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Chromosomal aberrations, clastogens vs aneugens

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1. ABSTRACT

Current anticancer therapy may be one of the most important exogenous sources of exposure to genotoxic agents in US, Japan, and Europe, where approximately 40–55 percent of the population is diagnosed with cancer at a certain point in their life. This review focuses on recent efforts to integrate a novel biomarker, gamma-H2AX, into anticancer drug screening to classify the mode of action (MoA) for genotoxic outcome into clastogenicity and aneugenicity, a distinction that has considerable impact on risk assessment and control strategy. The emerging biomarker gamma-H2AX is applicable to high throughput assay platforms and is therefore changing in vitro mammalian genotoxicity screening from traditional positive/negative selection to MoA elucidation. Because gamma-H2AX is not only a sensitive biomarker for DNA double strand break but is also induced by apoptosis, the key for successful screening is using additional biomarkers of caspase-3 and/or phosphorylated histone H3 to discriminate between relevant and irrelevant elevation of gamma-H2AX. Establishment of a standard methodology and a consensus threshold for its positive criteria will further support the application of gamma-H2AX to drug screening.

2. INTRODUCTION

Genomic integrity is essential for organizing and maintaining multicellular eukaryotes. DNA itself, replication of DNA, and segregation of chromosomes are affected by various disturbing factors like endogenous active metabolites, radiation, oxidative stress, and environmental chemicals. Activities that cause genetic alterations, such as DNA strand breaks, mutations, and structural and numerical aberrations of chromosomes, are known as genotoxicity that is relevant to carcinogenesis. Although millions of instances of DNA damage are generated in our bodies in daily life, exposure to artificial genotoxic agents from food additives, pesticides, cosmetic ingredients, pharmaceuticals, or pharmaceutical impurities are required to be strictly controlled.

An exception is cancer therapy. Cancer therapy using genotoxic anticancer drugs and radiation has been widely accepted because its therapeutic benefit is recognized to be superior to the genotoxic risk. Therapies with genotoxic agents cause DNA lesions in normal cells that increase the potential risk of therapy-related secondary malignant neoplasms (SMN). Patients who received chemotherapy with genotoxic topoisomerase II inhibitors for primary cancer had significantly shorter latency periods to SMN onset (1). Due to a striking increase in SMN after treatment with radioactive iodine therapy, the use of radiotherapy in the patient population with low-risk well-differentiated thyroid cancer is being reconsidered (2). As a result of improved survival rates and the continual aging of survivors, SMN in all cancer diagnosis increased from 9 percent in 1975-1979 to 19 percent in 2005-2009 in the United States (3).

Today, anticancer therapy may be one of the most important exogenous sources of exposure to genotoxic agents in US, Japan, and European countries, where approximately 40–55 percent of population is diagnosed with cancer at a certain point in their life (4-6). Patients used to accept the SMN risk for the sake of therapeutic benefit; however, pharmaceutical industries are trying to reduce the risk of SMN with new chemotherapeutics. In addition, the potential impact of genotoxic anticancer drugs on the environment is an emerging issue (7-12). It is increasingly important to understand the genotoxicity of anticancer drugs and to appropriately control therapeutic and environmental exposure to such agents.

This review focuses on recent efforts with the novel biomarker gamma-H2AX to identify the mode of action (MoA) for a positive genotoxic outcome in an in vitro screening for anticancer drug candidates and looks especially at how aneugens and clastogens are classified, which decides the control strategy for the compound. Aneugens cause genomic instability with numerical aberration of chromosomes via DNA replication stresses (13, 14). Clastogens cause structural aberrations of chromosomes, which potentially trigger mutations via repairing errors. I describe the basic strategy for estimating the acceptable levels of exposure to DNA-reactive clastogens and DNA-non-reactive aneugens, followed by a section on applying the recently emerging biomarker gamma-H2AX to discriminate the two activities.

