Application of the ToxTracker reporter assay in a mode of action approach for genetic toxicology assessment.

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Introduction

ToxTracker is a mammalian stem cell-based reporter assay that detects activation of specific cellular signalling pathways upon exposure to unknown compounds. ToxTracker contains six different GFP-tagged reporters that allow discrimination between induction of DNA damage, oxidative stress and protein damage in a single test (Hendriks *et al.*, Tox Sci 2016). ToxTracker can be particularly useful in an Adverse Outcome Pathway (AOP) approach for both genotoxic and non-genotoxic carcinogens.

Results

In an extensive validation study using 300 reference chemicals and >200 proprietary compounds, ToxTracker classified the genotoxic compounds with a sensitivity of 94% and specificity of 95%. By assessing the differential induction of the two DNA damage reporters, ToxTracker was able to discriminate between a mutagenic and clastogenic mechanism of genotoxicity. We also found that the assay could discriminate between a clastogenic and aneugenic mode of action by the selective induction of the DNA strand break reporter. Induction of this reporter was significantly slower (>12h) for the mitotic spindle poisons compared to clastogenic compounds (8h). Furthermore, by staining for phosphorylation of histone H3 and including a DNA stain for polyploidy in the reporter cell lines, ToxTracker can identify an aneugenic MOA by inhibition of cell cycle kinases.

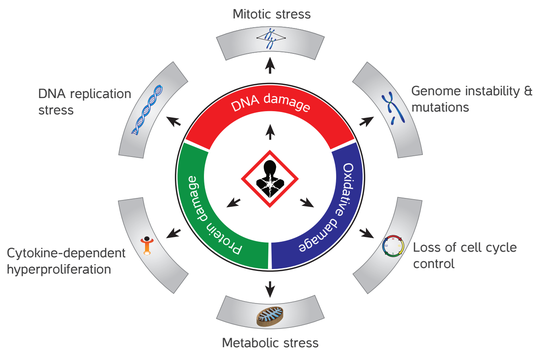
Conclusion

The integrative approach of the ToxTracker assay provides a unique tool for *in vitro* carcinogenic hazard identification of chemicals by unveiling activation of specific cellular signalling pathways upon exposure and deliver insight into the underlying mechanism of toxicity.

The ToxTracker reporter assay

- Stem cell-based reporter assay
- *In vitro* carcinogenicty hazard screening
- Insight into mechanisms of genotoxicity

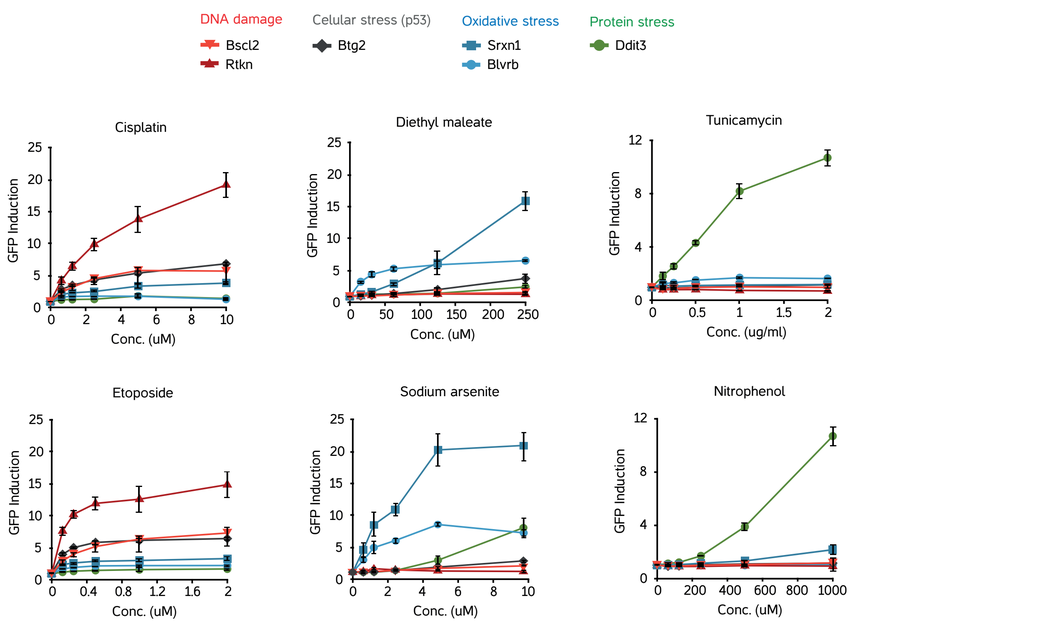
- Six independent GFP reporter cell lines
- High throughput detection by flow cytometry
- Discriminate between induction of DNA damage, oxidative stress and protein damage



