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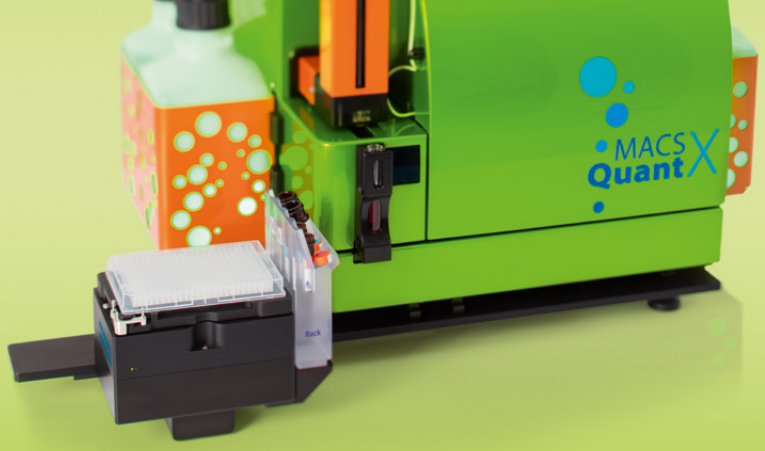

High-throughput ToxTracker® ACE genotoxicity assay on the MACSQuant® X flow cytometer

Background

Pharmaceutical, chemical, and cosmetic industries develop an increasing number of novel compounds each year. These compounds need to be assessed for potential adverse and carcinogenic properties, ideally in early phases of development. To test for mutagenicity and carcinogenicity, several standard *in vitro* and *in vivo* assays are currently in use, such as the Ames test and micronucleus assay. However, these standard assays suffer from a limited sensitivity and specificity and do not provide insights into the mode of action of compounds. Compounds can negatively affect cells by damaging DNA, by causing oxidative stress, or by harming proteins directly. To provide predictive measures of carcinogenicity and gain insight into the mode of action of tested compounds, the ToxTracker® assay was developed.

ToxTracker® is a mammalian stem cell–based reporter assay that detects the activation of specific cellular signaling pathways upon chemical exposure. It contains six different GFP-tagged reporters that allow for the discrimination between induction of DNA damage, oxidative stress, or protein damage in a single test.

In combination with the MACSQuant® X flow cytometer this assay can be performed in a fast, standardized, and automated manner. This workflow generates high-content information, which is both reproducible and reliable. The MACSQuant X is equipped with a syringe needle capable of volumetric pipetting, thereby enabling absolute counting from sample volumes starting at 5 µL. The instrument is fully automated in its calibration and compensation procedures, which, paired with the ToxTracker assay, pre-set templates, and instrument settings, significantly reduces experiment setup and optimization time.

To validate the ToxTracker assay for separating aneugenic from clastogenic DNA damaging agents and identify tubulin poisons as well as kinase inhibitors, we evaluated 3 compounds with known properties using an extension of this assay termed ToxTracker ACE (aneugen and clastogen evaluation). By adding the Hoechst DNA stain to the assay, we were able to analyze cell cycle profiles as well as aneuploidy and therefore better distinguish between different modes of action.

Compounds that act by directly interacting with DNA are known as clastogenic compounds. They give rise to DNA damage, mutations, and DNA rearrangements, and eventually induce cancer. However, these compounds generally do not cause aneuploidy. Exposure to aneugenic compounds, which act by interfering with mitosis, leads to the loss or gain of whole chromosomes. Such aneugenic compounds are generally not carcinogenic. Both, clastogenic and aneugenic compounds can give rise to a positive result in the micronucleus assay which is often used in the standard genotoxicity test battery. In most cases, additional experiments are required to differentiate between clastogens and aneugens. The ToxTracker assay identifies clastogenic compounds by measuring the activation of both markers for DNA damage, while aneugenic tubulin poisons only activate the marker for DNA double strand breaks. Another type of aneugenic compounds, aurora kinase inhibitors, do not activate any ToxTracker markers, even though they do cause aneuploidy. In this study, we present a rapid and simple assay for the collection of genotoxicity information that also provides insight into the mode of action of compounds, including discrimination between clastogenic and aneugenic compounds. The combination of the ToxTracker ACE assay and MACSQuant X flow cytometer enables the generation of high-quality, reliable results that are required for compound screening and safety studies in the field of drug discovery and development.

