No. 100390-1

SUPREME COURT
OF THE STATE OF WASHINGTON

Asha Singh, personally and as Personal Representative
of the Estate of Narendra P. Singh,

Petitioner,

v.

State of Washington, a government entity, and
University of Washington, a government entity,

Respondents.

MEMORANDUM OF *AMICUS CURIAE* DR. ROBERT H. HEFLICH, PH.D.

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A. INTRODUCTION

Often, one can immediately understand the stakes involved when it comes to preserving a respected person's life's work. Based on name recognition alone, the parties can speak for themselves. For instance, it goes without saying that a famous writer's diary should be preserved or that a popular politician's memoirs would have historical and cultural significance. But in the comparatively obscure field of genetic toxicology, the names of the leading figures like Dr. Narendra P. Singh are not so well known.

This memorandum will contextualize for this Court the stakes involved by relaying Dr. Singh's groundbreaking achievements in his field. The research and techniques he developed are known by every scientist in his field and used throughout the world. They are recommended to ensure the quality of the environment and the safety of food, drugs and medical devices by the World Health Organization, the Organization for Economic Cooperation and Development

(OECD), and the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use, to name a few. To comprehend the stakes and the public interests raised by Asha Singh's petition for review, *see* RAP 13.4(b)(4), this Court must understand who Dr. Singh was and what he accomplished during his lifetime. It was critical that his work be preserved so that future scientists, biotechnology companies, and most importantly the public at large could benefit from it.

This is true of all researchers at public institutions like the University of Washington ("UW"), who rely on the institution's promises to respect and to preserve their work. But it is especially true of Dr. Singh – one of the most influential researchers in UW's history.

B. INTEREST OF *AMICUS CURIAE*

The identity and interest of Dr. Robert H. Heflich in this action, as required by RAP 10.3(e), are set out in Dr. Heflich's motion for leave to submit this *amicus* memorandum. Dr. Heflich currently serves as the Director of the Division of

Genetic and Molecular Toxicology at the United States Food and Drug Administration.

C. STATEMENT OF THE CASE

Dr. Heflich adopts the statement of the case in Asha Singh's petition and prior briefing before Division I of the Court of Appeals.

D. ARGUMENT

(1) The Court Should Not Decide this Case in a Vacuum. It Should Instead Consider the Academic Context and Importance of Dr. Singh's Work to the Scientific Community.

Appellate courts should not decide cases in a vacuum. As this Court has explained, a court must instead "inform [itself], as best [it] can, of the probable impact with [its] decision may have upon the affairs of the people of this state." *State ex rel. Distilled Spirits Inst., Inc. v. Kinnear*, 80 Wn.2d 175, 187, 492 P.2d 1012 (1972). This often requires that a court consider "social, economic, and scientific facts," including scholarly works and academic articles, so it can understand the context of its decision.

Wyman v. Wallace, 94 Wn.2d 99, 102, 615 P.2d 452 (1980). Ensuring that courts are fully informed is so important that a court's refusal to take notice of academic sources is grounds for reversal. *Cameron v. Murray*, 151 Wn. App. 646, 657-60, 214 P.3d 150 (2009) (reversing a trial court's refusal to take judicial notice of "scientific articles linking underage drinking and aggression"), *review denied*, 168 Wn.2d 1018 (2010).[1]

This Court should understand the context of this dispute. This case does not involve a routine employment dispute between an employer and a typical employee. This case involves a prestigious institution, UW, who destroyed the research data of Dr. Singh – a preeminent scholar and trailblazer in his scientific field. Thus, although it might not be obvious that preserving Dr. Singh's work would be critical, this memorandum attempts to contextualize Dr. Singh's achievements for this Court, so it can

[1] Dr. Heflich submitted an *amicus* brief that the Court of Appeals failed to even mention in its opinion below.

understand the impact of this dispute on the relevant scientific field and community.

(2) Dr. Singh Was a Groundbreaking Scientist in His Field. His Work Near the End of his Life Likely Had Significant Scientific Value.

Dr. Singh developed a groundbreaking process by which he could detect DNA damage in human lymphocytes treated with very low levels of ionizing radiation. Narendra P. Singh et al., *A Simple Technique for Quantification of Low Levels of DNA Damage in Individual Cells*, 175 EXPERIMENTAL CELL RESEARCH 184, 184-91 (1988) (appendix). With the method, which was later called the "Comet assay," one could measure the DNA damage in single cells – something unheard of at the time, and something that is still unique to this assay. Thus, he could observe the different responses of the various cells that he scored – some responding quite markedly, some responding not at all – as one would expect from a heterogeneous population of blood cells. This ability to measure damage in different cell populations undoubtedly contributed to the sensitivity of the

method compared to other ways of measuring DNA damage, which average the responses of thousands up to millions of cells together.

Dr. Singh's process was a blockbuster breakthrough in the field of genetic toxicology; a simple technique that can measure DNA damage in virtually any cell, in animals, or in cells in culture, with exquisite sensitivity. His original peer-reviewed report has been cited at least 10,000 times, which might be a record for the field.

The Comet assay was a groundbreaking achievement that continues to affect the field to this day. *See, e.g.*, Emilio Rojas, *Preface to special issue on the 20th anniversary of the comet assay*, 681 MUTATION RESEARCH 1, 1-2 (2009) (appendix). Many people have made their careers using Dr. Singh's assay. Several companies have commercialized the technique by selling materials and reagents to perform the assay. And virtually all contract-research organizations offer the Comet assay as part of their testing portfolio. The assay is a key part of testing strategies

to identify carcinogens and mutagens, specifically in laboratory rats and mice. Regulatory agencies, including the Food and Drug Administration, recommend the assay for establishing the safety of human pharmaceuticals and other consumer products.

The method also has guidelines for its use that have been adopted by major international organizations. *See*, *e.g.*, *OECD Guidelines for the Testing of Chemicals*, OECD/OCDE, Jul. 29, 2016 (appendix); *Guidance for Industry: S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use*, U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES FOOD & DRUG ADMINISTRATION, Jun. 2012 (appendix). These guidelines and guidance documents are essentially treaty obligations between countries that standardize the assay so that it can be done by anyone in any country in the world, and that the data can be relied on for making safety decisions related to products and pharmaceuticals.

Besides Dr. Singh's original assay, scientists over the years have developed variants of the Comet assay that detect

specific forms of DNA damage. Scientists in Dr. Heflich's department recently developed a method that detects epigenetic changes, not true damage to the DNA, but changes in how the DNA is read. These types of changes are believed to contribute to cancer induction. In addition, the Comet assay recently has been adapted to 'chips' which are used for high-throughput, computer-controlled analysis of cellular DNA damage. Le P. Ngo et al., *Sensitive Comet Chip assay for screening potentially carcinogenic DNA adducts by trapping DNA repair intermediates*, NUCLEIC ACIDS RESEARCH, Vol. 48, No. 3, Dec. 11, 2019 (appendix). These Comet chips can be used to efficiently evaluate the safety of the literally hundreds of thousands of substances presently in commercial use for which little safety data are available. In sum, the Comet assay is *vital* to regulatory science and continues to affect the scientific world to this day. *See* Narendra P. Singh, *The comet assay: Reflections on its development, evolution and applications*, 767 MUTATION RESEARCH 23, 23-30 (2016) (appendix).

In recent years, Dr. Singh became enmeshed in the controversy about the DNA damage and tumors purportedly produced by the radiation emitted by cell phones. Using his science to tackle ongoing public health issues was a theme throughout his career, such as when he researched the effects of asbestos exposure. Very powerful interests are often arrayed against the somewhat plodding pace of scientific research, whose results can appear contradictory if not evaluated with expert judgement. One can imagine that a well-funded industry can find and present a case that supports their interests, and it may be difficult for a handful of scientists to be sufficiently convincing to fight these powerful interests. Such opposition would be demoralizing for a scientist like Dr. Singh.

Ultimately, Dr. Singh's research was vindicated by research conducted by the National Toxicology Program ("NTP"), a consortium formed by US government agencies that researches problems in toxicology that are broad or complex in nature. The NTP conducted a cancer bioassay on cell phone

radiation that took 10 years and costs millions of dollars to complete.[2] The study concluded that exposure to certain radio frequency radiation used by cell phones was associated with an increase in DNA damage and tumors in lab animals.[3] The results from the DNA damage study (which used the Comet assay) can be found in Smith-Roe, *et al.*, 61 ENVIRON. MOL. MUTAGENESIS 276-290 (2020) (appendix).

These results supported Dr. Singh's position in this controversy, but sadly, they were announced after his death. If he were still alive, Dr. Singh would most likely forgo a celebration and merely continue to systematically research the health effects of various products using the groundbreaking assay he developed three decades earlier.

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[2] *See* https://ntp.niehs.nih.gov/whatwestudy/topics/cellphones/index.html (last visited Dec. 15, 2021).

[3] These particular radio frequency radiations are no longer used in modern consumer cell phones.

This Court should understand that Dr. Singh was an unusual, dedicated, and remarkably insightful scientist. Over his career, he made remarkable discoveries, developed an important technology, and took courageous stands. This Court should appreciate that his name is known by virtually every toxicologist practicing today. The assay he developed almost single-handedly is at the core of the world's environmental and consumer product safety-assessment programs. At the very least, his life's work, lab notes, and any research materials that he left behind, including cell lines (such as the ARTN-103) that he was developing towards the end of his life, should have been preserved (and not destroyed) by the UW so that future scientists can appreciate what he accomplished during his lifetime and continue to benefit from his accomplishments after his death.

(3) Every Scientist Reasonably Expects His or Her Research to Be Preserved.

The Court should also recognize that within the scientific community generally, scientists are expected to keep meticulous

records of their work and have the expectation that their lab research will be preserved. I have worked in or around lab environments since beginning my undergraduate studies in biology in 1964. I currently oversee the Division of Genetic and Molecular Toxicology, an important research department at the United States Food and Drug Administration. A constant throughout my long career has been the basic tenet that lab research must be preserved and archived. Preservation is fundamental to the scientific process and progress.

Preserving lab data, logs, and even cell lines, as in this case, is vital. For example, preservation ensures results can be repeated and peer reviewed, a key part of the scientific method. Research must also be archived in sponsored studies to ensure the goals of the sponsor are met. And one can never be sure when something will be important for future applications; what may seem irrelevant or meaningless at first may someday lead to a significant breakthrough.

UW responded to my prior *amicus* brief by arguing that it was "required and privileged to safely dispose of all biological and hazardous waste remaining in the University laboratory where Dr. Singh worked after his unfortunate passing." UW ans. to amicus br. at 4. But as a well-respected, public research facility, it knows that it must preserve and archive important research even when cleaning a lab station.

Even setting aside Dr. Singh's preeminence in his field, every researcher at a university like UW reasonably expects that their research will be preserved. The Court should grant review in this case to ensure that expectation is protected in Washington.

E. CONCLUSION

This Court should grant review to address the issues of substantial public importance raised by Asha Singh in her petition brought on behalf of her late husband. RAP 13.4(b)(4). Dr. Singh was a giant in his field, a field that has benefited the public health immeasurably through his groundbreaking

research. UW should have recognized the importance of his work and the importance of preserving it for future applications.

This document contains 2,097 words, excluding the parts of the document exempted from the word count by RAP 18.17.

DATED this 18th day of January, 2022.

Respectfully submitted,

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APPENDIX

APPENDIX TO
AMICUS BRIEF

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Experimental Cell Research 175 (1988) 184–191

A Simple Technique for Quantitation of Low Levels of DNA Damage in Individual Cells[1]

NARENDRA P. SINGH,*[2] MICHAEL T. McCOY,* RAYMOND R. TICE,† and EDWARD L. SCHNEIDER*

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Human lymphocytes were either exposed to X-irradiation (25 to 200 rads) or treated with H_2O_2 (9.1 to 291 μM) at 4°C and the extent of DNA migration was measured using a single-cell microgel electrophoresis technique under alkaline conditions. Both agents induced a significant increase in DNA migration, beginning at the lowest dose evaluated. Migration patterns were relatively homogeneous among cells exposed to X-rays but heterogeneous among cells treated with H_2O_2. An analysis of repair kinetics following exposure to 200 rads X-rays was conducted with lymphocytes obtained from three individuals. The bulk of the DNA repair occurred within the first 15 min, while all of the repair was essentially complete by 120 min after exposure. However, some cells demonstrated no repair during this incubation period while other cells demonstrated DNA migration patterns indicative of more damage than that induced by the initial irradiation with X-rays. This technique appears to be sensitive and useful for detecting damage and repair in single cells.

Techniques which permit the sensitive detection of DNA damage have been useful in studies of environmental toxicology, carcinogenesis, and aging [1, 2]. Since the effects of environmental toxicants, cancer, and aging are often tissue and cell-type specific [3–5], it is important to develop techniques which can detect DNA damage in individual cells. Rydberg and Johanson [6] were the first to directly quantitate DNA damage in individual cells by lysing cells embedded in agarose on slides under mild alkali conditions to allow the partial unwinding of DNA. After neutralization, the cells are stained with acridine orange and the extent of DNA damage is quantitated by measuring the ratio of green (indicating double-stranded DNA) to red (indicating single-stranded DNA) fluorescence using a photometer. This technique, however, is not widely used as numerous critical steps are involved in the processing.

To improve the sensitivity for detecting DNA damage in isolated cells, the same laboratory [7] developed a microgel electrophoresis technique. In this technique, cells are embedded in agarose gel on microscope slides, lysed by detergents and high salt, and then electrophoresed for a short period under neutral conditions. Cells with increased DNA damage display increased migration of DNA from the nucleus toward the anode. The migrating DNA is quantitat-

[1] The U.S. Government's right to retain a nonexclusive royalty-free license in and to the copyright covering this paper, for governmental purposes, is acknowledged.

[2] To whom reprint requests should be addressed.

0014-4827/88 $03.00

ed by staining with ethidium bromide and by measuring the intensity of fluorescence at two fixed positions within the migration pattern with a microscope photometer. However, while the neutral conditions for lysis and electrophoresis permit the detection of double-stranded DNA breaks, they do not allow for the detection of single-stranded ones. Since many agents induce from 5- to 2000-fold more single-stranded than double-stranded breaks [8], neutral conditions are clearly not as sensitive as alkaline conditions in detecting DNA damage. Alkaline conditions would also result in the degradation of cellular RNA, which otherwise could interfere in the quantitation of the ethidium bromide-stained samples.

We have modified the microgel electrophoresis technique to permit an evaluation of DNA damage in single cells under alkaline conditions. This approach optimizes DNA denaturation and the migration of single-stranded DNA, thus permitting an evaluation of single-stranded DNA breaks and alkali-labile sites. Details of the technique and some of our studies to validate the applicability of the approach for measuring DNA damage and repair in single cells are presented here.

MATERIALS AND METHODS

Low-melting-temperature agarose was purchased from BRL (Gathersburg, MD); Triton X-100 from Bio-Rad laboratories (Richmond, CA); sodium sarcosinate, ethylenediaminetetraacetic acid, disodium salt (Na_2-EDTA), Tris base, and ethidium bromide from Sigma Chemical Company (St. Louis, MO); hydrogen peroxide (H_2O_2), 30% solution, from Fisher Scientific (Fair Lawn, NJ); phosphate-buffered saline (PBS), without calcium and magnesium, and RPMI 1640 medium from GIBCO (Grand Island, NY); lymphocyte separation medium from Litton Bionetics, Inc. (Charleston, SC); and TRI X 135, ASA 400, black and white film from Eastman Kodak (Rochester, NY).

Lymphocytes were separated from whole blood utilizing Ficoll–Hypaque lymphocyte separation medium, washed in RPMI 1640, and suspended in PBS at a concentration of 30 million cells/ml. The blood used in these DNA damage studies was obtained from the same adult male donor. From 1000 to 500,000 cells were mixed with 25 μl of 0.5% low melting temperature agarose at 37°C and then placed on a precleaned microscope slide (Curtin Matheson Scientific Inc., Houston, TX, USA, Cat No. 267-0960) which were already covered with thin layer of 0.5% normal melting agarose to promote even and firm attachment of second layer. The cell suspension was immediately covered with a No. 1 coverglass, and the slides were then kept at 4°C for 5 min to allow solidification of the agarose. After gently removing the coverglass, the slides were covered with a third layer of low melting agarose by using a coverglass and then placed horizontally in a steel tray and returned to 4°C. The cells embedded in the agarose on the slides were exposed to X-rays or to H_2O_2 within 20 min of their preparation.

For X-irradiation, a Phillips Model MG 300 X-ray machine (Ridge Instrument Company, Inc., Tucker, GA) was used at a dose rate of 200 rads/min. Slides were treated with various concentrations of H_2O_2 in cold PBS. In the initial damage studies, both X-ray and H_2O_2 treatments were kept at 4°C to avoid repair of damage induced by these agents. To assess the kinetics of DNA repair, lymphocytes isolated from blood obtained from three adult male individuals were exposed to 200 rads and then incubated for various times in RPMI 1640 (supplemented with 10% fetal bovine serum) at 37°C in a 5% CO_2:95% air incubator. Cells were centrifuged at 4°C, resuspended in a small volume of PBS, and mixed with agarose and slides were prepared as described before. After the solidification of the agarose covering, the slides were immersed in a lysing solution (1% sodium sarcosinate, 2.5 *M* NaCl, 100 m*M* Na^2-EDTA, 10 m*M* Tris, pH 10, and 1% Triton X-100, added fresh) for 1 h to lyse the cells and to permit DNA unfolding. The slides were then removed from the lysing solution and placed on a horizontal gel electrophoresis unit. The unit was filled with fresh electrophoretic buffer (1 m*M* Na_2-EDTA and 300 m*M* NaOH) to a level 0.25 cm above the slides. The slides were allowed to set in this high-pH buffer for 20 min to allow unwinding of DNA before electrophoresis. Electrophoresis was conducted for the next 20 min at 25 V using an electrophoresis compact power supply (International Biotechnologies, Inc., New Haven, CT).

All of the steps described above were conducted under yellow light or in the dark to prevent

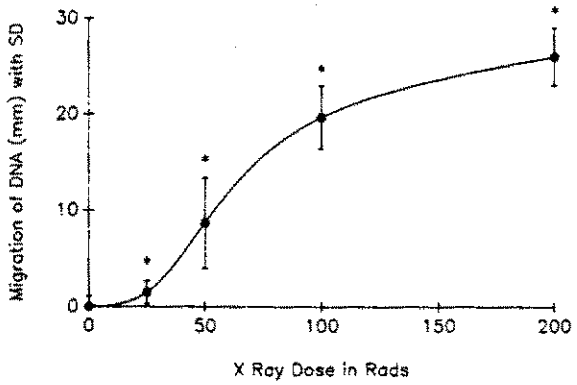

Fig. 1. Length of DNA migration as a function of X-ray dose. The average nuclear size of unexposed cells has been subtracted from each exposed group to obtain DNA migration. Each point represents the mean of 20 cells. The range bars indicate standard deviations. * indicates significantly different from control data at $P<0.0125$ (α of 0.05 Bonferroni corrected for four pairwise comparisons), based on Student t test using separate variances.

additional DNA damage. After electrophoresis, the slides were washed gently to remove alkali and detergents, which would interfere with ethidium bromide staining, by placing them horizontally and flooding them slowly with 0.4 *M* Tris, pH 7.5. After 5 min, the slides were stained by placing 25 µl of 20 µg/ml ethidium bromide in distilled water solution on each slide, and then covering the slide with a coverglass. Observations were made using an Axiomat microscope (Zeiss, RG), equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. Photomicrographs of single cells were taken at 400× magnification using TRI X 135 black and white film, ASA 400. DNA migration was determined on a negative photomicrograph by measuring the nuclear DNA and the migrating DNA in 20 randomly selected cells in each exposure group.

The effect of dose on the length of the DNA migration was analyzed using a one-tailed trend test, with the α level set at 0.05. For a determination of the regression coefficient, multiple linear regression analysis was used. To determine the lowest dose at which a significant increase in the length of migration occurred, multiple pairwise comparisons were conducted between the control data and each dose using Student t test, with the α level appropriately Bonferroni corrected for the number of comparisons made.

RESULTS AND DISCUSSION

A significant increase (one-tailed trend $P<0.001$) in the length of DNA migration was observed in human lymphocytes exposed to ionizing radiation over a dose range of 25 to 200 rads (Fig. 1). Photomicrographs of typical lymphocytes in control samples or exposed to X-rays are presented in Fig. 2. Under the electrophoretic conditions used, no migration of DNA occurred among the majority of the control cells and an approximately linear increase in the length of DNA migration was observed for doses between 25 and 100 rads (correlation coefficient $r=0.92$). By 200 rads, the length of migration appeared to plateau, while the extent of DNA damage in cells exposed to greater doses was too great to permit an accurate measurement of the migration pattern. At each dose of radiation, a relatively homogeneous response in the extent of DNA migration among cells was observed (Fig. 3).

In human lymphocytes exposed to H_2O_2, a significant increase in the migration of DNA occurred at concentrations between 9.1 and 291 µ*M* ($P<0.001$) (Fig. 4).

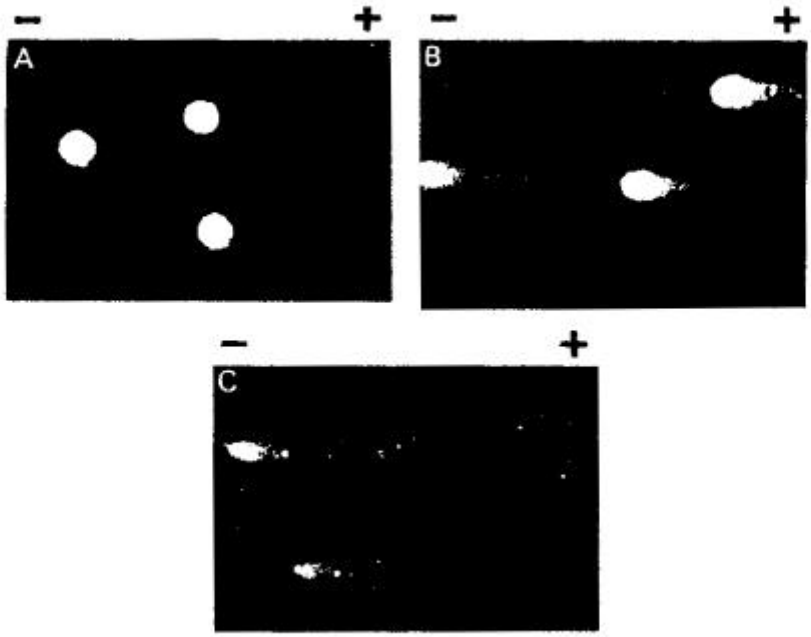

Fig. 2. Photomicrograph negatives of typical DNA migration patterns in (*A*) untreated human lymphocytes; (*B*) human lymphocytes exposed to 50 rads of X-rays; (*C*) human lymphocytes exposed to 100 rads of X-rays. Pictures were taken with TRI X 135, ASA 400, black and white film.

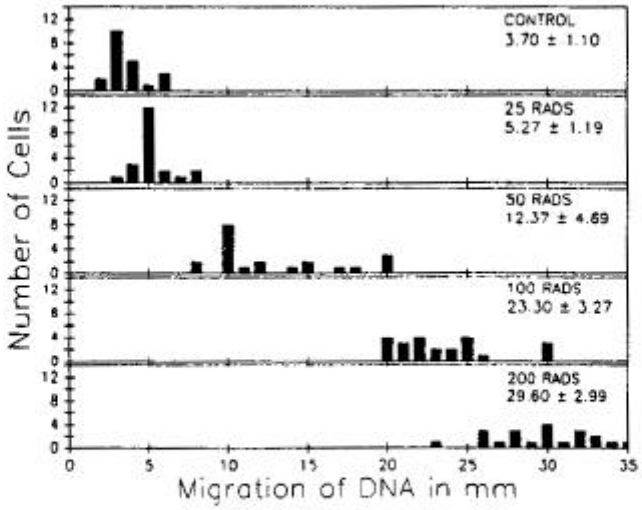

Fig. 3. Histogram of the distribution of the length of migration observed among 20 cells as a function of X-ray dose. The mean and standard deviation of each distribution are provided. The control data represent the mean nuclear size since no migration of DNA occurred. The length of migration among exposed cells includes the size of the nucleus. The width of each bar represents 1 mm.

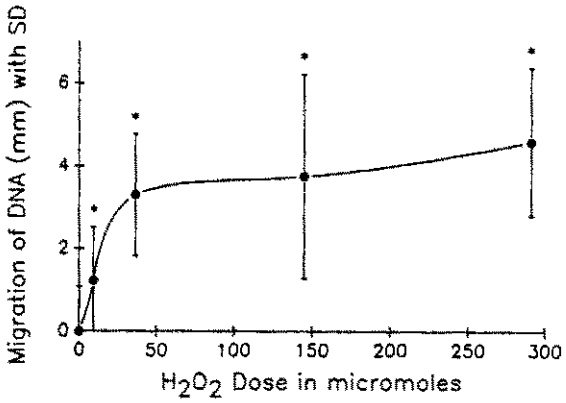

Fig. 4. Length of DNA migration as a function of H_2O_2 concentration. The average nuclear size of unexposed cells has been subtracted from each exposed group to obtain DNA migration. Each point represents the mean of 20 cells. The range bars indicate standard deviations. * indicates significantly different from control data at $P<0.0125$ (α of 0.05 Bonferroni corrected for four pairwise comparisons), based on Student t test using separate variances.

The extent of migration plateaued at H_2O_2 concentrations above 36.4 μM. However, in contrast to the relatively homogeneous DNA migration patterns observed for lymphocytes exposed to X-rays, extensive differences in the length of DNA migration, and thus in the extent of DNA damage, were observed among cells exposed to H_2O_2 (Fig. 5). There are several possible explanations for the differential response observed for these two agents. Individual cells may vary in their permeability to H_2O_2, their radical scavenging capabilities, the access of H_2O_2 or its metabolites to DNA, and other mechanisms which either enhance or

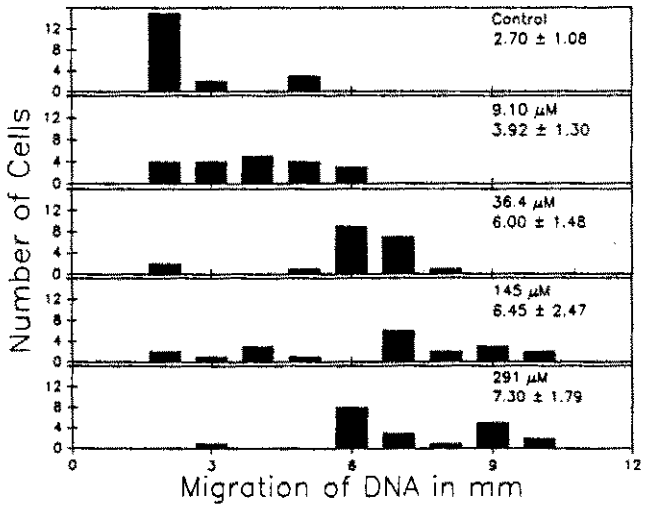

Fig. 5. Histogram of the distribution of the length of migration observed among 20 cells as a function of H_2O_2 concentration. The mean and standard deviation of each distribution are provided. The control data represent the mean nuclear size since no migration of DNA occurred. The length of migration among exposed cells included the size of the nucleus. The width of ear bar represents 1 mm.

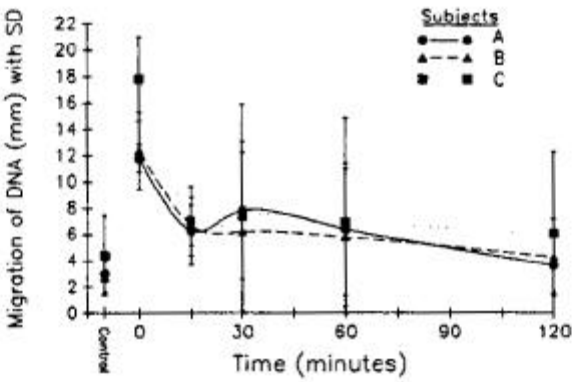

Fig. 6. Length of DNA migration in human lymphocytes exposed to 200 rads of X-rays as a function of postexposure incubation time. Each point represent the mean migration length, including nuclear size, for 20 cells, while the error bars indicate the standard deviation.

diminish the effects of H_2O_2. Whatever may be the mechanism for this differential response to these two agents, our data demonstrate the usefulness of this technique for examining DNA in individual cells.

To further examine the potential of this technique, human peripheral blood lymphocytes obtained from three donors were exposed to 200 rads of X-irradiation and incubated at 37°C in complete medium for from 15 to 120 min to assess the kinetics of DNA repair. In the lymphocytes from all three individuals, the bulk of the repair occcurred within the first 15 min, with a second, slower component that was essentially complete by the end of the 120-min incubation period (Fig. 6). However, there was considerable variability among cells in their ability to repair X-ray-induced DNA damage (Fig. 7). Even at 120 min after

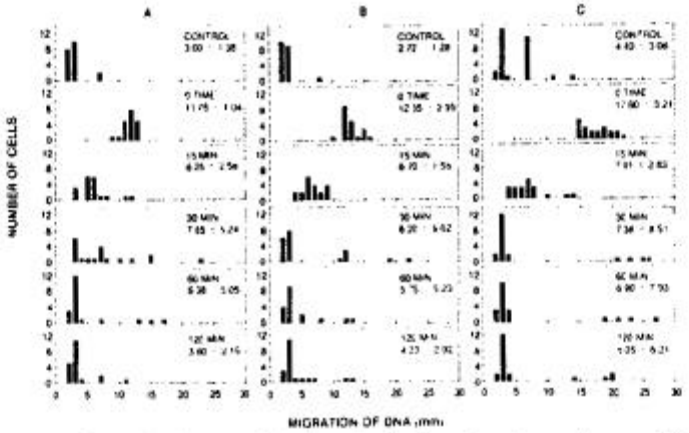

Fig. 7. Histogram of the distribution of the length of migration observed among 20 cells in three subjects as a function of postexposure incubation time after treatment with 200 rads of X-rays. The control data represent the mean nuclear size since no migration of DNA occurred. The length of migration among exposed cells includes the size of the nucleus. The mean and standard deviation of each distribution are provided. The width of each bar represents 1 mm.

treatment, the extent of DNA migration in some cells in each individual suggested a complete lack of repair capacity. Furthermore, some cells exhibited DNA migration patterns in length greater than that which occurred immediately after the X-ray treatment, which may be indicative of cell death.

Using the same microgel electrophoresis technique, but under neutral pH conditions, we were unable to detect any migration of DNA at up to 100 rads of X-rays. Under neutral conditions, DNA remains double-stranded and will migrate most readily in an electrophoretic field in regions of double-stranded breaks. The ratio of double-stranded to single-stranded DNA breaks is 1:20 for X-rays and 1:2000 for H_2O_2 [8]. Thus, it is clear that alkaline conditions will permit a more sensitive detection of DNA damage, including single- and double-stranded DNA breaks and alkali-labile regions, such as apurinic and apyrimidinic sites [9] and phosphotriesters [10].

Using two agents, X-rays and H_2O_2, at relatively low doses, we have demonstrated the detection of DNA damage in individual human lymphocytes. As few as 1 cell and as many as 500,000 cells can be placed on a single slide, making the technique applicable to microsampling procedures. For best results, the slides should be examined shortly after electrophoresis. The 37°C temperature for the agarose appears to be optimum for cell viability and for the adherence of the cells to the slides. The use of this temperature also seems to aid in the easy removal of the coverglass after the agarose has solidified at 4°C. Twenty minutes appears to be the optimum time for allowing the DNA to unwind under alkaline conditions. In our experiments, whole blood has been used with success and thus eliminates the necessity for lymphocyte isolation. Also, since the length of migration depends upon the percentage of agarose in the gel and upon the duration of electrophoresis, it should be possible by using either higher-percentage agarose gels or shorter electrophoretic times to quantitate greater amounts of DNA damage in single cells. Conversely, increasing the duration of electrophoresis would perhaps permit an evaluation of extremely low levels of DNA damage. Finally, quantitation of fluorescence intensity throughout the DNA migration pattern by the use of a microdensitometric or image-analyzing system should provide a more quantitative assessment of DNA damage.

In conclusion, we have developed a simple approach for the sensitive detection of DNA damage as well as the assessment of DNA repair in individual cells. In the applications described above, we have observed cellular heterogeneity both in the response to DNA damage (with H_2O_2) and in DNA repair. Further application of this approach should facilitate insight into these differences in cellular response to DNA damage.

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Preface

Special issue on the 20th anniversary of the comet assay☆

Twenty years ago, in a decade where the comet field was in the realm of physics, N.P. Singh and coworkers developed the versatile alkaline method of the comet assay to measure DNA damage. This method was developed to detect low levels of strand breaks with high sensitivity. Today the comet assay has become one of the most popular methods to assess DNA damage, with more than 4700 manuscripts published in more than 150 different journals (Fig. 1). The assay has had an impact in more than 20 areas of scientific knowledge, from mathematics to biochemistry, genetics, and molecular biology (Fig. 2), and it has been cited in more than 30,000 papers. The use of the assay continues to grow, which new applications for it being developed continuously.

It is with great pleasure that I present this volume of *Mutation Research—Reviews*, which contains detailed reviews on the state-of-the-art and new approaches in the use of the comet assay in diverse scientific areas. The topics include radiobiology (Olive), the characterization of the oxidative DNA damage and its repair (Collins), the effects of antioxidants and dietary factors on DNA damage (Cemeli et al.; Knasmuller), the combined use of FISH with the comet assay to study specific DNA repair mechanisms (Glei et al.; Spivak et al.), animal and human biomonitoring (Frenzilli et al.; Valverde and Rojas), and a new proposed protocol for the utilization of the comet assay to study genotoxicity in germ cells (Speit et al.).

Collectively, these reviews highlight the strengths and weaknesses of an assay that is widely used and becoming more robust year after year, providing directions for future research applications.

I would like to express my gratitude to all the people involved in the accomplishment of this Special Issue, especially to the authors for their generous and scholarly contributions and the reviewers for their time and effort. In addition, I want to thank the editors, David DeMarini and Mike Waters, for allowing me the opportunity to Guest Edit this anniversary issue on the comet assay. I am sincerely grateful for their assistance and support.

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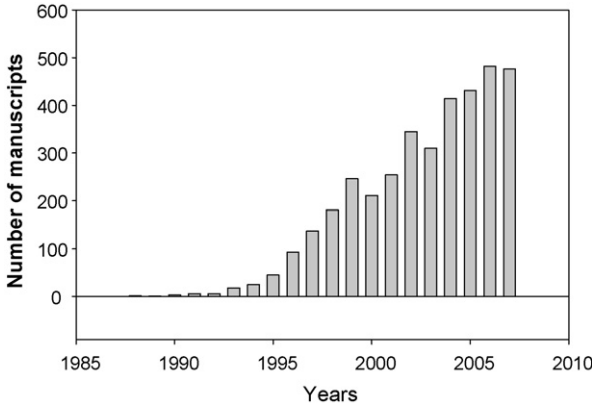

Fig. 1. Comet assay manuscript publication per year.

☆To feel ....
That one's life is a twinkle,
twenty years hardly reckon...

Alfredo La Pera.

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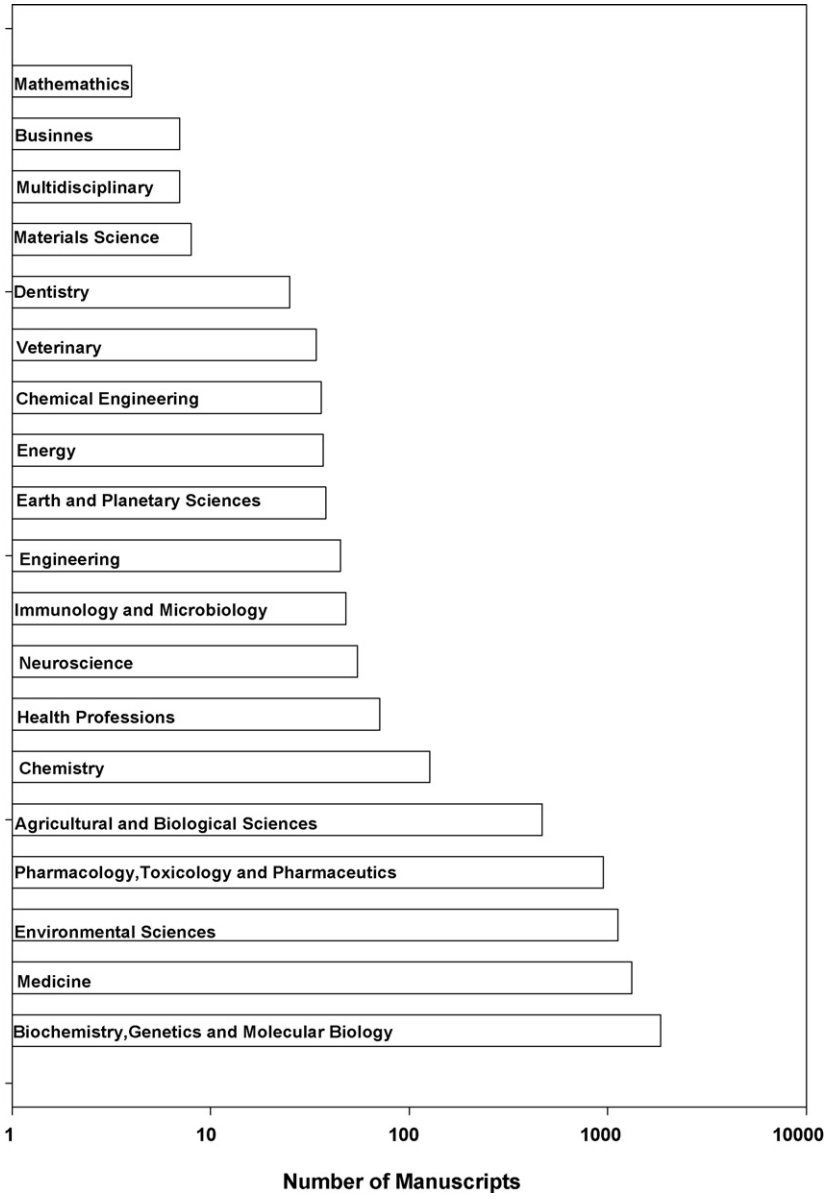

Fig. 2. Comet assay impact in different subject areas.

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Reflections in Mutation Research

The comet assay: Reflections on its development, evolution and applications☆

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ABSTRACT

The study of DNA damage and its repair is critical to our understanding of human aging and cancer. This review reflects on the development of a simple technique, now known as the comet assay, to study the accumulation of DNA damage and its repair. It describes my journey into aging research and the need for a method that sensitively quantifies DNA damage on a cell-by-cell basis and on a day-by-day basis. My inspirations, obstacles and successes on the path to developing this assay and improving its reliability and sensitivity are discussed. Recent modifications, applications, and the process of standardizing the technique are also described. What was once untried and unknown has become a technique used around the world for understanding and monitoring DNA damage. The comet assay's use has grown exponentially in the new millennium, as emphasis on studying biological phenomena at the single-cell level has increased. I and others have applied the technique across cell types (including germ cells) and species (including bacteria). As it enters new realms and gains clinical relevance, the comet assay may very well illuminate human aging and its prevention.

1. Introduction

By the time I began research, it was already accepted that to understand the process of aging and its causes, one had to see DNA. The scientists who laid the foundations for our field were scientists who found a way to see: whether Sutton [1] and Boveri who saw that genes had to be located on chromosomes, Franklin and Gosling [2] who saw the structure of DNA, or Tjio and Levan [3] who saw the true number of human chromosomes. The desire to see human aging with as much clarity as I could was always my main mission, and the development of the comet assay was a result of this desire. I always felt that, once seen, the secret of aging and its prevention could be found.

2. Scientific foundation in India

As a child, I thought that I would find the secret to aging and make my parents immortal, but I had no knowledge about research and no intention to pursue it. In July 1967, when I entered King George's Medical College (KGMC) in Lucknow, India, it was with the goal of becoming a family doctor in a village like the one that I had just left or a small town clinic. But KGMC was a unique place. Set on the Gomti River, it is a famously beautiful campus in a city known for its culture and courtliness. At the time, it was the top medical college in India, and its alumni, called Georgians, were top physicians, surgeons and researchers. It was also very well funded. I was exposed to new fields, taught by experts, and I had the opportunity to be in a lab. I stayed there for nearly ten years as a student, then post-graduate and finally as faculty.