Biomarker gene	Mode of action	Cellular pathway
Bscl2	Mutagenic DNA lesions	ATR/Chk1 DNA damage signaling
Rtkn	DNA double-strand breaks	NF-kB signaling
Srxn1	ROS production	Nrf2 antioxidant response
Blvrb	ROS production	Nrf2-independent
Ddit3	Protein damage	Unfolded protein response
Btg2	Cytotoxicity	p53 signaling

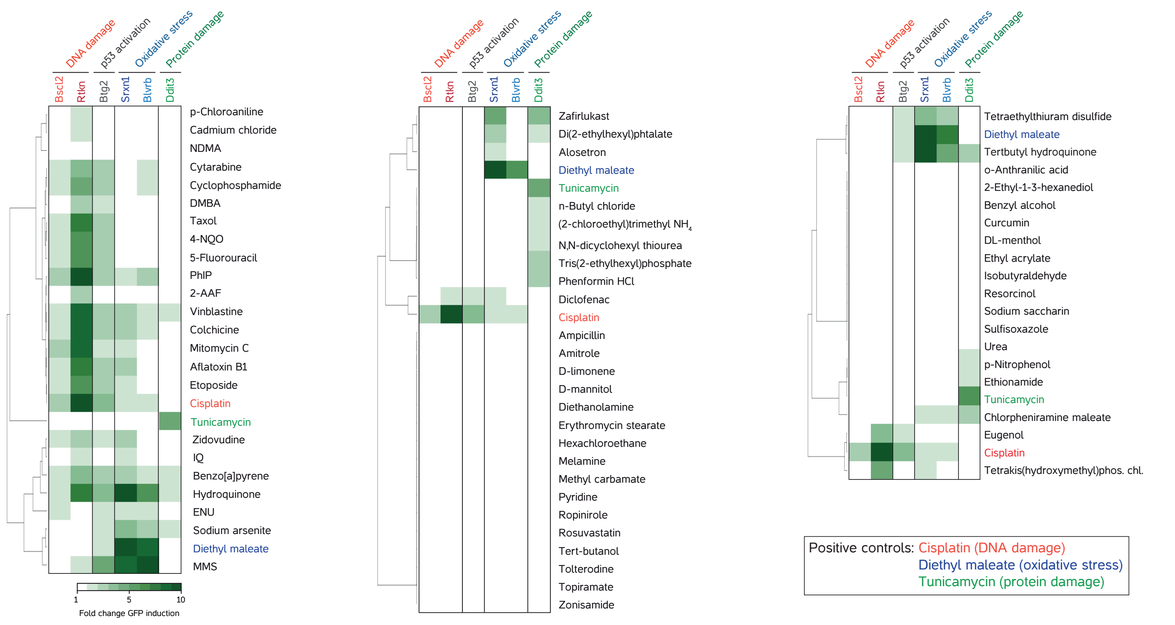
Test name	Sensitivity (%)	Specificity (%)
Regulatory		
Bacterial reversion (Ames)	60	77
Chromosome aberrations	70	55
Mammalian mutation	81	48
Screening		
ToxTracker	94	95
Ames MPF	58	63
GreenScreen HC	87	95

Validation of the ToxTracker assay



Selective activation of the ToxTracker reporter cell lines. GFP reporter cells were exposed to increasing concentrations of the DNA damaging agents cisplatin and etoposide, the oxidative stress-inducing agents diethyl maleate and sodium arsenite, and the UPR-activating compounds tunicamycin and nitrophenol. GFP induction levels in intact cells were determined by flow cytometry at 24 h. after initiation of the exposure. The relative cell survival was determined by cell count after 24 h. exposure using flow cytometry.

