Image analysis of mechanistic protein biomarkers for the characterization of genotoxicants: Aneugens, clastogens, and reactive oxygen species inducers

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Abstract
The early detection of genotoxicity contributes to cutting-edge drug discovery and development, requiring effective identification of genotoxic hazards posed by drugs while providing mode of action (MoA) information in a high throughput manner. In other words, there is a need to complement standard genotoxicity testing according to the test battery given in ICH S2(R1) with new in vitro tools, thereby contributing to a more in-depth analysis of genotoxic effects. Here, we report on a proof-of-concept MoA approach based on post-translational modifications of proteins (PTMs) indicative of clastogenic and aneugenic effects in TK6 cells using imaging technology (with automated analysis). Cells were exposed in a 96-well plate format with a panel of reference (geno)toxic compounds and subsequently analyzed at 4 and 24 hr to detect dose-dependent changes in PTMs, relevant for mechanistic analysis. All tested compounds that interfere with the spindle apparatus yielded a BubR1 (S640) (3/3) and phospho-histone H3 (S28) (7/9) positive dose–response reflecting aneugenicity, whereas compounds inducing DNA double-strand-breaks were associated with positive FANCD2 (S1404) and 53BP1 (S1778) responses pointing to clastogenicity (2/3). The biomarker p53 (K373) was able to distinguish genotoxicants from non-genotoxicants (2/4), while the induction of reactive oxygen species (ROS), potentially causing DNA damage, was associated with a positive Nrf2 (S40) response (2/2). This work demonstrates that genotoxicants and non-genotoxicants induce different biomarker responses in TK6 cells which can be used for reliable classification into MoA groups (aneugens/clastogens/non-genotoxicants/ROS inducers), supporting a more in-depth safety assessment of drug candidates.

KEYWORDS
aneugens, biomarkers, clastogens, genotoxicity, image analysis, ROS inducers

1 | INTRODUCTION

Genotoxicants exert their effects through different modes of action (MoA). Depending on the induced DNA damage (e.g., aneugenic, clastogenic effects), they trigger the complex DNA damage response (DDR) which involves the mechanistic protein biomarkers studied in this work. The identification of the MoA is a key part in risk assessment and provides guidance for an informed decision whether or not...
a threshold, that is, a safe, nongenotoxic dose can be determined for a genotoxicant based on an experimentally determined nonlinear dose–response relationship. Mechanistic information justifying the derivation of thresholds for genotoxicants may affect the further development of the drug candidate. Especially, kinase inhibitors need a clear characterization because in many cases their off-target-kinase activity is suspected to induce the unintended genotoxic effect (Fabbro, 2015; Santa-Gonzalez et al., 2016; Chondrou et al., 2018).

Basically, four MoA groups are relevant for drug discovery and development which can be categorized into subgroups based on their (sub)cellular target:

1. Clastogens (inducing structural chromosome aberrations): act by directly or indirectly interacting with DNA, resulting in DNA strand breaks. This MoA group includes alkylating agents, topoisomerase inhibitors (type I and II), and antimitotobolites.

2. Aneugens (inducing numerical chromosome aberrations): disturb the dynamics of the microtubules (MT) or damage other components of the mitosis machinery. This group includes stabilizers (result in polymerization of the MT) and/or destablizers (result in depolymerization of the MT). Kinase inhibitors may be aneugenic by targeting numerous cellular processes (e.g., proliferation, adhesion, transformation, and survival). Aneugens include (a) inhibitors of mitotic kinases (aurora, CDK1), (b) kinesin inhibitors: inhibition of motor proteins (Eg5 or CENP-E) and (c) multiprotein complex inhibitors: inhibition of enzyme complexes (APC or proteasome) (Rudolph et al., 2009; Chan et al., 2012; Juan et al., 2014; Potapova and Gorbsky, 2017).

3. Nongenotoxicants are compounds with no relevant genotoxicity in vivo. They comprise compounds with or without relevant increase in micronucleus frequency in mammalian cells in vitro. These compounds may still be (cyt)otoxic in vitro and/or in vivo. The identification and correct classification of nongenotoxicants is a challenge. Specific drugs may show simultaneously several mechanisms, exhibit tissue-specific toxicity but essentially are nongenotoxic. In addition, they may generate false positive results due to their cytotoxicity and a potential induction of genotoxic endpoints (phenotypic: e.g., micronuclei and molecular: e.g., p53) (Doktorova et al., 2014; Fowler et al., 2014; Lee et al., 2014; Pérez et al., 2016).

4. Reactive oxygen species (ROS) may cause DNA damage and genotoxicity. ROS inducers include compounds inducing genotoxic effects in vitro such as hydrogen peroxide and carbonyl cyanide m-chlorophenylhydrazone (CCCP). Data indicating an induction of ROS alone are not sufficient to demonstrate relevant genotoxic activity.

The aforementioned four MoA groups cannot be fully distinguished by the standard in vitro test battery and thus there is a need for MoA analysis to support the classification of unknown compounds. This MoA information can be obtained by state-of-the-art technologies such as single and multiplex methods on fluorescence-activated cell scanning (FACS), image analysis and toxicogenomics approaches (Nikolova et al., 2014; Li et al., 2015; Khoury et al., 2016; Bryce et al., 2017). Thus, numerous published works demonstrated that MoA analysis by quantifying transcripts, by analyzing (post-translationally modified) proteins using single- or multiplex methods are useful tools for identifying DNA damage and correctly classifying unknown compounds, especially when supported by data analysis tools. For example, several companies such as Litron Laboratories and Toxys developed biomarker assays for the characterization of genotoxic activities in a high-throughput format (Doktorova et al., 2014; Hendriks et al., 2016; Khoury et al., 2016; Bryce et al., 2017).

