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JES JOURNAL OF ENVIRONMENTAL SCIENCES www.jesc.ac.cn

Comparative cytotoxicity of fourteen trivalent and pentavalent arsenic species determined using real-time cell sensing☆

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ARTICLE INFO

Article history:
Received 10 October 2016
Revised 11 October 2016
Accepted 11 October 2016
Available online 24 October 2016

Keywords:
Arsenic species
Thio-arsenicals
Methylarsenicals
Phenylarsenicals
Toxicity
Real-time sensing

ABSTRACT

The occurrence of a large number of diverse arsenic species in the environment and in biological systems makes it important to compare their relative toxicity. The toxicity of arsenic species has been examined in various cell lines using different assays, making comparison difficult. We report real-time cell sensing of two human cell lines to examine the cytotoxicity of fourteen arsenic species: arsenite (As^{III}), monomethylarsonous acid (MMA^{III}) originating from the oxide and iodide forms, dimethylarsinous acid (DMA^{III}), dimethylarsinic glutathione ($DMAG^{III}$), phenylarsine oxide (PAO^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}), monomethyltrithioarsonate ($MMTTA^{V}$), dimethylmonothioarsinate ($DMMTA^{V}$), dimethyldithioarsinate ($DMDTA^{V}$), 3-nitro-4-hydroxyphenylarsonic acid (Roxarsone, Rox), and 4-aminobenzenearsenic acid (p-arsanilic acid, p-ASA). Cellular responses were measured in real time for 72 hr in human lung (A549) and bladder (T24) cells. IC_{50} values for the arsenicals were determined continuously over the exposure time, giving rise to IC_{50} histograms and unique cell response profiles. Arsenic accumulation and speciation were analyzed using inductively coupled plasma-mass spectrometry (ICP-MS). On the basis of the 24-hr IC_{50} values, the relative cytotoxicity of the tested arsenicals was in the following decreasing order: $PAO^{III} \gg MMA^{III} \geq DMA^{III} \geq DMAG^{III} \approx DMMTA^{V} \geq As^{III} \gg MMTTA^{V} > As^{V} > DMDTA^{V} > DMA^{V} > MMA^{V} \geq Rox \geq$ p-ASA. Stepwise shapes of cell response profiles for DMA^{III}, $DMAG^{III}$, and $DMMTA^{V}$ coincided with the conversion of these arsenicals to the less toxic pentavalent DMA^{V}. Dynamic monitoring of real-time cellular responses to fourteen arsenicals provided useful information for comparison of their relative cytotoxicity.

☆ This manuscript honors Dr. William R. Cullen for his extraordinary contributions to the field of arsenic chemistry.

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http://dx.doi.org/10.1016/j.jes.2016.10.004
1001-0742/

Introduction

Arsenic occurs naturally throughout the geosphere and is ubiquitous in the environment (Cullen and Reimer, 1989). Arsenic contamination of groundwater that serves as a source of human drinking water is a serious public health concern (NRC, 1999). Chronic consumption of inorganic arsenic at elevated concentrations is a known cause of skin, bladder, and lung cancers (NRC, 2001; IARC, 2012; Cohen et al., 2016), and has also been associated with kidney, liver, and prostate cancers (IARC, 2012) as well as several non-carcinogenic ailments including diabetes and reproductive, cardiovascular, and neurological diseases (Schuhmacher-Wolz et al., 2009; Hughes et al., 2011; Maull et al., 2012; Naujokas et al., 2013). In humans, inorganic arsenic is enzymatically biotransformed to several methylated metabolites. This pathway of inorganic arsenic metabolism is generally accepted to be: inorganic arsenate [As^{V}] → inorganic arsenite [As^{III}] → monomethylarsonic acid [MMA^{V}] → monomethylarsonous acid [MMA^{III}] → dimethylarsinic acid [DMA^{V}] → dimethylarsinous acid [DMA^{III}] (Challenger, 1945; Cullen et al., 1989; Le et al., 2000; Styblo et al., 2002; Vahter, 2002; Thomas et al., 2001, 2004, 2007; Cullen, 2014). Alternative pathways postulating glutathione- or protein-conjugated intermediates have also been proposed (Hayakawa et al., 2005; Naranmandura et al., 2006; Dheeman et al., 2014), although their chemical basis has been questioned (Cullen, 2014).

Arsenic cytotoxicity is dependent on its oxidation state and chemical structure (speciation). In general, trivalent arsenicals are more cytotoxic than pentavalent species. The methylated trivalent arsenicals, MMA^{III} and DMA^{III}, are more cytotoxic than the inorganic arsenicals, As^{III} and As^{V}, which are more cytotoxic than the methylated pentavalent arsenicals, MMA^{V} and DMA^{V} (Styblo et al., 2000; Petrick et al., 2000; Dopp et al., 2004; Nascimento et al., 2008; Charoensuk et al., 2009; Naranmandura et al., 2011). Historically, the focus of arsenic toxicity studies has been the examination of the oxygenated metabolites of inorganic arsenic. However, as analytical techniques have improved to increase sensitivity and specificity, several thiolated arsenic metabolites have been identified (Hansen et al., 2004; Wang et al., 2015; Chen et al., 2016; Sun et al., 2016). A class of thiol-containing arsenicals that were first identified as metabolites in seaweed-fed sheep (Hansen et al., 2004), are the pentavalent sulfur-containing arsenic species, such as dimethylmonothioarsinate [$DMMTA^{V}$], dimethyldithioarsinate [$DMDTA^{V}$], and monomethylmonothioarsonate [$MMMTA^{V}$]. These thio-arsenicals have been detected in human or animal urine as metabolites of inorganic arsenic (Hansen et al., 2004; Naranmandura et al., 2007a, 2013; Raml et al., 2007; Chen et al., 2016), and are believed to be formed from reactions between oxygenated arsenicals and hydrogen sulfide (Wang et al., 2015). Monomethyltrithioarsonate [$MMTTA^{V}$] is another thiol-containing pentavalent metabolite, but it has only been found as a metabolite of anaerobic microbiota *in vitro* (Pinyayev et al., 2011). Recent cytotoxicity analysis of these newly identified thiolated pentavalent arsenicals suggests that thiol conjugation can modulate arsenic toxicity, prompting inclusion of thiolated metabolites in this study. $DMMTA^{V}$ has been found to be as toxic as the trivalent species, As^{III} and DMA^{III}, in human cancer cell lines (Naranmandura et al., 2007b, 2009, 2011; Bartel et al., 2011). The trivalent glutathione conjugated arsenical, dimethylarsinic glutathione [$DMAG^{III}$], is suspected to play a key role in the transport of methylated arsenic species from the liver to the blood stream (Percy and Gailer, 2008). Reported IC_{50} values for $DMAG^{III}$ are equal to or less than those of As^{III} (Styblo et al., 2000; Vega et al., 2001).

While inorganic arsenic and its metabolites are often considered the most important from a human health perspective, other organoarsenic species have become topics of recent research interest. Two pentavalent phenyl arsenic species used in the poultry and pork industries are 3-nitro-4-hydroxyphenylarsonic acid (Roxarsone, Rox) and 4-aminobenzenearsenic acid (p-arsanilic acid, p-ASA). As livestock feed additives, Rox and p-ASA not only improve feed efficiency, allowing for faster weight gain, but also help control intestinal bacteria and parasites (Jones, 2007; Chen and Huang, 2012; Nachman et al., 2013). Both arsenicals have been phased out of use in the European Union and the United States; however, they are still used in many other countries (Kazi et al., 2013; Yao et al., 2013; Mafla et al., 2015; Mangalgiri et al., 2015; Wang and Cheng, 2015). Although several studies have shown that these arsenicals can accumulate in the tissue and organs of livestock (Aschbacher and Feil, 1991; Desheng and Niya, 2006; Nachman et al., 2013; Peng et al., 2014; Liu et al., 2015, 2016), little is known about the cytotoxicity of these pentavalent arsenic species to human cells. Another arsenic species that is used in laboratory research as a known inhibitor in various biochemical reactions to elucidate toxicity mechanisms is phenylarsine oxide [PAO^{III}]. This trivalent organoarsenic species is not naturally occurring, but it is found in the environment at sites contaminated with chemical warfare agents, as it is a degradation product of the chemical warfare agent diphenylarsine dichloride (also known as Pfiffikus) (Leermakers et al., 2006). Studies have shown PAO^{III} to be a potent cytotoxicant (Charoensuk et al., 2009).

