NA
Substance 13	+	-	NA	NA	-	-	-	-
Substance 14	+	-	NA	NA	-	-	-	-
Substance 15	+	-	NA	-	-	-	NA	NA
Substance 16	+	-	-	NA	-	-	NA	NA
Substance 17	+	-	NA	+	-	-	-	NA
Substance 18	+	-	NA	+	-	-	-	NA
Substance 19	+	-	-	+	-	-	-	NA
Substance 20	+	-	NA	-	-	-	-	-
Substance 21	+	NA	NA	NA	-	NA	-	NA
Substance 22	+	NA	NA	NA	-	NA	-	-
Substance 23	+	-	NA	NA	-	NA	-	NA
Substance 24	+	-	NA	NA	-	NA	-	-
Substance 25	-	-	NA	NA	-	NA	-	NA
Substance 26	-	-	NA	+	-	-	-	NA
Substance 27	-	-	-	NA	-	NA	NA	NA
Substance 28	-	-	NA	-	-	NA	-	NA
Substance 29	-	+	-	+	+	-	NA	NA

ToxTracker Reliably Anticipates *In Vivo* Genotoxicity Outcomes

- Most of the Ames-positive findings are **systematically negative** in ToxTracker
- ToxTracker negative: concordance in the Comet Assay

\+ Ames test → − ToxTracker → − Comet assay

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Cases demonstrate that early ToxTracker integration could have prevented *unnecessary in vivo* Comet assays by clarifying Ames false positives

ToxTracker MoA dataset

	without S9 (−S9) GENOTOXICITY Bscl2	Rtkn	Btg2	OXID. STRESS Srxn1	Blvrb	PROT. Ddit3	with S9 (+S9) GENOTOXICITY Bscl2	Rtkn	Btg2	OXID. STRESS Srxn1	Blvrb	PROT. Ddit3	GENOTOXICITY In vitro (TT)	In vivo
REACTIVE	2	3.2	3.7	6	6.2		1.9 P	2.9	4	4.8	6		Yes	Yes
REACTIVE		2.2	3	8.6	4.2			2.3	4	14	5.5		Yes	Yes
REACTIVE				3.2	3.7						2.7		No	No
REACTIVE			1.9	3.9	3.3	2.8					2.2		No	NA
REACTIVE				2.8	2.3					2.1	2.3		No	NA
REACTIVE			2.2	5.6	4.3						2.4		No	No
REACTIVE			2.6	9.8	3.7						2.1		No	No
REACTIVE			2.1	7.7	2.8	2.4							No	No
ACID				5		1.9							No	No
ACID				12.5		7							No	No
ACID													No	NA
BASIC			2.1	10	3.25	14				20		5	No	No
BASIC								1.7	2	2.8	2.5	3.3	Equiv.	No
BASIC			2.3	6.9		5.4		2.9	2.6	7 P	2	3.5	Yes	TPE
BASIC				5.7		6.2		2.5	2.1	5.8		4.5	Yes	TPE
BASIC													No	NA
DIRECT													No	No
DIRECT						2.1							No	NA
DISPERSE		2.1	2.4	5.9	3	3.37			2.3	3	2.8		Yes	No
DISPERSE													No	NA
SOLVENT													No	NA
VAT													No	NA
LEUCO				5.7	4.2	3.7				3.1	2.2		No	No

Fold induction (GFP reporter): 2 … >=10

weak / equivocal · negative · P = with precipitation · **TPE** = Testing Proposal

Reactive & Disperse Dyes:
- Functional group influences the direct or indirect MoA;
- Oxidative stress inducer;
- Metabolic activation (S9) ↓ activity

Basic Dyes:
- metabolic activation (S9) ↑ activity
- Oxidative stress inducer

Acid & Leuco Dyes:
- Generally not genotoxic;
- Oxidative stress inducer;
- Metabolic activation (S9) ↓ activity

Direct, Solvent, Vat Dyes:
Generally not genotoxic & no induction of any reporter genes

ETHICAL IMPACT: Safety with fewer animals

Without ToxTracker — all dyes to *in vivo*

> 3,000 animals

With ToxTracker strategy

≈ 780 animals

↓ up to ~80% reduction in animal use

> 2,000 animals avoided

51 ToxTracker-negative → 0 animals

14 positives → ≈ 780 animals (confirmatory only)

CONCLUSIONS: Move to mechanistically informative, human-relevant *in vitro* approaches

Reliable first screen

ToxTracker is a robust, high-throughput mechanistic assay providing MoA information and improving prediction of *in vivo* genotoxicity (soon OECD TG available!)

Smarter testing strategy

Integrated as an intermediate test between the Ames test and *in vivo* studies, it strengthens weight-of-evidence evaluations.

Fewer animal tests

Directly supports EU Commission and REACH's objective of reducing animal use, advancing regulatory acceptance of non-animal methods (3Rs).