I. ECVAM class 1 (*in vivo* genotoxins) II. ECVAM class 2 (Non-DNA-reactive chemicals) III. ECVAM class 3 (Non-DNA-reactive, *in vitro* pos.)

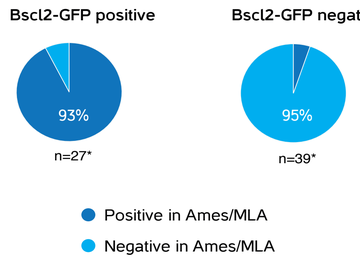


Positive controls: Cisplatin (DNA damage)
Diethyl maleate (oxidative stress)
Tunicamycin (protein damage)

Preferential activation of ToxTracker reporter cell lines. Induction levels of the different GFP reporters were calculated for all compounds at an equitoxic concentration that induced 50% cytotoxicity. GFP levels were determined by linear regression of the GFP induction data points of the two doses encompassing 50% cytotoxicity. In case the 50% cytotoxiucity levels was nogt reached, the GFP indcution at the maximum tested concentrations was used. Induction of the GFP reporter upon exposure to a selection of ECVAM-recommended carcinogens that (I) should be positive in an *in vitro* genotoxicity assay, (II) a selection of non-carcinogens that should be negative in an *in vitro* genotoxicity assay and (III) a collection of compounds that are non-carcinogens or non-genotoxic carcinogens but that occasionally scored positive in a conventional *in vitro*/*in vivo* genotoxicity test.

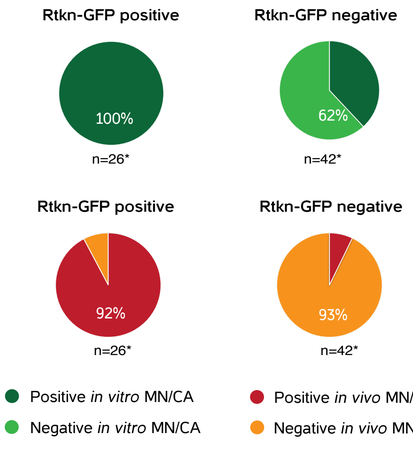
Mutagenicity and clastogenicity

- Bscl2-GFP reporter for promutagenic DNA lesions
- Correlation with bacterial (Ames) and mammalian (MLA) mutation assays



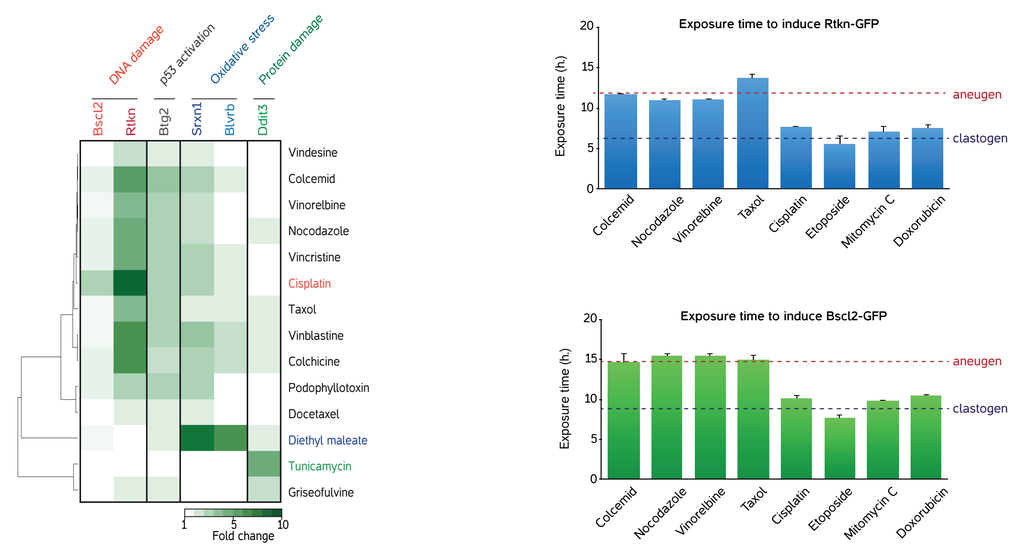
*Comparison with ECVAM library of reference compounds, Kirkland et al 2016

