Mechanistic Studies of Arsenic and Selenium Detoxification

by

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Abstract

Over 200 million people worldwide are exposed to the proven human carcinogen arsenic, due to contaminated drinking water. Animal studies have shown that arsenic and the essential trace element selenium can undergo mutual detoxification through the formation of the seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ which undergoes biliary excretion, resulting in fecal elimination of both compounds. The ATP-binding cassette (ABC) transporter multidrug resistance protein 2 (MRP2/*ABCC2*), localized to the canalicular surface of hepatocytes, is a transporter of this conjugate. $[(GS)_2AsSe]^-$ is also formed in animal red blood cells (RBCs), resulting in the sequestration of arsenic and selenium. In human cells, the influence of arsenic on selenium accumulation, and vice versa, is largely unknown. The objectives of this thesis were (a) to characterize arsenite and selenite uptake and metabolism in human RBCs (hRBCs), (b) to characterize the effects of selenite on the hepatobiliary efflux of arsenic in sandwich-cultured human hepatocytes (SCHH) and (c) to characterize the effects of different selenium species on arsenic cytotoxicity and accumulation in HepG2 cells. The overarching hypothesis was that selenium would alter arsenic metabolism and transport through the formation of $[(GS)_2AsSe]^-$. ^{75}Se-selenite uptake inhibition assays and experiments with heterologous overexpression systems showed selenite uptake in hRBCs to be mediated by the Cl^-/bicarbonate (HCO_3^-) anion exchanger 1 (AE1). ^{73}As-arsenite uptake inhibition assays showed arsenite uptake in hRBCs to be partially AQP3-mediated, while no evidence of GLUT1 involvement was found. Speciation analysis using X-ray absorption spectroscopy showed that 73% of the arsenic and selenium present in RBCs co-treated with arsenite and selenite was in the form of $[(GS)_2AsSe]^-$. The remaining 26% of As was present as $As(GS)_3$, providing the first evidence of the formation of both As-glutathione conjugates in human cells.

Consistent with published literature, all ^{73}As biliary efflux in SCHH was glutathione-dependent. Selenite either reduced or did not alter ^{73}As efflux across the sinusoidal and canalicular surfaces of SCHH. Preliminary data showed that selenide increases or does not change ^{73}As biliary efflux, ^{75}Se-selenide accumulation in hepatocytes was higher than ^{75}Se-selenite; and selenide was able to protect SCHH against arsenite-induced toxicity while, selenite only conferred protection when the SCHH preparation displayed a potential for arsenic biliary efflux. Further characterization of selenite and selenide uptake and cytotoxicity differences in HepG2 cells also showed a higher IC_{50} value for cells treated with arsenite + selenide (138 ± 76 μM) than arsenite + selenite (50 ± 6 μM). Cytotoxicity assays of arsenite + selenite and arsenite + selenide at different treatment concentration ratios revealed higher overall mutual antagonism of arsenite + selenide toxicity than arsenite + selenite. In comparison to ^{75}Se-selenite, HepG2 cells in suspension were at least 3-fold more efficient at accumulating selenium from reduced ^{75}Se-selenide, and its accumulation was further increased by arsenite. These results were corroborated by X-ray fluorescence imaging of HepG2 cells which also showed increased arsenic accumulation in the presence of selenide. These results from studies in SCHH and HepG2 cells are consistent with a greater intracellular availability of selenide relative to selenite for protection against arsenite, and the formation and retention of a less toxic product, likely $[(GS)_2AsSe]^-$.

Lastly, although arsenic-induced toxicities are known to display inter-individual variability, selenium supplementation trials are underway in arsenic-endemic regions in efforts to find cost-effective therapeutic solutions for arsenic-induced toxicities. As such, the focus of this thesis work was also to characterize single nucleotide polymorphic variants of MRP2 in order to gain a comprehensive understanding of the protective role of $[(GS)_2AsSe]^-$ not only through its sequestration in RBCs, but also its efflux from hepatocytes. This work provides support in understanding the the mechanistic aspects of arsenic and selenium cellular handling in humans.

