Genotoxicity evaluation using flow cytometry based micronucleus test in HepG2 cells

Humans are exposed to the contaminated environment which may contain known human or animal carcinogens and it was estimated that environmental chemicals contribute to 7.19% of cancers in humans (Goodson et al. 2015). Among the total number of Group 1 agents (known human carcinogens as per IARC classification), most belong to different classes of chemicals (Baan et al. 2009).

Polyaromatic hydrocarbons (PAHs) and metals are the major chemicals in the environment and have been associated with adverse health effects in humans. PAHs are formed during the incomplete combustion of organic materials and also present in cigarette smoke, vehicle exhaust, coal tar, and charcoal broiled foods. In 1775, Sir Percival Pott first observed an association between an increased incidence of scrotal cancer in chimney sweeps and exposure to soot (Pott 1775). Since then many epidemiological studies in PAH exposed workers have reported the increased incidence of cancers in humans (ATSDR 1995, Bosetti et al. 2007, Zhang et al. 2009, IARC 2010, Silverman et al. 2012, Kim et al. 2013).

There are no epidemiological data available for incidences of cancer followed by B[a]P exposure alone. But studies with experimental animals showed that B[a]P produced tumors in all tested species by different routes of exposure (IARC 2012a). Occupational exposure to B[a]P containing PAHs mixtures have been associated with increased risk of cancer (lung, bladder, skin and hematolymphatic system IARC 2010). Arsenic (As) and cadmium (Cd are known human carcinogens. Occupational and
environmental exposure to Cd causes lung, kidney, and prostate cancers (IARC 2012b). Arsenic exposure has been linked with lung, urinary bladder, skin and kidney cancers in humans (IARC 2012c).

Mixed contamination of PAHs, As, Cd, and Pb is elevated in the environment due to increased industrial and anthropogenic activities (Megharaj and Naidu 2008).

Benzo[a]pyrene, As, Cd, and Pb are top priority pollutants in the ATSDR substance priority list (ATSDR 2015) and also the major contaminants in various contaminated sites around the world (Megharaj and Naidu 2008, Panagos et al. 2013). The data on the co-genotoxicity of metals and B[a]P have so far been limited to binary mixtures of B[a]P with As or Cd and the co-genotoxic effects of metals are inconsistent in these studies which indicate synergistic or antagonistic effect of metals on the genotoxicity of B[a]P (Maier et al. 2002, Tran et al. 2002, Mukherjee et al. 2004, Fischer et al. 2005, Lewińska et al. 2007, Simon et al. 2014). There are several hundred PAHs among which 17 PAHs are prioritized due to their common occurrence in the contaminated sites and potential for human exposure (ATSDR 1995).

Simple hydrocarbons like naphthalene (Nap), phenanthrene (Phe) and pyrene (Pyr) are common non-carcinogenic PAHs found along with B[a]P in most of the contaminated sites (ATSDR 1995). Pyrene is one of the abundant hydrocarbons in PAHs mixtures and used as a biomarker of PAH exposure (Silins and Högberg 2011). Naphthalene is classified as Group 2B, Phenanthrene and Pyr are Group 3 carcinogen (IARC, 2010). The genotoxicity of PAHs mixtures or as a binary combination of B[a]P with other PAHs vary depending on the PAHs that are present in the mixtures and the reported findings include synergism, additivity or antagonism (Staal et al. 2008, Tarantini et al. 2009, Jarvis et al. 2013).

The In vitro micronucleus test has been routinely used to
screen the genotoxicity of pharmaceutical agents and environmental chemicals. This assay has higher throughput than the traditional genotoxicity assays and reliable MN measurement help to determine the genotoxicity of chemicals (Bryce et al. 2007).

In this study, HepG2 cells are used as a test system, since liver plays a major role in the metabolism of PAHs and is one of the target organs of metal toxicity. Apart from the inherent capacity of metabolic enzymes, these cells express p53 protein which will be useful to screen the p53 mediated DNA damage (Boehme et al. 2010, Zager et al. 2010) In our previous study, we have reported the genotoxicity of mixtures of As, Cd and PAHs in HepG2 cells (Peng et al. 2015). But this study evaluated only the binary mixtures of metals (As and Cd) with B[a]P and a quaternary combination of B[a]P with Nap, Phe, and Pyr.

