



The Automated FADU-Assay, a Potential High-Throughput *In Vitro* Method for Early Screening of DNA Breakage

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Summary

Genotoxicity tests are essential to identify compounds that have a potential to compromise not only the environment but also human and animal health, including compounds that increase the risk of cancer. At present, no single test is capable of detecting all types of genotoxic effects; therefore a battery of *in vitro* and, if positive, *in vivo* tests is necessary to determine the genotoxicity of a substance. However, the respective specificities of current assays have been criticized for their high percentage of false positive results. We recently published an automated version of the “Fluorimetric detection of Alkaline DNA Unwinding” (FADU) assay for measuring DNA strand breaks in human peripheral blood mononuclear cells or in cell lines. Using this new technology we show detection of DNA strand breaks in cells treated with several compounds known to induce DNA strand breaks by various mechanisms. We also tested toxic compounds that were not expected to induce DNA strand breaks; these were negative in the assay as expected. Finally, we included zinc oxide nanoparticles of high production volume to explore further fields of potential FADU applications. The main advantages of this assay are high reproducibility, easy handling, lack of operator bias, high-throughput, speed, and low cost.

Keywords: DNA strand breaks, method validation, nanoparticles, genotoxicity, clastogenicity

1 Introduction

Exposure to radiation, environmental agents, or medicinal drugs may induce different types of DNA damage in organisms, which may lead to mutations, genetic instability, and epigenetic alterations and may cause a variety of diseases, including cancer or even death. Current regulatory measures to identify genotoxic compounds include the following: bacterial reverse mutation (Ames) test, *in vitro* cell gene mutation test, *in vitro* chromosomal aberration test, *in vitro* mammalian cell micronucleus test, *in vitro* sister chromatid exchange test, *in vitro* unscheduled DNA synthesis test, *Saccharomyces cerevisiae* gene mutation assay, and *Saccharomyces cerevisiae* mitotic recombination assay. Multiple DNA repair pathways have evolved to minimize the negative effects of DNA damage. Several methods have been established to investigate DNA lesions and different DNA repair pathways in the cell. During the last decade, methods such as fluorescence *in situ* hybridization (FISH), TdT-mediated dUTP-biotin nick end labelling (TUNEL), and single cell gel electrophoresis (“comet”) assay have been improved and have been applied in different fields, including pharmacological, toxicological, epidemiological, and occupational health stud-

ies (Cordelli et al., 2005). The most commonly used method to measure DNA damage is the comet assay, which detects DNA strand breaks and alkaline labile sites in individual cells (Olive and Banáth, 2006).

There is a vast body of literature describing the use of the comet assay, but the results obtained by different research groups are controversial. The European Centre for the Validation of Alternative Methods (ECVAM) is working on the validation of the comet assay, and the European Comet Assay Validation Group (ECVAG) recently reported an inter-laboratory coefficient of variation (CV) of 47%, which could be reduced to 28% using reference standards (Forchhammer et al., 2010). There are large differences in the values of DNA damage reported for leukocytes from healthy humans, and it is not clear whether this is due to the use of different comet assay protocols or to some real biological differences between the populations studied in different countries (Møller, 2006). Many steps of the comet assay protocol (including slide preparation and electrophoresis) affect both intra-assay variability and inter-assay reproducibility (Møller et al., 2010). In general, automation of a method contributes to reducing the variability of results and increases reproducibility; furthermore, automated systems are characterized



by higher throughput. Several modifications of the comet assay have been reported over the past years (Kiskinis et al., 2002; Baert and Van Oostveldt, 2003; Morley et al., 2006; Cordelli et al., 2007; Wooda et al., 2010), however, its automation has only been successful in some of the experimental steps (Frieauff et al., 2001; Wooda et al., 2010).

It has been reported that each chemical that goes through the multiple tests required for registration can use up to 5,000 animals, or even 12,000 if the chemical is a pesticide. The cost of doing this for the 30,000 unregistered chemicals so that they comply with REACH has been estimated at between € 5 and € 10 billion (US\$ 6-12 billion) (Abbott, 2005). Testing a single chemical for carcinogenicity takes five years and involves 400 rats. Such tests are dramatically over-predictive: more than 50% of the results are positive, of which 90% are false positive (Gold et al., 2005). The relatively high percentage of false positive results for rodent carcinogenicity associated with clastogenicity assays is of considerable concern. As a consequence, human cell-based alternatives to clastogenicity assays are needed for early screening of human genotoxicity (Witte et al., 2007). Furthermore, the increasing demands for chemical safety assessment call for validation of alternative methods to reduce animal experimentation (Cordelli et al., 2007).

By using commonly accepted clastogenic as well as nonclastogenic but otherwise cytotoxic substances we assessed the suitability of the automated FADU assay to reliably detect DNA strand breaks. Going beyond this "proof of principle" approach, we also used ZnO nanoparticles to identify potential additional areas of application for the automated FADU technology. These nanoparticles have been shown to be cytotoxic and most likely are also able to induce DNA strand breaks.

