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Novel approach for the detection of alkylated bases using the enzyme-modified comet assay

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ABSTRACT

The enzyme-modified comet assay is widely used for the detection of oxidized DNA lesions. Here we describe for the first time the use of the human alkyladenine DNA glycosylase (hAAG) for the detection of alkylated bases. hAAG was titrated using untreated and methyl methanesulfonate (MMS)-treated TK-6 cells. The hAAG-modified comet assay was compared to the formamidopyrimidine DNA glycosylase (Fpg)-modified comet assay, widely used to detect oxidized lesions but that also detects ring-opened purines derived from some alkylated lesions, using cells treated with potassium bromate (oxidizing agent) or MMS. Moreover, neutral and alkaline lysis conditions were used to determine the nature of detected lesions. When alkaline lysis was employed (condition normally used), the level of hAAG-sensitive sites was higher than the Fpg-sensitive sites in MMS-treated cells and hAAG, unlike Fpg, did not detect oxidized bases. After neutral lysis, Fpg did not detect MMS-induced lesions; however, results obtained with hAAG remained unchanged. As expected, Fpg detected oxidized purines and imidazole ring-opened purines, derived from N7-methylguanines under alkaline conditions. It seems that hAAG detected N7-methylguanines, the ring-opened purines derived at high pH, and 3-methlyladenines. Specificity of hAAG towards different DNA lesions was evaluated using a multiplex oligonucleotide-cleavage assay, confirming the ability of hAAG to detect ethenoadenines and hypoxanthine. The hAAG-modified comet assay is a new tool for the detection of alkylated bases.

1. Introduction

Alkylation is the process of covalent bonding of an alkyl group to a broad range of biological molecules, including nucleic acids. Alkylation can occur by a simple addition reaction or by substitution of another functional group. Alkylating agents are typically highly reactive and unavoidable, as they are broadly ubiquitous, being present both in the environment and endogenously within living cells (Fu et al., 2012).

There are different sources of alkylating compounds in the environment, such as pollutants that may be present in food, water or air (e.g. some tropical plants and fungi produce chloromethane, and some methylating agents are generated in tobacco smoke or fuel combustion products) (Ballschmiter, 2003; Hamilton et al., 2003; Ma et al., 2019). Moreover, alkylating compounds comprise a major class of cytostatic drugs in cancer therapy (Hurley, 2002; Drabløs et al., 2004). Additionally, alkylating compounds can also be found endogenously, arising as byproducts of oxidative stress or during metabolism; for instance, S-adenosylmethionine, a physiological methyl radical donor in enzymatic reactions *in vivo* (Rydberg and Lindahl, 1982).

On DNA, alkylating agents may form adducts at all oxygen and nitrogen atoms of the DNA bases, generating a variety of lesions with different complexity and implications in living cells, including cytotoxicity and mutagenicity (Drabløs et al., 2004; Shrivastav et al., 2009). Depending on the position of these alterations in DNA, the adducts induced by alkylating agents may pose a threat to genome integrity, as an unrepaired or erroneously repaired DNA lesion may lead to a mutation (thereby promoting carcinogenic processes) and/or may block essential biological processes (DNA replication or transcription). Additionally, it is worth mentioning that some lesions can be processed into byproducts that can also be clastogenic or cytotoxic (Fu et al., 2012).

N-methylation adducts are the most common alkylated bases, N7-

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methylguanine being the predominant adduct, comprising between 60–80 % of the total alkylation lesions in DNA (Shrivastav et al., 2009). In general, these N-alkylations (e.g., N7- and N1-methylguanine or N3-methyladenine) are mainly cytotoxic (blocking DNA polymerases and DNA synthesis), being less mutagenic compared to O-alkylations (Kondo et al., 2010), which are generated to a much lesser extent and are of great biological significance. O6-methylguanine is a primary mutagenic lesion under most conditions of alkylation damage to DNA, as it induces G-A transitions during replication; and O4-methylthymine is a mispairing lesion that also presents mutagenic potential (Shrivastav et al., 2009; Fu et al., 2012).

Nowadays there are several methods available for measuring different lesions in DNA, such as chromatographic techniques, the comet assay, polymerase chain reaction assays, mass spectrometry, electrochemistry, radioactive labeling, immunochemical methods or different sequencing methods (reviewed in Himmelstein et al., 2009). However, all have some limitations and only a few are applicable to the detection of alkylation damage in DNA.

The alkaline comet assay (single cell gel electrophoresis) is a widely used method for measuring DNA damage at the single cell level (Azqueta and Collins, 2013). Particularly, it detects strand breaks (SB) and alkali-labile sites (ALS) such as apurinic or apyrimidinic (AP) sites. It is relatively simple, economical and very versatile, as it can be applied to almost any eukaryotic cell type or to disaggregated tissues. Briefly, cells are embedded in agarose on a microscope slide and then lysed to form nucleoids, which contain supercoiled DNA. Lysed cells are further subjected to alkaline pH incubation to unwind the DNA prior to electrophoresis. Finally, naked DNA of individual cells is evaluated under the microscope: if DNA contains any SB, supercoiling will be disrupted so part of the DNA will migrate during the electrophoresis giving a comet-like image; whereas if DNA remains intact, supercoiling will be preserved and migration will not occur.

During the last three decades, the assay has been modified to detect other lesions, mainly oxidized bases, by the use of DNA-repair enzymes (DNA glycosylases), such as formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III), which are the most frequently used. These enzymes are able to detect and remove the base, leaving an AP-site, which is then converted to a SB by an associated AP lyase activity of the enzyme (Azqueta and Collins, 2013). In practice, enzyme digestion is applied to the nucleoids that are formed after lysing the agarose-embedded cells. A digestion with enzyme buffer alone gives a measure of SBs and ALS; the difference between comet scores for the two gels, +buffer and +enzyme, indicates the frequency of 'net enzyme-sensitive sites' (Azqueta et al., 2013a). Traditionally, this enzyme-modified comet assay has been applied mainly for the detection of oxidized bases (Collins, 2014).

However, specific enzymes for the detection of alkylated bases have also been applied in combination with the comet assay. The enzyme 3-methyladenine DNA glycosylase II (AlkA) is a bacterial repair enzyme with 3-methyladenine as its main substrate. It was first reported in combination with the comet assay by Collins et al. (2001). Similarly, the enzyme 3-methyladenine DNA glycosylase (AlkD) is also a bacterial repair enzyme that was applied in combination with the comet assay for the first time by Hašplová et al. (2012). It is specific for 3-methyladenine and 7-methylguanine. However, according to our knowledge, these enzymes are not commercially available and so the majority of researchers do not have access to them or the facilities and knowledge to produce them. Actually, these enzymes have not been extensively used and there are no recent publications showing their use in combination with the assay; there are a total of 16 publications using AlkA, most of them from the same group, and only 2 publications using AlkD).

It is worth mentioning that Fpg, apart from detecting oxidized bases, also detects ring-opened purines derived from some alkylation lesions at alkaline conditions (i.e., during lysis at pH 10 in the comet assay) (Speit et al., 2004; Smith et al., 2006; Azqueta et al., 2013a; Hansen et al., 2018).