This study did not evaluate the genotoxicity of all possible mixture combinations of metals and PAHs. In addition, the MN was counted manually which was low in throughput. In this study, a flow cytometry based MN test was used to determine the individual and combined effects of As, Cd, Pb and PAHs (Nap, Phe and Pyr) on the genotoxicity of B[a]P in HepG2 cells.

The genotoxic effects were determined for the binary, ternary, quaternary and seven component mixtures of PAHs and metals. In addition, the individual and combined effects of Cd and Pb on the genotoxicity of As was determined. The AhR pathway plays a major role in the toxicity of PAHs (Baird et al. 2005, Galván et al. 2005, Nebert and Dalton 2006). In this study, the effects of As, Cd, Pb and PAHs (Nap, Phe and Pyr) on the AhR activation of B[a]P were determined using the HepG2 cell based chemically activated fluorescent (CAFLUX) assay.
Materials and methods

Chemicals

Cell culture medium DMEM (Dulbecco’s Modified Eagle Medium), Trypsin EDTA (0.25%), penicillin streptomycin solution and fetal bovine serum (FBS) were purchased from Gibco (Life Technologies Ltd, VIC, Australia). CellTiter 96 Aqueous One Solution Cell Proliferation Assay (G3581) was purchased from Promega Corporation, Madison, USA.

The In Vitro microflow kit was purchased from Litron laboratories Ltd, Rochester, NY, USA. The kit contains incomplete lysis solutions 1 and 2, nucleic acid dye A (ethidium monoazode, EMA), nucleic acid dye B (SYTOX® green nucleic acid stain), RNase solution and 10X buffer. PeakFlowTM green flow cytometry reference beads, 6 µm was purchased from Molecular robes (Life Technologies Ltd), USA. Benzo[a]pyrene (B[a]P), (CAS number: 50 32 8), naphthalene (CAS number: 91 20 3), phenanthrene (CAS number: 85 01 8), pyrene (CAS number: 129 00 0), cadmium chloride (CAS number: 10108 64 2), lead acetate (CAS number: 6080 56 4) and sodium arsenite (CAS number: 7784 46 5) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell culture

HepG2 cells (ATCC No. HB 8065) were obtained from ATCC (American type culture collection), Manassas, USA.

The cells were maintained as a subconfluent monolayer in 75 cm culture flask using the modified DMEM medium with 10% (v/v) FBS + penicillin streptomycin (50 Units/mL) and used for experiments after two weeks of thawing from cryopreserved stock. The cells were harvested using 0.25% Trypsin EDTA (prewarmed to 37ºC).

For cytotoxicity and MN study, the cells (1×10 cells/well)
were seeded into 96 well plates and incubated at 37 °C under 5% CO in an incubator (Heracell 150i CO incubator, Thermo Scientific, Australia) for 24 h before chemical treatment. Stock solutions of PAHs (B[a]P, Nap, Phe and Pyr) and metals (As, Cd and Pb) were prepared in DMSO and MilliQ water (18 MΩ.cm, Merck Millipore, VIC, Australia), respectively. The working solutions prepared in DMEM + FBS (10%) medium or DMEM alone were used for MN test and Ah CAFLUX assay, respectively. The working solutions were added to the plates reaching a final concentration of 0.5% v/v of vehicle control (DMSO or MilliQ water).

**In vitro flow cytometry micronucleus test**

The selected concentrations of individual MN study were 0 to 2 μM for B[a]P and Cd; 0 to 10 μM for As and 0 to 100 μM for Pb. Initially, the cytotoxicity of PAHs and metals were determined using the MTS (tetrazolium compound [3 (4,5 dimethylthiazolyl) (3 carboxymethoxyphenyl) (4 sulfophenyl) 2H tetrazolium]) assay. The cytotoxicity assay was carried out as described by Muthusamy et al. (2016b).