Materials and methods

To assess the ability of the ToxTracker ACE assay for distinguishing clastogens from aneugens and identifying tubulin poisons and kinase inhibitors, the assay was performed using three compounds with known properties. Briefly, the six ToxTracker reporter cell lines as well as wild type mouse stem cells were exposed to cisplatin (blastogenic compound), taxol (tubulin poison), and AMG900 (aurora kinase inhibitor). Subsequently, GFP induction was determined in reporter cell lines and cell cycle, as well as aneuploidy analyses were performed on DNA-stained wild type cells, after 4 and 24 hours respectively, both using the MACSQuant X flow cytometer.

Cell culture and treatments

C57BL/6 B4418 wild type mES cells were cultured in mES knockout medium (Gibco) containing 10% fetal calf serum (FCS), 2 mM glutamax, 1 mM sodium pyruvate, 100 mM ß-mercaptoethanol, and leukemia inhibitory factor (complete mES cell culture medium). Cells were propagated on irradiated primary mouse embryonic fibroblasts as feeders according to established protocols[1]. The ToxTracker® reporter cell lines were maintained in 200 µg/mL G418 in complete mES cell culture medium. For chemical exposure, cells were seeded 24 hours prior to exposure on gelatin-coated plates in complete mES cell culture medium in the absence of feeder cells, and subsequently exposed to the test compounds for 4 and 24 hours. Cells were exposed to 5 doses of the test compounds in 2-fold dilutions. Cisplatin was dissolved in PBS, taxol and AMG900 were dissolved in DMSO. Treatments were performed in triplicate biological experiments.

Detection of ToxTracker® reporters

ToxTracker analysis was performed as previously described[2]. In brief, GFP reporter expression was determined by flow cytometry on the MACSQuant® X flow cytometer. Following 24 hours of exposure, cells were washed with PBS, detached with trypsin, and suspended into PBS supplemented with 2% FCS, immediately followed by flow cytometry analysis. To keep cells in constant suspension, sample mixing was performed by the Orbital Shaker of the MACSQuant X. In addition, the integrated vibration needle was used to mix every single well before uptake. Reporter activity was determined by the mean fluorescence intensity (MFI) of 1×10^4 intact cells. Cell numbers were determined using the absolute cell count provided by the instrument. Activation of a reporter cell line was considered positive when, at any applied dose, exposure to a compound resulted in greater than 2-fold induction of GFP expression.

DNA staining

DNA staining was performed after 4 or 24 hours of compound exposure. Hoechst solution was diluted with medium and added to the wells to achieve a final dilution of 1:500. After a 30-minute incubation period at 37 °C, cells were washed twice in PBS, detached using trypsin, and suspended in PBS + 2% FCS. Cells were then collected by centrifugation and the supernatant was removed. Subsequently, cells were suspended in ACE buffer (Toxys®) and incubated for 10 min. Cells were fixed by adding glutaraldehyde. After a 10-minute incubation period, $NaBH_4$ was added to quench the glutaraldehyde. Samples were then immediately analyzed on the MACSQuant X. For the analysis of DNA content, the violet laser was set to linear. At this setting, the average intensity of G2/M cells is twice as high as for cells in G1. At least 1×10^4 events were analyzed per sample. Samples with more than 75% cell death were not considered for the analysis.

Cell cycle and aneuploidy analysis

To test whether ToxTracker could discriminate clastogens from aneugens and identify tubulin poisons and kinase inhibitors interfering with mitosis, the distribution of cell cycle phases and the fraction of aneuploid cells was analyzed using Flowlogic™ Software (fig. 1A, B).

