Evaluation of cytotoxicity assays and a high-throughput variant of the alkaline Comet assay in mouse lymphoma L5178Y cells

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Abstract

In the pharmaceutical industry, there is an increasing demand for rapid screening assays with a good predictivity for the regulatory tests. The Comet assay, or Single Cell Gel Electrophoresis Assay, is a fast, easy and relatively inexpensive method to visualize and measure DNA strand breaks in individual cells. In the early drug development, when the amount of the test substance is limited, the Comet assay must fulfil several criteria.

Since DNA damage is associated with cell death, cytotoxicity measurements should be evaluated concurrently with each Comet experiment. The purpose of the present study was to find a suitable cytotoxicity test in mouse lymphoma L5178Y (ML) cells and evaluate a high-throughput variant of the alkaline Comet assay (HT-ACA) in the same cell line. A modified protocol for the Comet assay, with inclusion of commercially available multi-well object slides was tested. The HT-ACA experiments were performed with four alkylating agents and the obtained results were then compared to Comet assay data previously obtained by the standard HT-ACA protocol of Comet Assay, currently in use at Safety Assessment, AstraZeneca.

Based on the results from experiments with different cytotoxicity tests, measurement of ATP levels was chosen as a suitable test in ML cells. The observed LOELs obtained from the modified HT-ACA were in good accordance with the standard HT-ACA protocol, albeit some differences were revealed. For instance, the modified version detected positive genotoxicity in concentrations two to five times lower than earlier had been found with the standard HT-ACA protocol. Even though the Comet assay has the potential to become a powerful complement to other genotoxicity tests, there are some bottlenecks in its performance, which need to be addressed.

1. Abbreviations

B(a)P	Benzo(a)pyrene
DMBA	9,10-dimetyl-1,2-benzanthracene
EthD-1	Ethidium homodimer-1
G6PD	Glucose 6-Phosphate Dehydrogenase
HT-ACA	High-throughput alkaline Comet assay
LMP agarose	Low Melting Point agarose
LOEL	Lowest-Observed-Effect-Level
ML cells	Mouse Lymphoma L5178Y cells
MMC	Mitomycin C
NDCN	Non-Detectable Cell Nuclei
NNP	N-nitrosopiperidine
4NQNO	4-nitroquinoline-N-oxide
SAS	Safety Assessment Sweden
TBDE	Trypan Blue Dye Exclusion

2. Preface

This study is my graduate project for a Master of Science degree in Biology at the Swedish University of Agricultural Science, Uppsala. The study was conducted at the Department of Genetic Toxicology, Safety Assessment R&D, AstraZeneca, Södertälje. The project is a part of developing a standardized protocol for the high-throughput variant of the Comet assay intended for use in the standard battery of genotoxicity tests performed at AstraZeneca. Examination of this project was performed at the Department of Biomedical Sciences and Veterinary Public Health, Division of Pathology, Pharmacology and Toxicology, SLU, Uppsala.

3. Introduction

Different kind of DNA damages e.g. point mutations, DNA-DNA or DNA-protein crosslinks and single or double strand breaks may appear when cells are exposed to genotoxic substances (Marzin 1999, Garberg *et al.* 1988). The DNA damage is one of the main events that give rise to mutations and chromosome brakeage, which might lead to development of cancer (Christmann *et al.* 2003). By monitoring DNA strand breaks as an indicator, potential genotoxic compounds can be discovered (Garberg *et al.* 1988).

In the pharmaceutical industry, there is an increasing demand for rapid screening assays with a good predictivity for the regulatory tests. A recent study by Kiskinis *et al.* has indicated that the Comet assay has the potential to become such an assay, especially during the early drug development when the amount of test substance is limited. This kind of genotoxicity tests are generally conducted between discovery and development phases.

Today, the pharmaceutical industry uses a battery of tests when test substances are analyzed for genotoxicity. These tests include two *in vitro* (the Ames test and the mouse lymphoma TK locus forward-mutation assay) and one *in vivo* test (the micronucleus assay). In case of equivocal or contradictory results from the standard test battery, other genotoxicity tests may be used for further evaluation.

The Comet assay, or Single Cell Gel Electrophoresis Assay, is a quick, simple and relatively inexpensive method to visualize and measure DNA strand breaks in individual cells. After exposure to chemicals, cells are embedded in agarose on a microscope slide and immersed in a lysis solution to remove lipids and proteins. DNA is then allowed to unwind under alkaline conditions. Thereafter an electrophoresis is run to attract broken, negatively charged DNA towards the anode. This will shape a "comet-like" figure with a distinct head and a tail, which contains the damaged or broken pieces of DNA. After staining with a fluorochrome (e.g. ethidium bromide), individual cells can be visualized in a fluorescent microscope (Marzin 1999, Comet Assay Interest Group 051201). A schematic overview of the Comet assay is provided in *Figure 1*.

In the agarose, cells form a cavity that after lysis is occupied by the DNA. Other biomolecules in the cell do not have the same high molecular weight as DNA and will hence diffuse through the agarose gel from the cavity into the lysis solution. The theory behind the migration of DNA is that when an electrical field is applied DNA is stretched out toward the anode but will still be fixed in the cavity. If DNA strand breaks are introduced the super-coiling of the DNA is relaxed and will provide a more distinct migration (Comet Assay Interest Group 051201, Ostling & Johanson 1984). DNA damage must therefore be converted into strand breaks that relax the supercoiled DNA or produce DNA fragments before detected in the Comet assay (Comet Assay Interest Group 051201).
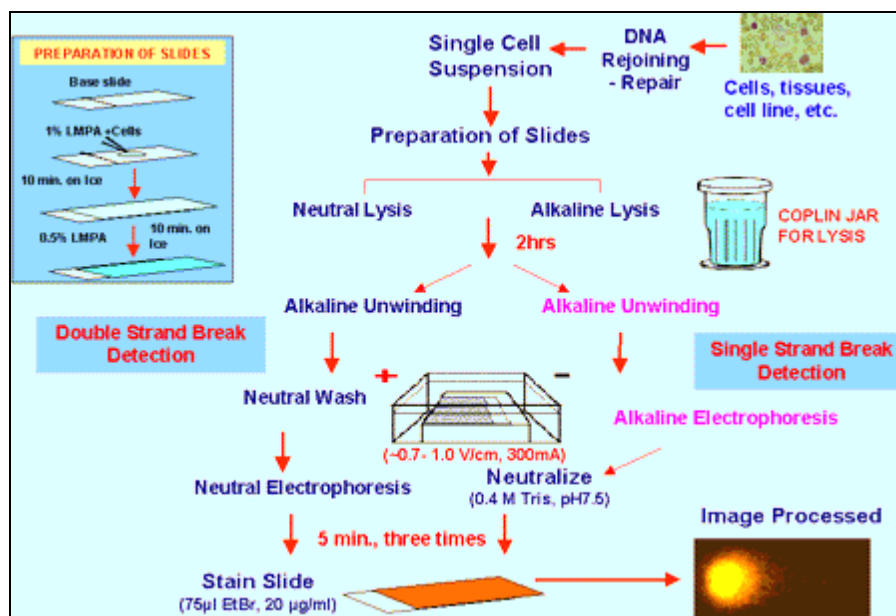

Figure 1: Methodology of the Comet assay. The alkaline variant on the right hand was used throughout the study (Comet Assay Forum 051207).

Individual digital images of cells in the Comet assay are captured, whereafter a variety of comet parameters can be analysed e.g. tail length, tail DNA content, tail moment etc. These parameters indicate the number of strand breaks present in the cell i.e. the higher the breakage frequency the more DNA is present in the tail of the comet (Marzin 1999). Hence genotoxicity is detected as an increase in a particular comet parameter e.g. the median comet length (Frei *et al.* 2001, Collins & Harrington 2002).

The Comet assay has become very popular due to its ability to measure DNA damage in almost any population of monodispersed cells and can be used under both *in vitro* and *in vivo* conditions (Frei *et al.* 2001, Andersson *et al.* 2003 and Singh *et al.* 1994). Modifications of the original method described by Ostling and Johanson have extended the types of DNA

damage that can be detected with this technique (Collins & Harrington 2002, Singh *et al.* 1994). In addition to single-stand breaks (SSB), the Comet assay can be used to detect double-strand breaks (DSB), alkali-labile sites (ALS), damage to DNA bases, DNA-DNA or DNA-protein cross-linking plus apoptotic fragments (Tice *et al.* 2000). The extent of DNA migration (tail length, % DNA in the tail) is generally believed to be related directly to fragment size and would be expected to be proportional to the frequency of strand breaks and ALS, and inversely proportional to the extent of DNA cross-linking (Comet Assay Interest Group 051201, Tice *et al.* 2000).

There are several variants of the Comet assay employing different pH conditions during unwinding and/or electrophoresis. DSB are the simplest DNA damage detected by the Comet assay and can easily be discovered by electrophoresis at neutral pH (7-8). SSB can not be produced unless the DNA strands are denatured. Therefore, unwinding the DNA at alkaline conditions (pH of 12.1-12.5) and subsequently an electrophoresis run at the same pH, allows the detection of SSB, cross-links and DSB. At pH >13, the assay detects SSB, DSB, ALS (which are transformed into SSB) and cross-linking (Comet Assay Interest Group 051201).

Since DNA damage is associated with cell death, cytotoxicity measurements should be evaluated concurrently with each Comet experiment. It may also be helpful, prior to the Comet experiment, to perform a preliminary range finding experiment with a cytotoxicity assay. There are a number of cytotoxicity methods to use either at the end of the exposure period (e.g., exclusion of a vital dye, ATP levels, release of various enzymes from the cytoplasm) or after certain incubation period for recovery (e.g., cell count). The general approach is to avoid proceeding with samples in the Comet assay that decrease viability (assessed by the trypan blue dye exclusion method) by more than 30% when compared to the concurrent control (Tice *et al.* 2000).