During the past years, valuable progress has been made in the development of biomarker assays (Ellinger-Ziegelbauer et al., 2009; Birrell et al., 2010; Doktorova et al., 2014; Williams et al., 2015; Hendriks et al., 2016; Khoury et al., 2016) also involving large (multidisciplinary) collaborative studies such as for the MultiFlow® method (Bryce et al., 2017). However, whereas commercial methods either provide (a) a more in-depth description of the MoA (Westerink et al., 2010) or (b) simultaneous cytotoxicity (Rajakrishna et al., 2014; Bryce et al., 2017), a technology fulfilling both these criteria (a) and (b) has not yet been fully developed to reach the level of broad and commercial application.

Thus, new research focuses on the identification of post-translational modifications of proteins (PTMs) subsequent to DNA damage. As PTMs are indicative of a specific DNA damage, they can be used to identify the MoA of a specific compound. A reversible or irreversible modification affects the reaction and functional properties of proteins within cellular processes. PTMs include modification types such as phosphorylation, acetylation, methylation, hydroxylation, glycosylation, and ubiquitination (Khoury et al., 2011).

Therefore, based on a literature review, DNA damage-associated markers indicative of different MoA were selected, as described below, and analyzed in this work.

1.1 | Phosphorylated BubR1

Besides Mad1-3, Mps1, Bub1 and Bub3, also BubR1 is a relevant checkpoint protein, which is co-localized with unattached kinetochores. Phosphorylations of BubR1 on sites S670 and S676 are best described regarding their functions and both show central activity for stable kinetochore-microtubule attachment. The work by Elowe et al. (2010) demonstrated that BubR1 (S670) was mainly phosphorylated during prometaphase. Phosphorylation of BubR1 (S670) is essential for error correction and kinetochore attachment. Further, in vitro data suggest that the phosphorylation status of BubR1 is important for checkpoint inhibition of the APC complex. Studies by Suijkerbuijk et al. (2012) and Elowe et al. (2010) demonstrated that cells exposed to nocodazole (NOC) and paclitaxel (PAC) exhibited increased BubR1 (S670) levels.

1.2 | Phospho-histone H3

The phosphorylation of histone H3 at serine 10 and 28 (pH 3 (S10)/pH 3 (S28)) is a key event in mitosis and meiosis. The pH 3 (S10) is associated with the G2 phase, whereas S28 is associated with the mitosis phase of the cell cycle (Van Hooser et al., 1998; Pacaud et al., 2015). Phosphorylation of histone H3 (S10) is regulated by the
JNK kinase (c-Jun N-terminal kinase), a subtype of the MAP family (mitogen-activated protein) kinase (Pacaud et al., 2015). In contrast to for example, γH2AX, pH 3 (S10) is not directly or obviously involved as PTM in the DDR and originally established itself as a mitotic marker for analysis of cell cycle progression (Veras et al., 2009; Tane et al., 2014; Mir et al., 2017; Shen et al., 2017). In addition to the reliable determination of the mitotic index (mitotic cells vs. total cell count), the marker pH 3 (S10) was investigated in combination with aneugenic substances. The work of Muehlbauer and Schuler (2005), who found a dose-dependent increase of pH 3 (S10) upon treatment with aneugenic substances and a dose-dependent decrease upon treatment with clastogenic substances, was a breakthrough in this context. If the cell is locked in the M-phase due to serious disturbances during chromosome segregation, pH 3 (S10) remains in the active state and therefore accumulates in the cell. Thus, pH 3 (S10) was established as a suitable marker for substances with an aneugenic mechanism (Gollapudi et al., 2016; Bryce et al., 2016; Khoury et al., 2016; Bryce et al., 2017).

1.3 | Acetylated tumor protein p53

The phosphoprotein p53 is a well-known tumor suppressor, which is mutated in 50% of human cancers (Soussi et al., 2006; Olivier et al., 2010). It has a crucial function in cellular response to genotoxic stress. Thus, it recognizes genomic damage and induces cell cycle arrest, thereby preventing damaged cells from proliferation. More than 150 target genes are up- or down-regulated by p53 (Appella and Anderson, 2001). The regulation of p53 is achieved by its abundance and post-translational modification. Healthy cells have a low amount of p53 and its degradation by proteasome results from ubiquitination, mainly via the ubiquitin ligase MDM2 (Haupt et al., 1997). In response to cellular stress, ubiquitination is inhibited and leads to p53 stabilization. Subsequently, p53 translocates to the nucleus as a transcription factor and stimulates the synthesis of cell cycle inhibitors to prevent replication of damaged genetic material. It is known that the acetylation of six lysines (K370, K372, K373, K381, and K382) is important for the stabilization of p53 (Liu et al., 1999; Brooks and Gu, 2003; Chung et al., 2014; Luo et al., 2017).

1.4 | Phosphorylated FANCD2

FANCD2 is a well-characterized mechanistic marker and accumulates after replication stress upon S-phase and after DNA damage (Howlett et al., 2005). The protein has multiple modification sites including serine 1404 phosphorylated by ATM. As demonstrated by Boisvert and Howlett (2014), clastogens such as mitomycin C or hydroxyurea cause increases of FANCD2 phosphorylated at S1404.

1.5 | Phosphorylated 53BP1

Besides the recruitment of γH2AX, MDC1, or BRCA1 upon double-strand breaks (DSBs), 53BP1 (S1778) is also mobilized as a central mediator in the NHEJ pathway and can be visualized after fluorescence staining as foci co-localized with γH2AX (Feng et al., 2015). Protein 53BP1 has several phosphorylation sites, including ATM- and ATR-mediated sites at S13, S25, S176, and S1778. The study by Lee et al. (2009) showed that 53BP1 (S1778) was more relevant for the repair process of DSBs than 53BP1 (S25).

1.6 | Phosphorylated Nrf2

Several works demonstrated Nrf2 activation due to a variety of stress inducers and pathological disorders to avoid oxidative damage (Talay et al., 2003; Ma, 2013; Jimenez-Blasco et al., 2015). Nrf2 is mainly controlled by Keap1, known as Keap1-Nrf2 defense system. Keap1 is modified at its cysteine residues by reactive oxygen species (ROS) or electrophilic agents, resulting in its inactivation. Consequently, Nrf2 is stabilized following translocation to the nucleus to activate cytoprotective cellular mechanisms (Ichimura et al., 2013). The work of Park et al. (2015) showed that exposure to CCCP, a well-known mitochondrial uncoupler, led to degraded or modified Keap-1 followed by activation of Nrf2.