Since the intensity of the G2/M peak in the Hoechst channel is approximately twice as high as that of the G1 peak, polyploid cells (>4n) were selected using a gate that starts after the G2/M peak (fig. 1C).

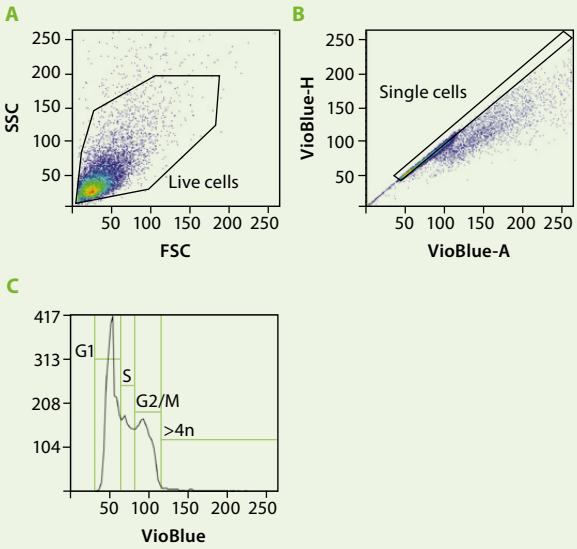

Figure 1: Gating strategy for cell cycle analysis. (A) Using the FSC-A and SSC-A on the x- and y-axes respectively, all living cells were selected. (B) Then, plotting the area for the Hoechst channel on the x-axis and the peak height on the y-axis, single cells were selected. Doublets were excluded in this gate, as they appear away from the diagonal (the area of their signal is not linearly related to the height). Sub-G1 cells, located at the bottom left part of the diagonal, were also excluded. (C) Exemplary cell cycle analysis.

Results

To test the specificity of ToxTracker ACE, the clastogenic compound (cisplatin), a tubulin poison (taxol), and an aurora kinase inhibitor (AMG900) were tested in the assay. Clastogenic compounds cause DNA damage and activate both markers for genotoxicity in ToxTracker[2]. Cisplatin is a DNA-damaging agent that causes bulky DNA adducts as well as inter- and intra-strand crosslinks. During replication, these crosslinks can be converted to DNA double strand breaks. As expected, cisplatin activated both markers for genotoxicity in ToxTracker (fig. 2A). After 4 hours of exposure, there was no effect on the cell cycle, compared to vehicle-treated cells (fig. 3A). After 24 hours of exposure to cisplatin, cells arrest in S phase due to replication stress and cell cycle arrest (fig. 3B, C). However, no aneuploid cells were observed.

As a typical aneugen, the tubulin poison taxol was tested. Tubulin is the main component of microtubuli, which play important roles in cell motility and mitosis. During mitosis, the mitotic spindle apparatus pulls apart the aligned chromosomes. Exposure to tubulin poisons arrests cells in mitosis and can lead to misaligned or lagging chromosomes. If mitosis proceeds, daughter cells can end up with an abnormal number of chromosomes (aneuploidy). Exposure to tubulin poisons leads to rapid cell cycle arrest. After 4 hours of exposure to taxol, cells accumulated in G2/M (fig. 3A). 24 hours of exposure resulted in the appearance of aneuploid cells, that increased in a

dose-dependent manner (fig. 3B, C). In ToxTracker, taxol most strongly activated the Rtkn-GFP marker for DNA double strand breaks (fig. 2A), and the Bscl2-GFP reporter only at higher cytotoxicity, as published previously[2]. For cell cycle, aneuploidy, and ToxTracker analyses, samples with less than 25% cell viability were not analyzed, since this level of cytotoxicity is too high for reliable marker activation and DNA content quantification.

Aurora kinases also play a role in mitosis by controlling chromatid segregation[3]. Their inhibition leads to arrest of the cells in mitosis and can cause polyploidy. In ToxTracker, treatment with AMG900 did not activate the markers for genotoxicity, even though it did cause aneuploidy.