In the present study, experiments with different cytotoxicity assays were performed in mouse lymphoma L5178Y (ML) cells. Thereafter, one of these tests was chosen to proceed to comparison experiments with the traditionally used trypan blue dye exclusion (TBDE) method. In addition, the concept of 96-well Comet assay, so called high-throughput variant of the alkaline Comet assay (HT-ACA), have been further developed in ML cells. The protocol of Kiskinis *et al.* was modified by inclusion of commercially available multi-well object slides instead of one sample per object slide. The modified protocol was tested with four

alkylating agents as test compounds. The obtained results were then compared to Comet assay data that were previously obtained by the standard HT-ACA protocol of Comet assay, currently in use at Safety Assessment Sweden (SAS).

4. Aims

The project was divided into two parts. The aim of the first part was to find a suitable cytotoxicity test in ML cells among three commercially available kits. These tests measures different biological markers, which are related to cytotoxicity. The most suitable of these tests was chosen for further evaluation experiments against the traditionally used TBDE method. Compounds with different cytotoxic effect were used in these experiments.

The aim of the second part was to compare results from the modified HT-ACA and the standard HT-ACA protocol of Comet assay in ML cells. The amount of the test substance is limited during the early drug development and therefore the Comet assay must fulfill several criteria to be useful as an *in vitro* screening assay. These criteria are listed below.

- Adaptability to a high-throughput format – preferably in a standard 96-well format that might be suitable for automation
- Simple and quick protocol – the assay should ideally be carried out by one person
- Reliability, versatility, low inter-experiment variability – standardized protocol and reagents, evenly (if possible) distributed workload
- Concurrent determination of cytotoxicity – must match the throughput of the Comet assay, be biologically relevant for the tested cell type and if possible allow simultaneous detection and discrimination of both apoptotic and necrotic events

5. Materials and methods

5.1. Cells and culture conditions

Mouse lymphoma L5178Y TK$^+$/$^-$ cells, subline 3.7.2C, were obtained from Dr. D. Clive, Burroughs Welcome Co., Research Triangle Park, N.C., USA and kept at -196°C. The cells were cultured in RPMI 1640 medium supplemented with PSGP (penicillin, streptomycin, L-glutamine and Na-pyruvate), 10% heat inactivated horse serum and Pluronic F68 from Gibco (Gaithersburg, MD) at +37°C in a humidified air incubator (95% relative humidity and 5% CO_2) using 80cm^2 cultivation flasks. A new culture was started every week from a fresh ampoule to minimize variations in the experiments due to differences in generation time and spontaneous mutations. Every working day the cell concentration in the ongoing culture was adjusted to $15 \cdot 10^4$ cells/mL. Cell concentration was measured with BECKHAM COULTER$^®$ Z2 Particle Count and Size Analyzer. During experiments the cell suspensions contained growth media complemented with only 3% heat inactivated horse serum (end concentration in culture).

5.2. Materials

Detailed buffer compositions and other solutions used throughout the experiments are provided in the Appendix. CellTiter-Glo$^®$ Luminescent Cell Viability Assay was purchased from Promega. Vybrant$^{™}$ Cytotoxicity Assay Kit and LIVE/DEAD$^®$ Viability/Cytotoxicity Kit for mammalian cells were obtained from Invitrogen. Double-distilled water was used all the way through the experiments. Other chemicals used in the study were of analytical grade and obtained from regular commercial sources.

Two different groups of compounds were used during comparison tests between ATP levels and TBDE, an alkylating agent (see below) in the presence and absence of metabolic activation and two aneugens (*Figure 2*). Aneugens inhibit cell division and cause cytotoxicity by interfering with the processes of mitosis and meiosis, without interacting with the DNA. They act by binding to tubulin and inhibiting its polymerization into microtubules, which prevent spindle formation in dividing cells and causes arrest at metaphase. They also inhibit

other cellular activities that involve microtubules like migration and transport (Rang *et al.* 2003, Timbrell 2001).

In evaluation experiments of the modified HT-ACA, test compounds with a variety of genotoxic effects were used (*Figure 2*). For some compounds, metabolic activation was needed. Alkylating agents contain chemical groups (alkyl groups) that cause DNA damage when interacting covalently with bases of the DNA. The alkyl group reacts preferably with nucleophilic oxygen and nitrogen atoms in the DNA molecule. Most agents have two alkylating groups, so called bifunctional alkylating agent, and can cross-link two nucleophilic sites and cause intra- or interchain cross-linking. This can interfere with transcription and replication (Rang *et al.* 2003, Timbrell 2001).
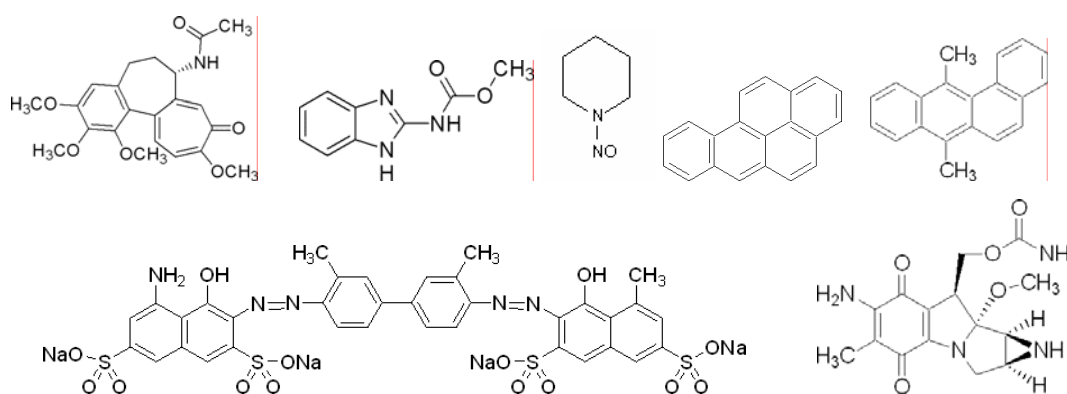

Figure 2: Chemical structure of test compounds used in evaluation experiments. At the top, starting form left: aneugens (colchicine, carbendazim), alkylating agents (NNP, B(a)P and DMBA). At the bottom, from left; alkylating agents (4NQNO) and bifunctional alkylating agent (MMC).

The S9 liver homogenate was prepared according to the method of Majeska and Matheson and was stored frozen at or below -70°C until use. The cofactor mix (see Appendix) was sterilized by filtration through a 0.2 μm Millipore filter and stored frozen at -80°C until use. On the day of treatment, the protein content was adjusted to 30 g/L by dilution with R0P before it was blended with the cofactor mix according to standard operating procedures at SAS (23 mL cofactor mix to 2 mL S9). The end concentration of S9 fraction in the cultures was 4%.

Incubation of cells was performed in 96-well microplate (Costar 3585) and luminescence and fluorescence measurements were conducted in FluoroNunc™/LumiNunc™ 137101.

5.3. Treatment of cells

Treatment of cells with the test compounds were all performed in 96-well microplates, with or without S9-mix. The plates contained series of concentrations of the test compound dissolved in DMSO (end concentration in culture 1%) or water. As controls served cells exposed to the solvent without test compound. The plate was incubated for 3 hours at +37°C on a tilted table in a humidified air incubator with 5% CO_2. During experiments with cytotoxicity assays some incubation periods were set to 10 minutes or 24 hours. In evaluation of cytotoxicity tests a heat-induced cell death experiment was conducted according to Van der Waal *et al.* The heat treatments were aimed to induce apoptosis and necrosis, thus inducing distinct difference in ATP levels. The ML cells were incubated at +43°C for 24 minutes or +45°C for 30 minutes that should result in the two processes of apoptosis and necrosis, respectively. After the heat induction, cells were incubated for 2.5-3 hours in +37°C in a humidified air incubator and results were compared to controls only exposed to +37°C.

The treatment of cells was optimized with respect to cell concentration and the end concentration of DMSO in culture. Too high cell density in the Comet assay would lead to a harder time to find and score individual cells. On the other hand, if the cell concentration was too low it took a lot of time to search for the widely spread cells. The final concentration of DMSO was chosen to 1%, because that concentration caused no cell toxicity.

In the experiments with the test compounds, dilution series were used. The dilution factor between stock 1 and 2 was 1.5. Two series of five concentrations with a 2-fold spacing were prepared from each stock solution. Aliquots of 200 μL, or 100 μL if metabolic activation was needed, of stock 1 and stock 2 were placed in all wells in columns 3 and 8. The remaining wells, except column 2, were filled with growth media without serum, 100 μL or 50 μL if S9-mix was required. 1:2 dilutions of the stock solutions were performed from column 3 to 7 and from column 8 to 12, using a multi-tip pipette, as described in *Figure 3*. Finally, 100 μL cell suspension ($48 \cdot 10^4$ cells/mL), or 70 μL cell suspension ($70 \cdot 10^4$ cells/mL) in growth media complemented with 3% serum (end concentration in culture) along with 80 μL S9-mix, were added to all wells except column 2. The total volume in each well was 200 μL and the final cell concentration was $24 \cdot 10^4$ cells/mL.
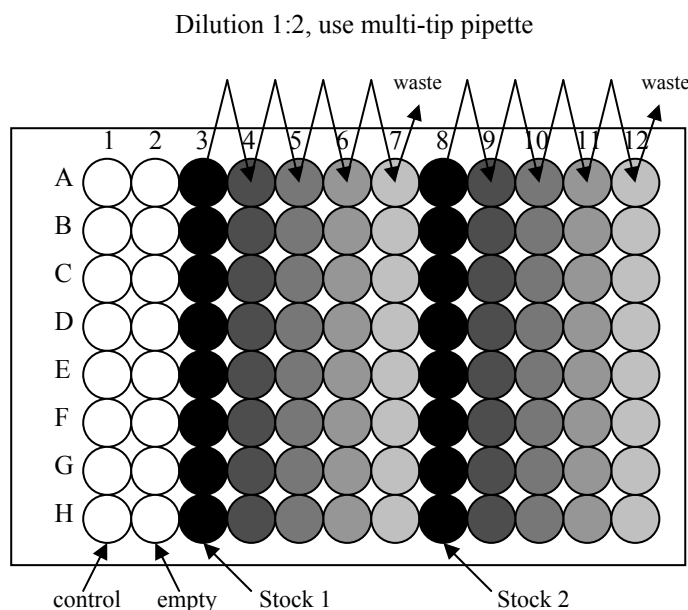
Figure 3: Layout of 96-well microplate. Two stock solutions were prepared for every test compound. The picture is based on information from Kiskinis *et al.*