Extensive data are available on the cytotoxicity of individual arsenicals. However, these data have been obtained using various assays on different cell lines. The species-dependent cytotoxicity and variations in different assays make it difficult to compare the relative cytotoxicity of different arsenic species. In addition, some arsenicals have therapeutic uses, as with the treatment of acute promyelocytic leukemia with arsenic trioxide (As_2O_3) (Shen et al., 1997; Wang et al., 2004; Chen et al., 2015) and refractory solid tumors with $DMAG^{III}$ (alternate names: S-dimethylarsino-glutathione, ZIO-101, and darinaparsin). Understanding the relative cytotoxicity of various arsenic species may direct their exploitation for further therapeutic investigation.

Real-time cell sensing is an impedance-based detection technique that can simultaneously perform 96 *in vitro* tests of cytotoxicity. Because this technique is label-free and dye-free, it is less invasive than traditional colorimetric cytotoxicity assays. It provides continuous monitoring, revealing more dynamic and complete cytotoxic response information, and its use has been demonstrated in chemical cytotoxicity testing (Solly et al., 2004; Xing et al., 2005; Boyd et al., 2008; Moe et al., 2016). It is also one of the cell-based *in vitro* assay technologies implemented in the United States Environmental Protection Agency ToxCast program to prioritize the vast number of environmental chemicals, many of which are already under heavy commercial use, for further toxicological testing through the development of

predictive *in vitro* assays (Xia et al., 2008; Judson et al., 2010). The features of high-throughput and real-time cytotoxicity monitoring make real-time cell sensing a desirable tool for prioritizing chemicals for surveillance and regulatory consideration.

The objective of this study is to examine the relative cytotoxicity and cellular responses of fourteen arsenic species in two human cell lines using a real-time cell sensing technique. We chose two cell lines, A549 and T24, for this study. A549 cells, derived from a human alveolar adenocarcinoma, have been shown to express arsenic (+3 oxidation state)-methyltransferase (As3MT) (Sumi et al., 2011). T24 cells, derived from a human urinary bladder carcinoma, lack the ability to methylate inorganic As^{III} (Drobna et al., 2005). The enzyme responsible for methylation is known to be As3MT, which catalyzes the transfer of a methyl group from the donor molecule, S-adenosylmethionine (Lin et al., 2002). Therefore, these two cell lines represent cells that are either able or unable to methylate arsenic. The species under investigation include the inorganic arsenicals [As^{III}, As^{V}], their trivalent methylarsenical metabolites [MMA^{III}, DMA^{III}], pentavalent methylarsenical metabolites [MMA^{V}, DMA^{V}], and thiolated metabolites [$DMAG^{III}$, $MMTTA^{V}$, $DMMTA^{V}$, $DMDTA^{V}$]. The study also included three phenylarsenicals [PAO^{III}, Rox, p-ASA]. We chose real-time cell sensing because it is able to provide real-time monitoring of cytotoxic responses of the test cells to the arsenicals. The dynamic monitoring provides information necessary to generate quantitative IC_{50} histograms, revealing important cytotoxic responses that are dependent on cell type, arsenic species, concentration, and exposure time. In addition, the determination of cytotoxic response using a single assay will allow for comparison of the relative cytotoxicity of the arsenic species under investigation. Hence, the results of this study will provide information that can be used to better inform the human health risk assessment and pharmaceutical application of arsenicals.

1. Experimental

1.1. Cell culture

Two cell lines, A549 and T24, were used in this study. The human lung carcinoma cell line, A549 (CCL-185; American Type Culture Collection (ATCC), Manassas, VA, USA), was cultured in RPMI 1640 media (Invitrogen, Burlington, ON, Canada). The human bladder carcinoma cell line, T24 (HTB-4; ATCC) was cultured in McCoy's 5A modified media (ATCC). Both media were supplemented with 10% fetal bovine serum (Sigma-Aldrich, Oakville, ON, Canada) and 1% penicillin–streptomycin (Invitrogen). The incubation conditions were maintained at 37°C, 5% CO_2, and 90% humidity. During the study, cells were sub-cultured twice weekly into standard 10 cm × 20 mm cell culture dishes (Corning Incorporated, Corning, NY, USA) containing fresh media, using 0.05% trypsin–EDTA (Invitrogen) for cell detachment.

1.2. Preparation of arsenicals

Solutions of As^{III} and As^{V} in deionized (DI) water were prepared from commercially available sodium arsenite and sodium arsenate (Sigma-Aldrich). Solutions of MMA^{III} in DI water were prepared from synthesized standards of methyldiiodoarsine (Millar et al., 1960; Zingaro, 1996; Cullen et al., 2016) and methylarsine oxide [MAO^{III}] (Cullen et al., 1989, 2016), and the solution of DMA^{III} in DI water was prepared from a synthesized standard of dimethyliodoarsine (Millar, 1960; Cullen et al., 2016). Solutions of $DMAG^{III}$, $DMMTA^{V}$, $MMTTA^{V}$, and $DMDTA^{V}$ in DI water were also prepared from synthesized standards prepared in our laboratory (Cullen et al., 2016). $DMAG^{III}$ stock solutions also contained 2% methanol (Fisher Scientific, Nepean, ON, Canada). Solutions of PAO^{III}, 3-nitro-4-hydroxyphenylarsonic acid [Rox], and 4-aminobenzenearsenic acid [p-ASA] in DI water were also prepared from commercially available standards (Sigma-Aldrich). PAO^{III} stock solutions contained 2% methanol, and p-ASA stock solutions contained up to 10% methanol. Table S1 in Appendix A presents a list of the prepared arsenic species with their chemical structure. Solutions were sterilized *via* filtration (0.22 μm) and the final concentration of arsenic in each solution was calibrated using an Agilent 7500ce inductively coupled plasma-mass spectrometry (ICP-MS) system (Agilent Technologies, Japan). As^{III}, As^{V}, and PAO^{III} stock solutions were stored at 4°C until use. Rox and p-ASA solutions re-precipitate over time in DI water at 4°C and had to be used within a week of preparation. All remaining arsenic solutions are unstable at 4°C and had to be prepared fresh the day of treatment.

1.3. Real-time cell sensing

The principles of the impedance-based real-time cell analysis (RTCA) system (ACEA Biosciences, San Diego, CA, USA) have been described previously (Xing et al., 2005; Boyd et al., 2008; Moe et al., 2016). The measured impedance is automatically converted to cell index (CI). Increases in CI result from an increase in the number of cells adhered to the microelectrodes (*via* cell proliferation), an increase in cell adhesion, or an increase in cell spreading. Decreases in CI occur when the number of cells decrease due to cell death, detachment from the microelectrodes, or morphological changes. Therefore, changes in CI can represent multiple cytological responses to the testing compound.