2 Material and methods

Compounds

Methyl-methanesulfonate (MMS), ethyl-methanesulfonate (EMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 4-nitroquinoline 1-oxide (4-NQO), vinblastine, staurosporine, rotenone, camptothecin, etoposide and hydroxyurea (HU) were purchased from Sigma-Aldrich Co. (Steinheim, Germany). MMS, EMS, MNNG, 4-NQO, staurosporine, camptothecin and etoposide were stored as stock solutions in 100% DMSO (Merck, Darmstadt, Germany) at -20°C. Aliquots were thawed and diluted with RPMI cell culture medium (Invitrogen, Darmstadt, Germany) before each experiment. The final DMSO concentration did not exceed 1%. HU, vinblastine, and rotenone were stored as dry substances at 8°C (HU) or room temperature (vinblastine and rotenone); all substances were dissolved in dH₂O or RPMI-1640 medium before each experiment.

Nanomaterial

For each experiment a suspension (1 mg/ml) of ZnO (ZncoxTM 10; IBUtec) nanoparticles (NP) was prepared freshly. Stock suspensions were sonicated in an ultrasound bath for 10 min-

utes and serial dilutions in bidistilled H₂O were prepared. Final concentrations in cell culture medium are given in µg/ml. A minimal characterization was provided by the manufacturer (see data sheet).

Cells

The human lymphoid cell line Jurkat (clone E61) was purchased from the American Type Culture Collection (ATCC), Wesel, Germany. Cells were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO₂ in a humidified atmosphere. Medium and supplements were purchased from Invitrogen (Darmstadt, Germany), FCS from Biochrom (Berlin, Germany).

The human alveolar epithelial cell line A549 (ATCC, CCL-185) was obtained from ATCC. Cells were grown in RPMI-1640 medium (Sigma-Aldrich, Munich, Germany) supplemented with 10% FCS, 0.2 mg/ml L-glutamine, 50 µg/ml penicillin, 50 µg/ml streptomycin, and 100 µg/ml neomycin at 37°C and in a 5% CO₂ atmosphere. Medium was replaced every third day and cells were passaged routinely once a week. Medium supplements were purchased from Invitrogen (Darmstadt, Germany), FCS from Lonza (Basel, Switzerland).

For preparation of human peripheral blood mononuclear cells, venous blood was taken from healthy volunteers aged 24-53 years. Peripheral blood mononuclear cells (PBMC) were isolated using Biocoll (Biochrom, Berlin, Germany) density gradient centrifugation.

Treatment

Jurkat cells were counted using a Neubauer chamber, pelleted (5 min, 200 g) and resuspended in fresh medium at 2 x 10⁶ cells per ml. Drug treatment was carried out in RPMI-1640 medium without FCS for 10, 20, or 30 min at various concentrations.

Human peripheral blood mononuclear cells (PBMC) were counted using a Neubauer chamber, pelleted (5 min, 200 g) and resuspended in fresh medium at 4 x 10⁶ cells per ml. 4-NQO, MMS and MNNG treatment was carried out in RPMI-1640 medium without FCS at 37°C at various concentrations. After 10, 20, or 30 min drug exposure DNA strand breaks were analyzed using the automated FADU assay. Staurosporine was added at a final concentration of 3 µM, cells were cultured in RPMI-1640 medium with 10% FCS at 37°C and analyzed using flow cytometry for apoptosis/necrosis and automated FADU for DNA strand breaks after 1.5, 4, 7, and 16 h, respectively.

A549 cells were detached from T75 cell culture flasks using trypsin/EDTA (Invitrogen, Darmstadt, Germany). Cells were counted using a Neubauer chamber, pelleted (5 min, 200 g) and resuspended in fresh medium at 4 x 10⁵ cells per ml. Each sample used 0.5 ml cell suspension and either 80 µl of the respective nanoparticle dilutions or 15 mM ethyl methanesulfonate (EMS) as a positive control. Cells were incubated for 5 min, 30 min, or 3 h at 37°C and 5% CO₂ in 2 ml Eppendorf cups or were used immediately. As a positive control, one sample was exposed to 6 Gy of irradiation.

Quantification of DNA strand breaks

We used the automated FADU assay (Moreno-Villanueva et al., 2009), a very sensitive *in vitro* method to assess DNA strand breaks and DNA repair. The detection of DNA strand breaks and repair is based on progressive DNA unwinding (denaturation) under highly controlled conditions of alkaline pH, time, and temperature. The starting points for the unwinding process are DNA strand interruptions, such as replication forks or chromosome ends, but also DNA strand breaks induced by reactive oxygen species (ROS), irradiation, or chemical compounds. For monitoring alkaline DNA unwinding, a commercially available fluorescent dye (Sybr[®]Green, Invitrogen, Darmstadt, Germany) is used as a marker for double stranded DNA. Fluorescence intensity is expressed as a percentage of fluorescence in control cells without induced DNA damage (P_0). A decrease in the fluorescence intensity of Sybr[®]Green indicates an increase of DNA unwinding and, consequently, a higher number of DNA strand breaks.