3. IMPACT OF GENOTOXIC MODE OF ACTION (MoA) ON THE CONTROL OF EXPOSURE LEVELS

3.1. DNA-reactive MoA

It is important to elucidate the MoA of genotoxic compounds because it has considerable impact on the risk assessment and control strategy (15-19). The consensus that DNA-reactive genotoxic carcinogens have no safety threshold has a long history; the non-threshold model was proposed in 1949 and supported by the Biological Effects of Atomic Radiation committee of the US National Academy of Science in 1956 (20, 21). Accordingly, even a trace amount of exposure to genotoxic compounds is recognized as causing a finite risk (22, 23). The currently adopted control strategy for DNA-reactive genotoxic agents is based on the non-threshold consensus, generally known as the concept of threshold of toxicological concern (TTC) (24, 25). TTC was derived from linear dose-response approximations of numerous compounds, including genotoxic carcinogens, and is considered to reduce lifetime cancer risk to less than 1/100,000 (24-29).

Recent understanding of the mechanisms maintaining genomic integrity has raised a question related to the non-threshold model as to whether endogenous DNA repair systems can effectively erase some kinds of low-level DNA lesions. Mechanisms that prevent the accumulation of genetic lesions are known collectively as DNA damage response (DDR) and involve expansion of sensor signals for DNA damage, transduction to kinase cascades, and activation of cell cycle checkpoints. Various DNA-repairing systems are employed during cell cycle arrest (30), and extensive DNA damage that cannot be repaired induces apoptosis of the cells to eliminate the erroneous genetic information. Then, the fate of each cell to survive or die is determined by a central player, p53, and relevant signaling components in order to maintain genomic stability (31, 32).

The non-threshold model was challenged in 2002 by non-linear dose-response results for the induction of carcinoma from vinyl acetate (33) and for the initiation of cancer from 2-amino-3,8-dimethylimidazo(4,5-f) quinoxaline (34). More reliable evidence for the existence of a safety threshold for a DNA-reactive genotoxin was recently provided using ethyl methanesulfonate and is based on numerous experimental data that indicate a “hockey stick” dose-response curve of genotoxic endpoint, detailed kinetics of blood concentration, and molecular understanding of the damage and repair processes (35-41). Even though sufficient evidence has not been gathered yet, some compounds with a DNA-reactive MoA are now considered to have safety thresholds (19, 42).

However, it is quite difficult to estimate an acceptable level of exposure for each genotoxic anticancer drug, because the standard non-clinical safety test battery for anticancer drugs does not include animal carcinogenicity studies that can provide dose-response increases in cancer frequency, which would give pivotal understanding. It is also difficult to comprehend the relationship between SMN and exposure to each genotoxic drug from clinical data because cancer therapy is usually composed of various genotoxic agents. There are also the technical difficulties involved in detecting slight responses at very low dose ranges. Thus, the current control strategy based on the concept of non-threshold using the generic virtual safe dose, TTC, has been widely accepted as a practical solution.

3.2. DNA non-reactive MoA

A genotoxic agent is considered to have a safety threshold if its MoA is DNA non-reactive (16, 43-45). This category includes agents with various types of pharmacological activity, including inhibition of DNA synthesis, production of reactive oxygen species, and inhibition of spindle function (46-48). Spindle inhibitory activity that causes numerical aberrations of chromosomes is known as aneugenicity and is the most common form of genomic instability in cancer cells (49). Aneugenicity causes birth defects, pregnancy wastage, and cancer (50). Anticancer drugs like taxanes and vinca alkaloids inhibit the assembly or disassembly of tubulin to induce aneuploidy cells.

Acceptable levels of exposure to compounds that have a DNA non-reactive MoA can be established based on a calculation provided by the WHO in Environmental Health Criteria 170 (51). The no-observed effect level or the lowest-observed-effect level gained from pivotal animal studies can be the starting point, from which an acceptable level is derived after application of various modifying factors to adjust species difference between experimental animal and human, human individual difference, short study duration, severe toxicity, and conversion to no-effect level. The methodology is widely used for various regulations to protect humans from environmental chemical hazards.

