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The Vitotox and ToxTracker assays: A two-test combination for quick and reliable assessment of genotoxic hazards

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ABSTRACT

To ensure safety for humans, it is essential to characterize the genotoxic potential of new chemical entities, such as pharmaceutical and cosmetic substances. In a first tier, a battery of *in vitro* tests is recommended by international regulatory agencies. However, these tests suffer from inadequate specificity: compounds may be wrongly categorized as genotoxic, resulting in unnecessary, time-consuming, and expensive *in vivo* follow-up testing. In the last decade, novel assays (notably, reporter-based assays) have been developed in an attempt to overcome these drawbacks. Here, we have investigated the performance of two *in vitro* reporter-based assays, Vitotox and ToxTracker. A set of reference compounds was selected to span a variety of mechanisms of genotoxic action and applicability domains (*e.g.*, pharmaceutical and cosmetic ingredients). Combining the performance of the two assays, we achieved 93% sensitivity and 79% specificity for prediction of gentoxicity for this set of compounds. Both assays permit quick high-throughput analysis of drug candidates, while requiring only small quantities of the test substances. Our study shows that these two assays, when combined, can be a reliable method for assessment of genotoxicity hazard.

1. Introduction

Genotoxicity is an indispensable endpoint for assessment of risk to humans, before new chemicals reach the market [1]. To characterize potential genotoxic effects, regulatory agencies usually accept the results of a battery of validated *in vitro* genotoxicity tests which cover gene mutations and both numerical and structural chromosome damage [2]. The sensitivity (ability to identify established genotoxic chemicals or "true positives") of these tests is high, but at the cost of limited specificity (ability to identify established non-genotoxic chemicals or "true negatives") [3,4]. Measures have been taken to improve the performance of these tests, but they remain relatively slow, laborious, and require large amounts of chemicals [5,6]. Industry is therefore seeking assays that could improve lead selection and early safety screening of novel drug candidates and cosmetics at both high sensitivity and high specificity.

Reporter-based assays rely on genetically engineered reporter strains consisting of a promoter of a gene known to be activated by genotoxic assault, fused with one or more reporter genes to facilitate measurement of the response. Most of these assays allow mechanism-based screening [7,8] and therefore might also permit discrimination between true and false positives. By combining a number of these reporter-based assays, several mechanisms of genotoxicity can be covered, which might lead to an overall improvement in genotoxicity assessment.

Here, we assess the ability of a combination of two commercially available reporter-based assays, the Vitotox and ToxTracker assays, to identify genotoxicity.

We selected 15 well-established genotoxic compounds, acting through several different mechanisms. Since "mechanism of genotoxic action" is irrelevant for non-genotoxic compounds, 15

Abbreviations: B[*a*]P, benzo[*a*]pyrene; Blvrb, biliverdin reductase B; Bscl2, berardinelli-seip congenital lipodystrophy 2; Btg2, B-cell translocation gene 2; CHF, chloramphenicol; CYC, cyclophosphamide; DAT, 2,4-diaminotoluene; Ddit3, DNA damage inducible transcript 3; GFP, green fluorescent protein; HBM, hydroxybenzomorpholine; mES, mouse embryonic stem cell; MIT, methylisothiazolinone; NAP, 1-naphthol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PBS, phosphate-buffered saline; Rtkn, rhotekin; S/N, signal-to-noise ratio; SCCS, Scientific Committee on Consumer Safety; Srxn1, sulfiredoxin 1; VIN, vinblastine.

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Table 1
In *vivo* genotoxic (GTX) compounds used as reference substances (micronucleus (MN); chromosome aberration (CA)) [31,32].

Abbreviation	Compound	CAS number	*In vitro* genotoxicity		*In vivo* genotoxicity	Main mechanism of action	Tested dose range Vitotox (mM)	Tested dose range ToxTracker (mM)
			Ames test	MN/CA test				
2NF	2-nitrofluorene	607-57-8	POS [14]	POS [14]	POS [14]	Bulky adduct formation [14]	0.015-1.5	0.094-1.5
AFB	Aflatoxin B1	1162-65-8	POS [14]	POS [14]	POS [14]	Bulky adduct formation [14]	0.00032-0.032	0.00063-0.01
B[α]P	Benzo[α]pyrene	50-32-8	POS [14]	POS[14]	POS [14]	Polycyclic aromatic hydrocarbon, bulky adduct formation [14]	0.004-0.4	0.0156-0.25
BLE	Bleomycin sulfate	9041-93-4	POS [31,32]	POS [31,32]	POS [31,32]	Radical generator causing DNA strand breaks (radiomimetic) [31,32]	0.0033-0.33	1.25-20
$CdCl_2$	Cadmium chloride	10108-64-2	NEG [14]	POS [14]	POS [14]	DNA repair inactivator, cell cycle inducer, p53 inhibitor [14]	0.01-1	0.0025-0.02
CHF	Chloramphenicol	56-75-7	NEG [14]	POS [14]	POS [14]	Clastogen that binds to DNA [14]	0.000045-0.0045	0.0625-1
CIS	Cisplatin	15663-27-1	POS [14]	POS [14]	POS [14]	Cross-linking agent [14]	0.01-1	0.00063-0.01
CYC	Cyclophosphamide	50-18-0	POS [14]	POS[14]	POS [14]	O6 and N7 alkylator (alkylating agent) [14]	0.1-10	0.006-0.1
DAT	2,4-diaminotoluene	95-80-7	POS [14]	POS [14]	POS [14]	Aromatic amine, DNA adduct formation [14]	0.05-5	0.313-2.5
ENU	1-ethyl-1-nitrosourea	759-73-9	POS [14]	POS [14]	POS [14]	O6 and N7 alkylator (alkylating agent) [14]	0.1-10	0.0125-0.2
ETO	Etoposide	33419-42-0	POS[14]	POS [14]	POS [14]	Topoisomerase II inhibitor [14]	0.01-1	0.00013-0.002
MMS	Methyl methanesulphonate	66-27-3	POS [14]	POS [14]	POS [14]	N7 alkylation, replication fork impairment [14]	0.01-1	0.031-0.5
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone	64091-91-4	POS [14]	POS [14]	POS [14]	Bulky adduct formation [14]	0.1-10	0.625-10
VIN	Vinblastine sulfate	143-67-9	NEG [31]	POS [31]	POS [31]	Tubulin polymerization inhibitor [31]	0.004-0.4	0.00625-0.1
ZID	Azidothymidine (Zidovudine)	30516-87-1	NEG [14]	POS [14]	POS [14]	Antimetabolite, nucleoside analogue [14]	0.05-5	0.0625-1