5.4. Cytotoxicity assays

5.4.1. Vybrant™ Cytotoxicity Assay Kit

The Vybrant™ Cytotoxicity Assay Kit monitors the release of the cytosolic enzyme glucose 6-phosphate dehydrogenase (G6PD) from damaged cells into the surrounding medium. G6PD catalyzes the reaction of NADP to its reduced form, NADPH, in the pentose phosphate pathway. The assay detects G6PD through a two-step enzymatic reaction that leads to the reduction of resazurin into red-fluorescent resorufin (*Figure 4*) measured by microplate spectrophometer (Spectramax M2, Molecular Devices). The fluorescence signal is pro-portional to the amount of G6PD released into the cell medium, and this release correlates with the number of dead cells in the sample (Product Information V-23111, Frank 2005). During exposure, all cell cultures contained $0.5 \cdot 10^4 - 2.5 \cdot 10^4$ cells/well in growth media. According to the manufacturer, the recommended cell concentration was between 500 and 25 000 cells/well. In experiments with Vybrant™ Cytotoxicity Assay Kit, the manufacturer's protocol was optimized in ML cells (e.g. cell concentration, incubation times, excitation and emission wavelengths). Tests with DMSO, a frequently used solvent for many of the test chemicals used in this study, was also investigated for its possible interaction with reagents in the assay. Experiments showed that DMSO interacted with reagents in the Vybrant™

Cytotoxicity Assay in a way that was not related to immediate cell toxicity, already at 10 minutes incubation time (with DMSO). Thus, the interference of DMSO with the enzymatic reaction was significant already at the concentration of 1 %, which is the regular final concentration of DMSO during the exposure period in the Comet assay.
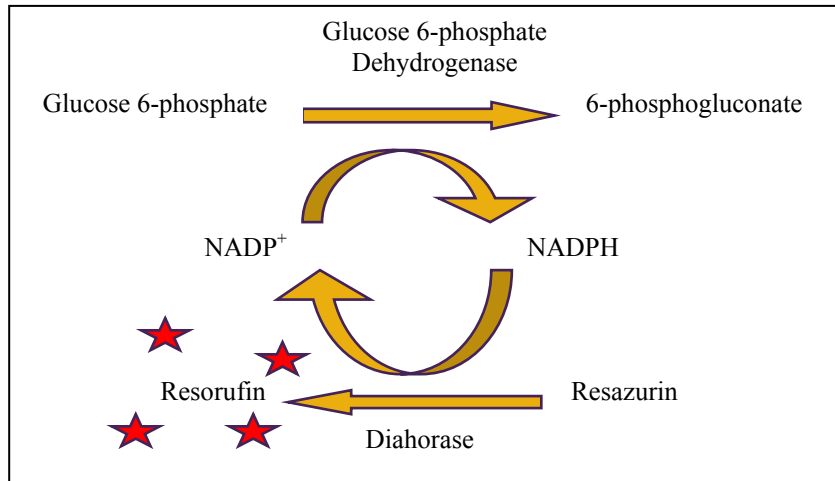

Figure 4: Principle of the two-step enzymatic assay for detection of glucose 6-phosphate dehydrogenase. Oxidation of glucose 6-phosphate by glucose 6-phosphate dehydrogenase results in the generation of NADPH, which in turn leads to the reduction of resazurin by diaphorase to yield fluorescent resorufin.

5.4.2. LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells

The LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells distinguishes between live and dead cells with two probes (calcein AM and ethidium homodimer-1) that measure intracellular esterase activity and plasma membrane integrity, respectively. Live cells were detected due to the presence of ubiquitous intracellular esterase that catalyses the conversion of the non-fluorescent calcein AM to the intensely fluorescent calcein. Calcein AM can cross an intact plasma membrane and the polyanionic dye calcein is well retained within live cells, producing an intense stable green fluorescence in live cells. Ethidium homodimer-1 (EthD-1) enters the damaged membranes of dead cells. Binding to nucleic acids of the probe produces a bright red fluorescence in dead cells (Product Information 03224). The fluorescence signals of the reactions were measured by microplate spectrophometer (Spectramax M2, Molecular Devices). Different cell concentrations were used during the experiments ranging between $0.5 \cdot 10^4 - 25 \cdot 10^4$ cells/well. Manufacturer recommended minimum 200-500 cells/well and not more than 10^6 cells/well. Excitation and emission spectra were analysed to locate optimal wavelengths for the fluorescent dyes in experiments with LIVE/DEAD® Viability/

Cytotoxicity Kit. They were determined to be 485/530 nm for calcein and 544/645 nm for EthD-1. The assay was performed in two steps. First, the presence of live and dead cells was determined by measuring the calcein and EthD-1 signals, respectively. Afterward, the total number of cells in the sample was revealed by lysis of all cells with 0.1% saponin. The overall results of pilot experiments with this assay were deemed as unsatisfactory. Addition of saponin to the cell suspension resulted only in a slight increase of the EthD-1 signal (results not shown). Moreover, serum-supplemented growth media can cause an increase in background caused by extracellular fluorescence due to hydrolysis of calcein AM. This means that cells have to be washed prior to the assay to remove or dilute serum esterase activity. The assay also includes an incubation period of 30-45 minutes after adding the reagents for stabilization of the signal. If this incubation occurs in D-PBS or HBSS, after attempts to remove serum from the cells, previous studies at SAS have shown that the condition becomes cytotoxic for the ML cells.

5.4.3. CellTiter-Glo™ Luminescent Cell Viability Assay

The regents of CellTiter-Glo™ assay were combined with cell samples according to the instructions of the manufacturer. This assay measures the ATP level which is used as an indicator for the presence of metabolically active cells. The reagent generates cell lysis and produces a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells present in culture. The luciferase reaction for the assay is shown in *Figure 5* (Technical Bulletin No. 288).
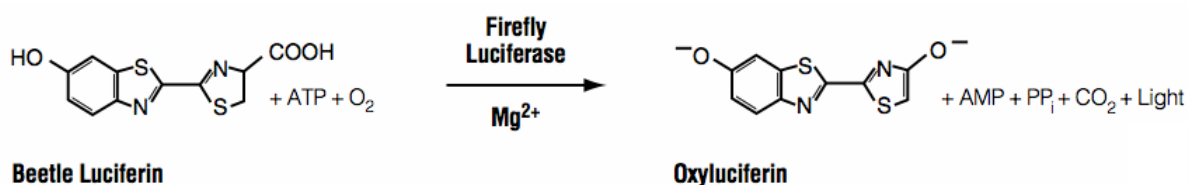

Figure 5: The luciferase reaction. Oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+}, ATP and molecular oxygen (Technical Bulletin No. 288).

During exposure, all cell cultures contained $0.5 \cdot 10^4 - 4 \cdot 10^4$ cells/well. According to the manufacturer, there is a linear relationship (R=0.99) between 0 and 50 000 cells/well in the 96-well format. The luminescence of the ATP-releasing reaction was detected by a microplate luminometer (Luminoscan Ascent; Labsystems). In the experiments with heat-induced cell

death a significant decrease of ATP levels were observed in the cells exposed to +45°C but no significant differences were observed between the control cells and cells pre-incubated at +43°C. The relationship between luminescence signal and cell concentration was found to be linear (R^2=1.00) in cell concentrations between $0.5 \cdot 10^4$ and $4 \cdot 10^4$ cells/well (results not shown).

5.4.4. Choice of cytotoxicity assay

Based on the results of pilot experiments the assay measuring ATP levels was chosen as a suitable cytotoxicity test in ML cells before the other two assays. It is more sensitive, faster and easier to perform and seems to be gentler to the ML cells. Whereas ATP levels reflect cytotoxic events both in presence and absence of membrane damage, the other two assays are limited to detection of membrane damage. Moreover, in the Vybrant™ assay, DMSO showed direct interference with the assay components regardless of incubation time. Furthermore, the prolonged incubation of ML cells in HBSS or D-PBS during the LIVE/DEAD® method is cytotoxic *per se*.

5.4.5. TBDE

In the TBDE method, cell samples were mixed with an equal volume of 0.4% trypan blue and counted in a light microscope. Cells with a ruptured membrane allowed dye uptake and was considered dead.

5.5. HT-ACA

Concentrations of test compounds that continued to genotoxicity tests in the Comet assay were selected after cell viability determination at the end of the 3 hours exposure. Hence, 100 μL of all cell samples were transferred from the 96-well incubation microplate to a new 96-well plate for measurement of cytotoxicity. According to Kiskinis *et al.* concentrations resulting in <70% (TBDE test) or <50% (ATP test) relative viability were considered to have

a too high cytotoxicity for analysis in the Comet assay. These thresholds were applied in this study.

The selected samples were transferred from the 96-well incubation microplate to Eppendorf tubes and washed in PBS once. Thereafter, the cells were mixed 1:10 with Trevigen LMAgarose and placed on slides. Two protocols for preparation of Comet slides for the HT-ACA were used (A and B). Protocol A was compared with B which is the standard HT-ACA protocol used at SAS.