Based on their cytotoxicity to HepG2 cells, the concentrations of metals and PAHs were selected for the MN study. The treatment was carried out for 1.5 – 2 normal doubling period of HepG2 cells (OECD 2014). The details of selected individual concentrations are provided in Table 1. Cell staining and lysis were carried out as instructed by the manufacturer, Litron Laboratories Ltd, Rochester, NY, USA. In brief, the plates were removed from the CO incubator after a treatment period of 48 50 h and placed on ice for 20 min.
Table 1. Concentrations of individual and mixtures of PAHs, As, Cd, and Pb of genotoxicity evaluation using flow 2 cytometry based micronucleus (MN) test in HepG2 cells.

The supernatant was carefully removed and nucleic acid dye A working solution (EMA + 1 x buffer solution) was added to each well. Then, the plates were exposed to light for photoactivation and the cells were washed with 1 x buffer solution. The complete lysis I solution (containing lysis solution + Sytox Green + RNase) was added to the cells and incubated at 37ºC for 1 h. Then, lysis II solution (lysis II + Sytox green + counting beads) was added, and the plates were further incubated at room temperature for 30 min and proceeded to flow cytometry analysis.

Flow Cytometry analysis

Flow cytometry analysis of MN was carried out using the BD LSR II analyser (Becton, Dickinson, and Company, BD Biosciences, San Jose, USA). The experimental template provided by Litron Laboratories was used for analysis. The cells were resuspended and protected from light during the analysis. Initially, vehicle control samples were used to adjust the voltage to capture the nuclei and an average 7,500–10,000 events/well were recorded.

The data analysis was carried out using BD FACSDiva TM Software (BD FACSDiva TM Software Reference Manual, Version 8.0.1, BD Biosciences, San Jose, USA). The MN values were expressed as frequency percent and calculated by dividing the

<table>
<thead>
<tr>
<th>Mixture type</th>
<th>B(a)P (µM)</th>
<th>As (µM)</th>
<th>Pb (µM)</th>
<th>Cd (µM)</th>
<th>Nap, Phe and Pyr (µM)</th>
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<tbody>
<tr>
<td>Individual</td>
<td>0.5, 1 and 2</td>
<td>2.5, 5, 10 and 20</td>
<td>12.5, 25 and 100</td>
<td>0.5, 1 and 2</td>
<td>5 and 15</td>
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<td>dose response study</td>
<td></td>
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<td></td>
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<tr>
<td>Binary mixtures</td>
<td>5, 10</td>
<td>25, 50</td>
<td>0.3, 1</td>
<td>5, 15</td>
<td></td>
</tr>
<tr>
<td>Ternary mixtures</td>
<td>2.5, 5, 12.5, 25</td>
<td>0.15, 0.5</td>
<td>2.5, 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quaternary mixtures</td>
<td>1.25</td>
<td>3.12</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAHs + metals</td>
<td>10</td>
<td>25, 50</td>
<td>0.3, 1</td>
<td></td>
<td></td>
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<tr>
<td>Ternary mixtures of</td>
<td>10</td>
<td>25</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>metals</td>
<td></td>
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</table>
number of events that fall within the “MN” region by the number of events that fall within the “Nucleated” region and multiplying by 100. The criterion to consider the positive response is that increase of greater than two fold in mean % MN in the individual or chemical mixtures treated groups compared to concurrent vehicle control values (Shi et al. 2010).

**Mixture experiments- Determination of effects of multi-component mixtures of PAHs and metals on MN formation**

The individual concentration response of the MN test showed that B[a]P and As gave a clear positive response for MN formation. The mixture experiments were designed to study the effects of individual or mixtures of As, Cd, and Pb/or PAHs on the genotoxicity of B[a]P. In this study, two independent experiments were conducted for binary, ternary, quaternary, and seven component mixtures of PAHs and metals.