non-genotoxic chemicals were chosen to represent various applicability domains, *e.g.*, drugs and cosmetic ingredients.

Vitotox is a high-throughput bacterial genotoxicity assay that detects genotoxic chemicals by monitoring activation of the bacterial SOS DNA-repair system. Two genetically engineered *Salmonella typhimurium* strains, TA104 recN2-4 (Genox strain) and TA104 pr1 (Cytox strain) are used. Vitotox is a rapid and simple assay thought to be useful as a "first-approach" screening assay. Extensive validation of the Vitotox assay revealed 91–96% concordance with the Ames test (recommended by regulatory agencies), but it is much faster (4–5 h *vs.* 2 days), less laborious, and requires less testing material compared to the Ames test and some other bacterial mutagenicity assays [9–11].

ToxTracker is a mouse embryonic stem cell (mES)-based *in vitro* test that uses six different green fluorescent protein (GFP) reporter cell lines to assess induction of DNA damage, oxidative stress, and protein unfolding upon chemical exposure. The genes encoding Berardinelli-Seip congenital lipodystrophy 2 (Bscl2) and rhotekin (Rtkn) proteins are biomarker genes for DNA damage; sulfiredoxin 1 (Srxn1) and biliverdin reductase B (Blvrb) detect oxidative stress; DNA damage-inducible transcript 3 (Ddit3) is a biomarker for the unfolded protein response; and B-cell translocation gene 2 (Btg2) indicates general cellular stress. As such, ToxTracker allows identification of various potentially toxic properties of a compound in a single assay [12,13]. For the purpose of this study, we focus on the two genes detecting genotoxic events, namely Bscl2 and Rtkn.

2. Materials and methods

2.1. Compound selection

To test the performance of the two *in vitro* reporter-based genotoxicity tests, 15 well-known *in vivo* genotoxicants and 15 *in vivo* non-genotoxicants were selected from various databases and peer-reviewed publications (Tables 1 and 2). The choice of compounds was based on a set of predefined criteria. For the genotoxicant group, the main prerequisites for inclusion were: (i) availability of *in vivo* genotoxicity data; (ii) availability of results of *in vitro* tests detecting gene mutations and clastogenic and aneugenic effects; (iii) diversity in the mechanisms of genotoxic action. The first two criteria were implemented to define the sensitivity and specificity of the chosen assays and the third criterion to challenge the performance of the tests when different mechanisms of genotoxicity are involved.

For the non-genotoxic group, so-called "false positive" compounds were included to challenge the Vitotox and ToxTracker assays, since low specificity (high number of false positives) is a vexing problem in the regulatory test battery [3,4]. By including these false-positive compounds in our test set, we can assess the specificity of the two assays, as is recommended by Kirkland et al. [14,15]. To select these false positive compounds, the Opinions of the European Commission's Scientific Committee on Consumer Safety (SCCS), readily available online, were consulted: ec.europa.eu/health/scientific_committees/consumer_safety/opinions/index_en.htm.

In these opinions, safety assessments of cosmetic ingredients by expert toxicologists are reported and mutagenicity/genotoxicity is one of the endpoints considered. Expert toxicologists judge whether the studies provided by the submitter(s) are of acceptable quality and have been based on accepted tests, before the safety of an ingredient is fully assessed. In the case of genotoxicity, when *in vivo* testing was still allowed in the cosmetic sector, judgements could be made by weighing *in vitro* and *in vivo* results. In some cases, when additional *in vitro* and *in vivo* tests of good quality (*e.g.*, according to OECD guidelines) showed clear negative results, a positive *in vitro* test result could be flagged as false positive. Four of these so-called false positive compounds were included in our data set, as shown in Table 4. We stress that this judgement was made by the members of the SCCS, not by us. For the non-genotoxic

Table 2
In vivo non-genotoxic compounds used as reference substances (micronucleus (MN); chromosome aberration (CA)) [33–35].