In protocol A, 20-well object slides (Trevigen), already pre-coated by the manufacturer, were used. 30 µL of the agarose-cell suspension were applied per well on pre-coated 20-well slides. About 7 000 cells/well were used in each experiment.

In protocol B, frosted microscope slides (Erie Scientific Company) were pre-coated with 1% agarose (400 µL). The excess agarose was scraped off and a bottom gel with 0.6% agarose (100 µL) was added. Thereafter, about $2 \cdot 10^4 - 7 \cdot 10^4$ cells were embedded in 0.6% low melting point (LMP) agarose on the slides and finally a top gel of 0.6% LMP agarose (75 µL) was layered on top.

The slides, from both protocols, were dried at +4°C until a 0.5 mm clear ring appeared at the edge of the sample area. Slides were placed in lysis solution for at least one hour at +4°C. The lysis solution used in protocol A was a pre-made Trevigen Lysis Solution and the composition of the lysis solution used for protocol B is specified in Appendix. After lysis the slides were placed in a horizontal gel electrophoresis unit in which the DNA samples were left to unwind at room temperature for 30 minutes in freshly prepared alkaline electrophoresis buffer pH >13 (see Appendix). The electrophoresis was run at room temperature for 20 minutes using field strength of 0.7 V/cm (301 mA, 25 V). Following electrophoresis, the slides were neutralised in neutralization buffer for 3x5 minutes and left to air-dry in room temperature. In protocol A, samples were also dehydrated in 70% ethanol for improved drying out. Before scoring, the dried microscope slides were stained with 5 µL/well of 20 µg/mL ethidium bromide in protocol A and 50 µL/slide in protocol B. The slides were stored at +4°C for at least 30 minutes before image analysis was performed. All steps from lysis until the end of the

image analysis were performed under reduced light in order to avoid unspecific DNA damage from UV light.

5.6. Image analysis

Slides were examined at 25x magnification in a fluorescence microscope (Leitz DIALUX 22EB) equipped with an excitation filter (515-560 nm) and a barrier filter (590 nm). Digital images were captured by a black and white charge-coupled device video camera (Ikegami) attached to the microscope and transferred to a PC-based image analysis system (Colourmorph v5.0; Perceptive Instruments, UK). Fifty "comets" per sample were randomly selected, avoiding the edges and eventually damaged parts of the gel, debris, superimposed comets and comets without a distinct "comet head" (i.e. so called "non-detectable cell nuclei" (NDCN), "clouds", "hedgehogs" or "ghost cells"). NDCN or "clouds" are cells exhibiting extensive DNA fragmentation to a point where no clear head/nucleus but only the tail is visible, most likely representing dead or dying cells. Albeit NDCN were excluded from the image analysis, their frequency was determined for each sample and used as an additional indicator of cytotoxicity.

5.7. Evaluation criteria

Based on extensive historical data of negative and positive controls, evaluation criteria for genotoxicity (positive, negative, equivocal) of the tested chemical in the Comet assay have been previously established (unpublished data, AstraZeneca). The protocol tested for the purpose of the modified HT-ACA differed only in the procedure of object slide preparation. As the conditions of lysis, unwinding and electrophoresis (steps with major influence on the obtained results) were similar for both protocols, the assumption that the evaluation criteria for protocol B (see below) could be applied also for protocol A. No formal statistical analysis was performed of the results. Significance levels were judged as obvious increase/decrease of a particular parameter.

1) A test chemical was considered to be positive

   a) If it produces an increase in the tail length of more than 6.0 μm or/and if the tail moment was more than 0.500 μm or/and if the fraction of DNA damaged cells was 16% or more compared with the concurrent solvent control in either or all of the above mentioned parameters.

   b) And it these effects are seen at two or more concentrations of the test compound in at least one trial.

   c) And if a positive dose trend is seen in consecutive doses among the cells measured in at least one trial.

   d) And these effects are found at a relative toxicity of 30% or less measured with trypan blue exclusion method.

   e) And these effects are found where a sufficient number of living cells, i.e. more than approximately 30%, remain after treatment.

   f) And these effects are found where a sufficient number of cell nuclei, i.e. more than approximately 40%, could be scored for DNA damage analysis.

2) A result was considered to be negative

   a) If no increase in tail length or tail moment were found and the test compound was tested up at least 10 mM.

   b) Or if the effects under 1a, 1b and 1c were found only at concentrations that give a relative toxicity of more than 30% measured with the trypan blue exclusion method or/and a relative decrease of ATP levels by more than 50%.

   c) Or if the effects under 1a, 1b and 1c were found only at concentrations where less than approximately 30% living cells remain after treatment.

   d) Or if the effects under 1a, 1b and 1c were found only at concentrations where less than approximately 40% of the cell nuclei could be scored for DNA damage.

   e) Or if the effects were only found at one concentration in one trail.

6. Results and discussion

6.1. Comparison between TBDE and ATP

A series of experiments were conducted for comparison between measuring ATP levels and the standard TBDE method. The aim of these experiments was to confirm the previously suggested 50% relative decrease of ATP as limiting cytotoxicity criteria in the Comet Assay (Kiskinis *et al.* 2002).

ML cells were exposed to NNP, colchicine and carbendazim. NNP was tested with and without metabolic activation during 3 hours and the two aneugens were tested without metabolic activation during 24 hours. At the end of the incubation period the ATP levels were determined. Thereafter the membrane integrity of the cells was assessed with the TBDE method. Results are expressed as % ATP content of control for ATP measurement and % viable cells of control for the TBDE method.

The correlation between measurement of ATP levels and the TBDE cytotoxicity data for NNP was good in the absence of metabolic activation since the criteria for both tests would have selected similar concentrations for further analysis in the Comet assay (*Figure 6*). In the presence of S9-mix there was a more rapid decrease. The decrease of the ATP levels was also dose-dependent. The highest dose would have been rejected according to the ATP levels. Based on the results of the TBDE method all doses would have been processed in the Comet assay (*Figure 6*). If the different results obtained with and without S9 mix is correct, the generally applied viability criteria, ≥70% (TBDE method) and ≥50% (ATP method), of the concurrent control should be changed. It should be noted, however, that in this experiment the activation system was not removed prior to ATP measurements. Hence the possible degradation of ATP due to presence of the S9-mix should be further evaluated in experiments.
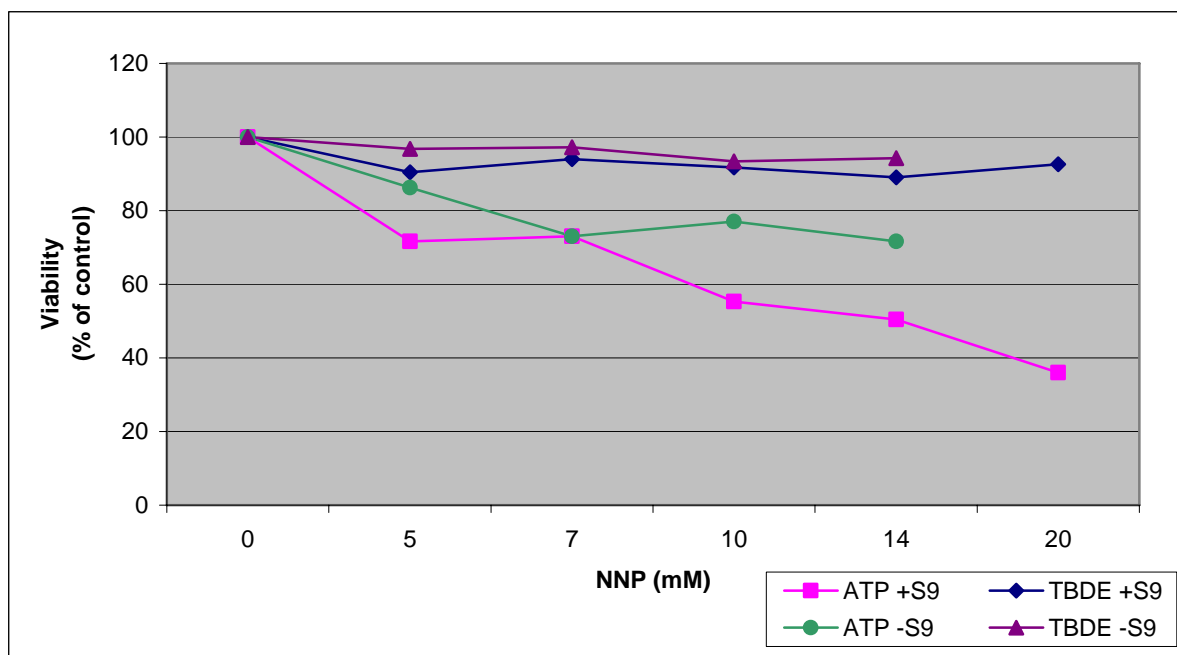
Figure 6: Effects on viability of ML cells exposed to serial concentrations of NNP in the absence and presence of S9-mix for 3 hours. Viability is measured by ATP levels and TBDE method. Results are expressed as the mean percentage of control (mean; n=4 for ATP and mean; n=2 for TBDE). As controls served cells exposed to the solvent without test compound.