The aim of this work was to evaluate the ability of the above-mentioned PTMs to distinguish between different MoA groups and thus to be used for the classification of drug candidates. To this end, up to 12 well-characterized, diverse genotoxicants and non-genotoxicants were submitted to analysis. The compounds were selected based on their expected MoA and the known biomarker function. Thus, the choice of compounds differed for the various biomarkers investigated and consequently, the total set of compounds was not studied with every candidate biomarker. Nevertheless, each biomarker was investigated using compounds with diverse MoA and thus with expected positive or negative responses, respectively.

2 | MATERIALS AND METHODS

2.1 | Test agents

A panel of routinely used reference genotoxicants (clastogens and aneugens) and nongenotoxicants as indicated in Table 1 were used in this work. The choice of the test agents including their concentration ranges was based on the publication by Bryce et al. (2017). Before use, compounds were solubilized in dimethyl sulfoxide (DMSO) and the final concentration in cell culture was 1%.

2.2 | Cell culture and treatments

The experiments were conducted in the human lymphoblastoid cell line TK6 (ATCC® CRL-8015). Due to its many advantages, such as genome stability, easy handling (suspension cells), p53 competence, efficient DNA repair and superior specificity in mammalian cell
genotoxicity tests, the cell line has proven well-suited for genotoxicity tests and is one of the most commonly used cell lines today (Lorge et al., 2016; Verma et al., 2017; Yamamoto et al., 2017). The cells were grown in a humid atmosphere at 37°C with 5% CO2. They were cultured in RPMI-1640 media (ATCC) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. On the day of treatment, cells were adjusted to $4 \times 10^5$ cells/ml and transferred in a volume of 100 μl/well in round bottom 96-well plates. Subsequently, formulations of test substances were added at a volume of 100 μl. In general, cells were exposed to defined concentrations (Table 1) for 4 and 24 hr.

2.3 Investigation of PTMs for the characterization of genotoxicants using image analysis

The putative biomarkers indicated in Table 2 were evaluated in a singleplex approach in two independent experimental runs with three technical replicates each to assess their suitability for distinguishing MoA groups (aneugenic/clastogenic/nongenotoxicants/ROS inducers).

Cells were centrifuged (80 g, 5 min) after 4 and 24 hr exposure, washed once with medium, resuspended, and transferred to a new 96-well plate with flat bottom. Then, cells were centrifuged and subsequently fixed with methanol at −20°C for 10 min. Afterwards, the cells were again centrifuged (80 g, 5 min) and washed with 100 μl PBS. Subsequently, the cells were blocked by addition of 10% goat serum in 1×TBST for 25 min at 37°C. Following this, the diluted primary antibodies indicated in Table 2 were added to the cells at a volume of 15 μl for 1 hr at 37°C. After the incubation period, the samples were washed twice with 100 μl 1× TBST. This was followed by a 30-min incubation at 37°C with the secondary antibody Alexa Fluor® 488 goat anti-rabbit IgG (1 μg/ml). For the studies with p53 (K373), no incubation with a secondary antibody was needed as a primary antibody labeled with Alexa Fluor® 647 was used. Following the

| Antibody | Product no. | Supplier | Final concentration (μg/ml) |
|----------|-------------|----------|-----------------------------|
| Alexa Fluor® 488 | A11034 | Thermo Fisher Scientific, Rockford, IL | 1.0 |
| Anti-Histon 3 (S28) monoclonal rabbit IgG | Ab32388 | Abcam, Cambridge, UK | 1.21 |
| Anti-BubR1 (S670) monoclonal rabbit IgG | Ab200062 | Abcam, Cambridge, UK | 1.152 |
| Anti-p53 (acetyl K373)-AlexaFluor® 647 monoclonal rabbit IgG | Ab206055 | Abcam, Cambridge, UK | 4.06 |
| Anti-FANCD2 (S1404) monoclonal rabbit IgG | Ab109542 | Abcam, Cambridge, UK | 5 |
| Anti-S3BP1 (S1778) polyclonal rabbit IgG | PA5-17462 | Thermo Fisher Scientific, Rockford, IL | 9.035 |
| Anti-Nrf2 (S40) monoclonal rabbit IgG | Ab76026 | Abcam, Cambridge, UK | 0.32 |

*CCC, carbonylcyanide-3-chlorophenyl hydrazone.

*Highest concentration corresponds to the OECD guideline recommended cytotoxicity limit of 55 ± 5% (RNC).
two-step washing with 1x TBST, the nuclei were stained with 100 μl DAPI (NucBlue® Fixed Cell ReadyProbes® reagent).

The fluorescence stained TK6 cells were analyzed by the ImageXpress® Micro XLS system (Molecular Devices, Molecular Devices GmbH Biberach a. d. Riß, Germany) with a 20x objective and automatically counted by the interactive software application “custom module editor”. Image analysis uses a segmentation process to define, separate, and identify objects. When recording, individual postlaser focus offsets were set for each of the wavelengths, considering the different distances of the signals from the bottom of the cavity. This application provides the user with a variety of segmentation and classification processes as well as cell measurement parameters for reading. Automated image analysis was performed either through the multiwavelength cell scoring software tool, which can simply be adjusted or via a custom segmentation process by using the interactive custom module editor (Figure 1). By using the custom module editor, an improved automated segmentation process can be used to remove fluorescence artifacts, which otherwise could be erroneously counted.