Quantification of apoptotic and necrotic cells

After treatment cells were centrifuged and resuspended in binding buffer (0.14 M NaCl, 2.5 mM CaCl₂, 0.01 M HEPES, pH 7.4) at 1×10^6 cells/ml. 2.5 μ l FITC Annexin V (Sigma, Germany) and 2.5 μ l propidium iodide (Sigma, Germany) were added to 50 μ l cell suspension. The cells were gently mixed and incubated for 15 min at RT in the dark. After incubation, 200 μ l of binding buffer was added and analyzed by flow cytometry within one hour. Two-color flow cytometric analyses were performed on a FACSsort (Becton-Dickinson). The fraction of early apoptotic cells was calculated by measuring the percentage of Annexin V-positive and PI-negative cells. The fraction of late-apoptotic and necrotic cells (including debris) was calculated by measuring the percentage of PI-positive cells.

Presentation of data

All experiments were carried out in quadruplicates (four wells for each experimental condition) and were independently re-

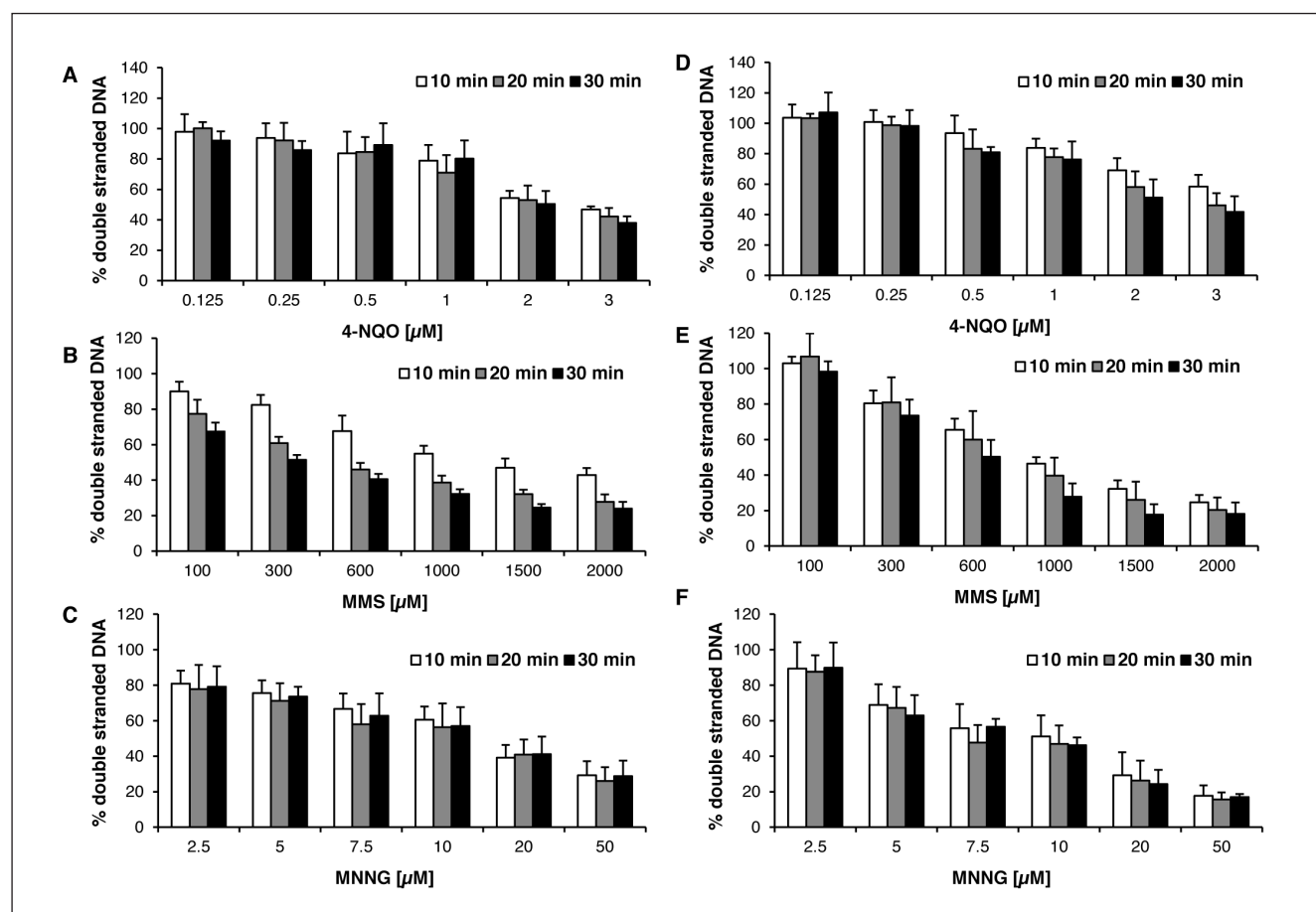


Fig. 1: DNA strand breaks induced by carcinogens

Jurkat cells (A,B,C) and PBMC (D,E,F) were treated with several concentrations of 4-NQO, MMS and MNNG for 10, 20, and 30 min at 37°C as indicated. DNA strand break formation was measured using the automated FADU assay. In all cases an increase in DNA strand breaks could be detected in a concentration-dependent manner, as revealed by diminished SybrGreen[®] fluorescence. In addition, time dependent DNA strand break formation could be observed after MMS treatment. Error bars represent standard deviations of three independent experiments. Statistical significance ($p < 0.05$ by two-way ANOVA with Bonferroni post-test) between exposure times was found only in Jurkat cells for MMS comparing 10 with 20 and 10 with 30 min.



peated at least three times (on separate days). Data are expressed as a percentage of the control value P₀ where the cells were not exposed to any of the agents tested. Bars represent the mean with standard deviations of at least three separate experiments. The average coefficient of variation was 8.98% for Jurkat cells and 13.3% for PBMC.

