**Assessment of the in vitro γH2AX assay by High Content Screening as a novel genotoxicity test: Application for the evaluation of single toxicants and mixtures**

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INTRODUCTION

There are multiple forms of DNA damage. The most deleterious lesions are double-strand breaks (DSBs) since no complementary strand is left intact to act as template during DNA repair.

Phosphorylation of the histone 2AX resulting in γH2AX occurs in the cell nucleus as an early response to DSBs. The quantification of γH2AX can be used as a measure of genotoxicity in vitro.

Here, we assessed the specificity and sensitivity of the in vitro γH2AX assay by High Content Screening (HCS) in bronchial epithelial BEAS-2B cells. Then we applied the assay in the evaluation of two cigarette smoke toxicants individually and in a binary mixture.

METHODS

- Initially, in vitro genotoxic positive and negative compounds with different DNA damage mechanisms were selected for treatment of BEAS-2B cells for 3 and 24 hr (Table 1). The maximum concentration tested was 1 mM when not limited by solubility.

<table>
<thead>
<tr>
<th>Direct DNA-damaging</th>
<th>Ethyl methanesulfonate, Mitomycin C, MNNG</th>
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</thead>
<tbody>
<tr>
<td>Reactive oxygen species generator</td>
<td>Bleomycin-sulfate</td>
</tr>
<tr>
<td>Topoisomerase inhibitor</td>
<td>Amacrine (m-AMSA), Ethoposide</td>
</tr>
<tr>
<td>Nucleotide/DNA synthesis inhibitor</td>
<td>Amsacrine, Mitomycin C</td>
</tr>
<tr>
<td>Anaglone</td>
<td>Ascorbic acid, t-Aminobenzoic acid, DMSO</td>
</tr>
<tr>
<td>Artifical in vitro positive</td>
<td>2,4-Dichlorophenol, D,L-menthol</td>
</tr>
<tr>
<td>In vitro negative</td>
<td>Acetaminophen, 5-Amino-1,2,4-triazole, Dimethyl formamide, Ethylene glycol, DMSO, Sodium chlorite</td>
</tr>
</tbody>
</table>

- Two tobacco-specific nitroamines: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosornicotinone (NNN), were then tested individually (in the presence and absence of Aroclor-induced rat S9 mix) for 3 hr. Acetylated NNN and NNN reactive precursors were also tested for 3 hr individually and in different mixture permutations.

Immunostaining for γH2AX was assessed in formaldehyde-fixed BEAS-2B cells set up in microplates. A primary antibody specific to the phosphorylated form of H2AX was used in conjunction with a secondary antibody labelled with DyLight™ 549 and Hoechst dye to stain the cell nuclei (Thermo Fisher). Images of treated and untreated cells were taken by the Cellomics ArrayScan® II platform (Fig. 1). At least 250 cells were scored per well. The ArrayScan® software v.6.6.1.4 was used to quantify the frequency of γH2AX, represented by fluorescence in intensity units (IU). Cell counts obtained during the immunostaining were used to calculate Relative Cell Counts (RCC). The RCC were included in the γH2AX frequency graphs as a cell viability indicator.

RESULTS

γH2AX

Each of the compounds known to interact directly with DNA resulted in a significant dose-related response compared to the vehicle-treated control (Fig. 2A). Compounds which induce DSBs by other mechanisms such as topoisomerase inhibition or ROS generation also showed a significant dose-response (Fig. 2B and C).

No significant increase in the frequency of γH2AX when compared to the vehicle-treated control was observed with any of the in vitro genotoxicity negative compounds.

Figures 2A, 2B, 2C: γH2AX frequency (solid lines) and RCC (dashed lines) after 3 and 24 hr exposure to [A] direct acting agent ethyl methanesulphonate; [B] ROS generator bleomycin-sulfate; [C] topoisomerase inhibitor etoposide. RCC error bars (n=3) are not included in the graphs for clarity. * Indicates minimum concentration showing a significant increase in γH2AX frequency compared to the vehicle-treated control.

Mixtures

NNK and NNN tested individually in the presence of S9 mix produced a negative response probably due to the lack of bioactivation (data not shown). Acetylated NNN and NNN reactive precursors did produce a dose-related response. NNK-acetate is the primary driver of the genotoxic response when present in mixture with NNN-acetate (Fig. 3).

Figures 3A, 3B, 3C: γH2AX frequency after 3 hr treatments with different ratios of TSNA mixtures. Asterisk (*) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after treatment with 100% NNK-acetate. Dagger (†), section sign ($) and double dagger (‡) indicate the minimum concentration that shows genotoxicity compared to the vehicle treated control after treatment with 75% NNK-acetate and 25% NNN-acetate, 50% NNK-acetate and 50% NNN-acetate and 25% NNK-acetate and 75% NNN-acetate respectively. Hash (#) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after treatment with 100% NNN-acetate.

CONCLUSIONS

- The in vitro γH2AX assay by HCS has shown potential as a novel genotoxicity assay and would be a useful pre-screening tool for testing large number of compounds such as cigarette smoke toxicants and mixtures thereof.

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