In this study, the experiments for binary, ternary, quaternary, and seven component mixtures of PAHs and metals were carried out in two steps. In step one, the experiments were conducted using the binary combinations of PAHs and metals. Based on results obtained from step 1, the next set of studies was extended to ternary to seven chemical mixtures combinations. In addition, the effects of Cd and Pb on the MN formation of As were also determined. The results of individual and different combinations of PAHs and metals are expressed as fold change in the MN frequency compared to the vehicle control. The details of selected concentrations of the mixture study are provided in Table 1. The cytotoxicity of the selected concentrations of the PAHs and metal mixtures were determined using the MTS assay.
Cell cycle analysis

The cell cycle parameters, G1, S and G2/M were analyzed using ModFit LT (version 3.2) Software (ModFit LT user guide), Verity Software House, ME, USA. The linear scale recording of nucleus fluorescence intensity from flow cytometry analysis was used for cell cycle analysis.

Determination of activation of AhR using HepG2 cell based

CAFLUX assay HepG2 cells transfected with the reporter plasmid, pGreen1.1 (dioxin responsive enhanced green fluorescent protein, EGFP) was used to determine the effects of individual or mixtures of B[a]P with metals or PAHs on the AhR. In our previous study, we have reported the procedure for transfection of HepG2 cells with the reporter plasmid, pGreen1.1 (Peng et al. 2015) Cell culture: The cells were maintained in a medium containing DMEM + 10% (v/v) FBS + G418 sulphate + penicillin streptomycin (50 Units/ml). On the day of experiments, cells (30,000 cells/well) were seeded into 96 well plate (Corning® 96 well flat clear bottom, sterile black polystyrene TC treated microplates, Corning Life Sciences, NY, USA) and incubated at 37 °C under 5% C 2 for 24 h. The individual dose response study of AhR activation was carried out for B[a]P, Nap, Phe, and Pyr.

For mixtures studies, the effects of metals (Cd (0 to1 µM), As (0 to10 µM) and Pb (0 to 50 µM)) and other PAHs ((Nap, Phe and Pyr, 0 to 15 µM) on the AhR activity of B[a]P (2 µM) was determined. After the chemical treatment, the cells were incubated at 33ºC under 5% CO for 72 h. It was observed that cells that are grown at 33 ºC results in several fold greater EGFP activity than cells incubated at 37 ºC (Zhao et al. 2010). After the treatment period of 72 h, the fluorescence intensity as a measurement of EGFP expression was recorded using FLUOstar Omega, BMG Labtech, VIC, Australia.
The fluorescence intensity of control and treated groups was presented after deduction of background fluorescence intensity.

**Statistical analysis**

The data from MN test, cell cycle analysis, and the AhR activation assay were analysed by “one way ANOVA followed by Turkey’s multiple comparison tests using GraphPad Prism version 6.00 for Windows”, GraphPad Software, La Jolla California USA, (www.graphpad.com). In case of MN test, the fold change in the MN frequency compared to the vehicle control were calculated for individual and chemical mixtures. The results of fold change in the MN frequency was used for statistical analysis and the fold change of MN frequency from chemical mixture treated group were compared against B[a]P or As depending on the type of chemical mixtures. A significant difference between the control and treated groups was evaluated at p <0.05.

**Results**

**Cytotoxicity**

The cytotoxicity of binary, ternary, quaternary and seven component mixtures of PAHs, As, Cd, and Pb are presented in Supplementary Material Figure S1. At the selected concentrations, the individual PAHs and metals were not toxic to HepG2 cells. The binary to multi component mixtures of PAHs, As, Cd, and Pb were found to be toxic to HepG2 cells (up to 40% reduction in cell viability). In the case of B[a]P and metals mixtures, the binary mixture of B[a]P + As and the ternary mixture of B[a]P + Cd + Pb caused 38 and 35% of reduction in cell viability, respectively. Among the B[a]P + PAHs mixtures, a maximum reduction of 39% in cell viability was observed with B[a]P + Phe and B[a]P + Nap + Phe mixtures. The quaternary mixtures of B[a]P with metals (26%) or PAHs
and multi component mixtures of PAHs and metals (34%) also reduced the cell viability of HepG2 cells.