Here we describe, for the first time, the use of the human enzyme alkyladenine DNA glycosylase (hAAG), a commercially available enzyme, in combination with the comet assay to detect alkylated bases on DNA of human lymphoblastoid cells (TK-6 cell line). This enzyme, also known as methylpurine DNA glycosylase (MPG) and alkyl-N-purine DNA glycosylase (ANPG), is the enzyme initiating the base excision repair (BER) pathway for the repair of alkylation adducts. In particular, it detects 3-methyladenine and 7-methylguanine (O'Connor, 1993). hAAG is a monofunctional glycosylase that catalyzes the hydrolysis of the N-glycosidic bond, releasing the N-alkyl-adduct from DNA and leaving an abasic site that can be detected using the comet assay (Lau et al., 1998). Moreover, the Fpg-modified and the hAAG-modified comet assay are compared under two different lysis conditions (i.e., pH 7 and pH 10) and the activity of hAAG in detecting other lesions was determined by using the Glyco-SPOT assay (multiplex oligonucleotide-cleavage assay).

2. Material and methods

2.1. Chemicals and reagents

Low melting point agarose, standard agarose, Triton X-100, Tris base, HEPES, Na_2EDTA, Bovine Serum Albumin (BSA), NaOH, KCl, potassium bromate ($KBrO_3$) methyl methanesulfonate (MMS) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. DPBS 1x for mixing cell suspensions with agarose was purchased from Gibco. DPBS without Ca^{+2} and Mg^{+2} 10x from Lonza was used to prepare PBS 1x washing solutions for comet assay slides. Dimethyl sulfoxide was purchased from PanReac AppliChem. All cell culture reagents were purchased from Gibco.

hAAG and Endo III were purchased from New England Biolabs (catalog number M0313S and M0268S respectively). Fpg from an overproducing *E. coli* strain was kindly provided by NorGenoTech AS (Oslo, Norway).

2.2. Cell culture

The human-derived lymphoblastoid TK-6 cell line was obtained from the American Type Culture Collection (ATCC). Cells were grown in RPMI (Roswell Park Memorial Institute, ref. A10491-01, Gibco) medium containing D-glucose, HEPES, L-glutamine, sodium bicarbonate and sodium pyruvate and supplemented with 10 % heat-inactivated fetal calf serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin (all from Gibco). Cells were maintained as a suspension culture in continuous agitation at 37 °C in a humidified atmosphere with 5 % CO_2. Cells were maintained in culture for no longer than 2 months.

2.3. Treatment and freezing of cells

TK-6 cells were seeded at 1×10^6 cells/mL in culture medium containing no serum and treated for 3 h with different non-cytotoxic concentrations of either MMS or $KBrO_3$ or their vehicles (DMSO and water respectively). Using these conditions, preliminary studies were performed to assess cytotoxicity employing the proliferation assay according to Azqueta et al. (2013a) and concentrations with a relative suspension growth (RSG) over 80 % were selected for further experiments. Concentrations for specific purposes are detailed in their respective sections. Cells were kept in continuous agitation at 37 °C in a humidified atmosphere with 5 % CO_2.

From this point, cells were kept cold to prevent DNA repair. After treatment, cells were centrifuged and washed twice with phosphate-buffered saline (PBS). Then cells were resuspended in culture medium containing 5 % DMSO and aliquoted in cryotubes at 1×10^6 cells/mL in 0.5 mL. Finally, cells were frozen by using the freezing container Mr. Frosty (Thermo Scientific, Nalgene). The container including the cryotubes was placed in a freezer at −80 °C at least overnight. After

that, cryotubes were transferred to boxes and kept at −80 °C until analysis.

2.4. hAAG-modified comet assay

2.4.1. Titration

The comet assay was performed using the medium-throughput format of 12-Gel Comet Assay Unit™ (Shaposhnikov et al., 2010) as previously described (Muruzabal et al., 2018). To titrate the enzyme, frozen cells (untreated and 1.25 μM MMS-treated cells; previous section) were employed as substrate. MMS was employed as it is a known alkylating agent. Frozen cells were quickly thawed by immersing the cryovial in a water bath at 37 °C and washed in 10 mL of cold PBS by centrifugation. Then cells were suspended in PBS at 2.5×10^5 cells/mL. For the preparation of the agarose minigels, 30 μL of cell suspension were mixed with 140 μL of 1 % low melting point agarose in PBS at 37 °C. After that, 12 droplets of 5 μL each of the corresponding cell suspension were placed on agarose-precoated slides. Slides were placed on the bottom metal holder of the 12-Gel Comet Assay Unit™ (NorGenoTech, Oslo, Norway), previously cooled in the fridge, which contains a template to set the minigels in certain positions (two rows of six).

Once gels were prepared, slides were immersed in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 0.1 M Tris base, pH 10 and 1 % Triton X-100) at 4 °C for 1 h. Prior to enzyme/buffer treatment, slides were washed three times, 5 min each at 4 °C with the reaction buffer of the enzyme (40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg/mL BSA, pH 8).

During the washes, hAAG was prepared by making serial dilutions: in preliminary studies a broad range of enzyme dilutions (i.e., from 1:100 to 1:1,000,000 using a dilution factor of 10) was employed and the range was then reduced to 1:300 − 1:100,000; with 1:3 and 1:3.33 dilution factors. The enzyme was diluted from the original stock (10,000 U/mL) using the reaction buffer. After washing with the enzyme reaction buffer, slides were transferred to a cold 12-Gel Comet Assay Unit™ (12-Gel Comet Assay Unit™, NorGenoTech, Oslo, Norway). These units allow differential treatments, as gels are isolated in wells on each slide. Units were placed on a cold metal plate to keep them cold during the enzyme or buffer addition to avoid enzymatic reactions until incubation. Thirty microliters of hAAG enzyme or reaction buffer were pipetted to each well and a clean slide was placed on top of the unit to cover all wells and prevent contamination and evaporation. The design of each 12-minigels slide was the same in all cases: 2 minigels were incubated with enzyme buffer alone and 5 concentrations of hAAG were tested on 2 minigels each. The 12-Gel Comet Assay Units™ were then transferred to a pre-heated moist box and placed in the incubator at 37 °C. To detect the optimal incubation time, initially different times of incubation were employed (15 min, 30 min, 45 min, 1 h and 1 h 15 min).

After incubation, the enzyme reaction was stopped by placing the units on a cold plate. Subsequently, slides were transferred to the electrophoresis tank for unwinding in electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH > 13) during 40 min at 4 °C. Afterwards, electrophoresis was carried out at 1.2 V/cm for 20 min (4 °C).

Following electrophoresis, slides were neutralized by washing them in PBS for 10 min and then in distilled water for 10 min. To dehydrate the gels for avoiding an edge effect (i.e. comets going in different angles) (Azqueta et al., 2013b), slides were immersed in 70 % ethanol for 15 min and then in absolute ethanol for 15 min. Finally, slides were air dried at room temperature overnight.