Abbreviation	Compound	CAS number	*In vitro* genotoxicity		*In vivo* genotoxicity	Applicability domain	Tested dose range Vitotox (mM)	Tested dose range ToxTracker (mM)
			Ames test	MN/CA test				
AMP	Ampicillin trihydrate	7177-48-2	NEG [14]	NEG [14]	NEG [14]	Drug/Beta-lactam antibiotic [14]	0.01-1	0.0625-1
ANP	4-amino-3-nitrophenol	610-81-1	POS [18]	POS [18]	NEG [18]	Oxidative hair dye [18]	0.01-1	0.0625-1
CAP	Caprolactam	105-60-2	NEG [33]	NEG [33]	NEG [33]	Synthetic polymer [33]	0.1-10	0.625-10
CLI	Climbazole	38083-17-9	NEG [34]	NEG [34]	NEG [34]	Drug/antifungal agent [34]	0.015-1.5	0.00625-0.1
CLO	Clonidine	4205-90-7	NEG [14]	NEG [14]	NEG [14]	Drug/α2 adrenergic agonist [14]	0.033-3.33	0.208-3.33
HBM	Hydroxybenzomorpholine	26021-57-8	POS [19]	NEG [19]	NEG [19]	Oxidative hair dye [19]	0.01-1	0.0625-1
MAN	d-mannitol	69-65-8	NEG [14]	NEG [14]	NEG [14]	Sweetener [14]	1-10	0.0625-1
MEL	Melatonin	73-31-4	NEG [35]	NEG [35]	NEG [35]	Dietary supplement [35]	0.005-0.5	0.031-0.5
MIT	Methylisothiazolinone	2682-20-4	NEG [20]	POS [20]	NEG [20]	Biocide and preservative [20]	0.00001-0.001	0.006-0.1
NaCl	Sodium chloride	7647-14-5	NEG [21]	NEG [21]	NEG [21]	Condiment/food preservative [21]	0.1-10	0.625-10
NAP	1-naphthol	90-15-3	NEG [17]	POS [17]	NEG [17]	Oxidative hair dye/ Insecticide [17]	0.005-0.5	0.025-0.4
NIF	Nifedipine	21829-25-4	NEG [14]	NEG [14]	NEG [14]	Drug/Antihypertensive, dihydropyridine calcium channel blocker [14]	0.01-1	0.019-0.3
SDF	Sodium diclofenac	15307-79-6	NEG [14]	NEG [14]	NEG [14]	Drug/Nonsteroidal anti-inflammatory drug [14]	0.05-5	0.0625-1
SOR	Sorbitol	50-70-4	NEG [21]	NEG [21]	NEG [21]	Sweetener/Sugar alcohol [21]	0.1-10	0.625-10
TOL	Tolbutamide	64-77-7	NEG [14]	NEG [14]	NEG [14]	Drug/ Hypoglycemic potassium channel blocker [14]	0.1-10	0.00625-0.1

Table 3
Overview of Vitotox and ToxTracker results, compared to the *in vitro* and *in vivo* genotoxicity results of the genotoxic compounds. CA = chromosome aberration; EQ = equivocal; MN = micronucleus; NEG = Negative result; POS = positive result.

Compound	*In vitro*		*In vivo*	Vitotox	ToxTracker
	Ames test	MN/CA test			
2NF	POS	POS	POS	NEG	NEG
AFB	POS	POS	POS	POS	POS
B[α]P	POS	POS	POS	POS	POS
BLE	POS	POS	POS	POS	POS
$CdCl_2$	NEG	POS	POS	NEG	EQ
CHF	NEG	POS	POS	NEG	EQ
CIS	POS	POS	POS	POS	POS
CYC	POS	POS	POS	NEG	POS
DAT	POS	POS	POS	POS	POS
ENU	POS	POS	POS	POS	POS
ETO	POS	POS	POS	POS	POS
MMS	POS	POS	POS	POS	POS
NNK	POS	POS	POS	POS	NEG
VIN	NEG	POS	POS	NEG	POS
ZID	NEG	POS	POS	POS	POS

Table 4
Overview of Vitotox and ToxTracker results, compared to the *in vitro* and *in vivo* genotoxicity results of the non-genotoxic compounds. CA = chromosome aberration; EQ = equivocal; MN = micronucleus; NEG = Negative result; POS = positive result.

Compound	*In vitro*		*In vivo*	Vitotox	ToxTracker
	Ames test	MN/CA test			
AMP	NEG	NEG	NEG	NEG	NEG
ANP	POS	POS	NEG	NEG	POS
CAP	NEG	NEG	NEG	NEG	NEG
CLI	NEG	NEG	NEG	NEG	NEG
CLO	NEG	NEG	NEG	NEG	NEG
HBM	POS	NEG	NEG	NEG	POS
MAN	NEG	NEG	NEG	NEG	NEG
MEL	NEG	NEG	NEG	NEG	NEG
MIT	NEG	POS	NEG	NEG	POS
NaCl	NEG	NEG	NEG	NEG	NEG
NAP	NEG	POS	NEG	NEG	EQ
NIF	NEG	NEG	NEG	NEG	NEG
SDF	NEG	NEG	NEG	NEG	NEG
SOR	NEG	NEG	NEG	NEG	NEG
TOL	NEG	NEG	NEG	NEG	NEG

group, the "mechanism of genotoxic action" criterion is obviously not relevant; the applicability domain of the compounds is added in order to ensure coverage of a broader "chemical space".

2.2. In vitro *reporter-based genotoxicity tests*

2.2.1. *Vitotox assay*

Salmonella typhimurium strains TA104 *rec*N 2–4 (Genox test strain) and TA104 *pr1* (Cytox control strain) were constructed and cultivated as described in [9] and on http://gentaur.com/toxi-vitotox.htm.

Strain checks confirmed the characteristics of *Salmonella* TA104. The overnight incubation of bacteria in suspension is performed in nutrient-rich medium (the exact composition is proprietary), in order to achieve conditions for exponential growth of the bacteria. Bacteria are then plated out at 2×10^8–2×10^9 CFUs/mL in 96-well plates. The subsequent incubation of the test compounds is carried out in nutrient-poor medium, in order to halt the growth of the bacteria and to have less variation in number of bacteria per well, once seeded.

Both the Genox and Cytox strains were exposed to three different concentrations, in 10-fold dilutions, of the test compounds, with and without the metabolic activation system (S9). The top test concentrations were chosen based on the results of the Cytox strain. The highest concentration showed a Cytox signal <0.8. The top concentrations of non-cytotoxic compounds were set at 10 mM or the solubility limit. An overview of the test concentration ranges is given in Tables 1 and 2. 4-Nitroquinoline-1-oxide (4-NQO) (no S9) and benzo[*a*]pyrene (B[*a*]P) + S9 were used as positive controls. Three independent experiments were performed per test compound. S9 mix was freshly prepared before each experiment. Genotoxicity and cytotoxicity were measured in a black 96-well plate, using a luminometer (Beckman Paradigm). Light emission was measured (1 s/well) every 5 min, during 4 h at 30 °C. It should be noted that, although the optimal temperature for the growth of the bacteria is 37 °C, during the actual measurement, 30 °C is used, as previous research has shown that the lux operon performs best at 27–32 °C [9,16].

