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Multi-component mixtures of polyaromatic hydrocarbons and heavy metal/loid(s) effects on Nrf2-Antioxidant Response Element (ARE) pathway in ARE reporter–HepG2 cells

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ABSTRACT

Exposure to polyaromatic hydrocarbons (PAHs) and heavy metal/loid(s) has been demonstrated to induce oxidative stress response in mammalian cells. The combined effect of PAHs and heavy metal/loid(s) on oxidative stress response has not been reported extensively. The Nrf2 antioxidant response pathway plays an important role in cellular antioxidant defense against oxidative stress induced cell damage. In this study, we have determined the combined effect of four PAHs (benzo[a]pyrene (B[a]P), naphthalene (Nap), phenanthrene (Phe) and pyrene (Pyr) and three heavy metal/loid(s) (arsenic (As), cadmium (Cd) and lead (Pb)) on the Nrf2 antioxidant pathway using the ARE reporter-HepG2 cell line. The mixture study was carried out for binary, ternary, quaternary and seven-chemical combinations of PAHs and heavy metal/loid(s). Initially, individual dose responses for PAHs (B[a]P, Nap, Phe and Pyr) and heavy metal/loid(s) (As, Cd and Pb) as well as their respective concentrations that induced an induction ratio of 1.5 ($EC_{IR1.5}$) were determined. The luciferase assay system was used to quantify the induction of the Nrf2 antioxidant pathway. The individual dose response study showed that both PAHs and heavy metal/loid(s) activated the Nrf2 antioxidant pathway in ARE reporter-HepG2 cells. Among these chemicals, Cd was the most potent inducer followed by B[a]P and As. Based on the individual dose response findings, PAHs and heavy metal/loid(s) were mixed at equipotent ratios using a fixed concentration ratio, and the effects of the mixtures of PAHs and heavy metal/loid(s) (binary to seven-component) on the Nrf2 antioxidant pathway were determined. The mixture effects were predicted by using concentration addition (CA) model. Overall, the results showed that the multi-component mixtures of PAHs and heavy metal/loid(s) induced oxidative stress response in ARE reporter-HepG2 cells, and that the CA model is an appropriate model to predict the interaction effect of these selected mixtures. A human cell line based reporter gene assay

system was successfully used to determine mixture effects of two groups of common contaminants on oxidative stress response pathways.

Keywords: Mixture toxicity, oxidative stress response, reporter gene assay system, Nrf2 antioxidant response pathway

72 **Abbreviations**

73 ARE - Antioxidant response element

74 As – Arsenic

75 ATSDR - Agency for Toxicological Substances and Disease Registry

76 B[a]P - Benzo[a]pyrene

77 CA - Concentration addition

78 Cd - Cadmium

79 DMSO - Dimethyl sulfoxide

80 $EC_{IR1.5}$ - Concentration that induces an induction ratio of 1.5

81 EDTA - Ethylene diamine tetra acetic acid

82 EPA - Environmental Protection Agency

83 EXP - Experimental values

84 h - Hour or hours

85 HEK- Human embryonic kidney cells

86 HepG2 - Human hepatocellular carcinoma cell

87 IA - Independent action

88 IARC - International Agency for Research on Cancer

IPQ - Index on prediction quality

IR - Induction ratio

Nap - Naphthalene

Nrf2 - Nuclear factor erythroid 2 (NFE2)-related factor 2

MCF-7- Michigan Cancer Foundation-7

min – Minute or minutes

MTS – Tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

PAHs - Polyaromatic hydrocarbons

Pb - Lead

PBS - Phosphate buffered saline

Phe - Phenanthrene

Pyr - Pyrene

ROS - Reactive oxygen species

µM - Micromolar

mM – Millimolar

WHO- World health organization

INTRODUCTION

Cellular exposure to chemicals either alone or in mixtures causes imbalance of reactive oxygen species (ROS) production, which may diminish the ability of cells to detoxify these ROS.[1-3] Oxidative stress induced by environmental stressors is associated with epidemiological diseases such as cancers, lung diseases, neurodegenerative disorders, atherosclerosis, rheumatoid arthritis, diabetes, cardiovascular diseases, stroke and aging.[4-6] Among the various classes of stressors, polyaromatic hydrocarbons (PAHs) and heavy metal/loid(s) are ubiquitous environmental pollutants of global concern. Both PAHs and heavy metal/loid(s) are known to cause a broad spectrum of toxic effects in humans.[7-8]

Chronic exposure to elevated levels of heavy metal/loid(s)s like arsenic (As), cadmium (Cd) and lead (Pb) of which As and Cd are classified as Group I carcinogens by IARC, can also cause adverse effects in the neurological, cardiovascular, hematological, gastrointestinal, musculoskeletal and immunological systems.[9-11] Both individual toxicity and human health risk assessment of these heavy metal/loid(s) have been extensively reviewed and reported by various international regulatory agencies such as WHO and US EPA. Oxidative stress is attributed as the unifying factor for metal/loid(s) toxicity.[12-14] Heavy metal/loid(s) induced oxidative stress may result in lipid peroxidation, damage to cellular protein and nucleic acids leading to a variety of cellular dysfunctions including cell death.[15-16]

Similarly, some PAHs are known human carcinogens and cause developmental and immunotoxicity. Most of the PAHs are indirect carcinogens and require metabolic activation to exert their toxicity. For example, benzo[a]pyrene (B[a]P), a potent Group I carcinogen, is metabolically activated by CYP1A1 and CYP 1B1 enzymes.[17] During the metabolism of B[a]P, free radicals are formed and these radicals can cause oxidative damage to the DNA.[18-20]

Naphthalene (Nap) is classified as a 2B carcinogen and associated with hemolytic anemia, cataract and respiratory disorders.[21] Oxidative stress plays an important role in naphthalene toxicity.[22] Phenanthrene (Phe) and pyrene (Pyr) are classified as Group 3 carcinogens.[23] There are no data available for Phe and Pyr toxicity to humans. These four PAHs are selected for the study because of their frequent occurrence at hazardous waste sites and potential human exposure. They are listed as priority pollutants by the US EPA.[24] Naphthalene, Phe and Pyr are included in the study due to their common occurrence as mixtures with B[a]P and also to determine their potential interaction effect with B[a]P.

Humans have developed elaborate antioxidant defense mechanisms to protect the cells against oxidative stress induced damage.[25] A major cellular defense mechanism against oxidative stress is activation of antioxidant genes that are involved in the detoxification and elimination of reactive oxidants by enhancing cellular antioxidant capacity.[26-27] Nrf2 (nuclear factor erythroid 2 (NFE2)-related factor 2) plays a pivotal role in protecting cells against oxidative stress through ARE-mediated expression and coordinated induction of antioxidant enzymes.[28-29] Cellular exposure to electrophilic chemicals activates the Nrf2 antioxidant pathway and measurement of Nrf2 pathway induction is considered as a reliable indicator of oxidative perturbation. Heavy metals like As[30-32], Cd[33-35] and Pb[36-37] have been reported to activate the Nrf2 antioxidant pathway followed by their exposure and the Nrf2 antioxidant defense mechanism also plays a major role against B[a]P induced carcinogenesis.[38]

PAHs and heavy metal/loid(s) often are co-occurred in the environment.[39-41] Amongst these environmental pollutants, As, Cd, Pb and B[a]P are top priority pollutants.[42] At elevated levels, these contaminants can cause serious health effects in humans and other organisms and oxidative stress is one of the common modes of action for these mixed contaminants. To the best of our

knowledge, there are no studies reported on the effect of mixtures of PAHs and heavy metal/loid(s) on the oxidative stress response.

In this study, the ARE reporter-HepG2 cell line is used to determine the interaction effect on the Nrf2 antioxidant pathway, an indicator for oxidative stress response. Liver is the major organ for environmental chemical metabolism and heavy metal/loid(s) are known to cause toxicity to liver cells.[43] HepG2 cells have been extensively used for toxicological research and their inherent metabolic capacity is useful to determine the toxicity of chemicals like PAHs.[44] These cells are highly differentiated and display many of the genotypic features of normal liver cells. In addition, the steady state maintenance of antioxidant defense is higher than that in primary hepatocytes.[45-46] Hence, the HepG2 cell line is used as a model for studying the mechanisms of oxidative stress.

The objective of this study is to determine, for the first time, the effects of up to seven-component mixture of PAHs and heavy metal/loid(s) on the Nrf2 antioxidant pathway using the ARE reporter-HepG2 cell line. The mixture effect is determined for binary, ternary, quaternary and seven-component mixtures of PAHs and heavy metal/loid(s).