For scoring the comets, each gel was stained with a 5 μL drop of 1 μg/mL of 4,6-diamidino- 2-phenylindole (DAPI) and a coverslip (24 × 60 mm) was placed on top to cover all the minigels of a slide. After 30 min of incubation with DAPI at room temperature, slides were analyzed using the semi-automated image analysis system Comet Assay IV (Perceptive Instruments) and 50 nuclei per gel, 100 per condition, were scored. The percentage of DNA in tail (or tail intensity) was used as descriptor for each comet, and the median percentage tail DNA of 100 comets was taken as the measure of DNA damage for each condition. Net enzyme-sensitive sites were calculated by subtracting the percentage tail DNA obtained with the buffer incubation alone from that obtained after hAAG incubation at different concentrations. To determine the optimal concentration and incubation time for the enzyme, relative activity of hAAG was calculated by subtracting the level of DNA damage of non-treated cells from the level of the MMS-treated ones.

Titration experiments using 12-Gel Comet Assay Unit™ were performed twice (independent experiments).

2.4.2. Measuring different levels of alkylated bases

The hAAG-modified comet assay using different concentrations of hAAG (i.e., 33.33, 10, 3.33. 0.33 and 0.1 U/mL) and 1 h of incubation was applied to cells containing different levels of alkylated lesions. In particular, cells treated with a range of non-cytotoxic concentrations of MMS (0, 1.25, 2.5, 3, 4 and 10 μM) were employed to test whether higher levels of DNA damage could be detected. At least three independent experiments (four in some cases) were performed using the 12-Gel Comet Assay Unit™ with each of the MMS concentration.

A concentration-response curve using the selected conditions for hAAG incubation, 10 U/mL of enzyme and 1 h of incubation time at 37 °C was constructed.

2.5. hAAG- vs Fpg-modified comet assay

2.5.1. Concentration-response experiments with MMS and $KBrO_3$

Performance of hAAG and a crude extract of Fpg (from an overproducing *E. coli* strain) was compared towards different substrates using the 12-Gel Comet Assay Unit™. The comet assay was performed as previously described using the optimized conditions for the incubation with hAAG (10 U/mL and 1 h at 37 °C) employing cells treated with either MMS (0, 5, 10 and 20 μM) or $KBrO_3$ (0, 0.313, 0.625 and 1.25 mM) for 3 h. Fpg was previously titrated (Muruzabal et al., 2018). The same reaction buffer was used for both enzymes. Two independent experiments were performed.

2.5.2. Activity in MMS- and $KBrO_3$- treated cells using neutral and alkaline lysis

Performance of hAAG and a crude extract of Fpg (from an overproducing *E. coli* strain) was studied using neutral and alkaline lysis conditions in the comet assay. These experiments were performed in a different laboratory using a high throughput (HTP) comet assay format with Gelbond® films (Cambrex, Rockland, ME) as a matrix. For this reason, the enzyme was also titrated with this format. This HTP version was performed according to Hansen et al., 2010 and Gutzkow et al., 2013 with some modifications; unless otherwise indicated recipes of the solutions and buffers were the same as explained in Section 2.4.1. Briefly, cells were mixed with 1 % low melting point agarose dissolved in PBS at 37 °C and moulded as 48 gels (4 μL each) per GelBond® film (being 0.82 % the final agarose concentration). The moulding process was performed over metallic plates that were previously cooled in the fridge at 4 °C. Films were immersed immediately in lysis solution (pH 10) for 1 h. Prior to enzyme incubation, films were washed three times (5 min each) with enzyme buffer at 4 °C. Commercial hAAG enzyme was titrated using five dilutions (1:1,000 − 1:100,000 using 1:3 and 1:3.33 as dilution factors) from the original stock using only 1 h as incubation time. Incubation was performed by immersing the films in trays with enzymes diluted on enzyme buffer for 1 h at 37 °C. After enzyme treatment, electrophoresis was performed at 0.85 V/cm at 4 °C and with a solution recirculation system. The remaining procedure was the same as aforementioned except for film staining for scoring. Staining was performed with 1:12,500 SYBR Gold nucleic-acid gel stain (Invitrogen, Oslo, Norway) in TE buffer (10 mM Trizma HCl and 1 mM Na_2EDTA, pH 8) for 20 min with shaking in the dark.

Once conditions were optimized, untreated cells and cells treated with either MMS (2.5, 5 or 10 μM) or $KBrO_3$ (0.5, 1 or 2 mM) for 3 h

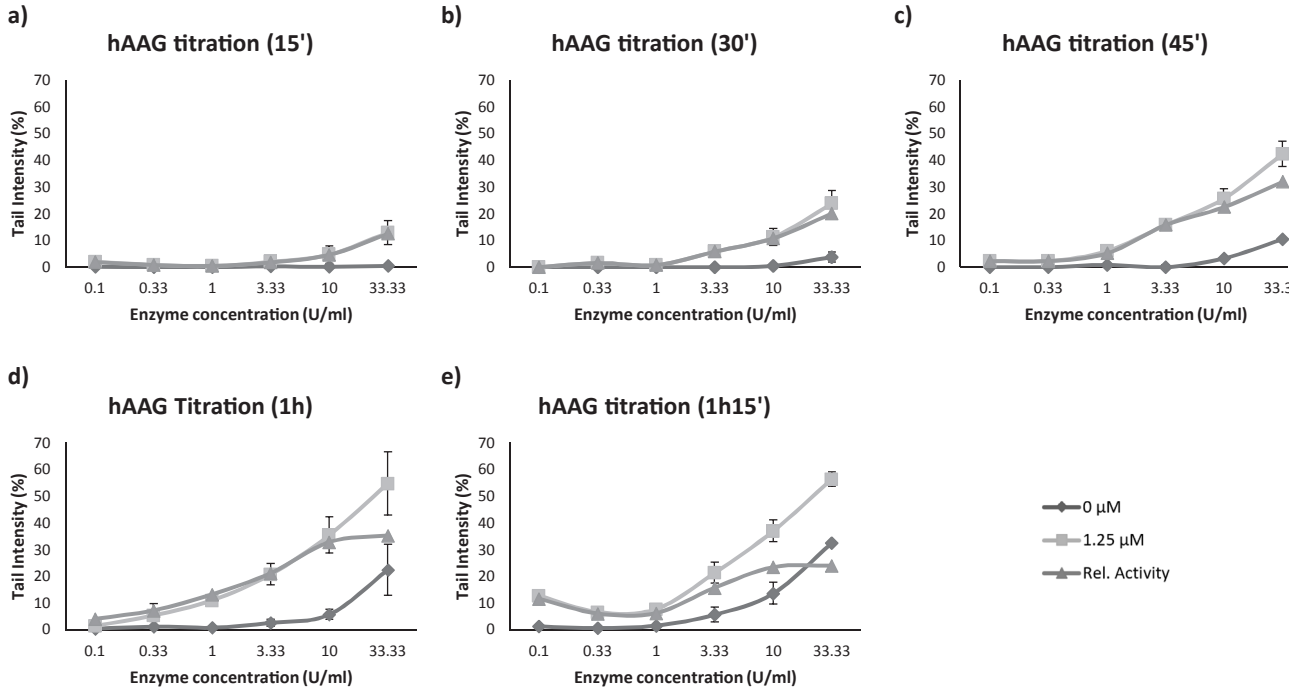

Fig. 1. *hAAG titration using the 12-Gel Comet Assay Unit™.* Titration curves for the different incubation times tested: a) 15 min, b) 30 min, c) 45 min, d) 1 h and e) 1 h and 15 min. Results of 0 and 1.25 µM MMS are expressed as net enzyme-sensitive sites (in terms of tail intensity); relative activity of hAAG was calculated by subtracting the level of DNA damage of non-treated cells from the level of treated ones. Data from two independent experiments are presented (mean and individual experimental values).