Preface

Chapter 2 of this thesis chapter has been submitted for publication as Gurnit Kaur, Warda Javed, Olena Ponomarenko, Diane P. Swanlund, Kamran Shekh, Kelly L. Summers, Angela Casini, Margot Wenzel, Joseph R. Casey, Emmanuelle Cordat, Ingrid J. Pickering, Graham. N. George, and Elaine M. Leslie., Human red blood cell uptake and sequestration of arsenite and selenite: evidence of seleno-bis(*S*-glutathionyl) arsinium ion formation in human cells. Biochemical Pharmacology (2020). Gurnit Kaur and Warda Javed are co-first authors on the manuscript. This research project was a collaboration led by Dr. Elaine Leslie, from the University of Alberta and the research groups of Dr. Graham George and Dr. Ingrid Pickering, from the University of Saskatchewan. Gurnit Kaur initiated all ^{73}As experiments (with technical replicates completed by Diane Swanlund), and was responsible for x-ray absorption spectroscopy (XAS) sample preparation and manuscript writing. Warda Javed completed all ^{75}Se experiments, with support from Diane Swanlund and Kamran Shekh. XAS analysis was performed by Olena Ponomarenko and Kelly Summers. Angela Casini (with assistance from Margot Wenzel) provided AQP3 inhibitors. Joseph Casey and Emmanuelle Cordat provided scientific insight, critical to experiment design. This research project received ethics approval from the University of Alberta Research Ethics Board, Project Name "Metabolism and Transport of Arsenic and Selenium by Human Red Blood Cells", protocol number 5614, January 27, 2010.

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Dedication

For my wonderful family.

And for those many families living in arsenic-endemic regions,
awaiting solutions.

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Lastly, thank you to the donors that have provided liver and blood samples, for your trust in research. Albeit a drop in a vast ocean, I hope that this work adds another piece to the giant puzzle of the arsenic-modulated interactome.

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List of Abbreviations

AE1	anion-exchanger 1
AMP	adenosine monophosphate
AQP3	aquaglyceroporin 3
AQP9	aquaglyceroporin 9
As	arsenic
As^{V}	arsenate
As^{III}	arsenite
$As(GS)_3$	arsenic triglutathione
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
Auphen	[Au (1,10-phenanthroline)Cl_2]Cl
BCA	bicinchoninic acid
BEI	biliary excretion index
BSA	bovine serum albumin
BSO	buthionine sulfoximine
CDF	5(6)-carboxy-2',7'-dichlorofluorescein
DJS	Dubin Johnson syndrome
DMA^{V}	dimethylarsinic acid
DMA^{III}	dimethylarsinous acid
DNDS	4,4'-dinitrosostilbene-2,2'-disulfonate
GLUT1	glucose transporter 1

$[(GS)_2AsSe]^-$	seleno-bis(*S*-glutathionyl) arsinium ion
GSH	glutathione
HA	hemagglutinin
HBSS	Hanks' balanced salt solution
H_2DIDS	4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSA	Highest Single Agent
HSe^-	hydrogen selenide
LDH	lactate dehydrogenase
MSD	membrane spanning domain
MeSeCys	methylselenocysteine
mAb	monoclonal antibody
MMA^V	monomethylarsonic acid
MMA^{III}	monomethylarsonous acid
$MMA(GS)_2$	monomethylarsenic diglutathione
$MMMTA^V$	monomethylmonothioarsonic acid
MOPS	3-(N-morpholino) propanesulfonic acid
MRPs	multidrug resistance proteins
MRP2	multidrug resistance protein 2
NaB	sodium butyrate
NBD	nucleotide binding domains
PBS	phosphate buffered saline
pAb	polyclonal antibody
PVDF	polyvinylidene difluoride

RIPA	radioimmunoprecipitation assay
RBCs	red blood cells
ROI	regions of interest
ROS	reactive oxygen species
SCHH	sandwich cultured human hepatocytes
Se	selenium
Se^{IV}	selenite
SeMet	selenomethionine
Si_3N_4	silicon nitride
SNVs	single nucleotide polymorphic variants
TM	transmembrane
XAS	X-ray absorption spectroscopy
XFI	X-ray fluorescence imaging

1. General Introduction

1.1. Arsenic Background/History

1.1.1 Uses of Arsenic

Throughout history, arsenic has an infamous reputation for being the poison of kings and the king of poisons [1,2]. However, arsenic is naturally present in the environment and has become ubiquitous to the human experience also. We are exposed to arsenic in our soil, in our water and in our medicines [3].