For each measurement, the signal-to-noise ratio (S/N), *i.e.*, the light production by exposed cells divided by the light production by non-exposed cells, was calculated. A compound is considered genotoxic if: (i) the maximum S/N ratio in the Genox-strain shows a clear dose-response relationship and (ii) there is a dose-response relationship in max S/N Genox to Cytox and it has a value >1.5. Genotoxicity is excluded when (i) the S/N increases very quickly during the first 20 min of the measurement (the SOS-response takes at least 20 min to start); (ii) both strains are strongly induced, even when Genox/Cytox >1.5; (iii) the maximum S/N for the Genox strain is <1.5, even when Genox/Cytox >1.5; (iv) if S/N is rapidly decreased below 0.8, there is a toxic effect [9].

2.2.2. *ToxTracker assay*

The mES GFP reporter cell lines were generated as previously described [12,13]. The cell lines were exposed to five different concentrations in 2-fold dilutions of each chemical for 24 h in gelatin-coated 96-well plates. Exposure concentrations were determined by a viability assay in which the relative cell survival was calculated as the ratio of intact treated cells *versus* untreated cells, as determined by flow cytometry. The highest test concentration induced 50–70% cytotoxicity. For compounds that did not affect cell viability, top exposure concentration = 10 mM was used. Tested concentration ranges are presented in Tables 1 and 2.

After 24 h exposure, cells were washed with phosphate buffered saline (PBS), trypsinized and resuspended in PBS + 2% FCS, immedi-

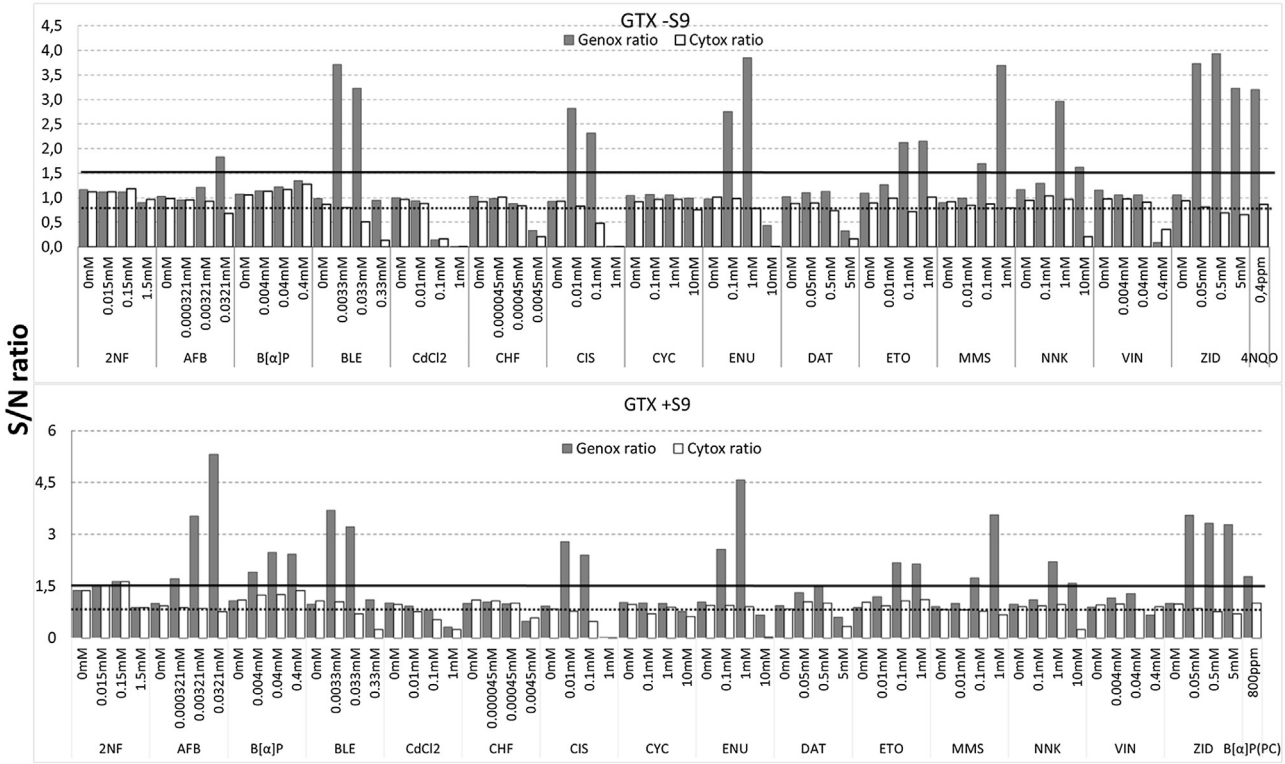

Fig. 1. Results of the Vitotox assay showing the changes of the Genox and Cytox ratios following exposure to 15 prototypical genotoxic (GTX) compounds with and without S9 (n = 3). Ratios displayed in the graph are the maximum signal-to-noise ratios per concentration, detected in a time span of 4 h. In order for a compound to be considered as positive, the Genox ratio should be above 1.5 (solid line) and the respective Cytox ratio in the range between 0.8 (dashed line) and 1.5. Additionally a dose response trend should be observed. Abbreviations: 2NF = 2-nitrofluorene; 4NQO = 4-nitroquinoline-1-oxide; AFB = aflatoxin B1; B[α]P = benzo[*a*]pyrene; BLE = bleomycin sulfate; $CdCl_2$ = cadmium chloride; CHF = chloramphenicol; CIS = cisplatin; CYC = cyclophosphamide; DAT = 2,4-diaminotoluene; ENU = 1-ethyl-1-nitrosourea ETO = etoposide; MMS = methyl methanesulphonate; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; VIN = vinblastine sulfate; ZID = azidothymidine (zidovudine).

ately followed by detection of the GFP reporters by flow cytometry (Guava easyCyte 6HT, EMD Millipore). Reporter activity was determined as the mean fluorescence intensity of 5000 intact cells. Activation of a reporter cell line was considered positive when exposure to a compound resulted in >2-fold induction of GFP expression. Compounds inducing a 1.5–2 fold change are considered as equivocal. Three independent experiments were performed per test compound. Cisplatin served as positive control for induction of DNA damage [12,13].