were employed as substrate. The HTP version of the comet assay employing GelBond® films was used following the protocol describe in Section 2.4.3 for the titration of hAAG. Additionally, lysis buffers with different pH were used. To do so, a duplicate of each film was prepared, and then half of the films were immersed in basic lysis solution (pH 10), and the remaining films were immersed in neutral lysis solution (pH 7) for 1 h; the remaining procedure was the same as previously described. hAAG enzyme was employed at 1 U/mL and Fpg at 0.5 µg/mL (as it was a home-made stock, the amount of units was not determined) and the incubation time with both enzymes was 1 h at 37 °C. Two independent experiments were performed.

2.6. Glyco-SPOT assay

The Glyco-SPOT assay is a multiplexed ODN (oligonucleotide) cleavage assay on support and it was used to simultaneously control the activity of hAAG toward several potential substrate lesions. 24-well glass slides (Streptavidin-coated, Xantec bioanalytics, Germany) functionalized with a panel of ODNs bearing different lesions (8oxoG paired with C -8oxoG-C-, A paired with 8oxoG -A-8oxoG-, ethenoadenine paired with T -EthA-T-, hypoxanthine paired with T -Hx-T-, tetrahydrofuran -abasic site stable analog- paired with A -THF-A-, thymine glycol paired with A -Tg-A-, and Uracil paired either with G or with A -U-G and U-A, respectively-) and labelled with a Cy3 at their end, were used as described in Candéias et al., 2010; Pons et al., 2010. Each ODN was immobilized in duplicate in each well together with a Control-ODN that contained no modification. Five different concentrations of hAAG were tested (final concentration: 0.007 U, 0.02 U, 0.07 U, 0.2 U, 0.6 U) in two different wells in the same enzyme-reaction buffer as employed for the enzyme-modified comet assay (for details see Section 2.4.1). As hAAG is a monofunctional enzyme, it requires an additional AP-endonuclease activity to cleave the abasic site resulting from the cleavage of the N-glycosidic bond (Lau et al., 1998). Consequently, the removal of the damaged base by hAAG was revealed through the addition of Endo III (0.5 U/well) that catalyzed the cleavage of the abasic site. (In the comet assay, cleavage is achieved by the alkaline conditions during unwinding and electrophoresis.) The activity of Endo III was initially titrated to select the most adapted concentration. Indeed, it was active only against thymine glycols. The excision reaction was run for 60 min at 37 °C under agitation (700 rpm). Then the slides were washed 2 × 5 min in 1XPBS containing 0.2 M NaCl - 0.05 % Tween 20 and dried by centrifugation.

For each spot, fluorescence was quantified at 532 nm wavelength using the Innoscan 710AL scanner from Innopsys (Toulouse, France) and the associated MAPIX software. Data were normalized as described using NormalizeIt software (Millau et al., 2008). To calculate the final cleavage rate of each ODN-containing lesion, the fluorescence of the control well, incubated with the excision buffer only, was taken as reference (100 % fluorescence). The data were also corrected by the control-ODN cleavage rate that remained below 10 %. Finally, the lesion-ODN cleavage percentage was 100 x (1 - percentage of fluorescence of lesion-ODN/percentage of fluorescence of control-ODN).

2.7. Statistics

The median percentage of DNA in tail for 50 comets was calculated for each of the duplicate minigels in each experiment; the mean of the two medians was then calculated. We show the mean percentage of DNA in tail for the duplicate experiments together with the individual experimental values in the titration experiments and in the experiments comparing the performance of hAAG- and Fpg-modified comet assay. The mean and the SD of the 3 or 4 independent experiments performed when testing the capability of hAAG to detect different levels of alkylated lesions are shown.

3. Results

3.1. hAAG-modified comet assay

3.1.1. Titration of hAAG

For hAAG titration, untreated and 1.25 μM MMS-treated cells were used as substrate and the assay was performed with the 12-Gel Comet Assay Unit™. To select an appropriate range of hAAG dilutions to test, preliminary studies were performed with a broad range of enzyme dilutions (data not shown) to select the final range presented here (0.1–33.33 U/mL).

Fig. 1 shows the titration curves of hAAG after 15, 30 and 45 min, 1 h and 1 h 15 min of incubation with nucleoids from MMS-treated and untreated cells. In this figure, net hAAG-sensitive sites obtained in treated and untreated together with the relative activity of the enzyme are presented. MMS-treated cells showed a clear enzyme concentration-response at all incubation times tested, especially after 45 min, and reached the maximum level of enzyme-sensitive sites detected at 1 h of incubation; after 1 h 15 min of incubation the detected DNA damage on treated cells did not increase further. Regarding non-treated cells, hAAG-sensitive sites were found at the highest concentration tested (33.33 U/mL) in all incubation times from 45 min onwards (Fig. 1*c*–*e*). A clear increase of breaks was also found at lower concentrations of enzyme after 1 h 15 min of incubation (Fig. 1*e*), suggesting a non-specific nuclease activity.

When studying the relative activity of the enzyme, a plateau was reached at almost 40 % of DNA in tail after 1 h of incubation time with the two highest concentrations tested (i.e. 10 and 33.33 U/mL) (Fig. 1*d*). Indeed, it was the only incubation time along with 1 h 15 min in which a plateau was reached. However, after 1 h 15 min of incubation, the plateau of relative activity was reached at lower levels of DNA damage (20 % of DNA in tail) (Fig. 1*e*).

Thus, the following conditions were selected as optimal for the hAAG-modified comet assay: 10 U/mL of enzyme and 1 h as incubation time. Thus, according to these results the range of hAAG-sensitive sites that can be detected goes from 0 to at least 30 % of DNA in tail.

3.1.2. Measuring different levels of alkylated bases

Following the titration experiments, to evaluate whether hAAG could detect high levels of alkylated lesions, the hAAG-modified comet assay was performed using a range of concentrations of the enzyme with cells treated with different non-cytotoxic concentrations of MMS (0, 1.25, 2.5, 4 and 10 μM, RSG > 80 %). These experiments were performed using the 12-Gel Comet Assay Unit™. Fig. 2 shows the net enzyme-sensitive sites obtained for each condition (Fig. 2*a*), the relative activity for each enzyme concentration (Fig. 2*b*) and the resulting concentration-response curve using the selected conditions for hAAG (i.e., 10 U/mL of enzyme and 1 h of incubation time at 37 °C) (Fig. 2*c*).