2.4 Statistical analysis

The investigations of the putative markers were consistently carried out in three technical replicates. The arithmetic means of these technical replicates were calculated and the two independent experiments were presented individually. Data in the figures are given as scatter dot plot graphs with means ± SD. To test for significant differences between groups, one-way ANOVA and for multiple comparisons, Dunnett test was used, with significance levels indicated in the figure legends. All statistics were run with the Software GraphPad Prism 8, version 8.01 (Graphpad Software). The statistical approach for evaluating the individual biomarkers was based on receiver operating characteristic (ROC) analyses. ROC curves describe the quality of a system and are therefore applied to the classification quality of the mechanistic PTM. In a first step, separate tables were created for each biomarker and exposure time (4 or 24 hr), using mean values of independent experiments with individual compounds as well as the respective class labels (e.g., for pH 3 (S28): aneugen versus non-aneugen (comprising clastogens, nongenotoxicants, ROS inducers)). ROC curves were then calculated using the software GraphPad Prism 8, version 8.01 (Graphpad Software). The area under the curve (AUC) was provided together with the ROC curves. In order to evaluate the use of individual biomarkers for the classification of compounds, the area under the curve (AUC) was rated according to the following classification: 0.90–1.00 = very good; 0.80–0.90 = good; 0.70–0.80 = moderate; 0.60–0.70 = weak; 0.50–0.60 = bad (Safari et al., 2016).
3 RESULTS

Using imaging technology, selected DDR associated PTMs were recorded in a singleplex approach after 4 and 24 hr in two independent experiments. The following Figure 2 shows immunofluorescence-stained TK6 cells, which represent the substance-induced effects on the investigated protein markers compared to the control. The dose–response relationships were presented separately for each investigated biomarker as scatter dot plots (Figures 3–8). In order to avoid a false classification resulting from a biomarker response due to cytotoxicity (relative cell count in a defined field), test concentrations were excluded which reduced the relative cell count below 40%. Finally, ROC analyses were performed for each biomarker to determine whether they are suitable for the identification and characterization of genotoxic substances and ROS inducers (Figures 3–8).

FIGURE 2 Investigations in immunofluorescence-stained TK6 cells for the identification of proteomic biomarkers. Substance-induced effects in TK6 cells on the quantity of (a) pH 3 (S28), (b) p53 (K373), (c) Nrf2 (S40), (d) BubR1 (S640), (e) FANCD2 (S1404), and (f) 53BP1 (S1178). Using a commercial high-content imager equipped with a 20x objective, nuclei were detected via DAPI (blue) and proteins via AlexaFluor 488® (green) or Cy5 (red).
3.1 | Presence of 53BP1 (S1778)

With the exception of 5-fluorouracil (5-FU), nuclei stained positive for phosphorylated 53BP1 (S1778)-foci increased strongly after treatment with the clastogenic compounds hydroxyurea (HU) and etoposide (ETO) at 4 and 24 hr. The aneugens vinblastine (VB) and AMG-900 affected this parameter in one experiment. The early time point (AUC of 0.8798) was more sensitive for detecting clastogens than the later time point (AUC of 0.6746) (Figure 3).
3.2 | Presence of BubR1 (S670)

The phosphorylation of the mitotic checkpoint protein BubR1 (S670) was distinctly increased after treatment with aneugenic substances (VB, GF, and AMG-900) at early and late time points. In contrast, both clastogenic compounds ETO and 5-FU as well as the nongenotoxicant imatinib (IMA) had no effect on the spindle protein BubR1 (S670). Thus, ROC analyses revealed that a very good classification result was achieved when using the later timepoint (AUC: 0.9558) in contrast to the early time point (AUC: 0.7868) (Figure 4).

3.3 | Presence of pH 3 (S28)

Using the mitotic protein pH 3 (S28), almost all aneugens were detected at early time points whereas the clastogen HU did not change pH 3 (S28) levels, except for ETO and the nongenotoxicant CCCP, which moderately increased the biomarker level in one experiment. In accordance, ROC analysis indicated that the early time point was very well suitable for identifying aneugens (AUC: 0.9687) in contrast to the later time point (AUC: 0.8561) (Figure 5).

**FIGURE 3**  Analysis of 53BP1 (S1778). Shown are the scatter dot plots for the biomarker 53BP1 (S1778) in TK6 cells treated for 4 hr (left) and 24 hr (right) with various concentrations (each represented as one dot) of the given reference compounds (mean with SD) as well as the respective ROC curves for both time points (based on mean of independent experiments). (a) and (b) plots represent independent repeat experiments and in (c) the ROC curves are given (black lines), with red dotted lines representing random guess. AUC values are also given in the plots. The substances are colored according to their MoA: clastogens = red dots, aneugens = blue dots, and nongenotoxicants = gray dots. (*, **, *** indicate significance with p < .05, p < .01, p < .001, p < .0001 respectively, vs. negative control group according to one-way ANOVA and Dunn’s post-test)
3.4 | Presence of Nrf2 (S40)

Figure 6 shows the dose–response relationships of Nrf2 (S40) upon treatment with reference substances. The oxidative stress-associated protein was increased over vehicle controls in three out of four experiments especially after treatment with the potent ROS-inducing substances $\text{H}_2\text{O}_2$, and CCCP pertaining to the other MoA groups. The effect size of the Nrf2 (S40) increase was largest after 4 hr as determined by an AUC of 0.9031 pointing to a good classification result, whereby 24 hr resulted in an AUC of 0.7423 (Figure 6).