**Micronucleus test**

Benzo[a]pyren was clearly positive for MN formation in HepG2 cells but other PAHs (Nap, Phe and Pyr) did not elicit positive responses. Among the metals, As was clearly positive for MN formation, and Cd and Pb showed a weak positive response effect for MN formation. The effects of individual PAHs and metals on MN formation in HepG2 cells are presented in Supplementary Material Figure S2.

**Effects of individual and mixtures of metals on the genotoxicity of B[a]P**

The binary mixture studies were conducted for mixtures of B[a]P (2 µM) with As (5 and 10 µM) or Cd (0.3 and 1 µM) or Pb (25 and 50 µM) (Figure 1). The binary mixtures of B[a]P with metals significantly increased the MN formation compared to B[a]P alone. At the selected low and high concentrations of mixtures the binary combinations of B[a]P and metals resulted in two and four fold increase of MN formation compared to B[a]P alone, respectively.
Figure 1. Effects of mixtures of B[a]P and metals on micronucleus (MN) formation in HepG2 cells. Results are expressed as fold change in MN formation compared to vehicle control. * indicates significant difference (p<0.05) compared to B[a]P alone. B[a]P benzo[a]pyrene, As arsenic, Cd cadmium and Pb lead. Values are mean ± SD, n= 4.

The ternary mixture studies were conducted using B[a]P (2 µM) with As + Cd (2.5 + 0.15 and 5 + 0.5 µM) or As + Pb (2.5 + 12.5 and 5 + 25 µM) or Cd + Pb (0.15 + 12.5 and 0.5 + 25 µM) (Figure 1). The ternary mixtures of B[a]P and metals increased the MN formation compared to B[a]P alone. Among the ternary mixtures, the mixture of B[a]P + As + Cd (2 + 5 + 0.50 µM) showed a maximum increase of 4.7 fold in the MN formation compared to B[a]P alone. The quaternary mixture of B[a]P + As + Cd + Pb did not show a significant increase in the MN formation (2 fold vs B[a]P alone) compared to B[a]P alone (Figure 1).

Effects of individual and mixtures of Nap, Phe and Pyr on the genotoxicity of B[a]P

The binary combination of B[a]P with Nap or Phe or Pyr and ternary mixtures (B[a]P + Nap + Phe, B[a]P + Nap + Pyr and B[a]P + Phe + Pyr) decreased the MN formation compared to B[a]P alone (up to 60% reduction in MN formation vs B[a]P
Figure 2. Effects of multi component mixture of PAHs and metals and mixtures of metal on micronucleus (MN) formation in HepG2 cells. Results are expressed as fold change in MN formation compared to vehicle control. * indicates significant difference (p<0.0) compared to B[a]P or As alone. B[a]P benzo[a]pyrene, As arsenic, Cd cadmium, Nap naphthalene, Pb lead, Phe phenanthrene and Pyr pyrene Values are mean ± SD, n=4.

The quaternary mixture (B[a]P + Nap + Phe + Pyr) and seven component mixtures (containing both PAHs and metals) did not
show significant difference in the MN formation compared to B[a]P alone (Figure 2). 3.1.3 Effects of Cd and Pb on the genotoxicity of As The binary and ternary mixtures of Cd and Pb with As increased the MN formation compared to As alone (Figure 2). Among the binary mixtures, As + Pb mixture showed a maximum of 2 fold increase in the MN formation compared to As alone. In the case of a ternary mixture of As + Cd + Pb, the observed increase of MN formation was less than those of binary mixtures (Figure 2).

**Effects of individual and mixtures of PAHs and metals on cell cycle parameters**

The effects of mixtures of PAHs and metals on cell cycle distribution and percentage of cells in different phases G1, S and G2/M in HepG2 cells are presented in Tables 1 to 2. The individual treatment of B[a]P alone reduced the cell population in G1 phase and increased the cell accumulation in G2/M phase compared to vehicle control, respectively. The binary combination of B[a]P with metals decreased the cell population in G1 phase (maximum of 39%) and mixtures of B[a]P with As or Cd (at higher concentration combination) increased the cell accumulation in G2/M phase (maximum of 60%) compared to B[a]P, respectively (Table 1).