During my post-graduate studies in the Department of Anatomy, I had the privilege of establishing a laboratory where I could study chromosomes under the microscope. My childhood desire to find the secret of aging was within my reach! I used to soak red kidney beans in water for 2 to 3 h, then blend and centrifuge them. I would remove the top supernatant layer using an ordinary pipet and syringe. This solution was rich in phytohemagglutinin and was used to stimulate human lymphocytes to divide. After using colchicine to arrest the cell cycle at metaphase, I could see a cell frozen in the midst of division. Finally, I had a chance to look at chromosomes, 46 of them. I ended up writing my thesis on chromosomal aberrations observed after treatments with hormones and antibiotics. During my Master's program, my supervisor, Professor Avinash Chandra Das, Chair of the Department of Anatomy, found funding to create a cytogenetics

☆ This article is part of the Reflections in Mutation Research series. To suggest topics and authors for Reflections, readers should contact the series editor, G.R. Hoffmann (ghoffmann@holycross.edu).

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laboratory and I was only too eager to set it up. This was the beginning of my journey into DNA damage and aging research.

Our conditions were not perfect: the room was a converted processing area for anatomy specimens and body parts. We were missing some key equipment but we found substitutes—I took a pressure cooker from our kitchen at home and this served as the autoclave for our glassware. Without having fully sterile conditions, I used to lose 90% of my cultures to contamination. I had a UV light and a glass chamber that I sterilized using the light. I had a water bath, light microscope and electric centrifuge but no incubator. Electricity outages were common, almost everyday occurrences, and they interrupted many experiments. Still, by aspirating rabbit bone marrow directly, using colchicine to arrest cell division in metaphase, and staining with Wright's or Giemsa stain, we were able to visualize chromosomes. I found effects of antibiotics (tetracycline, chloramphenicol) but not of hormones (testosterone, estrogen and progesterone) on rabbit chromosomes after 7 days of daily injections [4].

Eventually, I wanted to see DNA, not just chromosomes, but this goal exceeded the resources and knowledge at KGMC. In the fall of 1977, I visited the labs of Drs. Geeta Talukedar and Archana Sharma in Calcutta to learn autoradiography and unscheduled DNA synthesis (UDS). The incorporation of radioactive bases into damaged DNA during UDS allowed for the estimation of repair in DNA by visual grain counting. In 1978, I traveled to Bhabha Atomic Research Center in Bombay to learn mutagenesis in bacteria—the Ames test—with Drs. A.S. Aiyar and P.S. Chauhan. This allowed me to quantify the number of mutations induced by environmental chemicals. Still, even at Bhabha, they were not studying DNA damage directly. By the time I left Bombay, I had the notion that I would try to make an assay to directly measure a cell's DNA damage.

Wanting to work with DNA directly, I read any article that I could find on DNA damage, sister-chromatid exchange (SCE), alkaline elution and chromosomal aberrations. The medical library at KGMC had few scientific journals, so I used to read articles in the well-stocked archives of the National Botanic Garden and Central Drug Research Institute, both in Lucknow. On countless occasions, my wife would copy the articles by hand so that I could read and replicate experiments in the lab. After I had left Lucknow and arrived in America, I showed these handwritten copies of articles to their original authors. Ronald Hart was incredulous and amusedly took these papers around the labs at the National Center for Toxicological Research (NCTR). Painstakingly copied in blue ink were the articles of Drs. Nathan Shock, Ed Schneider, George Martin and Dr. Hart himself. I gained a lot of knowledge from this published work, and it inspired me toward new research directions and even life style changes. While I was still in India, Lester Packer's work on vitamin E's effect on WI-38 cells, making them immortal [5], inspired me to buy a bottle of vitamin E oil for daily ingestion.

3. Research training in the United States

Having taken advantage of all the resources available in India for studying DNA damage, I began to look for a post-doctoral fellowship. I wrote letters to every author outside of India whose work I had read and respected. Two positive responses came: one first from Dr. Hart and then one from Dr. Ed Schneider. I accepted Dr. Hart's offer as he was more of a basic researcher. The airplane ticket was equivalent to six months of my salary as a demonstrator in KGMC's anatomy department, where teaching medical students was my main job. I had to borrow money from my father and a fellow "Georgian," the co-author of my first publication, Dr. M.K. Tolani. I had never left India before, but a month after Dr. Hart's letter arrived, I traveled 12,500 miles—exactly half way around the earth.

I arrived at Ohio State University on the 10th of October, 1979, as a post-doctoral fellow. I had less than a hundred dollars in cash, a letter from Dr. Hart, and a suitcase filled with cashew nuts and raisins. As a vegetarian, I had no idea what I would find to eat in the United States. I was fortunate to have the best possible guide into American life; like a kindly grandmother, Mrs. Helen Dixon hosted many foreign postdocs in her large home near campus. She was my good friend and host for my entire time at OSU. My supervisor and the head of our lab, Dr. Hart was tall and vibrant with a booming laugh that conveyed positivity and progress. My main project at OSU was studying the effects of known carcinogens in rat tissue. The animals were sacrificed to estimate DNA damage in various organs. My approach was initially limited to mincing the organs with scalpels in a crisscrossing motion on frosted glass to get single-cell suspensions of the tissues that were then used for a variety of assessments. I spent many contented hours in the lab. I emerged to use OSU's playing fields and swimming pools, trying American-style football, diving or tennis. Many weekends were spent in the immigration offices of Cincinnati, where I struggled to obtain a temporary or permanent status that would allow me to stay in the country.

As I was finishing my postdoctoral fellowship at OSU, I was offered a position in Jefferson, Arkansas, in January of 1981. Dr. Hart had been appointed director of the NCTR, and he asked me to be part of his team. He had ambitious goals. Alongside Drs. Ming Chang and Angelo Turturro, I worked on assessing the effects of asbestos in vivo and in vitro [7]. I also developed a novel technique to infuse BrdU using an intraperitoneal catheter *in utero* in rats [8]. We then found stage-dependent effects of toxic agents on fetal development by studying SCEs in various tissues in embryos at various stages of development [9].

4. Formative ideas for the comet assay

When my appointment as a visiting scientist at NCTR ended, Dr. Steve D'Ambrosio offered me a position as Visiting Assistant Professor back at OSU in 1982. Returning to OSU resulted in my long-lasting research collaboration and friendship with Dr. Ralph Stephens. I learned more about staining DNA working with Dr. Stephens than I had ever known and that was a starting point for developing a new technique. We even published a methods paper showing differences in staining between live and dead cells [10]. By this time, I was familiar with several techniques for assessing DNA damage, including the alkaline sucrose gradient technique, which I had learned from Dr. Hart, and the UDS assay. As a postdoctoral fellow, I also became proficient in the nucleoid sedimentation technique, thanks to the guidance of Drs. Philip Lipetz and Ralph Stephens. In this technique, the nonionic detergent Triton X-100 was added to a high salt (2.5 M) solution for rapid lysis of cells.

Learning these techniques and knowing their drawbacks laid the foundation of ideas for a new technique. While I was still a postdoc, Dr. Douglas Brash, by chance, gave me a book chapter by Rydberg and Johanson [6]. Rydberg and Johanson's technique involved embedding lymphocytes in agarose gel, lysing cells with a solution of detergent (SDS) and EDTA on microscope slides, air drying cells in agarose, treating with an alkaline solution, and then immersing cells and gels in a neutralizing solution before staining with acridine orange. I studied the work overnight, and the next day Dr. Brash told me how to make agarose, mix it with the cells and solidify it on microscope slides. In this technique, the alkaline solution unwinds the DNA, which, after staining, appears as a halo in damaged cells. The intercalation of dye in double-stranded DNA is responsible for the green fluorescence, and the red fluorescence is due to the association of acridine orange along the single stranded DNA. Quantification of the ratio between green and red

was done with a special microscope that measured their intensity. The technique estimates DNA damage using a ratio of green to red fluorescence; it cannot quantify the number of DNA strand breaks, but it can be used as an index of DNA damage. However, the variability was so great that I could never properly visualize or assess induced DNA damage. I repeated the technique to the point of exhaustion, but the results seemed to be pH-dependent, concentration-dependent and time-dependent. I spent many hours in the zoology labs at OSU because Dr. Hart's lab, with its focus on alkaline sucrose gradient, had no fluorescence microscope. I liked the idea of embedding cells in agarose, but I still wanted a way to directly quantify DNA damage.

In May of 1982, I attended the First World Congress on Toxicology and Environmental Health in Washington, D.C. At the poster session of my work, I saw Dr. Raymond Tice. I was surprised to see his name-tag, and I asked him, "Are you the same Tice?" He smiled and said, "Yes, I'm the same Ray Tice." Incredulous that the man whose work I had read for so long would be visiting my poster, I asked again and got the same answer. Dr. Tice had been a Ph.D. student under Dr. Schneider, and we had common research interests. Thus began our collaboration. We exchanged phone calls and letters, and over the next ten years we would publish several papers [11–17], beginning with the 1988 paper that forms the basis of what is now known as the comet assay.

5. The path to the comet assay

In 1985, for several months after my appointment at OSU ended, I was jobless and I spent the time thinking of the ideal technique to assess DNA damage. I already knew I would embed cells in agarose as Rydberg and Johanson had done. At that time, I realized that I had three problems: isolation of living cells, embedding of cells, and lysis of cells. During this otherwise infertile, idle period, the idea came to me to electrophorese the cells in order to move the small, negatively-charged DNA pieces outside of the nucleus. Frustratingly, I had no lab or resources to test it. In a lucky stroke, Dr. Schneider called me from the University of Southern California (USC) in the fall of 1985 to tell me that he was going to the National Institutes of Health (NIH), and he asked me to join him there in the National Institute on Aging (NIA).

Dr. Schneider wanted someone in his lab to be trained in alkaline elution. He had found a perfect place and so for the last two months of the year, I went to Lausanne to learn alkaline elution in the laboratory of Dr. Peter Cerutti at the Swiss Institute for Experimental Cancer Research. Dr. Cerutti was a thorough teacher. At the end of my visit, he gave a dinner for me at his house. He had a spread of cheeses, breads and special foods. He offered me a spoonful of something very shiny, gray-white in color. He put it directly on my plate and I promptly ate it, inquiring only after it was in my mouth what it was. Caviar, he told me. I kept chewing and asked, "What is caviar?" Fish eggs, he replied. As a vegetarian, I was horrified and had to ask for the restroom! Dr. Cerutti was equally horrified. He thought he was offering me a real treat! What I learned in the lab, however, was an inspiration for me, and Dr. Cerutti would later make several visits to NIA to see our progress. I must have spoken of him often at home because my young daughter, when given a little yarn doll as a gift, promptly named it Peter Cerutti.

From Switzerland, I went back to NIA and published a paper on alkaline elution of sperm [12]. Still, I could see drawbacks to the alkaline-elution technique: it could have up to 30% variation in the same sample, even with the same cells under the same conditions. Although I was not satisfied with the technique, I did pick up the idea that sorting DNA according to molecular weight was viable and could be informative. Even while setting up Dr. Schneider's lab for alkaline elution in 1986, I remained eager to start working on the idea of alkaline microgel electrophoresis. I did many experiments applying current to cells in agarose, but I was not able to get rid of RNA or get the right resolution. Slowly, I was refining the method. I made microgels after isolating lymphocytes, lysing the cells in high salt with two detergents, and doing electrophoresis in highly alkaline solution. Lacking samples during these early days of development, I used my own blood, sometimes pricking my finger several times a day. I thought to precipitate the DNA after lysis and electrophoresis because localized DNA could be detected and measured more easily. I worked on precipitating DNA using ammonium acetate and ethanol combinations, spermine and ethanol combinations, and later, cetrimonium bromide (CTAB) to precipitate small amounts of DNA. I then washed the DNA in ethanol and dried the slides. In previous attempts, I had used a neutral solution with acridine orange. Now I tried an alkaline solution of ethidium bromide. It proved to be the most stable and sensitive.

I was gaining more knowledge about the structure of DNA under neutral and alkaline conditions, and I thought it would be more sensitive to use alkaline electrophoresis. As a bonus, RNA is degraded under alkaline conditions. The conditions also denatured DNA, revealing the breaks. I could easily see damage from X-rays, and for the first time I saw comet-like images with a streaming tail rather than a faint break here or there. I could not believe it! I was jubilant to see the tail, which I knew signified DNA (not RNA). I ran to tell everyone in the lab: Mike McCoy, Dr. Tice and Dr. Schneider. They had some concerns about whether the technique could be reproduced, and I started new experiments straightaway. I succeeded in showing a difference between controls and cells treated with 200 rads (2 Gy) of X-rays, but the goal remained to make the technique sensitive enough to detect damage caused by

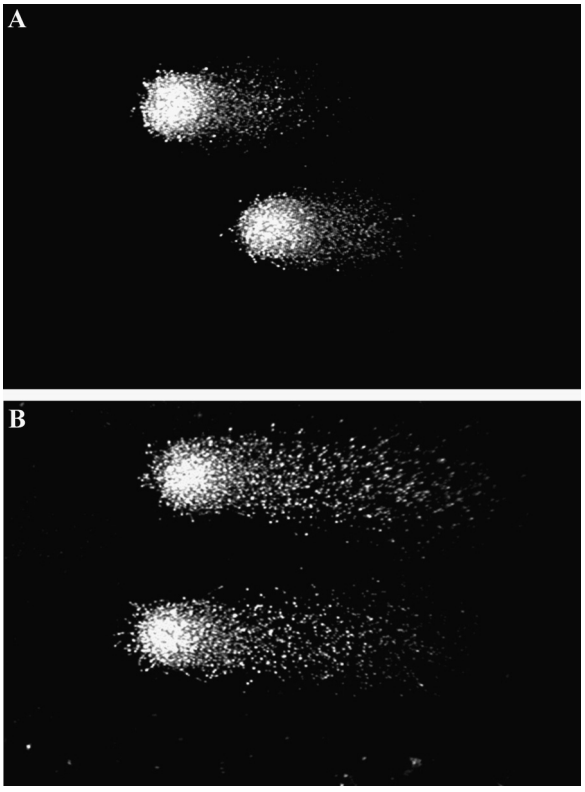

Fig 1. Comet assay. (A) shows two human leukocytes, representing an untreated control after single-cell gel electrophoresis. (B) shows two human leukocytes that had been irradiated with 100 rads (1 Gy) of X-rays in one minute. The comet-like tail consists of small fragments of DNA that arose by DNA strand breakage (dye: YOYO-1; magnification 400x).

25 rads (250 mGy) of X-rays. Taken from these early experiments, Fig. 1 shows control and irradiated human lymphocytes after microgel electrophoresis.

When I had completed a draft of my manuscript, Dr. Tice, who often came up from Integrated Laboratory Systems in Research Triangle Park, NC, to visit NIA, informed me that Ostling and Johanson had published similar work a few years earlier, in 1984. I went to the library soon after the meeting to read their paper. Ostling and Johanson [18] had added a novel step, electrophoresis, to the Rydberg and Johanson technique described earlier. Their new method, however, had two major disadvantages. First, due to the significant amount of RNA, estimation of the correct amount of DNA was not possible. When high quality agarose is properly made and layered with sufficient thickness on top of a layer of cells, the matrix retains DNA strands, RNA and small, broken fragments of DNA. I wanted to see DNA strands and broken pieces of DNA but not RNA. Second, sensitivity was limited by the conditions used for dissociation of the chromatin, which allowed DNA to maintain its tertiary and quaternary structures. Ostling and Johanson had used a neutral solution for cell lysis. DNA, with tertiary and quaternary structure intact, does not move in a predictable manner.

In the work that we were about to submit for publication, we had electrophoresed lysed cells under alkaline conditions to partially disrupt secondary structure and to remove the DNA's tertiary and quaternary structure. This allowed more predictable movement of DNA in the agarose. Alkaline conditions also degrade RNA and reveal more DNA lesions, including single-strand breaks, double-strand breaks, alkali-labile sites, etc., so they are more sensitive than neutral conditions that reveal only double-strand breaks. This is the basis of the comet assay's sensitivity. Ostling and Johanson were unable to detect less than 100 rads of damage, while we had detected significant changes at 25 rads. Finally, Ostling and Johanson had stained DNA with acridine orange and used a fluorescence ratio calculation at two points (nucleus and tail) as an index of DNA damage rather than migration distance. For these reasons, I knew that the technique that we were about to publish would be unique and sensitive. Some years later, after our publication of the 1988 paper, Dr. Karl-Johan Johanson came to my lab at the University of Washington with his colleague Dr. Britt-Marie Svedenstål to see the kind of research we were doing. He was a man of few words, but he was kind and tolerant and showed a true love of science.

6. Applications of the comet assay

Our 1988 paper on this technique [11] was, I felt, a big step in the right direction. My goal had always been to develop a technique to visualize aging but my larger aim was to elucidate the causes and mechanisms of aging. At this point, I integrated my original aim with the new technique. I thought that maybe the technique would be sensitive enough to see changes caused by aging. Using blood samples from NIA's Baltimore Longitudinal Study on Aging, we compared DNA damage levels in young and old individuals and found significant differences [13]. For the first time, I was able to observe changes in the DNA of a single cell due to aging. This had been the driving force behind my leaving my home institution in Lucknow, and I felt I had finally found my path.

I was thrilled by seeing the evidence of aging but the relationship was not as overwhelming as I had hoped, and I wanted to do a better study with more samples and different cell types. I thought of more experiments. It occurred to me that sperm should not be aging and that there should be zero damage. I looked at other cell types that, like sperm, had condensed chromatin and I found that chicken erythrocytes would offer similar condensation. So I drove from Baltimore to a farm in rural Maryland to get some fresh chicken blood. After finding extensive DNA breaks, we theorized that alkali-labile sites are a characteristic of condensed chromatin [14,15]. This was confirmed when we compared levels of DNA damage in mouse and human sperm [12].

Perhaps because I now had a newborn at home, one phenomenon particularly interested me: two adults, with relatively old cells, can produce a new baby with perfect, intact DNA. How does this happen? After seeing how many breaks were present in sperm cells, I speculated that the breaks could be repaired by meiotic proteins before fertilization in order to produce healthy new offspring. I became interested in recombinational repair and was particularly interested in the work of a Japanese scientist, Dr. Yasuo Hotta, who had isolated a recombinase protein. I wrote to Dr. Hotta to ask whether I could visit his lab to learn more about recombinases. He responded favorably and was kind enough to suggest a source of support. Through the generosity of the Japanese Society for the Promotion of Science, I was able to stay in Japan for two months. This was a wonderful experience both in the lab and outside of it. Dr. Hotta, his team and Dr. Takahiro Kunisada were ideal hosts, and I went away with friendships, a great deal of knowledge and some new ideas.

In 1989 I left NIH to be with my wife and young children in rural Washington State. At nearby Eastern Washington University, I continued to do DNA damage research [16,17], explored the relationship between DNA damage and disease, and observed DNA damage in an Alzheimer's model cell line. As an adjunct professor, I had a lab but no salary or budget for supplies or equipment. I wrote several unfunded grant proposals on aging, and after a year I was looking for a new position.

In 1991, with the help of Dr. Schneider, I moved to USC where I performed modifications of the technique (e.g., trypsinized and nontrypsinized cells) with various kinds of agarose (e.g., low melting point but high resolution). None of the adaptations provided enough sensitivity. My goal was to detect the minute changes of human life: exercise, X-rays, even deep inhalation. We made several technical modifications to further enhance sensitivity [19]. To free nuclear DNA of proteins, we introduced a proteinase-K step that could be applied after or during regular lysis. To apply a uniform electric field, which minimizes variation in DNA migration from cell to cell and slide to slide, we modified the electrophoretic unit and used a recirculating antioxidant-rich alkaline electrophoretic solution. I tried many different kinds of dyes that might make the technique more sensitive. I used to go around the nearby labs, looking to get a few drops of any unusual dye – anything I could get my hands on – "Are you using that? No? Can I borrow it?" Anything that I could not find, I ordered from the Sigma catalog. I tried 21 different dyes before settling on YOYO-1, an intense fluorescent dye that detects electrophoretically migrated DNA extremely well. These changes enabled us to detect significant DNA damage at doses as low as 5 rads (50 mGy) of gamma-rays [19].

I then wanted to see whether the assay could detect the effects of an extremely-low frequency (60-Hz) field. My family was now in Seattle, so I telephoned researchers and department heads at the University of Washington (UW) trying to find someone studying the effects of extremely low-frequency radiation. Dr. Arthur Guy, who was head of the Bioelectromagnetics Research Laboratory, referred me to Dr. Henry Lai. Dr. Lai told me that it was unlikely that a 60-Hz field could affect DNA because its energy level was so low, but he proposed that we look at radiofrequency radiation because its energy is higher. Enthusiastic about this possibility, I decided to leave USC and work with Dr. Lai without pay until we could secure funding. In 1994, we finished our first experiments. I prepared slides and flew with them back to USC to perform the analysis because we still did not have a fluorescence microscope with image analysis at UW. Using the comet assay, we were able to detect

increased DNA damage in brain cells of rats exposed to radiofrequency radiation at as low as 0.6 W/kg. The standard for cell phones in North America at that time was a maximum of 1.6 W/kg. The experiments with Dr. Lai on the effects of electromagnetic fields [21] and radiofrequency radiation [22] were the beginning of my longest scientific partnership, and Dr. Lai became both friend and mentor in my new environment at UW. On the basis of our studies on radiofrequency radiation, we obtained funding to do further studies and found that 60-Hz fields caused DNA damage [23–25] at a similar frequency to that used by cell phones. Unintentionally controversial, our findings were regarded as a challenge to the growing cell phone industry.

In 1995, we introduced the use of ethanol precipitation of migrated DNA in agarose to enhance the sensitivity of detection of DNA in microgels. This method also allowed slides to be preserved for future use. Our experimental design for these studies was simple. I taped microfuge tubes of lymphocytes to a wooden ruler at the 5, 10 and 20 cm marks. I placed the ruler next to a gamma ray source (technetium-99) and the data showed a clear dose response relationship [20]. Exposure at 4°C prevented DNA repair, resulting in unmitigated accumulation of DNA damage for the duration of the exposure. We were able to detect a significant increase in DNA single-strand breaks at a dose as low as 1 rad (10 mGy).

I also wanted to use the technique to study the effects of various common substances. Alcohol works as an antioxidant in leukocyte cultures and does not cause DNA damage; the story is very different in vivo where ethanol is metabolized into toxic acetaldehyde. In our work, we intubated rats with alcohol and dissected their brains to find the time kinetics of DNA damage. We found significant DNA damage from ethanol [26]. We also observed that the metabolite acetaldehyde is genotoxic [27] in human lymphocytes in vitro. I then thought that the same substances or experiences can be oxidant (damaging to DNA) or antioxidant depending on the existing defenses of an individual. I investigated the effects of antioxidants, such as vitamin C, on human lymphocytes, human diploid fibroblasts and MOLT-4 human leukemia cells and found a significant DNA damaging effect from moderate doses of sodium ascorbate [28].

At this point I felt the assay was sensitive enough to detect the minute changes that lead to aging and simple enough to be a regular part of my routine. In fact, I had incorporated the technique into my daily life. I would make small changes in lifestyle and test their effects; I did the comet assay on myself almost every day, after playing tennis, swimming, eating half a dozen carrots or trying a new vitamin regimen.

In 1995, a collaboration allowed us to see beyond the number of DNA breaks: Dr. A. T. Natarajan at Leiden University, an expert in chromosome hybridization, led a study combining the neutral comet assay with the FISH technique. This successful combination of techniques allowed us to see genes, centromeres and telomeres, and we were able to visualize the location of gene segments. For the first time I could see specific genes in the halo of the comet, where we identified the O^6-methylguanine-DNA methyltransferase gene [29].

After working for so many years with human chromosomes and DNA, in 1999 I directed my research toward bacteria. There were two reasons: (1) I wanted to know whether replicating *Escherichia coli*, having a theta (θ) shaped chromosome, would have one straight chromosome if broken. Only one double-strand break would be needed to do this and therefore, (2) I wanted to know the sensitivity of detecting only one double-strand break for testing antibiotics or chemicals. Neutral conditions were used to reveal only double-strand breaks, which are lethal in bacterial cells. The neutral comet assay revealed a simple and elegant demonstration of these breaks: an *E. coli* nucleoid with a single tail of DNA streaming behind it [30].

Our next iteration of the comet assay was only peripherally related to DNA damage: a sensitive method for visualization of apoptosis on a cell-by-cell basis. In the DNA diffusion assay [31], cells are lysed in alkaline detergent solution, embedded in agarose, and stained using my very favorite dye, YOYO. The technique also takes advantage of the numerous alkali-labile sites in DNA of damaged cells. Under alkaline conditions, these fragments of DNA diffuse outward from the nucleus and give apoptotic cells the appearance of a halo. Studying apoptosis, I realized, was crucial in studying how damaged cells are eliminated and thus, critical to studying healthy aging.

The versatility of a technique lies in its adaptability to a variety of tissues. Using the comet assay in collaboration with Dr. Norman Wolf of the Department of Pathology at UW, we were able to show increasing DNA damage with age and with light exposure in lens epithelial cells [32],which Dr. Wolf showed was related to cataract formation [33]. We also used an innovative method of dispersing a variety of tissues into single cell suspensions, including the kidney (one of the hardest tissues). Dispersion of tissues into single cells is required in many biological assays but the procedure often causes damage (e.g., the mincing method that I used as a post-doc!), and there was a need for a device to minimize DNA damage while still effectively dispersing tissue. I had earlier worked with a gentleman named Tim Hopkins, who designed a specialized and novel system, the Tissue Press [34]. A few years later he called me up with an unusual offer. He had a new device which was intended for use in immunizations and he wondered if this device, the Biojector, could be adapted for use with the comet assay. The CO_2 cartridge, which was the source of pressure in the syringe, rapidly dispersed any tissue into single cells through a narrow (<50 micron wide) hole with minimal procedural damage. Using this dispersion method, in 2001, we were able to show an increase in DNA damage with age in mouse kidney cells in collaboration with Dr. Wolf and Dr. George Martin. Dr. Martin was the first to correlate lifespan with cloning efficiency in the rat model [35] and one of the authors I looked up in the libraries in Lucknow, India. We were also able to quantify and calibrate this increase with such sensitivity that we could show the equivalent of 12 months of aging in terms of rads of X-rays and number of DNA double-strand breaks [36].

Yet, I still had not answered critical questions about the aging process. I had tried to assay DNA damage in human sperm since I had first developed the assay. No matter how much I tried, it did not move during electrophoresis. Even after 24 h and 400 rads or more of X-rays, I saw no DNA migration. Searching the literature, I read that sperm chromatin was highly condensed. The process of chromatin condensation requires crosslinks between DNA and proteins, such as protamines but also some histones. Using Proteinase-K in lysing solution to decondense chromatin finally allowed me to see an X-ray dose response in sperm exposed to radiation. In 1997, Dr. Stephens and I had introduced a neutral version of the assay to detect X-ray induced DNA damage in human lymphocytes [37]. In 1998, we used this neutral version of the assay to detect DNA double-strand breaks in sperm cells [38]. This neutral comet assay, using proteinase-K, sensitively detected DNA damage in sperm and I continue to use it in a variety of studies. For example, with Drs. Bhaskar Gollapudi and Sue Marty, we were able to show a relationship between p53 and levels of DNA damage in mouse sperm [39]. In collaboration with Dr. Charles Muller of the UW's Male Fertility Clinic, we showed a significant increase in DNA damage and a surprising decrease in apoptosis after the age of 35 [40]. This meant that men older than 35 had sperm with high levels of DNA damage that would not be eliminated by apoptosis and might go on to fertilize an ovum. This finding, labeled a "male biological clock," attracted high levels of scientific and media interest. For me, our work contradicted my earlier theory that gametes repair their DNA damage before

fertilization. Our findings led to new research directions that I would still like to pursue, specifically the fetal origins of adult disease.

Many researchers, including myself, had by this time shown relationships between mutagens and DNA strand breaks using the comet assay. However, my real work in environmental chemicals and DNA damage began with my collaborations with Dr. Russ Hauser at the Harvard School of Public Health who was principal investigator on a large study of phthalates (a class of chemicals found in a variety of household plastic products). Our ultimate goal was to study the effects of phthalates, PCBs and insecticides on sperm DNA. We found that urinary levels of these chemicals were associated with increased levels of sperm DNA damage [41]. Other studies with Drs. Hauser, Susan Duty and Zuying Chen investigated the comet assay in relation to fresh and flash-frozen semen samples [42], semen parameters [43] and insecticides [44]. A collaboration with Dr. John Wise [45] on environmental and occupational exposures to chemicals also contributed to toxicological applications of the technique. Several CDC and NIOSH studies have recently used the comet assay to study occupational exposures. In collaboration with Dr. Mark Toraason, we found increased DNA damage in the leukocytes of factory workers exposed to spray adhesive chemicals, such as bromopropane [46]. In collaboration with Dr. Mark Boeniger, we studied polycyclic aromatic hydrocarbons (e.g., benzopyrene; dimethylbenzanthracene) and DNA damage in auto repair workers. These studies prompted me to develop a protocol for the collection of samples in the field, their storage, and their shipment from the agency conducting the study (in our case, CDC and NIOSH) to a laboratory for freezing, thawing and assessment of DNA damage. This protocol was used by the CDC for a project headed by Dr. Mary Ann Butler to study workers exposed to Jet Fuel at US Air Force bases [47].

7. Refinement and new directions for the assay

Real refinement of the comet assay came through customization of the equipment. After experimenting with the electrophoretic units used in other techniques, in the mid-1990s I decided to make my own. In consultation with Ralph Stephens, I began to design a specialized unit. Early on, I would saw flat sheets of Lucite and glue them together in order to realize my designs but they had problems due to their inexpert construction. We found a skilled manufacturer and designer, Clive Ellard (Ellard Instrumentation). The new unit solved some of the recurrent problems in the technique and allowed greater sensitivity. I then started to modify slides, because frosted slides caused background with YOYO dye. We had used frosted slides for better attachment of agarose, but the uneven background from the frosting made it difficult to analyze the migrated DNA using an image analysis system. Two changes were made to address this problem: the use of a tray to simultaneously process eight slides and the use of newly designed slides with a clear window and frosted borders [30]. These changes enhanced the sensitivity of the technique to the point that we could visualize an individual DNA double-strand break in *E. coli* [30].

Finally, I have worked to attain ultimate sensitivity for assessing the extent of DNA damage. Considering the comet as only a head and tail may be simplistic. I had to consider the comet in three parts: head, body and tail. The body consists of relaxed loops of DNA, and the tail consists of broken pieces of DNA. Our latest refinement of the comet assay is designed to retain these broken pieces of DNA. The earliest comet assay studies used a single parameter: comet length. However, the most complete picture of DNA damage is offered by the inclusion of a variety of parameters. Dr. Peggy Olive developed the parameter "Tail Moment" to assess intensity of broken DNA fluorescence. We developed the parameter "Integrated Intensity" to account for the three-dimensional aspects of DNA migration. I have worked to incorporate such parameters in computerized image analysis programs. I once had to rely on my own macros and a camera hooked up to a fluorescent microscope and computer. Now a variety of advanced image analysis systems have been developed and a reliable, automated system for use in labs and clinics is on the horizon.

8. The comet assay comes of age

The comet assay has been modified, adapted and adopted for various purposes over the past 25 years. Even the name has changed through the years. Ostling and Johanson [18] called their technique "Microelectrophoresis." In our 1988 paper [11], we named the assay "the Microgel Electrophoresis technique." Soon after the publication of this paper, I was invited to North Carolina to help set up Ray Tice's lab at Integrated Laboratory Systems. Dr. Tice, his versatile and gentlemanly technician Paul Andrews, and I came up with a better name. We called the technique Single Cell Gel Electrophoresis or just Single Cell Gel (SCG). Shortly afterward, Dr. Peggy Olive and colleagues introduced the term "comet assay" [48], and that has rightly stuck for the last 25 years.

In this span, researchers have applied the comet assay to a variety of fields. Dr. Andrew Collins and colleagues introduced the assay's use in human biomonitoring, studying the possible amelioration of DNA damage by nutritional supplements [49] and repair enzymes such as endonuclease and formamidopyrimidine DNA glycosylase [50]. Dr. Awadhesh Jha and others have innovated ecotoxicological applications of the assay for use in wildlife and environmental monitoring [51]. My early collaborator, Ray Tice, has taken the lead, along with Drs. Diana Anderson, Emilio Rojas, Yu Sasaki and others, in validating the assay's use in genotoxicology [52]. There have been concerted and ongoing efforts to develop international standards for the assay, including those of the American, Japanese and European Centers for the Validation of Alternative Methods and principally of the European Comet Assay Validation Group. On the basis of work by these centers and the collaborative efforts of several international working groups on the comet assay, the Organisation for Economic Development and Co-operation (OECD) adopted test guidelines for the comet assay in 2014 (https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd-tg489-2014.pdf). The assay is now an accepted method for human biomonitoring according to FDA (http://www.fda.gov/downloads/Drugs/Guidances/ucm074931.pdf) and WHO guidelines. The comet assay has long been an online presence. An NIH list-serve group was established more than 20 years ago by Dr. T.S. Kumaravel (comet-assay@list.nih.gov), who brought knowledge of the assay to thousands of experienced and novice researchers. Dr. Alok Dhawan established an online repository of protocols, discussion and research related to the assay (http://www.cometassayindia.org/).

If the comet assay had a birthplace, it would be the labs of the National Institutes, the hub of basic science research in America. Yet, the reach of the technique has quickly expanded beyond these borders, and I have been able to observe its application in England, Hong Kong, Germany, India, Italy, and Korea. Seeing the technique used in many different kinds of labs was evidence to me of its simplicity and an indicator of its future.

From arachnids [53] to zebra mussels [54], the comet assay has been used in plants, animals and microorganisms of all types. It has been applied to every kind of research that I could have imagined and at least one that I would never have imagined – precisely estimating the time of death in homicides [55]. This post-mortem application never occurred to me! My original impetus for the development of the technique was the study of aging and the

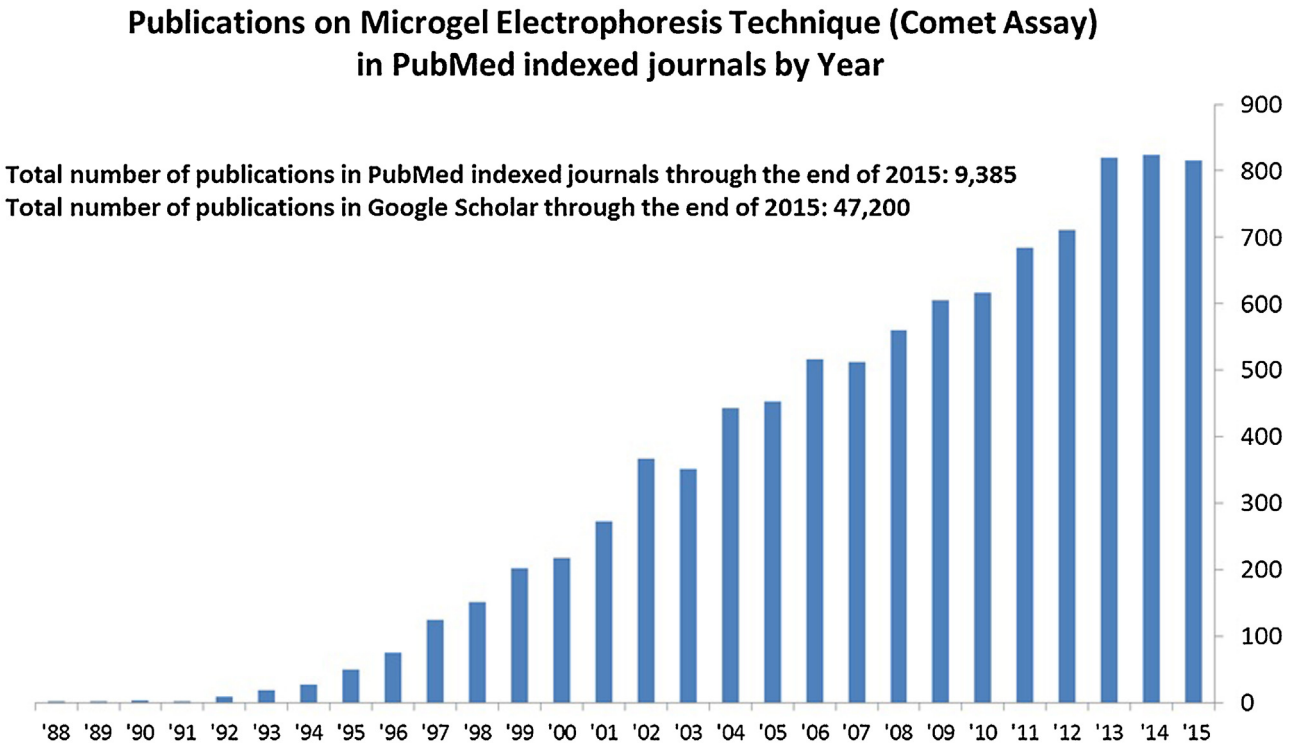

Fig. 2. Increasing numbers of publications using the microgel electrophoresis technique widely known as the comet assay. The numbers are publications in journals indexed by the National Library of Medicine's PubMed database since the description of the assay by Singh et al. in 1988 [11]. The search includes papers found using the search terms "comet assay," "microgel electrophoresis," or "single cell gel electrophoresis." Total numbers of publications are also shown for the exact search term "comet assay' in PubMed and Google Scholar.

extension of healthy human lifespan. I have worked mostly on studies in humans or animal models, but a variety of fascinating and significant research has been done in unusual organisms, wildlife and plants.

The past has been bright: the comet assay has detected DNA damage in a variety of organisms, tissues and cell types as a result of aging, disease and exposures. The recent emphasis on studying phenomena at the single-cell level will ensure its continuing relevance. As seen in Fig. 2, the number of publications using the technique has grown rapidly since 1988 and most rapidly in the last ten years. No other technique offers the same level of information in the same dramatic fashion: under the microscope we see those individual strands of DNA that form the basis of our existence, and we see their fragility as they break and trail out beyond their nucleus. It is a striking picture and one that is essential to understanding the health of our own species and a variety of others. As we develop ways to improve health and extend our lifespan, the future of the comet assay looks brighter still.

Conflict of interest

The author states that there are no conflicts of interest.

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Environmental and Molecular Mutagenesis 61:276–290 (2020)

Research Article

Evaluation of the Genotoxicity of Cell Phone Radiofrequency Radiation in Male and Female Rats and Mice Following Subchronic Exposure

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The National Toxicology Program tested two common radiofrequency radiation (RFR) modulations emitted by cellular telephones in a 2-year rodent cancer bioassay that included interim assessments of additional animals for genotoxicity endpoints. Male and female Hsd:Sprague Dawley SD rats and B6C3F1/N mice were exposed from Gestation day 5 or Postnatal day 35, respectively, to code division multiple access (CDMA) or global system for mobile modulations over 18 hr/day, at 10-min intervals, in reverberation chambers at specific absorption rates of 1.5, 3, or 6 W/kg (rats, 900 MHz) or 2.5, 5, or 10 W/kg (mice, 1,900 MHz). After 19 (rats) or 14 (mice) weeks of exposure, animals were examined for evidence of RFR-associated genotoxicity using two different measures. Using the alkaline (pH > 13) comet assay, DNA damage was assessed in cells from three brain regions, liver cells, and peripheral blood leukocytes; using the micronucleus assay, chromosomal damage was assessed in immature and mature peripheral blood erythrocytes. Results of the comet assay showed significant increases in DNA damage in the frontal cortex of male mice (both modulations), leukocytes of female mice (CDMA only), and hippocampus of male rats (CDMA only). Increases in DNA damage judged to be equivocal were observed in several other tissues of rats and mice. No significant increases in micronucleated red blood cells were observed in rats or mice. In conclusion, these results suggest that exposure to RFR is associated with an increase in DNA damage. Environ. Mol. Mutagen. 61:276–290, 2020.

Key words: DNA damage; micronucleus assay; comet assay; brain; Sprague Dawley; glioma

INTRODUCTION

Over the past two decades, cellular telephone use has become nearly ubiquitous worldwide; cell phone subscriptions numbered ~7.68 billion in 2017 according to the International Telecommunication Union (2017) with ~5.12 billion unique subscribers (GSMA Intelligence 2019). Radiofrequency radiation (RFR) is a form of electromagnetic radiation that ranges from 3 kHz to 300 GHz. Most cell phones transmit RFR signals within the 800–900 and 1,800–2,200 MHz ranges (International Agency for Research on Cancer [IARC] Working Group on the Evaluation of Carcinogenic Risks to Humans 2013).