4 hours of exposure to AMG900 lead to the arrest of cells in G2/M (fig. 3A). Aneuploid cells appeared after 24 hours of exposure (fig. 3B, C).

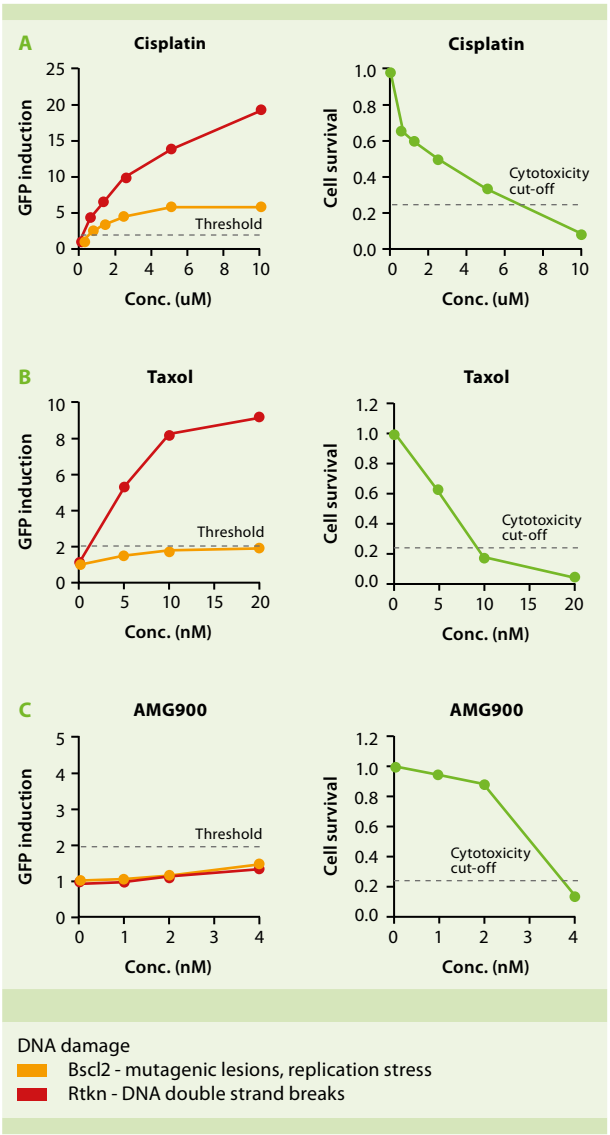

Figure 2: ToxTracker genotoxicity reporters. The assay was performed after 24 hours of exposure to the test compound. Cisplatin activated both markers for genotoxicity (A). The tubulin poison taxol only activated the marker for DNA double strand breaks (Rtkn-GFP), but not the marker for replication stress (Bscl2-GFP) (B). The aurora kinase inhibitor AMG900 did not activate the markers for genotoxicity at 50% cytotoxicity (C).