3.5 | Presence of p53 (K373)

The number of cells stained positive for acetylated p53 (K373) was specifically increased after 24 hr treatment with the

**FIGURE 4** Analysis of BubR1 (S670). Shown are the scatter dot plots for the biomarker BubR1 (S640) in TK6 cells treated for 4 hr (left) and 24 hr (right) with various concentrations (each represented as one dot) of the given reference compounds (mean with SD) as well as the respective ROC curves for both time points (based on mean of independent experiments). (a) and (b) plots represent independent repeat experiments and in (c) the ROC curves are given (black lines), with red dotted lines representing random guess. AUC values are also given in the plots. The substances are colored according to their MoA: clastogens = red dots, aneugens = blue dots and nongenotoxicants = gray dots. (*, **, *** , **** indicate significance with $p < .05, p < .01, p < .001, p < .0001$ respectively, vs. negative control group according to one-way ANOVA and Dunn’s post-test)
FIGURE 5  Analysis of pH 3 (S28). Shown are the scatter dot plots for the biomarker pH 3 (S28) in TK6 cells treated for 4 hr (left) and 24 hr (right) with various concentrations (each represented as one dot) of the given reference compounds (mean with SD) as well as the respective ROC curves for both time points (based on mean of independent experiments). (a) and (b) plots represent independent repeat experiments and in (c) the ROC curves are given (black lines), with red dotted lines representing random guess. AUC values are also given in the plots. The substances are colored according to their MoA: clastogens = red dots, aneugens = blue dots and nongenotoxicants = gray dots. (*, **, ***, **** indicate significance with $p < .05$, $p < .01$, $p < .001$, $p < .0001$ respectively, vs. negative control group according to one-way ANOVA and Dunn’s post-test).
**FIGURE 6** Analysis of Nrf2 (S40). Shown are the scatter dot plots for the biomarker Nrf2 (S40) in TK6 cells treated for 4 hr (left) and 24 hr (right) with various concentrations (each represented as one dot) of the given reference compounds (mean with SD) as well as the respective ROC curves for both time points (based on mean of independent experiments). (a) and (b) plots represent independent repeat experiments and in (c) the ROC curves are given (black lines), with red dotted lines representing random guess. AUC values are also given in the plots. The substances are colored according to their MoA: clastogens = red dots, aneugens = blue dots and nongenotoxicants = gray dots. (*, **, ---, **** indicate significance with $p < .05$, $p < .01$, $p < .001$, $p < .0001$ respectively, vs. negative control group according to one-way ANOVA and Dunn's post-test).
genotoxic compounds VB, AMG-900, ETO, and 5-FU whereby VB was not significant. The nongenotoxic substances IMA and CCCP did not affect significantly the biomarker. This is reflected in an AUC of 0.9987, pointing to a very good classification result compared to the early time point (AUC: 0.6607) (Figure 7).

3.6 | Presence of FANCD2 (S1404)

An increase of FANCD2 (S1404)-positive cells at both time points was characteristic for clastogens (ETO, HU, and 5-FU). The 24 hr time point was more suitable for identifying all tested clastogens in one experiment and yielded a good classification result.
DISCUSSION

Several studies were published over the past years demonstrating that the specificity of in vitro genotoxicity tests can be impaired by the use of p53-deficient mammalian cell lines and also by cytotoxicity measurements based on RCC (relative cell count) that might underestimate cytotoxicity compared to relative increase in cell count (RICC) or relative population doubling (RPD) (Kirkland et al., 2007; Fowler et al., 2014). Therefore, in order to improve the conventional in vitro methods, scientific workshops/committees such as ECVAM (European Centre for the Validation of Alternative Methods) or EFSA (European Food Safety Authority) recommended the use of...
p53-proficient human cell lines and more sensitive cytotoxicity metrics (RPD, RICC) as recommended, for example, in the pertinent OECD Test Guideline 487.

Moreover, novel in vitro biomarker assays have been developed to complement the standard battery given in ICH S2(R1) with in-depth MoA analysis. These advances contribute both to an improved genotoxicity assessment in drug development and discovery as well as to avoiding unnecessary animal testing (3Rs). Several companies such as Litron Laboratories (MultiFlow®) and Toxys (ToxTracker®) have developed such biomarker assays. Besides these activities, also toxicogenomics approaches demonstrated advances in the classification of (non)genotoxicants while presenting considerable workload (Ellinger-Ziegelbauer et al., 2009; Birrell et al., 2010; Williams et al., 2015). Previous studies have shown that in human HepG2 and TK6 cells it was possible to distinguish between genotoxic and non-genotoxic substances by gene expression analyses (Godderis et al., 2012; Li et al., 2015). However, these studies differ partly in the duration of substance treatment, cell models used, set of genes or markers and the statistical analysis to obtain the genomic signatures. All these differences may cause increased efforts for standardization prior to the use of toxicogenomics analyses complementing genotoxicity testing.

Consequently, the aim of the present work was the initial evaluation of selected mechanistic PTMs in a singleplex approach for the classification of future drug candidates according to their MoA, thus contributing to genotoxicity risk assessment. These mechanistic PTMs, selected by literature research, are mainly involved in DNA damage signaling pathways triggered by different MoA groups (aneugens/clastogens/pro-oxidants/nongenotoxicants): 53BP1 (S1778), FANCD2 (S1404), BubR1 (S670), pH 3 (S28), Nrf2 (S40), and p53 (K373). Thus, these PTMs were analyzed by image analysis in p53-competent TK6 cells after treatment with various concentrations of reference (non)genotoxicants representing different MoA groups, together with cytotoxicity data for obtaining RCC (cell count per visual field in a predefined area) in one workflow with the opportunity to multiplex MoA analyses. The compounds were selected based on their expected MoA and the known biomarker function. Further, we focused on chemicals which can be classified either "easily" (e.g., vinblastine) or "problematically" (5-FU) by existing methods such as the MultiFlow, as already published in Wilde et al. (2017) or in Bryce et al. (2017). Thus, the choice of compounds differed for the various biomarkers investigated and consequently, the total set of compounds was not studied with every candidate biomarker.

Mechanistic biomarkers for the identification of clastogenic substances: The PTMs 53BP1 (S1778) and FANCD2 (S1404) showed a characteristic response after treatment with clastogenic substances. The proportion of 53BP1 (S1778)-positive cells was increased at both time points after treatment with ETO and HU, but not after 5-FU treatment. The insensitivity of 53BP1 (S1778) to treatment with 5-FU is consistent with the biomarker γH2AX which similarly was not induced by 5-FU (Bryce et al., 2017; Wilde et al., 2017). It had already been demonstrated that the detection of 5-FU-induced DSBs in TK6 cells by recording γH2AX responses was not appropriate due to their metabolic constellation and p53 status (Oka et al., 2006; Khoury et al., 2011). In contrast, the marker FANCD2 (S1404) was favorable and detected all the clastogenic substances ETO, HU, and also—albeit with smaller effect size—5-FU, whose damage response was clearly different from the aneugenic and nongenotoxic substances.