4. GENOTOXICITY SCREENING FOR ANTICANCER DRUG CANDIDATES

4.1. *In vitro* micronucleus test (MNT)

A combination of the Ames test and an *in vitro* MNT includes all the essential endpoints for genotoxic activities, such as mutagenicity, clastogenicity, and aneugenicity (52). The combination is recognized as sufficiently predictive of *in vivo* genotoxic and carcinogenic activities (53) and has been widely used for the purpose of drug screening. The Ames test measures the elevation of bacterial mutation frequency induced by test compounds, and the MNT scores cells with micronuclei (MN), which are the result of structural or numerical chromosome aberrations generated in mammalian cells after treatment with test compounds. Activity that induces structural aberration of chromosomes is defined as clastogenicity, which is involved in both non-threshold and threshold MoA. The activity to induce numerical aberration of chromosome is defined as aneugenicity, which is involved in threshold MoA only. If a test compound is Amesnegative and MNT-positive, it is difficult to know whether the positive results suggest a mammal-specific non-threshold MoA or not.

Many canonical cytotoxic anticancer drugs target DNA itself and/or DNA replication factors, which tend to provide positive genotoxic results in any test system. Molecular target drugs, including kinase inhibitors, are often negative in the Ames test and positive in the *in vitro* MNT, only because kinase inhibitors tend to be specific to mammalian targets and inhibit off-target kinases including those functional in chromosomal segregation (54-56). Therefore, *in vitro* MNT is the pivotal assay in genotoxicity screening of molecular target anticancer drug candidates to date.

The methodology of an *in vitro* MNT has been well standardized (57) and is widely used to assess the genotoxicity of a range of chemicals (58). The endpoint of the MNT is the frequency of interphase cells with MN that are composed of chromosome fragments or whole chromosomes. The MN of a chromosomal fragment is generated by clastogens that break the DNA and chromosome, whereas the MN of a whole chromosome is generated by aneugens that induce mis-segregation of chromosomes into daughter cells (57, 58). The formation of MN by clastogens and aneugens is summarized in Figure 1. The MNT detects both clastogenic and aneugenic activity of test compounds (57-60).

4.2. MoA of anticancer drugs inducing MN

Variety of recent molecular target drug candidates for cancer therapy have aneugenic activity, e.g. inhibitors of HSP90 are aneugens, probably because its client proteins include microtubule-associated proteins that control chromosomal disjunction (61). Glycogen synthetase kinase inhibitors have an aneugenic MoA due to hypo-phosphorylation of client Aurola A kinase involved in spindle assembly and MAP4 involved in microtubule binding and stabilizing (62, 63). Several potential target kinases of anticancer drugs are also suggested to be involved in spindle dynamics (64-69). Therefore, aneugenicity has been a frequently observed genotoxic activity in anticancer drug screening.

Not only DNA-reactive but also DNA non-reactive MoA — for example, production of reactive oxygen species or inhibition of DNA synthesis — induce chromosome breakage, so clastogenicity is caused by both DNA-reactive and DNA non-reactive MoA. However, aneugenicity is induced by DNA non-reactive MoA only. Therefore, it is important to know whether a compound that shows positive in the MNT is an aneugen or not. Significant increase of kinetochore/centromere-positive MN (MN+) has been accepted as definitive evidence of an aneugenic MoA. The kinetochore/centromere staining is not free from failure to some extent, which may cause slight to moderate increase of kinetochore/centromere-negative MN (MN-), even after treatment with a pure aneugen. Empirically, the percentage of MN+ is less than 30 percent in cultured human lymphocytes and increases to 50 – 90 percent after treatment with aneugens (70). Thus, it is often difficult to assert that a clastogenic MoA is not involved in the positive response seen in the assay. Data sets for the presence of an aneugenic MoA and the lack of a clastogenic MoA are both required to support that the test compound is a pure aneugen and has a safety threshold. A negative result from a chromosomal aberration test is strong evidence for lack of clastogenicity. However, preparing the entire data set is too great a workload for the purposes of drug screening.