The experiment with the two aneugens showed that values from both ATP levels and the TBDE method followed the same course in cells exposed to colchicine for 24 hours (*Figure 7*). The lowest tested concentration was cytotoxic to the ML cells, but there was not any dose-dependent increase in cytotoxicity. A putative explanation of these results could be the immediate cytotoxic effect of colchicine on a subpopulation of ML cells while exerting a mitotic block on the rest of the cells that is still present after 24 hours. In this context, the abnormal p53 status of the ML cells would hamper the induction of apoptosis, which normally is a common result of cell cycle disruptions (Sherwood *et al.* 1994). The correlation between the cytotoxicity data from the two tests is rather poor in this case. Based on the ATP levels data none of the concentrations would have moved on to genotoxicity test in the Comet assay. The TBDE method, on the other hand, would allow proceeding with almost all the doses.
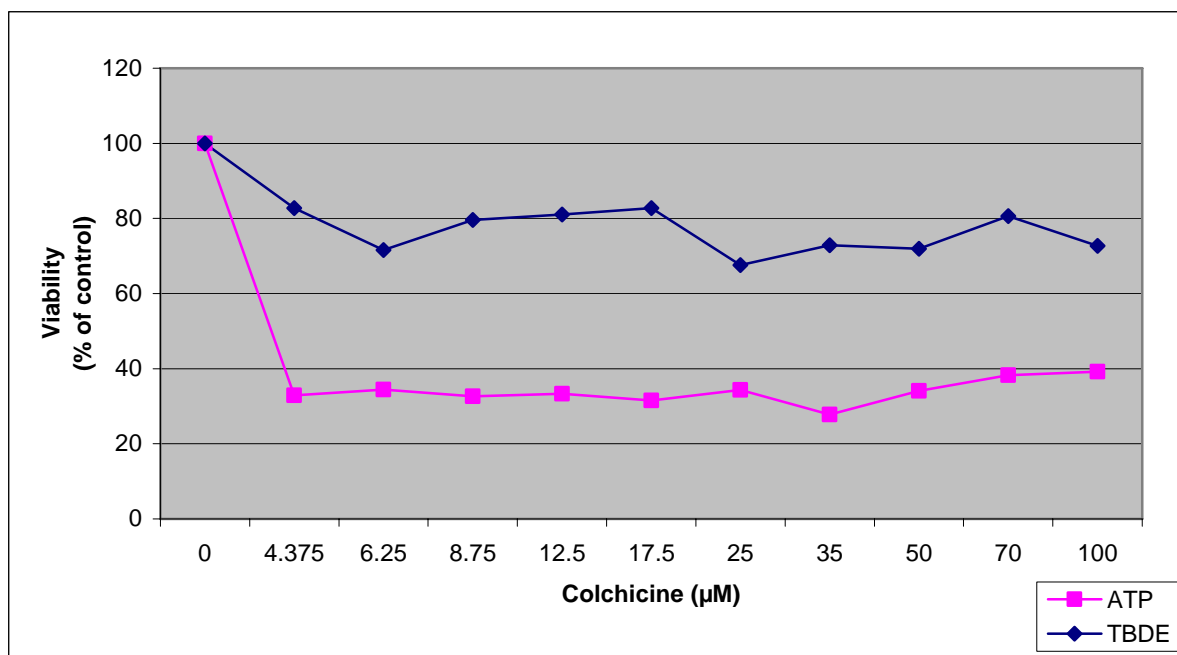
Figure 7: Effects on viability of ML cells exposed to serial concentrations of colchicine for 24 hours. Viability is measured by ATP levels and TBDE method. Results are expressed as the mean percentage of control (mean; n=8 for ATP and mean; n=2 for TBDE). As controls served cells exposed to the solvent without test compound.

Incubation of ML cells with carbendazim at concentrations 15 µM and higher during 24 hours resulted in loss of viability, as shown in *Figure 8*. No loss of viability could be seen at lower concentrations according to the results of both cytotoxicity tests. The results are in good agreement with previously obtained data (Smith 2005), where a significant reduction in both relative suspension growth and TBDE values for carbendazim-treated ML cells was seen at the same concentration. Unexpectedly, these lower concentrations had a stimulatory effect on ATP levels as compared with the untreated controls. The counting of cells in light microscope revealed an increased cell number in lower concentrations.
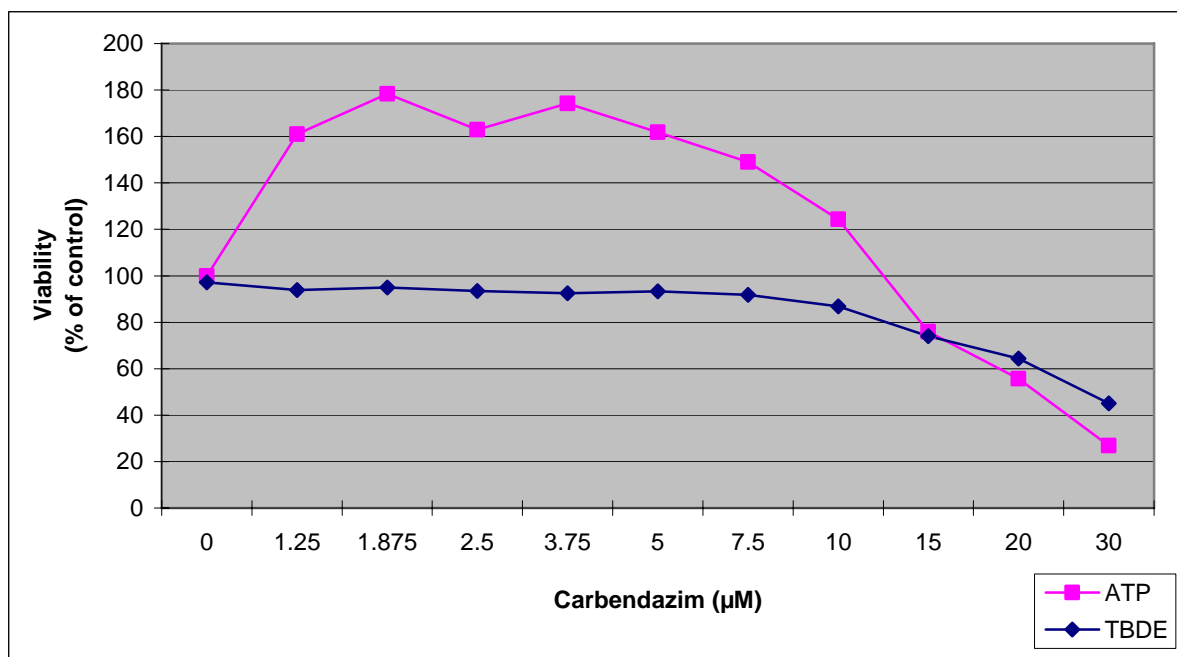
Figure 8: Effects on viability of ML cells exposed to serial concentrations of carbendazim for 24 hours. Viability is measured by ATP levels and TBDE method. Results are expressed as the mean percentage of control (mean; n=8 for ATP and mean; n=2 for TBDE). As controls served cells exposed to the solvent without test compound.

In summary, the comparison experiments showed a rather good agreement between TBDE method and ATP levels for assessment of cell viability. Based on the cytotoxicity data, 2/3 of the experiments showed that comparable doses would have proceeded to genotoxicity test in the Comet assay. While having a similar sensitivity, the time was reduced from a couple of hours for the TBDE method to only about 20 minutes to complete the measurement of ATP levels with CellTiter-Glo®.

6.2. Evaluation of the HT-ACA

4NQNO was the only test compound tested according to protocol B. In parallel with ATP measurements, cell viability for the three highest concentrations of 4NQNO (2.5, 3.5 and 5 μM) was also assessed with the TBDE method (*Figure 9*). Concentrations above 2.5 μM resulted in a steep decrease in viability according to results from both viability tests. All concentrations, except 5 μM, moved on to genotoxicity test in Comet assay. Five μM was not chosen because earlier studies at SAS have showed increased damage in another genotoxicity test at this concentration. In that genotoxicity test, which is part of the standard battery at SAS, 1.5 μM of 4NQNO is used as positive control.
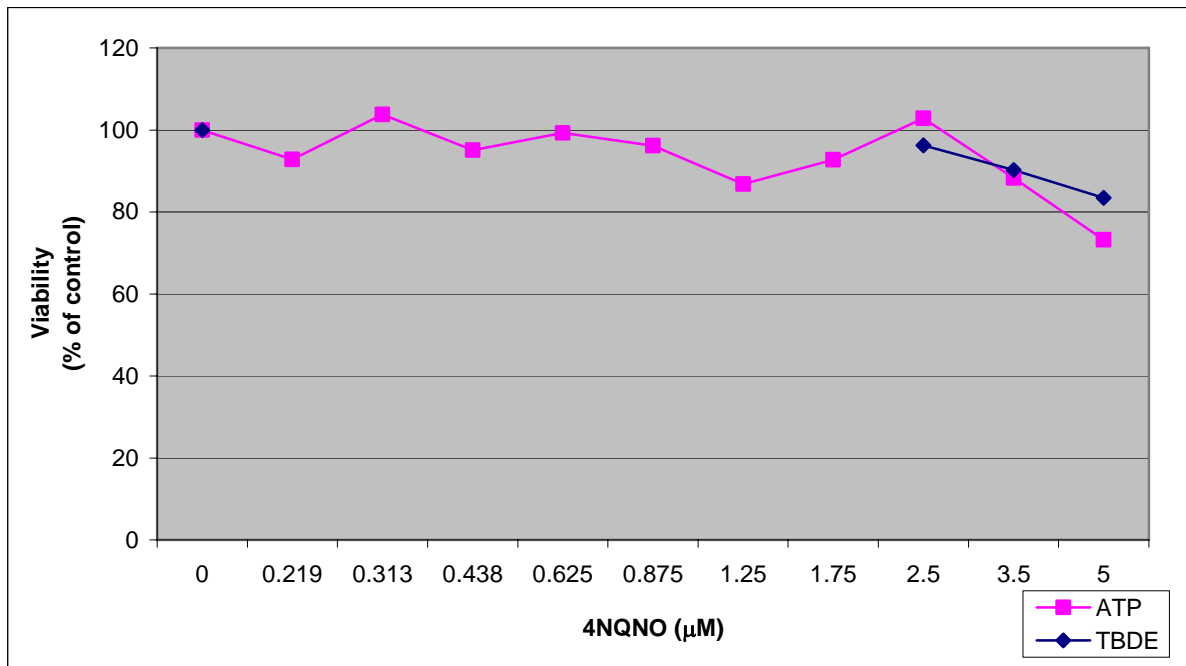
Figure 9: Effects on viability of ML cells exposed to serial concentrations of 4NQNO for 3 hours prior Comet assay. Viability is measured by ATP levels and TBDE method. Results are expressed as the mean percentage of control (mean; n=8 for ATP and mean; n=2 for TBDE). As controls served cells exposed to the solvent without test compound.