Mechanistic biomarkers for the identification of aneugenic substances: The investigated mitosis checkpoint proteins BubR1 (S670) and pH 3 (S28) were induced after treatment with aneugenic substances at early and late time points. In contrast, no changes were observed for these markers upon treatment with either clastogenic or nongenotoxic substances. Thus, they are able to distinguish aneugens from clastogens and nongenotoxicants. Suijkerbuij et al. (2012) reported that NOC and PAC-treated cells displayed increased BubR1 (S670) levels, which was confirmed in this study using the aneugens VB, AMG-900, and GF. The phosphorylation of pH 3 (S28) showed similarly good results to the modification of serine 10 as was demonstrated in recent work by Bryce et al., 2017 and Wilde et al., 2017, with the main difference that pH 3 (S28) and BubR1 (S670) showed an effect by AMG-900 already after 4 hr treatment compared to pH 3 (S10) which was not increased before 24 hr of treatment. Functionally, pH 3 (S28) and BubR1 (S670) are associated with the M-phase, whereas pH 3 (S10) is associated with the late S and G2 phases. Typically, AMG-900 is an aneugen (kinase inhibitor) and led to increased pH 3 (S10) population and polyploidy at later time point (Bryce et al., 2017). In this work, AMG-900 not only led to an increase of pH 3 (S28), but also BubR1 (S670) at the early time point. Thus, the changes observed for pH 3 (S28) reflect the expected interference of AMG-900 with mitosis and are generally consistent with earlier work on pH 3 (S10) (Bryce et al., 2017; Wilde et al., 2017), albeit with different kinetics. However, BubR1 (S670) may complement pH 3 as it is relevant to the regulation of spindle checkpoint (direct evidence of aneugenic MoA) while the frequently used pH 3 is indicative of mitosis (indirect evidence of aneugenic MoA). In order to confirm this, additional experiments are warranted.

Mechanistic biomarkers for the identification of genotoxic substances: the increase of acetylated p53 (K373)-positive cells was useful at the late time point for detecting the genotoxic compounds VB, AMG-900, ETO, and 5-FU and for distinguishing them from non-genotoxic substances such as IMA and CCCP. Thus, p53 (K373) may be used as a holistic marker for discriminating genotoxicants from nongenotoxicants with a high degree for classification, thereby increasing the specificity of in vitro genotoxicity testing. They may contribute to risk assessment of genotoxicants especially when combined with markers indicative of a specific MoA.

Mechanistic biomarkers for the identification of ROS inducers: The oxidative stress-associated protein Nrf2 (S40) was increased by all compounds tested after 24 hr of incubation. In contrast, after 4 hr of incubation, the effect size was largest for the ROS inducers H2O2 and CCCP while substances pertaining to the other MoA groups displayed no increase or an increase with much smaller effect size, respectively, in the two independent repeats. Thus, a genotoxic clastogen inducing an Nrf2 increase may be classified additionally as a ROS inducer, which is a widely accepted threshold-based mechanism.
of genotoxicity. However, given that nongenotoxicants may also induce Nrf2, prior classification of a compound as genotoxicant or nongenotoxicant is warranted for a sound interpretation of an Nrf2 increase, which can be followed up by combined acetylated p53 analysis which has implications on risk assessment. Nevertheless, the role of Nrf2 warrants further investigation of its ability to detect ROS inducers. This can be accomplished by including compounds such as menadione as well as benchmarking the Nrf2 (S40) data against 2',7'-dichlorofluorescein diacetate (DCFDA), a reagent for the detection of reactive oxygen species. So far, early (stable) increased Nrf2-positive cells were associated with ROS inducing compounds (H$_2$O$_2$) or cytotoxicity leading to ROS (CCCP).

Future method development studies will be designed to evaluate all endpoints across all chemicals studied in order to comprehensively assess the performance of the proposed methodology as a whole. As discussed, in recent years many activities have been initiated using biomarkers (transcriptional and protein modifications) for the identification and classification of genotoxicants to support genotoxicity (risk) assessment of drug candidates (Westerink et al., 2010; Hughes et al., 2012; Rajakrishna et al., 2014; Li et al., 2015; Williams et al., 2015; Hendriks et al., 2016; Khoury et al., 2016). The advantages of the early singleplex approach presented herein comprise the inclusion of biomarkers for (a) the identification of ROS inducers allowing us to refine the MoA information and (b) the identification of aneugens and clastogens with causal relationship to the MoA: BubR1, a marker for aneugens, is relevant to the regulation of spindle checkpoint. Similarly, the induction of 53BP1, a marker for clastogens, is a direct consequence of DSBs while the commonly used γH2AX is also associated with apoptosis, requiring a prior exclusion of apoptotic/necrotic cells in alternative flow cytometric assays (Johansson et al., 2017). This can be seen from results for the nongenotoxicant CCCP which was negative for 53BP1, while the clastogen hydroxyurea induced a strong effect, without prior exclusion of apoptotic cells. The same applies to FANCD2.

The comparison with recently available in vitro methods revealed that there are a few methods able to differentiate between aneugenic versus clastogens and genotoxicants versus nongenotoxicants (including labor-intensive toxicogenomics assays using large sets of genes) simultaneously determining cytotoxicity in one step (Ellinger-Ziegelbauer et al., 2009; Li et al., 2015; Lan et al., 2016; Bryce et al., 2018). Therefore, the current early work constitutes an important contribution to identifying mechanistic biomarkers complementing existing assays or representing new methodologies. Furthermore, the newly identified biomarkers presented herein help further improve the classification results, use causally related in addition to mere correlative effects for classification and further specify a genotoxicant’s MoA (e.g., clastogenic substances acting via ROS induction).