MATERIALS AND METHODS

Chemicals

Cell culture medium MEM (minimum essential medium), trypsin-EDTA (0.25%), penicillin-streptomycin solution, Geneticin® selective antibiotic (G418 sulfate), non-essential amino acids, sodium pyruvate (100 mM) and fetal bovine serum (FBS) were purchased from Gibco® (Life Technologies, VIC, Australia). CellTiter96® Aqueous One solution cell proliferation assay system (G3581), luciferase assay system (E1501) and luciferase cell culture lysis 5X reagent (E1531) were purchased from Promega Corporation, Madison, WI, 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), sodium arsenite (CAS number: 7784-46-5) and Tert-butylhydroquinone (t-BHQ) (1948-33-0) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell line

ARE reporter-HepG2 cell line (Catalog # 60513) was purchased from BPS Bioscience Inc., CA, USA. This ARE Reporter-HepG2 cell line contains a firefly luciferase gene under the control of ARE stably integrated into HepG2 cells. The reporter cells were maintained in 75 cm^2 culture flask containing MEM medium supplemented with 10% FBS, 1% non-essential amino acid, 1 mM sodium pyruvate, 1% penicillin/streptomycin and 600 µg/ml of Geneticin®. Cells were seeded into 96-well plates (Corning® 96 well flat clear bottom, sterile white polystyrene TC-treated microplates, Corning Life Sciences, NY, USA) at a density of 12000 cells/well. Cells were incubated at laboratory room temperature (24 ± 1 ºC) for 15 min following seeding for cell settling and incubated at 37 °C under 5% CO_2 in a humidified incubator for 24 h.

Chemical treatment

Stock dilutions of PAHs (B[a]P, Nap, Phe and Pyr) in DMSO and metal/loid(s) (As, Cd and Pb) in MilliQ water (18 MΩ.cm) (Merck Millipore, VIC, Australia) were prepared. Working solutions were prepared in MEM medium and added to the plates containing the cultured cells with final concentration of vehicle (DMSO or MilliQ water) at 0.5% v/v.

Cytotoxicity assay

Initially, the cytotoxicity of PAHs and heavy metal/loid(s) to ARE reporter-HepG2 cells were determined by measurement of cell viability using the MTS assay (CellTiter 96® aqueous one solution, Promega, Madison, WI, USA). The selected concentrations were 0, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µM of B[a]P, Nap, Phe and Pyr; 0, 0.156, 0.312, 0.625, 1.25, 2.5 5, 10 and 20 µM of Cd; 0, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM of As and 0, 2.34, 4.68, 9.37, 18.75, 37.5, 75 and 150 µM of Pb. The working solutions containing treatment chemicals and vehicle control were exposed in triplicate to ARE reporter-HepG2 cells for 24 h.

Cytotoxicity was determined using the CellTiter 96® Aqueous one solution. After a chemical treatment period of 24 h, the treatment medium was carefully aspirated using a multi-channel micropipette then 20 µL of MTS reagent and 80 µL of DMEM were added to each well and incubated for another 2-3 h. The absorbance was measured at 490 nm in a microplate reader (FLUOstar Omega, BMG Labtech, VIC, Australia).

Cell viability was calculated as shown in equation (1) after blanking.

$$\text{Cell viability (\%)} = \frac{\mathbf{100}\ \boldsymbol{X\ mean\ optical\ density\ (OD) in\ single\ chemical\ or\ mixtures\ treated\ samples}}{\boldsymbol{mean\ OD\ in\ vehicle\ control\ treated\ samples}} \text{ --- (1)}$$

Determination of Nrf2 antioxidant pathway activation

Preliminary experiments showed that the Nrf2 antioxidant pathway activation in ARE reporter-HepG2 cells was not proportional to dose levels at higher concentrations, and substantial decrease in the Nrf2 pathway activation was observed at concentrations near the cytotoxic level (data not shown). Based on these observations, the selected concentrations of individual dose response study on the Nrf2 antioxidant pathway were 0-5 µM of B[a]P; 0-15 µM of Nap, Phe and Pyr; 0-5 µM of As; 0-0.5 µM of Cd; 0-10 µM of Pb. Tert-butylhydroquinone (t-BHQ, 0-20

μM) was used as a positive control. The treatment chemicals and controls were exposed to ARE reporter-HepG2 cells for 24 h.

The Nrf2 pathway activation was quantified by using a luciferase assay system (catalogue # E1501) purchased from Promega Corporation, Madison, USA. The luciferase activity was determined as per manufacturer's instructions. In brief, the growth medium was removed from the plates using a multi-channel micropipette after a treatment period of 24 h and rinsed twice with phosphate buffered saline (PBS). Cell lysis buffer (1x lysis buffer was prepared from 5x lysis buffer), 20 μL/well, were added to each well and incubated for 5 min at room temperature. Then, luciferase assay reagent (luciferase assay buffer + lyophilized assay substrate), 100 μL/well was added to the lysed cells and luminescence was quantified by using a microtiter plate reader.

Oxidative stress response was measured as the difference between ARE luciferase reporter expression in chemical treated groups compared to that of vehicle control and was calculated using equation (2)

$$\text{Induction ratio (IR)} = \frac{Luminescence\ of\ treated\ wells}{average\ luminescence\ of\ vehicle\ control\ wells} \quad \text{---------- (2)}$$

The results showed that all the chemicals did not achieve a maximum induction of the Nrf2 antioxidant pathway. Based on these observations, the linear part of the concentration effect relationship was selected and concentration that induced IR of 1.5 ($EC_{IR1.5}$) was determined as described by Escher *et al.* (2013)[47]. In brief, the dose responses of individual chemicals and concentration that induces an induction ratio (IR) of 1.5 ($EC_{IR1.5}$) was determined using a linear regression method. The individual concentration effect relationship was determined using equation (3)

IR = 1 + slope x concentration---------- (3)

$EC_{IR1.5}$ was calculated using the linear regression method and using equation (4)

$$EC_{IR1.5} = \frac{0.5}{slope} \text{ ----------} \quad (4)$$

Mixture Experiments

Mixture experiments were conducted for binary, ternary, quaternary and seven-component mixtures of PAHs and heavy metal/loid(s). All the mixtures experiments were conducted at fixed ratio concentrations and chemicals were mixed at 1:1 an equipotent ratio based on their individual $EC_{IR1.5}$ value. The mixtures were diluted in 1:3 serial dilutions for six or seven times and full concentration response study was carried out. The chemical treatment was carried out in triplicate for each concentration and two or three independent experiments were conducted for the mixture interaction studies. The details of chemical mixtures and concentrations are provided in Table S1 of Supplementary Information.

Prediction of mixture effects by concentration addition (CA) model

Concentration addition (CA) and independent action (IA) models are commonly used for the prediction of chemical mixture toxicity[48] and the CA model is used for chemicals with the same mode of action. The single dose response studies showed that both PAHs and heavy metal/loid(s) induced the Nrf2 antioxidant pathway (same mode of action) in ARE reporter-HepG2 cells. Hence, the CA model was used to predict the mixture effects as described by Escher *et al.* (2013)[47] and uses equation (5)

$$EC_{IR1.5,CA} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{EC_{IR1.5,i}}} \text{ ----------} \quad (5)$$

$EC_{IR1.5,CA}$ is the concentration of the mixture, $EC_{IR1.5,i}$ is the concentration of component *i*, and p_i is the molar concentration ratio of the i^{th} component in the mixture.

The difference and quantitative relationship between predicted and observed effect is determined by an index on prediction quality (IPQ) [49]

If the predicted value is greater than the observed value, the prediction quality is determined by using equation (6)

For $EC_{predicted} > EC_{observed}$, $IPQ = \left(\frac{EC_{predicted}}{EC_{observed}}\right) - 1$ ---------- (6)

If the predicted value is less than observed value, the prediction quality is determined by using equation (7)

For $EC_{predicted} < EC_{observed}$, $IPQ = -\left(\frac{EC_{observed}}{EC_{predicted}}\right) + 1$ ---------- (7)

An IPQ value of zero indicates exact prediction by reference models, while IPQ value <0 indicates overestimation and value >0 indicates an underestimation of mixture effects.

Statistical analysis

Data were presented as mean ± SD for experimental and the predicted values. Statistical analysis was carried out using Graphpad Prism version 6.00 for windows (GraphPad software, Inc, CA, USA). Data was analyzed using "student's t-test" and the signficant different between experimental and CA prediction was evaluated at $p < 0.05$".

RESULTS

Cytotoxicity of PAHs and heavy metal/loid(s)

Heavy metal/loid(s) were found to be more toxic to ARE reporter-HepG2 cells than PAHs. Among the heavy metal/loid(s), Cd was more toxic with an IC_{50} value of 3.36 μM followed by As (IC_{50}=72 μM) and Pb (IC_{50}=108 μM). Benzo[a]pyrene was found to be toxic to HepG2 cells and a reduction in cell viability was observed at concentrations above 12.5 μM (max. of 30 % at 25 μM). At 100 μM, B[a]P was found to precipitate in the medium during the 24 h exposure period. The other PAHs were found to be nontoxic to ARE reporter-HepG2 cells up to the maximum feasible (soluble) concentrations (Table S2 of Supplementary Information).