As expected, the highest hAAG concentration tested (i.e. 33.33 U/mL) produced breaks in untreated cells, whereas no significant levels of enzyme-sensitive sites were detected at the remaining concentrations (Fig. 2*a*). However, a slight increase (5 % of DNA in tail) was observed using hAAG at 10 U/mL (Fig. 2*a)*.

Regarding MMS-treated cells, a clear concentration-response pattern was observed in all MMS concentrations tested. The assay was saturated at the highest MMS concentration (10 μM) from 3.33 U/mL of hAAG (Fig. 2*a*).

The relative activity of the enzyme was also calculated for each hAAG concentration at all conditions tested (Fig. 2*b*). As shown in the figure, a plateau is reached at 10 U/mL of hAAG in the case of MMS concentrations of 1.25 and 1.5 μM. With higher MMS-concentrations, the relative activity with 33.33 U/mL of enzyme decreased considerably; this effect is accounted for by the high levels of unspecific DNA damage in non-treated cells combined with the saturation of the assay in the treated ones.

A concentration-response curve showing the activity of hAAG under selected conditions (i.e., 10 U/mL and 1 h of incubation) against substrates treated with different MMS concentrations was also constructed (Fig. 2*c*). The figure shows that with 10 U/mL of hAAG it was possible to detect MMS-induced alkylating damage from low concentrations up to the upper detection limit of the assay (i.e., about 80 or 90 % of DNA in tail). Despite the marginal unspecific damage induced in non-treated cells, it was the only concentration able to detect such levels.

In these experiments, the enzyme reaction buffer alone did not induce DNA damage in any of the conditions tested (data not shown), which indicates the absence of SB.

3.2. hAAG- vs Fpg-modified comet assay

3.2.1. Concentration-response experiments with MMS and $KBrO_3$

The activities of hAAG and Fpg were compared using the 12-Gel Comet Assay Unit™. For this purpose, cells were treated at three non-toxic concentrations of either MMS, an alkylating agent, or $KBrO_3$, an oxidizing compound. The net enzyme-sensitive sites obtained with both enzymes are shown in Fig. 3.

When cells treated with different concentrations of MMS were analyzed (Fig. 3*a*), both enzymes showed a concentration-dependent increase in tail intensity representing the detection of alkylated lesions. However, hAAG detected considerably higher levels of DNA damage, as it reached the upper detection limit of the assay (i.e., about 80 or 90 % of DNA in tail), even at the lowest MMS concentration tested (5 μM). Regarding the oxidizing compound (Fig. 3*b*), a concentration-dependent increase in DNA damage was observed with Fpg, whereas no damage was detected with hAAG. As it can be seen in Fig. 3, in these experiments the enzyme reaction buffer alone did not induce DNA damage in any of the conditions tested, which indicates the absence of SB.

3.2.2. Activity in MMS- and $KBrO_3$-treated cells using neutral and alkaline lysis

A HTP format of the comet assay using GelBond® films was used to carry out these experiments. To apply the hAAG-modified comet assay in this format, titration experiments were carried out in untreated and MMS-treated cells using 1 h of incubation and 5 different hAAG concentrations (i.e., 0.1, 0.33, 1, 3.33 and 10 U/mL). Results showed that 1 U/mL obtained the highest relative activity without inducing unspecific damage in non-treated cells (data not shown). This hAAG concentration and 1 h of incubation were used in the following experiments.

The Fpg- and hAAG-modified comet assay was applied in MMS- and $KBrO_3$-treated cells using neutral and alkaline lysis solution to elucidate the type of lesions detected. Lysis at alkaline pH converts some alkylated bases into ring-opened purines (Speit et al., 2004). The net enzyme-sensitive sites obtained for both enzymes in each experimental condition are shown in Fig. 4. As can be observed, the pH during the lysis step did not affect the level of MMS-induced DNA lesions detected with hAAG at any of the concentrations tested (Fig. 4*a* and *c*). The net-hAAG sensitive sites increased depending on the MMS concentration. However, the concentration-dependent increase observed in the levels of Fpg-sensitive sites when using an alkaline lysis (*Fig. 4 a*) was not observed when using neutral lysis (Fig. 4*c*). In this case, the Fpg-sensitive sites detected with the different MMS concentrations tested were the same as in untreated cells.

To confirm that this reduction in the pH of the lysis solution did not affect oxidized lesions, cells were also treated with different concentrations of $KBrO_3$ (Fig. 4*b* and *d*). In this case, pH did not affect the levels of either hAAG- or Fpg-sensitive sites in $KBrO_3$-treated cells. As expected, hAAG did not detect the induced lesions whereas a concentration-dependent increase of net fpg-sensitive was observed.

Untreated cells in both conditions (MMS and $KBrO_3$ experiments) showed a higher level of Fpg-sensitive sites (around 15 % of DNA in tail) than hAAG-sensitive sites (0–3 % of DNA in tail). No SB were detected with any of the compounds at any of the tested concentrations