Taken together, the results presented herein look promising but warrant further investigations given that they reside on a proof-of-principle level. This was achieved using a small set of carefully chosen compounds that were considered difficult to classify based on prior work. The good to very good classification results (AUC) reported herein need to be corroborated in future work including a larger set of compounds, hopefully showing that these results will hold up across a more diverse chemical space. Further, the generated ROC curves represent the values of sensitivity to specificity and thereby might be used for determining thresholds after increasing the number of compounds in subsequent studies. Ultimately, the most valuable biomarkers can be experimentally combined into a multiplex approach for classifying unknown compounds in one experimental run and the subsequent submission of the data to a prediction model similar to the previous development of the MultiFlow® by Bryce and colleagues.

5 | CONCLUSION

In summary, the data presented herein on selected PTMs, which are functionally involved in the substance-induced DDR supported a reliable identification of aneugens (BubR1 (S670) and pH 3 (S28)), clastogens (53BP1 (S1778) and FANCD2 (S1404)), ROS inducers (Nrf2 (S40)) and a differentiation between genotoxicants and nongenotoxicants by acetylated p53 (K373) in a singleplex approach by high content imaging. To improve and validate the aforementioned PTMs by increasing the number of compounds and their future use for deriving mechanistic signatures of unknown drug candidates (clastogens, aneugens, nongenotoxicants, and ROS inducers), additional work is warranted given the proof-of-concept nature of this report. Further work will focus on including more chemicals, multiplexing the most valuable biomarkers into a single-step analysis method, and developing more sophisticated data analysis strategies that consider the multiple biomarkers simultaneously as opposed to one at a time as described herein. Nevertheless, the studies carried out in the present study showed that dose–response relationships (primarily high concentrations) and the choice of concentrations strongly influence the reliable identification of a biomarker. The findings presented herein showed that the drug characterization was improved using the evaluated PTMs and strongly support the combined use (multiplexed approach) of these identified biomarkers enabling a rapid screening of genotoxic hazard. It should be emphasized that novel in vitro methods help avoid in vivo follow-up tests in the context of ambiguous results and/or to identify potent geno(toxic) compounds early by the application as a screening method, so that by modifying the lead structure, less potent genotoxicants may be submitted to animal studies.

AUTHOR CONTRIBUTIONS

S.W., N.Q., and A.S. designed the study, interpreted the data, and created the manuscript. S.W. performed the experiments and analyzed the data. S.W. developed the automated image analysis algorithms.

DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

Appella, E. and Anderson, C.W. (2001) Post-translational modifications and activation of p53 by genotoxic stresses. European Journal of Biochemistry, 268, 2764–2772.
Birrell, L., Cahill, P., Hughes, C., Tate, M. and Walmsley, R.M. (2010) GADD45a-GFP GreenScreen HC assay results for the ECVAM recommended lists of genotoxic and non-genotoxic chemicals for assessment of new genotoxicity tests. *Mutation Research*, 695(1–2), 87–95.

Boisvert, R.A. and Howlett, N.G. (2014) The Fanconi anemia ID2 complex: dualing saxes at the crossroads. *Cell Cycle*, 13(19), 2999–3015.

Brooks, C.L. and Gu, W. (2003) Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. *Current Opinion in Cell Biology*, 15(2), 164–171.

Bryce, S.M., Bernacki, D.T., Bemis, J.C. and Dertinger, S.D. (2016) Genotoxic mode of action predictions from a multiplexed flow cytometric assay and a machine learning approach. *Environmental and Molecular Mutagenesis*, 57(3), 171–189.

Bryce, S.M., Bernacki, D.T., Bemis, J.C., Spellman, R.A., Engel, M.E., Schuler, M., Lorge, I., Heikkinen, P.T., Hemmann, U., Thybaud, V., Wilde, S., Queisser, N., Sutter, A., Zeller, A., Guérard, M., Kirkland, D. and Dertinger, S.D. (2017) Interlaboratory evaluation of a multiplexed high information content in vitro genotoxicity assay. *Environmental and Molecular Mutagenesis*, 58(3), 146–161.

Bryce, S.M., Bernacki, D.T., Smith-Roe, S.L., Witt, K.L., Bemis, J.C. and Dertinger, S.D. (2018) Investigating the generalizability of the Multi-Flow® DNA damage assay and several companion machine learning models with a set of 103 diverse test chemicals. *Toxicological Sciences*, 162(1), 146–166.

Chan, K.S., Koh, C.G. and Li, H.Y. (2012) Mitosis-targeted anti-cancer therapies: where they stand. *Cell Death & Disease*, 3, e41 e111.

Chondrou, V., Trochoutsou, K., Panayides, A., Efthimiou, M., Stephanou, G. and Demopoulos, N.A. (2018) Combined study on clastogenic, aneugenic and apoptotic properties of doxorubicin in human cells in vitro. *Journal of Biological Research*, 25, 17.

Chung, S.K., Zhu, S., Xu, Y. and Fu, X. (2014) Functional analysis of the acetylation of human p53 in DNA damage response. *Protein & Cell*, 5 (7), 544–551.

Doktorova, T.Y., Ates, G., Vinken, M., Vanhaecke, T. and Rogiers, V. (2014) Way forward in case of a false positive in vitro genotoxicity result for a cosmetic substance? *Toxicology In Vitro*, 28(1), 54–59.

Ellinger-Zieglerbauer, H., Aubrecht, J., Kleinjans, J.C. and Ahr, H.J. (2009) Application of toxicogenomics to study mechanisms of genotoxicity and carcinogenicity. *Toxicology Letters*, 186, 36–44.

Elowe, S., Dulla, K., Uldschmid, A., Li, X., Dou, Z. and Nigg, E.A. (2010) Uncoupling of the spindle-checkpoint and chromosome-congression functions of BubR1. *Journal of Cell Science*, 123(Pt 1), 84–94.

Fabbro, D. (2015) 25 years of small molecular weight kinase inhibitors: potentials and limitations. *Molecular Pharmacology*, 87(5), 766–775.