4.3. Traditional biomarker for aneugenicity

How to distinguish aneugens from clastogens in the MNT has been discussed at length. Matsuoka *et al* (71) proposed that the frequency of polynuclear or mitotic cells in an *in vitro* MNT was a good biomarker for classifying aneugens and clastogens. Increase in multinucleated cells in an *in vitro* MNT was reported as a potential indicator of an aneugenic MoA (72). One of

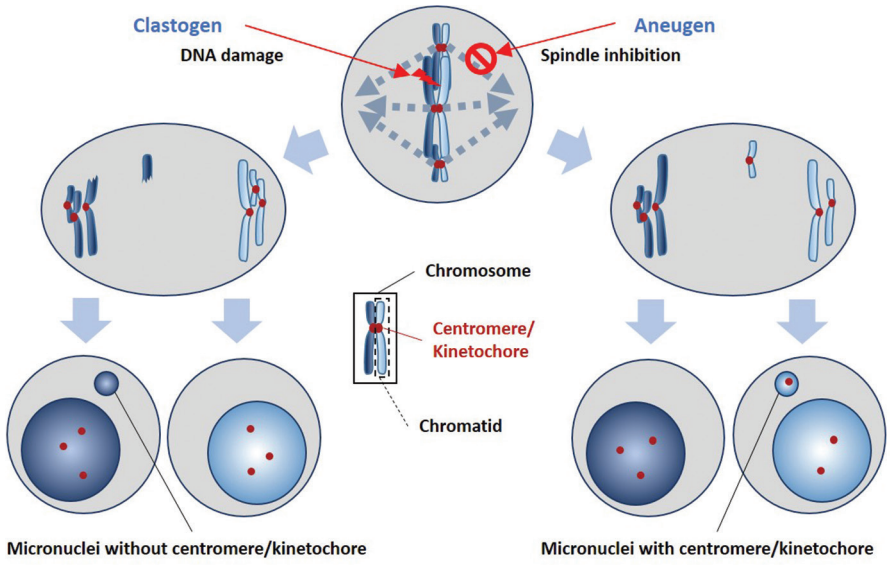

Figure 1. Mode of action for induction of micronuclei, clastogenic and aneugenic pathways.

the standard protocols for *in vitro* MNT (57) includes treatment with cytochalasin B that blocks cell division. Cytochalasin B produces binuclear cells that undergo cell cycle after exposure to test chemicals and are therefore considered suitable for analyzing MN cells. Rosefort *et al* examined both binuclear and mononuclear cells for MN and found that aneugens increased MN in binuclear and mononuclear cells, while clastogens increased in binuclear cells only (73). Hashimoto *et al* (74) observed that aneugens induced larger MN than clastogens, which was proposed as a useful index to classify aneugens and clastogens. All these proposals are useful to suggest involvement of aneugenicity with lack of clastogenicity.

5. EMERGING BIOMARKER FOR DNA DAMAGE, GAMMA-H2AX

5.1. Gamma-H2AX

A new surrogate marker for DNA damage, gamma-H2AX, could provide helpful information of a genotoxic MoA. Gamma-H2AX is a phosphorylated form of histone protein. Histone core proteins, H2A, H2B, H3 and H4, compose an octamer with two of each subunit to form a basic subunit of chromatin structure that supports 140 bp DNA, and these proteins receive various chemical modifications like phosphorylation, methylation, acetylation, and ubiquitination, some of which participate in DDR and its consequent DNA-repairing processes (75-78). A member of the H2A histone protein family is H2AX, which includes an additional SQRY motif at the c-terminal domain (79).

H2AX was found to be an important component of DDR, especially in sensing and repairing DNA double strand breaks (DSB) (80). Gamma-H2AX, a phosphorylated form of H2AX at serine 139 (81), is a binding interface of the DSB-repairing components (82). A DSB is sensed by the Mre11, Rad50, and Nbs1 complex (MRN complex), the MRN complex phosphorylates ATM, activated ATM phosphorylates H2AX into gamma-H2AX, gamma-H2AX recruits the MRN complex, MRN complex amplifies gamma-H2AX to more than 2 Mbp in the chromatin region around a DSB locus, which then forms a microscopically visible focus under immunofluorescent staining with an anti-gamma-H2AX antibody (81, 83-89).

