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H2AX phosphorylation as a genotoxicity endpoint

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ABSTRACT

The γH2AX focus assay, based on phosphorylation of the variant histone protein H2AX, was evaluated as a genotoxicity test in immortalised wild-type mouse embryonic fibroblasts (MEFs) treated for 4 h with a panel of reference compounds routinely used in genotoxicity testing. The topoisomerase II poison etoposide (0.006–60 μg/ml), the alkylating agent methyl methanesulfonate (1.3–65 μg/ml) and the direct DNA-damaging agent bleomycin (0.1–10 μg/ml) all produced a positive concentration–response relationship. The non-genotoxic compounds ampicillin (0.035–3500 μg/ml) and sodium chloride (0.058–580 μg/ml) showed no such response with increased concentrations. The H2AX phosphorylation results were compared with the outcome of two standard *in vitro* genotoxicity tests, namely the micronucleus and comet assays. Compounds that produced measurable DNA damage in the focus assay generated micronuclei at comparable concentrations. In this study, the focus assay identified genotoxic agents with the same specificity as the comet assay.

These results were substantiated when H2AX phosphorylation was analysed using flow cytometry in the murine cell line L5178Y, growing in suspension. The data were in concordance with the manual scoring focus assay. To further this investigation, the γH2AX flow cytometry was compared to the *in vitro* micronucleus flow cytometry and mouse lymphoma assay using the same cell population after MMS treatment. The median γH2AX value increased significantly above the control at all four MMS concentrations tested. The percentage of micronucleus events in the *in vitro* micronucleus flow test and the mutation frequency in the mouse lymphoma assay were also significantly increased at each MMS concentration. The current data indicate that H2AX phosphorylation could be used as a biomarker of genotoxicity, which could predict the outcome of *in vitro* mammalian cell genotoxicity assays.

1. Introduction

A specific variant of the H2A histone protein, H2A family member X, or H2AX, was first reported in 1980 by West and Bonner [1]. Bonner, along with Pantazis in 1981 [2], went on to show that H2AX can be phosphorylated and acetylated. The rapid phosphorylation of the carboxyl-tail of H2AX at Serine 139 [3], to become γH2AX, occurs in response to DNA damage and can be visualised by the formation of nuclear foci at DNA double-strand breaks (DSBs) [4,5]. It has been demonstrated that one γH2AX focus is equivalent to one double-strand break [6], at least when less than 100–150 DSBs are produced [7].

H2AX is phosphorylated by members of the phosphatidylinositol 3-kinase-like kinase (PIKK) family, which includes Ataxia-Telangiectasia Mutated (ATM), Ataxia Telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) [8–10]. Enzymes of the PIKK family are involved in DNA-damage response, DNA recombination and cell-cycle progression [11]. At sites of DSBs, ATM is activated by auto-phosphorylation at Ser1981 [12]. The phosphorylation of H2AX occurs over a large region of chromatin surrounding the DSB, estimated to be in the order of a megabase [4]. This expanded phosphorylation is the result of a positive feedback loop, which is essential to the accumulation of repair proteins and other ATM substrates that are required downstream in the DNA-damage response [4,13,14].

The use of γH2AX as a marker for DNA damage and because of its pharmacological interests, is very popular as it is deemed to be highly sensitive. Zhou et al. [15] evaluated the use of γH2AX-focus formation to detect DNA damage by treating cell lines with various cell stressors. The data indicated that formation of γH2AX foci could be used to evaluate DNA damage; however, 2-acetyl-aminofluorene produced mixed results in different cell types. γH2AX has been used as an indicator of cell killing by chemotherapeutic agents [16] and as a marker of DSBs in A549 human pulmonary adenocarcinoma cells when exposed to tobacco smoke [17]. Clinical researchers have also used γH2AX as a tool to evaluate therapy

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outcomes in patients [18–20] and for diagnosis of biopsy samples [21].

Early characterisation of the genotoxic potential of a chemical or pharmaceutical is essential in drug development. To standardise the practise of chemical hazard identification, the International Conference on Harmonisation (ICH) recommended a set of "guidelines for testing pharmaceuticals for genetic toxicity" [22]. A battery of complementary tests was suggested: the Ames bacterial mutagenesis assay, an *in vitro* cytogenetic assay and an *in vivo* test for genetic damage. In addition to assays being conducted for regulatory requirements, 'pre-screening' assays are essential to assess the genotoxic effect of chemicals early in the development of new drugs to reduce late-stage attrition.

The formation of micronuclei after drug treatment can be used to assess the clastogenic nature of a compound. The reliability of this assay has been thoroughly validated [23–26] and it has been further developed to increase its throughput using flow cytometry, with varying degrees of success [27–30]. The alkaline comet assay is a DNA-damage assay that is also used both *in vitro* and *in vivo*. It has the ability to detect both single-stranded and double-stranded DNA breaks, the majority of a-purinic and a-pyrimidinic sites and alkali-labile DNA adducts [31–33].