Cells were seeded into 96-well or 16-well E-plates of the 96×- or 16×-RTCA systems (ACEA Biosciences) at pre-calibrated concentrations that allowed for a CI of 1 to be reached between 18 and 24 hr after seeding to standardize the treatment time of replicate experiments. A549 cells were seeded at 4000–4500 cells per well and T24 cells at 3500–4000 cells per well. When a CI of 1 was reached, the arsenic solutions described above (Sec. 1.2) were serially diluted in the respective media of the A549 and T24 cell lines to achieve the proper dose range for quantitative analysis. Two hundred microliters of each treatment concentration was added to triplicate wells. Negative controls (untreated media) and solvent controls (methanol) were added at a volume of 200 μL to triplicate wells. After treatment, CI was measured at hourly intervals for at least 72 hr post-exposure. A minimum of three separate experimental runs with all corresponding negative and solvent controls were performed for each arsenic species on each cell line ($n \geq 3$). CI scales of response profiles were normalized using multiplication factors for easier visual display of results.

1.4. Accumulation of total arsenic in A549 and T24 cells

Cells were seeded into standard 6-well culture dishes (Greiner BioOne, Oakville, ON, Canada) at concentrations that had previously been determined to allow the cells to reach 50%–60% confluency between 18 and 24 hr growth. A549 cells were seeded at a density of 4.5×10^4 cells/mL and T24 cells at 4.0×10^4 cells/mL. When the cells reached the proper confluency, the As^{III}, MMA^{III}, and DMA^{III} stock solutions described above (Sec. 1.2) were serially diluted in media to produce concentrations equivalent to the 24 hr IC_{50} value and ½ IC_{50} value, as determined using RTCA. The old media in each well was aspirated and replaced with the treated media, with triplicate wells prepared for each concentration. The control wells (without arsenic treatment) were also prepared in triplicate. Cells were incubated for 24 hr.

After 24 hr exposure, cells were washed twice with phosphate buffered saline (PBS; Gibco), detached using 0.05% trypsin–EDTA, and suspended in fresh media. Cells were pelleted by centrifugation at 1700 r/min for 3 min and washed with ice-cold PBS. The cells were then counted using a hemocytometer. After pelleting again at 1000 r/min for 10 min, the PBS was carefully aspirated to avoid disturbing the pellet. The cell pellets were then resuspended in 2% HNO_3 (Fisher Scientific) and sonicated in a water sonicator (Fisher Scientific) for 30 min for lysis. After pelleting the lysed cells at 2500 r/min for 30 min, the supernatant (containing the intracellular components) was carefully collected to avoid disturbing the pellet and placed into a fresh 0.5 mL micro-centrifuge tube (Fisher Scientific). Because these samples were prepared for total arsenic analysis, samples were parafilmed and stored at 4°C until analysis by ICP-MS with either an ELAN 6000 ICP-MS system (PerkinElmer, Waltham, MA, USA) or an Agilent 7500ce ICP-MS system.

1.5. Determination of arsenic species in media of treated A549 cells

A549 cells were seeded into standard 96-well culture dishes (Corning) at a density of 4.5×10^4 cells/mL. When the cells reached 50%–60% confluency, the As^{III}, MMA^{III}, DMA^{III}, $DMAG^{III}$, As^{V}, $MMTTA^{V}$, $DMMTA^{V}$, and $DMDTA^{V}$ stock solutions (Sec. 1.2) were serially diluted in media to produce concentrations equivalent to the 24 hr IC_{50} values for A549 cells determined using RTCA. Due to the available quantity of $DMDTA^{V}$ standard, a treatment concentration of only the ¼ IC_{50} value (1.05 mmol/L) was prepared. Each treatment concentration was added to 24 wells, and 150 μL of media from 3 replicate wells was collected at each of 8 post-exposure time points. The selected time points for analysis were at the time of treatment (0 hr) and at 3, 6, 9, 12, 24, 36, 48, and 56 hr post-exposure.

High-performance liquid chromatography (HPLC) (Agilent 1100 series; Agilent Technologies) separation of arsenic species was performed on a Prodigy™ ODS-3 column (3 μm particle size, 100 Å, 100 × 4.6 mm; Phenomenex, Torrance, CA, USA). The mobile phase was prepared as follows: 5 mmol/L tetrabutylammonium hydroxide, 200 mmol/L malonic acid, and 5% methanol, with the pH adjusted to 5.85. The flow rate was maintained at 1.2 mL/min for the entire 6 min. The column temperature was maintained at 50°C. The injection volume was 30 μL. An Agilent 7500ce ICP-MS system was used as the detector.

1.6. Data analysis

The determination of IC_{50} values over time and the statistical analysis of data (two-way analysis of variance with Bonferroni post-tests, *t*-test) were performed using Prism 5.0 (Graph Pad Software Inc., San Diego, CA, USA). IC_{50} values were defined as the concentration of arsenical that resulted in a 50% reduction in normalized CI as compared to the normalized CI of non-treated control cells at a given time point. Values are expressed as the mean ± the standard error of the mean (SEM).

2. Results and discussion

2.1. Cytotoxicity testing of inorganic arsenicals, As^{III} and As^{V}

To illustrate the real-time cell sensing method for cytotoxicity assessment of the fourteen arsenicals, we first tested the inorganic arsenicals, As^{III} and As^{V}. Fig. 1 shows the response of A549 and T24 cells to As^{III} and As^{V} exposure. The top four graphs in Fig. 1 show the normalized cell index (CI) over time after arsenic treatment and represent typical real-time response profiles of As^{III} and As^{V} in A549 (Fig. 1a) and T24 (Fig. 1b) cells. The concentration-dependent cytotoxicity was observed for A549 cells (Fig. 1a, top graph) between 40 and 250 μmol/L As^{III} and for T24 cells (Fig. 1b, top graph) between 1 and 75 μmol/L As^{III}. Likewise, the dose–response for As^{V} in A549 cells (Fig. 1a, middle graph) was in the mmol/L range (0.1–4 mmol/L) and in the μmol/L range (20–500 μmol/L) for T24 cells (Fig. 1b, middle graph). These results indicate that T24 cells are more sensitive than A549 cells to both inorganic arsenicals.

The cell-dependent and species-dependent cytotoxicity of arsenic is further demonstrated by the quantitative IC_{50} histograms (in μmol/L, mean ± SEM), as shown in the bottom two graphs of Fig. 1. The IC_{50} histograms were obtained from the corresponding real-time response profiles (Fig. 1 top four graphs) and represent the concentration of arsenical that results in a 50% reduction in CI compared to the untreated control cells at a given time point. The data at 24-hr exposure in Fig. 1 are indicated with a dotted line. IC_{50} histograms are a unique feature of real-time cell sensing, as they are difficult to obtain using conventional endpoint cytotoxicity assays. These histograms show that As^{III} has significantly lower IC_{50} values throughout the exposure period, indicating that As^{III} is more cytotoxic than As^{V} in both cell lines. These results are consistent with previous reports comparing the cytotoxicity of As^{III} and As^{V} (Styblo et al., 2000; Vega et al., 2001; Hirano et al., 2003; Charoensuk et al., 2009).