The number of gamma-H2AX foci is considered to be a surrogate marker for the extent of DNA damage because it is in proportion to the exposure levels of the cells to ionizing radiation (90). Not only DSB but also single strand breaks after treatment with UV irradiation (91), camptothecin (92), and reactive oxygen species (93) induce gamma-H2AX in the process of repairing DNA lesions. Gamma-H2AX is a universal biomarker for a wide range of DNA damage (94).

Various experimental platforms are available for detecting gamma-H2AX: flow cytometry, microscopic focus counting, and immunoblotting (95). Microscopic scoring of gamma-H2AX foci in each cell and measurement of the fluorescence corresponding to gamma-H2AX in flow cytometry have been the gold standards during this decade, though higher throughput and/or automated methods have been proposed recently (96-100). It is interesting that the morphology of gamma-H2AX induced by DNA lesions differs from that induced by apoptosis. When apoptotic cells are immunostained with anti-gamma-H2AX antibody, pan-nuclear staining is seen, not gamma-H2AX foci (101). It is known that pan-nuclear gamma-H2AX exhibits a

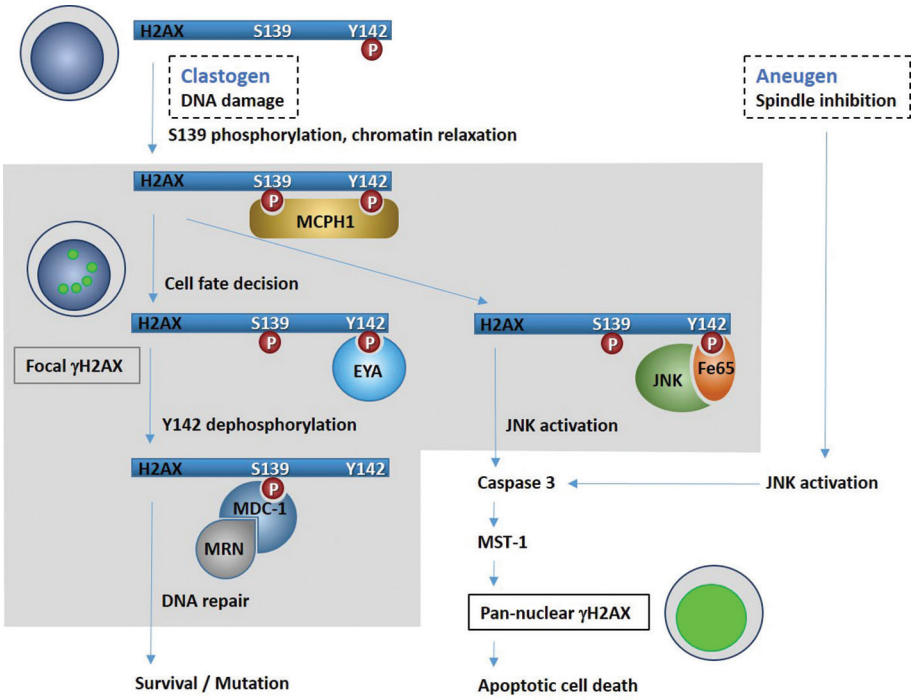

Figure 2. Formation of focal and pan-nuclear gamma-H2AX after exposure to clastogen or aneugen.

higher level of fluorescence than gamma-H2AX foci under immunofluorescent staining (102).

5.2. Mechanism to induce focal and pan-nuclear gamma-H2AX

It has not been elucidated how the focal and pan-nucleic phosphorylation of H2AX are determined after treatment with clastogens and aneugens. Pathways currently recognized to be relevant to the different morphology of gamma-H2AX are summarized in Figure 2. Gamma-H2AX foci were formed very quickly within three minutes after gamma-radiation in cancer cells (83). In the early stage of DDR, microcephalin (MCPH1/BRIT1) binds to doubly phosphorylated H2AX at S139 and tyrosine 142 (Y142) that has been constitutively phosphorylated (103). MCPH1 relaxes the regional chromatin structure around DNA lesions (104).