Activation of Nrf2 antioxidant pathway by PAHs and heavy metal/loid(s)

The individual dose response studies showed that all chemicals including the four PAHs and three heavy metal/loid(s) activated the Nrf2 antioxidant pathway. Among these seven chemicals, Cd was the most potent inducer with $EC_{IR1.5}$ of 0.58 μM. Both B[a]P and As have similar potency with $EC_{IR1.5}$ value of 0.93 and 1.1 μM respectively. The $EC_{IR1.5}$ values for individual chemicals and dose response curves are presented in the Supplementary Information (Table S2, Figures S1 and S2 respectively).

Mixtures of PAHs and heavy metal/loid(s) effect on the Nrf2 antioxidant pathway

The effect of mixture combinations of binary to seven-component mixtures of PAHs and heavy metal/loid(s) on activation of Nrf2 antioxidant pathway in the ARE reporter-HepG2 cells are given in Table 1. The experimental and predicted $EC_{IR1.5}$ values, IPQ values and 95% confidence intervals (CI) for experimental and CA predicted values are presented in Table 1 and Figures 1-6 which show the dose response effects of multi-component mixtures of PAHs and heavy metal/loid(s) on the Nrf2 antioxidant pathway in ARE reporter-HepG2 cells.

312 **Binary mixtures of PAHs and heavy metal/loid(s)**
313
314 The binary mixtures of heavy metal/loid(s) (As + Cd, As + Pb and Cd + Pb), B[a]P + heavy
315 metal/loid(s) (B[a]P + As, B[a]P + Cd and B[a]P + Pb) and B[a]P + PAHs (B[a]P + Nap, B[a]P
316 + Phe and B[a]P + Pyr) showed varying potencies of activating the Nrf2 antioxidant pathway in
317 ARE reporter-HepG2 cells (Table 1 and Figures 1-3). The mixture of As + Cd showed a higher
318 induction effect on the Nrf2 pathway compared to other heavy metal/loid(s) mixtures with
319 $EC_{IR1.5}$ 0.70 μM followed by As + Pb ($EC_{IR1.5}$ - 1.63 μM) and Cd + Pb ($EC_{IR1.5}$ - 2.2 μM). A
320 maximum induction ratio of 2.4 was observed with mixtures of As + Cd and As + Pb. In the case
321 of B[a]P and heavy metal/loid(s) mixtures, B[a]P + Cd showed higher induction effect ($EC_{IR1.5}$ of
322 0.45 μM) compared to that of other mixtures and the maximum induction ratio observed for all
323 three mixtures is almost equal (max. of 3.3). Among the binary mixtures of B[a]P + other PAHs,
324 mixture of B[a]P + Pyr had a higher induction effect ($EC_{IR1.5}$ 1.56 μM) and also showed a
325 maximum induction ratio of 3.3 compared to that of other B[a]P + PAHs mixtures. There was no
326 significant difference between experimental and predicted values for all the mixtures except for
327 the As + Pb mixture and the IPQ value is less than one for all mixtures. There were overlaps of
328 95% confidence intervals between predicted and observed value for all the mixtures (Table 1).

329 **Ternary, quaternary and seven chemical mixtures of PAHs and heavy metal/loid(s)**
330
331 The ternary mixture of B[a]P + Cd + As has a higher induction effect ($EC_{IR1.5}$ 0.45 μM) with a
332 maximum induction ratio of 5 compared to other ternary mixtures. In general, the induction
333 effect is higher for ternary mixtures of B[a]P + heavy metal/loid(s) compared to that of ternary
334 mixtures of As + Cd + Pb and B[a]P + other PAHs. The ternary mixture of As + Cd +Pb showed
335 less induction effect with an $EC_{IR1.5}$ of 1.87 μM. There was no significant difference between the

observed and predicted effect for ternary mixtures except for B[a]P + As + Cd (p = 0.0088) and B[a]P + As + Pb (p = 0.003). The experimental values for these mixtures were less than that of predicted values and therefore the mixture effect is underestimated.

Quaternary and seven-component mixtures also induced the Nrf2 antioxidant pathway with a maximum induction ratio of 6 observed with seven-component mixtures. There was no significant difference between the observed and predicted effect for these mixtures except for B[a]P + As + Cd + Pb (p= 0.0131). The IPQ value is less than one for all mixtures (ternary to seven-component mixtures) with overlapping of 95% confidence intervals observed between the predicted and observed values for all mixtures (Table 1).

DISCUSSION

Oxidative stress has been implicated with the pathophysiology of various systemic diseases and mechanisms of action of chemical toxicity. Most importantly, oxidative stress plays a crucial role in carcinogenesis.[1, 50] and environmental agents are one of the main exogenous sources for ROS production. Measurement of oxidative stress response is a sensitive endpoint for chemical exposure and various methods used for its determination include direct measurement of the ROS, oxidative damage to biomolecules and detection of antioxidant levels.[51-52] Most of these methods are technically laborious and lack specificity and the National Research Council (NRC, 2007[53]) recommends the development of rapid, economical cell based and high throughput assays to determine perturbation of cellular response using *in vitro* assays for better understanding of human diseases. Measurement of stress response pathways (Nrf2 antioxidant pathway) has been identified as a toxic pathway indicator[53] and human cell based reporter gene assays have been developed to measure the Nrf2 antioxidant pathway as a means of monitoring oxidative stress

response. These assays measure the ARE activation using luciferase reporter gene, which is preferred as a screening tool due its rapidness and stable transfection which helps to define mode of action.[36, 54] This bioassay has been used to screen pharmaceutical molecules for Nrf2 activation[55] and profiling of environmental chemicals.[56] The Nrf2 luciferase assay is designed to measure changes in transcriptional activity of Nrf2, where the Nrf2 binds to ARE and regulates genes involved in cytoprotection. The Nrf2-responsive luciferase construct monitors increase or decrease in the transcriptional activity of Nrf2 and activity of the ARE pathway. Therefore, the changes of luciferase expression in the chemical treated cells provide a sensitive measure of changes in the Nrf2 activity. Methods such as real time PCR can provide information about gene expression and steady-state level of transcription which is influenced by transcriptional activity and mRNA instability.[36] Thus cell based assays are preferred to monitor the Nrf2 pathway activity and hence chemically induced changes in oxidative stress response.

The Nrf2-reporter gene assays have been developed using various immortalized cell lines, including HEK293T, MCF7, A172, A549, HepG2 [36] and Huh cell lines.[57] The results from these studies have shown that Nrf2 activity profiles vary between the cell lines, due to the origin of tissues, cellular subtype and culture conditions. Thus, the results obtained by using a particular cell line (HepG2 cells) can be correlated with the response of the liver in the body, although the results do not necessarily provide a complete picture of biological response and other factors like changes in temperature, pH, and luciferase buffer may also affect the luminescence signal.

In this study, we have determined the effect of up to seven component mixtures on the Nrf2 antioxidant pathway using ARE reporter-HepG2 cells. The results showed that both heavy metal/loid(s) and PAHs activated the Nrf2 antioxidant pathway. The role of Nrf2 antioxidant pathway in heavy metal/loid(s) and B[a]P toxicity has been reported.[58, 59] This present study

shows that non-carcinogenic PAHs like Nap, Phe and Pyr can also induce a positive response in activation of the Nrf2 antioxidant pathway. There are no studies available in the literature reporting these PAHs effects on the Nrf2 antioxidant pathway.

Our results also show that multi-component mixtures of PAHs and heavy metal/loid(s) displayed various degrees of activity on the Nrf2 antioxidant pathway in ARE reporter-HepG2 cells. In the case of binary mixtures, the B[a]P + Cd mixture had a higher induction effect compared to that of other combinations. Among+ the binary mixtures, B[a]P with heavy metal/loid(s) showed higher induction of the Nrf2 pathway compared to mixtures of metal/loid(s) and B[a]P + PAHs and a similar trend was observed with ternary mixtures, where B[a]P + Cd + As showed a higher induction effect than that of other ternary mixtures. The observed $EC_{IR1.5}$ value for seven-component mixtures was higher than that of lower order mixtures (binary and ternary). This could be due the mixtures of both potent inducers (Cd, B[a]P, As and Pb) and less active chemicals (Nap, Phe and Pyr). Various reports indicated that combined exposure of As, Cd and/or Pb or in combination with other metals increased the oxidative stress response compared to their individual response. [60-62] Cd was found to enhance the Nrf2 antioxidant pathway and total glutathione level of B[a]P when compared to that of B[a]P alone.[63] Arsenic and Pb also have a synergistic effect on oxidative stress response in combination with B[a]P.[64-65] These studies did not use any prediction model to determine the interaction and interpretation was based on statistical difference between individual and mixture groups. There are no detailed reports available for these mixtures at higher order (ternary mixture and above). For the first time, we report here oxidative stress response data for ternary, quaternary and seven-component mixtures containing four PAHs and three heavy metal/loid(s).

CA and IA models are commonly used to predict mixture toxicity, and these models are used for chemicals with similar and dissimilar modes of action respectively. We have used only the CA model in this study to predict the mixtures effect on the Nrf2 antioxidant pathway as individual PAHs and metal/loids showed the same mode of action (Nrf2 pathway activation). The CA model is the preferred reference model for risk assessment of mixtures consisting of both similar and dissimilar acting chemicals[66] and this model is considered as a general solution for mixture risk assessment.[67] This prediction model (CA) has been used to predict the mixture effect of different classes of chemicals, including pharmaceuticals and pesticides on oxidative stress response using the AREc32 cell line.[47] The mixtures of pharmaceuticals and pesticides showed induction activity in the AREc assay and the mixture effect is well predicted by the CA model.