2.2. Cytotoxicity of methylated and thiolated arsenic metabolites

Fig. 2 presents the response profiles of A549 and T24 cells exposed to the methylated and thiolated arsenicals, MMA^{III}, DMA^{III}, $DMAG^{III}$, $MMTTA^{V}$, $DMMTA^{V}$, and $DMDTA^{V}$. These

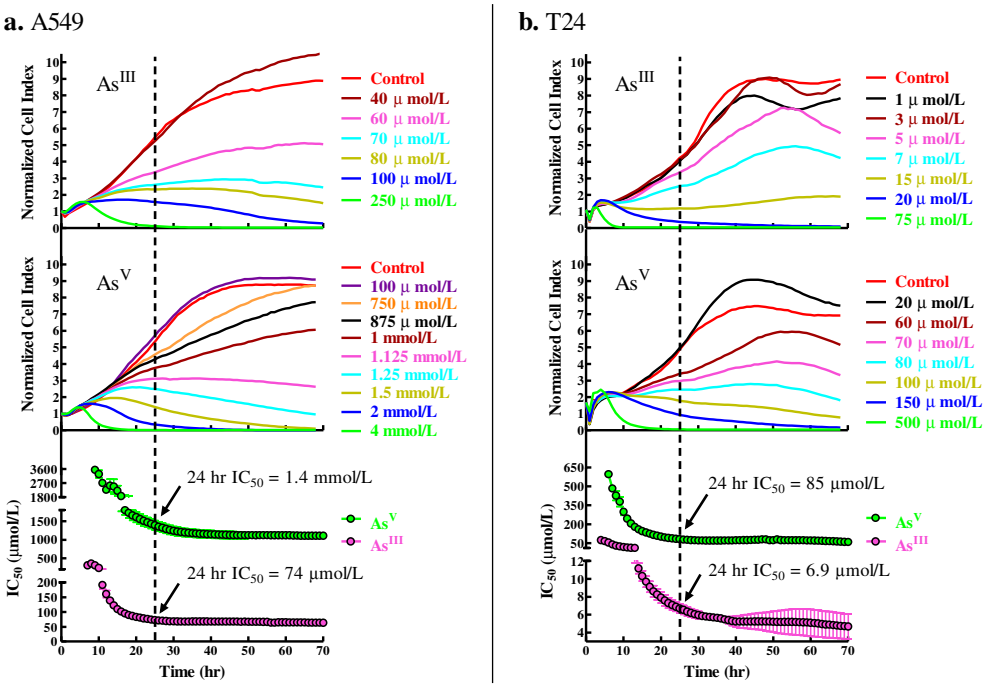

Fig. 1 – Normalized cell index (CI) and IC_{50} values (μmol/L) obtained from continuous real-time cell sensing of (a) A549 and (b) T24 cells demonstrating the qualitative and quantitative cellular response characterization of As^{III} and As^{V} exposure. The hourly dose–response data from the response profiles of CI over time are used to generate temporal IC_{50} histograms of the hourly IC_{50} values (μmol/L mean ± SEM) over the exposure period. The data collected at 24 hr are indicated with a dotted line. As^{III}: arsenite; As^{V}: arsenate.

response profiles demonstrate the concentration-, cell-, and species-dependent nature of arsenical cytotoxicity. To test the cytotoxicity of MMA^{III}, both the methyldiiodoarsine (labeled hereafter as MMA^{III}) and MAO^{III} forms were used, as both form the same MMA^{III} species in solution. Therefore, similar cytotoxicity of these species is expected. Indeed, our results (Fig. 2a and c) show similar response profiles when both starting forms of MMA^{III} were tested.

The response profiles of DMA^{III} (Fig. 2b) show a stepwise shape in the concentration range of 7.5–10 μmol/L for A549 cells and 5–7 μmol/L for T24 cells. The profile shapes of the thiolated trivalent arsenic species, $DMAG^{III}$ (Fig. 2d), in both cell lines are similar to those of DMA^{III} (Fig. 2b). The stepwise profile shape is visible in the concentrations of $DMAG^{III}$ between 10 and 25 μmol/L for A549 cells and between 2.5 and 6 μmol/L for T24 cells. The stepwise response may be related to the conversion of DMA^{III} and $DMAG^{III}$ to DMA^{V} in solution over the testing period, as will be discussed later.

The response profiles for the pentavalent thiolated arsenic metabolites, $MMTTA^{V}$ (Fig. 2e), $DMMTA^{V}$ (Fig. 2f), and $DMDTA^{V}$ (Fig. 2g), also show distinct species-dependent responses in both A549 and T24 cells. $DMMTA^{V}$ (Fig. 2f) shows stepwise profile shapes similar to those of DMA^{III} (Fig. 2b) and $DMAG^{III}$ (Fig. 2d). In A549 cells, this stepwise profile shape is seen with the concentrations between 25 and 30 μmol/L $DMMTA^{V}$ (Fig. 2f), while in T24 cells, it is seen with concentrations between 5 and 7 μmol/L $DMMTA^{V}$ (Fig. 2f). The shape of the response profiles for $MMTTA^{V}$ (Fig. 2e) and $DMDTA^{V}$ (Fig. 2g) are characterized by an initial increase in CI followed by a rapid decrease. Although the reasons for the distinct profiles are not known, the possibility of different biological effects or the involvement of the conversion of arsenic species during cell incubation cannot be ruled out.

Real-time cell sensing of A549 and T24 cells incubated with PAO^{III} (Fig. 3a), Rox (Fig. 3b), and p-ASA (Fig. 3c) shows that the trivalent PAO^{III} is much more cytotoxic than the two pentavalent phenylarsenicals. PAO^{III} is highly cytotoxic with a dose–response in the nmol/L range, consistent with a previous report (Charoensuk et al., 2009). Rox and p-ASA exhibit a dose–response in the mmol/L range, five to six orders of magnitudes higher than PAO^{III}. These results are consistent with previous cytotoxicity studies reporting p-ASA dose–response ranges of 0.05–50 mmol/L in rat primary hepatocytes (Yuan et al., 2006) and 1–25 mmol/L in mouse embryonic stem cells (Kang et al., 2014), both determined using the tetrazolium (MTT) colorimetric assay.

To quantitatively compare the relative cytotoxicity of the tested arsenicals, temporal IC_{50} histograms were generated from the response profiles for each arsenical tested in both the A549 and T24 cell lines. As an example to illustrate the range of IC_{50} values that were determined amongst the tested arsenicals, Fig. 4 shows the IC_{50} values over time for the most cytotoxic arsenical, PAO^{III}, in comparison to inorganic As^{III} and the much less cytotoxic Rox, in both A549 (Fig. 4a) and T24 (Fig. 4b) cells. These results demonstrate the strong influence of arsenic speciation on cytotoxicity, as the IC_{50} values range from nmol/L levels for PAO^{III} to μmol/L for As^{III} and mmol/L for Rox. IC_{50} histograms of the other tested arsenic species are