Concern exists as to whether cell phone RFR frequencies are capable of adversely affecting human health. Although some epidemiological studies suggest that cell phone use might increase the risk for certain brain

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cancers, such as gliomas and acoustic neuromas (a,k,a, vestibular schwannomas), the odds ratios for these increased risks are quite low (INTERPHONE Study Group 2010; Cardis et al. 2011; Hardell et al. 2011; Larjavaara et al. 2011; Sato et al. 2011; Hardell and Carlberg 2015). Conclusions drawn from these observations may be premature, as cell phone use has become commonplace only within the past two decades, a period of time that may be insufficient to accurately assess cancer-related outcomes. Results of previous rodent cancer studies conducted with a variety of RFR exposures and durations are inconsistent and inconclusive, and many of these studies used experimental protocols with important limitations, indicating a need for a more definitive study (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2013).

Additionally, extensive reviews of the literature on the genotoxicity of various frequencies and modulations of RFR have concluded that evidence for RFR-associated genotoxicity is inconsistent and weak (Brusick et al. 1998; Ruediger 2009; Verschaeve et al. 2010), and some key studies reporting RFR-associated genotoxicity in human cell lines could not be replicated (Speit et al. 2013). As with the cancer studies, interpretations of the genotoxicity studies, particularly those performed *in vivo*, have also been limited by issues of experimental design. In 2013, after reviewing the available data, the IARC classified radiofrequency electromagnetic fields (RF-EMF), which include the RFR wavelength range, as "possibly carcinogenic to humans (Group 2B)," based on limited evidence in experimental animals and limited evidence in humans on the association between RF-EMF and cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2013).

To help inform human health risk assessments, the National Toxicology Program (NTP) designed and conducted a 2-year rodent cancer study of cell phone RFR, using code division multiple access (CDMA) or global system for mobile (GSM) modulations, the principal modulations used in the United States (CDMA and GSM) and in the rest of the world (GSM). GSM and CDMA are second-generation (2G) and third-generation (3G) technologies, respectively, and they differ in the method in which information is incorporated and transmitted within frequency bands. The previous inconsistent genotoxicity and tumorigenicity findings that have been reported following RFR exposure could be due in part to the immense and unique technical challenges inherent in studying the effects of non-ionizing radiation, including RFR (Capstick et al. 2017; Gong et al. 2017). To address these challenges and provide data to clarify possible adverse biological effects of cell phone RFR exposure, the NTP took into account numerous variables and parameters in designing its rodent cancer bioassay. Key features included construction of custom-designed reverberation chambers that exposed animals to a clearly defined, statistically homogenous radiofrequency field, that shielded animals from all other sources of RFR, and eliminated the need for restraint, a method commonly employed by other researchers for point-source exposures (Capstick et al. 2017; Gong et al. 2017). Animals were housed inside the reverberation chambers and exposed to RFR for a total of 9 hr 10 min per day in 10-min on/off cycles (over the course of an ~18 hr period) at frequencies with modulations being used in cellular networks (Capstick et al. 2017). In addition, the exposure levels selected for this study were based on the results of previously conducted dosimetry studies and thermal pilot studies that demonstrated no measurable hyperthermia in rats and mice at the exposure levels chosen for this study (Gong et al. 2017; Wyde et al. 2018).

In the NTP study design, Sprague Dawley rats and B6C3F1/N mice of both sexes were whole-body exposed to RFR (CDMA or GSM modulations). Rats were exposed *in utero* beginning on Gestation day 5 (GD5), and mice were exposed beginning at 5 weeks of age. After a total of 19 weeks of exposure for rats and 14 weeks for mice, subsets of 5 rats and 5 mice of each sex from each exposure group were removed from the ongoing 2-year cancer bioassay after subchronic exposure and assessed for DNA damage using the comet assay, and for changes in chromosomal structure and/or number using the peripheral blood erythrocyte micronuclei (MN) assay. For the comet assay, cells from three functionally distinct structures of the brain (frontal cortex, hippocampus, and cerebellum), along with liver cells and peripheral blood leukocytes were assessed. Brain tissue was analyzed in the comet assay due to concerns that RFR may increase risk for brain cancer in humans, whereas liver cells and blood leukocytes were selected for analysis as these cells are part of typical analyses conducted at the NTP for DNA damage.

MATERIALS AND METHODS

Animal Husbandry

Time-mated Hsd:Sprague Dawley SD rats (11–14 weeks of age) (Harlan, Indianapolis, IN) were received on GD2 at the laboratory (Illinois Institute of Technology Research Institute; IITRI, Chicago, IL). After littering, male and female pups were housed with their dams until weaning on Postnatal day 28 (PND28). During the perinatal phase, rats were fed irradiated NIH-07 wafers; from weaning until study completion, rats were fed irradiated NTP-2000 rodent diet (Zeigler Brothers, Gardners, PA). Male and female B6C3F1/N mice (Taconic, Germantown, NY) were received at 3–4 weeks of age. Mice were quarantined for 10–14 days and were 5–6 weeks of age at the start of exposure. Mice were fed irradiated NTP-2000 rodent diet. All animals were provided food and tap water (city of Chicago, IL, municipal supply) *ad libitum*. During the studies, animal health was monitored according to the NTP sentinel animal program. Mice, and rats after weaning, were housed individually in solid polycarbonate cages with irradiated hardwood bedding (Sani-chips, P.J. Murphy, Montville, NJ) within custom designed, stainless steel reverberation chambers. Environmental conditions were set to maintain a 12-hr light/dark

cycle, a temperature of 72 ± 3°F, a humidity range of 50 ± 15%, and >10 air changes/hr.

Animal use was in accordance with the U.S. Public Health Service policy on humane care and use of laboratory animals and the Guide for the Care and Use of Laboratory Animals (National Research Council 1996). Animal housing facilities were accredited by the Association for Assessment and Accreditation of Laboratory Animal Care; all procedures were approved by the IITRI Institutional Animal Care and Use Committee. The RFR exposures performed at IITRI were in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21CFR, Part 58). Animals were euthanized by CO_2 asphyxiation.

Reverberation Chambers

Reverberation chambers were self-contained rooms that were designed to house unrestrained animals in cages and expose them to a uniform field of RFR (GSM or CDMA) and to shield animals from all outside RFR. Detailed descriptions of the design of the reverberation chambers and the RFR exposure system are provided in Capstick et al. (2017) and Gong et al. (2017). Uniformity of the RFR field was achieved by installing excitation antennas with rotating horizontal and vertical reflective surface paddles to ensure uniform distribution of statistically homogenous RFR fields within the volume of the chambers. Therefore, animals were exposed to all polarizations of RFR fields from all directions regardless of their posture or orientation to the antenna. Animals were housed one per cage to prevent interference in energy absorption. Cages, cage racks, and materials used to deliver food and water to the animals were designed to minimize interference with RFR exposure. Chamber design and animal housing were developed in collaboration with the National Institute of Standards and Technology (NIST) and the Foundation for Research on Information Technologies in Society (IT'IS). RFR field intensity, uniformity, quality of modulation, and numerous other parameters were validated by NIST, and consistency of exposure was monitored in real time by IT'IS. Further evaluation of the exposure systems is presented in NTP Technical Reports 595 (NTP 2018a) and 596 (NTP 2018b).

Dosimetry, Specific Absorption Rates, and Exposure Regimen

Briefly, in pilot studies, body temperatures were monitored using subcutaneously implanted temperature chips (Wyde et al. 2018). Both young and older animals were tested for the possibility of thermal effects from radiation. An upper limit of 1°C was set as an acceptable increase in body temperature. Models predicted that thermoregulation might not be maintained in rats exposed to an specific absorption rate (SAR) > 6.0 W/kg, delivered at a frequency of 900 MHz, and in mice exposed to an SAR > 10.0 W/kg, delivered at a frequency of 1,900 MHz (Gong et al. 2017; Wyde et al. 2018). Thus, these were selected as the highest exposure levels in the current study, and the two lower exposures were each reduced by half. Due to technical constraints, body temperatures could not be monitored in the current study.

Rats were exposed to SARs of 0, 1.5, 3.0, or 6.0 W/kg (CDMA or GSM) RFR (900 MHz) beginning *in utero* at GD5 and continuing through gestation (~2 weeks) until weaning at PND28. Exposures continued for 14 weeks after weaning. Mice were exposed to SARs of 0, 2.5, 5.0, or 10.0 W/kg (CDMA or GSM) RFR (1,900 MHz) for 14 weeks beginning at 5–6 weeks of age. Rat exposures were initiated at the time of implantation (GD5) to simulate whole-life exposures in humans, but because B6C3F1/N mice are poor and unpredictable breeders, this animal model is not suitable for whole-life exposure assessments. Exposures ran daily from 11:00 A.M. to 2:00 P.M. and from 3:40 P.M. to 7:00 A.M., with RFR cycling on and off every 10 min, resulting in a total duration of exposure of 9 hr 10 min per 24-hr period. This exposure schedule accommodated two daily intervals for animal care. Animals were housed individually in a total of 21 reverberation chambers, 7 for the mice and 14 for the rats. Each reverberation chamber emitted one power level for one modulation. Male and female mice, due to similarity in weight and size, were exposed together in 7 reverberation chambers. In contrast, due to gender-related differences in weight and size, male and female rats were exposed in separate chambers, thus requiring 14 chambers. To control for possible positional differences in RFR field strength, cages were rotated in the racks weekly. Because SAR is dependent on body weight, the energy used to emit RFR was adjusted twice weekly for rats and once weekly for mice based on the average weight of all animals in an exposure chamber.

The sham control rats and mice were housed in reverberation chambers without activation of RFR. One group of five animals of each sex/species served as the sham control for both CDMA and GSM exposures.

Tissue Sample Collection

On the day of necropsy, RFR exposure ceased at 7 A.M. Necropsies were performed in two shifts. For each species, 35 male animals (5 controls, 15 exposed to CDMA, and 15 exposed to GSM) were necropsied 1.5–4 hr after cessation of exposure and 35 female animals (5 controls, 15 exposed to CDMA, and 15 exposed to GSM) were necropsied approximately 4.5–7 hr after cessation of exposure. Animals were necropsied in the following order: one animal from each dose group starting with the sham exposed group, moving through each dose group for each RFR modulation in turn, then rotating back to the sham control group; animals were necropsied in numerical order within each dose group. Five tissues were collected from each animal for the comet assay. One blood sample per animal collected by retro-orbital bleeding was divided into two aliquots: one for the comet assay and the other for the MN assay.

For the comet assay, 50 μL of blood were transferred to a tube containing 1 mL of freshly prepared cold mincing buffer (Mg^{+2}, Ca^{+2}, and phenol free Hank's Balanced Salt Solution [Life Technologies, Carlsbad, CA] with 20 mM ethylenediamine tetraacetic acid (EDTA) pH 10.0 and 10% vol/vol fresh dimethyl sulfoxide [DMSO]) pH 7.47. The liver and the hippocampus, cerebellum, and frontal cortex sections of the brain were removed, rinsed with cold mincing buffer, and held on ice (≤5 min) until processed. Small portions (3–4 mm^3) of each tissue were placed in tubes containing cold mincing solution and rapidly minced until finely dispersed. Blood and minced tissue samples were immediately flash frozen in liquid nitrogen and transferred to a −80°C freezer for a minimum of 1 week until shipment by overnight air courier on dry ice to the analytical laboratory (ILS, Research Triangle Park, NC).

For the MN assay, blood samples (~200 μL) were placed into EDTA tubes and immediately refrigerated. The samples were sent on the day of collection to ILS on cold packs via overnight air courier. Upon arrival, samples were diluted in anticoagulant (heparin) and fixed in ice-cold methanol (Sigma-Aldrich, St. Louis, MO) according to instructions provided with the MicroFlow^{PLUS} Kit (Litron Laboratories, Rochester, NY). Fixed samples were stored in a −80°C freezer for at least 3 days prior to analysis by flow cytometry.

Comet Assay

Slides were prepared and analyzed as described previously (Hobbs et al. 2012; Recio et al. 2012) with some modifications. In a laboratory with controlled humidity (≤60%), samples were thawed on ice and a portion of the cell suspension was diluted with 0.5% low melting point agarose (Lonza, Walkersville, MD) dissolved in Dulbecco's phosphate buffer (Ca^{+2}, Mg^{+2}, and phenol free) at 37°C and layered onto each well of a 2-well CometSlide™ (Trevigen, Gaithersburg, MD). Slides were prepared one tissue at a time, such that 35 slides were prepared at a time in 3 batches of 10 and 1 batch of 5, and each batch was immediately refrigerated to solidify the agarose and prevent deterioration of the samples. Once all slides per tissue had been prepared and refrigerated for at least 20 min (typically ≤2 hr for completion of an entire set), the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM tris

[hydroxymethyl]aminomethane, pH 10, containing freshly added 10% DMSO, and 1% Triton X-100) overnight with refrigeration. After rinsing in 0.4 M Trizma base (pH 7.5), slides were treated with cold alkali solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 20 min to allow DNA unwinding, electrophoresed at 4–9°C for 20 min at 25 V (0.7 V/cm), with a current of approximately 300 mA, neutralized with Trizma base, dehydrated in absolute ethanol (Pharmco-AAPER, Shelbyville, KY), and air-dried. Slides from the same species, sex, and tissue were run together during electrophoresis and were placed randomly into the electrophoresis tank by exposure level and modulation to control for any possible variations in electrical field. Slides were stored at room temperature in a desiccator (relative humidity ≤60%) until stained and scored. NaCl, Na_2EDTA, Triton X-100, DMSO, and Trizma base were purchased from Sigma-Aldrich; NaOH was purchased from Fisher Scientific (Pittsburgh, PA).

After staining with SYBR® Gold (Molecular Probes, Life Technologies, Grand Island, NY), slides, independently coded to mask treatment, were scored using Comet Assay IV Imaging Software, Version 4.3.1 (Perceptive Instruments, Suffolk, UK). DNA migration was quantified as % tail DNA (OECD 2016). Comets were classified as scorable, nonscorable, or "hedgehog." Comets were classified as hedgehogs if they had no easily defined head, that is, all DNA appeared to be in the tail, or the head and tail appeared separated. Initially, % tail DNA was determined for 100 scorable comet figures per animal/tissue, standard practice at the time the study was conducted (prior to OECD Guideline 489). In addition, the frequency of hedgehogs was determined by tabulating the number of hedgehogs per 100 cells per animal/tissue, but hedgehog frequencies were not analyzed for statistical significance, in accordance with OECD Guideline 489. Although it has been proposed that hedgehogs are apoptotic cells, some studies strongly suggest that hedgehogs represent cells with high levels of repairable DNA damage (Rundell et al. 2003; Lorenzo et al. 2013), and it remains uncertain in the field as to what hedgehogs represent.

In the initial scoring of the rat samples, we noted that the range of % tail DNA values appeared truncated at ~ 65%. To better understand this observation, we reanalyzed the rat slides, scoring 150 cells/tissue/animal, as recommended by the OECD guideline (OECD 2016). In this second scoring exercise, we included analysis of scorable comet images that, upon visual inspection, appeared to be hedgehogs to determine if this affected the capture of DNA damage levels between 65 and 100% tail DNA. For the 150-cell scoring method, because the % hedgehogs were not independently determined, the value was estimated by dividing the number of comets with ≥90% tail DNA by 150. Several mouse tissues were also

TABLE I. DNA damage in Male Sprague Dawley Rats Exposed to CDMA-Modulated Cell Phone Radiofrequency Radiation (900 MHz) for 19 Weeks[a]

	Dose (W/kg)	% Tail DNA (100 cells)[b]	*P* value[c]	% Hedgehogs (100 cells)[b]	% Tail DNA (150 cells)[b]	*P* value	% Hedgehogs (150 cells)[b,d]
Frontal cortex							
	0[e]	6.18 ± 0.72		2.00 ± 0.71	9.73 ± 0.81		0.27 ± 0.27
CDMA	1.5	6.00 ± 0.48	1.000	1.00 ± 0.77	8.24 ± 0.39	1.000	0.13 ± 0.13
	3.0	9.51 ± 1.17	0.081	10.60 ± 3.89	18.77 ± 3.27	0.043	2.53 ± 1.29
	6.0	12.78 ± 3.96	0.049	12.20 ± 6.84	23.62 ± 8.66	0.092	3.20 ± 1.72
		P = 0.004			*P* = 0.005[f]		
Hippocampus							
	0	5.88 ± 0.39		3.40 ± 1.21	8.99 ± 1.55		1.07 ± 0.45
CDMA	1.5	8.06 ± 1.20	0.135	3.80 ± 2.33	12.27 ± 2.21	0.244	0.40 ± 0.27
	3.0	8.16 ± 0.98	0.151	6.20 ± 2.56	15.46 ± 2.25	0.107	2.53 ± 0.90
	6.0	10.42 ± 2.18	0.019	4.40 ± 2.98	16.77 ± 5.44	0.069	2.40 ± 1.44
		P = 0.014			*P* = 0.043		
Cerebellum							
	0	5.57 ± 0.92		0.40 ± 0.24	4.90 ± 0.82		0 ± 0
CDMA	1.5	5.60 ± 0.71	1.000	1.80 ± 0.80	6.33 ± 1.00	0.681	0.27 ± 0.16
	3.0	10.70 ± 3.66	0.504	9.40 ± 6.81	13.75 ± 6.01	0.504	2.93 ± 2.20
	6.0	10.58 ± 3.52	0.731	8.00 ± 3.91	15.86 ± 5.91	0.163	2.40 ± 1.07
		P = 0.156			*P* = 0.061		
Liver							
	0	13.81 ± 2.88		33.60 ± 17.89	25.71 ± 8.71		1.73 ± 1.73
CDMA	1.5	22.99 ± 2.77	0.081	68.60 ± 15.70	55.41 ± 7.91	0.136	14.67 ± 5.57
	3.0	16.04 ± 2.14	0.098	7.80 ± 0.86	19.11 ± 2.28	0.164	0.80 ± 0.49
	6.0	20.79 ± 3.10	0.057	41.10 ± 14.80	40.01 ± 7.90	0.114	9.07 ± 7.10
		P = 0.154			*P* = 0.385		
Peripheral blood leukocytes							
	0	1.48 ± 0.29		0.20 ± 0.20	0.69 ± 0.20		0 ± 0
CDMA	1.5	1.22 ± 0.45	0.596	0.80 ± 0.80	1.16 ± 0.47	0.295	0 ± 0
	3.0	2.13 ± 0.34	0.156	0.40 ± 0.40	1.83 ± 0.74	0.121	0.13 ± 0.13
	6.0	2.08 ± 0.43	0.166	1.40 ± 1.17	2.57 ± 0.80	0.026	0 ± 0
		P = 0.071			*P* = 0.012		

[a]Exposure began *in utero* on GD5.
[b]Mean ± SE.
[c]Pairwise comparison with the sham control group; exposed group values are significant at $P \le 0.025$ by Williams' or Dunn's test.
[d]A comet figure was considered a hedgehog if ≥90% DNA was in the tail. % Hedgehogs = number of comets with ≥90% tail DNA/150.
[e]Sham control; no exposure to CDMA-modulated cell phone RFR.
[f]Dose-related trend derived from one-tailed linear regression or Jonckheere's test; the trend is significant when $P \le 0.025$.

reevaluated using the 150-cell method for comparison. Although there was no concurrent positive control group (as is standard for all NTP chronic and subchronic animal toxicity tests), slides made with human lymphoblastoid TK6 cells treated with ethyl methanesulfonate were processed in parallel with each tissue set as an internal technical control for slide preparation, staining, and electrophoresis.

Micronucleus Assay

Flow cytometric analysis of red blood cells was performed using MicroFlow^{PLUS} Kit reagents and a FACSCalibur™ dual-laser bench top system (Becton Dickinson Biosciences, San Jose, CA) as described previously (Witt et al. 2008) and was consistent with OECD Test Guideline 474 (OECD 2014). Briefly, both immature erythrocytes (reticulocytes, RET) and mature erythrocytes were analyzed for the presence of MN. For each sample, 20,000 (±2,000) RET were analyzed and ~1×10^6 mature erythrocytes were enumerated concurrently during micronucleated–RET (MN-RET) analysis, allowing for calculation of the percentage of RET (%RET) among total erythrocytes as a measure of bone marrow toxicity.

Data analysis

Data from both the comet and the MN assays, presented as mean ± standard error (SE), were analyzed using the same statistical methods (Kissling et al. 2007). Mean % tail DNA was calculated for each tissue per animal; likewise, mean MN–RET and MN–erythrocytes per 1,000 cells, as well as %RET, were calculated for each animal. Levene's test was used to determine if variances among treatment groups were equal at significance level 0.05. When variances were equal, linear regression analysis was used to test for trend and Williams' test was used to evaluate pairwise differences between each treated group and the control. When variances were unequal, Jonckheere's test was used to evaluate linear trend and Dunn's test was used to assess the significance of pairwise differences of each treated group with the control group. To maintain the overall significance level at 0.05, the trend as well as the pairwise differences were declared statistically significant if $P < 0.025$. A result was considered positive if the trend test was significant and at least one dose group was significantly elevated over the control, or if two or more dose groups were significantly increased over the corresponding control. A response was considered equivocal if only

TABLE II. DNA Damage in Male Sprague Dawley Rats Exposed to GSM-Modulated Cell Phone Radiofrequency Radiation (900 MHz) for 19 Weeks[a]

	Dose (W/kg)	% Tail DNA (100 cells)[b]	*P* value	% Hedgehogs (100 cells)[b]	% Tail DNA (150 cells)[b]	*P* value[c]	% Hedgehogs (150 cells)[b,d]
Frontal cortex							
	0[e]	6.18 ± 0.72		2.00 ± 0.71	9.73 ± 0.81		0.27 ± 0.27
GSM	1.5	6.98 ± 0.42	0.465	1.40 ± 0.51	11.96 ± 1.65	0.634	0.40 ± 0.27
	3.0	8.66 ± 1.96	0.247	8.20 ± 2.69	17.98 ± 5.12	0.545	1.20 ± 0.57
	6.0	6.30 ± 0.32	1.000	3.00 ± 1.55	9.57 ± 1.57	1.000	1.30 ± 0.13
		$P = 0.343$			$P = 0.500$[f]		
Hippocampus							
	0	5.88 ± 0.39		3.40 ± 1.21	8.99 ± 1.55		1.07 ± 0.45
GSM	1.5	11.82 ± 2.68	0.092	4.80 ± 2.84	17.24 ± 4.09	0.186	0.27 ± 0.16
	3.0	9.64 ± 1.27	0.111	4.80 ± 1.53	14.77 ± 2.54	0.227	1.47 ± 0.57
	6.0	11.69 ± 3.92	0.072	10.20 ± 7.98	21.32 ± 9.55	0.080	3.60 ± 2.03
		$P = 0.103$			$P = 0.076$		
Cerebellum							
	0	5.57 ± 0.92		0.40 ± 0.24	4.90 ± 0.82		0 ± 0
GSM	1.5	7.36 ± 2.48	0.295	2.40 ± 1.91	9.43 ± 4.69	0.190	1.33 ± 1.17
	3.0	6.37 ± 0.77	0.354	3.40 ± 1.17	8.66 ± 2.17	0.232	1.47 ± 0.68
	6.0	8.48 ± 1.85	0.149	5.00 ± 2.86	12.11 ± 3.89	0.088	1.07 ± 1.07
		$P = 0.132$			$P = 0.076$		
Liver							
	0	13.81 ± 2.88		33.60 ± 17.89	25.71 ± 8.71		1.73 ± 1.73
GSM	1.5	13.26 ± 2.38	0.547	21.00 ± 12.30	23.27 ± 9.43	0.539	4.13 ± 3.64
	3.0	13.09 ± 2.32	0.634	28.40 ± 15.07	25.15 ± 8.43	0.604	0.40 ± 0.40
	6.0	14.49 ± 2.71	0.536	24.80 ± 16.13	28.25 ± 10.55	0.534	4.93 ± 3.94
		$P = 0.404$			$P = 0.390$		
Peripheral blood leukocytes							
	0	1.48 ± 0.29		0.20 ± 0.20	0.69 ± 0.20		0 ± 0
GSM	1.5	1.83 ± 0.63	0.352	3.20 ± 2.71	3.97 ± 2.75	0.146	0.27 ± 0.27
	3.0	1.78 ± 0.33	0.419	1.20 ± 0.49	1.97 ± 0.35	0.021	0 ± 0
	6.0	1.50 ± 0.27	0.446	0.40 ± 0.24	1.28 ± 0.23	0.272	0 ± 0
		$P = 0.550$			$P = 0.089$		

[a]Exposure began *in utero* on GD5.
[b]Mean ± SE.
[c]Pairwise comparison with the sham control group; exposed group values are significant at $P \le 0.025$ by Williams' or Dunn's test.
[d]A comet figure was considered a hedgehog if ≥90% DNA was in the tail. % Hedgehogs = number of comets with ≥90% tail DNA/150.
[e]Sham control; no exposure to GSM-modulated cell phone RFR.
[f]Dose-related trend derived from one-tailed linear regression or Jonckheere's test; the trend is significant when $P \le 0.025$.

the trend test was significant or only a single dose group was significantly increased over the control. In the absence of either a significant trend or a significantly elevated dose group, the result was considered negative.

RESULTS

Comet Assay

Eight hundred tissue samples were analyzed for % tail DNA in the comet assay. The mean % tail DNA, SE, and statistical outcomes for pairwise and trend comparisons are shown for all 40 sets of tissues (5 tissues × 8 conditions of the study) in Tables 1–8. Results are reported based on the standard 100-cell scoring approach in use at the time that the data were collected. Data obtained using the 150-cell scoring approach (OECD 2016) are noted for the few instances where results differed between the two methods. In addition, results that were either positive or equivocal are presented in figures to illustrate interanimal variability in response, and to compare the 100- versus 150-cell scoring results (Figs. 1–3). Samples were not removed from analysis unless a technical issue was identified with acquisition of the sample, or if the result was considered to be biologically implausible, as apparent outliers or influential data points could represent true biological variability. Of the 800 tissue samples that were analyzed for % tail DNA, three samples were omitted from analysis. Two samples, female rat hippocampal tissue exposed to 1.5 W/kg GSM and female rat hippocampal tissue exposed to 3.0 W/kg, were omitted due to a labeling error that occurred during necropsy. A sample of hippocampal tissue from a sham-exposed female rat was

TABLE III. DNA Damage in Female Sprague Dawley Rats Exposed to CDMA-Modulated Cell Phone Radiofrequency Radiation (900 MHz) for 19 Weeks[a]

	Dose (W/kg)	% Tail DNA (100 cells)[b]	*P* value	% Hedgehogs (100 cells)[b]	% Tail DNA (150 cells)[b]	*P* value[c]	% Hedgehogs (150 cells)[b,d]
Frontal cortex							
	0[e]	7.03 ± 1.21		3.80 ± 1.46	12.23 ± 2.18		0.40 ± 0.16
CDMA	1.5	12.70 ± 5.15	0.205	19.00 ± 15.04	25.37 ± 12.96	0.782	8.67 ± 7.67
	3.0	9.50 ± 2.27	0.249	9.80 ± 5.12	18.70 ± 5.28	0.634	1.87 ± 0.88
	6.0	13.00 ± 3.63	0.150	25.40 ± 11.44	33.49 ± 11.14	0.092	7.20 ± 5.62
		$P = 0.166$[f]			$P = 0.035$		
Hippocampus							
	0[g]	13.14 ± 1.20		9.00 ± 2.58	18.08 ± 1.30		0.83 ± 0.32
CDMA	1.5	14.94 ± 0.70	0.346	8.40 ± 1.96	20.58 ± 2.06	0.531	1.07 ± 0.34
	3.0	15.24 ± 1.97	0.379	9.40 ± 2.89	20.63 ± 1.92	0.382	1.33 ± 0.21
	6.0	19.11 ± 5.27	0.126	21.20 ± 11.12	29.55 ± 9.44	0.218	6.53 ± 5.23
		$P = 0.080$			$P = 0.068$		
Cerebellum							
	0	5.94 ± 0.98		3.80 ± 1.07	4.93 ± 1.09		0 ± 0
CDMA	1.5	4.91 ± 0.58	0.671	2.00 ± 1.05	4.61 ± 1.61	0.621	0.53 ± 0.53
	3.0	5.46 ± 0.83	0.747	2.00 ± 0.63	3.89 ± 0.43	0.709	0.13 ± 0.13
	6.0	5.86 ± 0.84	0.650	1.20 ± 0.37	5.88 ± 0.63	0.342	0.27 ± 0.16
		$P = 0.421$			$P = 0.249$		
Liver							
	0	10.09 ± 0.87		7.00 ± 1.87	12.41 ± 1.64		0.13 ± 0.13
CDMA	1.5	15.26 ± 3.35	0.634	33.40 ± 15.11	26.15 ± 8.57	0.145	4.00 ± 3.67
	3.0	11.49 ± 2.05	1.000	12.40 ± 3.59	16.17 ± 2.17	0.176	0.67 ± 0.42
	6.0	18.35 ± 3.44	0.163	31.40 ± 12.33	26.65 ± 6.91	0.059	2.00 ± 1.17
		$P = 0.113$			$P = 0.102$		
Peripheral blood leukocytes							
	0	3.15 ± 0.40		0.20 ± 0.20	3.32 ± 0.09		0.13 ± 0.13
CDMA	1.5	3.77 ± 1.19	0.371	1.20 ± 0.80	4.45 ± 1.53	1.000	0.40 ± 0.27
	3.0	4.13 ± 0.54	0.361	0.40 ± 0.40	3.94 ± 0.40	0.465	0.13 ± 0.13
	6.0	6.06 ± 2.18	0.082	9.80 ± 8.81	12.76 ± 7.59	0.028	2.93 ± 2.77
		$P = 0.048$			$P = 0.013$		

[a]Exposure began *in utero* on GD5.
[b]Mean ± SE.
[c]Pairwise comparison with the sham control group; exposed group values are significant at $P \le 0.025$ by Williams' or Dunn's test.
[d]A comet figure was considered a hedgehog if ≥90% DNA was in the tail. % Hedgehogs = number of comets with ≥90% tail DNA/150.
[e]Sham control; no exposure to CDMA-modulated cell phone RFR.
[f]Dose-related trend derived from one-tailed linear regression or Jonckheere's test; the trend is significant when $P \le 0.025$.
[g]$n = 4$.

omitted because it had a biologically implausible value of 56.1% tail DNA.

In rats, the only clear positive result was observed in hippocampus cells of male rats exposed to the CDMA modulation when evaluated using the 100-cell scoring approach (Table I; Fig. 1A,B). Although the levels of DNA damage in hippocampus cells were also increased in an exposure-related fashion using the 150-cell scoring approach, the increases did not meet our criteria for statistical significance (Table I). Equivocal results were obtained for the frontal cortex (CDMA) of male rats using both scoring approaches (Table I; Fig. 2A, B). For male rat blood leukocytes (both modulations), results from scoring 100 cells were negative; however, equivocal responses were seen with the 150-cell method based on a significant trend test ($P = 0.012$) or pairwise test ($P = 0.021$) for CDMA- and GSM-exposed rats, respectively (Tables I and II). No statistically significant increases in % tail DNA were observed in any of the samples from female rats exposed to either modulation (Tables III and IV). Although it would appear that an equivocal result was obtained for CMDA-exposed female rat blood leukocytes using the 150-cell scoring approach (Table III), this result was driven by a single animal in the high exposure (6 W/kg) group.

In mice, positive results were obtained with both scoring approaches in frontal cortex of male mice (CDMA and GSM) (Tables V and VI; Fig. 3A–D) and blood leukocytes of female mice (CDMA) (Table VII; Fig. 3E,F). Scoring 150 cells resulted in a positive response in liver of female mice exposed to CDMA; a similar pattern of response was seen with the 100-cell scoring method, but none of the increases met our criteria for significance (Table VII). No

TABLE IV. DNA Damage in Female Sprague Dawley Rats Exposed to GSM-Modulated Cell Phone Radiofrequency Radiation (900 MHz) for 19 Weeks[a]

	Dose (W/kg)	% Tail DNA (100 cells)[b]	*P* value	% Hedgehogs (100 cells)[b]	% Tail DNA (150 cells)[b]	*P* value[c]	% Hedgehogs (150 cells)[b,d]
Frontal cortex							
	0[e]	7.03 ± 1.21		3.80 ± 1.46	12.23 ± 2.18		0.40 ± 0.16
GSM	1.5	4.87 ± 0.47	0.820	2.20 ± 0.73	6.28 ± 1.00	0.856	0 ± 0
	3.0	6.18 ± 0.67	0.843	5.60 ± 2.36	9.83 ± 1.11	0.877	0.67 ± 0.21
	6.0	6.74 ± 0.74	0.723	6.40 ± 2.73	13.74 ± 2.79	0.376	0.13 ± 0.13
		$P = 0.386$			$P = 0.137$[f]		
Hippocampus							
	0[g]	13.14 ± 1.20		9.00 ± 2.58	18.08 ± 1.30		0.83 ± 0.32
GSM	1.5[g]	13.22 ± 1.56	0.936	7.25 ± 3.20	17.54 ± 3.59	1.000	1.50 ± 1.29
	3.0[g]	17.67 ± 3.64	0.351	19.50 ± 7.89	28.08 ± 7.00	0.662	3.66 ± 1.40
	6.0	13.21 ± 1.03	1.000	10.00 ± 3.81	18.19 ± 3.35	1.000	2.93 ± 1.53
		$P = 0.334$			$P = 0.534$		
Cerebellum							
	0	5.94 ± 0.98		3.80 ± 1.07	4.93 ± 1.09		0 ± 0
GSM	1.5	5.69 ± 0.75	0.662	2.00 ± 0.71	5.11 ± 0.63	0.731	0 ± 0
	3.0	4.62 ± 0.85	0.749	0.60 ± 0.24	3.51 ± 0.74	1.000	0 ± 0
	6.0	6.62 ± 0.96	0.381	2.40 ± 1.03	6.54 ± 2.33	1.000	0.27 ± 0.16
		$P = 0.302$			$P = 0.705$		
Liver							
	0	10.09 ± 0.87		7.00 ± 1.87	12.41 ± 1.64		0.13 ± 0.13
GSM	1.5	9.91 ± 2.60	1.000	13.20 ± 11.23	17.05 ± 7.24	1.000	0.93 ± 0.62
	3.0	9.46 ± 2.07	1.000	17.00 ± 14.76	14.06 ± 5.68	1.000	0.27 ± 0.16
	6.0	18.99 ± 6.20	1.000	35.20 ± 19.42	26.03 ± 10.69	1.000	4.00 ± 3.23
		$P = 0.394$			$P = 0.580$		
Peripheral blood leukocytes							
	0	3.15 ± 0.40		0.20 ± 0.20	3.32 ± 0.09		0.13 ± 0.13
GSM	1.5	2.80 ± 0.33	0.593	0.80 ± 0.49	3.07 ± 0.43	1.000	0.27 ± 0.16
	3.0	3.39 ± 0.68	0.447	0.60 ± 0.24	2.82 ± 0.52	1.000	0.13 ± 0.13
	6.0	3.93 ± 0.63	0.203	1.00 ± 0.32	3.86 ± 0.76	1.000	0.40 ± 0.16
		$P = 0.093$			$P = 0.580$		

[a]Exposure began in utero on GD5.
[b]Mean ± SE.
[c]Pairwise comparison with the sham control group; exposed group values are significant at $P \le 0.025$ by Williams' or Dunn's test.
[d]A comet figure was considered a hedgehog if ≥90% DNA was in the tail. % Hedgehogs = number of comets with ≥90% tail DNA/150.
[e]Sham control; no exposure to GSM-modulated cell phone RFR.
[f]Dose-related trend derived from one-tailed linear regression or Jonckheere's test; the trend is significant when $P \le 0.025$.
[g]$n = 4$.

statistically significant increases in % tail DNA were observed in any of the samples from female mice exposed to the GSM modulation (Table VIII).

In general, for those data sets that were scored using both methods (100- and 150-cell scoring methods), similar conclusions were reached when considering positive or equivocal results (see Supporting Information Fig. S1A–D for examples) except for hippocampus from male rats (CDMA) (Table I), blood leukocytes from male rats (CDMA and GSM) (Tables I and II), and liver from female mice (CDMA) (Table VII). In summary, 8 of 40 tissue sets exhibited positive or equivocal results when assessed using the 100- or 150-cell scoring approaches.

In all instances, where both methods were used, the 150-cell method that included all scorable cells, even those that visually appeared to be hedgehogs before software analysis, revealed a much broader spectrum of DNA damage than the 100-cell method that excluded all apparent hedgehogs (Supporting Information Figs. S2A–D and S3A–D).

We noticed considerable interanimal variability in % tail DNA in both sexes of mice and rats. To rule out any influence from technical artifacts or protocol features, % tail DNA values for all tissues and % hedgehogs for the rat tissues were correlated to the position of slides in the electrophoresis chambers, the interval from exposure cessation to tissue collection, and the date of slide preparation. No patterns in the level of observed DNA damage emerged for any of these variables. To investigate the interanimal variability more closely, we plotted the % tail DNA response data for all tissues using the 100-cell data

TABLE V. DNA Damage in Male B6C3F1/N Mice Exposed to CDMA-Modulated Cell Phone Radiofrequency Radiation (1,900 MHz) for 14 Weeks[a]

	Dose (W/kg)	% Tail DNA (100 cells)[b]	*P* value[c]	% Hedgehogs (100 cells)[b]	% Tail DNA (150 cells)[b]	*P* value	% Hedgehogs (150 cell)[d]
Frontal cortex							
	0[e]	0.63 ± 0.08		0.40 ± 0.24	1.32 ± 0.21		0 ± 0
CDMA	2.5	3.46 ± 0.65	0.014	0.60 ± 0.40	4.52 ± 0.57	0.131	0 ± 0
	5.0	5.88 ± 1.06	0.001	0.60 ± 0.24	6.06 ± 0.96	0.018	0 ± 0
	10.0	8.85 ± 1.09	0.001	4.40 ± 1.69	10.04 ± 2.08	0.001	0.53 ± 0.39
		P = 0.001[f]			*P* = 0.001		
Hippocampus							
	0	7.69 ± 2.00		1.20 ± 0.58			
CDMA	2.5	9.59 ± 4.33	0.521	5.40 ± 2.11			
	5.0	6.44 ± 1.21	0.606	2.80 ± 0.97			
	10.0	6.38 ± 0.93	0.641	4.40 ± 2.27			
		P = 0.740					
Cerebellum							
	0	5.48 ± 1.30		1.80 ± 0.80			
CDMA	2.5	7.35 ± 2.47	0.339	4.40 ± 2.06			
	5.0	7.87 ± 2.80	0.404	4.60 ± 2.34			
	10.0	5.43 ± 2.43	0.431	1.60 ± 0.93			
		P = 0.554					
Liver							
	0	16.30 ± 2.21		6.80 ± 2.82			
CDMA	2.5	20.27 ± 5.53	1.000	21.60 ± 16.88			
	5.0	16.15 ± 1.15	1.000	11.00 ± 3.77			
	10.0	16.43 ± 0.83	1.000	7.20 ± 1.11			
		P = 0.368					
Peripheral blood leukocytes		1.60 ± 0.68		0.40 ± 0.24			
	0						
		2.10 ± 0.50	0.449	1.20 ± 0.58			
CDMA	2.5	1.30 ± 0.28	0.527	0.40 ± 0.24			
	5.0	2.86 ± 0.26	0.046	1.40 ± 0.87			
	10.0						
		P = 0.057					

[a]Exposure began at ~5 weeks of age.
[b]Mean ± SE.
[c]Pairwise comparison with the sham control group; exposed group values are significant at $P \le 0.025$ by Williams' or Dunn's test.
[d]A comet figure was considered a hedgehog if ≥90% DNA was in the tail. % Hedgehogs = number of comets with ≥90% tail DNA/150.
[e]Sham control; no exposure to CDMA-modulated cell phone RFR.
[f]Dose-related trend derived from one-tailed linear regression or Jonckheere's test; the trend is significant when $P \le 0.025$.

set. The median % tail DNA was included in each plot as a measure of central tendency in the distribution (see Supporting Information Fig. S4A–D). We found that % tail DNA values were relatively small (<5%) in blood leukocytes in both sexes and species, while the other four tissues exhibited a much greater interanimal variability in response with % tail DNA values that exceeded 30% in some cases. Female mice generally displayed less variability in response than male mice in the hippocampus, cerebellum, and liver. Female rats exposed to RFR also seemed to show less variability in response than male rats exposed to RFR in the cerebellum.