DNA migration parameters showed a clear dose-dependent curve (*Figure 10*), especially for tail moment. The frequency of NDCN was non-significant. The observed LOEL value (0.219 µM, tail moment) corresponds well with the historical value (0.250 µM)
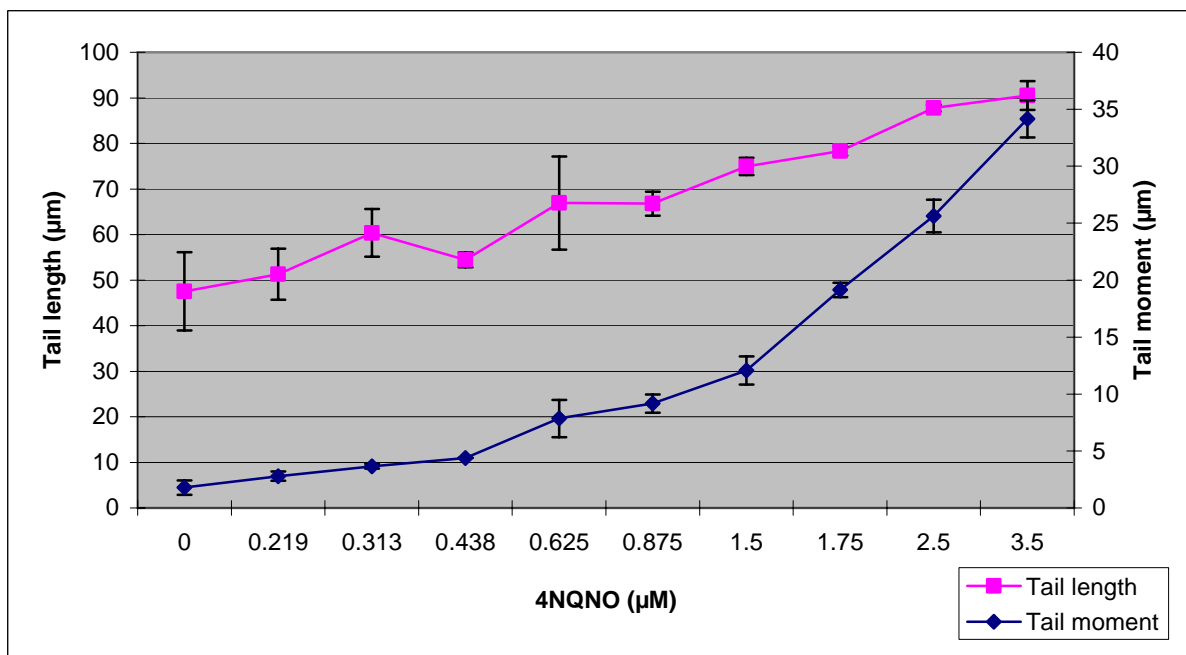

Figure 10: Parameters obtained in the Comet assay according to protocol B. ML cells exposed to serial concentrations of 4NQNO for 3 hours. Results are expressed as the mean tail length and tail moment (mean ± SD; n=50 cells). As controls served cells exposed to the solvent without test compound.

MMC was tested according to protocol A and was the only cross-linking agent used in this study. All concentrations of MMC, except for the highest one (800 µM), showed unchanged ATP levels as compared to the solvent control (*Figure 11*). All doses moved on to geno-toxicity test in the Comet assay.
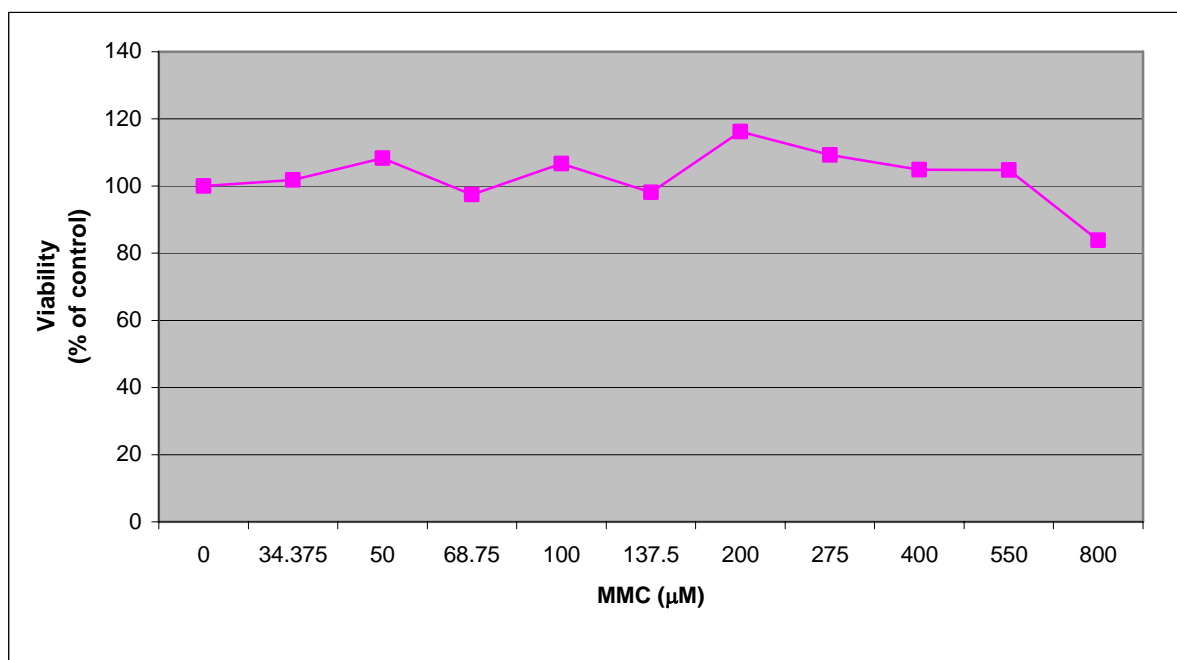

Figure 11: Effects on viability of ML cells exposed to serial concentrations of MMC for 3 hours prior Comet assay. The viability is measured by ATP levels. Results are expressed as the mean percentage of control (mean; n=5). As controls served cells exposed to the solvent without test compound.

Results from the Comet measurements (*Figure 12*) indicate a slight increase of DNA migration in tail length for doses up to 50 µM and in tail moment up to 100 µM. At higher concentrations, a significant reduction of DNA migration was observed as compared to the solvent control. No NDCN were detected during analysis. Due to problems with gel slippage, only one sample for 68.75 µM was captured during image analysis.
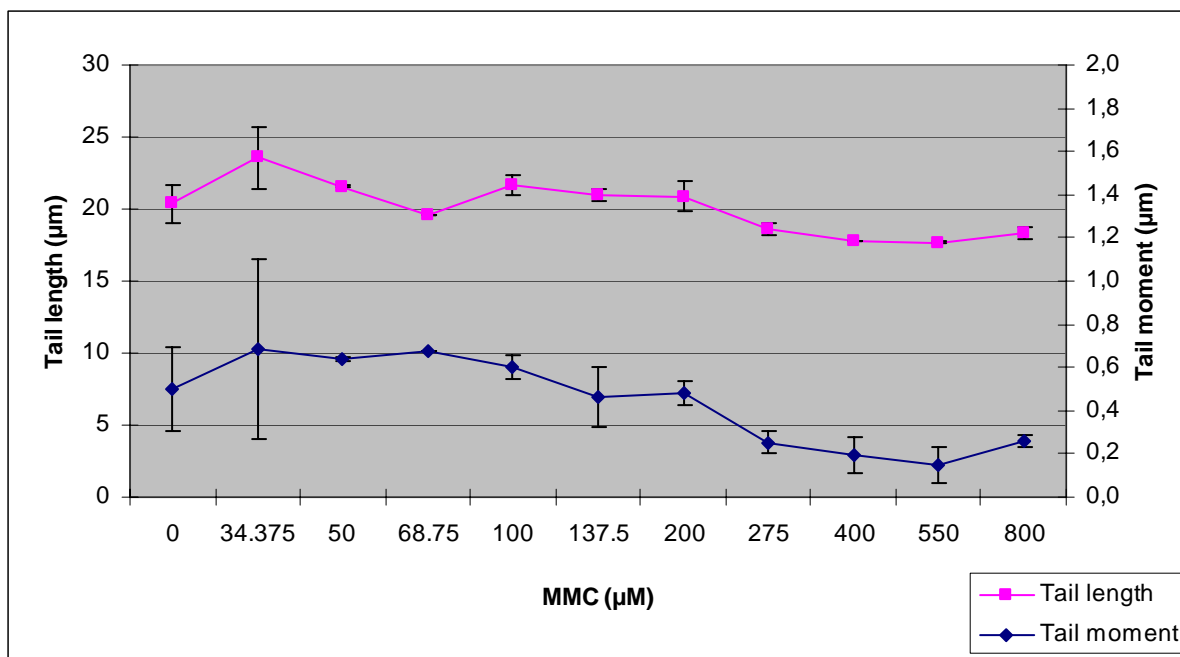
Figure 12: Parameters obtained in the Comet assay according to protocol A. ML cells exposed to serial concentrations of MMC for 3 hours. Results are expressed as the mean tail length and tail moment (mean ± SD; n=50 cells). As controls served cells exposed to the solvent without test compound.