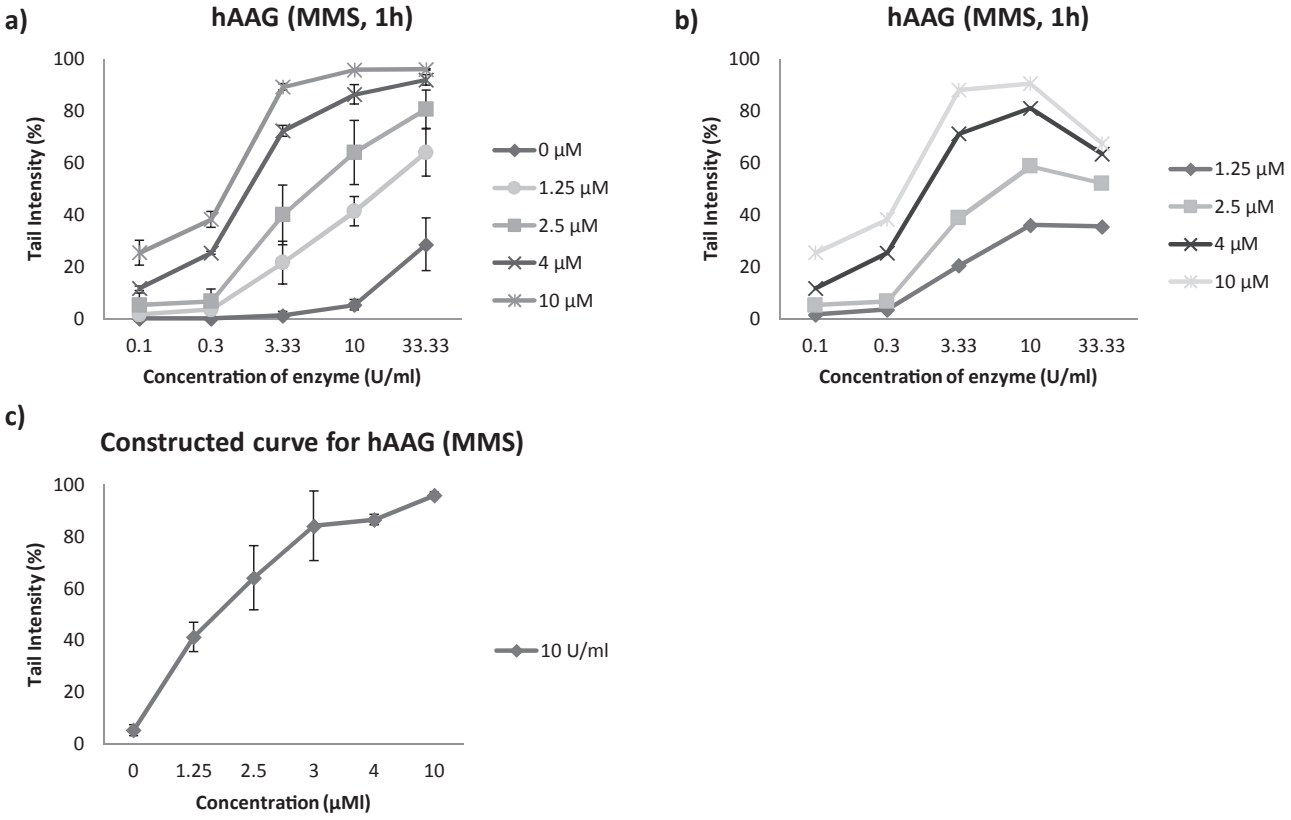

Fig. 2. *Measuring different levels of alkylated damage*. a) net enzyme-sensitive sites (in terms of tail intensity) obtained for each MMS concentration tested; b) relative activity of each enzyme concentration for each MMS concentration tested and c) MMS concentration-dependent curve of the net hAAG-sensitive sites using the selected conditions (10 U/mL and 1 h of incubation). Data from three independent experiments (4 in some cases) are presented (Mean ± SD).

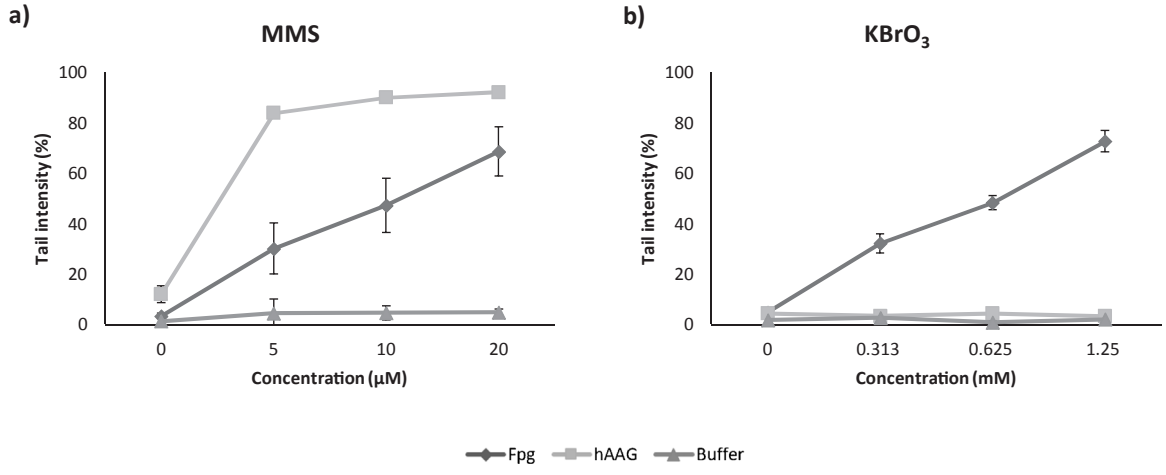

Fig. 3. *Concentration-response curves after performing the hAAG- and Fpg-modified comet assay in $KBrO_3$- and MMS- treated cells*. Results are expressed as net enzyme-sensitive sites (in terms of tail intensity) and the level of SB induced by the buffer alone ("Buffer"); a) MMS curve and b) $KBrO_3$ curve. Data from two independent experiments are presented (Mean and individual experimental values).

(Fig. 4). Indeed, SB levels were not affected by the variations in the pH of the lysis solution with any of the compounds at the concentrations tested.

3.3. Glyco-SPOT assay

Incubation of the different lesions with increasing concentrations of hAAG + Endonuclease III (Endo III) 0.5 U resulted essentially in the cleavage of ethenoadenine and thymine glycol. Hypoxanthine, which is also a known substrate for hAAG, was also cleaved but only at the highest hAAG concentration (Fig. 5).

4. Discussion

Here, we describe for the first time the use of hAAG in combination with the comet assay for the detection of alkylated bases on DNA. Particularly, hAAG detects 3-methyladenine and 7-methylguanine (O'Connor, 1993; and according to the enzyme specification). Some studies have also reported that hAAG also detects 1-methylguanine (Lee et al., 2009). Recently, we showed the importance of titrating the