- Rtkn-GFP reporter for DNA double strand breaks
- Correlation with *in vitro* and *in vivo* micronucleus and chromosome abberation tests



The ToxTracker genotoxicity reporters indicate a mutagenicand clastogenic mode of action. The Bscl2-GFP reporter is activaed by DNA replication stress in respone to promutagenic DNA lesion. The Rtkn-GFP reporters is activated by NF-kB following induction of DNA double strand breaks. Activation of the Bslc2-GFP genotoxicity reporter was compared results from the bacterial (Ames) or mammalian (MLA) mutation assays. Activation of the Rtkn-GFP reporter was compared with the conventional *in vitro* and *in vivo* micronucleus (MN) and chromosome abberation (CA) assays. Comparison between the ToxTracker reporters and standard genotoxicity assays was done for 68 established genotoxic and non-genotoxic compounds that were selected by ECVAM.

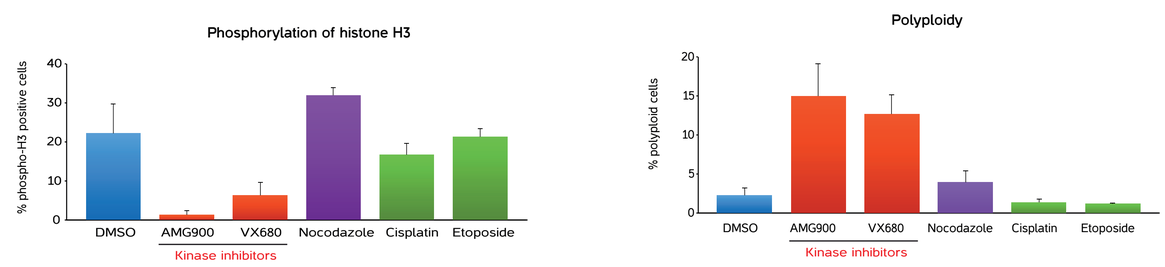
Clastogenicity and aneugenicity



Differential responses of the Bscl2 and Rtkn-GFP reporters allows discrimination between clastogenic and aneugenic compounds. ToxTracker reporter cell lines were exposed to a selection of microtubule disrupting agents. Induction levels of the different GFP reporters were calculated for all compounds at an equitoxic concentration that induced 50% cytotoxicity. The kinetics of Bscl2-GFP and Rtkn-GFP reporter induction was determined following exposure to clastogenic compounds (cisplatin, etoposide, mitomycin C, doxorubicin) or mitotic spindle poisons (colcemid, nocodazole, vinorelbine, taxol). GFP induction was determined after 4, 8, 12, 16 and 24 h. exposure. Exposure times that resulted in a 1.5-fold increase in GFP signal for the Bscl2-GFP and Rtkn-GFP reporters after exposure to various clastogenic and aneugenic compounds were calculated by linear regression of the exposure time data points of the two doses encompassing 1.5 fold GFP induction data points. The dashed lines indicat the thresholds for classification as clastogen or aneugen.

Kinase inhibitors

- ToxTracker reporter cell lines
- Antibody staining for phosphorylated histone H3
- DNA staining for cell cycle analysis and polyploidy
- Analysis by flow cytometry



ToxTracker combined with additional cellular stress markers allow identification of Aurora kinase inhibitors. ToxTracker reporter cell lines were exposed to the Aurora kinase inhibitors AMG900 or VX680, the microtubule disruption agent nocodazole and the DNA damaging agents cisplatin and etoposide. After 6h exposure, cells were fixed and stained with antibodies against phosphorylated histone H3. Histone H3 is phosphorylated by Aurora kinases during mitosis. Alternatively, after 24h cells were fixed and DNA was stained with propidium iodide. DNA content, cell cycle analysis and phosphorylation of histone H3 was performed using flow cytometry. Reduction of phospho-H3 levels and polyploidy are considered hallmarks of inhibition of cell cycle kinases. The Aurora kinase inhibitors AMG900 and VX680 strongly reduced phospo-H3 levels and induced polyploidy. In contrast, nocodazole arrested cells in mitosis and therefore increase phosho-H3 levels. The DNA damaging agents cisplatin and etoposide did not influence phosphorylation of H3 or DNA content of the cells.