H2AX phosphorylation was evaluated as a marker for genotoxicity in two different murine cell types. This study used two different methods of assessing H2AX phosphorylation to assess the response to genotoxic and non-genotoxic agents: microscope-based manual scoring of γH2AX foci and a high-throughput method using flow cytometry. The manual scoring of γH2AX foci in the focus assay was directly compared with the alkaline comet and micronucleus assays, which are routinely used as genotoxic endpoints in the pharmaceutical industry. Although H2AX phosphorylation was determined to be a useful marker for genotoxicity comparable with manual scoring of micronucleus and comet slides, no distinct advantage was noted for the focus assay to be a viable genotoxicity screening assay. Therefore, H2AX phosphorylation was analysed using flow cytometry in a commonly used single-cell suspension cell line, L5178Y. The results were in concordance with the manual scoring data. When compared with the *in vitro* flow micronucleus assay [28] and mouse lymphoma assay [34] using the same cell population after MMS treatment, all three endpoints were significantly induced for all concentrations of MMS tested.

2. Results

2.1. Manual scoring in mouse embryonic fibroblasts

2.1.1. Genotoxins increased γH2AX phosphorylation in a concentration-dependent manner

An increase in H2AX phosphorylation, as visualised by γH2AX foci, was found in response to increasing concentrations of etoposide, bleomycin or methyl methanesulfonate (MMS). The concentration-dependent increase in the mean of the median number of γH2AX foci per nucleus was seen at all concentrations tested (Table 1). A statistically significant increase in γH2AX foci occurs from 0.6 μg/ml etoposide ($p < 0.01$, one-way repeated measures ANOVA). The number of foci increased further when the concentration of etoposide was increased to 6 μg/ml and 60 μg/ml. The positive concentration–response with the radiomimetic bleomycin demonstrated an increase in the mean of the median number of γH2AX foci at the lowest concentration, 0.1 μg/ml, to a statistically

Table 1
Mean results of three independent experiments following 4-h treatments with etoposide, bleomycin, MMS, sodium chloride and ampicillin in mouse embryonic fibroblasts for γH2AX focus assay, alkaline comet assay and micronucleus assay.

Compound	Concentration (μg/ml)	Foci number	Fold increase	Integrated fluorescence (×10^5)	Fold increase	Tail moment	Fold increase	% MN	Fold increase
Etoposide	0	15	–	5.1	–	2.1	–	1.7	–
Etoposide	0.006	19	1.3	5.5	1.1	2.4	1.1	1.9	1.1
Etoposide	0.06	31	2.1	10	2.0	2.4	1.1	3.3	1.9
Etoposide	0.6	66*	4.4	35	6.9	3.5	1.7	7.1*	4.2
Etoposide	6	98*	6.5	55	10.8	15.0*	7.1	9.5*	5.6
Etoposide	60	108*	7.2	147	28.8	19.2*	9.1	17.9*	10.5
Bleomycin	0	29	–	32	–	1.7	–	1.7	–
Bleomycin	0.1	49	1.7	35	1.1	1.5	0.9	3.5*	2.1
Bleomycin	0.5	41	1.4	32	1.0	2.0	1.2	4.2*	2.5
Bleomycin	1	54*	1.9	39	1.2	2.8	1.6	7.3*	4.3
Bleomycin	5	60*	2.1	56*	1.8	3.3	1.9	9.7*	5.7
Bleomycin	10	69*	2.4	90*	2.8	4.0	2.4	12.7*	7.5
MMS	0	10	–	3.1	–	1.2	–	2.0	–
MMS	1.3	26	2.6	5.4	1.7	1.1	0.9	1.7	0.9
MMS	6.5	27	2.7	7.3	2.4	2.2	1.8	2.5	1.3
MMS	13	53*	5.3	12	3.9	2.2	1.8	2.3	1.2
MMS	32.5	46*	4.6	10	3.2	2.5	2.1	7.9*	4.0
MMS	65	98*	9.8	34*	11.0	2.1	1.8	9.8*	4.9
Sodium chloride	0	11	–	4.9	–	1.5	–	2.0	–
Sodium chloride	0.058	17	1.5	5.6	1.1	0.9	0.6	2.5	1.3
Sodium chloride	0.58	8	0.7	2.7	0.6	1.1	0.7	1.9	1.0
Sodium chloride	5.8	23	2.1	5.5	1.1	0.6	0.4	2.0	1.0
Sodium chloride	58	19	1.7	7	1.4	0.8	0.5	1.9	1.0
Sodium chloride	580	21	1.9	4	0.8	1.3	0.9	1.7	0.9
Ampicillin	0	11	–	4.9	–	2.2	–	2.1	–
Ampicillin	0.035	12	1.1	5.9	1.2	–	–	–	–
Ampicillin	0.35	15	1.4	6.8	1.4	1.6	0.7	1.6	0.8
Ampicillin	3.5	11	1.0	3.8	0.8	1.1	0.5	2.2	1.0
Ampicillin	35	10	0.9	2.6	0.5	1.0	0.4	2.2	1.0
Ampicillin	350	14	1.3	4.3	0.9	1.5	0.7	1.8	0.9
Ampicillin	3500	–	–	–	–	0.4	0.2	2.3	1.1

The three assays were analysed by manual scoring of slides.
* Statistical significance $p < 0.05$.