The present study shows there was no significant difference between observed and predicted $EC_{IR1.5}$ value for 15 out of 19 mixtures and an overlap of 95% confidence interval between experimental and predicted values was observed for all mixtures. The observed $EC_{IR1.5}$ for the mixture of As + Pb, ternary mixtures of B[a]P + Cd + As and B[a]P + As + Pb, and quaternary mixture of B[a]P and heavy metal/loid(s) showed significant differences with CA prediction and the $EC_{IR1.5}$ values for these mixtures were less than that of predicted values suggesting that the CA model under predicted the interaction effect for these mixtures. A closer examination of the predicted response showed that the CA model tends to underestimate the interaction effect for most of the mixtures at lower concentrations. The predicted response of binary mixtures of B[a]P + heavy metal/loid(s), As + Cd, Cd + Pb and B[a]P + other PAHs was underestimated at lower order combinations, and the same trend of underestimation at lower combinations was observed for ternary, quaternary and seven chemical mixtures. For a few mixtures like binary mixtures of B[a]P + heavy metal/loid(s), ternary mixture of B[a]P + Cd+ As, B[a]P + Nap + Pyr, B[a]P +

Phe + Pyr and B[a]P + As + Cd + Pb, the effect was underestimated at greater concentrations. An IPQ compares the difference between observed effects and predicted by models (CA and IA) and indicates the accuracy of predictions of the models. [49] An IPQ value of < 0 or > 0 indicates an over or underestimation respectively of mixture effects and values of -1 and +1 indicates over or under prediction by the prediction models. In our study, the IPQ value is close to zero for 5 out of 19 mixtures, less than 0.5 for 8 out of 19 mixtures and less than 1 for the remaining six mixtures which indicates acceptable agreement between predicted and observed effects. In the case of mixtures which showed significant differences between the predicted responses by the CA model, the IPQ values are less than 1 for all four mixtures and overlaps of 95% CI intervals between predicted $EC_{IR1.5}$ values were observed. This indicates acceptable agreement between observed and predicted effects. Based on these findings, we can conclude that the CA model can be used to predict the interaction between PAHs and heavy metal/loid(s) on the Nrf2 antioxidant pathway. In general and with the exceptions stated above, concentration addition may be appropriate for the risk assessment of B[a]P, Nap, Phe, Pyr, As, Cd and Pb mixtures.

CONCLUSIONS

A human cell line based reporter gene assay system (ARE reporter-HepG2 cells) has been successfully used to determine the chemical mixtures effect on oxidative stress response. This is the first report on the effects of individual and up to seven-component mixtures of PAHs (B[a]P, Nap, Phe and Pyr) and heavy metal/loid(s) (As, Cd and Pb) on the Nrf2 antioxidant pathway in ARE reporter-HepG2 cells. Individual and multi-component mixtures of PAHs and heavy metal/loid(s) activated the Nrf2 antioxidant pathway in ARE reporter-HepG2 cells. The CA model appears to be an appropriate model to predict these selected mixtures effect on oxidative stress response pathway. PAHs and heavy metal/loid(s) mixtures effect on the oxidative response

pathway can be utilized as an adjunct tool to inform health risk assessment. However, its adoption can be strengthened by the incorporation of a suite of other biological end points (AhR activation, cytotoxicity and genotoxicity) which forms part of our ongoing research.

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CONFLICT OF INTEREST

There is no conflict of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article is attached.

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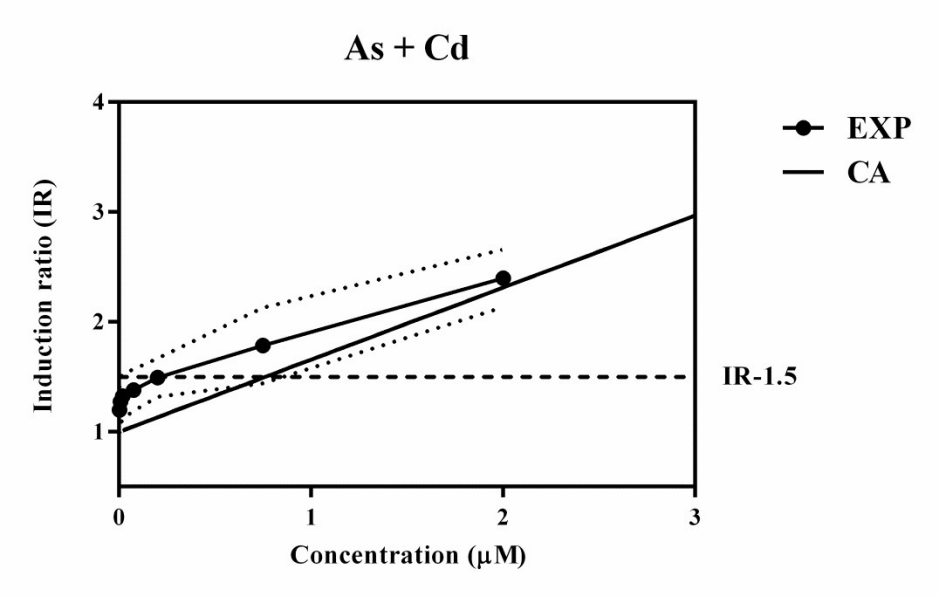
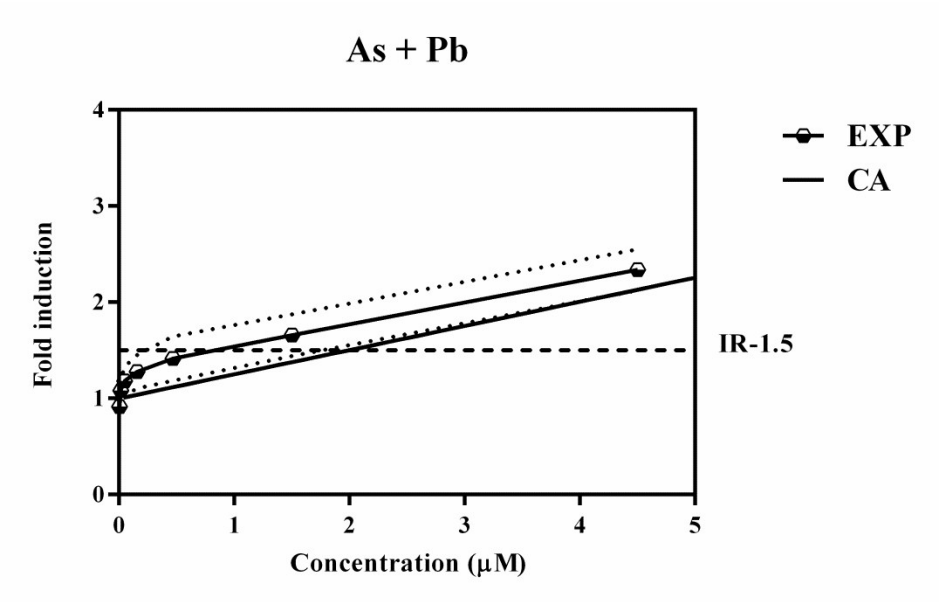
Table 1. The multi-component mixtures of PAHs (benzo[a]pyrene (B[a]P), naphthalene (Nap), phenanthrene (Phe), and pyrene (Pyr)) and heavy metal/loid(s) (arsenic (As), cadmium (Cd), lead (Pb)) on activation of Nrf2 antioxidant pathway in ARE reporter- HepG2 cells