3 Results

3.1 DNA strand breaks induced by carcinogens (MNNG, MMS, 4-NQO)

Both MMS and MNNG modify DNA by adding methyl groups to a number of nucleophilic sites on the DNA bases; MNNG produces a greater percentage of O-methyl adducts (Wyatt and Pittman, 2006).

The carcinogenic and mutagenic properties of 4-nitroquinoline 1-oxide (4-NQO) were first reported in 1957 (Nakahara et al., 1957). 4-NQO is thought to elicit its carcinogenicity by producing DNA adducts after being metabolized to 4-hydroxyaminoquinoline 1-oxide, which induces 8-hydroxydeoxyguanosine (8-OHdG), i.e. by oxidative damage (Arima et

al., 2006). 4-NQO also produces DNA single strand breaks (Baohong et al., 2005).

The DNA strand breaks detected after 10, 20, and 30 min of treatment with 4-NQO (A,D) or MMS (B,E) or MNNG (C,F) are shown in Figure 1. The decrease in fluorescence intensity with increasing drug concentrations indicates a concentration-dependent accumulation of DNA strand breaks. A time dependency in DNA strand break formation was only observed for MMS treatment.

3.2 DNA strand breaks induced by anti-tumor drugs (camptothecin, etoposide, hydroxyurea, vinblastine)

Camptothecin and etoposide are anti-tumor drugs that belong to the class of topoisomerase inhibitors. The cytotoxicity of these drugs stems from their ability to stabilize a covalent complex between DNA and DNA topoisomerase I and II, respectively, which results in a high level of DNA damage (Rothenberg, 1997; Montecucco and Biamonti, 2006). As an antineoplastic agent, the specific action of HU is on ribonucleotide reductase, whose action is to reduce ribonucleotides to deoxyribonucleotides. HU impedes the latter reaction and thus limits DNA