Feng, L., Li, N., Li, Y., Wang, J., Gao, M., Wang, W. and Chen, J. (2015) Cell cycle-dependent inhibition of 53BP1 signaling by BRCA1. *Cell Discovery*, 1, 15019.

Fowler, P., Smith, R., Smith, K., Young, J., Jeffrey, L., Carmichael, P., Kirkland, D. and Pfuhrer, S. (2014) Reduction of misaligning (“false”) positive results in mammalian cell genotoxicity assays: III: sensitivity of human cell types to known genotoxic agents. *Mutation Research, Genetic Toxicology and Environmental Mutagenesis*, 767, 28–36.

Godderis, L., Thomas, R., Hubbard, A.E., Tabish, A.M., Hoet, P., Zhang, L., Smith, M.T., Veulemans, H. and CM, M.H. (2012) Effect of chemical mutagens and carcinogens on gene expression profiles in human TK6 cells. *PLoS One*, 7(6), e39205.

Gollapudi, P., Hasegawa, L.S. and Eastmond, D.A. (2014) A comparative study of the aneugenic and polyploidy-inducing effects of fisetin and two model Aurora kinase inhibitors. *Mutation Research, Genetic Toxicology and Environmental Mutagenesis*, 767, 37–43.

Haupt, Y., Maya, R., Kazaz, A. and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature*, 387, 296–299.

Hendriks, G., van de Water, B., Schoonen, W. and Vrielings, H. (2016) The extended ToxTracker assay discriminates between induction of DNA damage, oxidative stress, and protein misfolding. *Toxicological Sciences*, 150, 190–203.

Howlett, N.G., Taniguchi, T., Durkin, S.G., D’Andrea, A.D. and Glover, T.W. (2005) The Fanconi anemia pathway is required for the DNA replication stress response and for the regulation of common fragile site stability. *Human Molecular Genetics*, 14(5), 693–701.

Hughes, C., Rabinowitz, A., Tate, M., Birrell, L., Allsup, J., Billington, N. and Walmsley, R.M. (2012) Development of a high-throughput Gaussian luciferase reporter assay for the activation of the GADD45a gene by mutagens, promutagens, clastogens, and aneugens. *Journal of Biomolecular Screening*, 17(10), 1302–1315.

Ichimura, Y., Waguri, S., Sou, Y.S., Kageyama, S., Hasegawa, J., Ishimura, R., Saito, T., Yang, Y., Kouno, T., Fukutomi, T., Hoshii, T., Hirao, A., Takagi, K., Mizushima, T., Motohashi, H., Lee, M.S., Yoshimori, T., Tanaka, K., Yamamoto, M. and Komatsu, M. (2013) Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. *Molecular Cell*, 51(5), 618–631.

Jimenez-Blasco, D., Santofimia-Castaño, P., Gonzalez, A., Almeida, A. and Bolaños, J.P. (2015) Astrocyte NMDA receptor’s activity sustains neuronal survival through a Cdk5-Nrf2 pathway. *Cell Death and Differentiation*, 22(11), 1877–1889.

Johansson, P., Fath, E., Ek, T., and Hammarsten, O. (2017) Validation of a flow cytometry-based detection of γ-H2AX, to measure DNA damage for clinical applications. *Cytometry Part B - Clinical Cytometry*, 92(6), 534–540.

Juan, G., Bush, T.L., Ma, C., Manoukian, R., Chung, G., Hawkins, J.M., Zoog, S., Kendall, R., Radinsky, R., Loberg, R., Friberg, G. and Payton, M. (2014) AMG 900, a potent inhibitor of aurora kinases causes pharmacodynamic changes in p-histone H3 immunoreactivity in human tumor xenografts and proliferating mouse tissues. *Journal of Translational Medicine*, 12, 307.

Khoury, G.A., Baliban, R.C. and Floudas, C.A. (2011) Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. *Scientific Reports*, 1, 90.

Khoury, L., Zalko, D. and Audebert, M. (2016) Evaluation of four human cell lines with distinct biotransformation properties for genotoxic screening. *Mutagenesis*, 31(1), 83–96.

Kirkland, D., Pfuhler, S., Tweets, D., Aardema, M., Corvi, R., Darroudi, F., Elhajouji, A., Glatt, H., Hastwell, P., Hayashi, M., Kaper, P., Kirchner, S., Lynch, A., Marzin, D., Maurici, D., Meunier, J.R., Müller, L., Nohyne, G., Parry, J., Parry, E., Thybaud, V., Tice, R., van Benthem, J., Vanparys, P. and White, P. (2007) How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: report of an ECVAM workshop. *Mutation Research*, 628(1), 31–55.

Lan, J., Gou, N., Rahman, S.M., Gao, C., He, M. and Gu, A.Z. (2016) A quantitative toxicogenomics assay for high-throughput and mechanistic genotoxicity assessment and screening of environmental pollutants. *Environmental Science & Technology*, 50(6), 3202–3214.

Lee, J.H., Cheong, H.M., Kang, M.Y., Kim, S.Y. and Kang, Y. (2009) Ser1778 of 53BP1 plays a role in DNA double-strand break repairs. *Korean Journal of Physiology & Pharmacology*, 13(5), 343–348.

Lee, W.J., Kim, S.C., Lee, S.J., Lee, J., Park, J.H., Yu, K.S., Lim, J. and Kwon, S.W. (2014) Investigating the different mechanisms of genotoxic and non-genotoxic carcinogens by a gene set analysis. *PLoS One*, 9(1), e86700.

Li, H.H., Hyduke, D.R., Chen, R., Heard, P., Yau, C.L., Aubrecht, J. and Fornace, A.J., Jr. (2015) Development of a toxicogenomics signature for genotoxicity using a dose-optimization and informatics strategy in human cells: optimization and informatics strategy in human cells. *Environmental and Molecular Mutagenesis*, 56(6), 505–519.

Liu, L., Scolnick, D.M., Trievel, R.C., Zhang, H.B., Marmorstein, R., Halazonetis, T.D. and Berger, S.L. (1999) p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. *Molecular and Cellular Biology*, 19(2), 1202–1209.