Chemical mixtures	$EC_{IR1.5\ exp}$ (μM)	95% CI	$EC_{IR1.5\ CA}$ (μM)	95% CI	IPQ	Observed maximum induction ratio
Binary mixtures						
As + Cd	0.70 ± 0.24	0.09-1.3	0.90 ± 0.21	0.39-1.4	0.29	2.4
As + Pb*	1.63 ± 0.31	0.87-2.4	2.33 ± 0.21	1.8-2.9	0.43	2.4
Cd + Pb	2.2 ± 0.44	1.17-3.2	2.0 ± 0.21	1.52-2.6	-0.09	1.7
B[a]P + As	0.67 ± 0.25	0.05-1.3	1.04 ± 0.31	0.27-1.8	0.78	3.3
B[a]P + Cd	0.45 ± 0.16	0.04-0.9	0.80 ± 0.34	0.04-1.7	0.81	3.0
B[a]P + Pb	1.11 ± 0.34	0.26-2.0	2.13 ± 0.25	1.5-2.8	0.92	3.3
B[a]P + Nap	1.86 ± 0.45	0.70-3.0	1.76 ± 0.45	0.64-2.9	-0.06	2.5
B[a]P + Phe	2.1 ± 0.74	0.25-3.9	1.73 ± 0.37	0.81-2.7	-0.21	2.4
B[a]P + Pyr	1.56 ± 0.80	0.17-3.5	1.88 ± 0.54	0.54-3.2	0.21	3.3
Ternary mixtures						
As + Cd + Pb	1.87 ± 0.55	0.50-3.2	1.70 ± 0.36	0.80-2.6	-0.09	2.3
B[a]P + As + Cd*	0.45 ± 0.16	0.05-0.9	0.79 ± 0.19	0.29-1.3	0.74	5.0
B[a]P + As + Pb*	1.22 ± 0.33	0.41-2.0	1.77 ± 0.32	0.97-2.6	0.45	3.4
B[a]P + Cd + Pb	1.00 ± 0.09	0.77-1.2	1.60 ± 0.27	0.93-2.3	0.60	3.3
B[a]P + Nap + Phe	1.5 ± 0.16	0.02-3.0	2.1 ± 0.83	0.6-3.5	0.39	4.4
B[a]P + Nap + Pyr	1.37 ± 0.23	0.13-3.4	1.94 ± 0.14	0.66-3.2	0.42	4.7
B[a]P + Phe + Pyr	1.34 ± 0.22	0.02-3.3	1.87± 0.05	1.4-2.33	0.40	4.8
Quaternary mixtures						
B[a]P + As + Cd + Pb*	0.77 ± 0.12	0.46-1.1	1.47 ± 0.25	0.84-2.1	0.91	5.1
B[a]P + Nap + Phe + Pyr	1.96 ± 0.07	1.31-2.6	2.06 ± 0.29	0.14-4.6	0.05	4.6
All seven chemicals	2.06 ± 0.40	0.18-3.7	1.87 ± 0.06	1.3-2.4	-0.09	6.0

Values are mean ± SD for $EC_{IR1.5\ exp}$ and $EC_{IR1.5\ CA}$; n= 9 for experimental data of all mixtures except for ternary and quaternary mixtures of B[a]P + other PAHs and seven-chemical combinations, where n=6); EXP - experimental; CA - concentration addition; CI – confidence interval; IPQ - index on prediction quality; , IPQ value <0 indicates overestimation; values >0 indicates an underestimation; $EC_{IR1.5}$ - concentration that results in 1.5 fold of induction in luciferase assay; * - statistical significance ($p<0.05$) between predicted and experimental $EC_{IR1.5}$ values.





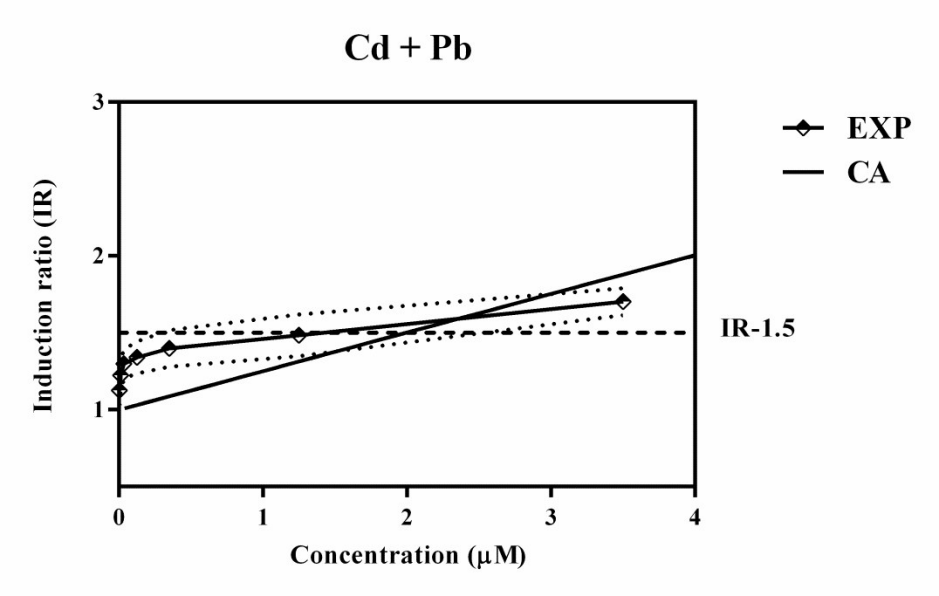
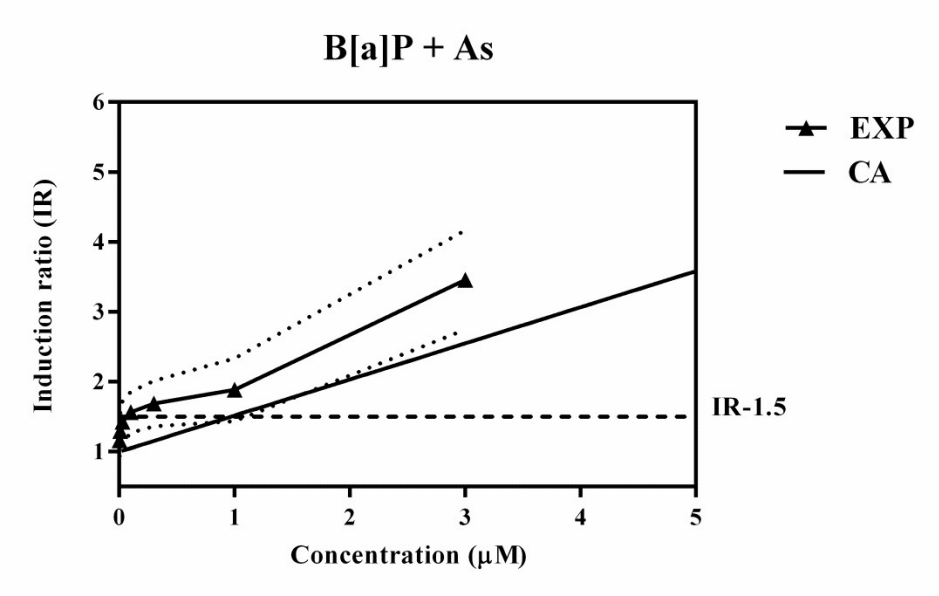
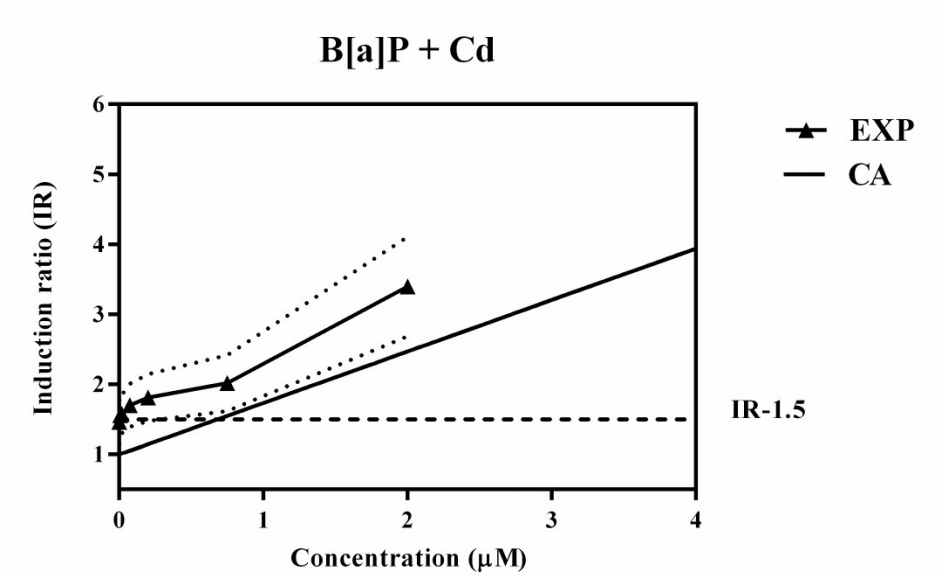


Figure 1. Dose response of binary mixtures of heavy metal/loid(s) for activation of Nrf2 antioxidant pathway in ARE reporter-HepG2 cells after 24 h exposure. IR- induction ratio; where CA denotes dose response predicted by concentration addition model; EXP denotes experimental data. The experimental data were from three independent experiments in triplicate for each exposure concentration. Values are expressed as mean and dashed line indicates 95% confidence interval.