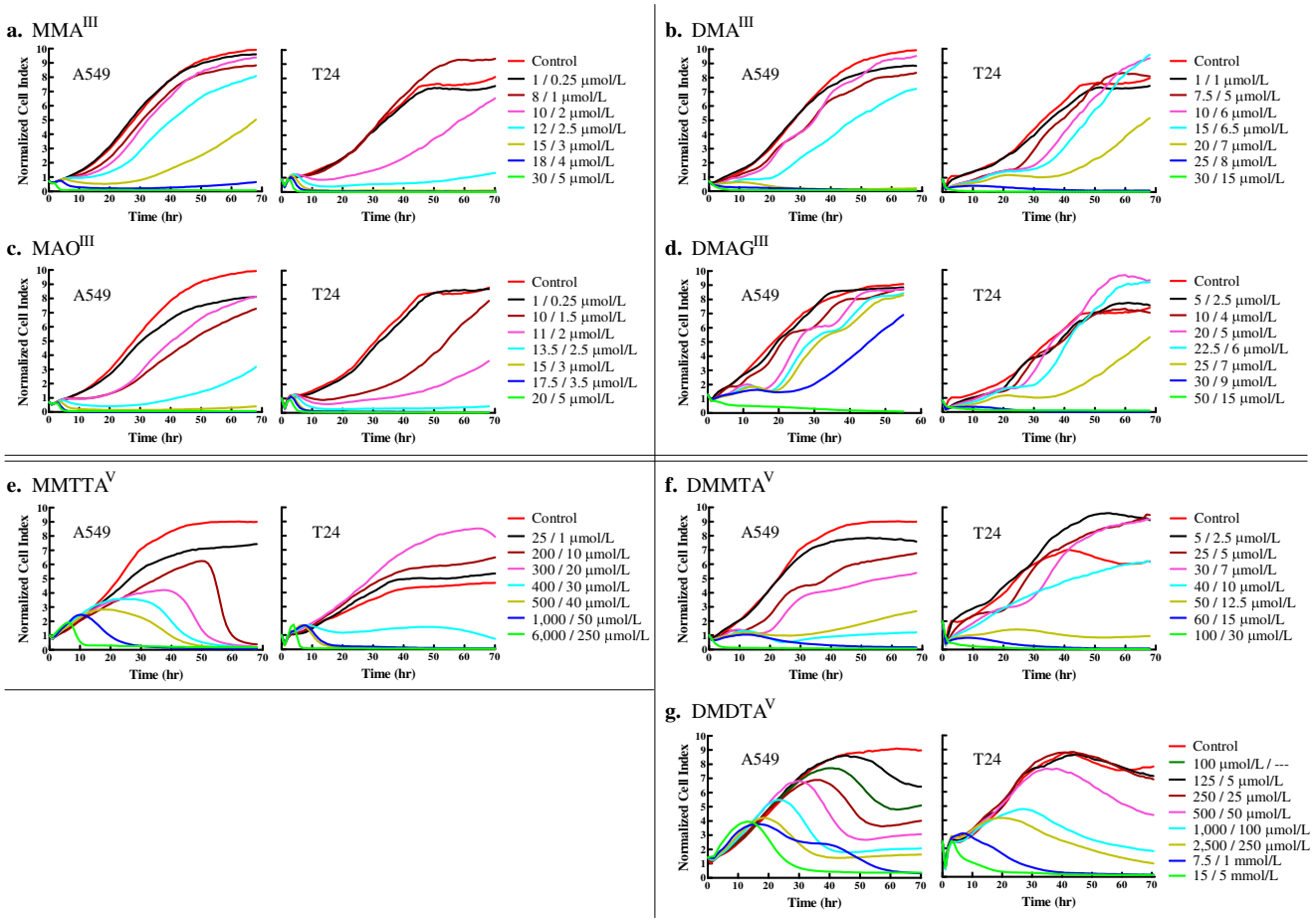

Fig. 2 – Response profiles of the normalized cell index (CI) over time for A549 and T24 cells exposed to (a) MMA^{III}, (b) DMA^{III}, (c) MAO^{III}, (d) $DMAG^{III}$, (e) $MMTTA^{V}$, (f) $DMMTA^{V}$, and (g) $DMDTA^{V}$. The tested concentration range for each arsenical is included for both A549 (left) and T24 (right) cell lines. Untreated control cells are indicated in red in each response profile. MMA^{III}: monomethylarsonous acid; DMA^{III}: dimethylarsinous acid; MAO^{III}: methylarsine oxide; $DMAG^{III}$: dimethylarsinic glutathione; $MMTTA^{V}$: monomethyltrithioarsonate; $DMMTA^{V}$: dimethylmonothioarsinate; $DMDTA^{V}$: dimethyldithioarsinate.

shown in Appendix A Fig. S1. From continuous real-time cell sensing, these results provide information on the toxicity of each arsenic species to the cells over the entire 72-hr incubation period.

2.3. *Accumulation of arsenic in A549 and T24 cells*

To explore whether cellular uptake and retention of arsenic contribute to the observed differences in cytotoxicity between A549 and T24 cells, we first determined the intracellular concentrations of total arsenic in each cell line after 24 hr exposure to As^{III}, MMA^{III}, or DMA^{III}. Table 1 shows the average amounts of arsenic in A549 and T24 cells after the cells were incubated with arsenic species at concentrations equivalent to their respective 24-hr IC_{50} or ½ IC_{50} values. The average number of 4.35×10^8 arsenic atoms/cell in T24 cells treated with 6.9 μmol/L As^{III} was almost twice the amount (2.55×10^8 As atoms/cell) found in A549 cells treated with 76.6 μmol/L As^{III}. These results suggest that T24 cells are capable of more efficient uptake and/or retention of As^{III} than A549 cells. The concentrations of intracellular arsenic in cells treated with the 24-hr IC_{50} value concentrations of MMA^{III} were similar in both cell lines at around 0.40×10^8 arsenic atoms/cell. Because the concentration of MMA^{III} present in the cell culture medium of the treated T24 cells (1.9 μmol/L) was nearly seven times lower than in the medium of the treated A549 cells (13.6 μmol/L), the similar amounts in the cells suggest that T24 cells are also capable of more efficient uptake and/or retention of arsenic than A549 cells exposed to MMA^{III}. In the case of incubation with DMA^{III}, T24 cells contained 0.44×10^8 arsenic atoms/cell after 24-hr incubation with 6.4 μmol/L μM DMA^{III}, while A549 cells contained 0.80×10^8 arsenic atoms/cell after 24-hr incubation with 14.1 μmol/L DMA^{III}, indicating similar accumulation of total arsenic (Table 1).

Another reason for the observed differences in the cytotoxicity between A549 and T24 cells may be related to the differences in the ability of these cells to methylate arsenic. The enzymatic methylation of inorganic arsenic is an important step in arsenic metabolism to promote its elimination. The enzyme responsible for methylation is

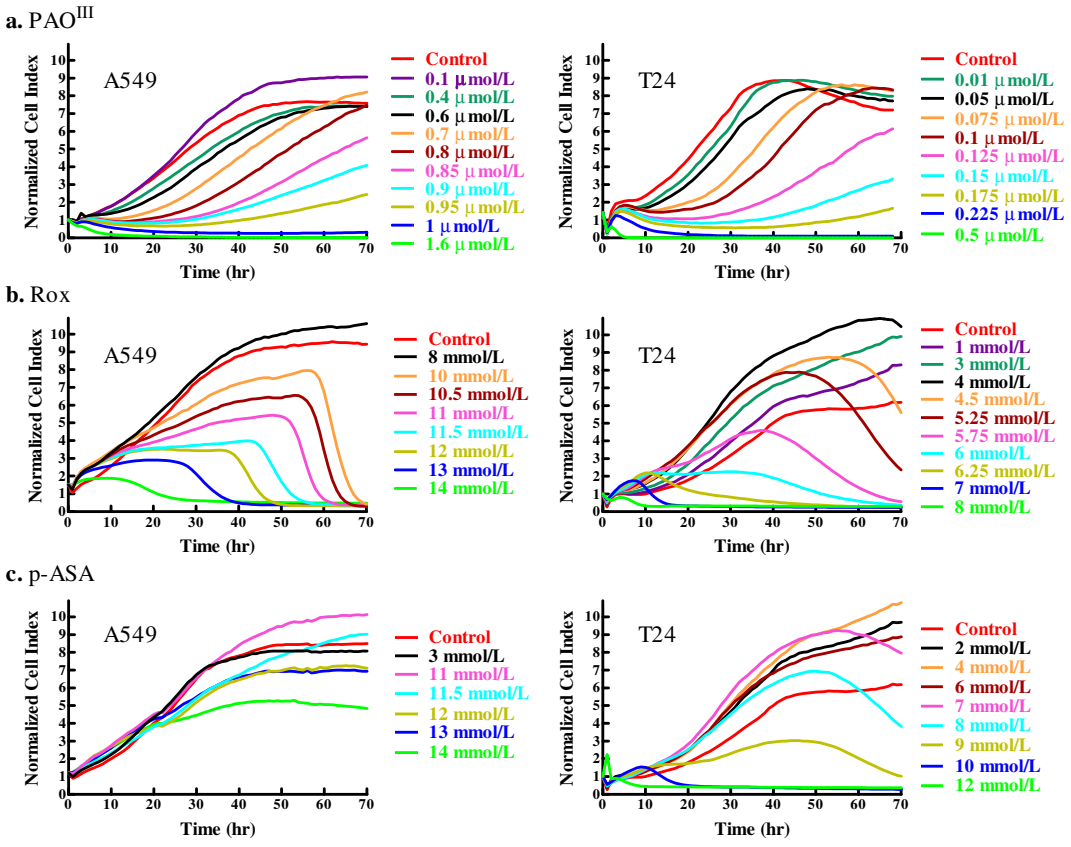

Fig. 3 – Response profiles of the normalized cell index (CI) over time for A549 and T24 cells exposed to (a) PAO^{III}, (b) Rox, and (c) p-ASA. PAO^{III}: phenylarsine oxide; Rox: Roxarsone; p-ASA: p-arsanilic acid.