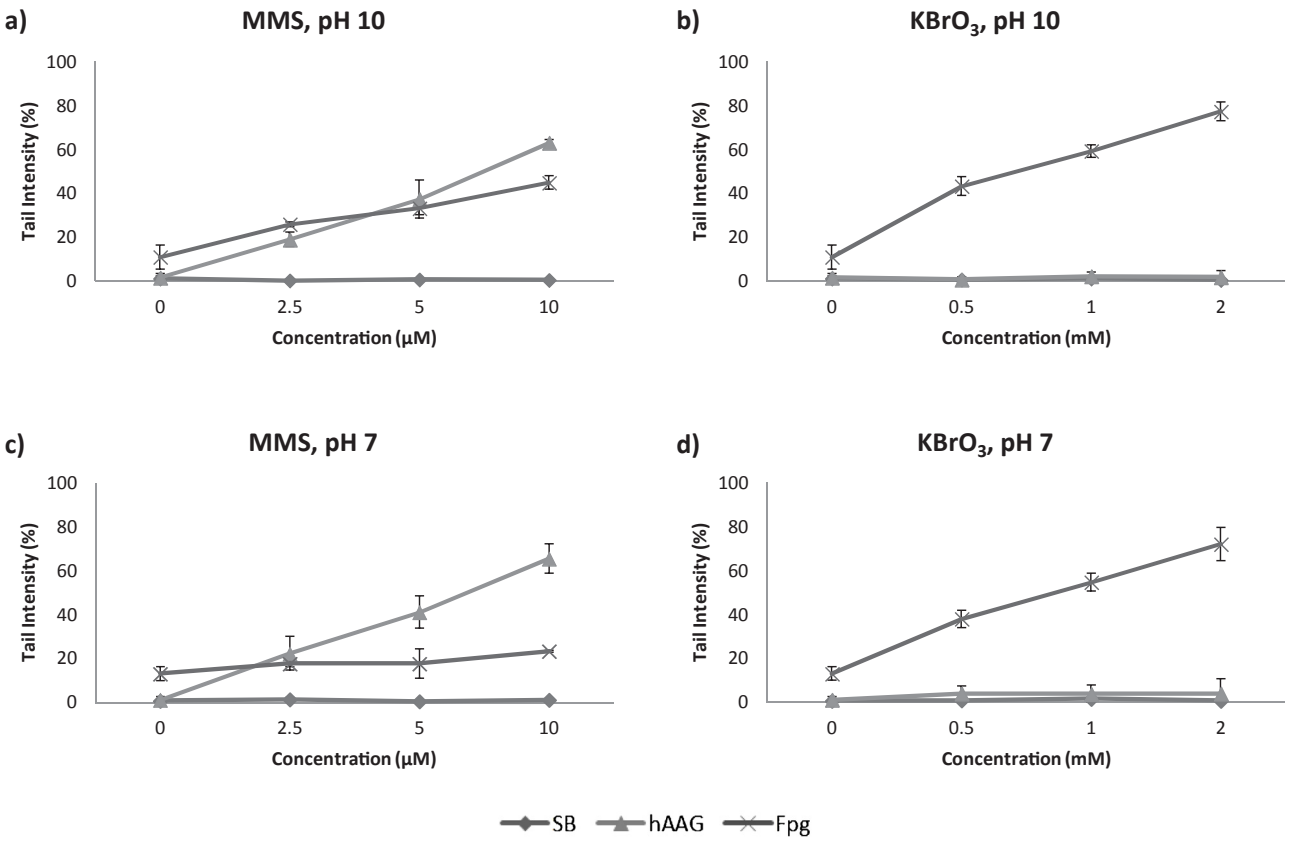

Fig. 4. *Activity of hAAG and Fpg in MMS- and $KBrO_3$-treated cells using neutral and alkaline lysis.* Results are expressed as net enzyme-sensitive sites (in terms of tail intensity) obtained for both enzymes and the SB obtained for each experimental condition. Alkaline lysis results with each compound are shown in a) and b); neutral lysis results are shown in c) and d). Data from two independent experiments are presented (Mean and individual experimental values).

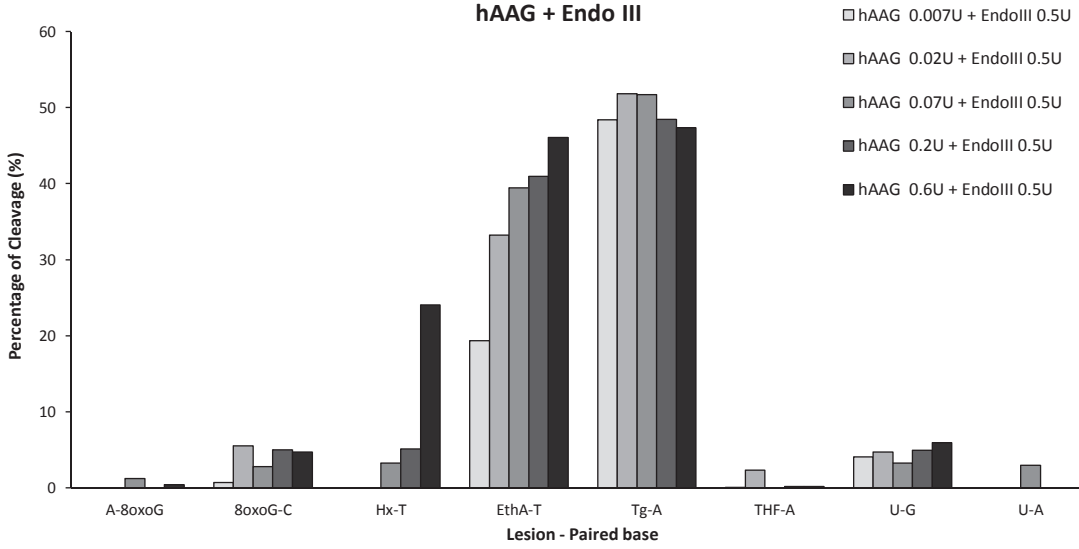

Fig. 5. *Percentage of cleavage induced by the hAAG (+Endo III) in the different DNA lesions included in the Glyco-SPOT assay*. A-8oxoG: A paired with 8oxoG; 8oxoG-C: 8oxoG paired with C; Hx-T: hypoxanthine paired with T; EthA-T: ethenoadenine paired with T; Tg-A: thymine glycol paired with A; THF-A: tetrahydrofuran -abasic site stable analog- paired with A; U-G: uracil paired with G; and U-A: uracil paired with A.

enzymes employed with the comet assay, taking into account the variations in comet assay format used (e.g., 2 gels/slide, 12 minigels/slide, etc) and incubation procedure (Muruzabal et al., 2018). Therefore titration experiments were conducted to determine the optimal hAAG concentration and time of incubation for detecting the maximum amount of enzyme-sensitive lesions without inducing non-specific breaks.

In this work, we have titrated commercial hAAG enzyme using the 12 minigels/slide format, and the 12-Gel Comet Assay Unit™ for incubation, in untreated and MMS-treated TK-6 cells. MMS is a known monoalkylating agent that induces mainly 7-methylguanine and 3-methlyladenine (Beranek, 1990). hAAG was successfully applied in

combination with the comet assay, and the optimal activity was obtained using 10 U/mL hAAG and 1 h of incubation; higher enzyme concentrations and longer time of incubation induced unspecific damage (Fig. 1). These conditions allow the detection of a wide range of alkylating damage, from the very low control levels up to the saturation level of the comet assay (Fig. 2*c*).

It is known that some alkylated lesions (i.e, N7-methylguanines) are prone to be converted into ring-opened purines at alkaline pH (Speit et al., 2004; Smith et al., 2006). Indeed, Gates (2009) reviewed different publications in which alkaline conditions were employed to accelerate the formation of alkyl-Fapy derivatives. This is observed when using the (alkaline) Fpg-modified comet assay. Fpg is a bifunctional enzyme able to detect oxidized purines (mainly 8-oxoguanines) and imidazole ring-opened purines (or formamidopyrimidines-Fapy-) (Boiteux et al., 1990, 1992). However the Fpg-modified comet assay detects DNA lesions induced by the alkylating agent MMS; this is due to the conversion of alkylated lesions into ring-opened purines during the lysis, which is performed at pH 10 (Speit et al., 2004; Azqueta et al., 2013a; Hansen et al., 2018). This was demonstrated by Hansen et al. (2018) by comparing the results obtained when using the Fpg-modified comet assay on MMS-treated human lymphocytes with lysis at pH 7.5 and pH 10. Fpg-sensitive sites were concentration-dependent when performing lysis at pH 10, but no Fpg-sensitive sites were detected when the lysis step was performed at pH 7.5. This effect was not seen when using the photosensitizer Ro 19-8022 plus light to induce 8-oxoguanines, as the same levels of Fpg-sensitive sites were obtained at both pHs. This clearly indicates that MMS-induced lesions (alkylated bases) are converted into Fpg-detectable lesions (i.e. ring-opened purines) during the lysis step at high pH and, as Hansen et al. (2018) pointed out, the process is likely to be both pH- and time-dependent. As expected, Fig. 3 shows how Fpg detected $KBrO_3$-induced lesions (i.e. 8-oxo-guanines) and MMS-induced lesions that are converted into ring-opened purines (i.e. N7-methylguanines) in a concentration-dependent manner. The same figures also show how hAAG is not able to detect oxidized bases, but it detects a higher level of alkylated bases than Fpg.