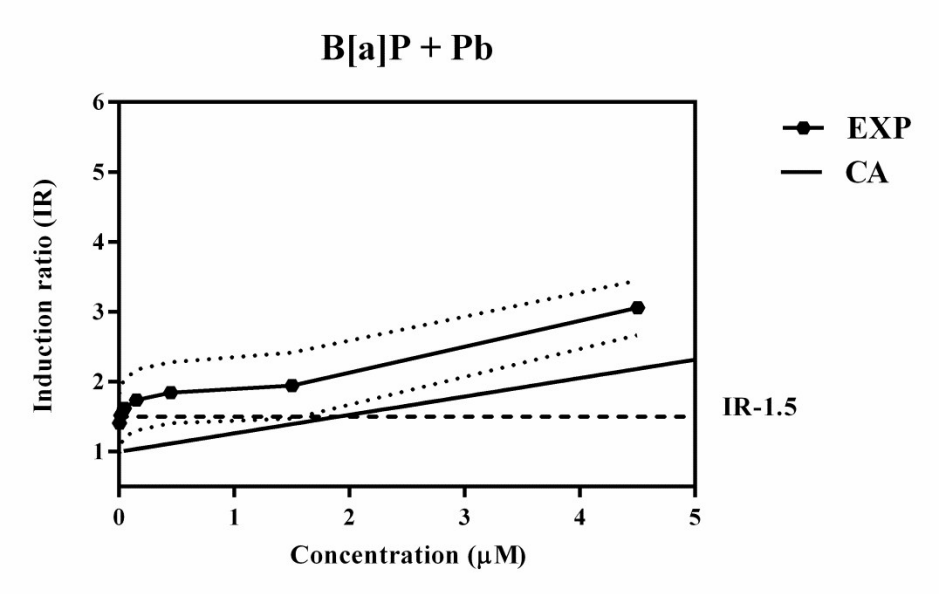
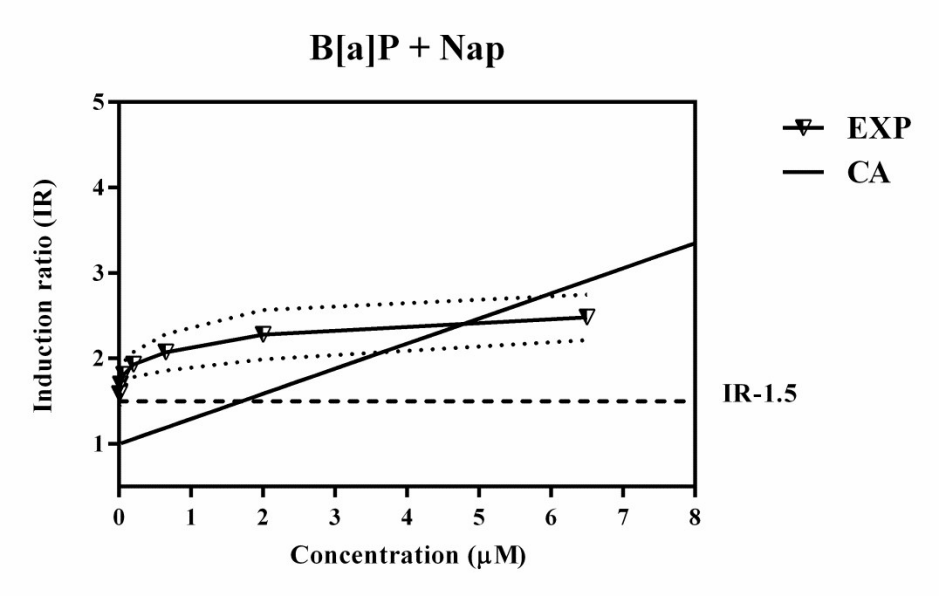
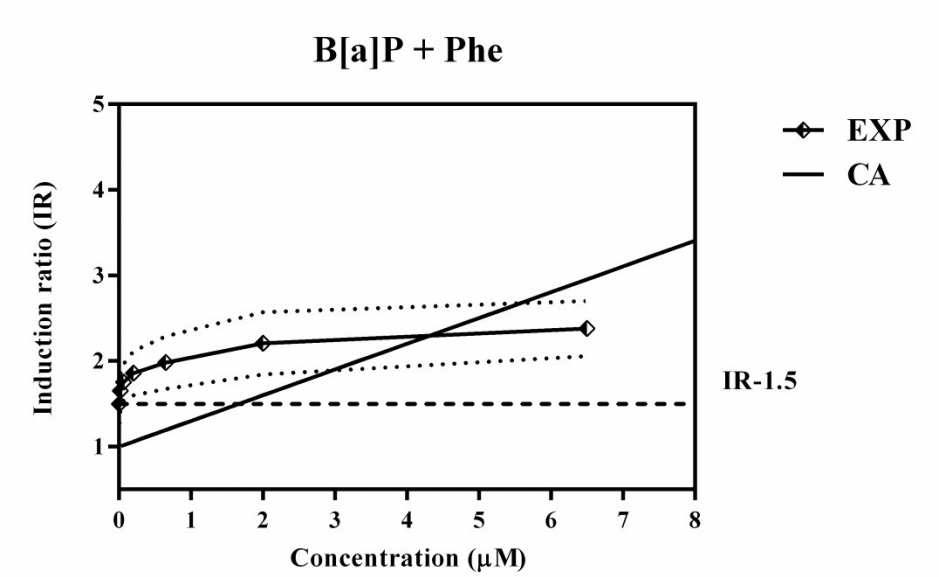


Figure 2. Dose response of binary mixtures of B[a]P and As, Cd or Pb for activation of Nrf2 antioxidant pathway in ARE reporter-HepG2 cells after 24 h exposure. IR- induction ratio; where CA denotes dose response predicted by concentration addition model; EXP denotes experimental data. The experimental data were from three independent experiments in triplicate for each exposure concentration. Values are expressed as mean and dashed line indicates 95% confidence interval.





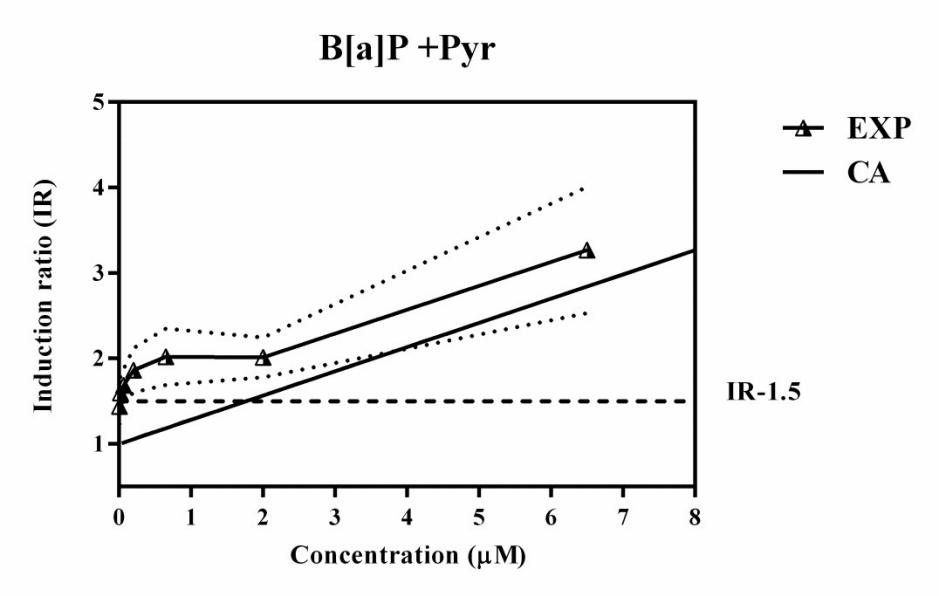
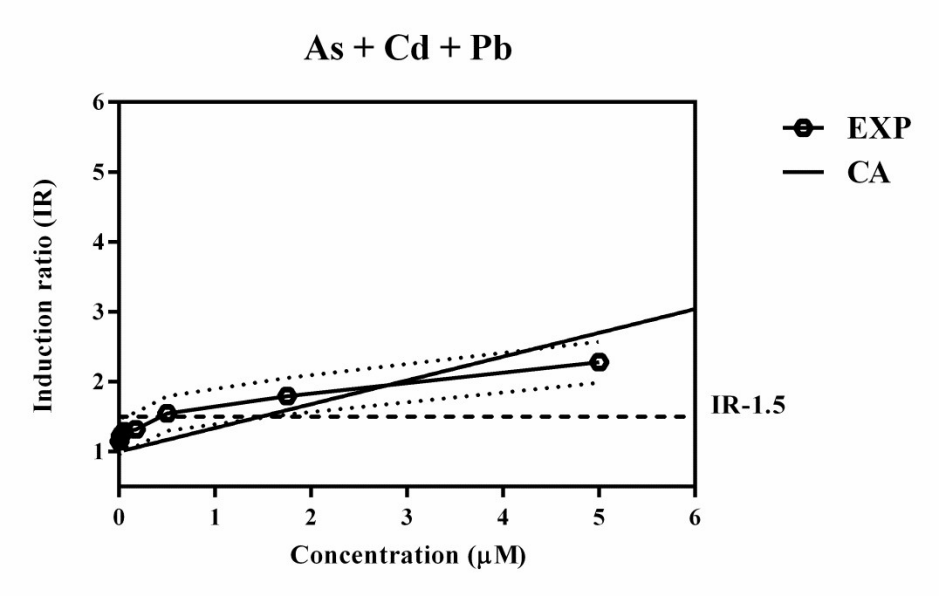
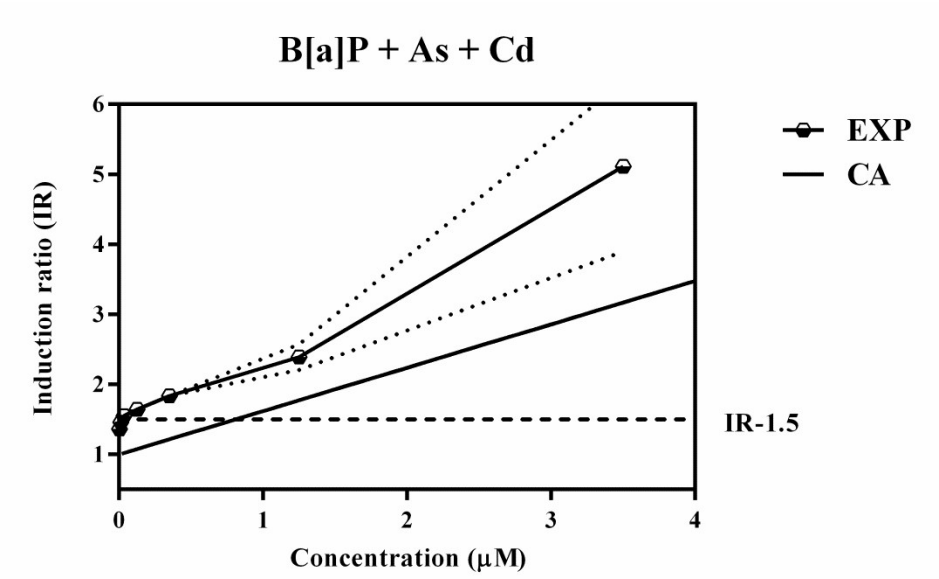