Micronucleus Assay

The MN assay data are reported in Supporting Information Tables S1 and S2. For male mice exposed to CDMA, although a significant trend was observed for MN–RET (P = 0.013), the absolute increase was quite small (the mean MN–RET for sham exposure was 2.55 vs. 2.93 for the 10 W/kg exposure) and within the laboratory's historical control range (1.66–3.06), and no corresponding increase was observed in the mature erythrocyte population that should be in steady-state equilibrium after continuous subchronic exposure. Thus, the overall MN assay result for male mice exposed to CDMA was considered to be negative. No other significant effects were seen in rats or mice exposed to either modulation of RFR.

RFR Exposure

The power levels for RFR exposure were adjusted based on the average weight of all animals in a chamber. Due to normal variations in animal weights, the actual SAR in individual animals differed slightly among animals in the

TABLE VI. DNA Damage in Male B6C3F1/N Mice Exposed to GSM-Modulated Cell Phone Radiofrequency Radiation (1,900 MHz) for 14 Weeks[a]

	Dose (W/kg)	% Tail DNA (100 cells)[b]	P value[c]	% Hedgehogs (100 cells)[b]	% Tail DNA (150 cells)[b]	P value	% Hedgehogs (150 cell)[d]
Frontal cortex							
	0[e]	0.63 ± 0.08		0.40 ± 0.24	1.32 ± 0.21		0 ± 0
GSM	2.5	1.71 ± 0.46	0.081	1.80 ± 0.97	4.25 ± 1.20	0.063	0.13 ± 0.13
	5.0	1.39 ± 0.15	0.081	1.60 ± 0.81	3.69 ± 0.53	0.063	0 ± 0
	10.0	3.73 ± 0.65	0.001	1.00 ± 0.45	5.60 ± 1.28	0.006	0.13 ± 0.13
		P = 0.001[f]			P = 0.004		
Hippocampus							
	0	7.69 ± 2.00		1.20 ± 0.58			
GSM	2.5	8.74 ± 1.93	0.514	5.40 ± 2.11			
	5.0	7.17 ± 1.08	0.598	2.20 ± 0.97			
	10.0	6.90 ± 1.19	0.633	5.40 ± 2.54			
		P = 0.720					
Cerebellum							
	0	5.48 ± 1.30		1.80 ± 0.80			
GSM	2.5	3.66 ± 0.30	0.831	3.00 ± 1.38			
	5.0	3.90 ± 0.59	0.896	1.80 ± 0.92			
	10.0	3.85 ± 1.08	0.919	3.40 ± 1.50			
		P = 0.838					
Liver							
	0	16.30 ± 2.21		6.80 ± 2.82			
GSM	2.5	17.66 ± 1.89	0.469	8.20 ± 3.84			
	5.0	15.40 ± 1.20	0.549	6.60 ± 1.96			
	10.0	18.94 ± 2.00	0.213	12.80 ± 4.40			
		P = 0.198					
Peripheral blood leukocytes							
	0	1.60 ± 0.68		0.40 ± 0.24			
GSM	2.5	1.85 ± 0.96	0.416	1.20 ± 1.20			
	5.0	1.75 ± 0.37	0.491	1.00 ± 0.55			
	10.0	1.85 ± 0.24	0.494	0.80 ± 0.58			
		P = 0.408					

[a]Exposure began at ~5 weeks of age.
[b]Mean ± SE.
[c]Pairwise comparison with the sham control group; exposed group values are significant at $P \le 0.025$ by Williams' or Dunn's test.
[d]A comet figure was considered a hedgehog if ≥90% DNA was in the tail. % Hedgehogs = number of comets with ≥90% tail DNA/150.
[e]Sham control; no exposure to GSM-modulated cell phone RFR.
[f]Dose-related trend derived from one-tailed linear regression or Jonckheere's test; the trend is significant when $P \le 0.025$.

same exposure chamber (Wyde et al. 2018). These minor deviations were considered to have negligible effect, as no correlations between actual individual animal SAR and comet assay outcomes were seen in any of several tissues, including brain, that were examined to evaluate possible associations (data not shown).

DISCUSSION

The two main RFR modulations used for cellular telephone communication worldwide, CDMA and GSM, were tested by the NTP in the 2-year rodent cancer bioassay. The reverberation chambers used to expose the animals for the bioassay were designed by physicists and engineers from NIST and IT'IS in collaboration with the NTP to overcome confounding factors that have limited the interpretation of other RFR studies. As a component of the bioassay, we examined the potential for RFR to induce DNA damage as measured by the comet assay and chromosomal damage as measured by the peripheral blood erythrocyte MN assay. Although results of the MN assays were negative, significant increases in the levels of DNA damage measured by the comet assay were seen in several tissues from rats and mice, indicating that RFR may be capable of causing increases in DNA damage.

DNA damage was primarily observed in brain tissue from male rats and mice exposed to RFR. Using the 100-cell scoring approach, the hippocampus of CDMA-exposed male rats showed a significant, exposure-related increase in % tail DNA, while no tissues in exposed female rats were found to have significant increases in % tail DNA compared to controls. Male mice exhibited significant CDMA exposure-related increases in % tail DNA compared to controls at all exposure levels in the frontal cortex,

TABLE VII. DNA Damage in Female B6C3F1/N Mice Exposed to CDMA-Modulated Cell Phone Radiofrequency Radiation (1,900 MHz) for 14 Weeks[a]

	Dose (W/kg)	% Tail DNA (100 cells)[b]	*P* value[c]	% Hedgehogs (100 cells)[b]	% Tail DNA (150 cells)[b]	*P* value	% Hedgehogs (150 cell)[d]
Frontal cortex							
	0[e]	8.11 ± 2.13		3.40 ± 1.47			
CDMA	2.5	4.88 ± 0.55	0.911	0.80 ± 0.49			
	5.0	4.89 ± 0.57	0.955	1.20 ± 0.49			
	10.0	4.80 ± 0.90	0.968	0.80 ± 0.58			
		P = 0.935[f]					
Hippocampus							
	0	8.15 ± 1.65		2.60 ± 1.69			
CDMA	2.5	5.76 ± 1.00	0.839	1.80 ± 0.80			
	5.0	5.22 ± 1.02	0.903	1.20 ± 0.58			
	10.0	5.34 ± 1.82	0.925	2.20 ± 0.97			
		P = 0.892					
Cerebellum							
	0	5.88 ± 0.85		0.20 ± 0.20			
CDMA	2.5	6.78 ± 1.67	0.296	1.75 ± 1.03			
	5.0	8.39 ± 1.13	0.194	0.20 ± 0.20			
	10.0	6.73 ± 0.77	0.207	0.40 ± 0.40			
		P = 0.298					
Liver							
	0	5.48 ± 0.60		0.60 ± 0.40	4.34 ± 0.60		0 ± 0
CDMA	2.5	7.54 ± 0.90	0.034	1.00 ± 0.45	6.20 ± 0.99	0.050	0 ± 0
	5.0	7.36 ± 0.72	0.041	4.40 ± 2.11	8.30 ± 0.92	0.009	0 ± 0
	10.0	7.63 ± 0.59	0.030	2.00 ± 0.77	6.14 ± 0.26	0.009	0 ± 0
		P = 0.050			*P* = 0.100		
Peripheral blood leukocytes							
	0	1.03 ± 0.13		0.20 ± 0.20	2.15 ± 0.08		0 ± 0
CDMA	2.5	2.52 ± 0.54	0.020	2.00 ± 1.14	3.62 ± 0.66	0.011	0 ± 0
	5.0	1.71 ± 0.37	0.024	0 ± 0	3.39 ± 0.45	0.015	0.13 ± 0.13
	10.0	2.20 ± 0.19	0.018	0.20 ± 0.20	2.45 ± 0.24	0.428	0 ± 0
		P = 0.085			*P* = 0.173		

[a]Exposure began at ~5 weeks of age.
[b]Mean ± SE.
[c]Pairwise comparison with the sham control group; exposed group values are significant at $P \le 0.025$ by Williams' or Dunn's test.
[d]A comet figure was considered a hedgehog if ≥90% DNA was in the tail. % Hedgehogs = number of comets with ≥90% tail DNA/150.
[e]Sham control; no exposure to CDMA-modulated cell phone RFR.
[f]Dose-related trend derived from one-tailed linear regression or Jonckheere's test; the trend is significant when $P \le 0.025$.

and a GSM exposure-related increase in % tail DNA compared to controls at the highest exposure level in the frontal cortex. Female mice showed small, but statistically significant, increases in % tail DNA compared to controls at all exposure levels in blood. No other potentially exposure-related patterns were apparent based on visual inspection of the % tail DNA data (see Figs. 1–3). A larger number of animals per treatment group may have improved the ability to detect increases in DNA damage; however, the size of the reverberation chambers limited the number of animals that could be used for genetic toxicity testing to 5 per treatment group, which is the standard for comet assay studies conducted at the NTP and consistent with OECD recommendations (Hartmann et al. 2003; OECD 2016).

A limitation in this study is the absence of histopathological assessment for indications of inflammation and cytotoxicity. Although histopathology was not performed on the animals used for genetic toxicity studies, an additional set of animals was removed from the 2-year cancer bioassay for histopathological evaluation at the same time as the animals used for the genetic toxicity studies. No evidence of neoplastic lesions or nonneoplastic lesions, such as inflammation or necrosis was observed in the brains or livers of these animals, which could be attributable to RFR exposure (NTP 2018a; 2018b). Furthermore, RFR-induced inflammation and necrosis were not observed in the brains or livers of rats or mice at the end of the 2-year cancer bioassay (NTP 2018a; 2018b).

The NTP bioassay was designed to evaluate nonthermal effects of cell phone RFR exposure, which meant that body temperature could not change more than 1°C under our exposure conditions. To meet that requirement, pilot studies conducted to establish acceptable SARs for the bioassay indicated that no body temperature increases over 1°C would be expected in rats (including pregnant rats) or mice at exposures up to 6.0 or 10.0 W/kg, respectively (Wyde

TABLE VIII. DNA Damage in Female B6C3F1/N Mice Following Exposure to GSM-Modulated Cell Phone Radiofrequency Radiation (1,900 MHz) for 14 Weeks[a]

	Dose (W/kg)	% Tail DNA (100 cells)[b]	*P* value[c]	% Hedgehogs (100 cells)[b]	% Tail DNA (150 cells)[b]	*P* value	% Hedgehogs (150 cell)[d]
Frontal cortex							
	0[e]	8.11 ± 2.13		3.40 ± 1.47			
GSM	2.5	7.33 ± 0.90	0.657	1.00 ± 0.45			
	5.0	7.69 ± 1.98	0.744	2.00 ± 0.84			
	10.0	5.74 ± 0.62	0.779	1.00 ± 0.32			
		P = 0.861[f]					
Hippocampus							
	0	8.15 ± 1.65		2.60 ± 1.69			
GSM	2.5	6.23 ± 1.00	0.866	0.80 ± 0.58			
	5.0	4.54 ± 1.29	0.923	1.20 ± 0.58			
	10.0	5.22 ± 1.23	0.942	1.60 ± 1.36			
		P = 0.933					
Cerebellum							
	0	5.88 ± 0.85		0.20 ± 0.20			
GSM	2.5	6.56 ± 1.22	1.000	1.20 ± 0.73			
	5.0	5.26 ± 0.59	1.000	0.60 ± 0.40			
	10.0	6.54 ± 1.71	1.000	1.80 ± 0.73			
		P = 0.606					
Liver							
	0	5.48 ± 0.60		0.60 ± 0.40	4.34 ± 0.60		0 ± 0
GSM	2.5	7.06 ± 0.61	0.096	3.40 ± 1.17	7.44 ± 0.48	0.027	0 ± 0
	5.0	6.36 ± 0.25	0.117	1.20 ± 0.37	5.45 ± 0.96	0.032	0 ± 0
	10.0	6.47 ± 0.79	0.124	2.60 ± 1.33	6.52 ± 0.75	0.030	0 ± 0
		P = 0.249			*P* = 0.133		
Peripheral blood leukocytes							
	0	1.03 ± 0.13		0.20 ± 0.20	2.15 ± 0.08		0 ± 0
GSM	2.5	1.25 ± 0.44	0.335	0.20 ± 0.20	2.58 ± 0.35	0.504	0 ± 0
	5.0	1.17 ± 0.08	0.400	0 ± 0	2.23 ± 0.19	1.000	0 ± 0
	10.0	1.32 ± 0.34	0.316	0 ± 0	2.28 ± 0.51	1.000	0 ± 0
		P = 0.266			*P* = 0.657		

[a]Exposure began at ~5 weeks of age.
[b]Mean ± SE.
[c]Pairwise comparison with the sham control group; exposed group values are significant at $P \le 0.025$ by Williams' or Dunn's test.
[d]A comet figure was considered a hedgehog if ≥90% DNA was in the tail. % Hedgehogs = number of comets with ≥90% tail DNA/150.
[e]Sham control; no exposure to GSM-modulated cell phone RFR.
[f]Dose-related trend derived from one-tailed linear regression or Jonckheere's test; the trend is significant when $P \le 0.025$.

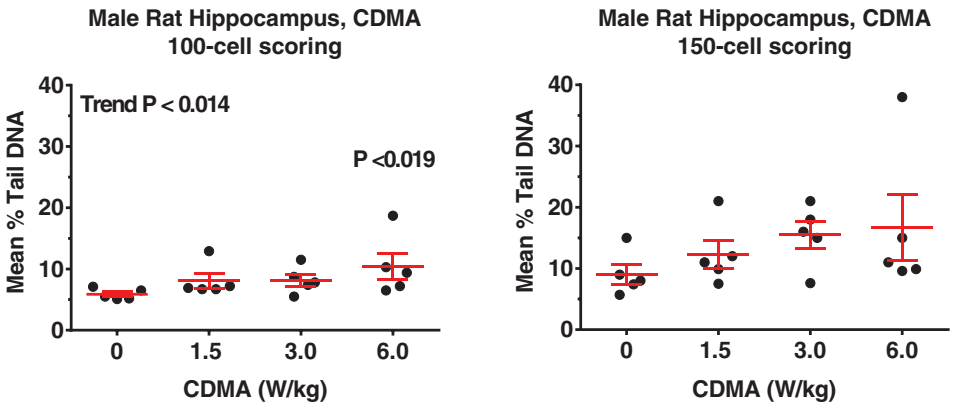

Fig. 1. (A,B) Male rat hippocampus, CDMA, was the only rat tissue judged to be positive in the comet assay when using the 100-cell scoring approach (A). Central horizontal bar indicates mean % tail DNA; upper and lower error bars indicate SE.

et al. 2018). Therefore, we consider it unlikely that thermal effects were a confounding factor for our genetic toxicity tests, although more work in general is needed to clarify the thermal effects of RFR on different tissues, and the degree to which increases in body or tissue temperature affect genomic integrity. Few studies have closely examined the relationship between increased body temperature and induction of DNA damage in mice, and there is almost no information on this relationship in rats. In one study in which the body temperatures of mice were closely monitored, an increase of ~2°C was required before increases in micronuclei were detected (Asanami and Shimono 1997).

Little is known about the mechanism by which RFR could induce DNA damage in the absence of heating. Unlike ionizing radiation or ultraviolet light, the radiation emitted by cell phones is not sufficiently energetic, by several orders of magnitude, to directly damage macromolecules (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2013). Calculations by physicists and engineers suggest that RFR would not have an appreciable effect on biological systems at nonthermal levels of exposure, primarily due to the damping effects of water molecules (Adair 2002; 2003; Sheppard et al. 2008; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2013). However, our results and the results of other experiments suggest that nonthermal exposure of cells or whole organisms to RFR may result in measurable genotoxic effects, despite varied and weak responses across studies overall (Brusick et al. 1998; Ruediger 2009; Verschaeve et al. 2010). Induction of

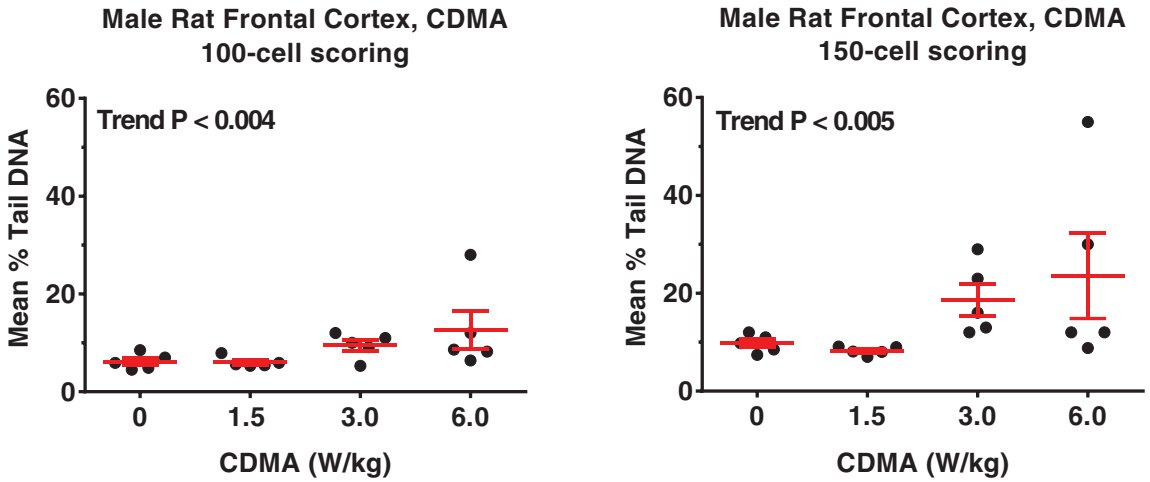

Fig. 2. (A,B) Male rat frontal cortex, CDMA, was judged to be equivocal in the comet assay using the 100-cell scoring approach (A); a similar result was obtained using the 150-cell scoring approach (B). Central horizontal bar indicates mean % tail DNA; upper and lower error bars indicate SE.

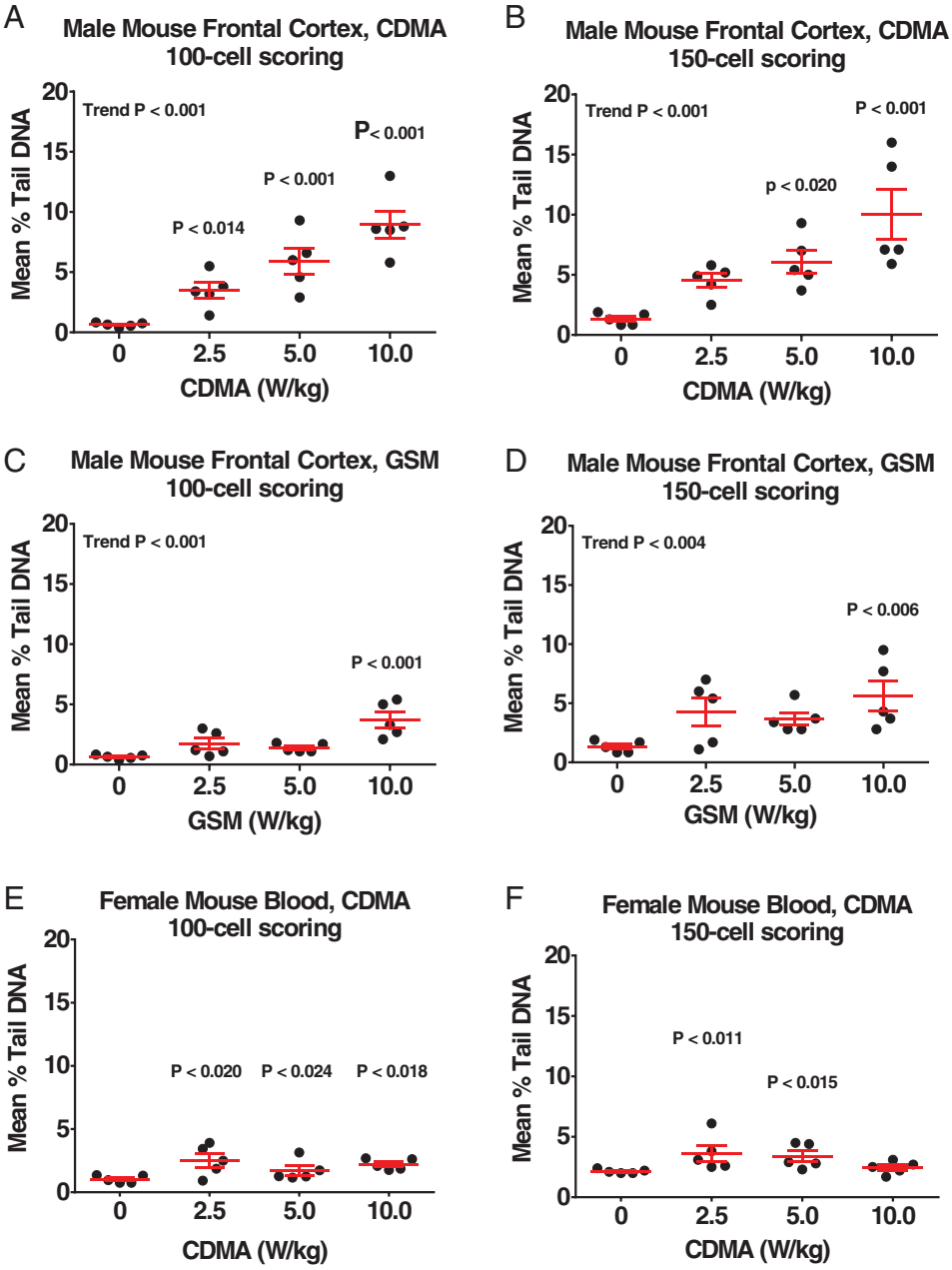

Fig. 3. (A–F) Mouse tissues judged to be positive in the comet assay using the 100-cell scoring approach. Central horizontal bar indicates mean % tail DNA; upper and lower error bars indicate SE.

oxygen radicals or interference with DNA repair processes has been proposed as possible mechanisms by which RFR could cause DNA damage (Ruediger 2009; Yakymenko et al. 2015).

NTP Technical Reports on the results of the 2-year cancer bioassay for exposure to RFR for rats (TR 595) and mice (TR 596) were finalized, peer reviewed, and made publicly available in 2018. The NTP concluded that results demonstrated clear evidence of carcinogenic activity of cell phone RFR (both modulations) based on incidences of malignant schwannomas of the heart in male rats. Malignant gliomas in the brain were also observed in male rats exposed to cell phone RFR and were considered to be related to exposure. Female rats exhibited malignant schwannomas of the heart and malignant gliomas, but incidences of these tumors were considered equivocal. The observation that cell phone RFR affects heart and brain tissue in Sprague Dawley rats after long-term exposure was replicated in a similar study (that used only the GSM modulation) by the Ramazzini Institute (Falcioni

et al. 2018). The gliomas and schwannomas observed in rats are similar to the tumor types reported in some epidemiology studies to be associated with cell phone use. The NTP bioassay findings in mice, in which different organs were affected compared to rats, were considered equivocal. Notably, spontaneous and chemically induced brain tumors are rare in rats (Sills et al. 1999), and as of 2019, only 12 out of approximately 600 test articles have shown evidence of an increase in brain tumor incidence in rats in NTP bioassays.

The U.S. Federal Communications Commission has set a guideline limit for RFR requiring that mobile devices emit an SAR of less than of 1.6 W/kg as measured in a volume containing 1 g of tissue absorbing the signal. In contrast, animals in the NTP studies received whole-body exposure to higher levels of RFR to identify potential target organs and to characterize toxicity. The highest exposure of 6 W/kg in rats and 10 W/kg in mice, for a total of 9 h 10 min a day (achieved by cycling for 10 min on, 10 min off over 18 h 20 min), produced higher exposures than experienced by humans under normal cellular phone use conditions. Thus, whether the findings in the NTP animal studies (eg, malignant gliomas in the brain and malignant schwannomas in the hearts of male rats; increased levels of DNA damage in hippocampal cells of male rats and the frontal cortex of male mice) indicate a potential for adverse health outcomes in humans remains a question. Because one of the most important questions prompted by our results concerns the mechanism(s) by which RFR might induce biological effects, follow-up studies by the NTP to investigate mechanisms of genetic damage associated with RFR exposure are underway.

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AUTHOR CONTRIBUTIONS

K.L.W., M.E.W., G.E.K., R.R.T., and J.R.B. designed the study. S.L.S., G.E.K., K.R.S., and K.L.W. analyzed the data; S.L.S., G.E.K., K.R.S., and K.L.W. prepared the manuscript with input from C.A.H., K.G.S., R.R.T., and J.R.B. K.G.S and A.S.G. collected the tissue samples at IITRI and, together with J.W.W., conducted the genetic toxicity assays under the supervision of C.A.H. G.E.K. and K.R.S. performed statistical analyses of the data. M.E.W., M.D.S., R.R.T., C.A.H., and J.R.B. contributed important intellectual input to the study and to the manuscript, and all authors read and approved the manuscript.

CONFLICT OF INTERESTS

The authors have no competing financial interests.

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Sensitive CometChip assay for screening potentially carcinogenic DNA adducts by trapping DNA repair intermediates

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ABSTRACT

Genotoxicity testing is critical for predicting adverse effects of pharmaceutical, industrial, and environmental chemicals. The alkaline comet assay is an established method for detecting DNA strand breaks, however, the assay does not detect potentially carcinogenic bulky adducts that can arise when metabolic enzymes convert pro-carcinogens into a highly DNA reactive products. To overcome this, we use DNA synthesis inhibitors (hydroxyurea and 1-β-D-arabinofuranosyl cytosine) to trap single strand breaks that are formed during nucleotide excision repair, which primarily removes bulky lesions. In this way, comet-undetectable bulky lesions are converted into comet-detectable single strand breaks. Moreover, we use HepaRG™ cells to recapitulate *in vivo* metabolic capacity, and leverage the CometChip platform (a higher throughput more sensitive comet assay) to create the 'HepaCometChip', enabling the detection of bulky genotoxic lesions that are missed by current genotoxicity screens. The HepaCometChip thus provides a broadly effective approach for detection of bulky DNA adducts.

INTRODUCTION

Injury to genetic material can lead to debilitating heritable diseases, cancer, neurodegeneration and accelerated aging (1–4). Therefore, regulatory agencies worldwide require that all pharmaceuticals be tested for their genotoxic potential (https://www.fda.gov/media/71980/download). In contrast, despite the fact that >2000 new chemicals are being produced by industry every year (https://ntp.niehs.nih.gov/annualreport/2017/2017annualreportdownloadpdf.pdf), the vast majority of these industrial chemicals have not been tested for their genotoxic potential. A major barrier to such testing is the need for a high throughput (HT) sensitive assay for DNA damage in mammalian cells (5). Although there have been recent advances in HT assays for genotoxicity (6), most of these technologies depend on indirect measures of DNA damage, such as phosphorylation of histones [e.g. γH2AX formation (7)] or gene induction [i.e. p53 activation (8,9)]. While there are several methods for direct detection of DNA damage (e.g. alkaline elution

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and mass spectrometry), these assays are laborious and low throughput. The alkaline comet assay is a promising platform as it detects single-strand breaks (SSBs; for a list of abbreviations, see Supplemental Table S1), abasic sites and other alkali sensitive sites. However, the assay has a critical blind spot, due to its inability to detect bulky DNA lesions, a class of lesions that are often carcinogenic (3,10,11). Here, we describe methods to overcome this limitation.

The comet assay

The comet assay is an established method for detecting DNA strand breaks, and is based upon the underlying principle that fragmented DNA migrates more readily through an agarose matrix under electrophoresis compared to intact DNA. The comet assay works because nuclear DNA is normally highly supercoiled and thus does not readily migrate, while loops and fragments migrate more readily through the agarose matrix (12,13). The result is a comet-like shape, where the percent DNA in the comet tail is proportional to the levels of DNA strand breaks.

While the comet assay is relatively simple and sensitive, it is low-throughput, it has poor reproducibility, and the imaging and analysis methods are laborious. To overcome these limitations, the CometChip was previously developed (14,15). The basis for the CometChip is an agarose microwell array. Briefly, cells are loaded into microwells by gravity, and excess cells are removed by shear force (Figure 1). By creating a mammalian cell microarray, overlapping comets are prevented, and the comets lie on a shared focal plane. As a result, it is possible to capture multiple comets (>50) in a single image rather than imaging each comet individually as is done for the traditional comet assay. With automated image analysis and reduced experimental noise, the CometChip provides >1000-fold improvement in throughput, increased robustness and increased sensitivity (14–18).

The comet assay can be performed using either neutral or alkaline conditions. Under alkaline conditions (pH > 13), SSBs release superhelical tension, enabling migration of DNA loops. Alkaline conditions also lead to SSBs at abasic sites and other alkali sensitive sites, which contribute to DNA migration. While broadly useful, alkaline comet conditions suffer from a major shortcoming, which is that the assay can only detect strand breaks that directly impact DNA migration and not base modifications or bulky DNA adducts. This is a significant limitation because many environmental carcinogens cause bulky DNA base adducts (3,10,11). Unrepaired adducts can block replication and transcription, which contributes to cell-cycle arrest, mutations, and cell death (19), ultimately contributing to carcinogenesis (20–24). In fact, high levels of bulky DNA adducts correlate with an increased risk of cancer in humans (25,26). Although the traditional alkaline comet assay does not detect DNA base lesions directly, they can be detected indirectly when acted upon by repair enzymes (27). For example, base excision repair (BER) enzymes remove damaged bases, cleave the backbone, synthesize across the gap, and ligate the DNA. As such, damaged bases lead to SSBs as requisite DNA repair intermediates, and these intermediates can be detected using the comet assay. Importantly, enzymes in the BER pathway can act independently, such that the subsequent SSB resolution steps are rate-limiting once the damaged base has been removed (28–30), leading to an accumulation of SSB intermediates that are detectable using the comet assay (27).

While many base lesions are repaired by BER, bulky lesions are repaired primarily by nucleotide excision repair (NER) (10,11,31,32) (Figure 2A). The NER pathway is highly coordinated in both prokaryotes and eukaryotes (31,33). In eukaryotes, the NER pathway operates by assembling more than a dozen different proteins prior to commencing repair (34). As such, once repair is initiated, the process is extremely efficient, thus minimizing the presence of SSB intermediates (35). Specifically, NER involves two major steps: endonucleolytic cleavage 5′ to the adduct, repair synthesis and endonucleolytic cleavage 3′ to the adduct (11,35), which together result in removal of an oligonucleotide containing the offending lesion. The incision 5′ to the damage site is made by the ERCC1-XPF endonuclease and is followed by recruitment of DNA polymerases and initiation of DNA repair synthesis. Repair synthesis creates a flap 3′ to the original lesion, which is then cleaved by the structure-specific endonuclease XPG, prior to completion of repair synthesis and ligation (36,37). NER SSB intermediates can be detected using the alkaline comet assay (38–40), but the required preassembly of the repair complex means that the signal is very weak due to the ephemeral nature of the NER SSB intermediates.

Detecting bulky lesions using CometChip

Here, we exploit methods for inhibiting repair synthesis as a means for prolonging the presence of NER SSB intermediates. Specifically, our approach is to use hydroxyurea (HU) and 1-β-D-arabinofuranosyl cytosine (AraC), which inhibit NER (41–44). HU depletes the deoxyribonucleotide triphosphate (dNTP) pool by inhibiting the activity of ribonucleotide reductase (45–49). AraC is a deoxycytidine structural analog (50), which can be incorporated into DNA (51–54), inhibiting DNA elongation by DNA polymerases and causing early chain termination (51,55–58). Despite the potential utility of HU/AraC as a means for making the comet assay more sensitive (39,59–64), little has been done to leverage and/or validate the utility of this approach. Developing an effective CometChip-based assay for detecting bulky lesions has the potential to give rise to a valuable tool for rapid detection of carcinogenic bulky lesions. Therefore, leveraging the high-throughput nature of CometChip, we set out to develop a rapid and sensitive assay for bulky lesions by using HU/AraC to trap NER repair intermediates and reveal SSBs generated during NER.

While bulky lesions can be created by direct chemical reactions with DNA, some chemicals lead indirectly to the creation of bulky lesions. For example, polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (B[a]P) and aflatoxin B_1 (AFB_1), do not react with DNA unless they are rendered reactive by metabolic enzymes (a.k.a. metabolic activation). In the human body, foreign substances (xenobiotics) are extensively metabolized, mainly by hepatocytes in the liver (65). Metabolism can convert a lipophilic molecule into a soluble molecule, thus aiding in its excretion. In some

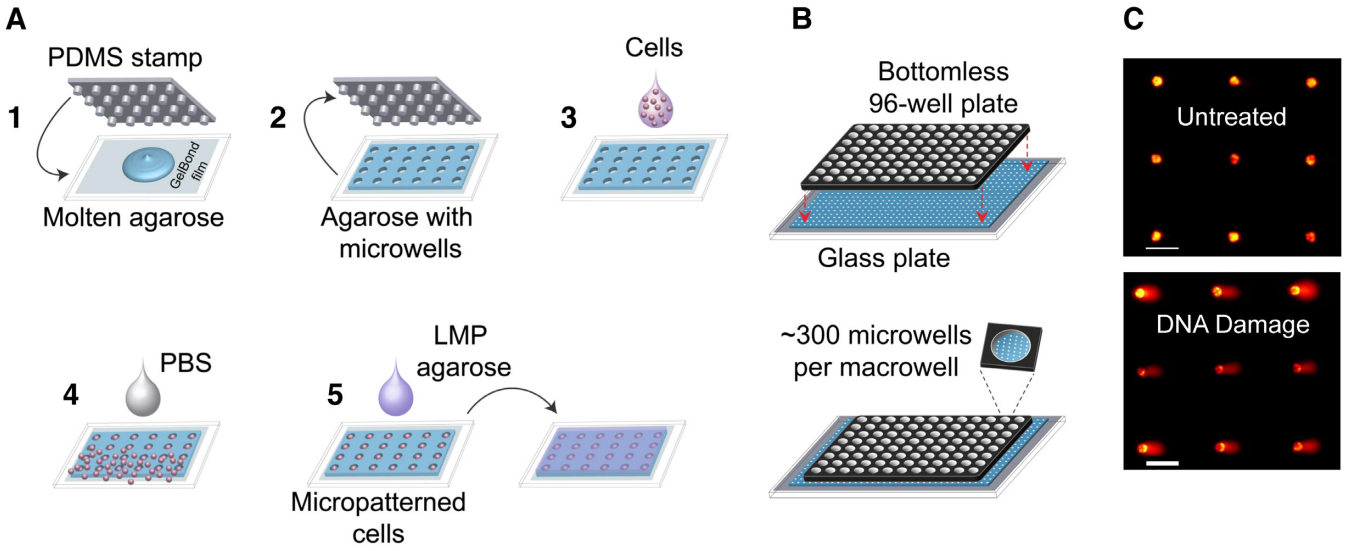

Figure 1. CometChip for high-throughput assessment of DNA damage. (**A**) CometChip fabrication. 1) A PDMS stamp with an array of micropegs is pressed into molten agarose. 2) Once the agarose gelates, the stamp is lifted to reveal an array of microwells (~40–50 μm in both diameter and depth, spaced 240 μm from each other). 3) Cells in suspension are loaded directly into microwells via gravity. 4) Excess cells are washed off by shear force, revealing an array of micropatterned cells. 5) Low-melting point (LMP) agarose kept molten at ~37°C is placed on top of the micropatterned cells and allowed to gelate by a brief incubation at 4°C (~2 min). (**B**) Macrowells are formed by clamping a bottomless 96-well plate on top of a microwell array. The bottom surface of each macrowell contains ~300 microwells. Macrowells can be used both to load multiple cell types at the same time and to perform parallel treatments. (**C**) Example fluorescent images of comets on alkaline CometChip. Images were taken at 4X magnification. Each image can capture ~60–100 comet images. Upper: untreated TK6 cells yield comets with little to no tail. Lower: comets from TK6 cells treated with a high dose of a DNA damaging agent (50 μM H_2O_2) have visibly large tails. Scale bars = 100 μm.

cases, metabolism can lead to formation of highly reactive and toxic intermediates. In fact, liver toxicity is a major problem in drug development and for public health. Drug-induced liver injury (DILI) remains a common cause for drug withdrawal from the market and is the most common cause of acute liver failure and death. In addition, the liver remains the most frequent target organ in rodents for >500 environmental chemicals tested as part of the EPA Integrated Risk Information System (IRIS) (www.epa.gov/iris) (27). Therefore, in order for chemical toxicity assessment to be physiologically relevant, it is essential to have a testing system that can provide biologically relevant levels of xenobiotic metabolism.

The biotransformation process of xenobiotics includes oxidation/reduction of parent chemicals, increasing their hydrophilicity by adding polar groups (such as hydroxyl or amine) and endogenous polar compounds, making them more soluble and thus more easily cleared via the bloodstream (65,66). The cytochrome P450 enzymes (CYP450s), or microsomal mixed-function mono-oxygenases localized to the endoplasmic reticulum, account for ~75% of all phase I enzymes (66) and are involved in ~95% of oxidative biotransformation (65). Phase I enzyme biotransformation creates functional groups that are then substrates for conjugation to water soluble molecules (e.g. glucuronic acid, sulfate or the tripeptide glutathione) by Phase II enzymes, greatly increasing the polarity of the metabolites from phase I and suitable for excretion (65). Given that oxidation products of CYP450s can become DNA reactive, assessment of chemical genotoxicity needs to take into consideration the genotoxic potential of both the parent chemicals and their metabolites. As an example, the DNA damaging effects of B[a]P, a major public health hazard that may lead to hundreds of thousands of cancer cases each year, would be entirely missed in laboratory tests were it not for metabolic activation. HepaRG cells can undergo extensive differentiation, exhibiting hepatocyte-like morphology as well as displaying substantial liver-specific functions. Here, we have developed the HepaCometChip, an enhanced CometChip (15) platform for genotoxicity screening, by incorporating the use of two DNA repair synthesis inhibitors, HU and AraC (to enable persistence of NER SSB intermediates) and HepaRG™ cells (to enable metabolic activation).