When auto-fluorescence was suspected, this was tested by exposing wild type mES (n = 3) to the same concentrations as used in the ToxTracker. The mean fluorescence caused by the compound was then subtracted from the ToxTracker results for the respective compound. Auto-fluorescence corrections were performed for 1-naphthol (NAP), hydroxybenzomorpholine (HBM), and 2,4-diaminotoluene (DAT).

3. Results

3.1. Vitotox performance data

Ten of the 15 *in vivo* genotoxicants were clearly identified as genotoxic within the acceptable levels of cytotoxicity; thus, sensitivity = 67% (Fig. 1). The compounds which failed to be identified as positive were: (i) vinblastin (VIN), which is known to induce its genotoxic effects *via* disruption of the mitotic spindle of the cell, a feature absent in bacteria; (ii) $CdCl_2$, a metal salt that is usually not detected by bacteria; (iii) 2-nitrofluorene (2NF), which requires nitroreductase-catalyzed metabolism to its genotoxic nitroso- or hydroxylamino- derivative; (iv) chloramphenicol (CHF), an antibiotic which presumably causes bacterial toxicity; and (v) cyclophosphamide (CYC). The limitations of the Vitotox assay seem mainly to reflect the inevitable limitations of a bacterial test system, similar to the Ames test. However, 2NF is an exception. In the Ames test, 2NF is a strong frameshift mutagen – indeed, it is a positive control for strain TA98.

The specificity of Vitotox was 100%, which implies that all 15 *in vivo* non-genotoxicants were correctly identified, including the so-called false positive compounds (Fig. 2). Although the compounds NAP, 4-amino-3-nitrophenol (ANP), HBM, and methylisothiazolinone (MIT) show *in vitro* positive results, they have been assessed by the SCCS as non-genotoxic, after additional *in vitro* and/or *in vivo* testing [17–20] and therefore are labeled as "false positive". Graphs of the responses to the individual compounds can be found in the Supplementary data.

3.2. ToxTracker performance data

As the purpose of this study is detection of genotoxicity, our focus was on the Bscl2-GFP and Rtkn-GFP biomarkers, which are controlled by the ATR/chk1 DNA replication stress and NF-kB-associated DNA damage signaling pathways, respectively. The results indicate that 13 of the 15 *in vivo* genotoxicants tested up-regulated at least one of the two genes more than 1.5-fold, corresponding to a sensitivity = 87%, when considering the equivocal results for CHF and $CdCl_2$ to be positive (Figs. 3 and 4). Leaving out those equivocal results would give sensitivity = 85% (11/13 compounds correctly predicted as positive).

To err on the side of safety, equivocal results should be considered positive. However, since further testing of equivocal compounds (in other test systems) is needed to reach a clear decision, it seems reasonable also to calculate sensitivity and specificity