In the case of ternary mixtures, B[a]P + As + Cd decreased the cell population in G1 phase and increased the cell accumulation in G2/M phase, respectively. The other ternary mixtures of B[a]P and metals (B[a]P + As + Pb and B[a]P + Cd + Pb) decreased the cell numbers in G1 phase and increased the cell population in S phase, respectively. The treatment related changes of increase and decrease of the cell population in G1 and G2/M phase were observed with a quaternary mixture of B[a]P + As + Cd + Pb, respectively (Table 1). The mixtures of B[a]P with Nap, Phe and Pyr did not cause any changes in the cell cycle parameters (G1, S and G2/M phases) compared to B[a]P alone (Table 2).
Similarly, quaternary and seven component mixtures did not result in any changes in cell cycle parameters compared to B[a]P alone. The metal mixtures, As with Cd or Pb (binary combination of As + Pb (10 +50 ), As + Cd (10 +1 µM) and As +Cd + Pb (10 +25 + 0.5 µM) decreased the cell population in G1 phase and increased the cell accumulation at G2/M phase, respectively (Table 2).

Table 2: Effects of mixtures of B[a]P and metals on cell cycle parameters in HepG2 cells

<table>
<thead>
<tr>
<th>Effects of individual PAHs on activation of AhR</th>
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<tbody>
<tr>
<td>The individual PAHs activated the AhR in HepG2 cell based CAFLUX assay. Among the PAHs, B[a]P was a potent inducer of the AhR. The remaining three PAHs (Nap, Phe and Pyr) also activated the AhR, but the observed effect were less than that of B[a]P (Supplementary Material Figure S3).</td>
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</table>

Effects of individual and mixtures of B[a]P and metals on activation of AhR

Individual (As or Cd or Pb) or combinations (As + Cd or As + Pb or Cd + Pb) of metals did not activate the AhR in HepG2 CAFLUX assay. The binary (B[a]P + As or Cd or Pb) or ternary
mixtures (B[a]P + (As + Cd) or (As + Pb) or (Cd + Pb) of B[a]P and metals significantly increased the activation of AhR compared to B[a]P alone (Figure 3). Among these mixtures, the mixture of B[a]P + As and B[a]P + As + Pb resulted in a maximum of 17% increase in the AhR activity compared to B[a]P alone.
Figure 3. Effect of arsenic (As), cadmium (Cd) and lead (Pb) on AhR activation of B[a]P in HepG2 cell based CAFLUX (chemical activated fluorescence gene expression) assay. The changes in the expression of enhanced green fluorescent protein (EGFP) indicate activation of AhR and measured as fluorescence intensity (FI). * indicates significance (p<0.0) compared to B[a]P. Values are mean ± SD, n=3.

**Effects of individual and mixtures of B[a]P, Nap, Phe, and Pyr on activation of AhR**

The binary combination of B[a]P with Nap, Phe and Pyr (B[a]P + Nap or Phe or Pyr) or ternary combinations (B[a]P + (Nap + Phe) or (Nap + Pyr) or (Phe + Pyr)) reduced the activation of AhR compared to B[a]P alone (Figure 4). The mixtures of B[a]P + Phe and B[a]P Nap + Phe resulted in 16 and 12% reduction in the AhR activity compared to B[a]P alone, respectively.
Figure 3. Effects of individual and mixtures of naphthalene (Nap), phenanthrene (Phe) and pyrene (Pyr) on AhR activation of B[a]P in HepG2 cell based CAFLUX (chemical activated fluorescence gene expression) assay. The changes in the
expression of enhanced green fluorescent protein (EGFP) indicate activation of AhR and measured as fluorescence intensity (FI). * indicates significance ($p<0.0$) compared to B[a]P. Values are mean ± SD, n=3.