The carcinogens AFB_1 and B[a]P are used as model DNA damaging agents that require metabolic activation to be reactive with DNA. The mycotoxin AFB_1 is produced by the molds *Aspergillus flavus* and *A. parasiticus*, which are frequent contaminants in peanuts and maize in certain regions of the world (67). Several CYP450 enzymes (68), such as CYP3A4 and CYP1A2, are known to oxidize AFB_1, producing a number of metabolites (69–72). The highly mutagenic metabolite, AFB_1-exo-8,9-epoxide, readily reacts with the N7 position of guanine to form DNA adducts. The most ubiquitous are AFB_1-N7-dG and AFB_1-Fapy-dG (21,22). In addition to AFB_1, we also elected to study B[a]P, another human carcinogen (http://monographs.iarc.fr/ENG/Classification/latest_classif.php). Common routes of exposure include breathing in fuel exhaust, cigarette smoke, and burning wood smoke, or consuming charred meat or other types of charred food (24). B[a]P, like many other PAHs, is an inducer of the CYP1 family (CYP1A1, CYP1A2, and CYP1B1) (73). B[a]P is also metabolically activated by the CYP1 family. The most genotoxic metabolite is the diolepoxide (+)-anti-B[a]*P*-7,8-diol-9,10-epoxide (BPDE),

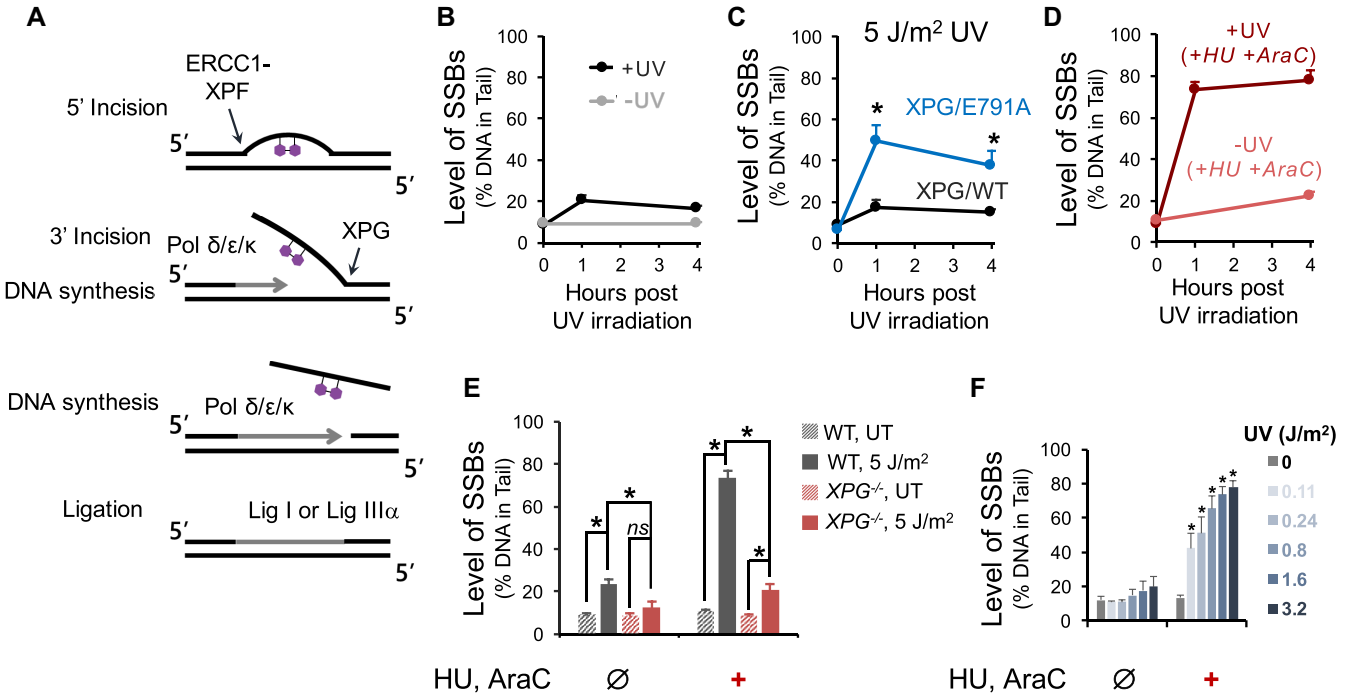

Figure 2. Analysis of SSBs by the alkaline CometChip as a measure of UV-induced lesions. Cells were pre-incubated with 10 mM GSH for 40 min at 37°C before UV irradiation and kept in the presence of 10 mM GSH for subsequent incubation following UV exposure. (**A**) Simplified schematic of NER of a UV-induced pyrimidine dimer. (**B**) Comparison of SSB levels in human skin fibroblast cell line (XPG/WT) between untreated cells (−UV) and cells irradiated with 5 J/m^2 UV-C (+UV). (**C**) SSB levels in XPG/WT and XPG/E791A cells up to four hours following 5 J/m^2 UV-C exposure. *$P < 0.05$, two-way ANOVA with post hoc analysis by Bonferroni test (between XPG/WT and XPG/E791A at each time point). (**D**) SSBs in XPG/WT cells incubated with DNA repair synthesis inhibitors, HU and AraC. Cells were pre-incubated with 1 mM HU, and 10 μM AraC for 40 min at 37°C, irradiated with 5 J/m^2 UV-C (dark red line), and then incubated with the same HU and AraC concentrations for up to 4 h after exposure. Untreated control cells were kept in the same HU and AraC conditions (light red line). (**E**) Contribution of NER SSB intermediates to detected SSBs. XPG/WT and XPG-deficient cells were exposed to 5 J/m^2 UV-C and allowed to repair for one hour following irradiation. Cells were either incubated with the repair synthesis inhibitors (1 mM HU, 10 μM AraC) for 40 min prior to UV irradiation and one hour of repair after exposure (+) or were incubated in regular medium without the inhibitors (Ø). ns: not statistically significant, *$P < 0.05$ (Student's *t*-test, two-tailed, paired). (**F**) HU/AraC approach reveals dose-response to UV exposure. TK6 cells were irradiated with indicated doses of UV-C and analyzed for SSBs 1 h following exposure. Cells were either incubated with the repair synthesis inhibitors (1 mM HU, 10 μM AraC) for 40 min prior to UV irradiation and 1 h of repair after exposure (+) or were incubated in regular medium without the inhibitors (Ø). *$P < 0.05$, one-way ANOVA with post hoc analysis by Dunnett's multiple comparison test (between each UV dose and the untreated control). $n \geq 3$. Error bars are standard error of the mean.

which covalently binds to the exocyclic N^2 of guanine. Both AFB_1-N7-dG and BPDE-DNA lesions highly distort the double helix and are excised by NER (24,74).

In this study, we report the development of the HepaCometChip, a HT screening platform that is highly effective for detecting repair intermediates of bulky lesions. We leveraged the existing high-throughput CometChip platform (15,17) and knowledge of how bulky lesions are normally repaired, in order to trap repair intermediates and use these intermediates as indicators of the presence of bulky lesions (10,11,32). In addition, we exploited metabolically competent human hepatic cells to account for metabolic processes that can convert non-reactive molecules into DNA-reactive molecules that form bulky lesions (65,66). Further, using specific CYP450 inhibitors, we validated that the detected SSBs are primarily dependent upon metabolic activation of B[a]P and AFB_1. We also showed that inhibition of NER initiation results in a reduction in SSB levels, indicating that NER intermediates contribute significantly to SSBs detected using the HepaCometChip. Furthermore, we demonstrated that the platform has superior sensitivity compared to the conventional alkaline comet procedure by performing a small screen of nine known *in vivo* genotoxic agents. Taken together, we have leveraged the HT advantage of CometChip, the enhanced comet sensitivity in the presence of HU/AraC, and the metabolic capacity of HepaRG™ cells to develop the HepaCometChip screening platform for single strand breaks induced by the metabolism of xenobiotics. This platform provides an unprecedented rapid and sensitive tool to help overcome vital limitations in current genotoxicity testing performed by regulatory agencies, public health researchers, and pharmaceutical companies.

MATERIALS AND METHODS

Chemicals

Sodium bicarbonate solution (7.5% $NaHCO_3$, S8761), dimethyl sulfoxide (DMSO, D2650), reduced L-glutathione (GSH, G6013), hydroxyurea (HU, H8627) and cytosine arabinoside (AraC, C1768) were obtained from Millipore-Sigma, St Louis, MO. GSH solution (10 mM) was prepared by dissolving GSH powder in warm culture medium and used within 30 min of preparation. Stock solutions of 1000× HU (1 M) and 1000× AraC (10 mM) were prepared

by dissolving crystal HU and AraC in cell culture grade water and stored at −20°C. Other chemicals (Supplementary Table S2) were purchased in powder form from Millipore-Sigma, St Louis, MO and dissolved to prepare stock solutions that were stored at −20°C.

Cell culture

Dulbecco's phosphate-buffered saline (PBS), high-glucose Dulbecco's modified Eagle's medium (DMEM, high glucose, 11965092), RMPI-1640 with GlutaMAX™ (61870), 200 mM L-glutamine (A2916801), 10 000 U/ml Pen-Strep (15140), 100× GlutaMAX™ supplement (35050061), William's E Medium (WEM, A1217601), HepaRG™ Thaw, Plate, & General Purpose Medium Supplement (HPRG670), HepaRG™ Maintenance/Metabolism Medium Supplement (HPRG620), 0.25% Trypsin-EDTA with phenol red (25200), and 96-well plate coated with collagen I (A1142803) were purchased from ThermoFisher Scientific, Waltham, MA. Fetal bovine serum was obtained from Atlanta Biologicals, Inc., Flowery Branch, GA.

All cells were cultured in an incubator set at 37°C with 5% CO_2. TK6 (75,76), a human B-lymphoblastoid cell line, was a gift from W.G. Thilly (Department of Biological Engineering, Massachusetts Institute of Technology). TK6 cells were cultured in RPMI 1640 medium with GlutaMAX™ supplemented with 100 U/ml Pen-Strep. The XPG cell lines were gifts from O.D. Scharer (Institute for Basic Science, Center for Genomic Integrity, Ulsan, Korea). These include XPG-deficient, XPG/WT, and XPG/E791A. The XPG-deficient cell line was obtained from SV40-transformation of the primary human skin fibroblasts from patient XPCS1RO (77). XPG/WT and XPG/E791A cells were obtained from the stable transfection of the lentiviral vector containing XPG WT cDNA or XPG-E791A cDNA in the XPG-deficient cell line (35). The XPG cell lines were cultured as previously described (35,77).

HepG2 (ATCC® HB-8065™), an immortalized cell line derived from human hepatocellular carcinoma, was obtained from the American Type Culture Collection (Manassas, VA). HepG2 cells were cultured in high-glucose DMEM supplemented with 10% FBS, 1× GlutaMAX™, and 100 U/ml Pen-Strep. For chemical exposures, exponentially growing cells were plated in a tissue cultured treated 96-well plate two days before treatment.

Cryopreserved HepaRG™ (HPRGC10), a terminally differentiated hepatic cell line, was purchased from ThermoFisher Scientific (Waltham, MA). HepaRG™ was thawed and cultured according to manufacturer's instructions. Briefly, the general purpose working medium was WEM supplemented with 1X GlutaMAX™ and 1X HepaRG™ Thaw, Plate, & General Purpose Medium Supplement. The metabolism working medium was WEM supplemented with 1X GlutaMAX™ and HepaRG™ Maintenance/Metabolism Medium Supplement. HepaRG™ cells were thawed in the general purpose working medium and plated in a 96-well plate coated with collagen I at 100 000 cells/well. One day after plating, the general purpose working medium was changed to the metabolism working medium. The metabolism working medium was renewed on day 4 and day 6 after plating. On day 7, the cells were treated in the metabolism working medium.

To obtain a cell suspension for the XPG cell lines, HepG2 and HepaRG, the monolayer culture was incubated with 0.25% Trypsin–EDTA at 37°C. For XPG cell lines, the incubation time was 1–2 min. For HepG2 and HepaRG, the incubation time was 5–10 min. Detached cells were then suspended in complete working media. Cell viability and cell number were analyzed using an automated Trypan Blue exclusion system [Vi-CELL™ cell counter (Beckman Coulter Life Sciences, Brea, CA, USA)].

CometChip fabrication

Sylgar™ 184 silicone elastomer kit (102092-312) and bottomless 96-well plates (82050-714) were purchased from VWR, Radnor, PA. GelBond® Film (53761) was obtained from Lonza, Portsmouth, NH. UltraPure™ agarose (16500100) and UltraPure™ low melting point agarose (16520100) were purchased from ThermoFisher Scientific, Waltham, MA. The microwells were fabricated as described previously (14–16,78). Briefly, 1% (w/v) agarose solution in PBS was prepared. A polydimethylsiloxane (PDMS) stamp with an array of micropegs was fabricated using the Sylgar™ 184 kit as described previously (15). The stamp was pressed into the molten agarose solution on top of the hydrophilic side of a sheet of GelBond® film. The agarose was allowed to gelate at room temperature for ∼15 min. The stamp was removed to reveal an array of microwells with ∼40–50 μm in both diameter and depth. The microwells were spaced ∼240 μm apart. A bottomless 96-well plate was pressed on top of the agarose chip to form 96 macrowells, each with an array of ∼300 microwells at its base.

To load cells into microwells, ∼2000–200 000 cells in suspension were placed into each macrowell, and the chip was incubated at 37°C in the presence of 5% CO_2 for 15 min. Cell were loaded into microwells by gravity, and excess cells were then washed off with PBS by shear force. The chip was covered with a layer of overlay agarose (1% w/v low-melting point agarose solution in PBS, kept molten at 43°C until use). For complete gelation of the overlay agarose, the chip was kept at room temperature for two minutes followed by 2 min at 4°C.

Trypan Blue exclusion test for cell viability

HepaRG™ cell viability was determined using an automated Trypan Blue exclusion system [Vi-CELL™ cell counter (Beckman Coulter Life Sciences, Brea, CA, USA)]. HepaRG™ cells were incubated with various doses of HU/AraC in triplicates for 24 h at 37°C in the presence of 5% CO_2. A vehicle control (1% DMSO) was included. The number of viable cells was recorded for each dose of HU/AraC and % control viability calculated.

Alkaline comet assay

The alkaline comet assay was performed as previously (15,79). Sodium chloride (NaCl, 7581), disodium EDTA (Na_2EDTA, 4931), and sodium hydroxide pellets (NaOH, 7708) were purchased from VWR, Radnor, PA. Trizma®

base (T1503), Trizma® HCl (T5941) and Triton X-100 (X-100) were obtained from MilliporeSigma, St. Louis, MO. 10 000× SYBR™ Gold nucleic acid gel stain was obtained from ThermoFisher Scientific, Waltham, MA.

The alkaline lysis buffer (pH ∼ 10) was a solution of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma® base, and 1% (v/v) Triton X-100 dissolved in deionized H_2O (dI H_2O). The alkaline unwinding buffer (pH ∼ 13.5) was prepared by diluting NaOH and Na_2EDTA stock solutions in dI H_2O to final concentrations of 0.3 M and 1 mM, respectively. The neutralization buffer (pH ∼ 7.5) was prepared by dissolving Trizma® HCl in distilled H_2O to a final concentration of 0.4 M.

Cells encapsulated in CometChip were lysed in the alkaline lysis buffer overnight at 4°C. The nuclei were unwound in the alkaline unwinding buffer for 40 min at 4°C, and the DNA was electrophoresed in the same buffer at the same temperature for 30 min at 1 V/cm and ∼300 mA. The CometChip was then washed three times in neutralization buffer by submerging for 5 min each time.

The DNA on CometChip was stained for 15 min at room temperature with 1× of SYBR™ Gold diluted in PBS, protected from light. Fluorescent images of the comets were captured at 40× magnification using an epifluorescence microscope (Nikon Eclipse 80i, Nikon Instruments, Inc., Melville, NY, USA) with a 480 nm excitation filter. Image acquisition was achieved by automatic scanning using a motorized XY stage. Comet images were automatically analyzed using Guicometanalyzer, a custom software developed in MATLAB (The MathWorks Inc., Natick, MA, USA) as previously described (15). For each condition, 100 comets or more were analysed. Outputs from Guicometanalyzer were processed and imported to a spreadsheet (Microsoft Excel, Microsoft Office Suite 2016) using Comet2Excel, an in-house software developed in Python (Python Software Foundation, Python version 2.7.10). Software is freely available upon request.

Liver perfusion and hepatocyte culture on CometChip. Gibco® Antibiotic-Antimycotic (15240, 100×) was purchased from ThermoFisher Scientific, Waltham, MA. Insulin-transferrin-sodium selenite supplement (ITS) (11074547001), aprotinin (A3428), HEPES (H4034), dexamethasone (D4902), and Percoll® (P4937) were obtained from MilliporeSigma, St. Louis, MO. Isolation medium was WEM supplemented with 1X GlutaMAX™, 1× Gibco® Antibioti-Antimycotic, 10 μg/ml IST, 1 μg/ml aprotinin, 10 mM HEPES, 0.1 μM dexamethasone and 10% FBS. Maintenance medium was the same as isolation medium, but without 10% FBS.

Primary mouse hepatocytes were obtained from 10 to 14 weeks old C57Bl6 mice using a standard two-step collagenase liver perfusion procedure with minor changes (80,81). The isolated cells were suspended in the isolation medium and were enriched for viable hepatocytes by centrifugation using a 45% Percoll® solution. Cell viability and cell number were analyzed using an automated Trypan Blue exclusion system [Vi-CELL™ cell counter (Beckman Coulter Life Sciences, Brea, CA)]. The perfusion procedure yielded ∼20–50 million cells per liver and ∼80–90% cell viability.

Microwell array in agarose chip was fabricated as described above with some changes. Specifically, the 1% w/v agarose solution and the overlay agarose solution were supplemented with 2× Gibco® Antibiotic–Antimycotic. Hepatocytes in suspension were loaded into microwells by incubating at 37°C for a maximum of 10 minutes. After the agarose overlay step, cells were incubated in isolation medium (50 μl/macrowell) at 37°C in the presence of 5% CO_2. After 4 h, isolation medium was exchanged for maintenance medium, and cells were incubated overnight at 37°C in the presence of 5% CO_2. After the overnight incubation, chemical treatments were performed in the maintenance medium (50 μl/macrowell).

HU/AraC treatments

HepaRG™ were treated with varying concentrations of HU/AraC in a 96-well plate for 24 h at 37°C in the presence of 5% CO_2. The treated cells were embedded onto a CometChip and the level of damage induced by HU/AraC analyzed by Alkaline comet assay. For this experiment, the comets were analysed using Trevigen® Comet Analysis Software.

Ultraviolet (UV) light exposure. Prior to UV irradiation, cells embedded in CometChip were incubated for 40 min at 37°C in working medium supplemented with 10 mM glutathione. Exposure to 254 nm UV light radiation (UVC) was administered via a handheld UV lamp that had a dose-rate of 14 J/m^2/s at a distance of 7.6 cm (UVP 95001614, ThermoFisher Scientific, Waltham, MA, USA). The UV irradiation procedure was carried out in the dark at 4°C.

HU/AraC approach to query UV-induced bulky adducts. A combination of 1 mM HU and 10 μM AraC was used to inhibit NER repair synthesis. Before UV exposure, cells were pre-treated with HU/AraC prepared in working medium supplemented with 10 mM GSH for 40 min at 37°C. Following UV exposure, cells were incubated in working medium supplemented with 10 mM GSH and HU/AraC for 1 h and 4 h at 37°C, and analyzed by alkaline comet.

AFB_1 and B[a]P treatments

For each dose of the test compound, a 200× solution was prepared by diluting the stock solution (4 mM AFB_1 or 20 mM B[a]P) in DMSO. A vehicle control condition was included by diluting DMSO in cell culture medium to get a final concentration of 0.5%. Cells were incubated with the test compound for 24 h at 37°C in the presence of 5% CO_2. To reveal the level of DNA damage induced by the test compound, cells were also exposed to HU/AraC.

HU/AraC approach in chemical screen of nine known genotoxins. HepaRG™ were incubated with the test compound for 24 h at 37°C in the presence of 5% CO_2. A vehicle control (1% DMSO) was included.

HU/AraC approach to test for genotoxicity of artesunate. HepG2 were incubated with the test compound for 24 h at 37°C in the presence of 5% CO_2. A vehicle control (0.07% sodium bicarbonate) was included.

Inhibition of AFB_1 or B[a]P metabolic activation

5 μM KET or 25 μM ANF was added to culture medium at the start of the AFB_1 or B[a]P treatment. The remaining steps were similar to the AFB_1 and B[a]P treatments.

Gamma radiation

Cells embedded in CometChip were placed on ice and irradiated with 0, 0.9, 1.8, 3.6, 5.4, 7.2 and 9 Gy of gamma rays from a Cs 137 radiation source with a dose rate of 0.9 Gy/min. The irradiated cells were submerged into lysis buffer overnight and the alkaline CometChip assay performed as described above. Standard curves with the numbers of SSBs induced at each radiation dose versus the change in % Tail DNA were generated and the slopes of the curves (SSBs induced/change in % tail DNA) used to estimate the number of SSBs induced at the highest concentrations of B[a]P and AFB_1 for in each cell line.

CellTiter-Glo® assay (CTG®) for cell viability

The CTG® luminescent cell viability assay kit (G7570) was obtained from Promega, Madison, WI. Cell viability after 24 h of chemical treatment was measured according to the manufacturer's instructions. Luminescent signals were recorded using a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA, USA) at room temperature. The emission bracket was from 360 to 750 nm. Control wells with no cells were included to obtain background luminescence, which was then subtracted from the signal measured in the sample wells.

CYP450-Glo™ assays for CYP3A4 and CYP1A2 activity

The CYP450-Glo™ CYP3A4 assay with luciferin-IPA (V9001) and the CYP450-Glo™ CYP1A2 assay with luciferin-1A2 (V8421) were purchased from Promega, Madison, WI. The activity levels of CYP3A4 and CYP1A2 in cells were measured according to the manufacturer's protocol for non-lytic cell-based assays. Luminescent signals were recorded using a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA, USA). Control wells with no cells were included to obtain background luminescence. The net signal for each sample was obtained by subtracting the background luminescence value.

CYP3A4 or CYP1A2 activity per cell can be obtained by normalizing CYP450-Glo™ values with cell numbers. After a sample was analyzed for CYP3A4 or CYP1A2 activity, the CTG® assay was performed to obtain an estimate of the number of viable cells in the sample. The result from CYP450-Glo™ was then divided by the CTG® value to obtain the average CYP3A4 or CYP1A2 activity per cell.

RESULTS

Application of HU/AraC for sensitive detection of bulky DNA adducts using the comet assay

Many environmental carcinogens, such as ultraviolet light, PAHs, and heterocyclic amines, induce DNA lesions that are considered to be 'bulky' (10,11). Bulky adducts thermodynamically destabilize the double helix, which helps to enable NER recognition (11). After the NER machinery has been assembled at the site of the DNA lesion, incisions are made to excise a 22–30 nucleotide fragment containing the lesion (32). As a consequence, SSBs are requisite intermediates of the pathway, and these intermediates are, in principle, detectable using the alkaline comet assay. However, it has been reported that the frequency of NER associated SSBs is relatively low, even for high UV doses (82). Poor detection of NER intermediates is consistent with SSBs being transient and thus difficult to detect with the alkaline comet assay. A major goal was therefore to render SSBs longer-lived, making them easier to detect, thus enabling their use as a metric for the presence of bulky lesions.

To study NER-induced SSBs, we first performed the traditional alkaline comet procedure using CometChip (15,16,78) (Figure 1). In order to investigate the sensitivity of the CometChip to detect NER-induced SSBs at sites of bulky DNA lesions, we exposed immortalized human fibroblasts to UV-C, which induces mostly CPDs and 6–4PPs (32), known substrates of NER (Figure 2A). After exposure to UV-C, cells were allowed to repair for up to 4 h. As shown in Figure 2B, the level of DNA SSBs analyzed by the alkaline comet assay increases slightly following UV exposure, reaching a maximum of ∼20% tail DNA after 1 h. This level of damage is only marginally above the basal damage level (∼10% tail DNA) and well below the alkaline comet assay's saturation level (∼75% tail DNA), indicating that the assay is relatively insensitive to bulky adducts.

The levels of SSBs are a function of both break generation (incision), gap filling (synthesis) and ligation. Therefore, inhibiting gap filling or ligation can theoretically increase the level of SSBs, thereby improving the assay's sensitivity. To test the possibility that inhibition of ligation increases sensitivity of the assay, we exploited XPG/E791A mutant cells, for which NER intermediates are predicted to persist. Specifically, the E791A mutation renders the XPG enzyme catalytically inactive, but still allows assembly of the NER machinery (35,83). Because the 5′ incision by ERCC1-XPF is not affected (35), XPG/E791A cells are able to generate NER SSB intermediates. However, XPG-E791A is not able to cleave 3′ to the damage site, leading to a 5′ overhang and preventing ligation (35). Consistent with this model, we observed a significant increase in the level of SSBs following exposure to UV (Figure 2C), agreeing with previous studies (35,83,84).

Given that inhibition of NER completion via catalytic inactivation of XPG leads to increased NER intermediates, it follows that the sensitivity of the assay should similarly be increased via inhibition of the gap filling that precedes ligation. In fact, classic SSB detection methods, such as the alkaline sucrose sedimentation technique (43,64) and alkaline elution assay (85), employ the use of DNA repair synthesis inhibitors to increase the levels of SSBs resulting from initiation of NER at sites of UV-induced DNA damage. We adapted this technique by employing a combination of the DNA replication inhibitors HU and AraC for the alkaline CometChip assay, similar to previously described studies (38–40,86,87). We performed a Trypan Blue exclusion test to determine HU/AraC toxicity, and a Comet assay to de-

termine level of DNA damage induced by HU/AraC. We chose a concentration of 1 mM HU and 10 μM AraC, for which there is at least 80% survival and no statistically significant change in DNA damage in HepaRG™ cells (Supplemental Figure S1). In the presence of HU/AraC, the level of accumulated SSBs reaches a steady state of ∼73–78% tail DNA after 1 h following UV exposure (Figure 2D), which is approximately four times higher than the control condition. Since ∼75% tail DNA is approximately the alkaline comet assay's saturation limit, it is possible that an even higher number of SSBs are generated.

NER intermediates contribute to UV-induced SSBs detected by HU/AraC

To formally test the hypothesis that HU/AraC leads to accumulation of NER-driven SSBs, we evaluated the level of UV-induced SSBs in cells that are not able to perform NER. Complete lack of XPG prevents formation of the pre-incision complex, thus preventing incision (35). For WT cells, UV alone leads to a slight increase in SSBs as expected (Figure 2E). This level is significantly reduced in the absence of XPG. In the presence of HU/AraC, the level of SSBs is greatly increased in WT cells. In contrast, cells completely lacking XPG show only a slight increase in SSBs (Figure 2E), demonstrating that almost all of the SSBs detected using HU/AraC are NER intermediates.

To address the generalizability of the approach, we performed a dose-response experiment. TK6 cells were irradiated with various doses of UV-C and allowed to repair in the presence of HU/AraC for one hour after UV exposure (to maximize SSB accumulation). While control cells treated without HU/AraC show a trend but no significant increase in SSBs, cells treated with HU/AraC show a strong increase in SSBs in a dose-dependent fashion (Figure 2F). Furthermore, given that UV is a direct acting DNA damaging agent, cells with and without the ability to undergo metabolic activation are anticipated to respond similarly to UV, which is indeed the case (Supplemental Figure S2).

Application of HU/AraC to detect DNA damage induced by metabolic activation of AFB_1 and B[a]P

Given the importance of metabolism for converting non-genotoxic compounds into genotoxic agents, we set out to create a CometChip procedure that is compatible with endogenous biotransformation to form reactive metabolites. To study DNA damage in physiologically relevant metabolic conditions, we developed the HepaCometChip by combining the HU/AraC approach with metabolically competent human cells. We tested the efficacy of the method by treating HepaRG™ and HepG2 with the carcinogens AFB_1 and B[a]P, both of which are known to become DNA reactive upon metabolic activation by CYP450s and to form bulky adducts recognized by NER. To control for metabolic activation, we included a negative control cell line, TK6, which does not express CYP450s (88).

We first looked at the levels of SSBs induced by AFB_1. In HepaRG™ cells, there is a strong dose-response relationship to AFB_1 treatment only in the presence of HU/AraC (Figure 3A). In contrast, the metabolically incompetent TK6 cells display no response to AFB_1 treatment (Figure 3A), supporting the role of metabolic activation in SSB formation. It should be noted that HU/AraC leads to a slight increase in SSBs in the absence of AFB_1, which is likely due to detection of spontaneous repair. Next, we looked at HepG2 cells, which are commonly used for studies that require metabolic activation. We found that in the presence of HU/AraC, there is a relatively small increase in SSBs induced by AFB_1 compared to the dose-response observed in HepaRG™ cells. This result is consistent with the fact that HepG2 cells are known to express CYP450s at a much lower level compared to HepaRG™ cells (89–100). In fact, we measured the activity levels of two enzymes essential in metabolic activation of AFB_1, CYP3A4 and CYP1A2, and found that HepG2 exhibits much lower levels of both of these CYP450s. Specifically, HepG2 shows >100-fold lower CYP3A4 activity (Supplemental Figure S3) and >10-fold lower CYP1A2 activity compared to HepaRG™ cells (Supplemental Figure S4).

Similar to AFB_1 treatment, B[a]P also does not induce SSBs in the metabolically incompetent TK6 cells (88). In contrast, in the presence of HU/AraC, there is a dose-dependent increase of SSBs in both HepaRG™ and HepG2 cells (Figure 3B). These results are consistent with the fact that both of these cell lines are known to have an inducible CYP1 system, supporting the role of metabolic activation in B[a]P-induced DNA damage. In fact, the activity level of CYP1A2, one of the key metabolic enzymes of B[a]P, is induced in a dose-dependent manner by B[a]P in both HepaRG™ and HepG2 cells (Supplemental Figure S4). Importantly, HepaRG™ cells display overall higher levels of SSBs compared to HepG2 (Figure 3B) consistent with the observation that HepaRG™ cells express >10-fold higher CYP1A2 activity level compared to HepG2 cells (Supplemental Figure S4).

Calibration results using gamma radiation

To provide more quantitative estimates for the number of SSBs (bulky adduct repair intermediates) detected by the alkaline CometChip assay, we performed a dose-response experiment using gamma radiation for which the strand breaks induced per Gy are known in order to obtain a standard curve for each cell line. TK6, HepG2 and HepaRG™ cells were irradiated with various doses of gamma radiation, and the levels of SSBs were assessed using the alkaline CometChip. We observed a dose-dependent increase in % Tail DNA (indicative of SSBs) in each cell line (Supplemental Figure S5A). Note that the dose response curve is distinct for each cell line, indicating that the same number of SSBs result in different levels of %tail DNA in different cell types. We then estimated the number of SSBs/cell induced by the highest concentrations of B[a]P and AFB_1 in each cell line using calibration curves from the radiation data. To generate calibration curves, we first converted the radiation doses into the number of SSBs (induced by radiation) using the estimation that 1Gy of gamma radiation induces 1000 SSBs (101). Next, we calculated the induced % Tail DNA by correcting the background (non-radiated cells) in all the radiation treated results. The slope of the standard curve (SSBs induced/change in % Tail DNA) was

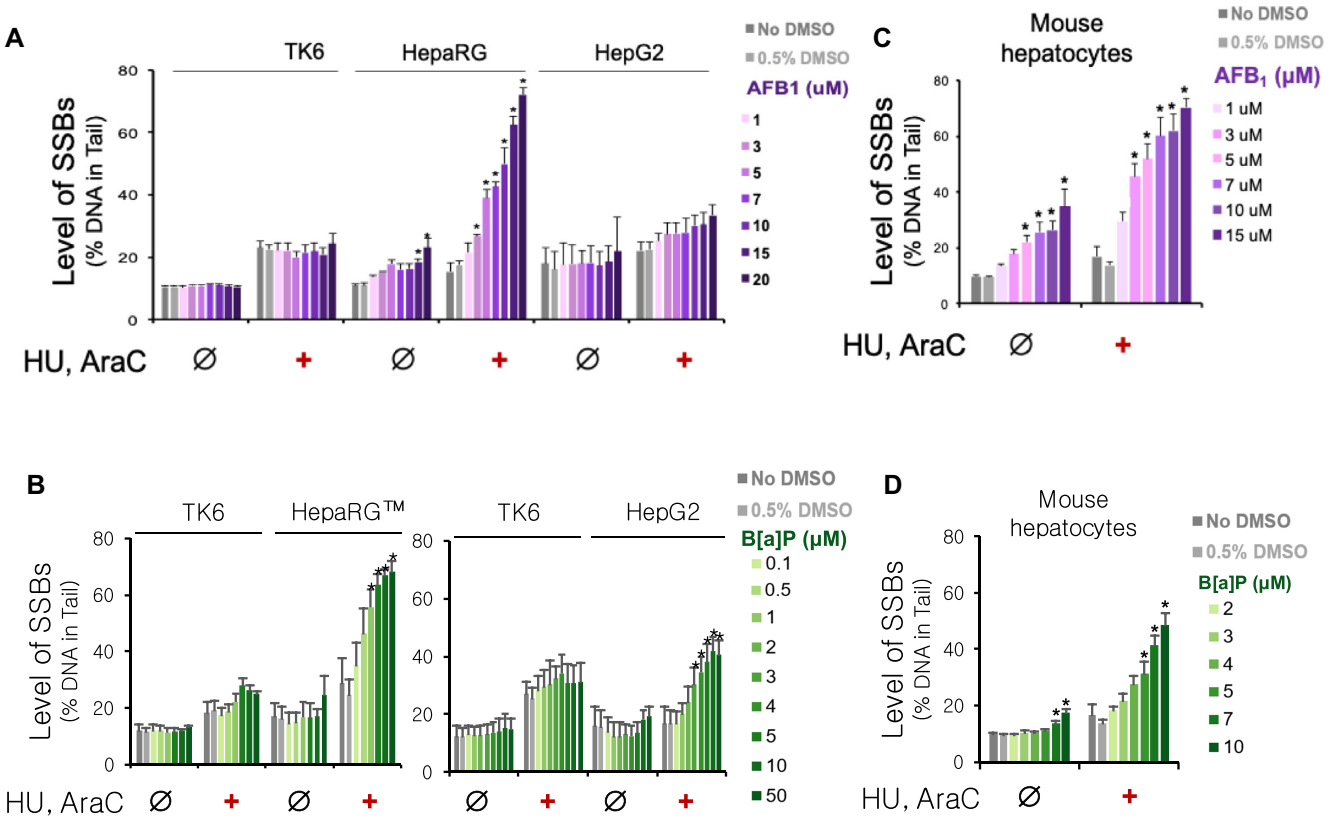

Figure 3. Application of HU/AraC approach on alkaline CometChip to detect DNA damage induced by metabolic activation of AFB_1 and B[a]P. Cells were treated with either AFB_1 or B[a]P in the absence (Ø) or presence (+) of 1 mM HU and 10 μM AraC for 24 h at 37°C and analyzed with the alkaline CometChip. (**A**) Dose-response to AFB_1 in TK6, HepaRG™ (same-day treatment), and HepG2. All three cell lines were treated in parallel. (**B**) Dose-response to B[a]P in TK6, HepaRG™ (day-7 treatment, see Materials and Methods), and HepG2. HepaRG™ and HepG2 cells were treated on different days. TK6 was analyzed in parallel as a control for each treatment. $n \geq 3$. Error bars are standard errors of the means. (**C** and **D**) Dose-response to AFB_1 (C) and B[a]P (D) in primary mouse hepatocytes. All data represent the average of six mice (C57Bl6, 10–14 weeks old). Error bars are standard error of the mean. *$P < 0.05$, one-way ANOVA with post hoc analysis by Dunnett's multiple comparison test [between treated dose and vehicle control (0.5% DMSO)].

then used to estimate the number of SSBs induced by B[a]P and AFB_1 (Supplemental Figure S5B, and Table S3). The results show that the presence of HU/AraC reveals thousands of SSBs induced by B[a]P and AFB_1 in HepaRG and HepG2 cells, indicative of thousands of bulky lesions that are missed without HU/AraC. Also, the correction for background damage resulted in negative numbers in a few cases where there is very little or no induced damage. The negative numbers are therefore an artefact of the calculation and are considered to reflect no change in DNA damage compared to background.

Application of HU/AraC in primary mouse hepatocytes

Primary hepatocytes are the gold standard for metabolism studies (102). Therefore, we wanted to test the efficacy of HU/AraC in these cells. Mouse hepatocytes were isolated using a standard two-step collagenase liver perfusion (80,81). The cells were immediately loaded into CometChip microwells and allowed to recover overnight before AFB_1 or B[a]P treatment. Unexpectedly, we observed that AFB_1 induces SSBs that are detectable even in the absence of HU/AraC (Figure 3C). A potential reason is because AFB_1 induces a mixture of DNA lesions that are repaired not only by NER but also by other pathways where SSB intermediates are less rapidly resolved. For example, a recent study shows that AFB_1-Fapy-dG is partially repaired by BER (recognized and excised by the glycosylase NEIL1) in mammalian cells (103), and it has been shown that BER intermediates are readily detected, even without HU/AraC (104–106). Importantly, HU/AraC greatly increases the overall level of SSBs (Figure 3C), consistent with trapping NER intermediates. In the case of B[a]P treatment, there is a significant, but relatively small, increase in SSBs in the absence of HU/AraC, whereas the addition of HU/AraC reveals a remarkably strong dose–response to B[a]P (Figure 3D). Together, these results show that HU/AraC works well with primary mouse hepatocytes and can be used to enhance detection of DNA damage induced by chemicals that form bulky lesions following metabolic activation.

Metabolic activation modulates the level of SSBs detected using HU/AraC approach

To further validate that SSBs induced by AFB_1 and B[a]P are due to metabolic activation of the carcinogens, we exploited CYP450 inhibitors. Specifically, we tested the possibility that inhibition of CYP450 enzymes would reduce the

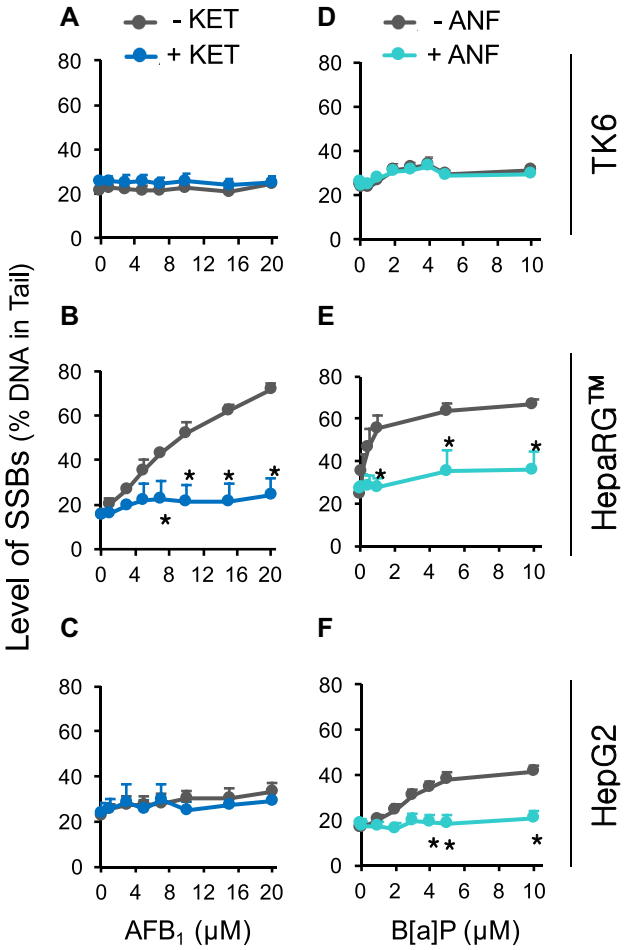

Figure 4. Role of metabolic activation in induction of SSBs by AFB_1 and B[a]P. Cells were treated with AFB_1 or B[a]P for 24 h in the presence of 1 mM HU and 10 μM AraC and analyzed with the alkaline CometChip. To inhibit AFB_1 metabolic activation, 5 μM KET was added to AFB_1 treatment (blue lines in (**A**), (**B**) and (**C**)). To inhibit B[a]P bioactivation, 25 μM ANF was added to B[a]P treatment (teal lines in (**D**), (**E**) and (**F**)). Gray lines represent treatment conditions without KET and ANF. (A) and (D) TK6 cells. (B) HepaRG™ cells (same-day treatment). (E) HepaRG™ (day-7 treatment). (C) and (F) HepG2 cells. $n \geq 3$. Error bars are standard error of the mean. * $P < 0.05$, two-way ANOVA with post hoc analysis by Bonferroni test.

level of DNA adducts, which in turn would suppress the formation of NER-induced SSBs. To reduce metabolic activation of AFB_1, we treated cells with ketoconazole (KET), a potent inhibitor of CYP3A4 (38,107–110). At 5 μM KET, CYP3A4 activity is reduced by ∼100-fold in HepaRG™ cells and by ∼10-fold in HepG2 cells (Supplemental Figure S3). As expected, KET reduces CYP3A4 activity regardless of HU/AraC (Supplemental Figure S3), and KET does not induce DNA damage in TK6, HepaRG™, or HepG2 cells (Figure 4A–C and Supplemental Figure S6 for untreated cells). When HepaRG™ cells are exposed to AFB_1, there is a significant increase in the level of SSBs, as expected. However, in the presence of KET, the level of SSBs is reduced to near background levels (Figure 4B, Supplemental Table S3), indicating that AFB_1 requires metabolic activation prior to formation of DNA damage. As expected, there is no significant increase in SSBs for TK6 or HepG2, and thus there is no impact of KET (Figures 3A, 4A, C and Supplemental Table S3). Together, these results show that HepaRG™ cells have the ability to metabolically activate AFB_1 and that the vast majority of AFB_1-induced damage is due to CYP3A4 activity.

B[a]P, like many other PAHs, is an inducer of the aryl hydrocarbon receptor (AhR) that regulates a number of phase I and phase II enzymes, including the CYP1 family (73). To inhibit the metabolism of B[a]P, we used α-naphthoflavone (ANF), which binds to AhR and inhibits its activation, thereby preventing the upregulation of the CYP1 family (73,111,112). In addition, ANF is also a potent antagonist of CYP1A2 (113,114). We observed that B[a]P induces CYP1A2 activity in HepaRG™ and HepG2 (Supplemental Figure S4, grey bars), consistent with activation of the AhR receptor. In the presence of 25 μM ANF, induction of CYP1A2 is prevented (Supplemental Figure S4), and ANF by itself does not induce DNA damage in TK6, HepaRG™, or HepG2 (Figure 4D–F and Supplemental Figure S6). Significantly, the same dose of ANF reduces the levels of B[a]P-induced SSBs to near background levels in both HepaRG™ and HepG2 (Figure 4E, F and Supplemental Table S3). These results show that, like AFB_1, DNA damage induced by B[a]P is dependent on metabolic activation.