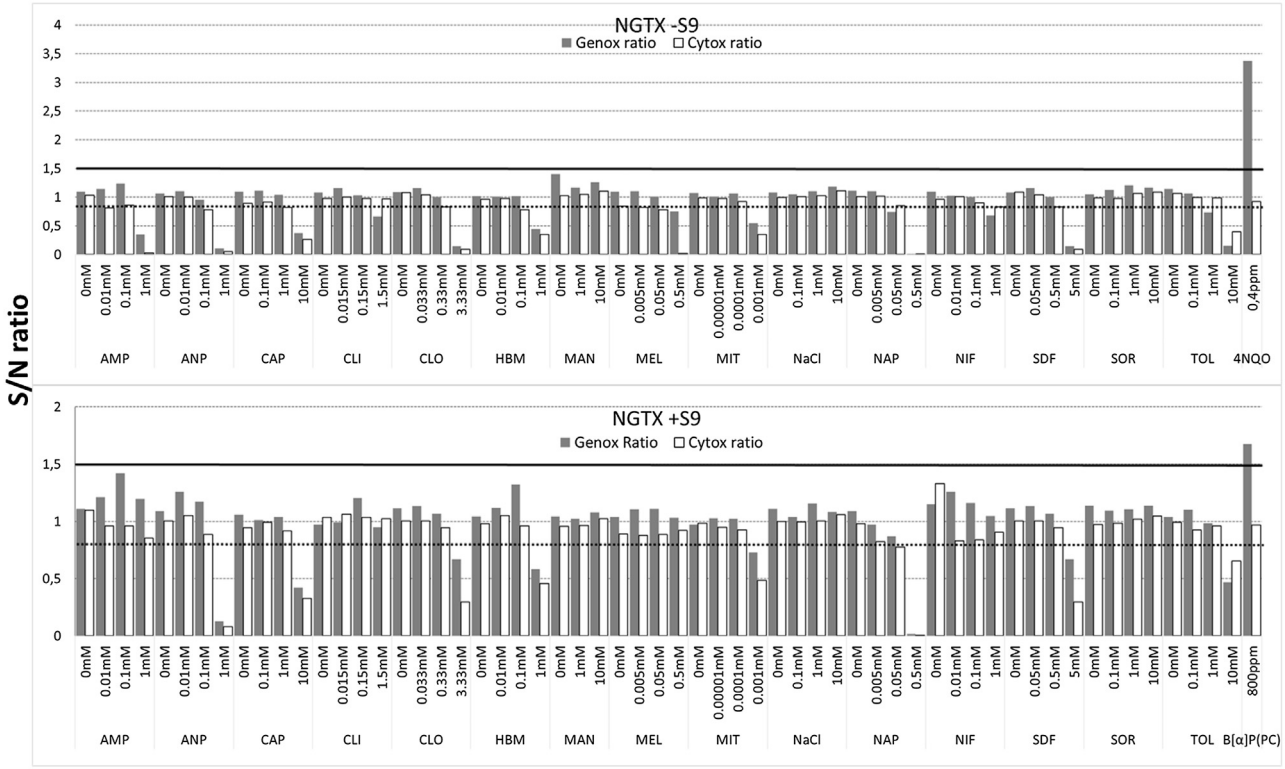

Fig. 2. Results of the Vitotox assay showing the changes of the Genox and Cytox ratios following exposure to 15 prototypical non-genotoxic (NGTX) compounds with and without S9 (n = 3). Ratios displayed in the graph are the maximum signal-to-noise ratios per concentration, detected in a time span of 4 h. In order for a compound to be considered as negative, the Genox ratio should be below 1.5 (solid line) and the respective Cytox ratio in the range between 0.8 (dashed line) and 1.5. Abbreviations: 4NQO = 4-nitroquinoline-1-oxide; AMP = ampicillin trihydrate; ANP = 4-amino-3-nitrophenol; B[α]P = benzo[α]pyrene; CAP = caprolactam; CLI = climbazole; CLO = clonidine; HBM = hydroxybenzomorpholine; MAN = d-mannitol; MEL = melatonin; MIT = methylisothiazolinone; NaCl = sodium chloride; NAP = 1-naphthol; NIF = nifedipine; SDF = sodium diclofenac; SOR = sorbitol; TOL = tolbutamide.

of the test systems omitting compounds that did not give a clear-cut answer. Therefore, we have applied both calculations in this manuscript.

The compounds that failed to induce either of the biomarker genes were 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 2NF. The specificity of the ToxTracker for this set of compounds is 73%, when considering the equivocal result for NAP to be positive. Leaving out the equivocal result would lead to a specificity of 79% (11/14 compounds correctly predicted as negative). The compounds HBM, ANP, NAP, and MIT are equivocal or positive in ToxTracker, yet negative *in vivo*, and thus are considered false positive compounds; they would require follow-up testing with other *in vitro* or *in vivo* tests. Graphs of the data for the individual compounds are presented in the Supplementary data.

3.3. *Performance in a combined approach: Vitotox plus ToxTracker*

In order to cover more mechanisms of genotoxicity and thus increase sensitivity, a battery of complementary tests can be applied. Indeed, by combining Vitotox and ToxTracker data, and considering the result positive when either one or both assays was positive, only one compound was missed: sensitivity = 93%. (We considered an equivocal test result in ToxTracker as positive, although follow-up would be required.)

The specificity of the Vitotox/ToxTracker combination was 73% (11/15) when equivocal ToxTracker results were considered positive (Table 3). If the equivocal ToxTracker test results were omitted, the specificity of the combination increases to 79% (11/14). An overview of the results is presented in Tables 3 and 4.

4. Discussion

In the development of pharmaceutical and cosmetic compounds, one of the criteria to select candidates for downstream development is their toxicological profile, including genotoxicity, which may indicate carcinogenicity [2] or risk of hereditary diseases or birth defects [21]. Currently, several *in vitro* assays, such as the Ames test (OECD 471) and the micronucleus test (OECD 487), are commonly accepted by regulatory agencies [2]. During the drug development process, difficulties arise when a shortlist must be made from thousands of candidate compounds. Rapid, high-throughput assays requiring only small amounts of compound are preferred [8,22]. Additionally, for cosmetics, follow-up *in vivo* tests are no longer allowed, according to European Regulation 1223/2009/EC. Consequently, to prove a safe toxicological profile for a candidate, it is necessary to gather as much information as possible on that compound, to use in a weight-of-evidence analysis [23].

Reporter-based assays for genotoxicity screening include GreenScreen HC [24], Anthem's Genotoxicity screen [25], and several luciferase-based assays developed by Westerink and colleagues [22]. Such assays allow automated and selective screening and, to a certain extent, provide mechanistic information [7]. Understanding the mechanism of genotoxicity can help to reduce the number of false positives. Here, we have tested two reporter-based assays: Vitotox, which monitors activation of the bacterial DNA damage response, and ToxTracker, which detects activation of different DNA damage responses in mammalian stem cells. Our limited validation study of the Vitotox assay with a selection of well-established genotoxic and non-genotoxic compounds reveals sensitivity = 67%, comparable to earlier validation studies [11] and