The status of phosphorylation of Y142 determines whether the cell's fate is survival or apoptosis (103). Phosphorylated forms of S139 and Y142 were seen after radiation; more than 8 hours later, Y142 was greatly dephosphorylated, and S139 was still highly phosphorylated (105). The developmentally regulated transcriptional co-factor Eya is a tyrosine phosphatase (106) that is recruited in response to DDR, is co-localized with gamma-H2AX foci, interacts with H2AX only when S139 is phosphorylated, and dephosphorylates Y142 after exposure to ionizing radiation (103). The mediator of DNA damage checkpoint protein 1 (MDC1), which cannot bind to S139 when H2AX is doubly phosphorylated at S139 and Y142 (107), directly binds to phosphorylated S139 after dephosphorylation on Y142 and recruits DNA-repairing components such as 53BP1, BRCA1, and MRN complex (108, 109). Therefore dephosphorylation of Y142 is necessary for the recruitment of these repairing factors (103). Because the DNA damage repairing process is a response to DNA errors, focal gamma-H2AX can be understood as a marker for genotoxic risk potential.

When Y142 keeps its phosphorylated form after exposure to clastogens, MDC1 does not coordinate its key function of recruiting DNA-repairing factors (103, 107). H2AX that is doubly phosphorylated at S139 and Y142 binds Fe65 instead of MDC1 (103). Fe65 is an adaptor protein with specific domains interacting with a range of proteins to form functional complexes of the proteins (110). During DDR generated by gamma-irradiation, Fe65 was suggested to bind and activate c-Jun N-terminal kinase (JNK) (103), which is also activated by aneugens. Spindle inhibitors, such as paclitaxel and vinblastine, are aneugens causing cell cycle arrest that trigger activated JNK (111, 112).

The JNK family of MAP kinases initiates the signaling cascade of apoptosis in the death receptor-mediated extrinsic pathway and the mitochondrial intrinsic one (113). The active form of JNK causes the release of mitochondrial cytochrome c into cytoplasm to activate caspase-3 (114) that triggers the apoptotic cascade including pan-nuclear phosphorylation of H2AX at S139 (103, 115). DNA fragmentation in apoptosis

induces phosphorylation of S139 (116). A detailed observation of the kinetics of gamma-H2AX in apoptosis indicated that the phosphorylation was initiated at the periphery of the nucleus to form a gamma-H2AX ring that finally resulted in pan-nucleic staining with anti-gamma-H2AX antibody (94, 117).

In the context summarized in Figure 2, pan-nuclear gamma-H2AX suggests that the apoptosis cascade is running in the cells triggered either by irreparable DNA damage by clastogens or by cell cycle arrest with aneugens. Pan-nuclear gamma-H2AX is a biomarker for dying cells; therefore it can be recognized as irrelevant to human risk.

5.3. Gamma-H2AX for classification of clastogens and aneugens

We first proposed the use of gamma-H2AX to classify clastogens and aneugens, and the influence of apoptosis at the International Conference on Environmental Mutagenesis in 2009 (118, 119). Reference compounds of 6 aneugens, 8 clastogens, and 10 cytotoxicants without genotoxicity were examined for induction of gamma-H2AX in CHL, CHO, and V79 cell lines. The result in cell ELISA was that none of the aneugens elevated gamma-H2AX while all the clastogens increased gamma-H2AX in a concentration-dependent manner, which supports the use of gamma-H2AX to classify clastogens from aneugens (96). However, the amount of gamma-H2AX did not decrease in parallel with cytotoxicity after treatment with aneugens, whereas the decreases in gamma-H2AX and survival cells correlated after treatment with cytotoxic compounds without genotoxicity (96). It is considered that the amount of gamma-H2AX after treatment with aneugens was affected by apoptotic gamma-H2AX in the cell ELISA, though the study indicated the potential use of gamma-H2AX to classify clastogens and aneugens.