Contribution of NER to SSB formation

Having shown that bulky lesions can be detected in primary mouse hepatocytes (Figure 3C and D), it is thus possible to exploit mouse models lacking key NER proteins. In order to directly test the role of NER in promoting SSBs, we used an $Xpa^{-/-}$ mouse model to completely abolish NER (115). The XPA protein is an essential component of the NER preincision complex, interacting with a number of NER proteins (e.g. TFIIH, RPA, ERCC1-XPF and PCNA) to enable incision (11). While there is a clear dose response in WT cells showing increased SSBs following exposure to AFB_1 in the presence of HU/AraC (Figure 5A, right), in $Xpa^{-/-}$ cells, AFB_1-induced SSBs are greatly reduced (Figure 5A, right). Similarly, B[a]P induces SSBs in WT cells, but not significantly in $Xpa^{-/-}$ cells (Figure 5B, right). These results indicate that NER intermediates contribute to most of the SSBs induced by AFB_1 and B[a]P. Interestingly, for AFB_1, even without HU/AraC, there is nevertheless a statistically significant increase in SSBs relative to untreated cells for both WT and $Xpa^{-/-}$ cells, albeit small in magnitude. This observation is consistent with the possibility that NER-independent enzymes contribute to AFB_1-induced SSBs. Since it is known that AFB_1 induces oxidative stress (116–118), and that oxidative lesions are repaired by BER it is possible that BER of oxidative lesions contributes to the low level of NER-independent SSBs.

HepaCometChip is a sensitive assay for genotoxic agents

For performance assessment of *in vitro* genotoxicity tests, the European Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) published recommendations of chemicals that should give either positive results or nega-

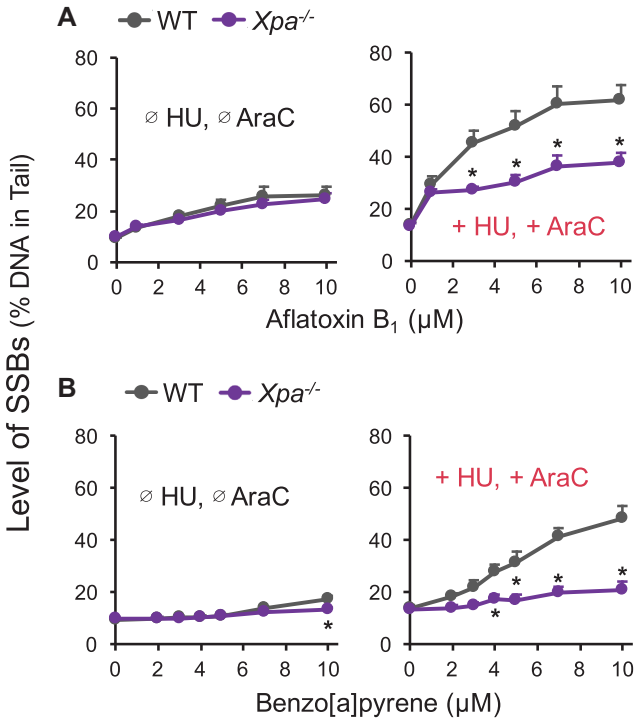

Figure 5. Application of $Xpa^{-/-}$ mouse hepatocytes to study the contribution of NER intermediates to SSBs detected by HU/AraC approach. Primary hepatocytes from six pairs of WT and $Xpa^{-/-}$ mice (C57Bl6, 10–14 weeks old) were isolated via two-step collagenase liver perfusion and incubated at 37°C overnight on CometChip (see Materials and Methods). Cells were then treated with AFB_1 (**A**) or B[a]P (**B**) for 24 hours in the absence (Ø) or presence (+) of 1 mM HU and 10 μM AraC and analyzed with the alkaline CometChip. Each data point is the average of six mice. Error bars are standard error of the mean. $*P < 0.05$, two-way ANOVA with post hoc analysis by Bonferroni test.

tive results in an *in vitro* test (119,120). To assess the sensitivity of the HepaCometChip, we treated HepaRG™ cells with nine known *in vivo* genotoxic agents from Group 1 of the ECVAM's recommendation (120) (Supplemental Table S2, No. 4–12) and compared the levels of SSBs in the absence and presence of HU/AraC. Remarkably, whereas only one chemical shows a positive result for DNA damage in the absence of HU/AraC (namely N-nitrosodimethylamine, or NDMA) (Table 1, third and fourth columns), the presence of the repair inhibitors reveals significant DNA damage for seven (out of nine) known positive compounds (Table 1, fifth and sixth columns). Thus, inhibition of NER completion converts seven false negatives (in the absence of HU/AraC) into correct positives (using HU/AraC). In addition, four of the test compounds are known to be metabolically activated [cyclophosphamide, B[a]P, NDMA, and 2,4-diaminotoluene (2,4-DAT) (120)], and all four are scored as positives using HU/AraC with the HepaRG™ cells. Among the seven chemicals that were scored as positives for DNA damage in the presence of HU/AraC, high levels of cytotoxicity were observed for hydroquinone (HQ) and chloramphenicol (CAM) (Supplemental Figure S7F and H). Although there is no formal threshold for cell viability in scoring the comet assay, cytotoxicity may contribute to DNA fragmentation, which can lead to overestimation of genotoxicity (79). If a 50% cell viability threshold is applied, then HQ and the top dose of CAM will be excluded from the positive results. In the case of etoposide, while statistical significance is only achieved with HU/AraC, the dose response trends appear to be similar in both the absence and presence of HU/AraC (Supplemental Figure S7A).

The two compounds that showed negative results are *p*-chloroaniline (PCA) and cisplatin. PCA is used in a number of industrial processes, such as dye production. Although PCA is an *in vivo* genotoxic agent (119,120), a rodent carcinogen, and a possible human carcinogen (Table 1), there are conflicting data about PCA's DNA damaging potential (WHO CICAD report 2003, https://www.who.int/ipcs/publications/cicad/en/cicad48.pdf). Notably, although the highest dose (5 mM) induces ∼50% cell death, there is nevertheless no significant increase in DNA damage (Supplemental Figure S7D). These results suggest that the *in vivo* genotoxic potential of PCA depends on processes that are independent from formation of DNA damage. Cisplatin was also negative. Cisplatin is a commonly used platinum-based chemotherapeutic for many cancers, including bladder, ovarian, head and neck, and non-small-cell lung cancer (121–124). Upon entering the cell, the chloride ligands hydrolyze, generating aquated cisplatin that can bind to the N7 atom of purine bases to form intra- and interstrand crosslinks (121). It is well established that crosslinks inhibit DNA migration in the comet assay, therefore a negative result for cisplatin is expected based on its mechanism of action (125).

In the absence of HU/AraC, only NDMA shows positive results for DNA damage (Table 1, third and fourth columns). NDMA is bioactivated in the body mainly by CYP2E1 (126) to yield an α-hydroxymethyl nitrosamine that forms a reactive methyl diazonium ion, which methylates nucleobases via S_N1 nucleophilic substitution (WHO CICAD report 2002, https://www.who.int/ipcs/publications/cicad/en/cicad38.pdf). Methylated bases are repaired primarily by the base excision repair (BER) pathway. As BER intermediates (including abasic sites and SSBs) are readily detected by the alkaline comet assay, the observation that NDMA yields a positive result in the absence of HU/AraC is therefore expected.

As another example of how the combination HU/AraC can be applied to study genotoxicity, we tested the DNA damaging potential of a classic antimalarial therapeutic, artesunate (127). We found that HU/AraC reveals a wider range of genotoxic artesunate doses compared to the traditional assay (Supplemental Figure S8). The results are consistent with previous studies showing that artesunate causes DNA damage in mammalian cells (127).

Taken together, the repair synthesis inhibitors HU and AraC significantly improve and extend the sensitivity of the alkaline comet assay. In the context of chemical genotoxicity testing, we propose the use of the alkaline CometChip with HepaRG™ cells in the presence of HU/AraC (HepaCometChip), as a screening platform to achieve high throughput and significantly decreased false negative rates.

Table 1. Comparisons among the Ames assay, traditional alkaline comet assay conditions, and conditions that trap NER intermediates (+ HU + AraC) in a genotoxic screen of nine known *in vivo* genotoxins (120). Statistical significance is designated as '+' and '−'. Dose ranges for statistically significant results are indicated

	Genotoxicity					
		Alkaline CometChip HepaRG™		Alkaline CometChip HepaRG™ (+ HU + AraC)		Carcinogenicity
In vivo genotoxins	Ames	+/−	'+' dose range	+/−	'+' dose range	IARC classification (92)
Etoposide	+ (134)	−		+	10 μM	Group 1
2,4-DAT	+ (135)	−		+	10 mM	Group 2B
CP	+ (135)	−		+	5–10 mM	Group 1
PCA	+ (135)	−		−		Group 2B
NDMA	+ (135)	+	2.5 - 20 mM	+	2.5 - 20 mM	Group 2A
HQ	− (135)	−		+	0.33 mM	Group 3
B[a]P	+ (135)	−		+	5–10 μM	Group 1
CAM	− (136)	−		+	3.1 mM	Group 2A
Cisplatin	+ (137)	−		−		Group 2A

IARC classification: Group 1: human carcinogen, Group 2A: probably human carcinogen, Group 2B: possible human carcinogen, Group 3: not classifiable as to its carcinogenicity to humans.

DISCUSSION

Despite their known carcinogenicity, to date, no high throughput methods had been developed for detecting bulky DNA lesions. Here, we have leveraged the comet assay to overcome this limitation, thus opening doors to improved detection of potential carcinogens. To increase the broad sensitivity of the alkaline comet assay, we combined the metabolic capacity of hepatic cells with small molecule inhibitors of NER repair synthesis so that bulky lesions can be formed and, through aborted repair, converted into detectable SSBs. We also performed the comet assay using the CometChip, thus achieving far greater throughput and sensitivity (15,16). The HepaCometChip enables rapid and sensitive detection of DNA damaging agents that create bulky lesions.

Here, we studied three carcinogenic DNA damaging agents known to create bulky lesions, namely UV-C, AFB_1 and B[a]P. To specifically test whether the SSBs are the result of NER activity, we used cells that were completely lacking key enzymes required for NER initiation. In the absence of XPG, UV-induced SSBs are virtually abolished. Further, primary mouse hepatocytes lacking Xpa similarly showed a dramatic reduction in SSBs induced by AFB_1 and B[a]P. Together, these results show definitively that bulky lesions can be detected by formation of downstream NER intermediates.

A significant barrier to the detection of DNA damaging agents is the frequent requirement for metabolic activation. Many pro-carcinogens are converted into DNA reactive metabolites by CYP450s. Nevertheless, most current genotoxicity screens are performed with cell types that do not support metabolic activation, leading to a blind spot when screening for potential carcinogens. To overcome this limitation, we incorporated hepatic cells into the platform. When HepaRG™ and HepG2 were treated with either AFB_1 or B[a]P, we observed a significant increase in NER intermediates, in sharp contrast to TK6 cells that are not capable of metabolic activation. To formally test the approach of harnessing metabolism to convert AFB_1 and B[a]P into chemicals that can damage DNA, we used known inhibitors of CYP450 activity. Specifically, using CYP450 inhibitors in HepaRG™ and HepG2 cells, we verified that formation of SSBs upon AFB_1 and B[a]P treatments is dependent on the activity of CYP3A4 and CYP1A2, respectively. Taken together, these results show that our HepaCometChip platform captures relevant CYP450 activities that are required for detecting metabolically activated DNA damaging agents. Interestingly, these results also point to the ability to use the HepaCometChip as a means for probing the specific roles of CYP450s in inducing genotoxicity. As a potential application for novel compounds with unknown metabolism, the HepaCometChip platform can be used to screen a panel of CYP450 inhibitors to differentiate between parent- and metabolite-based genotoxicity and to determine the contribution of specific CYP450s.

In these studies, we also compared HepaRG™ and HepG2 cells for their efficacy in detecting bulky lesions. With its broad-spectrum metabolism and its high basal and inducible metabolic enzyme levels, HepaRG™ has the potential to be a robust and reliable cell model for genotoxicity testing. We found that HepaRG™ exhibit orders of magnitude higher activity levels of CYP3A4 and CYP1A2, consistent with the difference in gene expression levels found in other studies (91,92,97). Importantly, we also observed that the higher CYP3A4 and CYP1A2 activities in HepaRG™ cells translate to higher levels of DNA damage induced by AFB_1 and B[a]P compared to HepG2. Furthermore, a recent study utilized both HepaRG™ and HepG2 cells on CometChip to test genotoxicity of a variety of chemicals with varying metabolic capacity and found that CometChip assay on HepaRG™ cells was more effective in detecting genotoxic carcinogens requiring metabolic activation (128). Together, these results point to the use of HepaRG™ cells on the CometChip as an effective strategy for broad detection of metabolically activated DNA damaging agents.

Primary hepatocytes are a critical tool in toxicity testing and metabolism studies. Here, we investigated the efficacy of studying primary hepatocytes using the CometChip. We demonstrated that immediately after isolation, primary mouse hepatocytes can be easily loaded onto the CometChip, exposed to genotoxic agents and analyzed for

SSBs directly on chip. Published methods for studies of primary hepatocytes often involve two-dimensional culturing on tissue culture dishes (102). By loading directly onto the CometChip, analysis of primary hepatocytes is simplified and eliminates the need to detach the cells from culturing vessels (usually via trypsinization), thus minimizing the stress on the cells. We demonstrated here that the hepatocytes on CometChip maintain their ability to metabolically activate the carcinogens AFB_1 and B[a]P, indicating that CometChip can potentially be used as a suitable culturing platform for primary hepatocytes. Because CometChip is fabricated with agarose, which provides a hydrophilic and neutrally charged surface, we expect that culturing primary hepatocytes on-chip will yield similar results compared to ultra-low attachment plates (e.g. Corning® Ultra-Low Attachment Spheroid Microplates) that are routinely used for hepatocyte spheroid formation and culture (129). Given the efficacy of this approach for studies of mouse hepatocytes, we anticipate that these methods would be equally effective for future studies of primary human hepatocytes.

Although we have shown that we can leverage NER intermediates as an indicator of bulky lesions, it remains possible that HU/AraC may also increase sensitivity for the detection of lesions repaired by other repair pathways (such as BER). Intriguingly, while HU/AraC is exploited here as a way to trap NER intermediates, ostensibly to convert undetectable bulky lesions into detectable single strand breaks, the concept of revealing base lesions via conversion to strand breaks is not new. Extensive work has been done to exploit purified glycosylases as a tool for expanding the sensitivity of the comet assay. To accomplish this, after lysis, the DNA of pre-comets is incubated with glycosylases that convert undetectable base lesions into single strand breaks (130,131). As one example, Fpg has been used extensively to convert its substrates (including 8-oxoguanine) into detectable strand breaks for comet analysis (131–133). Unlike BER (wherein damaged bases can be converted to strand breaks using a single purified bifunctional glycosylase), many proteins need to be present in order for NER to cleave the backbone near the site of the lesion, making *in vitro* studies complex. For this reason, in this work, we have focused on exploiting NER capacity that is inherent to live cells as a way to reveal bulky lesions.

As a screening tool for genotoxicity, having a platform that detects a broad range of DNA damaging agents is a great asset. In fact, to test the efficacy of the HepaCometChip platform for screening potential carcinogens, we compared the traditional comet assay (without HU/AraC) to the HepaCometChip for nine known genotoxic agents. A positive result was observed for seven agents using the HepaCometChip, all of which were missed using the traditional comet assay. PCA is a known genotoxic agent *in vivo*, but its mechanism of action is not well understood. The observation that PCA is negative on the HepaCometChip suggests that it may be an indirect acting genotoxic agent, e.g. one that does not directly damage DNA. The other genotoxin negative on the HepaCometChip was cisplatin, which forms interstrand crosslinks. It is well established that crosslinks inhibit DNA migration. Therefore, the negative result for cisplatin is consistent with its mode of action. Taken together, the combination of leveraging hepatocyte metabolism and DNA repair synthesis inhibition provides a highly sensitive approach for detecting DNA damaging agents that show a false negative result using the traditional comet assay.

With regard to limitations, the HepaCometChip is not as sensitive as assays that detect specific DNA lesions for which there is prior knowledge of adduct structures, such as HPLC and mass spectrometry. However, for applications where the structure is not known in advance of the assay, the HepaCometChip is preferable. In addition, for primary screens, it is generally the case that relatively high doses of an agent can be tested, making sensitivity less important. Nevertheless, for some small molecule libraries where compound quantities are limited, the amount of compound available could lead to formation of adducts that are below the level of detection. Finally, one other limitation is that HU/AraC, while effective for trapping NER intermediates, might potentially also trap intermediates formed during BER. This lack of specificity could be considered to be a strength, however, since as a screening tool, having a broad sensitivity can be advantageous. Further, for experiments where it is important to know if a DNA adduct is repaired specifically via NER, cell lines deficient in essential NER components could be useful as a means for determination of NER's contribution.

In conclusion, using a combination of the DNA repair synthesis inhibitors HU and AraC and a metabolically competent human cell line HepaRG™, we developed a CometChip platform for HT genotoxicity testing that has exquisite sensitivity for bulky DNA adducts. The platform can be used as a powerful HT tool for screening large chemical libraries, with applications in safety testing for both public health and the pharmaceutical industry. The use of HU/AraC together with CometChip is also a promising tool for clinical applications, where DNA damage levels can be monitored as a surrogate endpoint for tumor response. Taken together, the HepaCometChip fills a gap in genotoxicity testing by capturing agents that are negative using traditional comet analysis, and as such will serve as a useful tool for a broad range of applications.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Vivo Mammalian Alkaline Comet Assay

INTRODUCTION

1. The *in vivo* alkaline comet (single cell gel electrophoresis) assay (hereafter called simply the comet assay) is used for the detection of DNA strand breaks in cells or nuclei isolated from multiple tissues of animals, usually rodents, that have been exposed to potentially genotoxic material(s). The comet assay has been reviewed and recommendations have been published by various expert groups (1) (2) (3) (4) (5) (6) (7) (8) (9) (10). This Test Guideline is part of a series of Test Guidelines on genetic toxicology. A document that provides succinct information on genetic toxicology testing and an overview of the recent changes that were made to these Test Guidelines has been developed (11).

2. The purpose of the comet assay is to identify substances that cause DNA damage. Under alkaline conditions (>pH 13), the comet assay can detect single and double stranded breaks, resulting, for example, from direct interactions with DNA, alkali labile sites or as a consequence of transient DNA strand breaks resulting from DNA excision repair. These strand breaks may be repaired, resulting in no persistent effect, may be lethal to the cell, or may be fixed into a mutation resulting in a permanent viable change. They may also lead to chromosomal damage which is also associated with many human diseases including cancer.

3. A formal validation trial of the *in vivo* rodent comet assay was performed in 2006-2012, coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM), in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) (12). This Test Guideline includes the recommended use and limitations of the comet assay, and is based on the final protocol (12) used in the validation trial, and on additional relevant published and unpublished (laboratories proprietary) data.

4. Definitions of key terms are set out in Annex 1. It is noted that many different platforms can be used for this assay (microscope slides, gel spots, 96-well plates etc.). For convenience the term “slide” is used throughout the remainder of this document but encompasses all of the other platforms.

This Guideline was adopted by the OECD Council by written procedure on 29 July 2016 [C(2016)103].

INITIAL CONSIDERATIONS AND LIMITATIONS

5. The comet assay is a method for measuring DNA strand breaks in eukaryotic cells. Single cells/nuclei embedded in agarose on a slide are lysed with detergent and high salt concentration. This lysis step digests the cellular and nuclear membranes and allows the release of coiled DNA loops generally called nucleoids and DNA fragments. Electrophoresis at high pH results in structures resembling comets, which, by using appropriate fluorescent stains, can be observed by fluorescence microscopy; DNA fragments migrate away from the "head" into the "tail" based on their size, and the intensity of the comet tail relative to the total intensity (head plus tail) reflects the amount of DNA breakage (13) (14) (15).

6. The *in vivo* alkaline comet assay is especially relevant to assess genotoxic hazard in that the assay's responses are dependent upon *in vivo* ADME (absorption, distribution, metabolism and excretion), and also on DNA repair processes. These may vary among species, among tissues and among the types of DNA damage.

7. To fulfil animal welfare requirements, in particular the reduction in animal usage (3Rs - Reduction, Refinement, Replacement - principles), this assay can also be integrated with other toxicological studies, e.g., repeated dose toxicity studies (10) (16) (17), or the endpoint can be combined with other genotoxicity endpoints such as the *in vivo* mammalian erythrocyte micronucleus assay (18) (19) (20). The comet assay is most often performed in rodents, although it has been applied to other mammalian and non-mammalian species. The use of non-rodent species should be scientifically and ethically justified on a case-by-case basis and it is strongly recommended that the comet assay only be performed on species other than rodents as part of another toxicity study and not as a standalone test.

8. The selection of route of exposure and tissue(s) to be studied should be determined based on all available/existing knowledge of the test chemicals e.g. intended/expected route of human exposure, metabolism and distribution, potential for site-of-contact effects, structural alerts, other genotoxicity or toxicity data, and the purpose of the study. Thus, where appropriate, the genotoxic potential of the test chemicals can be assayed in the target tissue(s) of carcinogenic and/or other toxic effects. The assay is also considered useful for further investigation of genotoxicity detected by an *in vitro* system. It is appropriate to perform an *in vivo* comet assay in a tissue of interest when it can be reasonably expected that the tissue of interest will be adequately exposed.

9. The assay has been most extensively validated in somatic tissues of male rats in collaborative studies such as the JaCVAM trial (12) and in Rothfuss et al. 2010 (10). The liver and stomach were used in the JaCVAM international validation trial. The liver, because it is the most active organ in metabolism of substances and also frequently a target organ for carcinogenicity. The stomach, because it is usually first site of contact for substances after oral exposure, although other areas of the gastro-intestinal tract such as the duodenum and jejunum should also be considered as site-of-contact tissues and may be considered more relevant for humans than the rodent glandular stomach. Care should be taken to ensure that such tissues are not exposed to excessively high test substance concentrations (21). The technique is in principle applicable to any tissue from which analysable single cell/nuclei suspensions can be derived. Proprietary data from several laboratories demonstrate its successful application to many different tissues, and there are many publications showing the applicability of the technique to organs or tissues other than liver and stomach, e.g., jejunum (22), kidney (23) (24), skin (25) (26)**,** or urinary bladder (27) (28), lungs and bronchoalveolar lavage cells (relevant for studies of inhaled substances) (29) (30), and tests have also been performed in multiple organs (31) (32).

10. Whilst there may be an interest in genotoxic effects in germ cells, it should be noted that the

standard alkaline comet assay as described in this guideline is not considered appropriate to measure DNA strand breaks in mature germ cells. Since high and variable background levels in DNA damage were reported in a literature review on the use of the comet assay for germ cell genotoxicity (33), protocol modifications together with improved standardization and validation studies are deemed necessary before the comet assay on mature germ cells (e.g. sperm) can be included in the test guideline. In addition, the recommended exposure regimen described in this guideline is not optimal and longer exposures or sampling times would be necessary for a meaningful analysis of DNA strand breaks in mature sperm. Genotoxic effects as measured by the comet assay in testicular cells at different stages of differentiation have been described in the literature (34) (35). However, it should be noted that gonads contain a mixture of somatic and germ cells. For this reason, positive results in whole gonad (testis) are not necessarily reflective of germ cell damage; nevertheless, they indicate that tested chemical(s) and/or its metabolites have reached the gonad.

11. Cross-links cannot be reliably detected with the standard experimental conditions of the comet assay. Under certain modified experimental conditions, DNA-DNA and DNA-protein crosslinks, and other base modifications such as oxidized bases might be detected (23) (36) (37) (38) (39). But further work would be needed to adequately characterize the necessary protocol modifications. Thus detection of cross linking agents is not the primary purpose of the assay as described here. The assay is not appropriate, even with modifications, for detecting aneugens.

12. Due to the current status of knowledge, several additional limitations (see Annex 3) are associated with the *in vivo* comet assay. It is expected that the Test Guideline will be reviewed in the future and if necessary revised in light of experience gained.

13. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE METHOD

14. Animals are exposed to the test chemical by an appropriate route. A detailed description of dosing and sampling is given in paragraphs 36-40. At the selected sampling time(s), the tissues of interest are dissected and single cells/nuclei suspensions are prepared (*in situ* perfusion may be performed where considered useful e.g. liver) and embedded in soft agar so as to immobilize them on slides. Cells/nuclei are treated with lysis buffer to remove cellular and/or nuclear membrane, and exposed to strong alkali e.g., pH≥13 to allow DNA unwinding and release of relaxed DNA loops and fragments. The nuclear DNA in the agar is then subjected to electrophoresis. Normal non-fragmented DNA molecules remain in the position where the nuclear DNA had been in the agar, while any fragmented DNA and relaxed DNA loops would migrate towards the anode. After electrophoresis, the DNA is visualized using an appropriate fluorescent stain. Preparations should be analysed using a microscope and full or semi-automated image analysis systems. The extent of DNA that has migrated during electrophoresis and the migration distance reflects the amount and size of DNA fragments. There are several endpoints for the comet assay. The DNA content in the tail (% tail DNA or % tail intensity) has been recommended to assess DNA damage (12) (40) (41) (42). After analysis of a sufficient number of nuclei, the data are analysed with appropriate methods to judge the assay results.

15. It should be noted that altering various aspects of the methodology, including sample preparation, electrophoresis conditions, visual analysis parameters (e.g. stain intensity, microscope bulb light intensity,

and use of microscope filters and camera dynamics) and ambient conditions (e.g. background lighting), have been investigated and may affect DNA migration (43) (44) (45) (46).

VERIFICATION OF LABORATORY PROFICIENCY

16. Each laboratory should establish experimental competency in the comet assay by demonstrating the ability to obtain single cell or nuclei suspensions of sufficient quality for each target tissue(s) for each species used. The quality of the preparations will be evaluated firstly by the % tail DNA for vehicle treated animals falling within a reproducible low range. Current data suggest that the group mean % tail DNA (based on mean of medians - see paragraph 57 for details of these terms) in the rat liver should be preferably not exceed 6%, which would be consistent with the values in the JaCVAM validation trial (12) and from other published and proprietary data. There are not enough data at this time to make recommendations about optimum or acceptable ranges for other tissues. This doesn't preclude the use of other tissues if justified. The test report should provide appropriate review of the performance of the comet assay in these tissues in relation to the published literature or from proprietary data. Firstly, a low range of %tail DNA in controls is desirable to provide sufficient dynamic range to detect a positive effect. Secondly, each laboratory should be able to reproduce expected responses for direct mutagens and pro-mutagens, with different modes of action as suggested in Table 1 (paragraph 29).

17. Positive substances may be selected, for example from the JaCVAM validation trial (12) or from other published data (see paragraph 9), if appropriate, with justification, and demonstrating clear positive responses in the tissues of interest. The ability to detect weak effects of known mutagens e.g. EMS at low doses, should also be demonstrated, for example by establishing dose-response relationships with appropriate numbers and spacing of doses. Initial efforts should focus on establishing proficiency with the most commonly used tissues e.g. the rodent liver, where comparison with existing data and expected results may be made (12). Data from other tissues e.g. stomach/duodenum/jejunum, blood etc. could be collected at the same time. The laboratory needs to demonstrate proficiency with each individual tissue in each species they are planning to study, and will need to demonstrate that an acceptable positive response with a known mutagen (e.g. EMS) can be obtained in that tissue.

18. Vehicle/negative control data should be collected so as to demonstrate reproducibility of negative data responses, and to ensure that the technical aspects of the assay were properly controlled or to suggest the need to re-establish historical control ranges (see paragraph 22).

19. It should be noted, that whilst multiple tissues can be collected at necropsy and processed for comet analysis, the laboratory needs to be proficient in harvesting multiple tissues from a single animal, thereby ensuring that any potential DNA lesion is not lost and comet analysis is not compromised. The length of time from euthanasia to removal of tissues for processing may be critical (see paragraph 44).

20. Animal welfare must be considered whilst developing proficiency in this test and therefore tissues from animals used in other tests can be used when developing competence in the various aspects of the test. Furthermore, it may not be necessary to conduct a full study during the stages of establishing a new test guideline method in a laboratory and fewer animals or test concentrations can be used when developing the necessary skills.

Historical control data

21. During the course of the proficiency investigations, the laboratory should build a historical database to establish positive and negative control ranges and distributions for relevant tissues and species. Recommendations on how to build and use the historical data (i.e. criteria for inclusion and exclusion of data in historical data and the acceptability criteria for a given experiment) can be found in the literature (47). Different tissues and different species, as well as different vehicles and routes of administrations, may give different negative control % tail DNA values. It is therefore important to establish negative control ranges for each tissue and species. Laboratories should use quality control methods, such as control charts (e.g. C-charts or X-bar charts (48)), to identify how variable their data are, and to show that the methodology is 'under control' in their laboratory. Selection of appropriate positive control substances, dose ranges and experimental conditions (e.g. electrophoresis conditions) may need also to be optimised for the detection of weak effects (see paragraph 17).

22. Any changes to the experimental protocol should be considered in terms of their consistency with the laboratory's existing historical control databases. Any major inconsistencies should result in the establishment of a new historical control database.

DESCRIPTION OF THE METHOD

Preparations

Selection of animal species

23. Common laboratory strains of healthy young adult rodents (6-10 weeks old at start of treatment though slightly older animals are also acceptable) are normally used. The choice of rodent species should be based on (i) species used in other toxicity studies (to be able to correlate data and to allow integrated studies), (ii) species that developed tumours in a carcinogenicity study (when investigating the mechanism of carcinogenesis), or (iii) species with the most relevant metabolism for humans, if known. Rats are routinely used in this test. However, other species can be used if ethically and scientifically justified.

Animal housing and feeding conditions

24. For rodents, the temperature in the experimental animal room ideally should be 22°C (±3°C). The relative humidity ideally should be 50-60%, being at least 30% and preferably not exceeding 70% other than during room cleaning. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test chemical when administered by this route. Rodents should be housed in small groups (usually no more than five) of the same sex if no aggressive behaviour is expected. Animals may be housed individually only if scientifically justified. Solid floors should be used wherever possible as mesh floors can cause serious injury (49). Appropriate environmental enrichment must be provided.

Preparation of the animals

25. Animals are randomly assigned to the control and treatment groups. The animals are identified uniquely and acclimated to the laboratory conditions for at least five days before the start of treatment. The least invasive method of uniquely identifying animals must be used. Appropriate methods include ringing, tagging, micro-chipping and biometric identification. Toe and ear clipping are not scientifically justified in these tests. Cages should be arranged in such a way that possible effects due to cage placement are

minimized. At the commencement of the study, the weight variation of animals should be minimal and not exceed ± 20%.

Preparation of doses

26. Solid test chemicals should be dissolved or suspended in appropriate vehicles or admixed in diet or drinking water prior to dosing of the animals. Liquid test chemicals may be dosed directly or diluted prior to dosing. For inhalation exposures, test chemicals can be administered as gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties (50) (51).

27. Fresh preparations of the test chemical should be employed unless stability data demonstrate the acceptability of storage and define the appropriate storage conditions.

Test Conditions

Vehicle

28. The vehicle should not produce toxic effects at the dose volumes used, and should not be suspected of chemical reaction with the test substances. If other than well-known vehicles are used, their inclusion should be supported with reference data indicating their compatibility in terms of test animals, route of administration and endpoint. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first. It should be noted that some vehicles (particularly viscous vehicles) can induce inflammation and increase background levels of DNA strand breaks at the site of contact, particularly with multiple administrations.

Controls

Positive controls

29. At this time, a group of a minimum of 3 analysable animals of one sex, or of each sex if both are used (see paragraph 32), treated with a positive control substance should normally be included with each test. In future, it may be possible to demonstrate adequate proficiency to reduce the need for positive controls. If multiple sampling times are used (e.g. with a single administration protocol) it is only necessary to include positive controls at one of the sampling times, but a balanced design should be ensured (see paragraph 48). It is not necessary to administer concurrent positive control substances by the same route as the test chemical, although it is important that the same route should be used when measuring site-of-contact effects. The positive control substances should be shown to induce DNA strand breaks in all of the tissues of interest for the test chemical, and EMS is likely to be the positive control of choice since it has produced DNA strand breaks in all tissues that have been studied. The doses of the positive control substances should be selected so as to produce moderate effects that critically assess the performance and sensitivity of the assay and could be based on dose-response curves established by the laboratory during the demonstration of proficiency. The % tail DNA in concurrent positive control animals should be consistent with the pre-established laboratory range for each individual tissue and sampling time for that species (see paragraph 16). Examples of positive control substances and some of their target tissues (in rodents) are included in Table 1. Substances other than those given in Table 1 can be selected if scientifically justified.

Table 1: Examples of positive control substances and some of their target tissues

Substances and CAS RN No.
Ethyl methanesulfonate (CAS RN 62-50-0) for any tissue
Ethyl nitrosourea (CAS RN 759-73-9) for liver and stomach, duodenum or jejunum
Methyl methanesulfonate (CAS RN 66-27-3) for liver, stomach, duodenum or jejunum, lung and bronchoalveolar lavage (BAL) cells, kidney, bladder, lung, testis and bone marrow/blood
N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (CAS RN: 70-25-7) for stomach, duodenum or jejunum
1,2-Dimethylhydrazine 2HCl (CAS RN 306-37-6) for liver and intestine
N-methyl-*N*-nitrosourea (CAS RN 684-93-5) for liver, bone marrow, blood, kidney, stomach, jejunum, and brain.

Negative controls

30. A group of negative control animals, treated with vehicle alone, and otherwise treated in the same way as the treatment groups, should be included with each test for every sampling time and tissue. The % tail DNA in negative control animals should be within the pre-established laboratory background range for each individual tissue and sampling time for that species (see paragraph 16). In the absence of historical or published control data showing that no deleterious or genotoxic effects are induced by the chosen vehicle, by the number of administrations or by the route of administration, initial studies should be performed prior to conducting the full study, in order to establish acceptability of the vehicle control.

PROCEDURE

Number and Sex of Animals

31. Although there is little data on female animals from which to make comparison between sexes in relation to the comet assay, in general, other *in vivo* genotoxicity responses are similar between male and female animals and therefore most studies could be performed in either sex. Data demonstrating relevant differences between males and females (e.g. differences in systemic toxicity, metabolism, bioavailability, etc. including e.g. in a range-finding study) encourage the use of both sexes. In this case, it may be appropriate to perform a study in both sexes e.g. as part of a repeated dose toxicity study. It might be appropriate to use the factorial design in case both sexes are used. Details on how to analyse the data using this design are given in Annex 2.

32. Group sizes at study initiation (and during establishment of proficiency) should be established with the aim of providing a minimum of 5 analysable animals of one sex, or of each sex if both are used, per group (less in the concurrent positive control group – see paragraph 29). Where human exposure to chemicals may be sex-specific, as for example with some pharmaceuticals, the test should be performed with the appropriate sex. As a guide to maximum typical animal requirements, a study conducted according the parameters established in paragraph 33 with three dose groups and concurrent negative and positive controls (each group composed of five animals of a single sex) would require between 25 and 35 animals.

Treatment schedule

33. Animals should be given daily treatments over a duration of 2 or more days (i.e. two or more treatments at approximately 24 hour intervals), and samples should be collected once at 2-6 h (or at the Tmax) after the last treatment (12). Samples from extended dose regimens (e.g., 28-day daily dosing) are acceptable. Successful combination of the comet and the erythrocyte micronucleus test has been demonstrated (10) (19). However careful consideration should be given to the logistics involved in tissue sampling for comet analysis alongside the requirements of tissue sampling for other types of toxicological assessments. Harvest 24 hours after the last dose, which is typical of a general toxicity study, is not appropriate in most cases (see paragraph 40 on sampling time). The use of other treatment and sampling schedules should be justified (see annex 3). For example single treatment with multiple sampling could be used however, it should be noted that more animals will be required for a study with a single administration study because of the need for multiple sampling times, but on occasions this may be preferable, e.g. when the test chemical induces excessive toxicity following repeated administrations.

34. Whatever way the test is performed, it is acceptable as long as the test chemical gives a positive response or, for a negative study, as long as direct or indirect evidence supportive of exposure of, or toxicity to, the target tissue(s) has been demonstrated or if the limit dose is achieved (see paragraph 37):

35. Test chemicals also may be administered as a split dose, i.e., two treatments on the same day separated by no more than 2-3 hours, to facilitate administering a large volume. Under these circumstances, the sampling time should be scheduled based on the time of the last dosing (see paragraph 40).

Dose Levels

36. If a preliminary range-finding study is performed because there are no suitable data available from other relevant studies to aid in dose selection, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study according to current approaches for conducting dose range-finding studies. The study should aim to identify the maximum tolerated dose (MTD), defined as the dose inducing slight toxic effects relative to the duration of the study period (for example, clear clinical signs such as abnormal behaviour or reactions, minor body weight depression or target tissue cytotoxicity), but not death or evidence of pain, suffering or distress necessitating euthanasia. For a non-toxic test chemical, with an administration period of 14 days or more, the maximum (limit) dose is 1000 mg/kg bodyweight/day. For administration periods of less than 14 days the maximum (limit) dose is 2000 mg/kg bodyweight/day. For certain types of test chemicals (e.g. human pharmaceuticals) covered by specific regulations these limits may vary.

37. Substances that exhibit saturation of toxicokinetic properties, or induce detoxification processes that may lead to a decrease in exposure after long-term administration, may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis.

38. For both acute and sub-acute versions of the comet assay, in addition to the maximum dose (MTD, maximum feasible dose, maximum exposure or limit dose) a descending sequence of at least two additional appropriately spaced dose levels (preferably separated by less than $\sqrt{10}$) should be selected for each sampling time to demonstrate dose-related responses. However, the dose levels used should also preferably cover a range from the maximum to one producing little or no toxicity. When target tissue toxicity is observed at all dose levels tested, further study at non-toxic doses is advisable (see paragraphs 54-55). Studies intending to more fully investigate the shape of the dose-response curve may require

additional dose group(s).

Administration of Doses

39. The anticipated route of human exposure should be considered when designing an assay. Therefore, routes of exposure such as dietary, drinking water, topical, subcutaneous, intravenous, oral (by gavage), inhalation, intratracheal, or implantation may be chosen as justified. In any case the route should be chosen to ensure adequate exposure of the target tissue(s). Intraperitoneal injection is generally not recommended since it is not a typical relevant route of human exposure, and should only be used with specific justification (e.g. some positive control substances, for investigative purposes, or for some drugs that are administered by the intraperitoneal route). The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 1 mL/100 g body weight, except in the case of aqueous solutions where 2 mL/100g body weight may be used. The use of volumes greater than this (if permitted by animal welfare legislation) should be justified. Wherever possible different dose levels should be achieved by adjusting the concentration of the dosing formulation to ensure a constant volume in relation to body weight at all dose levels.

Sampling Time

40. The sampling time is a critical variable because it is determined by the period needed for the test chemicals to reach maximum concentration in the target tissue and for DNA strand breaks to be induced but before those breaks are removed, repaired or lead to cell death. The persistence of some of the lesions that lead to the DNA strand breaks detected by the comet assay may be very short, at least for some substances tested *in vitro* (52) (53). Accordingly, if such transient DNA lesions are suspected, measures should be taken to mitigate their loss by ensuring that tissues are sampled sufficiently early, possibly earlier than the default times given below. The optimum sampling time(s) may be substance- or route-specific resulting in, for example, rapid tissue exposure with intravenous administration or inhalation exposure. Accordingly, where available, sampling times should be determined from kinetic data (e.g., the time (Tmax) at which the peak plasma or tissue concentration (Cmax) is achieved, or at the steady state for multiple administrations). In the absence of kinetic data a suitable compromise for the measurement of genotoxicity is to sample at 2-6 h after the last treatment for two or more treatments, or at both 2-6 and 16-26 h after a single administration, although care should be taken to necropsy all animals at the same time after the last (or only) dose. Information on the appearance of toxic effects in target organs (if available) may also be used to select appropriate sampling times.

Observations

41. General clinical observations related to the health of the animals should be made and recorded at least once a day preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing (54). At least twice daily, all animals should be observed for morbidity and mortality. For longer duration studies, all animals should be weighed at least once a week, and at completion of the test period. Food consumption should be measured at each change of food and at least weekly. If the test chemical is administered via the drinking water, water consumption should be measured at each change of water and at least weekly. Animals exhibiting non-lethal indicators of excessive toxicity should be euthanized prior to completion of the test period, and are generally not used for comet analysis.