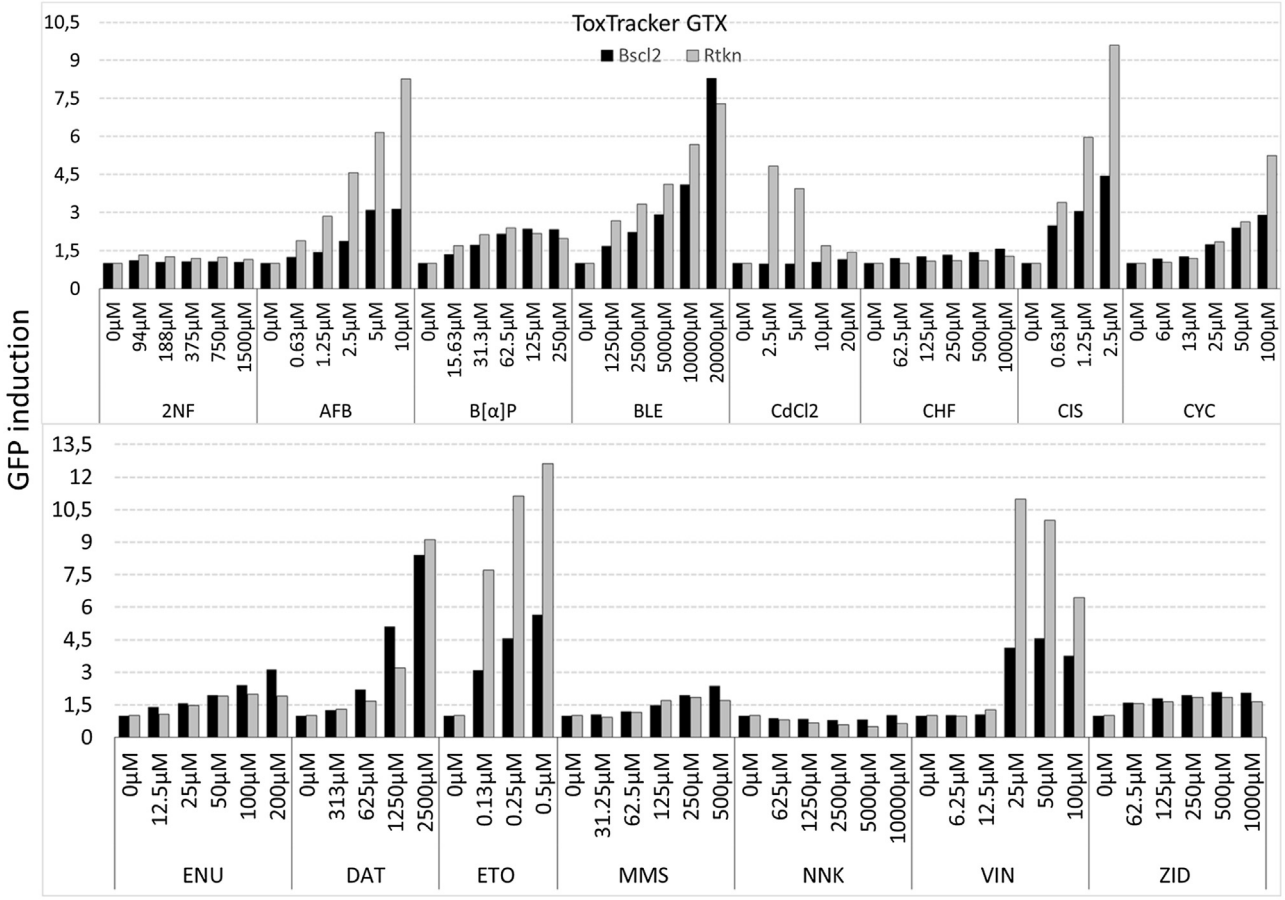

Fig. 3. Toxtracker results after exposure to 15 *in vivo* genotoxicants (GTX) (n = 3). The threshold for positivity is set at a GFP induction of 1.5 in comparison to the control (0 μM). Abbreviations: 2NF = 2-nitrofluorene; AFB = aflatoxin B1; B[α]P = benzo[*a*]pyrene; BLE = bleomycin sulfate; $CdCl_2$ = cadmium chloride; CHF = chloramphenicol; CIS = cisplatin; CYC = cyclophosphamide; DAT = 2,4-diaminotoluene; ENU = 1-ethyl-1-nitrosourea ETO = etoposide; MMS = methyl methanesulphonate; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; VIN = vinblastine sulfate; ZID = azidothymidine (zidovudine).

comparable to that of the Ames test (60%). Incorrect predictions by Vitotox may be ascribed to general limitations of bacterial assays. With 100% detection of true negatives, Vitotox seems to display higher specificity than the Ames test (74%) [3].

For ToxTracker, sensitivity = 87%, which seems to be somewhat higher than for other *in vitro* mammalian cell tests. Sensitivities reported for the *in vitro* micronucleus test (OECD 487) and the mouse lymphoma assay (OECD 476) are 80.9% and 80.8%, respectively [3]. With regard to specificity, ToxTracker (79%) outperforms the micronucleus test (54%) and mouse lymphoma assay (48%) [3]. Corrections were made for auto-fluorescence, but might not completely eliminate the effect. Therefore, further testing of compounds showing signs of auto-fluorescence (*e.g.*, DAT, HBM, and NAP) would be recommended.

The numbers of compounds tested in various validation studies differ, and of course this may affect the results. Also, comparing the performance of Vitotox and ToxTracker with the performance of the Ames test and the *in vitro* MN test based only on the set of compounds used in this study would be biased, since these compounds were specifically chosen based on their Ames and *in vitro* MN results.

While the individual performance statistics for Vitotox and ToxTracker has been reported previously, no results have yet been presented for the combination. The observed 93% sensitivity is higher than that reported for regulatory batteries [3] or for the combination of Vitotox with RadarScreen (85%), a reporter-based prescreening yeast clastogenicity assay [11]. The specificity of 79% for the Vitotox + ToxTracker combination is higher than that of the two-test combination recommended for regulatory purposes. For instance, as reported by Kirkland et al. (2005), combining the Ames test with the *in vitro* micronucleus test leads to a specificity of only 32%. However, in the study of Westerink et al. (2009), for Vitotox + RadarScreen, specificity = 81% is reported. Although the Vitotox + RadarScreen combination shows a good performance, lately there is strong emphasis on using mammalian cells that are p53 proficient [26], as is the case for ToxTracker.