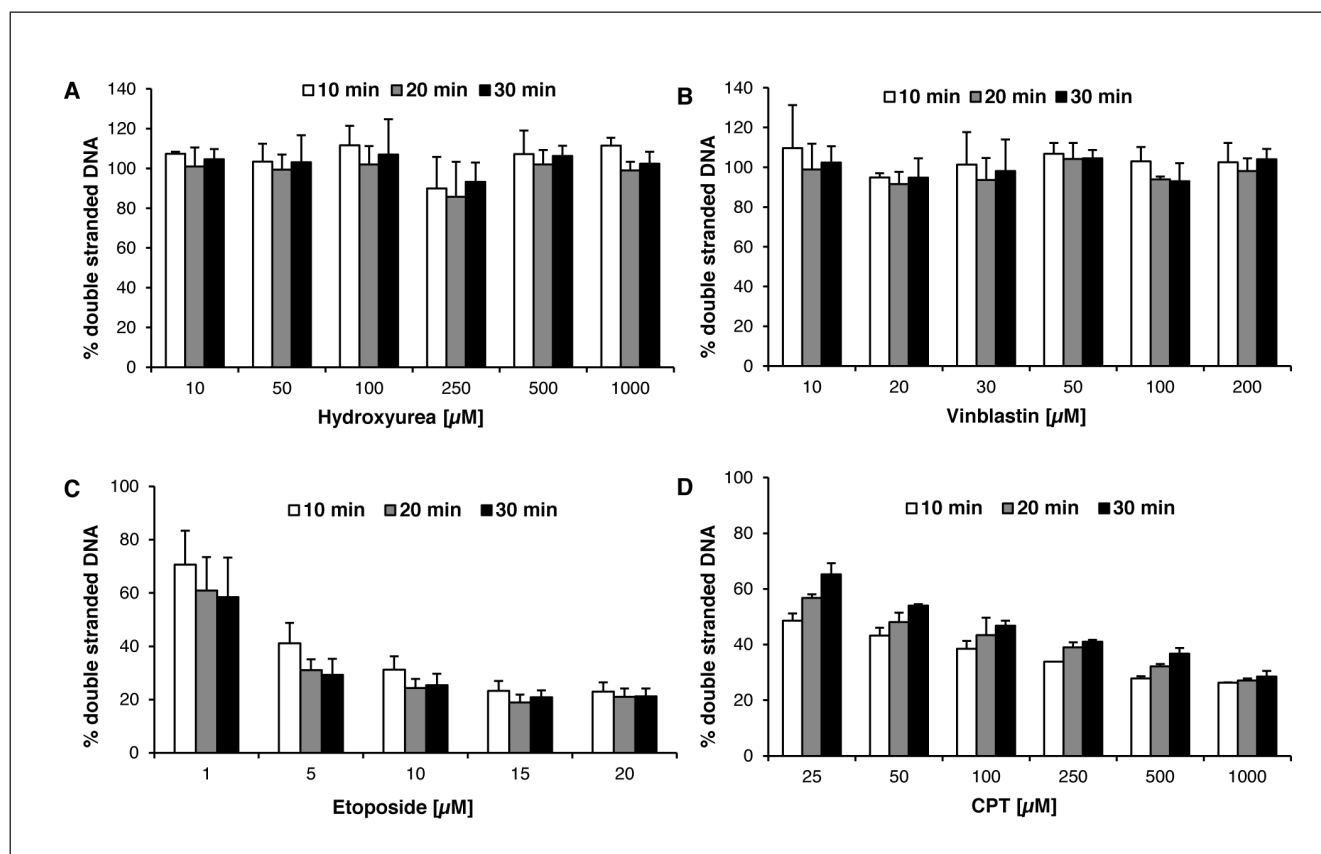


Fig. 2: Strand breaks induced by anti-tumor drugs

Jurkat cells were treated with several concentrations of hydroxyurea, vinblastine, etoposide, or camptothecin (CPT) for 10, 20, and 30 min at 37°C. DNA strand break formation was measured using the automated FADU assay. In the case of etoposide and camptothecin, an increase of DNA strand breaks could be detected in a concentration-dependent manner. By contrast, for hydroxyurea and vinblastine, no DNA strand breaks were detectable after 30 min drug exposure. Error bars represent standard deviations of three independent experiments.

biosynthesis. This makes HU an S-phase-specific cytotoxic and antineoplastic agent that interrupts the cell cycle at the G1 and S phases (Jones et al., 2009). Repair of DNA damaged by chemicals or irradiation may also be inhibited by hydroxyurea (Yarbro, 1992). Vinblastine provokes the destabilization of microtubules at minus ends and the stabilization at plus ends and may contribute to the altered function of mitotic spindle microtubules (Panda et al., 1996). This leads to mitotic arrest and subsequent cell death by apoptosis (Kolomeichuk et al., 2008). Not all types of anti-tumor drugs induce DNA strand breaks. Since HU provokes predominantly cell cycle arrest and vinblastine only acts during cell division, formation of DNA strand breaks is not expected after short-term exposure (Fig. 2A,B). In the case of etoposide and camptothecin, the inhibition of topoisomerase induces DNA strand breaks, which can be detected in a concentration-dependent manner (Fig. 2C,D).

3.3 Toxicity without DNA strand breaks (rotenone)

The mitochondrial complex I inhibitor rotenone induces apoptosis by enhancing mitochondrial ROS production (Li et al., 2003). In Jurkat cells treated with rotenone at concentrations from 1 to 6 μM , no DNA strand break formation was detectable after 10, 20, and 30 min of drug exposure (Fig. 3A). In contrast, rotenone-induced cytotoxicity could be demonstrated using flow cytometry (Fig. 3B).

3.4 DNA strand breaks induced during apoptosis (staurosporine)

Staurosporine-treated PBMC are known to show a decrease in mitochondrial membrane potential ($\Delta\Psi\text{m}$) with cytochrome-c release and a clear caspase 3 activation inducing apoptosis (Louagie et al., 1999). We treated PBMC with 3 μM staurosporine and analyzed, in parallel, DNA strand break for-