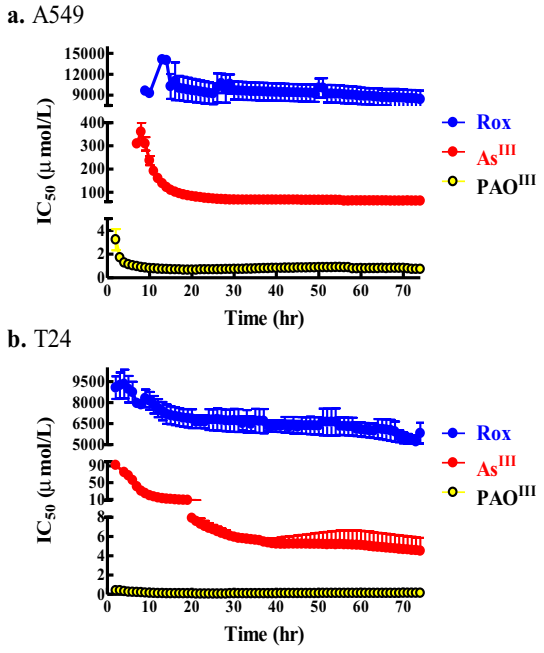

Fig. 4 – Temporal IC_{50} histograms of the hourly IC_{50} values (μmol/L mean ± SEM) for (a) A549 and (b) T24 cells exposed to PAO^{III}, inorganic As^{III}, and Rox. PAO^{III}: phenylarsine oxide; As^{III}: arsenite; Rox: Roxarsone.

known to be arsenic (+3 oxidation state)-methyltransferase (As3MT), which catalyzes the transfer of a methyl group from the donor molecule, S-adenosylmethionine (AdoMet) (Lin et al., 2002). In the pathway of oxidative methylation, As3MT catalyzes the conversion of As^{III} to MMA^{V} and MMA^{III} to DMA^{V}. A549 cells, derived from a human alveolar adenocarcinoma, have been shown to express As3MT (Sumi et al., 2011). However, T24 cells, derived from a human urinary bladder carcinoma, lack the ability to methylate inorganic As^{III} (Drobna et al., 2005). Hence, the observed difference in cytotoxicity and accumulation of the arsenicals in T24 and A549 cells is likely influenced by the inability of T24 cells to methylate As^{III}. The higher cytotoxicity observed in T24 cells exposed to As^{III} or MMA^{III} is consistent with the fact that T24 cells lack the enzyme to catalyze the conversion of As^{III} to the less toxic MMA^{V}, or the conversion of MMA^{III} to the less toxic DMA^{V}. It is also known that methylation affects clearance of arsenic from cells and tissues. Previous reports have shown higher accumulation of total arsenic in non-methylating *versus* methylating cell lines (Styblo et al., 2000; Dopp et al., 2005, 2008, 2010). Retention of arsenic species in tissues of As3MT knockout mice was also higher than in the wild-type mice (Drobna et al., 2009; Chen et al., 2011; Naranmandura et al., 2013; Currier et al., 2016). Thus, it is reasonable that T24 cells would have higher accumulation of arsenic after exposure to As^{III} or MMA^{III} than A549 cells, as observed in our results (Table 1).

Table 1 – Concentrations of total arsenic in A549 and T24 cells after 24-hr exposure to As^{III}, MMA^{III}, or DMA^{III} at their respective 24-hr IC_{50} and ½ IC_{50} concentrations.

		A549		T24	
		As in medium *	As in cells	As in medium	As in cells
			As atoms/cell ($\times 10^6$)		As atoms/cell ($\times 10^6$)
Control		–	2.2 ± 0.7	–	1.5 ± 0.6
As^{III}	IC_{50}	76.6 μmol/L	255 ± 9	6.9 μmol/L	435 ± 43
	½ IC_{50}	38.3 μmol/L	59 ± 21	3.45 μmol/L	114 ± 9.3
MMA^{III}	IC_{50}	13.6 μmol/L	36 ± 3	1.9 μmol/L	40 ± 5
	½ IC_{50}	6.8 μmol/L	6.8 ± 0.6	0.95 μmol/L	5.3 ± 0.4
DMA^{III}	IC_{50}	14.1 μmol/L	80 ± 7	6.4 μmol/L	44 ± 15
	½ IC_{50}	7.05 μmol/L	23 ± 4	3.2 μmol/L	22 ± 6

AsIII: arsenite; MMA^{III}: monomethylarsonous acid; DMA^{III}: dimethylarsinous acid.
As atoms/cell ($\times 10^6$) values were mean ± SEM (n = 3).
* As in medium represents the treatment concentrations of arsenic species that corresponded to IC_{50} and ½ IC_{50} values.

2.4. Arsenic speciation in culture media over time

Because the trivalent methylarsenicals are not stable at room temperature and can be oxidized to the pentavalent methylarsenicals (Gong et al., 2001), we determined arsenic speciation in the cell culture medium of A549 cells over time. Fig. 5 shows the concentrations of the main arsenic species detected in the culture media of A549 cells incubated with As^{III}, MMA^{III}, DMA^{III}, $DMAG^{III}$, As^{V}, $MMTTA^{V}$, $DMMTA^{V}$, and $DMDTA^{V}$. The initial concentrations of these arsenic species were equivalent to their respective 24-hr IC_{50} values determined using real-time cell sensing, and the concentrations of arsenic species over time were determined using HPLC–ICP-MS (*e.g.*, Fig. S2 in Appendix A). These results show that