Extensive DNA damage that cannot be repaired induces apoptosis to eliminate erroneous genetic information. The protective system with the central player p53 and relevant signaling components determines the fate of each cell with DNA lesions to survive or die (31, 32). Clastogens are therefore potent inducers of apoptosis, which in turn induces gamma-H2AX (117). A study using inhibitors and knockout mutant cells exposed to UVA indicated that JNK phosphorylated H2AX at Ser139, thereby regulating DNA degradation via the caspase-3/CAD pathway (114). Gamma-H2AX was induced in Jurkat cells after treatment with anti-Fas antibody when apoptotic fragmentation of DNA occurred (117). The presence of the anti-Fas monoclonal antibody, which is not genotoxic, suggested that gamma-H2AX was formed in the process of apoptosis, irrespective of DNA damage.

In an *in vivo* environment, apoptotic cells externalize “eat me” signal molecules, probably phosphatidylserine, amino sugars, intercellular adhesion molecule-3, or calreticulin, to the cell surface and are quickly engulfed by macrophages (120). Because there was no system to exclude apoptotic cells from culture wells, we observed gamma-H2AX that derived from a mixture of apoptosis and DNA damage in the *in vitro* screening systems. Therefore, the technology to distinguish between apoptosis-derived and DNA damage–derived gamma-H2AX was key for applying gamma-H2AX to the classification of aneugens and clastogens.

Harada *et al* (121) added caspase-3 staining in the *in vitro* gamma-H2AX assay with human lymphoblastoid TK6 cells to further classify gamma-H2AX-positive (gamma-H2AX+) cells into caspase-3-positive (gamma-H2AX+/caspase-3+) for apoptosis and caspase-3-negative (gamma-H2AX+/caspase-3-) for DNA-damaged populations. The two clastogens, mitomycin C and etoposide, caused a significant increase of gamma H2AX+/caspase-3- cells but did not increase gamma-H2AX+/caspase-3+ cells significantly. The two aneugens, vinblastine and paclitaxel, both increased gamma-H2AX+/caspase-3+ cells corresponding to the increase in apoptosis, and slightly increased gamma-H2AX+/caspase-3- cells. The results indicated that the aneugens predominantly induced apoptotic gamma-H2AX while the clastogens mainly induced non-apoptotic gamma-H2AX under the test condition of 24 hours treatment in the study. The contribution of apoptosis to the generation of gamma-H2AX was different between clastogens and aneugens, suggesting that concurrent staining with a biomarker for apoptosis could be a solution to the problem.

5.4. High throughput screening with gamma-H2AX

Bryce *et al* (122) have proposed a series of biomarkers — gamma-H2AX, phosphorylated histone H3 (pH3), ATP, and polyploidy — as simplified endpoints to understand the MoA involved in positive responses from *in vitro* MN tests with TK6 cells. The biomarkers were sought based on well-designed experiments with 12 biological endpoints, including gamma-H2AX and caspase-3. The endpoints were measured after cells had been treated with a testing set of 10 chemicals (clastogens, aneugens, or non-genotoxic cytotoxicants) to identify the biomarkers essential for classifying a MoA. A combination of the identified biomarkers was examined using another testing set of 10 chemicals (6 clastogens, three aneugens, and one cytotoxicant). The results suggested that gamma-H2AX is particularly effective for measuring clastogenicity and that pH3, an M phase marker, is a reliable biomarker for aneugenicity. A collaborative study with the clastogenic etoposide, the aneugenic noscapine, and the cytotoxic tunicamycin also suggested that pH3 is a helpful marker for aneugens (123). The usefulness of a combined

assessment with gamma-H2AX and pH3 has been further supported by a later study (100).

In the above studies, caspase 3 was examined as an apoptosis marker but was concluded to be less effective than other endpoints (122). Instead, pH3 and polyploidy were recognized as good biomarkers for an aneugenic MoA (100, 122, 123). Because those studies attempted to classify clastogens, aneugens, and cytotoxicants using a test set that included apoptosis inducers like an anti-FAS antibody, tunicamycin (124), tributyltin (125) and caffeine (126) within the reference cytotoxicants, it is reasonable that caspase 3 was not useful for classifying into the three categories. Aneugens often cause cell cycle arrest at M-phase that can be identified by the pH3 biomarker, and thus pH3 is a good indicator for an aneugenic MoA.