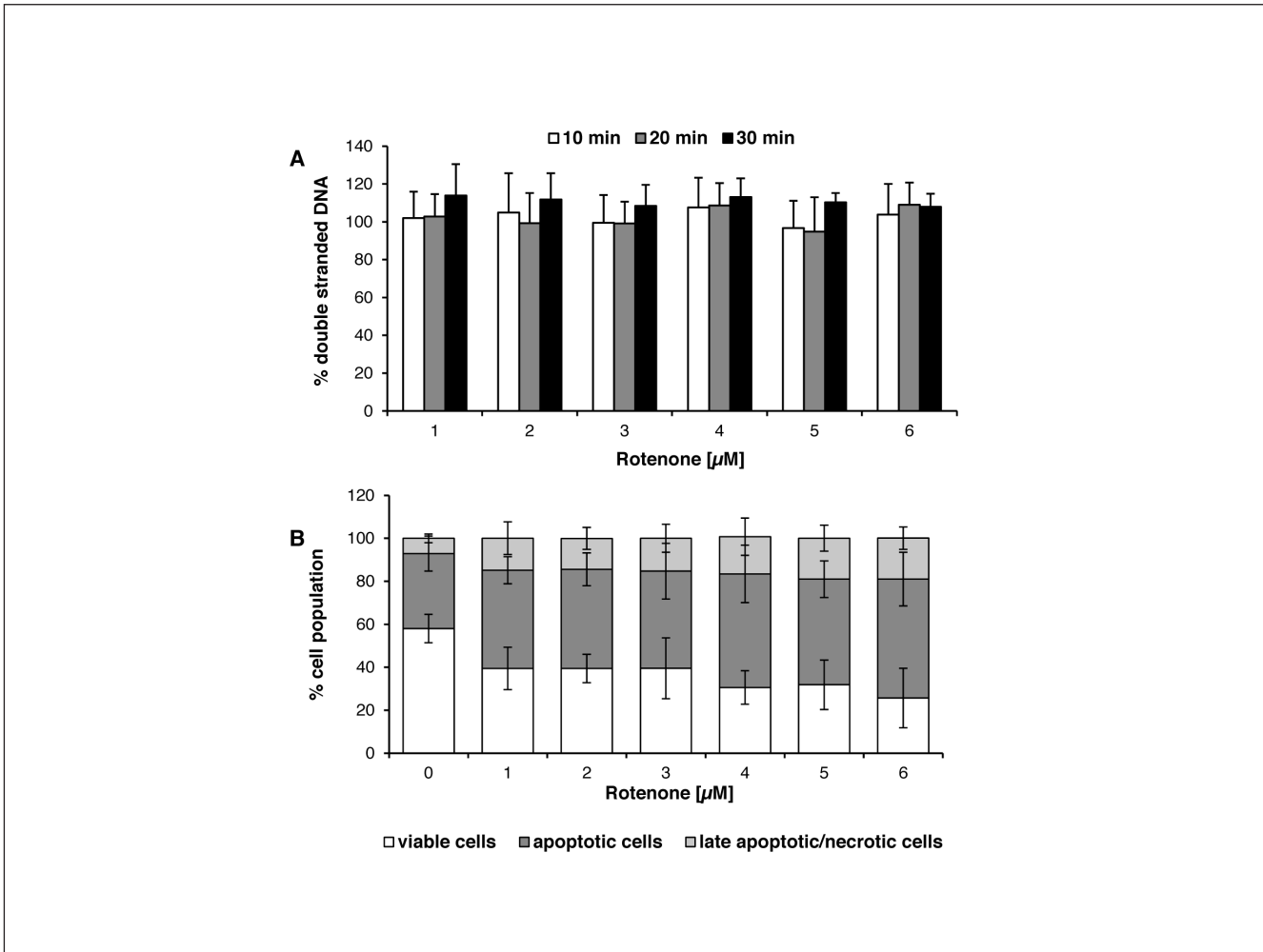


Fig. 3: Toxicity without DNA strand breaks

Jurkat cells were treated with several concentrations of rotenone for 10, 20 and 30 min at 37°C. DNA strand break formation was measured using the automated FADU assay (A). For flow cytometry analysis cells were incubated with the same rotenone concentrations as used for the FADU assay. Rotenone was removed from medium by centrifugation after 30 minutes and cells were resuspended in fresh medium supplemented with 10% FCS. The percentage of viable cells was measured after 24 h (B). No significant DNA strand break induction could be measured while a decrease of viable cells could be detected with increasing rotenone concentrations. Error bars represent standard deviations of three independent experiments.



mation by FADU and number of apoptotic/necrotic cells by flow cytometry. The results indicate an increase in both DNA strand breaks and number of apoptotic/necrotic cells in a time-dependent manner (Fig. 4A,B). Staurosporine did not induce significant DNA strand breaks in PBMC after 10, 20 or 30 minutes of treatment (Supplementary Fig. A at www.altex-edition.org)

3.5 Clastogenicity of nanoparticles (ZnO)

ZnO nanoparticles are widely used in a broad range of applications, including manufacturing of rubber, cosmetics, pigments, food additives, medicine, chemical fibers, electronics, paints, and solar cells (Lin et al., 2009; Song et al., 2010). Most *in vitro* studies show a rather high toxicity of ZnO nanoparticles towards cells of different tissues and organisms. The concentration-response relationship reveals a very steep curve where toxic effects abruptly start at concentrations between 10 and 20 $\mu\text{g}/\text{ml}$. Although the exact mechanism is not fully understood and remains controversial, zinc ions and reactive oxygen

species may be involved in the toxic effects (Xia et al., 2008; Song et al., 2010). We detected DNA strand break induction by ZnO nanoparticles after treatment of A549 cells at 160 $\mu\text{g}/\text{ml}$ for 3 h (Fig. 5).

4 Discussion

The determination of mutagenic risk of chemicals relies mainly on results from mutagenicity tests. Results from indicator tests (i.e., tests measuring effects related to the process of mutagenesis, such as DNA damage, recombination and repair) can provide additional useful information in the context of extended genotoxicity testing (Brendler-Schwaab et al., 2005). As genetic toxicity of a candidate molecule for a new drug, pesticide, food additive, etc. can be a reason for termination of development, there has been pressure to generate high-throughput screening methods that can predict what may happen in the regulatory screening battery (Parry, 2006). We have previously established