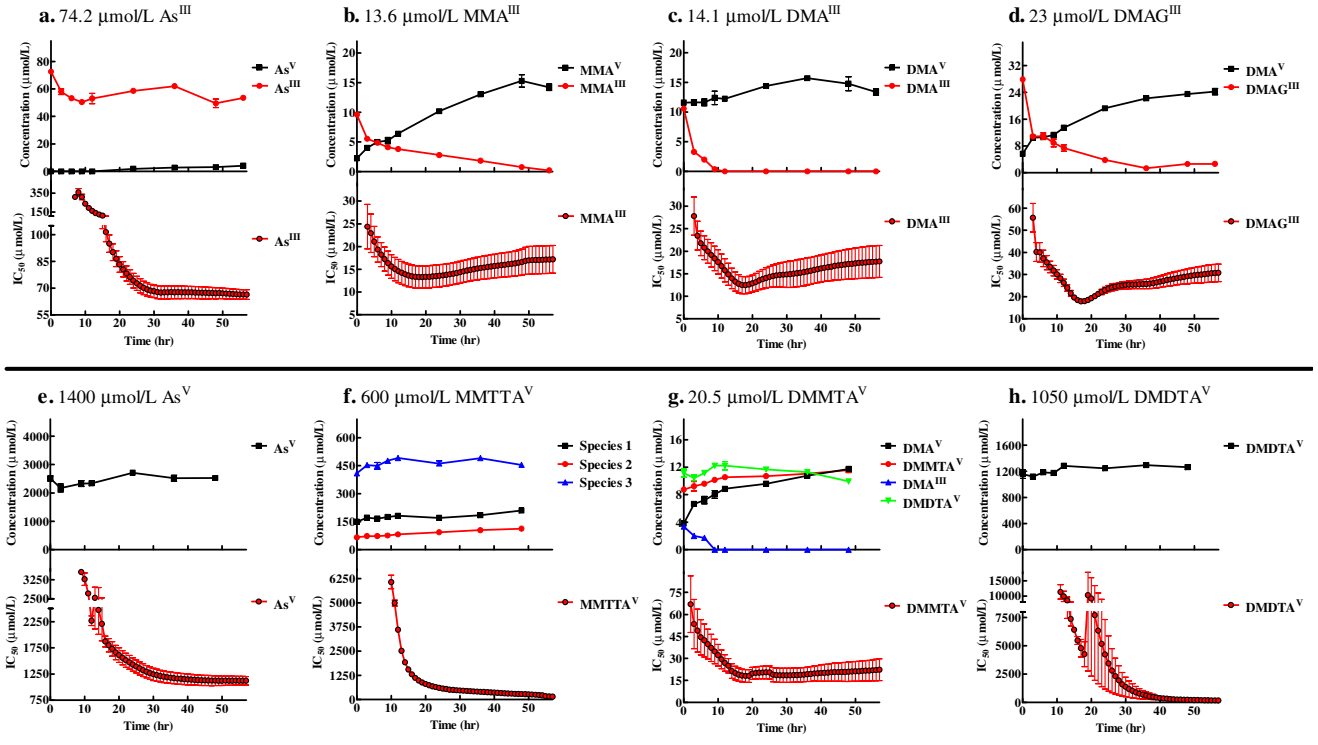

Fig. 5 – Concentrations of arsenic species present in the culture media of A549 cells (top graphs) and IC_{50} histograms (bottom graphs) over the same incubation period and incubation temperature (37°C). Parallel experiments were carried out by incubating A549 cells with the arsenic compound at a concentration equivalent to its respective 24-hr IC_{50} value. The media was collected from A549 cells treated with (a) 74.2 μmol/L As^{III}, (b) 13.6 μmol/L MMA^{III}, (c) 14.1 μmol/L DMA^{III}, (d) 23 μmol/L $DMAG^{III}$, (e) 1400 μmol/L As^{V}, (f) 600 μmol/L $MMTTA^{V}$, (g) 20.5 μmol/L $DMMTA^{V}$, and (h) 1050 μmol/L $DMDTA^{V}$. Arsenic species were determined using HPLC–ICP-MS. As^{III}: arsenite; MMA^{III}: monomethylarsonous acid; DMA^{III}: dimethylarsinous acid; $DMAG^{III}$: dimethylarsinic glutathione; As^{V}: arsenate; $MMTTA^{V}$: monomethyltrithioarsonate; $DMMTA^{V}$: dimethylmonothioarsinate; $DMDTA^{V}$: dimethyldithioarsinate.

the inorganic arsenicals As^{III} (Fig. 5a) and As^{V} (Fig. 5e) were relatively stable over the entire incubation period at 37°C. The pentavalent thiolated arsenicals $MMTTA^{V}$, $DMMTA^{V}$, and $DMDTA^{V}$ also appeared to be stable, although $MMTTA^{V}$ and $DMMTA^{V}$ contained mixtures of arsenic species (Fig. S2 in Appendix A). Importantly, the three trivalent methylarsenicals, MMA^{III} (Fig. 5b), DMA^{III} (Fig. 5c), and $DMAG^{III}$ (Fig. 5d), were oxidized over time. MMA^{III} was converted to the less toxic pentavalent MMA^{V}, with complete conversion by 56 hr exposure. DMA^{III} and $DMAG^{III}$ were both converted to the less toxic DMA^{V} over time. A complete conversion of DMA^{III} to DMA^{V} was observed by 9 hr post-exposure, while the conversion of $DMAG^{III}$ to DMA^{V} was complete after 36 hr. Therefore, in the tests of cytotoxicity of MMA^{III}, DMA^{III}, and $DMAG^{III}$, the actual concentrations of these highly toxic arsenic species experienced by the cells decreased over time. During the incubation period, the cells were actually exposed to a mixture of both the more toxic trivalent methylarsenicals and the less toxic pentavalent methylarsenicals. It is possible that the conversion of the more toxic arsenic species to the less toxic arsenic species in the culture medium could contribute, at least in part, to the observed unique response profiles of CI over time (Fig. 2).

For further comparison, the IC_{50} histograms (bottom graphs in Fig. 5) are shown along with the HPLC–ICP-MS results of the arsenic species detected in the culture media (top graphs in Fig. 5). The arsenicals that did not undergo conversion over time (As^{III}, As^{V}, $MMTTA^{V}$, $DMDTA^{V}$) had decreasing CI values over time (Figs. 1 and 2) and correspondingly, decreasing IC_{50} values over time (Fig. 5). With no observed conversion, these arsenicals maintained their potency over the exposure period. However, the arsenic species (MMA^{III}, DMA^{III}, $DMAG^{III}$, $DMMTA^{V}$) that exhibited conversion to less toxic arsenicals over time showed increasing CI values over time for mid-dose range treatments (Fig. 2), which resulted in increasing IC_{50} values over time after 20 hr exposure (Fig. 5). The more gradual increase in IC_{50} values over time for $DMMTA^{V}$ in comparison to MMA^{III}, DMA^{III}, and $DMAG^{III}$ is consistent with the partial conversion exhibited by $DMMTA^{V}$. These results show that the unique IC_{50} histograms for MMA^{III} (Fig. 5b), DMA^{III} (Fig. 5c), and $DMAG^{III}$ (Fig. 5d) coincide with the conversion of the more toxic trivalent methylarsenicals to the less toxic pentavalent methylarsenicals. These results are understandable because the cells were actually exposed to the pentavalent arsenicals of lower toxicity during the later incubation time.

2.5. Cytotoxicity ranking of arsenic species

The 24-hr and 48-hr IC_{50} values for the fourteen arsenicals examined in this study are summarized in Table 2. Our IC_{50} values determined using the real-time cell sensing technique were consistent with results from other arsenic studies using traditional end-point cytotoxicity assays. The 24-hr IC_{50} value of 74 ± 4 μmol/L for As^{III} in the A549 cell line was in good agreement with previously reported values of around 100 μmol/L for the same cell line, determined using MTT and cell counting assays (Talbot et al., 2008; Chiang and Tsou, 2009). The 24-hr IC_{50} values for MMA^{III}, DMA^{III}, and As^{III} in T24 cells determined using the real-time cell sensing technique were also consistent with results of MTT tests, where the potency of these arsenicals was MMA^{III} (2.5 μmol/L) > DMA^{III}, As^{III} (>10 μmol/L) (Styblo et al., 2002). Our quantitative results were also consistent with Bartel et al. (2011), who reported IC_{70} values in A549 cells after 24 hr exposure to arsenicals using cell counting methods. They found DMA^{III} (5.1 μmol/L) ≈ MMA^{III} (5.6 μmol/L) > $DMMTA^{V}$ (12.1 μmol/L) > As^{III} (57.2 μmol/L) (Bartel et al., 2011), in comparison to our IC_{50} values: MMA^{III} (14 μmol/L) ≈ DMA^{III} (14 μmol/L) > $DMMTA^{V}$ (20 μmol/L) > As^{III} (74 μmol/L). A second study from the Schwerdtle group

Table 2 – The 24-hr and 48-hr IC_{50} values (μmol/L mean ± SEM) for fourteen arsenicals in A549 and T24 cells ($n \geq 3$).