Tissue Collection

42. Since it is possible to study induction of DNA strand breaks (comets) in virtually any tissue, the rationale for selection of tissue(s) to be collected should be clearly defined and based upon the reason for conducting the study together with any existing ADME, genotoxicity, carcinogenicity or other toxicity data for the test substances under investigation. Important factors for consideration should include the route of administration (based on likely human exposure route(s)), the predicted tissue distribution and absorption, the role of metabolism and the possible mechanism of action of the test substances. The liver has been the tissue most frequently studied and for which there are the most data. Therefore, in the absence of any background information, and if no specific tissues of interest are identified, sampling the liver would be justified as this is a primary site of xenobiotic metabolism and is often highly exposed to both parent substance(s) and metabolite(s). In some cases examination of a site of direct contact (for example, for orally-administered substances the glandular stomach or duodenum/jejunum, or for inhaled substances the lungs) may be most relevant. Additional or alternative tissues should be selected based on the specific reasons for the test is being conducted but it may be useful to examine multiple tissues in the same animals providing the laboratory has demonstrated proficiency with those tissues and competency in handling multiple tissues at the same time.

Preparation of specimens

43. For the processes described in the following paragraphs (44-49) it is important that all solutions or stable suspensions should be used within their expiration date, or should be freshly prepared if needed. Also in the following paragraphs, the times taken to (i) remove each tissue after necropsy, (ii) process each tissue into cell/nuclei suspensions, and (iii) process the suspension and prepare the slides are all considered critical variables (see Definitions, Annex 1), and acceptable lengths of time for each of these steps should have been determined during establishment of the method and demonstration of proficiency.

44. Animals will be euthanised, consistent with effective animal welfare legislation and 3Rs principles, at the appropriate time(s) after the last treatment with a test chemical. Selected tissue(s) is removed, dissected, and a portion is collected for the comet assay, whilst at the same time a section from the same part of the tissue should be cut and placed in formaldehyde solution or appropriate fixative for possible histopathology analysis (see paragraph 55) according to standard methods (12). The tissue for the comet assay is placed into mincing buffer, rinsed sufficiently with cold mincing buffer to remove residual blood, and stored in ice-cold mincing buffer until processed. In situ perfusion may also be performed, e.g. for liver, kidney.

45. Many published methods exist for cell/nuclei isolation. These include mincing of tissues such as liver and kidney, scraping mucosal surfaces in the case of the gastro-intestinal tract, homogenization and enzymic digestion. The JaCVAM validation trial only studied isolated cells, and therefore in terms of establishing the method and being able to refer to the JaCVAM trial data for demonstration of proficiency, isolated cells are preferred. However, it has been shown that there was no essential difference in the assay result whether isolated cells or nuclei were used (8). Also different methods to isolate cells/nuclei (e.g., homogenizing, mincing, enzymic digestion and mesh filtration) gave comparable results (55). Consequently either isolated cells or isolated nuclei can be used. A laboratory should thoroughly evaluate and validate tissue-specific methods of single cell/nuclei isolation. As discussed in paragraph 40, the persistence of some of the lesions that lead to the DNA strand breaks detected by the comet assay may be very short (52) (53). Therefore, whatever method is used to prepare the single cell/nuclei suspensions, it is important that tissues are processed as soon as possible after the animals have been euthanised and placed in conditions that reduce the removal of lesions (e.g. by maintaining the tissue at low temperature). The cell suspensions should be kept ice-cold until ready for use, so that minimal inter-sample variation and appropriate positive and negative control responses can be demonstrated.

Preparation of slides

46. Slide preparation should be done as soon as possible (ideally within one hour) after single cell/nuclei preparation, but the temperature and time between animal death and slide preparation should be tightly controlled and validated under the laboratory's conditions. The volume of the cell suspension added to low melting point agarose (usually 0.5-1.0%) to make the slides should not reduce the percentage of low melting point agarose to less than 0.45%. The optimum cell density will be determined by the image analysis system used for scoring comets.

Lysis

47. Lysis conditions are also a critical variable and may interfere with the strand breaks resulting from specific types of DNA modifications (certain DNA alkylations and base adducts). It is therefore recommended that the lysis conditions be kept as constant as possible for all slides within an experiment. Once prepared, the slides should be immersed in chilled lysing solution for at least one hour (or overnight) at around 2-8^{o}C under subdued lighting conditions e.g. yellow light (or light proof) that avoid exposure to white light that may contain UV components. After this incubation period, the slides should be rinsed to remove residual detergent and salts prior to the alkali unwinding step. This can be done using purified water, neutralization buffer or phosphate buffer. Electrophoresis buffer can also be used. This would maintain the alkaline conditions in the electrophoresis chamber.

Unwinding and electrophoresis

48. Slides should be randomly placed onto the platform of a submarine-type electrophoresis unit containing sufficient electrophoresis solution such that the surfaces of the slides are completely covered (the depth of covering should also be consistent from run to run). In other type of comet assay electrophoresis units i.e. with active cooling, circulation and high capacity power supply a higher solution covering will result in higher electric current while the voltage is kept constant. A balanced design should be used to place slides in the electrophoresis tank to mitigate the effects of any trends or edge effect within the tank and to minimize batch-to-batch variability, i.e., in each electrophoresis run, there should be the same number of slides from each animal in the study and samples from the different dosage groups, negative and positive controls, should be included. The slides should be left for at least 20 minutes for the DNA to unwind, and then subjected to electrophoresis under controlled conditions that will maximize the sensitivity and dynamic range of the assay (i.e. lead to acceptable levels of % tail DNA for negative and positive controls that maximize sensitivity). The level of DNA migration is linearly associated with the duration of electrophoresis, and also with the potential (V/cm). Based on the JaCVAM trial this could be 0.7 V/cm for at least 20 minutes. The duration of electrophoresis is considered a critical variable and the electrophoresis time should be set to optimize the dynamic range. Longer electrophoresis times (e.g. 30 or 40 minutes to maximize sensitivity) usually lead to stronger positive responses with known mutagens. However longer electrophoresis times may also lead to excessive migration in control samples. In each experiment the voltage should be kept constant, and the variability in the other parameters should be within a narrow and specified range, for example in the JaCVAM trial 0.7 V/cm delivered a starting current of 300 mA. The depth of buffer should be adjusted to achieve the required conditions and maintained throughout the experiment. The current at the start and end of the electrophoresis period should be recorded. The optimum conditions should therefore be determined during the initial demonstration of proficiency in the laboratory concerned with each tissue studied. The temperature of the electrophoresis solution through unwinding and electrophoresis should be maintained at a low temperature, usually 2-10^{o}C (10). The temperature of the electrophoresis solution at the start of unwinding, the start of electrophoresis, and the end of electrophoresis should be recorded.

49. After completion of electrophoresis, the slides should be immersed/rinsed in the neutralization buffer for at least 5 minutes. Gels can be stained and scored "fresh" (e.g. within 1-2 days) or can be dehydrated for later scoring (e.g. within 1-2 weeks after staining) (56). However, the conditions should be validated during the demonstration of proficiency and historical data should be obtained and retained separately for each of these conditions. In case of the latter, slides should be dehydrated by immersion into absolute ethanol for at least 5 minutes, allowed to air dry, and then stored, either at room temperature or in a container in a refrigerator until scored.

Methods of Measurement

50. Comets should be scored quantitatively using an automated or semi-automated image-analysis system. The slides will be stained with an appropriate fluorescent stain e.g., SYBR Gold, Green I, propidium iodide or ethidium bromide and measured at a suitable magnification (e.g., 200x) on a microscope equipped with epi-fluorescence and appropriate detectors or a digital (e.g. CCD) camera.

51. Cells may be classified into three categories as described in the atlas of comet images (57), namely scorable, non-scorable and "hedgehog" (see paragraph 56 for further discussion). Only scorable cells (clearly defined head and tail with no interference with neighbouring cells) should be scored for % tail DNA to avoid artefacts. There is no need to report the frequency of non-scorable cells. The frequency of hedgehogs should be determined based on the visual scoring (since the absence of a clearly-defined head will mean they are not readily detected by image analysis) of at least 150 cells per sample (see paragraph 56 for further discussion) and separately documented.

52. All slides for analysis, including those of positive and negative controls, should be independently coded and scored "blinded" so the scorer is unaware of the treatment condition. For each sample (per tissue per animal), at least 150 cells (excluding hedgehogs – see paragraph 56) should be analysed. Scoring 150 cells per animal in at least 5 animals per dose (less in the concurrent positive control – see paragraph 29) provides adequate statistical power according to the analysis of Smith et al, 2008 (5). If slides are used, this could be from 2 or 3 slides scored per sample when five animals per group are used. Several areas of the slide should be observed at a density that ensures there is no overlapping of tails. Scoring at the edge of slides should be avoided.

53. DNA strand breaks in the comet assay can be measured by independent endpoints such as % tail DNA, tail length and tail moment. All three measurements can be made if the appropriate image software analyser system is used. However, the % tail DNA (also known as % tail intensity) is recommended for the evaluation and interpretation of results (12) (40) (41) (42), and is determined by the DNA fragment intensity in the tail expressed as a percentage of the cell's total intensity (13).

Tissue damage and cytotoxicity

54. Positive findings in the comet assay may not be solely due to genotoxicity, target tissue toxicity may also result in increases in DNA migration (12) (41). Conversely, low or moderate cytotoxicity is often seen with known genotoxins (12), showing that it is not possible to distinguish DNA migration induced by genotoxicity versus that induced by cytotoxicity in the comet assay alone. However, where increases in DNA migration are observed, it is recommended that an examination of one or more indicators of cytotoxicity is performed as this can aid in interpretation of the findings. Increases in DNA migration in the presence of clear evidence of cytotoxicity should be interpreted with caution.

55. Many measures of cytotoxicty have been proposed and of these histopathological changes are considered a relevant measure of tissue toxicity. Observations such as inflammation, cell infiltration, apoptotic or necrotic changes have been associated with increases in DNA migration, however, as demonstrated by the JaCVAM validation trial (12) no definitive list of histopathological changes that are always associated with increased DNA migration is available. Changes in clinical chemistry measures (e.g. AST, ALT), can also provide useful information on tissue damage and additional indicators such as caspase activation, TUNEL stain, Annexin V stain, etc. may also be considered. However, there are limited published data where the latter have been used for *in vivo* studies and some may be less reliable than others.

56. Hedgehogs (or clouds, ghost cells) are cells that exhibit a microscopic image consisting of a small or non-existent head, and large diffuse tails and are considered to be heavily damaged cells, although the etiology of the hedgehogs is uncertain (see Annex 3). Due to their appearance, % tail DNA measurements by image analysis are unreliable and therefore hedgehogs should be evaluated separately. The occurrence of hedgehogs should be noted and reported and any relevant increase thought to be due to the test chemical should be investigated and interpreted with care. Knowledge of the potential mode of action of the test substances may help with such considerations.

DATA AND REPORTING

Treatment of Results

57. The animal is the experimental unit and therefore both individual animal data and summarized results should be presented in tabular form. Due to the hierarchical nature of the data it is recommended that the median %tail DNA for each slide is determined and the mean of the median values is calculated for each animal (12). The mean of the individual animal means is then determined to give a group mean. All of these values should be included in the report. Alternative approaches (see paragraph 53) may be used if scientifically and statistically justified. Statistical analysis can be done using a variety of approaches (58) (59) (60) (61). When selecting the statistical methods to be used, the need for transformation (e.g. log or square root) of the data and/or addition of a small number (e.g. 0.001) to all (even non-zero) values to mitigate the effects of zero cell values, should be considered as discussed in the above references. Details of analysis of treatment/sex interactions when both sexes are used, and subsequent analysis of data where either differences or no differences are found is given in Annex 2. Data on toxicity and clinical signs should also be reported.

Acceptability Criteria

58. Acceptance of a test is based on the following criteria:

a. The concurrent negative control is considered acceptable for addition to the laboratory historical negative control database as described in paragraph 16

b. Concurrent positive controls (see paragraph 29) should induce responses that are compatible with those generated in the historical positive control database and produce a statistically significant increase compared with the concurrent negative control.

c. Adequate numbers of cells and doses have been analysed (paragraphs 50 and 36-38).

d. The criteria for the selection of highest dose are consistent with those described in paragraph 36.

Evaluation and Interpretation of Results

59. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if:

a) at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control,

b) the increase is dose-related when evaluated with an appropriate trend test,

c) any of the results are outside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations.

When all of these criteria are met, the test chemical is then considered able to induce DNA strand breakage in the tissues studied in this test system. If only one or two of these criteria are satisfied, see paragraph 62.

60. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if:

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

b) there is no concentration-related increase when evaluated with an appropriate trend test

c) all results are inside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations

d) direct or indirect evidence supportive of exposure of, or toxicity to, the target tissue(s) has been demonstrated.

The test chemical is then considered unable to induce DNA strand breakage in the tissues studied in this test system.

61. There is no requirement for verification of a clearly positive or negative response.

62. In case the response is neither clearly negative nor clearly positive (i.e. not all the criteria listed in paragraphs 59 or 60 are met) and in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations conducted, if scientifically justified. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using optimised experimental conditions (e.g. dose spacing, other routes of administration, other sampling times or other tissues) could be useful.

63. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results, and will therefore be concluded as equivocal.

64. To assess the biological relevance of a positive or equivocal result, information on cytotoxicity at the target tissue is required (see paragraphs 54-55). Where positive or equivocal findings are observed

solely in the presence of clear evidence of cytotoxicity, the study would be concluded as equivocal for genotoxicity unless there is enough information that is supportive of a definitive conclusion. In cases of a negative study outcome where there are signs of toxicity at all doses tested, further study at non-toxic doses may be advisable.

Test Report

65. The test report should include the following information:

Test chemical:

- source, lot number if available;
- stability of the test chemical, limit date for use, or date for re-analysis if known;

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent/vehicle:

- justification for choice of solvent/vehicle;
- solubility and stability of the test chemical in the solvent/vehicle, if known;
- preparation of dose formulations;
- analytical determinations on formulations (e.g., stability, homogeneity, nominal concentrations);

Test animals:

- species/strain used and scientific and ethical justifications for the choice;
- number, age and sex of animals;
- source, housing conditions, diet, enrichment, etc.;
- individual weight of the animals at the start and at the end of the test, including body weight range, mean and standard deviation for each group;

Test conditions:

- positive and negative (vehicle/solvent) control data;
- results from the range-finding study (if conducted);
- rationale for dose level selection;
- details of test chemical preparation;
- details of the administration of the test chemical;
- rationale for route of administration;
- site of injection (for subcutaneous or intravenous studies);
- methods for sample preparation, where available, histopathological analyses, especially for a substance giving a positive comet response;

- rationale for tissue selection;
- methods for verifying that the test chemical reached the target tissue, or general circulation, if negative results are obtained;
- actual dose (mg/kg body weight/day) calculated from diet/drinking water test chemical concentration (ppm) and consumption, if applicable;
- details of diet and water quality;
- detailed description of treatment and sampling schedules and justifications for the choices (e.g. toxicokinetic data, where available);
- method of pain relief, analgesia;
- method of euthanasia;
- procedures for isolating and preserving tissues;
- methods for preparing single cell/nucleus suspension;
- source and lot numbers of all reagents (where possible);
- methods for evaluating cytotoxicity;
- electrophoresis conditions;
- staining techniques used; and
- methods for scoring and measuring comets;

Results:

- General clinical observations, if any, prior to and throughout the test period for each animal;
- evidence of cytotoxicity if performed;
- for studies longer than one week: Individual body weights during the study, including body weight range, mean and standard deviation for each group; food consumption;
- dose-response relationship, where evident;
- for each tissue/animal, the % tail DNA (or other measures, if chosen) and median values per slide, mean values per animal and mean values per group;
- concurrent and historical negative control data with ranges, means/medians and standard deviations for each tissue evaluated;
- concurrent and historical positive control data;
- for tissues other than liver, a dose-response curve using the positive control. This can be from data collected during the demonstration of proficiency (see paragraphs 16-17) and should be accompanied by a justification, with citations to current literature, for the appropriateness of the magnitude and scatter of the responses to the controls in that tissue;
- statistical analyses and methods applied; and
- criteria for considering a response as positive, negative or equivocal;
- frequency of hedgehogs in each group and per animal;

Discussion of the results

Conclusion

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ANNEX 1

Definitions

Alkaline single cell gel electrophoresis: Sensitive technique for the detection of primary DNA damage at the level of individual cell/nucleus

Comet: The shape that nucleoids adopt after submitted to one electrophoretic field, due to its similarity to comets: the head is the nucleus and the tail is constituted by the DNA migrating out of the nucleus in the electric field.

A critical variable/parameter: This is a protocol variable for which a small change can have a large impact on the conclusion of the assay. Critical variables can be tissue-specific. Critical variables should not be altered, especially within a test, without consideration of how the alteration will alter an assay response, for example as indicated by the magnitude and variability in positive and negative controls. The test report should list alterations of critical variables made during the test or compared to the standard protocol for the laboratory and provide a justification for each alteration.

Tail intensity or % tail DNA: This corresponds to the intensity of the comet tail relative to the total intensity (head plus tail). It reflects the amount of DNA breakage, expressed as a percentage.

ANNEX 2

The Factorial Design for Identifying Sex Differences in the *in vivo* Comet Assay

The factorial design and its analysis

1. In this design, a minimum of 5 males and 5 females are tested at each concentration level resulting in a design using a minimum of 40 animals (20 males and 20 females, plus relevant positive controls.)

2. The design, which is one of the simpler factorial designs, is equivalent to a two-way analysis of variance with sex and concentration level as the main effects. The data can be analysed using many standard statistical software packages such as SPSS, SAS, STATA, Genstat as well as using R.

3. The analysis partitions the variability in the dataset into that between the sexes, between the concentrations and that related to the interaction between the sexes and the concentrations. Each of the terms is tested against an estimate of the variability between the replicate animals within the groups of animals of the same sex given the same concentration. Full details of the underlying methodology are available in many standard statistical textbooks (see references) and in the 'help' facilities provided with statistical packages.

4. The analysis proceeds by inspecting the sex x concentration interaction term in the ANOVA table[1]. In the absence of a significant interaction term the combined values across sexes or across concentration levels provide valid statistical tests between the levels based upon the pooled within group variability term of the ANOVA.

5. The analysis continues by partitioning the estimate of the between concentrations variability into contrasts which provide for a test for linear and quadratic contrasts of the responses across the concentration levels. When there is a significant sex x concentration interaction this term can also be partitioned into linear x sex and quadratic x sex interaction contrasts. These terms provide tests of whether the concentration responses are parallel for the two sexes or whether there is a differential response between the two sexes.

6. The estimate of the pooled within group variability can be used to provide pair-wise tests of the difference between means. These comparisons could be made between the means for the two sexes and between the means for the different concentration level such as for comparisons with the negative control levels. In those cases where there is a significant interaction comparisons can be made between the means of different concentrations within a sex or between the means of the sexes at the same concentration.

[1] Statisticians who take a modelling approach such as using General Linear Models (GLMs) may approach the analysis in a different but comparable way but will not necessarily derive the traditional ANOVA table which dates back to algorithmic approaches to calculating the statistics developed in a pre-computer age.

References

There are many statistical textbooks which discuss the theory, design, methodology, analysis and interpretation of factorial designs ranging from the simplest two factor analyses to the more complex forms used in Design of Experiment methodology. The following is a non-exhaustive list. Some books provide worked examples of comparable designs, in some cases with code for running the analyses using various software packages.

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ANNEX 3

Current Limitations of the Assay

Due to the current status of knowledge, several limitations are associated with the *in vivo* comet assay. It is expected that these limitations will be reduced or more narrowly defined as there is more experience with application of the assay to answer safety issues in a regulatory context.

1. Some types of DNA damage may be short-lived, i.e. may be repaired too quickly to be observed 24 hours or more after the last dose. There is no identifiable list of the types of short-lived damages, nor of the substances which are likely to cause this type of damage, nor is it known over what time period this type of damage can be detected. The optimum sampling time(s) may also be substance- or route-specific and sampling times should be determined from kinetic data (for example the time, T_{max}, at which the peak plasma or tissue concentration is achieved), when such data are available. Most of the validation studies supporting this guideline specified necropsy 2 or 3 hours following administration of the final dose. Most studies in the published literature describe administration of the final dose between 2 and 6 hours prior to sacrifice. Therefore these experiences were used as the basis for the recommendation in the test guideline that, in the absence of data indicating otherwise, the final dose should be administered at a specified time point between 2 and 6 hours prior to necropsy.

2. There are no identifiable study data that examine the sensitivity of the test for the detection of short-lived DNA damage following administration in food or drinking water compared to administration by gavage. DNA damage has been detected following administration in feed and drinking water, but there are relatively few such reports compared to the much greater experience with gavage and i.p. administration. Thus the sensitivity of the assay may be reduced for substances which induce short-lived damage administered through feed or drinking water.

3. No inter-laboratory studies have been conducted in tissues other than liver and stomach, therefore no recommendation has been established for how to achieve a sensitive and reproducible response in tissues other than liver, such as expected positive and negative control ranges. For the liver, agreement on setting a lower limit to the negative control value also could not be reached.

4. Although there are several publications demonstrating the confounding effect of cytotoxicity *in vitro*, very little data have been published *in vivo* and therefore no single measure of cytotoxicity could be recommended. Histopathological changes such as inflammation, cell infiltration, apoptotic or necrotic changes have been associated with increases in DNA migration however, as demonstrated by the JaCVAM validation trial (OECD, 2014), these changes do not always result in positive comet findings and consequently no definitive list of histopathological changes that are always associated with increased DNA migration is available. Hedgehogs (or clouds, ghost cells) have previously been suggested as an indicator of cytotoxicity, however, the etiology of the hedgehogs is uncertain. Data exist which suggest that they can be caused by substance-related cytotoxicity, mechanical/enzyme-induced damage initiated during sample preparation (Guerard et al., 2014) and/or a more extreme effect of test chemical genotoxicity. Other data seem to show they are due to extensive, but perhaps repairable DNA damage (Lorenzo et al., 2013).

5. Tissues or cell nuclei have been successfully frozen for later analysis. This usually results in a measurable effect on the response to the vehicle and positive control (Recio at al., 2010; Recio at al., 2012; Jackson at al., 2013). If used, the laboratory should demonstrate competency in freezing

methodologies and confirm acceptable low ranges of % tail DNA in target tissues of vehicle treated animals, and that positive responses can still be detected. In the literature, the freezing of tissues has been described using different methods. However, currently there is no agreement on how to best freeze and thaw tissues, and how to assess whether a potentially altered response may affect the sensitivity of the test.

6. Recent work demonstrates that the list of critical variables is expected to continue to become shorter and the parameters for critical variables more precisely defined (Guerard et al., 2014).

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Guidance for Industry

S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

June 2012
ICH

Guidance for Industry

S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use

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Contains Nonbinding Recommendations

Guidance for Industry[1]

S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use

This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. INTRODUCTION (1)[2]

A. Objectives of the Guidance (1.1)

This guidance combines and replaces two ICH guidances, *S2A Specific Aspects for Regulatory Genotoxicity Tests for Pharmaceuticals* (ICH S2A guidance) and *S2B Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals* (ICH S2B guidance). The purpose of the revision is to provide guidance on optimizing the standard genetic toxicology battery for prediction of potential human risks, and on interpreting results, with the goal of improving risk characterization for carcinogenic effects that have their basis in changes in the genetic material. The revised guidance describes internationally agreed-upon standards for follow-up testing and interpretation of positive results in vitro and in vivo in the standard genetic toxicology battery, including assessment of nonrelevant findings. This guidance is intended to apply only to products being developed as human pharmaceuticals.

B. Background (1.2)

The recommendations from the latest Organization for Economic Co-operation and Development (OECD) guidelines and the reports from the International Workshops on Genotoxicity Testing (IWGT) have been considered where relevant. In certain cases, the recommendations in this

[1] This guidance was developed within the Safety Implementation Working Group of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at *Step 4* of the ICH process, November 2011. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan, and the United States.

[2] Arabic numbers reflect the organizational breakdown of the document endorsed by the ICH Steering Committee at Step 4 of the ICH process, November 2011.

guidance are different from the OECD or the IWGT recommendations, and are noted in the text. The following notes for guidance should be applied in conjunction with other ICH guidances.

C. Scope of the Guidance (1.3)

The focus of this guidance is testing of new *small molecule* drug substances, and the guidance does not apply to biologics. Advice on the timing of the studies relative to clinical development is provided in the ICH guidance *M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals* (ICH M3(R2) guidance).[3] The recommendations in the guidance should be applied in conjunction with other ICH guidances.

D. General Principles (1.4)

Genotoxicity tests can be defined as in vitro and in vivo tests designed to detect compounds that induce genetic damage by various mechanisms. These tests enable hazard identification with respect to damage to DNA and its fixation. Fixation of damage to DNA in the form of gene mutations, larger scale chromosomal damage, or recombination is generally considered to be essential for heritable effects and in the multistep process of malignancy, a complex process in which genetic changes might possibly play only a part. Numerical chromosome changes have also been associated with tumorigenesis and can indicate a potential for aneuploidy in germ cells. Compounds that are positive in tests that detect such kinds of damage have the potential to be human carcinogens and/or mutagens. Because the relationship between exposure to particular chemicals and carcinogenesis is established for humans, although a similar relationship has been difficult to prove for heritable diseases, genotoxicity tests have been used mainly for the prediction of carcinogenicity. Nevertheless, because germ line mutations are clearly associated with human disease, the suspicion that a compound might induce heritable effects is considered to be just as serious as the suspicion that a compound might induce cancer. In addition, the outcome of genotoxicity tests can be valuable for interpreting carcinogenicity studies.

II. THE STANDARD TEST BATTERY FOR GENOTOXICITY (2)

A. Rationale (2.1)

Registration of pharmaceuticals requires a comprehensive assessment of their genotoxic potential. Extensive reviews have shown that many compounds that are mutagenic in the bacterial reverse mutation (Ames) test are rodent carcinogens. Addition of in vitro mammalian tests increases sensitivity for detection of rodent carcinogens and broadens the spectrum of genetic events detected, but also decreases the specificity of prediction (i.e., increases the incidence of positive results that do not correlate with rodent carcinogenicity). Nevertheless, a battery approach is still reasonable because no single test is capable of detecting all genotoxic mechanisms relevant in tumorigenesis.

[3] We update guidances periodically. To make sure you have the most recent version of a guidance, check the FDA Drugs guidance Web page at *http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm*.

The general features of a standard test battery are as follows:

i. Assessment of mutagenicity in a bacterial reverse gene mutation test. This test has been shown to detect relevant genetic changes and the majority of genotoxic rodent and human carcinogens.

ii. Genotoxicity should also be evaluated in mammalian cells in vitro and/or in vivo as follows.

Several in vitro mammalian cell systems are widely used and can be considered sufficiently validated: the in vitro metaphase chromosome aberration assay, the in vitro micronucleus assay (note 1) and the mouse lymphoma L5178Y cell Tk (thymidine kinase) gene mutation assay (MLA). These three assays are currently considered equally appropriate and therefore interchangeable for measurement of chromosomal damage when used together with other genotoxicity tests in a standard battery for testing of pharmaceuticals, if the test protocols recommended in this guidance are used. In vivo test(s) are included in the test battery because some agents are mutagenic in vivo but not in vitro (note 2) and because it is desirable to include assays that account for such factors as absorption, distribution, metabolism, and excretion. The choice of an analysis either of micronuclei in erythrocytes (in blood or bone marrow), or of chromosome aberrations in metaphase cells in bone marrow, is currently included for this reason (note 3). Lymphocytes cultured from treated animals can also be used for cytogenetic analysis, although experience with such analyses is less widespread.

In vitro and in vivo tests that measure chromosomal aberrations in metaphase cells can detect a wide spectrum of changes in chromosomal integrity. Breakage of chromatids or chromosomes can result in micronucleus formation if an acentric fragment is produced; therefore, assays that detect either chromosomal aberrations or micronuclei are considered appropriate for detecting clastogens. Micronuclei can also result from lagging of one or more whole chromosome(s) at anaphase and thus micronucleus tests have the potential to detect some aneuploidy inducers. The MLA detects mutations in the Tk gene that result from both gene mutations and chromosome damage. There is some evidence that MLA can also detect chromosome loss.

There are several additional in vivo assays that can be used in the battery or as follow-up tests to develop weight of evidence in assessing results of in vitro or in vivo assays (see below). Negative results in appropriate in vivo assays (usually two), with adequate justification for the endpoints measured, and demonstration of exposure (see section IV.D (4.4)) are generally considered sufficient to demonstrate absence of significant genotoxic risk.

B. Description of the Two Options for the Standard Battery (2.2)

The following two options for the standard battery are considered equally suitable (see note 4):

Option 1

i. A test for gene mutation in bacteria.

ii. A cytogenetic test for chromosomal damage (the in vitro metaphase chromosome aberration test or in vitro micronucleus test), or an in vitro mouse lymphoma Tk gene mutation assay.

iii. An in vivo test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells.

Option 2

i. A test for gene mutation in bacteria.

ii. An in vivo assessment of genotoxicity with two different tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second in vivo assay. Typically, this would be a DNA strand breakage assay in liver, unless otherwise justified (see below; also section IV.B (4.2) and note 12).

There is more historical experience with Option 1, partly because it is based on the ICH S2A guidance and the ICH S2B guidance. Nevertheless, the reasoning behind considering Options 1 and 2 equally suitable is as follows: When a positive result occurs in an in vitro mammalian cell assay, clearly negative results in two well-conducted in vivo assays, in appropriate tissues and with demonstrated adequate exposure, are considered sufficient evidence for lack of genotoxic potential in vivo (see section V.D.1.i (5.4.1.1) below). Thus, a test strategy in which two in vivo assays are conducted is the same strategy that should be used to follow up a positive result in vitro (see note 4).

Under both standard battery options, either acute or repeat-dose study designs in vivo can be used. In case of repeated administrations, attempts should be made to incorporate the genotoxicity endpoints into toxicity studies, if scientifically justified. When more than one endpoint is evaluated in vivo, it is preferable that they are incorporated into a single study. Often sufficient information on the likely suitability of the doses for the repeat-dose toxicology study is available before the study begins and can be used to determine whether an acute or an integrated test would be suitable.

For compounds that give negative results, the completion of either option of the standard test battery, performed and evaluated in accordance with current recommendations, will usually provide sufficient assurance of the absence of genotoxic activity and no additional tests are warranted. Compounds that give positive results in the standard test battery might, depending on their therapeutic use, need to be tested more extensively (see section V (5)).

There are several in vivo assays that can be used as the second part of the in vivo assessment under Option 2 (see section IV.B (4.2)), some of which can be integrated into repeat-dose toxicology studies. The liver is typically the preferred tissue because of exposure and metabolizing capacity, but choice of in vivo tissue and assay should be based on factors such as any knowledge of the potential mechanism, of the metabolism in vivo, or of the exposed tissues thought to be relevant.

Information on numerical changes can be derived from the mammalian cell assays in vitro and from the micronucleus assays in vitro or in vivo. Elements of the standard protocols that can indicate such potential are elevations in the mitotic index, polyploidy induction, and micronucleus evaluation. There is also experimental evidence that spindle poisons can be detected in MLA. The preferred in vivo cytogenetic test under Option 2 is the micronucleus assay, not a chromosome aberration assay, to include more direct capability for detection of chromosome loss (potential for aneuploidy).

The suggested standard set of tests does not imply that other genotoxicity tests are generally considered inadequate or inappropriate. Additional tests can be used for further investigation of genotoxicity test results obtained in the standard battery (see sections IV.B (4.2) and V (5)). Alternative species, including nonrodents, can also be used if indicated, and if sufficiently validated.

Under conditions in which one or more tests in the standard battery cannot be employed for technical reasons, alternative validated tests can serve as substitutes, provided sufficient scientific justification is given.

C. Modifications to the Test Battery (2.3)

The following sections describe situations where modification of the standard test battery might be advisable.

1. Exploratory Clinical Studies (2.3.1)

For certain exploratory clinical studies, fewer genotoxicity assays or different criteria for justification of the maximum dose in vivo might apply (see ICH M3(R2) guidance).

2. Testing Compounds That Are Toxic to Bacteria (2.3.2)

In cases where compounds are highly toxic to bacteria (e.g., some antibiotics), the bacterial reverse mutation (Ames) test should still be carried out, just as cytotoxic compounds are tested in mammalian cells, because mutagenicity can occur at lower, less toxic concentrations. In such cases, any one of the in vitro mammalian cell assays should also be done (i.e., Option 1 should be followed).

3. Compounds Bearing Structural Alerts for Genotoxic Activity (2.3.3)

Structurally alerting compounds (note 5) are usually detectable in the standard test battery since the majority of *structural alerts* are defined in relation to bacterial mutagenicity. A few chemical classes are known to be more easily detected in mammalian cell chromosome damage assays than bacterial mutation assays. Thus, negative results in either test battery with a compound that has a structural alert is usually considered sufficient assurance of a lack of genotoxicity. However, for compounds bearing certain specific structural alerts, modification to standard protocols can be appropriate (note 5). The choice of additional test(s) or protocol

modification(s) depends on the chemical nature, the known reactivity, and any metabolism data on the structurally alerting compound in question.

4. Limitations to the Use of In Vivo Tests (2.3.4)

There are compounds for which many in vivo tests (typically in bone marrow, blood, or liver) do not provide additional useful information. These include compounds for which data on toxicokinetics or pharmacokinetics indicate that the compounds are not systemically absorbed and therefore are not available to the target tissues. Examples of such compounds are some radioimaging agents, aluminum-based antacids, some compounds given by inhalation, and some dermally or other topically applied pharmaceuticals. In cases where a modification of the route of administration does not provide sufficient target tissue exposure, and no suitable genotoxicity assay is available in the most exposed tissue, it might be appropriate to base the evaluation only on in vitro testing. In some cases, evaluation of genotoxic effects at the site of contact can be warranted, although such assays have not yet been widely used (note 6).

D. Detection of Germ Cell Mutagens (2.4)

Results of comparative studies have shown that, in a qualitative sense, most germ cell mutagens are likely to be detected as genotoxic in somatic cell tests so that negative results of in vivo somatic cell genotoxicity tests generally indicate the absence of germ cell effects.

III. RECOMMENDATIONS FOR IN VITRO TESTS (3)

A. Test Repetition and Interpretation (3.1)

Reproducibility of experimental results is an essential component of research involving novel methods or unexpected findings; however, the routine testing of drugs with standard, widely used genotoxicity tests often does not call for replication. These tests are sufficiently well-characterized and have sufficient internal controls that repetition of a clearly positive or negative assay is not usually warranted. Ideally, it should be possible to declare test results clearly negative or clearly positive. However, test results sometimes do not fit the predetermined criteria for a positive or negative call and therefore are declared *equivocal*. The application of statistical methods can aid in data interpretation; however, adequate biological interpretation is of critical importance. An equivocal test that is repeated might result in (1) a clearly positive outcome, and thus an overall positive result; (2) a negative outcome, so that the result is not reproducible and overall negative, or (3) another equivocal result, with a final conclusion that remains equivocal.

B. Recommended Protocol for the Bacterial Mutation Assay (3.2)

Advice on the protocols is given in the OECD guidelines (1997) and the IWGT report (Gatehouse et al., 1994).

1. Selection of Top Dose Level (3.2.1)

Maximum dose level

The maximum dose level recommended is 5000 micrograms (µg)/plate (or 5 microliters (µL)/plate for liquid test substance) when not limited by solubility or cytotoxicity.

Limit of solubility

For bacterial cultures, precipitating doses are scored, provided precipitate does not interfere with scoring, toxicity is not limiting the evaluation of the assay, and the top concentration does not exceed 5000 µg/plate (or 5 µL/plate for liquid test substance). If no cytotoxicity is observed, then the lowest precipitating dose should be used as the top dose scored. If dose-related cytotoxicity or mutagenicity is noted, irrespective of solubility, the top dose scored should be based on cytotoxicity as described below.

Limit of cytotoxicity

In the Ames test, the doses scored should show evidence of significant toxicity, but without exceeding a top dose of 5000 µg/plate. Toxicity might be detected by a reduction in the number of revertants, and/or clearing or diminution of the background lawn.

2. Study Design/Test Protocol (3.2.2)

The recommended set of bacterial strains (OECD) includes those that detect base substitution and frameshift mutations as follows:

- *Salmonella typhimurium* TA98
- *Salmonella typhimurium* TA100
- *Salmonella typhimurium* TA1535
- *Salmonella typhimurium* TA1537 or TA97 or TA97a
- *Salmonella typhimurium* TA102 or *Escherichia coli* WP2 *uvrA* or *Escherichia coli* WP2 *uvrA* (pKM101)

One difference from the OECD and the IWGT recommendations is that based on experience with testing pharmaceuticals, a single bacterial mutation (Ames) test is considered sufficient when it is clearly negative or positive, and is carried out with a fully adequate protocol, including all strains with and without metabolic activation, a suitable dose range that fulfills criteria for top dose selection, and appropriate positive and negative controls. Also, for testing pharmaceuticals, either the plate incorporation or the pre-incubation method is considered appropriate for this single experiment (note 7). Equivocal or weak positive results might indicate that it would be appropriate to repeat the test, possibly with a modified protocol such as appropriate spacing of dose levels.

C. Recommended Protocols for the Mammalian Cell Assays (3.3)

Advice on the protocols is given in the OECD guidelines (1997) and the IWGT publications (e.g., Kirsch-Volders et al., 2003; Moore et al., 2006); advice on interpreting MLA results is also

given (Moore et al., 2006), including use of a global evaluation factor. Several differences from these recommendations are noted here for testing pharmaceuticals, notably for selection of the top concentration (see details below).

1. Selection of Top Concentration (3.3.1)

Maximum concentration

The maximum top concentration recommended is 1 millimolar (mM) or 0.5 milligram (mg)/milliliter (mL), whichever is lower, when not limited by solubility in solvent or culture medium or by cytotoxicity (note 8).

Limit of solubility

When solubility is limiting the ability to achieve the maximal concentration, the maximum concentration, if not limited by cytotoxicity, should be the lowest concentration at which minimal precipitate is visible in cultures, provided there is no interference with scoring. Evaluation of precipitation can be done by naked eye or by methods such as light microscopy, noting precipitate that persists or appears during culture (by the end of treatment).

Cytotoxicity

For in vitro cytogenetic assays for metaphase chromosome aberrations or for micronuclei, cytotoxicity should not exceed a reduction of about 50 percent in cell growth (notes 9 and 10). For the MLA, at the top dose there should be 80 to 90 percent cytotoxicity as measured by a relative total growth (RTG) between 20 to 10 percent (note 9).

2. Study Design/Test Protocols (3.3.2)

For the cytogenetic evaluation of chromosomal damage in metaphase cells in vitro, the test protocol should include the conduct of tests with and without metabolic activation, with appropriate positive and negative controls. Treatment with the test articles should be for 3 to 6 hours with a sampling time approximately 1.5 normal cell cycles from the beginning of the treatment. A continuous treatment without metabolic activation up to the sampling time of approximately 1.5 normal cell cycles should be conducted in case of negative or equivocal results for both short treatments, with and without metabolic activation. The same principles apply to the in vitro micronucleus assay, except that the sampling time is typically 1.5 to 2 normal cell cycles from the beginning of treatment to allow cells to complete mitosis and enter the next interphase. For both in vitro cytogenetic assays, there might be a need to modify the protocol for certain types of chemicals that could be more readily detected by longer treatment, delayed sampling times, or recovery periods (e.g., some nucleoside analogues and some nitrosamines). In the metaphase aberration assay, information on the ploidy status should be obtained by recording the incidence of polyploid (including endoreduplicated) metaphases as a percentage of the number of metaphase cells. For MLA, the test protocol should include the conduct of tests with and without metabolic activation, with appropriate positive and negative controls, where the treatment with the test article is for 3 to 4 hours. A continuous treatment

without metabolic activation for approximately 24 hours should be conducted in case of a negative or equivocal result for both short treatments, with and without metabolic activation. A standard MLA should include (1) the incorporation of positive controls that induce mainly small colonies and (2) colony sizing for positive controls, solvent controls, and at least one positive test compound concentration (should any exist), including the culture that gave the greatest mutant frequency.

For mammalian cell assays in vitro, built-in confirmatory elements, such as those outlined above (e.g., different treatment lengths, tests with and without metabolic activation), should be used. Following such testing, further confirmatory testing in the case of clearly negative or positive test results is not usually warranted. Equivocal or weak positive results might call for repeating tests, possibly with a modified protocol such as appropriate spacing of the test concentrations.