Here we have repeated the experiments performed by Hansen et al. (2018), comparing the performance of the Fpg- and the hAAG-modified comet assays using lysis solution at pH 7 and 10. In this work we used untreated, MMS- and $KBrO_3$- treated TK-6 cells. Results obtained when using the Fpg-modified comet assay are the same as the ones obtained by Hansen et al. (2018): MMS-induced lesions were not detected at pH 7 but detected at pH 10, while no differences were observed in the $KBrO_3$-treated cells (Fig. 4). However, the levels of hAAG-sensitive sites detected were the same independently of the pH of the lysis solution. This demonstrates that hAAG is detecting either lesions that are stable at pH 10 (so it is not detecting N7-methylguanines at all) or it is also detecting ring-opened purines. Considering all the data, the most plausible hypothesis is that hAAG is detecting N7-methylguanines, the ring-opened purines derived at high pH, and the 3-methyladenines, not detected by the Fpg-modified comet assay. (It is worth mentioning that 7-methylguanine occurs at much higher frequency than other alkylated bases (Beranek, 1990)). Clearly, hAAG does not detect 8-oxoguanines.

Figs. 3 and 4 show the results obtained when using the Fpg- and the hAAG-modified comet assay in untreated, MMS- and $KBrO_3$-treated cells in two laboratories using a similar protocol but different formats (12 minigels/slide and Gelbond® films respectively). Experiments were performed using the same batch of cells and same operator (data presented in Fig. 3 were obtained in one laboratory and data presented in Fig. 4 in the other one). hAAG was the same in both laboratories, and it was titrated in each of the laboratories, whereas Fpg was different for each laboratory (both were crude extracts of *E. coli* strains overproducing Fpg but coming from different batches). Fpg is commonly used in both laboratories and it was titrated before this work was carried out (data not shown). As can be observed, results obtained when performing the Fpg- and hAAG-modified comet assay in $KBrO_3$-treated cells are quite similar. However, differences arise when using similar concentrations of MMS. In the case of the Fpg-sensitive sites, it should be noted that the basal level in non-treated cells is very different in both laboratories, from non-detected lesions (Fig. 3) to 10–15 % DNA in tail, corresponding to the net Fpg-sensitive sites (Fig. 4); so detected net Fpg-sensitive sites induced by $KBrO_3$ are a bit lower in the case of Fig. 4 compared with Fig. 3, though the crude values are very similar. In the case of hAAG-sensitive sites, there is a considerable difference between both laboratories; basal levels are similar (non-detected lesions in both laboratories) but there is a huge difference in the levels detected in MMS-treated cells. It seems that this is not due to the difference in hAAG concentration used after the titration experiment (10 U/mL of hAAG in the 12 minigels/slide format -Fig. 3- and 1 U/mL of hAAG in the HTP -Fig. 4-). As can be observed in Fig. 4, there is enough enzyme to detect at least 60 % of net hAAG-sensitive sites (detected in the 10 μM MMS-treated cells) and it only detects about 40 % in cells treated with 5 μM MMS. Most likely, the cell treatment may differ from one laboratory to the other. In any case, the level of hAAG-sensitive sites is higher than the Fpg-sensitive sites in MMS treated cells in all cases.

The objective of the lysis is to remove all the soluble components of the cells and leave naked DNA in form of nucleoids (supercoiled DNA). Detergent (i.e, Triton X-100) included in the lysis solution breaks the membranes, both the cellular and the nuclear, while the high concentration of salts (i.e., NaCl at > 2 M) removes the proteins, including the histones. High pH may help in destabilizing all the cellular component, which is not really needed. However, high pH converts some non-detectable DNA lesions (in terms of comet assay) into others that are detected; on one hand making the assay more sensitive and, on the other, making more difficult the interpretation of the results in mechanistic studies (in which the primary lesions are wanted to be detected). According to results obtained in this work (Fig. 4) and in Hansen et al., 2018, it seems that performing the lysis at neutral pH gives reliable results in terms of Fpg-sensitive sites as oxidized lesions, hAAG-sensitive sites as alkylated lesions and SB; although more studies are needed in this direction. In any case, more experiments are needed to study the effect of high pH of lysis on other DNA lesions.

As mentioned in the introduction, N-alkylations are seen as being mainly cytotoxic, as they are relatively less mutagenic than O-alkylations (Kondo et al., 2010). However, here we have observed the induction of N-alkylations at non-cytotoxic concentrations. A high level of hAAG-sensitive sites, reaching the saturation level of the comet assay, was detected at 5 and 10 μM MMS (Fig. 3). These concentrations induce a RSG between 90 and 100 % (data not show). (At 20 μM MMS the RSG was close to 80 %.) Moreover MMS, which as mentioned before induces N-alkylations, is positive in several mutagenicity assays (Kirkland et al., 2016).

It is worth mentioning that hAAG is also able to detect non-alkylated lesions such as deaminated purine lesions (i.e., hypoxanthine and xanthine) and the lipid peroxidation-derived adduct 1,N6-ethenoadenine (Lee et al., 2009; Taylor et al., 2018). The deamination of bases is of great interest, as there is evidence of the mutation potential of hypoxanthine (DeVito et al., 2017). Regarding 1,N6-ethenoadenine, it can be produced either endogenously (i.e., by lipid peroxidation and/or reactive oxygen and nitrogen species) or by the metabolism of xenobiotics (e.g., vinyl chloride). Etheno-DNA adducts may have a causal role in the initiation and progression of carcinogenesis, as ethenobases produce mainly pair substitution mutations (Nair et al., 1999; Kennedy et al., 2019). To explore this possibility, the incision capability of hAAG towards these and other oxidized DNA lesions was studied using the Glyco-SPOT assay. In this work we have observed that hAAG + Endo III cleaved ethenoadenine, thymine glycol and hypoxanthine at the highest concentration tested (Fig. 5). (The cleavage of thymine glycol was due to the activity of Endo III and not to hAAG - data not shown.) hAAG + Endo III were not able to detect the other lesions included in the Glyco-SPOT assay (i.e., A or C paired with 8oxoG, abasic sites, and uracil paired with G or A).

It should be noted that in the comet assay it is not necessary to

complement hAAG with Endo III or any other enzyme with AP-endonuclease activity for the cleavage. This is explained because in the comet assay, after enzyme incubation the unwinding step is performed at pH > 13. Thus, alkaline conditions allow the cleavage of the AP-sites generated during hAAG incubation into SB, therefore emulating AP-endonuclease activity.

Ethenoadenines and hypoxanthine may also be detected by the hAAG-modified comet assay and this should be taken into account when interpreting the results. Nair et al. (1999) elucidated the effect of dietary fat intake on endogenous DNA damage and the protective effect of antioxidants in terms of production of ethenoadenines by lipid peroxidation. Thus, the use of hAAG in combination with the comet assay in human biomonitoring studies may have special interest.

In conclusion, we have shown for the first time the use of hAAG enzyme in combination with the comet assay for the detection of alkylation damage to DNA, although other lesions may also be detected, such as hypoxanthine and ethenoadenines. Our results support the application of this enzyme in combination with the comet assay, not only for genotoxicity assessment (*in vitro* and *in vivo*) but also in other fields such as human and environmental biomonitoring and ecotoxicology.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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