	A549		T24	
	24-hr	48-hr	24-hr	48-hr
PAO^{III}	0.7 ± 0.1^{a}	0.9 ± 0.1^{q}	0.08 ± 0.01^{j}	$0.11 \pm 0.01^{z,*}$
MAO^{III}	12 ± 2^{b}	14 ± 2^{r}	1.2 ± 0.4^{k}	1.1 ± 0.2^{aa}
MMA^{III}	$14 \pm 2^{b,c}$	$17 \pm 3^{r,s}$	1.9 ± 0.1^{k}	2.2 ± 0.2^{bb}
DMA^{III}	$14 \pm 2^{b,c}$	$17 \pm 3^{r,s}$	5 ± 1^{l}	5 ± 1^{cc}
$DMAG^{III}$	23 ± 1^{c}	29 ± 3^{s}	5 ± 1^{l}	5 ± 1^{cc}
$DMMTA^{V}$	$20 \pm 4^{b,c}$	$21 \pm 7^{r,s}$	6 ± 1^{l}	8 ± 2^{cc}
As^{III}	74 ± 4^{d}	67 ± 3^{t}	6.9 ± 0.5^{l}	5 ± 1^{cc}
$MMTTA^{V}$	600 ± 79^{e}	$280 \pm 22^{u,*}$	25 ± 2^{m}	29 ± 5^{dd}
As^{V}	1400 ± 130^{f}	1100 ± 93^{v}	85 ± 6^{n}	80 ± 8^{ee}
$DMDTA^{V}$	$4300 \pm 3000^{‡}$	230 ± 74^{u}	$342 \pm 95^{‡}$	$63 \pm 13^{ee,*}$
DMA^{V}	6030 ± 390^{g}	$3300 \pm 170^{w,*}$	1500 ± 210^{o}	$560 \pm 30^{ff,*}$
MMA^{V}	28000 ± 1020^{i}	25000 ± 280^{y}	2600 ± 350^{p}	2600 ± 240^{gg}
Rox	$9300 \pm 1600^{g,h}$	9300 ± 1200^{x}	6800 ± 740^{h}	6400 ± 620^{x}
p-ASA	Undetermined	Undetermined	6300 ± 3200	7200 ± 1900

PAO^{III}: phenylarsine oxide; MAO^{III}: methylarsine oxide; MMA^{III}: monomethylarsonous acid; DMA^{III}: dimethylarsinous acid; $DMAG^{III}$: dimethylarsinic glutathione; $DMMTA^{V}$: dimethylmonothioarsinate; As^{III}: arsenite; $MMTTA^{V}$: monomethyltrithioarsonate; As^{V}: arsenate; $DMDTA^{V}$: dimethyldithioarsinate; DMA^{V}: dimethylarsinic acid; MMA^{V}: monomethylarsonic acid; Rox: Roxarsone; p-ASA: p-arsanilic acid.

$^{a–gg}$: Denotes significant difference ($p < 0.05$), two-way analysis of variance of arsenical and cell line IC_{50} values at each time point with Bonferroni post-tests.

*: Denotes significant difference ($p < 0.05$), unpaired t-test of 24-hr and 48-hr IC_{50} values.

‡: Value not included in analysis (variable standard deviation for 24-hr IC_{50} value).

determined 24-hr IC_{70} values in A549 cells using a cellular dehydrogenase assay and found $DMAG^{III}$ (5.8 μmol/L) > $DMMTA^{V}$ (20.5 μmol/L) > As^{III} (97.0 μmol/L) (Leffers et al., 2013), which compare to our 24-hr IC_{50} values of $DMAG^{III}$ (23 μmol/L) ≈ $DMMTA^{V}$ (20 μmol/L) > As^{III} (74 μmol/L). Our IC_{50} values for $DMAG^{III}$ were consistent with previous reports that found the cytotoxicity of $DMAG^{III}$ was similar to that of As^{III} in most of the tested cell lines (Styblo et al., 2000).

Statistical analysis of the IC_{50} values at each individual time point (24 and 48 hr) quantitatively demonstrated cell- and species-dependent cytotoxicity, as the IC_{50} values were determined to be statistically different ($p < 0.05$) across both the arsenical and cell line tested (two-way analysis of variance with Bonferroni post-tests). With the exception of Rox, IC_{50} values of T24 cells were significantly lower than IC_{50} values of A549 cells for all arsenicals at both time points (Table 2). On the basis of the 24-hr IC_{50} values in Table 2, the relative toxicities of arsenicals to A549 cells are: $PAO^{III} \gg MMA^{III} \approx DMA^{III} \geq DMMTA^{V} \geq DMAG^{III} > As^{III} \gg MMTTA^{V} > As^{V} > DMDTA^{V} > DMA^{V} > Rox > MMA^{V}$. In T24 cells, the relative toxicities are: $PAO^{III} \gg MMA^{III} > DMA^{III} \approx DMAG^{III} \approx DMMTA^{V} \approx As^{III} \gg MMTTA^{V} > As^{V} > DMDTA^{V} > DMA^{V} > MMA^{V} > Rox \approx$ p-ASA.

In general, we found that the trivalent arsenicals were more cytotoxic than most pentavalent species, supportive of the trend reported in the literature (Styblo et al., 1997, 2000; Lin et al., 1999). $DMMTA^{V}$, a pentavalent thioarsenical, was more cytotoxic than As^{III} in the A549 cell line, and was equally cytotoxic as As^{III} in the T24 cell line, consistent with other studies (Naranmandura et al., 2007b, 2009, 2011; Bartel et al., 2011; Leffers et al., 2013). Of the pentavalent thio-arsenicals examined in this study, $DMMTA^{V}$ was also the most cytotoxic in both cell lines, with a 24-hr IC_{50} value either one or two orders of magnitude lower than those of the other tested thio-arsenicals, $MMTTA^{V}$ and $DMDTA^{V}$.

3. Conclusions

The cytotoxicity of more than a dozen arsenic compounds is dependent on the cell type, the arsenic species, and the concentration and exposure time of the arsenic species. The two selected cell lines represent those expressing As3MT (A549) or lacking this enzyme (T24) necessary for efficient arsenic methylation. The real-time cell sensing method enables continuous monitoring of the dynamic changes in CI, providing qualitative and quantitative information for the assessment of arsenical cytotoxicity in real time. The IC_{50} histograms show the relative cytotoxicity of the fourteen arsenic compounds in the test cell lines over the entire incubation period. The continuous monitoring over the entire cell incubation period reveals unique profiles that may suggest different processes/mechanisms involved in the cellular toxicity. Although the mechanisms responsible for the observed differences in temporal profiles are not fully understood, the results coincide with the conversion of the more toxic trivalent species MMA^{III}, DMA^{III}, and $DMAG^{III}$ to the less toxic pentavalent species MMA^{V} and DMA^{V}. Further studies are needed to understand the reasons for the unique response profiles and the mechanisms of arsenic toxicity.

Acknowledgments

This study was supported by Alberta Health, Alberta Innovates, the Canada Research Chairs Program, the Canadian Institutes of Health Research (CIHR), and the Natural Sciences and Engineering Research Council (NSERC) of Canada. The authors acknowledge Dr. Hongsui Sun for synthesizing many of the arsenic compounds used in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.10.004.

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