3. Positive Controls (3.3.3)

Concurrent positive controls are important, but *in vitro* mammalian cell tests for genetic toxicity are sufficiently standardized that use of positive controls can generally be confined to a positive control with metabolic activation (when it is done concurrently with the non-activated test) to demonstrate the activity of the metabolic activation system and the responsiveness of the test system.

IV. RECOMMENDATIONS FOR IN VIVO TESTS (4)

A. Tests for the Detection of Chromosome Damage In Vivo (4.1)

Either the analysis of chromosomal aberrations or the measurement of micronucleated polychromatic erythrocytes in bone marrow cells in vivo is considered appropriate for the detection of clastogens. Both rats and mice are considered appropriate for use in the bone marrow micronucleus test. Micronuclei can also be measured in immature (e.g., polychromatic) erythrocytes in peripheral blood in the mouse, or in the newly formed reticulocytes in rat blood (note 3). Likewise, immature erythrocytes can be used from any other species that has shown an adequate sensitivity to detect clastogens/aneuploidy inducers in bone marrow or peripheral blood (note 3). Systems for automated analysis (image analysis and flow cytometry) can be used if appropriately validated (OECD, 1997; Hayashi et al., 2000; 2007). Chromosomal aberrations can also be analyzed in peripheral lymphocytes cultured from treated rodents (note 11).

B. Other In Vivo Genotoxicity Tests (4.2)

The same in vivo tests described as the second test in the standard battery (Option 2) can be used as follow-up tests to develop weight of evidence in assessing results of in vitro or in vivo assays (notes 11 and 12). Although the type of effect seen in vitro and any knowledge of the mechanism can help guide the choice of in vivo assay, investigation of chromosomal aberrations or of gene mutations in endogenous genes is not feasible with standard methods in most tissues. Although mutation can be measured in transgenes in rodents, this entails prolonged treatment (e.g., 28 days) to allow for mutation expression, fixation, and accumulation, especially in tissues with little cell division (see note 12). Thus the second in vivo assay will often evaluate a DNA

damage endpoint as a surrogate. Assays with the most published experience and advice on protocols include the DNA strand break assays, such as the single cell gel electrophoresis (*Comet*) assay and alkaline elution assay, the in vivo transgenic mouse mutation assays and DNA covalent binding assays (all of which can be applied in many tissues (note 12)), and the liver unscheduled DNA synthesis (UDS) assay.

C. Dose Selection for In Vivo Assays (4.3)

Typically, three dose levels are analyzed (Hayashi et al., 2007).

1. Short-term Studies (4.3.1)

For short-term (usually 1 to 3 administrations) studies, the top dose recommended for genotoxicity assays is a limit dose of 2000 mg/kilogram (kg), if this is tolerated, or a maximum tolerated dose defined (for example, for the micronucleus assay (OECD)) as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Similar recommendations have been made for the Comet assay (Hartmann et al., 2003) and transgenic mutation assay (Heddle et al., 2000). Suppression of bone marrow red blood cell production should also be taken into account in dose selection. Lower doses are generally spaced at approximately two- to three-fold intervals below this dose.

2. Multiple Administration Studies (4.3.2)

Option 1 Battery: When the in vivo genotoxicity test is integrated into a multiple administration toxicology study, the doses are generally considered appropriate when the toxicology study meets the criteria for an adequate study to support human clinical trials; this can differ from dose selection criteria in the OECD guidelines for the in vivo micronucleus assay. This applies when the in vitro mammalian cell test is negative (or *nonrelevant positive* (see section V (5))).

Follow-up studies or Option 2 battery: When carrying out follow-up studies to address any indication of genotoxicity, or when using Option 2 with no in vitro mammalian cell assay, several factors should be evaluated to determine whether the top dose is appropriate for genotoxicity evaluation. Any one of the criteria listed below is considered sufficient to demonstrate that the top dose in a toxicology study (typically in rats) is appropriate for micronucleus analysis and for other genotoxicity evaluation:

i. Maximum feasible dose (MFD) based on physicochemical properties of the drug in the vehicle (provided the MFD in that vehicle is similar to that achievable with acute administration; note 13).

ii. Limit dose of 1000 mg/kg for studies of 14 days or longer, if this is tolerated.

iii. Maximal possible exposure demonstrated either by reaching a plateau/saturation in exposure or by compound accumulation. In contrast, substantial reduction in exposure to parent drug with time (e.g., ≥ 50% reduction from initial exposure) can disqualify the study (unless a blood sample taken in the first few days is available). If

this is seen in one sex, generally the sex with reduced exposure would not be scored at the end of the study, unless there is enhanced exposure to a metabolite of interest.

iv. Top dose is ≥ 50 percent of the top dose that would be used for acute administration, i.e., close to the minimum lethal dose, if such acute data are available for other reasons. (The top dose for acute administration micronucleus tests is currently described in the OECD guidelines as the dose above which lethality would be expected; similar guidance is given (e.g., Hartmann et al., 2003) for other in vivo assays.)

Selection of a top dose based only on an exposure margin (multiple over clinical exposure) without toxicity is not considered sufficient justification.

3. Testing Compounds That Are Toxic for Blood or Marrow (4.3.3)

Many compounds that induce aneuploidy, such as potent spindle poisons, are detectable in in vivo micronucleus assays in bone marrow or blood only within a narrow range of doses approaching toxic doses. This is also true for some clastogens. If toxicological data indicate severe toxicity to the red blood cell lineage (e.g., marked suppression of PCEs (polychromatic erythrocytes) or reticulocytes), doses scored should be spaced not more than about two fold below the top, cytotoxic dose. If suitable doses are not included in a multiweek study, additional data that could contribute to the detection of aneugens and some toxic clastogens could be derived from any one of the following:

i. Early blood sampling (at 3 to 4 days) is advisable when there are marked increases in toxicity with increasing treatment time. For example, when blood or bone marrow is used for micronucleus measurement in a multiweek study (e.g., 28 days), and reticulocytes are scored, marked hematotoxicity can affect the ability to detect micronuclei (i.e., a dose that induces detectable increases in micronuclei after acute treatment might be too toxic to analyze after multiple treatments (Hamada et al., 2001)). The early sample can be used to provide assurance that clastogens and potential aneugens are detected (but see notes 14 and 15).

ii. An in vitro mammalian cell micronucleus assay.

iii. An acute bone marrow micronucleus assay.

D. Demonstration of Target Tissue Exposure for Negative In Vivo Test Results (4.4)

In vivo tests have an important role in genotoxicity test strategies. The value of in vivo results is directly related to the demonstration of adequate exposure of the target tissue to the test compound. This is especially true for negative in vivo test results when in vitro test(s) have shown convincing evidence of genotoxicity, or when no in vitro mammalian cell assay is used. Evidence of adequate exposure could include toxicity in the tissue in question, or toxicokinetic data as described in the following section.

1. When an In Vitro Genotoxicity Test Is Positive (or Not Done) (4.4.1)

Assessments of in vivo exposure should be made at the top dose or other relevant doses using the same species, strain, and dosing route used in the genotoxicity assay. When genotoxicity is measured in toxicology assays, exposure information is generally available as part of the toxicology assessment.

Demonstration of in vivo exposure should be made by any of the following measurements:

i. Cytotoxicity

   a. For cytogenetic assays: By obtaining a significant change in the proportion of immature erythrocytes among total erythrocytes in the tissue used (bone marrow or blood) at the doses and sampling times used in the micronucleus test or by measuring a significant reduction in mitotic index for the chromosomal aberration assay.

   b. For other in vivo genotoxicity assays: Toxicity in the liver or tissue being assessed (e.g., by histopathological evaluation or blood biochemistry toxicity indicators).

ii. Exposure

   a. Measurement of drug-related material either in blood or plasma. The bone marrow is a well-perfused tissue, and levels of drug-related materials in blood or plasma are generally similar to those observed in bone marrow. The liver is expected to be exposed for drugs with systemic exposure regardless of the route of administration.

   b. Direct measurement of drug-related material in target tissue, or autoradiographic assessment of tissue exposure.

If systemic exposure is similar to or lower than expected clinical exposure, alternative strategies might be called for such as:

(i) Use of a different route of administration;
(ii) Use of a different species with higher exposure;
(iii) Use of a different tissue or assay (see section II.C.4 (2.3.4), Limitations to the Use of Standard In Vivo Tests).

When adequate exposure cannot be achieved (e.g., with compounds showing very poor target tissue availability), conventional in vivo genotoxicity tests have little value.

2. When In Vitro Genotoxicity Tests Are Negative (4.4.2)

If in vitro tests do not show genotoxic potential, in vivo (systemic) exposure can be assessed by any of the methods above, or can be assumed from the results of standard absorption, distribution, metabolism, and excretion (ADME) studies in rodents done for other purposes.

E. Sampling Times for In Vivo Assays (4.5)

Selection of the sampling time in the in vivo MN (micronucleus), chromosomal aberration, and UDS test should follow OECD (1997).

When micronucleus analysis is integrated into multiweek studies, sampling of blood or bone marrow can be done the day after the final administration (see recommendation for additional blood sampling time in section III.C.3 (3.3.3) above).

For other genotoxicity assays, sampling time should be selected as appropriate for the endpoint measured; for example, DNA damage/strand break measurements are usually made a few (e.g., 2 to 6) hours after the last administration for the multiple daily administration. In the case of single administration, two sampling times should be used: a few hours and 24 hours after the treatment.

In principle, studies of any length can be considered appropriate, provided the top dose/exposure is adequate.

F. Number of Animals Analyzed (4.6)

The number of animals analyzed is determined by current recommendations for the micronucleus assay (OECD) or other genotoxicity assays and generally does not include all the animals treated for a toxicology study. Animals used for genotoxicity analyses should be randomly selected from the group used for the toxicology study.

G. Use of Male/Female Rodents in In Vivo Genotoxicity Tests (4.7)

If sex-specific drugs are to be tested, then the assay can be done in the appropriate sex. In vivo tests with the acute protocol can generally be carried out in only one sex. For acute tests, both sexes should be considered only if any existing toxicity, metabolism, or exposure (Cmax (peak concentration) or AUC (area under the plasma concentration curve)) data indicate a toxicologically meaningful sex difference in the species being used. Otherwise, the use of males alone is considered appropriate for acute genotoxicity tests. When the genotoxicity test is integrated into a repeat-dose toxicology study in two sexes, samples can be collected from both sexes, but a single sex can be scored if there is no substantial sex difference evident in toxicity/metabolism. The dose levels for the sex(es) scored should meet the criteria for appropriate dose levels (sections IV.C.2 (4.3.2) and IV.C.3 (4.3.3)).

Similar principles can be applied for other established in vivo genotoxicity tests.

H. Route of Administration (4.8)

The route of administration is generally the expected clinical route (e.g., oral, intravenous, or subcutaneous) but can be modified if appropriate to obtain systemic exposure (e.g., for topically applied compounds (see section II.C.4 (2.3.4)).

I. Use of Positive Controls for In Vivo Studies (4.9)

For in vivo studies, it is considered sufficient to treat animals with a positive control only periodically, and not concurrently with every assay, after a laboratory has established competence in the use of the assay (note 16).

V. GUIDANCE ON EVALUATION OF TEST RESULTS AND ON FOLLOW-UP TEST STATEGIES (5)

Comparative trials have shown conclusively that each in vitro test system generates both false negative and false positive results in relation to predicting rodent carcinogenicity. Genotoxicity test batteries (of in vitro and in vivo tests) detect carcinogens that are thought to act primarily via a mechanism involving direct genetic damage, such as the majority of known human carcinogens. Therefore, these batteries are not expected to detect nongenotoxic carcinogens. Experimental conditions, such as the limited capability of the in vitro metabolic activation systems, can lead to false negative results in in vitro tests. The test battery approach is designed to reduce the risk of false negative results for compounds with genotoxic potential. On the other hand a positive result in any assay for genotoxicity does not always mean that the test compound poses a genotoxic/carcinogenic hazard to humans.

Although positive in vitro data could indicate intrinsic genotoxic properties of a drug, appropriate in vivo data determine the biological significance of these in vitro signals in most cases. Also, because there are several indirect mechanisms of genotoxicity that operate only above certain concentrations, it is possible to establish a safe level (threshold) for classes of drugs with evidence for such mechanisms (see section V.B (5.2) below; see also Müller and Kasper, 2000; Scott et al., 1991; Thybaud et al., 2007).

A. Assessment of Biological Relevance (5.1)

The recommendations below assume that the test has been conducted using appropriate conditions such as spacing of doses and levels of toxicity.

Small increases in apparent genotoxicity in vitro or in vivo should first be assessed for reproducibility and biological significance. Examples of results that are not considered biologically meaningful include:

i. Small increases that are statistically significant compared with the negative or solvent control values but are within the confidence intervals of the appropriate historical control values for the testing facility

ii. Weak/equivocal responses that are not reproducible

If either of the above conditions applies, the weight of evidence indicates a lack of genotoxic potential, the test is considered negative or the findings not biologically relevant, and no further testing is called for.

B. Evaluation of Results Obtained in In Vitro Tests (5.2)

In evaluating positive results, especially for the microbial mutagenicity test, the purity of the test compound should be considered to determine whether the positive result could be attributable to a contaminant.

1. Evaluation of Positive Results Obtained In Vitro in a Bacterial Mutation Assay (5.2.1)

Because positive results in the Ames test are thought to indicate DNA reactivity, extensive follow-up testing to assess the in vivo mutagenic and carcinogenic potential would be warranted to assess the potential risk for treatment of patients, unless justified by appropriate risk-benefit analysis.

There are some well-characterized examples of artifactual increases in colonies that are not truly revertants. These increases can occur due to contamination with amino acids (i.e., providing histidine for *Salmonella typhimurium* strains or tryptophan for *Escherichia coli* strains), so that the bacterial reversion assay is not suitable for testing a peptide that is likely to degrade. Certain cases exist where positive results in bacterial mutation assays might be shown not to indicate genotoxic potential in vivo in humans, for example, when bacterial-specific metabolism occurs, such as activation by bacterial nitroreductases.

2. Evaluation of Positive Results Obtained In Vitro in Mammalian Cell Assays (5.2.1)

Recommendations for assessing weight of evidence and follow-up testing for positive genotoxicity results are discussed in IWGT reports (e.g., Thybaud et al., 2007). In addition, the scientific literature gives a number of conditions that can lead to a positive in vitro result of questionable relevance. Therefore, any in vitro positive test result should be evaluated based on an assessment of the weight of evidence as indicated below. This list is not exhaustive, but is given as an aid to decision-making.

i. The conditions do not occur in vivo (pH; osmolality; precipitates).
(Note that the 1 mM limit avoids increases in osmolality, and that if the test compound alters pH, it is advisable to adjust pH to the normal pH of untreated cultures at the time of treatment).

ii. The effect occurs only at the most toxic concentrations.
-in the MLA increases, at ≥ 80 percent reduction in RTG
-for in vitro cytogenetic assays, when growth is suppressed by ≥ 50 percent

If any of the above conditions apply, the weight of evidence indicates a lack of genotoxic potential; the standard battery (Option 1) can be followed. Thus, a single in vivo test is considered sufficient.

3. Evaluation of In Vitro Negative Results (5.2.3)

For in vitro negative results, further testing should be considered in special cases, such as the following (the examples given are not exhaustive, but are given as an aid to decision-making): the structure or known metabolism of the compound indicates that standard techniques for in vitro metabolic activation (e.g., rodent liver S9) might be inadequate; the structure or known activity of the compound indicates that the use of other test methods/systems might be appropriate.

C. Evaluation of Results Obtained From In Vivo Tests (5.3)

In vivo tests have the advantage of taking into account absorption, distribution, and excretion, which are not factors in in vitro tests, but are potentially relevant to human use. In addition, metabolism is likely to be more relevant in vivo compared to the systems normally used in vitro. If the in vivo and in vitro results do not agree, then the difference should be considered/explained on a case-by-case basis (e.g., a difference in metabolism; rapid and efficient excretion of a compound in vivo).

In vivo genotoxicity tests also have the potential to give misleading positive results that do not indicate true genotoxicity. As examples:

(i) Increases in micronuclei can occur without administration of any genotoxic agent, due to disturbance in erythropoiesis (Tweats et al., 2007, I).

(ii) DNA adduct data should be interpreted in the light of the known background level of endogenous adducts.

(iii) Indirect, toxicity-related effects could influence the results of the DNA strand break assays (e.g., alkaline elution and Comet assays).

Thus, it is important to take into account all the toxicological and hematological findings when evaluating the genotoxicity data (note 15). Indirect effects related to toxicological changes could have a safety margin and might not be clinically relevant.

D. Follow-up Strategies for Positive Results (5.4)

1. Follow-up to Findings In Vitro in Mammalian Cell Tests (5.4.1)

The following discussion assumes negative results in the Ames bacterial mutation assay.

i. Mechanistic/in vivo follow-up (5.4.1.1)

When there is insufficient weight of evidence to indicate lack of relevance, recommended follow-up for positive mammalian cell assays would be to provide experimental evidence, either by additional in vitro studies (see item *a* below) **or** by carrying out two appropriate in vivo assays (see item *b* below), as follows:

a. Mechanistic information that contributes to a weight of evidence for a lack of relevant genotoxicity is often generated in vitro, for example evidence that a test compound that induces chromosome aberrations or mutations in the MLA is not a DNA damaging agent (e.g., other negative mutation/DNA damage tests in addition to the Ames test; structural considerations), or evidence for an indirect mechanism that might not be relevant in vivo or might have a threshold (e.g., inhibition of DNA synthesis; reactive oxygen species produced only at high concentrations) (Galloway et al., 1998; Scott et al., 1991; Müller and Kasper, 2000). Similar studies can be used to follow up a positive result in the in vitro micronucleus assay, or in this case, evidence can include a known mechanism that indicates chromosome loss/aneuploidy, or centromere staining experiments (note 17) that indicate chromosome loss. Polyploidy is a common finding in chromosome aberration assays in vitro. Although aneugens can induce polyploidy, polyploidy alone does not indicate aneugenic potential and can simply indicate cell cycle perturbation; it is also commonly associated with increasing cytotoxicity. If polyploidy, but no structural chromosome breakage, is seen in an in vitro assay, generally a negative in vivo micronucleus assay with assurance of appropriate exposure would provide sufficient assurance of lack of potential for aneuploidy induction.

   If the above mechanistic information and weight of evidence supports the lack of relevant genotoxicity, only a single in vivo test with appropriate evidence of exposure is called for to establish the lack of genotoxic activity. This is typically a cytogenetic assay, and the micronucleus assay in vivo is called for when following up potential for chromosome loss.

   If there is not sufficient weight of evidence or mechanistic information to rule out relevant genotoxic potential, two in vivo tests are generally called for, with appropriate endpoints and in appropriate tissues (usually two different tissues), and with an emphasis on obtaining sufficient exposure in the in vivo models.

Or

b. Two appropriate in vivo assays should be done, usually with different tissues, and with supporting demonstration of exposure.

In summary, negative results in appropriate in vivo assays, with adequate justification for the endpoints measured and demonstration of exposure (see section IV.D.1 (4.4.1)) are considered sufficient to demonstrate absence of significant genotoxic risk.

ii. Follow-up to an in vitro positive result that is dependent upon S9 activation (5.4.1.2)

When positive results are seen only in the presence of the S9 activation system, it should first be verified that metabolic activation is responsible and not some other difference in conditions (e.g., low or no serum in the S9 mix, compared with ≥ 10 percent serum in the non-activated incubations). The follow-up strategy is then aimed at determining the relevance of the results in vitro to conditions in vivo, and will generally focus on in vivo studies in liver (note 18).

2. Follow-up to a Positive In Vivo Micronucleus Assay (5.4.2)

If there is an increase in micronuclei in vivo, all the toxicological data should be evaluated to determine whether a nongenotoxic effect could be the cause or a contributing factor (note 15). If nonspecific effects of disturbed erythropoiesis or physiology (such as hypo/hyperthermia) are suspected, an in vivo assay for chromosome aberrations might be more appropriate. If a *real* increase is suspected, strategies should be used to demonstrate whether the increase is due to chromosome loss or chromosome breakage (note 17). There is evidence that aneuploidy induction (e.g., with spindle poisons) follows a nonlinear dose response. Thus, it might be possible to determine that there is a threshold exposure below which chromosome loss is not expected and to determine whether an appropriate safety margin exists compared with clinical exposure.

In conclusion, the assessment of the genotoxic potential of a compound should take into account the totality of the findings and acknowledge the intrinsic values and limitations of both in vitro and in vivo tests.

E. Follow-up Genotoxicity Testing in Relation to Tumor Findings in a Carcinogenicity Bioassay (5.5)

Additional genotoxicity testing in appropriate models can be conducted for compounds that were negative in the standard test battery but which have shown increases in tumors in carcinogenicity bioassay(s) with insufficient evidence to establish a nongenotoxic mechanism. To help understand the mode of action, additional testing can include modified conditions for metabolic activation in in vitro tests or can include in vivo tests measuring genetic damage in target organs of tumor induction, such as DNA strand break assays (e.g., comet or alkaline elution assays), liver UDS test, DNA covalent binding (e.g., by ^{32}P-postlabeling), mutation induction in transgenes, or molecular characterization of genetic changes in tumor-related genes (Kasper et al., 2007).

VI. NOTES (6)

Note 1. The in vitro micronucleus assay has been widely evaluated in international collaborative studies (Kirsch-Volders et al., 2003), is validated by the European Center for the Validation of Alternative Methods (ECVAM) (Corvi et al., 2008), and is the subject of an OECD guideline 487 (2010).

Note 2. There is a small but significant number of genotoxic carcinogens that are reliably detected by the bone marrow tests for chromosomal damage but have yielded negative/weak/conflicting results in the in vitro tests outlined in the standard battery options. Carcinogens such as procarbazine, hydroquinone, urethane, and benzene fall into this category. Some other examples from a survey of companies are described by Tweats et al., 2007, II.

Note 3. In principle, micronuclei in hematopoietic cells can be evaluated in bone marrow from any species, and in blood from species that do not filter out circulating micronucleated erythrocytes in the spleen. In laboratory mice, micronuclei can be measured in polychromatic erythrocytes in blood, and mature (normochromatic) erythrocytes can be used when mice are treated continuously for about 4 weeks or more. Although rats rapidly remove micronucleated erythrocytes from the circulation, it has been established that micronucleus induction by a range of clastogens and aneugens can be detected in rat blood reticulocytes (Wakata et al., 1998; Hamada et al., 2001). Rat blood can be used for micronucleus analysis, provided methods are used to ensure analysis of the newly formed reticulocytes (Hayashi et al., 2007; MacGregor et al., 2006) and the sample size is sufficiently large to provide appropriate statistical sensitivity, given the lower micronucleus levels in rat blood than in bone marrow (Kissling et al., 2007). Whichever method is chosen, bone marrow or blood, automated or manual analysis, each laboratory should determine the appropriate minimum sample size to ensure that scoring error is maintained below the level of animal-to-animal variation.

Some experience is now available for micronucleus induction in the dog and rhesus monkey (Harper et al., 2007; Hotchkiss et al., 2008). One example where such alternative species might be useful would be in evaluation of a human metabolite that was not sufficiently represented in rodents but was formed in the dog or monkey.

Note 4. Although the two options in the battery are equally suitable, specific knowledge about an individual test compound can indicate that one option is preferable. For example, if systemic exposure in animal models is equal to or less than anticipated clinical exposure, in vitro assays should be employed: Option 1 (see also sections II.C.4 (2.3.4) and IV.D.1 (4.4.1)). On the other hand, Option 2, including a test in liver, is recommended in cases where short-lived reactive metabolites are expected to be generated in the liver.

Note 5. Certain structurally alerting molecular entities are recognized as being causally related to the carcinogenic and/or mutagenic potential of chemicals. Examples of structural alerts include alkylating electrophilic centers, unstable epoxides, aromatic amines, azo-structures, *N*-nitroso groups, and aromatic nitro-groups (Ashby and Paton, 1994). For some classes of compounds with specific structural alerts, it is established that specific protocol

modifications/additional tests are important for optimum detection of genotoxicity (e.g., molecules containing an azo-group, glycosides, compounds such as nitroimidazoles requiring nitroreduction for activation, compounds such as phenacetin requiring a different rodent S9 for metabolic activation).

Note 6. There is some experience with in vivo assays for micronucleus induction in skin and colon (Hayashi et al., 2007), and DNA damage assays in these tissues can also be an appropriate substitute.

Note 7. A few chemicals are more easily detectable either with plate-incorporation or with pre-incubation methods, though differences are typically quantitative rather than qualitative (Gatehouse et al., 1994). Experience in the pharmaceutical industry where drugs have been tested in both protocols has not resulted in different results for the two methods, and, in the IWGT report (Gatehouse et al., 1994), the examples of chemical classes listed as more easily detectable in the pre-incubation protocol are generally not pharmaceuticals and are positive in in vivo genotoxicity tests in liver. These include short chain aliphatic nitrosamines, divalent metals, aldehydes (e.g., formaldehyde, crotonaldehyde), azo dyes (e.g., butter yellow), pyrrolizidine alkaloids, allyl compounds (allyl isothiocyanate, allyl chloride), and nitro (aromatic, aliphatic) compounds.

Note 8. The rationale for a maximum concentration of 1 mM for in vitro mammalian cell assays includes the following: The test battery includes the Ames test and an in vivo assay. This battery optimizes the detection of genotoxic carcinogens without relying on any individual assay alone. There is a very low likelihood of compounds of concern (DNA damaging carcinogens) that are not detected in Ames test or in vivo genotoxicity assay, but are detectable in an in vitro mammalian assay only above 1 mM. Second, a limit of 1 mM maintains the element of hazard identification, being higher than clinical exposures to known pharmaceuticals, including those that concentrate in tissues (Goodman & Gilman, 2001), and is also higher than the levels generally achievable in preclinical studies in vivo. Certain drugs are known to require quite high clinical exposures for therapeutic effect, e.g., nucleoside analogs and some antibiotics. Although comparison of potency with existing drugs can be of interest to sponsors, perhaps even above the 1 mM limit, it is ultimately the in vivo tests that determine relevance for human safety. For pharmaceuticals with unusually low molecular weight (e.g., less than 200) higher test concentrations should be considered.

Note 9. Although some genotoxic carcinogens are not detectable in in vitro genotoxicity assays unless the concentrations tested induce some degree of cytotoxicity, DNA damaging agents are generally detectable with only moderate levels of toxicity (Greenwood et al., 2004). As cytotoxicity increases, mechanisms other than direct DNA damage by a compound or its metabolites can lead to *positive* results that are related to cytotoxicity and not genotoxicity. Such indirect induction of DNA damage secondary to damage to non-DNA targets is more likely to occur above a certain concentration threshold. The disruption of cellular processes is not expected to occur at lower, pharmacologically relevant concentrations.

In cytogenetic assays, even weak clastogens that are known to be carcinogens are positive without exceeding a 50 percent reduction in cell counts. On the other hand, compounds that are

not DNA damaging, mutagenic, or carcinogenic can induce chromosome breakage at toxic concentrations. For both in vitro cytogenetic assays, the chromosome aberration assay and the in vitro micronucleus assay, a limit of about 50 percent growth reduction is considered appropriate.

For cytogenetic assays in cell lines, measurement of cell population growth over time (by measuring the change in cell number during culture relative to control, e.g., by the method referred to as population doubling (PD (see note 10)), has been shown to be a useful measure of cytotoxicity, as it is known that cell numbers can underestimate toxicity. For lymphocyte cultures, an inhibition of proliferation not exceeding about 50 percent is considered sufficient; this can be measured by mitotic index (MI) for metaphase aberration assays and by an index based on cytokinesis block for in vitro micronucleus assays. In addition, for the in vitro micronucleus assay, since micronuclei are scored in the interphase subsequent to a mitotic division, it is important to verify that cells have progressed through the cell cycle. This can be done by use of cytochalasin B to allow nuclear division but not cell division, so that micronuclei can be scored in binucleate cells (the preferred method for lymphocytes). For cell lines, other methods to demonstrate cell proliferation, including cell population growth over time (PD) as described above, can be used (Kirsch-Volders et al., 2003).

For MLA, appropriate sensitivity is achieved by limiting the top concentration to one with close to 20 percent Relative Total Growth (RTG) (10 to 20%) both for soft agar and for microwell methods (Moore et al., 2002). Reviews of published data using the current criteria found very few chemicals that were positive in MLA only at concentrations with less than 20 percent RTG and that were rodent carcinogens, and convincing evidence of genotoxic carcinogenesis for this category is lacking. The consensus is that caution is appropriate in interpreting results when increases in mutation are seen only below 20 percent RTG, and a result would not be considered positive if the increase in mutant fraction occurred only at ≤ 10 percent RTG.

In conclusion, caution is appropriate in interpreting positive results obtained as reduction in growth/survival approaches or exceeds 50 percent for cytogenetics assays or 80 percent for MLA. It is acknowledged that the evaluation of cells treated at these levels of cytotoxicity/clonal survival can result in greater sensitivity but bears an increased risk of nonrelevant positive results. The battery approach for genotoxicity is designed to ensure appropriate sensitivity without relying on single in vitro mammalian cell tests at high cytotoxicity.

To obtain an appropriate toxicity range, a preliminary range-finding assay over a broad range of concentrations is useful, but in the genotoxicity assay it is often critical to use multiple concentrations that are spaced quite closely (less than two-fold dilutions). Extra concentrations can be tested but not all concentrations need be evaluated for genotoxicity. It is not intended that multiple experiments be carried out to reach exactly 50 percent reduction in growth, for example, or exactly 80 percent reduction in RTG.

Note 10. For in vitro cytogenetic assays, it is appropriate to use a measure of relative cell growth to assess toxicity because cell counts can underestimate toxicity (Greenwood et al., 2004). Using calculated population doublings (see glossary) to estimate the 50 percent growth reduction level, it was demonstrated that the frequency of positive results with compounds that are not mutagenic

or carcinogenic is reduced, while agents that act via direct interaction with DNA are reliably positive.

Note 11. In certain cases, it can be useful to examine chromosome aberrations at metaphase in lymphocytes cultured from test animals after one or more administrations of test compound, just as bone marrow metaphase cells can be used. Because circulating lymphocytes are not replicating, agents that require replication for their genotoxic effect (e.g., some nucleoside analogs) are not expected to be detected in this cell type. Because some lymphocytes are relatively long-lived, in principle there is the potential for accumulation of unrepaired DNA damage in vivo that would give rise to aberrations when the cells are stimulated to divide in vitro. The in vivo lymphocyte assay can be useful in following up indications of clastogenicity, but in general another tissue such as liver is a more informative supplement to the micronucleus assay in hematopoietic cells because exposure to drug and metabolite(s) is often higher in liver.

Note 12. The inclusion of a second in vivo assay in the battery is to provide assurance of lack of genotoxicity by use of a tissue that is well exposed to a drug and/or its metabolites; a small number of carcinogens that are considered genotoxic gave positive results in a test in liver but were negative in a cytogenetics assay in vivo in bone marrow. These examples likely reflect a lack of appropriate metabolic activity or lack of reactive intermediates delivered to the hematopoietic cells of the bone marrow.

Assays for DNA strand breaks, DNA adducts, and mutations in transgenes have the advantage that they can be applied in many tissues. Internationally agreed protocols are not yet in place for all the in vivo assays, although considerable experience and published data and protocol recommendations exist for DNA strand break assays (Comet and alkaline elution assays), DNA adduct (covalent binding) measurements, and transgenic rodent mutation assays, in addition to the UDS assay. For a compound that is positive in vitro in the MLA and induces predominantly large colonies, and is also shown not to induce chromosome breakage in an in vitro metaphase assay, an in vivo assay for mutation, such as a transgenic mouse mutation assay, should be considered in preference to a DNA strand break assay. The UDS assay is considered useful mainly for compounds that induce bulky DNA adducts or are positive in the Ames test. Because cytotoxicity induces DNA strand breakage, careful cytotoxicity assessment is needed to avoid confounding the results of DNA strand break assays. This has been well-characterized for the in vitro alkaline elution test (Storer et al., 1996) but not yet fully validated for the Comet assay. In principle, the DNA strand break assays can be used in repeat-dose toxicology assays with appropriate dose levels and sampling times.

Because liver of mature animals is not a highly mitotic tissue, often a non-cytogenetic endpoint is used for the second assay; but when dividing hepatocytes are present, such as after partial hepatectomy, or in young rats (Hayashi et al., 2007), micronucleus analysis in liver is possible, and detects known genotoxic compounds.

Note 13. For common vehicles like aqueous methyl cellulose, this would usually be appropriate, but for vehicles such as Tween 80, the volume that can be administered could be as much as 30 fold lower than that given acutely.

Note 14. Caution is appropriate if the toxicological study design includes additional blood sampling, e.g., for measurement of exposure. Such bleeding could perturb the results of micronucleus analysis since erythropoiesis stimulated by bleeding can lead to increases in micronucleated erythrocytes.

Note 15. Increases in micronuclei can occur without administration of any genotoxic agent, due to disturbance in erythropoiesis (such as regenerative anemia; extramedullary hematopoiesis), stress, and hypo- and hyperthermia (reviewed by Tweats et al., 2007, I). In blood, changes in spleen function that affect clearance of micronucleated cells from the blood could lead to small increases in circulating micronucleated red blood cells.

Note 16. Positive controls for either short-term or repeat-dose genotoxicity studies:
For micronucleus (and other cytogenetic) assays, the purpose of the positive control is to verify that the individuals scoring the slides can reliably detect increases in micronuclei. This can be accomplished by use of samples from periodic studies (every few months) of small groups of animals (one sex) given acute treatment with a positive control. For manual scoring, such slides can be included in coded slides scored from each study. Positive control slides should not be obvious to readers based on their staining properties or micronucleus frequency. For automated scoring, appropriate quality control samples should be used with each assay.

For other in vivo genotoxicity assays, the purpose of positive controls is to demonstrate reliable detection of an increase in DNA damage/mutagenicity using the assay in the chosen species, tissue, and protocol. After a laboratory has demonstrated that it can consistently detect appropriate positive control compounds in multiple independent experiments, carrying out positive control experiments periodically is generally sufficient provided experimental conditions are not changed. However, currently it is considered that for the Comet assay, concurrent positive controls are advisable.

Note 17. Determination of whether micronucleus induction is due primarily to chromosome loss or to chromosome breakage could include staining micronuclei in vitro or in vivo to determine whether centromeres are present, e.g., using fluorescent in situ hybridization (FISH) with probes for DNA sequences in the centromeric region, or a labeled antibody to kinetochore proteins. If the majority of induced micronuclei are centromere positive, this suggests chromosome loss. (Note that even potent tubule poisons like colchicine and vinblastine do not produce 100% kinetochore positive micronuclei, but more typically 70 to 80 percent, and are accepted as primarily aneugens for assessing risk). An alternative approach is to carry out an in vitro or in vivo assay for metaphase structural aberrations; if negative, this would imply that micronucleus induction is related to chromosome loss.

Note 18. Standard induced S9 mix has higher activation capacity than human S9, and lacks phase two detoxification capability unless specific cofactors are supplied. Also, nonspecific activation can occur in vitro with high test substrate concentrations (see Kirkland et al., 2007). Genotoxicity testing with human S9 or other human-relevant activation systems can be helpful. Analysis of the metabolite profile in the genotoxicity test incubations for comparison with known metabolite profiles in preclinical species (in uninduced microsomes or hepatocytes, or in vivo) or in preparations from humans can also help determine the relevance of test results (Ku et

al., 2007), and follow-up studies will usually focus on in vivo testing in liver. A compound that gives positive results in vitro with S9 might not induce genotoxicity in vivo because the metabolite is not formed, is formed in very small quantities, or is metabolically detoxified or rapidly excreted, indicating a lack of risk in vivo.

VII. GLOSSARY (7)

Alkaline elution assay: See *DNA strand break assay.*

Aneuploidy: Numerical deviation of the modal number of chromosomes in a cell or organism.

Base substitution: The substitution of one or more base(s) for another in the nucleotide sequence. This can lead to an altered protein.

Cell proliferation: The ability of cells to divide and to form daughter cells.

Centromere/kinetochore: Structures in chromosomes essential for association of sister chromatids and for attachment of spindle fibers that move daughter chromosomes to the poles and ensure inclusion in daughter nuclei.

Clastogen: An agent that produces structural breakage of chromosomes, usually detectable by light microscopy.

Cloning efficiency: The efficiency of single cells to form clones. It is usually measured after seeding low numbers of cells in a suitable environment.

Comet assay: See *DNA strand break assay.*

Culture confluency: A quantification of the cell density in a culture by visual inspection.

Cytogenetic evaluation: Chromosome structure analysis in mitosis or meiosis by light microscopy or micronucleus analysis.

DNA adduct: Product of covalent binding of a chemical to DNA.

DNA repair: Reconstitution of the original DNA sequence after DNA damage.

DNA strand breaks: Single or double strand scissions in the DNA.

DNA strand break assay: Alkaline treatment that converts certain types of DNA lesions into strand breaks that can be detected by the alkaline elution technique, measuring migration rate through a filter, or by the single cell gel electrophoresis or Comet test (in which cells embedded in a thin layer of gel on a microscope slide are subjected to electric current, causing shorter pieces of DNA to migrate out of the nucleus into a *Comet tail*). The extent of DNA migration is measured visually under the microscope on stained cells.

Frameshift mutation: A mutation (change in the genetic code) in which one base or two adjacent bases are added to (inserted in) or deleted from the nucleotide sequence of a gene. This can lead to an altered or truncated protein.

Gene mutation: A detectable permanent change within a single gene or its regulating sequences. The changes can be point mutations, insertions, or deletions.

Genetic endpoint: The precise type or class of genetic change investigated (e.g., gene mutations, chromosomal aberrations, DNA strand breaks, DNA repair, DNA adduct formation).

Genotoxicity: A broad term that refers to any deleterious change in the genetic material regardless of the mechanism by which the change is induced.

Micronucleus*:* Particle in a cell that contains nuclear DNA; it might contain a whole chromosome(s) or a broken centric or acentric part(s) of a chromosome(s).

Mitotic index: Percentage of cells in the different stages of mitosis amongst the cells not in mitosis (interphase) in a preparation (slide).

Numerical chromosome changes: Chromosome numbers different from the original haploid or diploid set of chromosomes; for cell lines, chromosome numbers different from the modal chromosome set.

Plasmid: Genetic element in addition to the normal bacterial genome. A plasmid might be inserted into the host chromosome or form an extra-chromosomal element.

Point mutations: Changes in the genetic codes, usually confined to a single DNA base pair.

Polychromatic erythrocyte: An immature erythrocyte in an intermediate stage of development that still contains ribosomes and, as such, can be distinguished from mature normochromatic erythrocytes (lacking ribosomes) by stains selective for RNA.

Polyploidy: Numerical deviation of the modal number of chromosomes in a cell, with approximately whole multiples of the haploid number. Endoreduplication is a morphological form of polyploidy in which chromosome pairs are associated at metaphase as *diplochromosomes*.

Population doubling or culture growth: This can be calculated in different ways; one example of an appropriate formula is: Population doublings (PDs) = the log of the ratio of the final count (N) to the starting (baseline) count (Xo), divided by the log of 2. That is: $PD = [\log(N \div Xo)] \div \log 2$.

Recombination: Breakage and balanced or unbalanced rejoining of DNA.

RTG (relative total growth): This measure of cytotoxicity takes the relative suspension growth (based on cell loss and cell growth from the beginning of treatment to the second day post-treatment) and multiplies it by the relative plating efficiency at the time of cloning for mutant quantization.

Single cell gel electrophoresis assay: Comet assay. See *DNA strand break assay*.

Survival (in the context of mutagenicity testing): Proportion of living cells among dead cells, usually determined by staining or colony counting methods after a certain treatment interval.

Transgene: An exogenous or foreign gene inserted into the host genome, either into somatic cells or germ line cells.

Unscheduled DNA synthesis (UDS): DNA synthesis that occurs at some stage in the cell cycle other than S-phase in response to DNA damage. It is usually associated with DNA excision repair.

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DECLARATION OF SERVICE

On said day below I electronically served a true and accurate copy of the ***Memorandum of Amicus Curiae Dr. Robert H. Heflich, Ph.D.*** in Supreme Court Cause No. 100390-1 to the following:

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I declare under penalty of perjury under the laws of the State of Washington and the United States that the foregoing is true and correct.

DATED: January 18, 2022, at Seattle, Washington.

/s/ Matt J. Albers
Matt J. Albers, Paralegal
Talmadge/Fitzpatrick

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