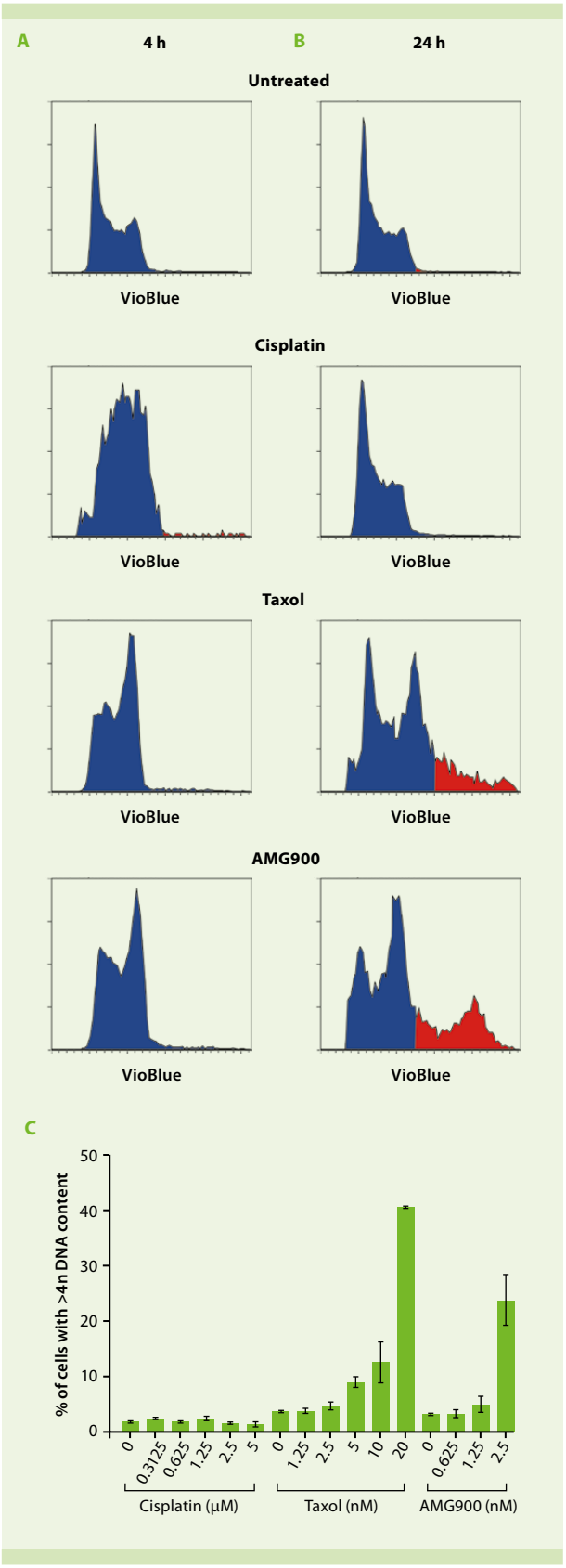

Figure 3: Cell cycle and aneuploidy analyses. (A, B) Exposure to cisplatin arrested cells in S phase after 24 hours of exposure. Taxol arrested cells in G2/M after 4 hours of exposure and caused aneuploidy after 24 hours. AMG900 clearly arrested cells in G2/M after 4 hours of exposure and caused aneuploidy after 24 hours. Aneuploid cells (>4n DNA content) shown in red. (C) Quantification of the number of cells with more than 4n DNA content. Samples with more than 30% cytotoxicity were excluded from this analysis.

For cell cycle and aneuploidy analyses with ToxTracker®, samples with less than 30% cell viability were not analyzed, since this level of cytotoxicity is too high to observe reliable marker activation and DNA content quantification. For AMG900, the two highest doses (above 3 nM) were excluded.

Conclusions

The ToxTracker ACE assay, that combines the ToxTracker reporters for DNA damage, oxidative stress, and protein damage with cell cycle analysis and polyploidy detection, can identify genotoxic compounds with a clastogenic or aneugenic mode-of-action. The distinction between these two classes of compounds is important for regulatory purposes, as different approaches for risk assessment are used for clastogenic and aneugenic compounds[4,5].
By testing archetype clastogenic and aneugenic compounds, we confirm that aneugenic compounds cause aneuploidy, while clastogenic compounds do not. Furthermore, aneugenic compounds show a characteristic accumulation of cells in G2/M phase after 4 hours of compound exposure. With the inclusion of the DNA stain, ToxTracker can therefore be used to detect genotoxicity as well as distinguish aneugens from clastogens.
The results also demonstrate that the combination of the ToxTracker assay with the MACSQuant® X flow cytometer enables a rapid, simple collection of genotoxicity information that is required for compound screening and safety studies in the field of drug discovery. The 96-well format allows high-throughput screening of compounds at multiple doses, generating large amounts of high-quality data.

References

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