The results are in accordance with the previously published data on the genotoxic properties of MMC (Warren *et al.* 1998). MMC is a cytotoxic chemotherapeutic agent that causes DNA damage in the form of DNA cross-links as well as a variety of DNA monoadducts. In addition, MMC is known to induce p53. As crosslinks represent about 10-15% of the total MMC adducts, it would seem likely that the observed increase of DNA migration at lower doses originates from the repair of DNA monoadducts. At higher doses the frequency of DNA crosslinks would reach a level where it would influence the migration of DNA from the nucleus.

DT-diaphorase can reduce MMC to a reactive intermediate that is a bifunktional alkylating agent. This intermediate crosslinks DNA and produces SSB (Parkinson 2001). No activation system was used in this experiment consequently results may be different using an activation system.

B(a)P and DMBA were run together on the same 96-well plate during the experiments and they were the only compounds that were tested with metabolic activation. The cytotoxicity profiles for B(a)P and DMBA, as determined by ATP levels, are shown in *Figures 13* and *14*. Although already the lowest tested concentration (4.375 μM) of B(a)P was moderately cytotoxic, increasing concentrations did not result in increasing cytotoxicity. All concentrations of B(a)P moved on to genotoxicity test in Comet assay. DMBA, on the other hand, became cytotoxic to the ML cells first at 20 μM, here increasing concentrations of the chemical were paralleled with steep decrease of the ATP levels. All concentrations, except 40 and 60 μM, moved on to genotoxicity test in Comet assay. The highest dose, 60 μM, failed according to the evaluation criteria and 40 μM was not chosen because earlier studies have showed increased damage in another genotoxicity test at this concentration.
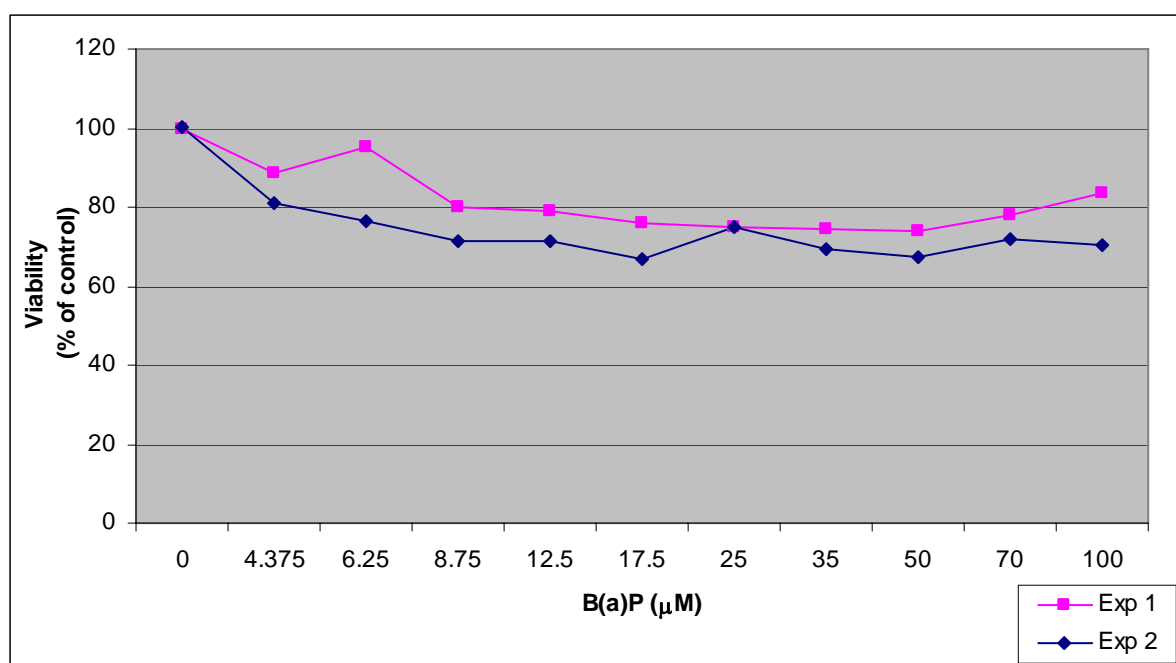

Figure 13: Effects on viability of ML cells exposed to serial concentrations of B(a)P in the presence of S9-mix for 3 hours prior Comet assay. The viability is measured by ATP levels from two independent experiments. Results are expressed as the mean percentage of control (mean; n≥4). As controls served cells exposed to the solvent without test compound.
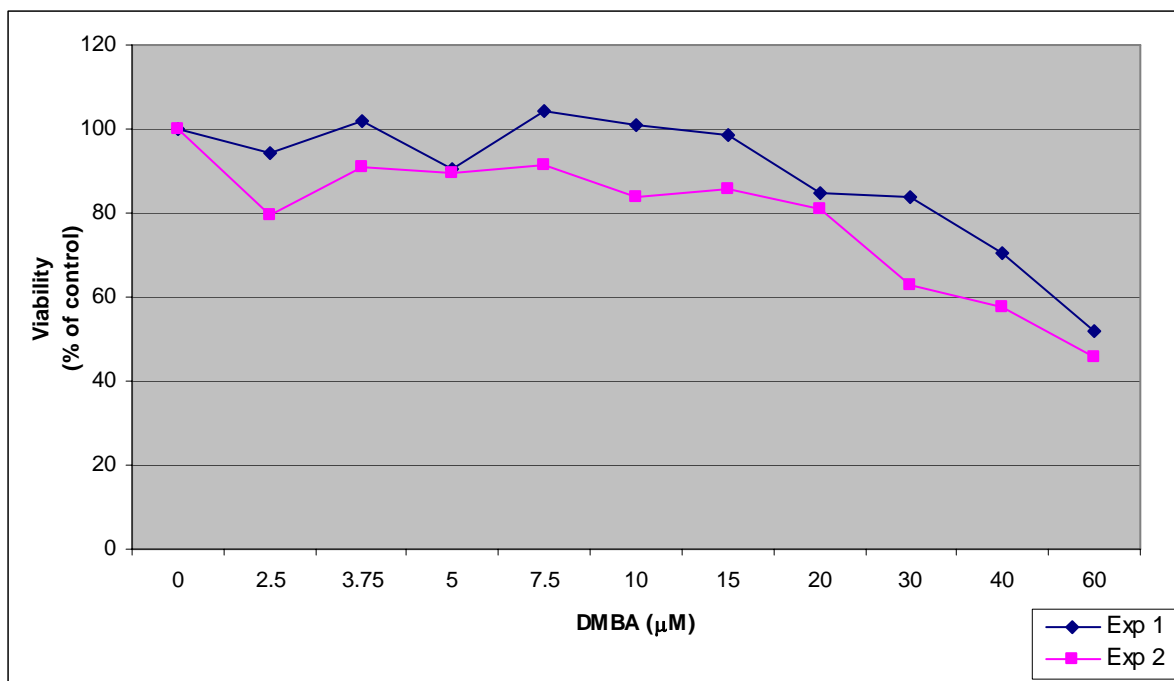
Figure 14: Effects on viability of ML cells exposed to serial concentrations of DMBA in the presence of S9-mix for 3 hours prior Comet assay. The viability is measured by ATP levels from two independent experiments. Results are expressed as the mean percentage of control (mean; n≥3). As controls served cells exposed to the solvent without test compound.

Comet data from protocol A for B(a)P and DMBA are shown in *Figures 15* and *16*. Both B(a)P and DMBA showed a slight dose-response curve in the Comet results. A few, up to 5%, NDCN were detected during analysis for both chemicals.
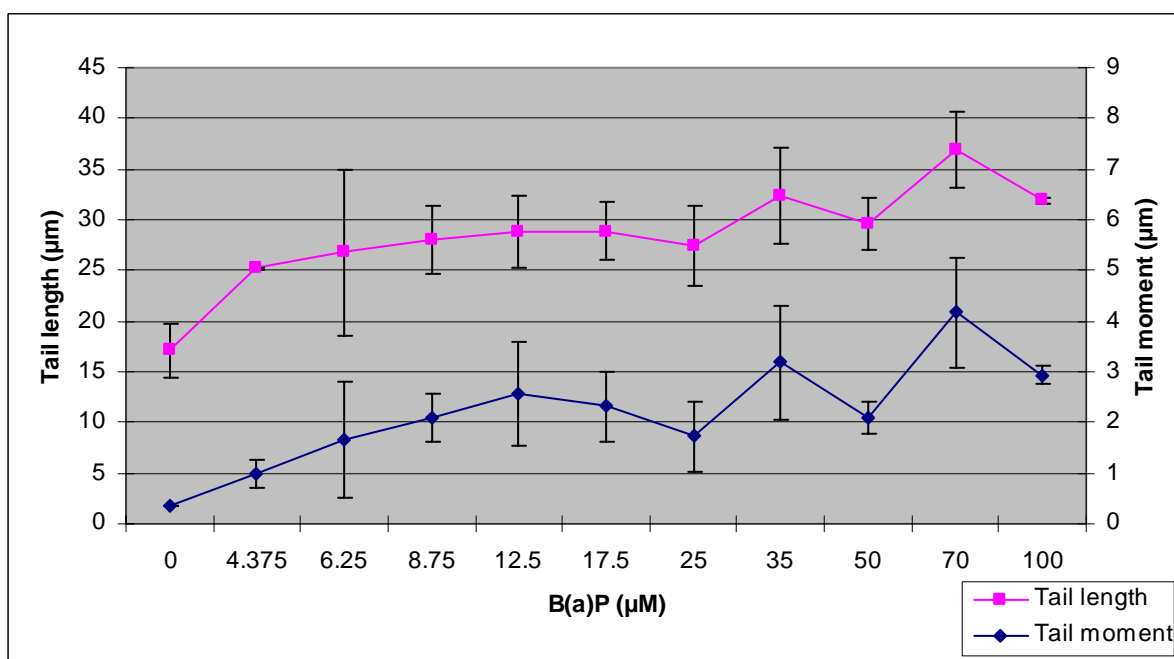