Figure 3. Dose response of binary mixtures of B[a]P and Nap, Phe and Pyr for activation of Nrf2 antioxidant pathway in ARE reporter-HepG2 cells after 24 h exposure. IR- induction ratio; where CA denotes dose response predicted by concentration addition model; EXP denotes experimental data. The experimental data were from three independent experiments in triplicate for each exposure concentration. Values are expressed as mean and dashed line indicates 95% confidence interval.





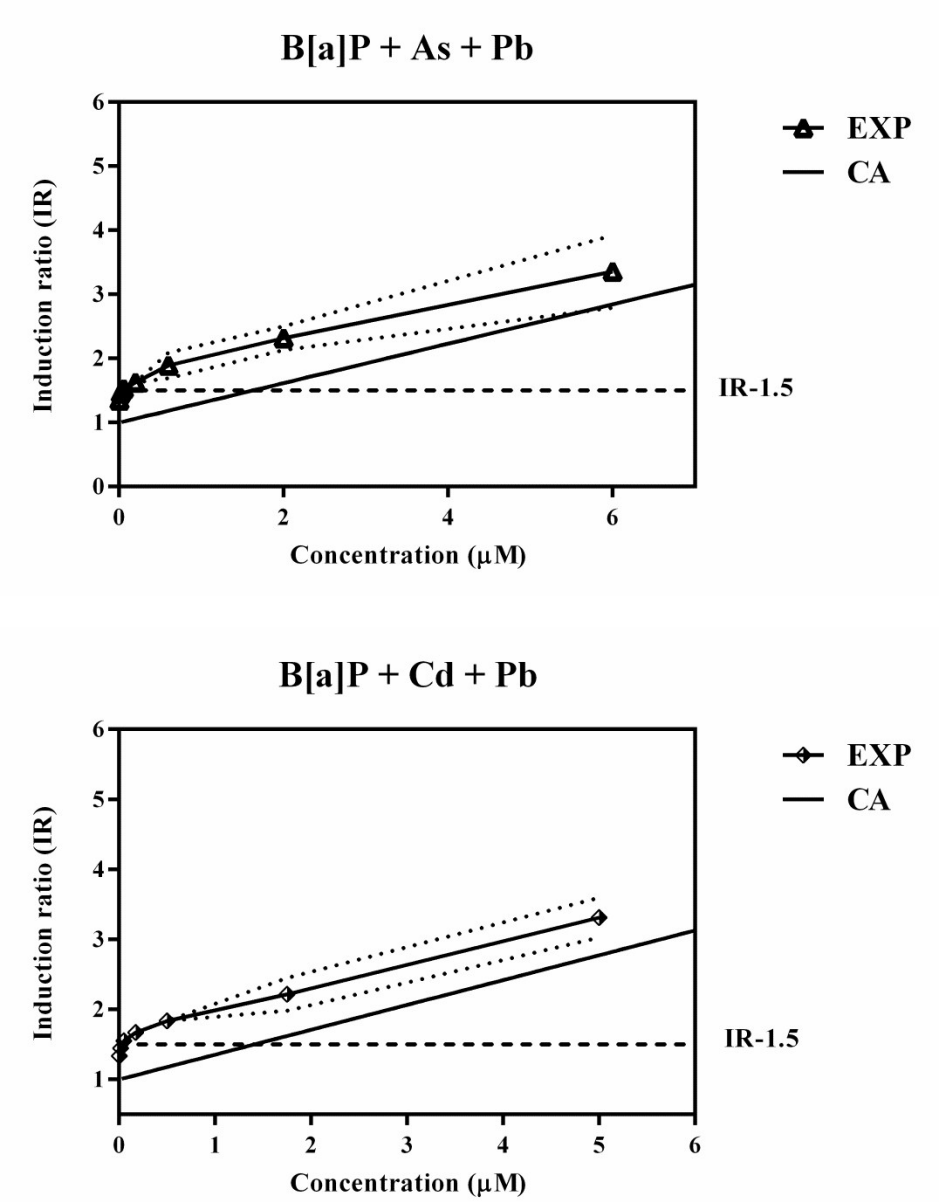
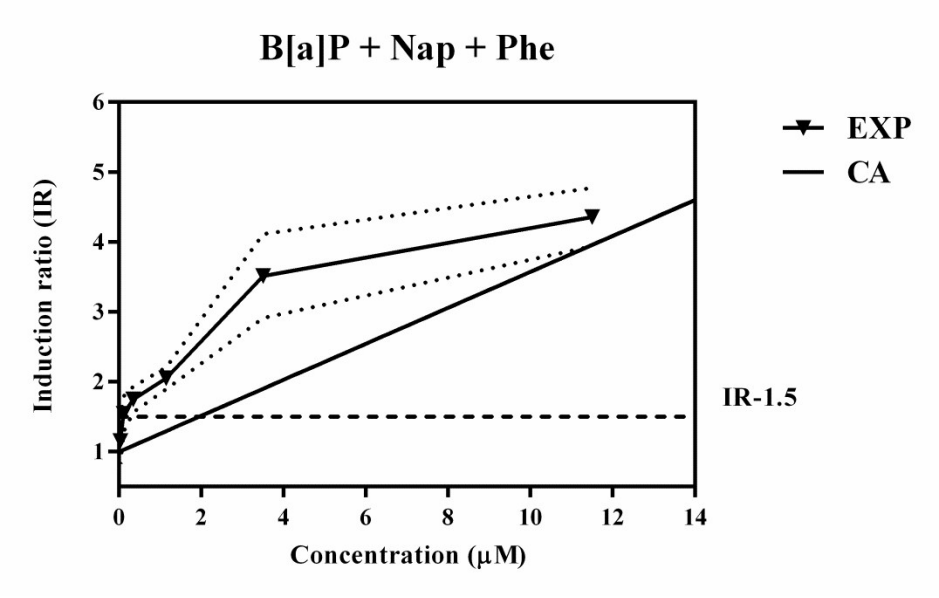
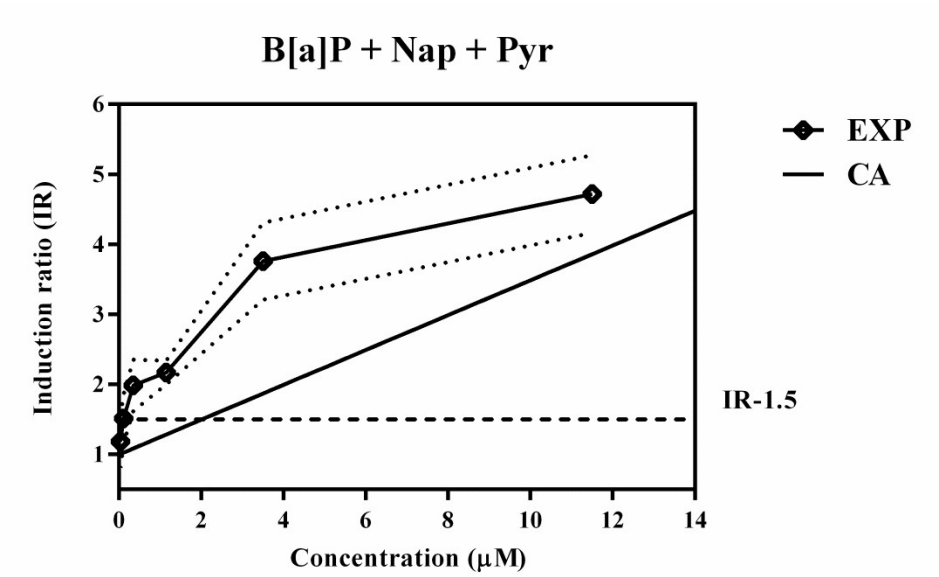


Figure 4. Dose response of ternary mixtures of B[a]P, As, Cd and/or Pb for activation for activation of Nrf2 antioxidant pathway in ARE reporter-HepG2 cells after 24 h exposure. IR- induction ratio; where CA denotes dose response predicted by concentration addition model; EXP denotes experimental data. The experimental data were from three independent experiments in triplicate for each exposure concentration. Values are expressed as mean and dashed line indicates 95% confidence interval.