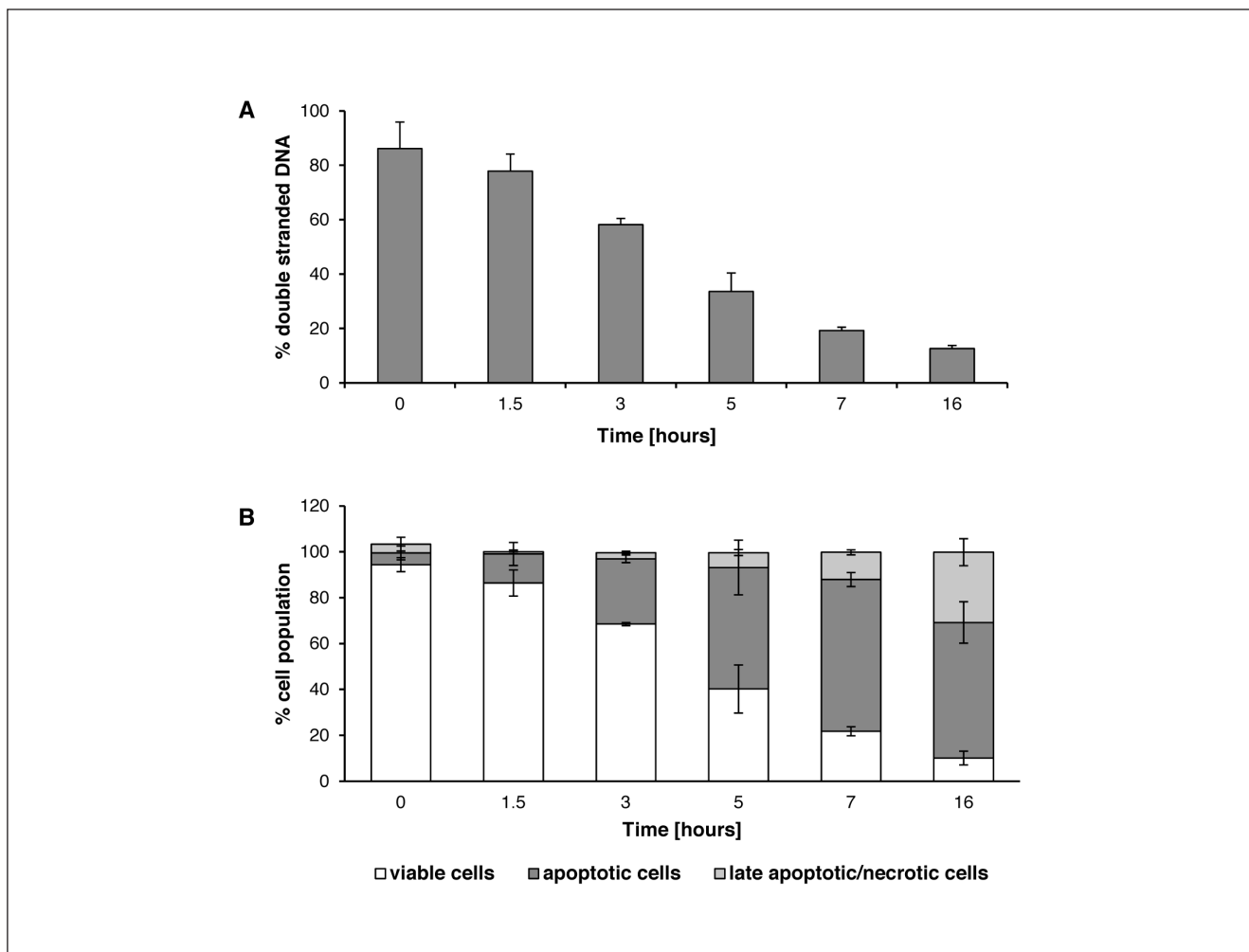


Fig. 4: DNA strand breaks induced by apoptosis

PBMC were freshly isolated from healthy donors and incubated with 3 μM staurosporine for 1.5, 3, 5, 7, and 16 h, respectively, at 37°C. DNA strand breaks (A) and the percentage of viable/early apoptotic/late apoptotic plus necrotic cells (B) were measured after 1.5, 3, 5, 7, and 16 h, respectively. Error bars represent standard deviations of two independent experiments.

a modified and automated version of the “*Fluorimetric detection of Alkaline DNA Unwinding*” assay (Moreno-Villanueva et al., 2009). Here we demonstrate its applicability in the early screening of potentially genotoxic compounds using well known toxic substances classified in different groups, depending on their mode of action.

Our results indicate not only a high reproducibility in measuring DNA strand breaks but also provide additional information on DNA damage and repair kinetics. We observed that MMS induced DNA strand breaks are significantly different between 10 and 20 and between 10 and 30 min exposure but not between 20 and 30 min. This could be due to time-dependent spontaneous hydrolysis of MMS, or due to DNA repair activity. It is, however, beyond the scope of the present paper to resolve the mechanism. By contrast, upon MNNG or 4-NQO treatment the numbers of DNA strand breaks detected were not significantly different after 10, 20, or 30 minutes exposure (Fig. 1), probably due to very rapid formation of DNA damage and initiation of repair. In order to correlate strand break frequency with cytotoxicity we performed flow cytometry to monitor cell death in Jurkat cells and PBMC treated with MMS, MNNG and 4-NQO under the same conditions as used for the FADU experiments (Supplementary Fig. B, C at www.altex-edition.org). We also observed that 30 min of *in vitro* treatment with low camptothecin concentrations led to less strand break formation than after 10 min drug incubation (Fig. 2D). This effect could be due to the capacity of the cells to repair the damaged DNA or to some other mechanism of reversibility of CPT-in-

duced DNA damage. In such a case, restricting the analysis to just a few time points of exposure could lead to false negative results.

We also measured DNA strand breaks induced by apoptotic mechanisms using staurosporine as a general apoptosis inducer (Fig. 4) and, as the field of ZnO nanoparticle applications is extremely broad and toxicity data – especially regarding genotoxicity – are urgently needed, we included nano-ZnO in our study. We detected DNA strand breaks after 3 h exposure of A549 cells to 160 $\mu\text{g/ml}$ ZnO nanoparticles (Fig. 5) following a simple and short protocol. Very similar results have been reported in the same cell line using the comet assay (Karlsson et al., 2008), although some interference with the comet assay cannot be excluded and needs further consideration (Karlsson, 2010). ZnO nanoparticles did not interfere with the FADU assay (data not shown).