Additional information provided by caspase-3 may still be useful for minimizing results of a screening that are misleading because some kinase inhibitor aneugens have no M-phase arresting activity and a low level of DSB may increase polyploidy. Checkpoint functions in the cell cycle were previously suggested to be incomplete (14, 127). Although BubR1 kinase is a central factor of spindle assembly checkpoint that protects from aneuploidy (128), this checkpoint can be bypassed before repairing small number DNA lesions, such as those with less than 20 DSB, probably because such slight DNA lesions did not activate BubR1 (14). The incomplete function of cell cycle arrest allows low level DNA damage to remain unrepaired during the transition to M phase, causes frailer cytokinesis, and results in polyploidy in the majority of cells (14). This suggests that, although increased polyploidy or pH3 are often seen after treatment with aneugens and are indicative of an aneugenic MOA, they may not be specific biomarkers for an aneugenic MoA.

6. DISCUSSION

Since 2005 when the comprehensive analysis of accumulated animal carcinogenicity and *in vitro* genotoxicity data revealed that the chromosomal aberration test had very low negative predictive values of 44.9. percent (only 61 negative in 136 non-carcinogenic compounds (129)), efforts have been made to reduce this unacceptably high rate of false positives. The methods known to be effective in decreasing misleading false positives are reduction of the maximum test concentration and selection of p53-functional cells to mitigate apoptotic influence (130-133). When 25 compounds were retrospectively re-evaluated for false-positive results in 2016 at lower concentration ranges, 12 compounds were found to be negative (134). These efforts have improved regulatory science.

However, it is questionable whether an assay can be accepted for use as a risk assessment or hazard identification tool based on higher concordance between *in vitro* genotoxicity assays and *in vivo* carcinogenicity tests. Accelerated cell proliferation, immunosuppression, and endocrine disruption are recognized as other plausible causes of carcinogenesis; therefore, as genotoxicity is not the sole reason for positive carcinogenicity in animal studies, the predictive power of any genotoxicity test cannot be very high. The fact that many animal cancers seen in carcinogenicity studies are not relevant to human risk may suggest the limited importance of predicting animal carcinogenicity from *in vitro* genotoxicity. Therefore, the most important part of an *in vitro* genotoxicity assessment for cancer risk in human is understanding the MoA and considering human relevance. Though the Ames test has had an indisputable impact on *in vitro* assessment, cytogenetic assays providing information on the MoA are also an important source of information.

The biomarker gamma-H2AX is changing *in vitro* mammalian genotoxicity screening from the traditional positive/negative selection to MoA elucidation. We can use various assay formats, including microscopic observation, flow cytometry, and automated image analysis, with various biomarkers of cell cycle and apoptosis, and convenient assay kits are commercially available. However, a standard methodology for the gamma-H2AX assay and a consensus threshold for positive criteria have not been established yet. At present, the most reliable classification of apoptotic and non-apoptotic induction of gamma-H2AX may be morphological discrimination by microscopy. Integrating caspase 3 and/or pH3 into the gamma-H2AX assay would provide reliable support for understanding the MoA, but is subject to the following two provisos: because induction of caspase 3 does not completely correlate with apoptotic phosphorylation of H2AX, a consensus on the criterion for irrelevant elevation of gamma-H2AX is needed; and because pH3 is not always indicative of an aneugenic MoA, a consensus on how to interpret low or no elevation of pH3 is needed. Achieving consensus on these considerations requires an interlaboratory validation study with standard reference compounds for clastogenicity, aneugenicity and cytotoxicity. A validation study of this nature would enable regulatory schemes to be established for using the new assay results for MoA-based risk assessment.

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Abbreviations: MN, micronucleus; DDR, DNA damage response; MoA, mode of action; SMN, secondary malignant neoplasm; TTC, threshold of toxicological concern

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