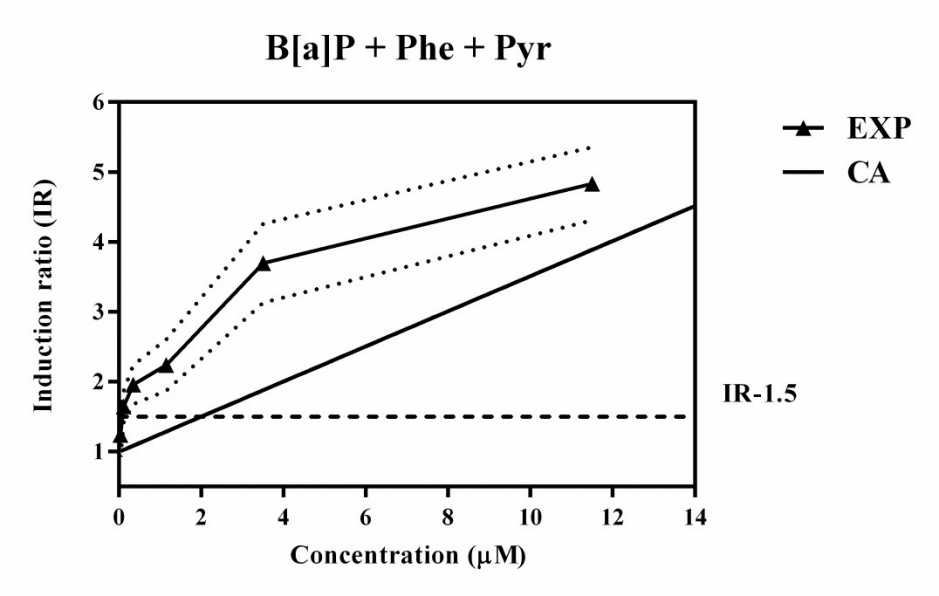
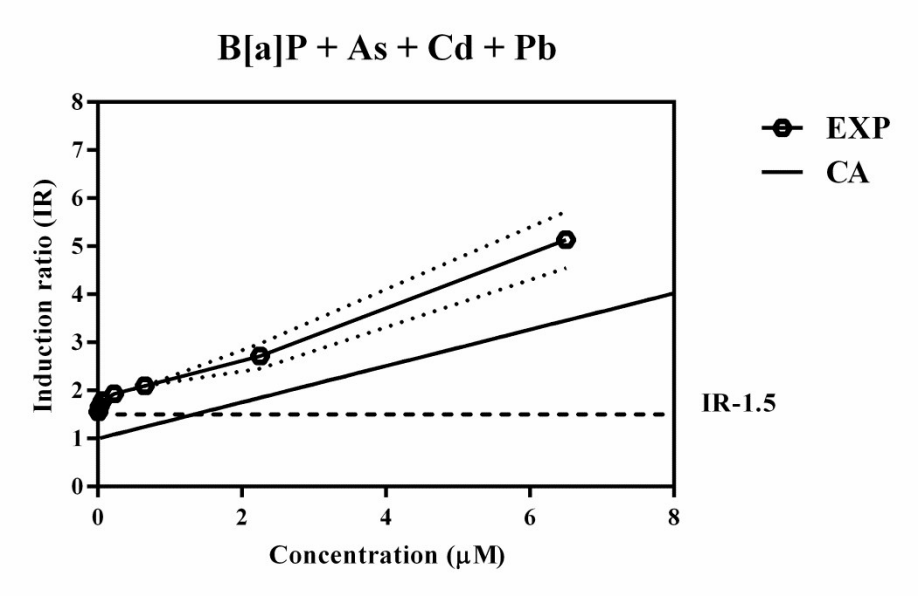
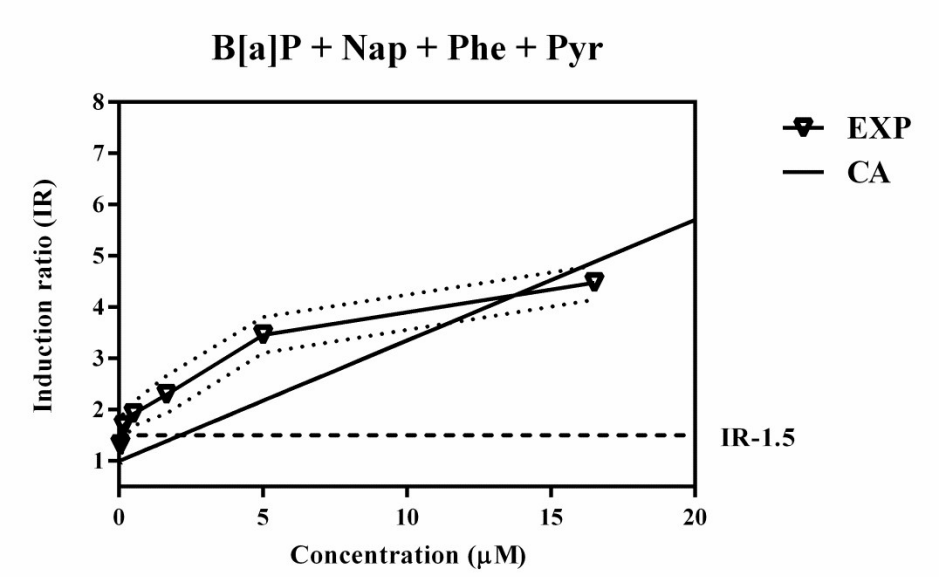


Figure 5. Dose response of ternary mixtures of B[a]P, Nap, Phe and Pyr for activation for activation of Nrf2 antioxidant pathway in ARE reporter-HepG2 cells after 24 h exposure. IR- induction ratio; where CA denotes dose response predicted by concentration addition model; EXP denotes experimental data. The experimental data were from two independent experiments in triplicate for each exposure concentration. Values are expressed as mean and dashed line indicates 95% confidence interval.





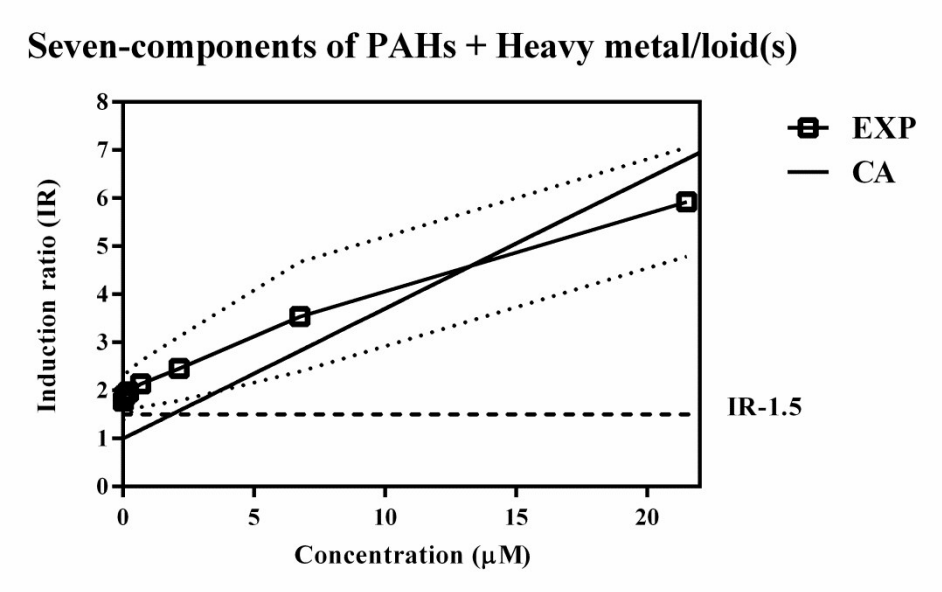


Figure 6: Dose response of quaternary and seven chemical mixtures of PAHs and heavy metal/loid(s) for activation for activation of Nrf2 antioxidant pathway in ARE reporter-HepG2 cells after 24 h exposure. IR- induction ratio; where CA denotes dose response predicted by concentration addition model; EXP denotes experimental data. The experimental data were from two or three independent experiments in triplicate for B[a]P + Nap + Phe + Pyr, seven chemical mixtures and B[a]P + As + Cd + Pb respectively. Values are expressed as mean and dashed line indicates 95% confidence interval.

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Highlights:

The effect of mixtures of PAHs and heavy metal/loid(s) on the Nrf2 antioxidant pathway in HepG2-ARE cells was determined as an indicator of oxidative stress response.