A drawback for both the Vitotox and ToxTracker assays, however, is their failure to detect the nitroarene, 2NF. In the Vitotox assay, this might be due to compromised nitroreductase activity arising during modification of the TA104 bacterial strain, although this possibility has not yet been tested directly. In general, for 2NF, contradictory results can be found in the literature. For instance, contradictory results have been reported in nitroreductase-proficient *Escherichia coli* [27] and *Salmonella* strains [28]. In a forward mutation assay with TA98 and TA100, 2NF was reported negative, even though it was positive in TA98 and TA100 in the Ames test [29]. Furthermore, a study of four nitro-group containing aromatic amines in TA100, TA104, TA4001, and TA4006 showed that factors other than nitroreductase activity play a role in mutagenic specificity: not all of the nitro-group containing aromatic amines could be identified by each of the four nitroreductase-proficient strains [30]. These examples show that one must be cautious in interpreting the results for nitro-group containing aromatic amines in *in vitro* assays, emphasizing the need for integrated testing strategies and for decision-making based on weight-of-evidence analysis.

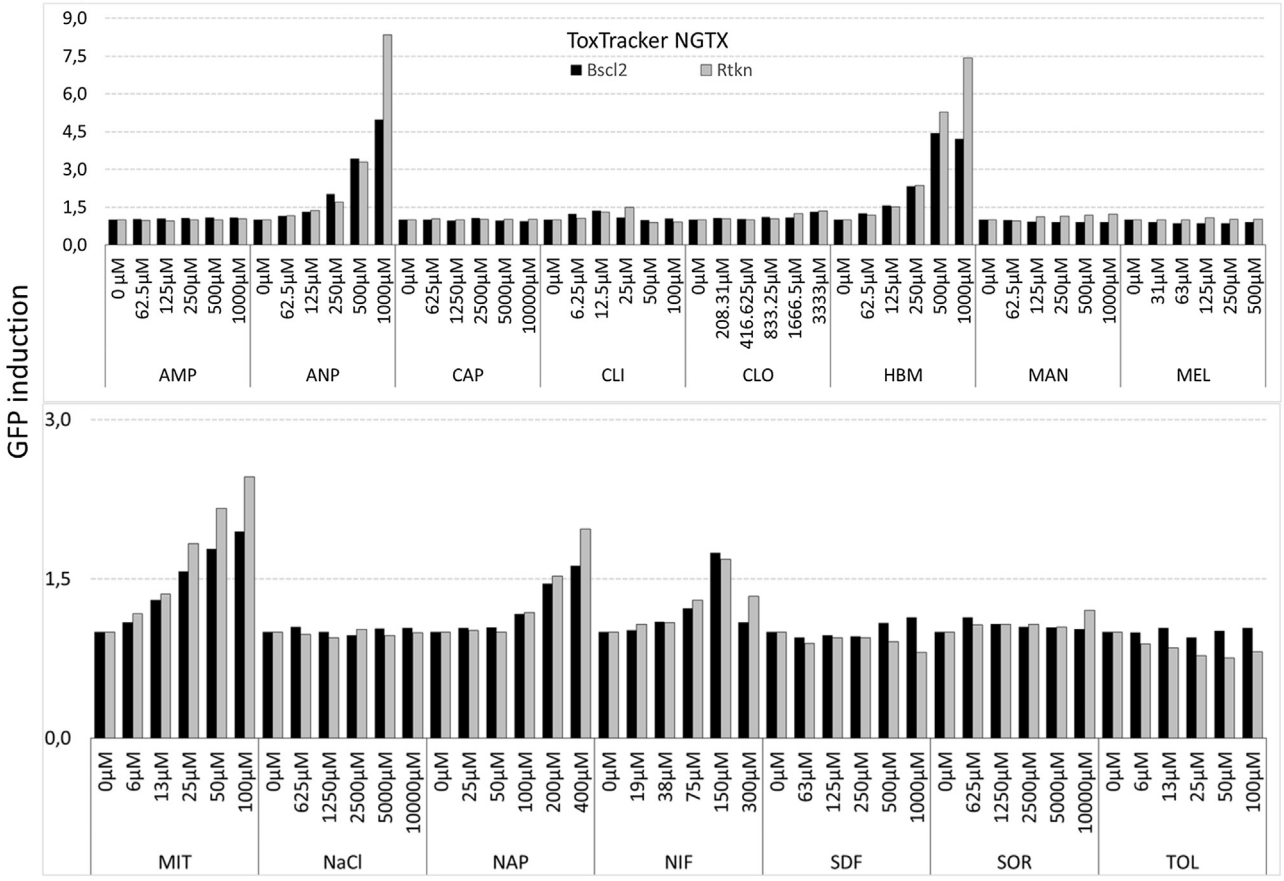

Fig. 4. ToxTracker results after exposure to 15 *in vivo* non-genotoxicants (NGTX) (n = 3). The threshold for positivity is set at a GFP induction of 1.5 in comparison to the control (0 μM). Abbreviations: AMP = ampicillin trihydrate; ANP = 4-amino-3-nitrophenol; CAP = caprolactam; CLI = climbazole; CLO = clonidine; HBM = hydroxybenzomorpholine; MAN = d-mannitol; MEL = melatonin; MIT = methylisothiazolinone; NaCl = sodium chloride; NAP = 1-naphthol; NIF = nifedipine; SDF = sodium diclofenac; SOR = sorbitol; TOL = tolbutamide.

For compounds showing intense auto-fluorescence, ToxTracker may not be the first-choice *in vitro* genotoxicity test. Assays with other detection systems, such as bioluminescence, might be more suitable [22,25].

5. Conclusions

The number of compounds tested in this manuscript is insufficient to state with confidence that the Vitotox + ToxTracker combination is better than the regulatory two-test battery, but it is worthwhile to investigate further the role of reporter-based tests in a regulatory setting. Indeed, other reporter-based assays, such as RadarScreen [11] and GreenScreen HC [24], have also proven to be valuable tools. Currently, reporter-based tests are all positioned in the pre-regulatory screening phase, exploiting their speed and their requirement of only small quantities of compound to support decision making for downstream development. However, in view of the results already available, it seems worthwhile to validate these assays further and to consider adding them to the regulatory testing "toolbox".

Conflict of interest

GH and RD are employees of Toxys B.V., the company that markets the ToxTracker assay.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrgentox.2016.09.005.

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