Figure 15: Parameters obtained in the Comet assay according to protocol A. ML cells exposed to serial concentrations of B(a)P for 3 hours in the presence of S9-mix. Results are expressed as the mean tail length and tail moment (mean ± SD; n=50 cells). As controls served cells exposed to the solvent without test compound.
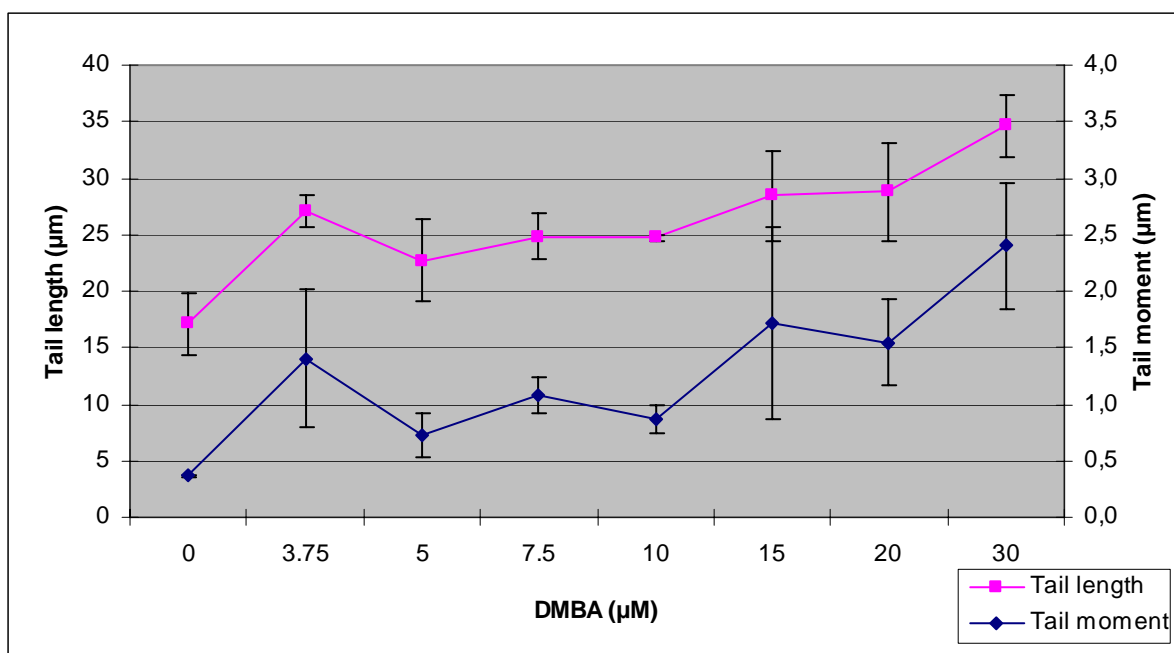
Figure 16: Parameters obtained in the Comet assay according to protocol A. ML cells exposed to serial concentrations of DMBA for 3 hours in the presence of S9-mix. Results are expressed as the mean tail length and tail moment (mean ± SD; n=50 cells). As controls served cells exposed to the solvent without test compound.

The results from the evaluation exercise of the modified high-throughput variant of Comet assay are summarized in *Table 1*. Observed LOELs obtained from the modified HT-ACA were in good accordance with the standard HT-ACA protocol, albeit some differences were revealed. For instance, the modified HT-ACA detected positive genotoxicity in concentrations two (B(a)P) to five (DMBA) times lower than earlier had been found with the standard HT-ACA protocol. Results from the present study are in contrast to the previously published evaluation of HT-ACA (Kiskinis *et al.* 2002), where the dose-response curves were similar for both tests but the absolute values for tail moment were lower in the high throughput variant (Kiskinis *et al.* 2002).

It should be noted, however, that even though the modified HT-ACA detected genotoxicity in lower concentrations, the dose response-curves tend to become saturated. This is especially true in the case of chemicals in need of metabolic activation. The most likely cause for the observed saturation for B(a)P and DMBA, seems to be depletion of cofactors or bad S9-mix preparation. The presence of proteins during electrophoresis is also known to influence the migration of DNA, therefore a remaining protein contamination from the S9-mix can cause a saturation of the dose-response curve.

Table 1: Data of historical LOELs from the standard HT-ACA protocol (unpublished data, AstraZeneca) and LOELs obtained in this study with the modified HT-ACA protocol.

Direct mutagens (-S9)	Historical LOEL (mM)	LOEL (mM)	Parameter
4-nitroquinoline-N-oxide (4NQNO)	0.00025 (-S9)	0.000219 (-S9)	Tail moment
Mitomycin C (MMC)	No effect (-S9)	0.275 (-S9)	Cross linking LOEL

Promutagens (+S9)	Historical LOEL (mM)	LOEL (mM)	Parameter
Benzo(a)pyrene (B(a)P)	0.00998 (+S9)	0.004375 (+S9)	Tail length, tail moment, rel. fraction of cells with DNA damage
9,10-dimethyl-1,2-benzanthracene (DMBA)	0.02 (+S9)	0.00375 (+S9)	Tail length, tail moment, rel. fraction of cells with DNA damage

7. Summary and future perspectives

The Comet assay has the potential to become a powerful complement to other genotoxicity tests during the early drug development. The results of this study showed equivalent or more sensitive outcomes for the modified high throughput variant than the standard HT-ACA protocol for the Comet assay. Still, there are some bottlenecks in the performance of this method and the choice of cytotoxicity test, which need to be addressed.

Cytotoxicity test

- Cytotoxicity marker - other investigators have concluded that measurements of ATP levels may be the more accurate parameter for cytotoxicity. The ATP level is an indicator of the functional integrity of living cells and any injury or cell death will lead to a corresponding decrease in cytoplasmatic ATP content (Crouch *et al.* 1993). In the case of the traditionally used TBDE method, only cells with a damaged membrane will allow uptake of dye and be considered dead. Nevertheless, according to Elia *et al.* certain compounds induce acute cytotoxicity without having an immediate effect on membrane integrity. A good thing to keep in mind is that the viability tests measures different types of cytotoxicity. Methods should be chosen according to what effect one is looking for. The most relevant approach would be to first measure ATP level changes in all samples and look for membrane damage with TBDE only in samples

with a significant decrease in ATP. If no significant decrease in ATP levels is present, there is probably no relevant cytotoxic damage either.

- Reproducibility - when comparing the results from the TBDE method and measurement of ATP levels (CellTiter-Glo®) using the generally applied viability values, ≥70% (TBDE method) and ≥50% (ATP levels) of the concurrent control, almost the same concentrations of the compounds were selected for further analysis in the Comet assay. Additional experiments are needed to establish if there is a good correlation between the tests.

HT-ACA

- Type of object slide – with use of multi-well object slides the number of samples that can be run at the same time has been greatly increased. At present, the equipment available at SAS allows simultaneous run of 21 object slides.

- Sample wash – samples selected to proceed in the Comet assay were moved from the 96-well plate to Eppendorf tubes to be washed in PBS. This is a time-requiring step posing a serious limitation for the throughput of the method (handling a great number of samples, limitations of centrifuge space). Thus, the washing steps should be optimized, preferentially using a centrifuge for 96-well plates and a multi-well pipette or performed twice.

- Cell density and focusing – it is rather laborious to find the 50 comets manually and adjust focus on the microscope each time. The cell concentration and volume of agarose-cell suspension applied per well should be optimized. Proper adjustment of the later is especially important in order to bring all the cells, after drying the agarose gel, to the same optical plane.

- Scoring procedure – the capture and evaluation of images would be greatly enhanced using a more modern version of the image analysis system. For instance, in the most recent version of the Comet assay software (Perceptive Instruments) there is no need to position a frame around each comet (from which background values are obtained). Here, a "one-click" scoring algorithm is used instead, resulting in increased speed of scoring.

- Evaluation of results – the Excel macros presently used to evaluate Comet assay results at SAS are designed to accommodate DNA migration parameters from 21

samples. A typical number of samples from a HT-ACA experiment would be around 70-90. Thus, the Excel macros must be improved.

- Reproducibility - only one single experiment per chemical was performed by the modified HT-ACA protocol.

- Evaluation criteria – the experiments in this study should be repeated using a standardized protocol in order to verify if the evaluation criteria previously developed for the standard HT-ACA protocol of the Comet assay can be applied in the modified HT-ACA protocol. Furthermore, new criteria must be found for cross-linking chemicals to find out when they should be considered as positive.

8. Acknowledgement

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10. Appendix

1%, 0.6% agarose   **0.6% LMP agarose**
Agarose in PBS   LMP agarose in PBS

Cofactor solution for S9-mix pH 7.2
3.9 mg NADP/mL R0P
16.7 mg Sodium isocitrate/mL R0P

200 mM EDTA
EDTA in water

Electrophoresis buffer pH >13
300 mM NaOH
1 mM Na2 EDTA

20 μg/mL ethidium bromide
Ethidium bromide in water

Lysis solution pH 10
2.5 M NaCl
100 mM EDTA
10 mM Trizma-Bas

10% DMSO
1% Triton X-100 } added before use

10M NaOH
Sodium hydroxide pellets in water

Neutralization buffer pH 7.5
0.4 M Trizma Base

PSGP
100 mL 10 000 units/mL penicillin and 10 000 μg/mL streptomycin in 85% saline
50 mL 200 mM L-Glutamine
100 mL 100 mM Sodium pyruvate

R10P (cell cultivation media supplemented with 10% serum)
500 mL RPMI 1640 (Dutch modification without L-Glutamine)
25 mL PSGP
5.5 mL Pluronic F68
55 mL heat inactivated horse serum

R0P (cell cultivation media without serum)
500 mL RPMI 1640 (Dutch modification without L-Glutamine)
25 mL PSGP
5 mL Pluronic F68