Recent publications report, on the one hand, that dissolution and hence Zn ions are the main cause of ZnO NP toxicity (Xia et al., 2008; Song et al., 2010; Deng et al., 2009). On the other hand, researchers find that ZnO NP toxicity is not due to dissolution effects (Lin et al., 2009; Moos et al., 2010) or that Zn ions cannot solely account for the observed results – at least not at concentrations exceeding the dissolution equilibrium (Gojova et al., 2007; Deng et al., 2009). Further experiments considering ZnO NP dissolution under the given circumstances will be needed to clarify this point.

These results, taken together, demonstrate the broad applicability of a very easy and rapid protocol for detecting DNA

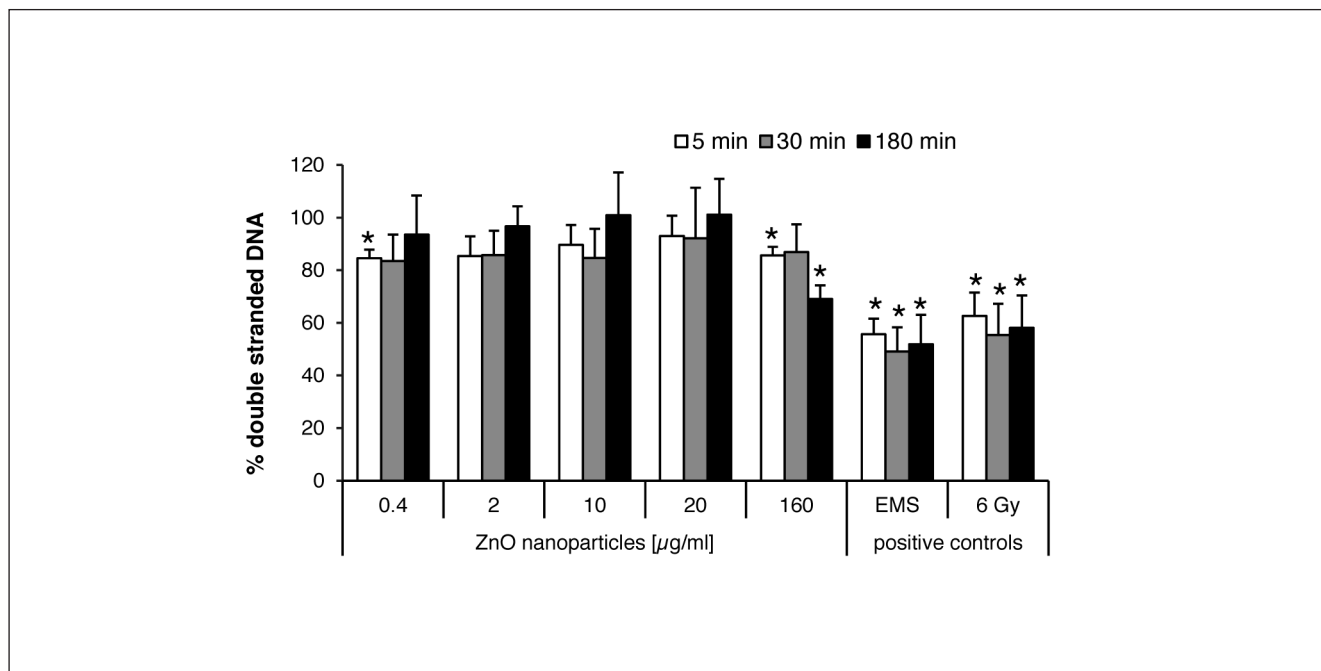


Fig. 5: Clastogenicity of nanoparticles

A549 cells were incubated for 5 (white bars), 30 (grey bars), or 180 min (black bars) at 37°C with 0.4, 2, 10, 20, and 160 $\mu\text{g/ml}$ ZnO nanoparticles. As a positive control, two samples were exposed to 15 mM ethyl methanesulfonate (EMS) or 6 Gy of X-irradiation, respectively. The final cell titer was 10^5 cells/ml. A significant induction of DNA strand breaks was detected after treatment with 160 $\mu\text{g/ml}$ for 180 min. Error bars represent standard deviations of three independent experiments.

*The values are significantly different from control (Student’s t-test, $p < 0.01$).



strand break formation, which was applied to all substances tested, independent of their mechanism of action. We have selected compounds that are well documented in the literature either to induce or not induce DNA strand break formation, and our data indeed met the prediction. In general, automation of a method helps reduce variability of the results and increases reproducibility; furthermore, automated systems provide for a higher throughput. The number of false positive or false negative results in animal testing could also be reduced by enabling use of human cells.

Recently, the automated FADU-assay has been used in different contexts (Kappes et al., 2008; Huljic et al., 2008; Mangerich et al., 2010) using different cell models. Compared to the comet assay, the automated FADU-assay is characterized by increased reproducibility, higher throughput, and lower demand for manpower and overall cost. Its use could contribute valuable information to a wide range of pharmacological, toxicological, epidemiological, and occupational health studies.

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