1.1.2. Exposure Scenarios

1.1.2.1. Therapeutic

As early as the year 2000 BC, arsenic has been used therapeutically [4]. Hippocrates, Aristotle and Paracelsus have all been known to use arsenic in medicinal treatments. Fowler's solution, a solution of 1% potassium arsenite was used in the treatment of syphilis, psoriasis, asthma, and malaria (Figure 1-1) [5]. The first arsenic-containing commercial drug to be licensed was by the German company called Hoescht, under the brand name of Arsphenamine/Salvarsan to treat syphilis (Figure 1-1) [6,7]. It was the most widely prescribed drug at one point and very effective, but was taken off the market when penicillin came out in the 1940s [7].

Since the early 2000s, arsenic trioxide (ATO) has been used to treat acute promyelocytic leukemia (APL) (Figure 1-1) [8]. The pathogenesis of APL is characterized by the fusion of the promyelocytic leukemia/retinoic acid receptor alpha (PML/RARα) genes, leading to inhibition of myeloid cell development and thus, increased levels of abnormal promyelocytes [9,10]. Due to the high affinity of arsenite for thiol groups of proteins, ATO targets the ring finger of the PML protein, resulting in the degradation of the PML/RARα fusion protein [11]. When used in combination with all-trans retinoic acid (ATRA), the remission rate is greater than 90% [12]. Common side effects with ATO use include

hyperleukocytosis and cardiac arrythmia, with interindividual variability due to differences in arsenic accumulation, metabolism and efflux [13].

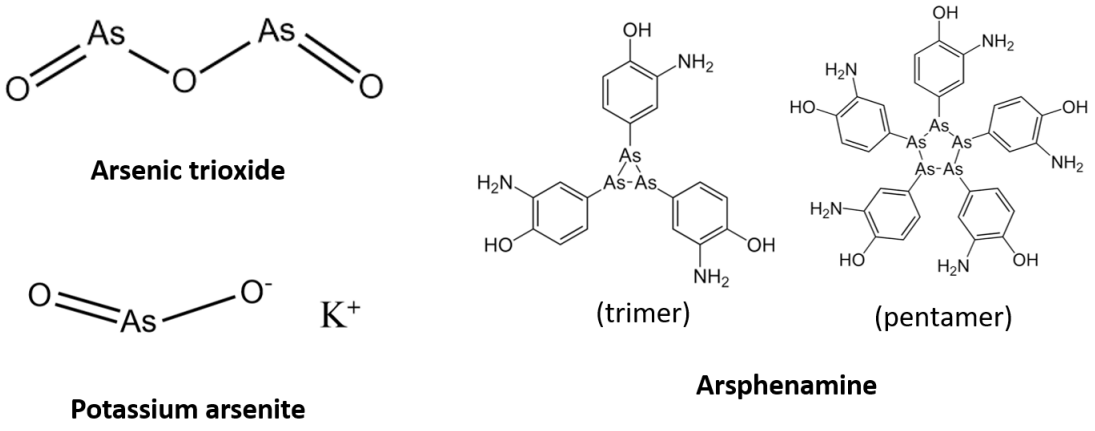

Figure 1-1. Chemical structures of arsenic compounds used in therapeutics.

1.1.2.2. Diet and Water

Millions of people worldwide are exposed to unacceptable levels of arsenic in their drinking water [14]. Arsenic is naturally found in the environment and can often leach into the groundwater supply through the weathering of rocks or when the arsenic deposits are disturbed. The presence of high levels of arsenic in aquifers is a public health problem in many countries [15]. Contaminated drinking water has been reported in North America, Latin America, Taiwan, China (Inner Mongolia and Xinjiang), India (West Bengal), Bangladesh, affecting the health of more than 200 million people worldwide [3,16–20].

Rice and cereal grown using contaminated groundwater can also be a source of arsenic contamination [21]. From all food types, rice contains the highest levels of inorganic arsenic contamination [21,22]. This is likely due to the paddy farming of rice, which requires rice to be grown in flooded water bodies. Arsenic is highly soluble in water, when rice is grown in contaminated irrigation water, arsenic is easily

taken up by the root and accumulated in the grains [22]. In the United States, arsenic levels in rice and rice-based products can range from 58 to 160 μg/L [23].

Arsenic containing pesticides were used on fruit orchards and other crops until the 1950s [24]. Although these pesticides have gradually been phased out, arsenic residue remains in soil and is assumed to be the reason for arsenic content in fruits [24,25].

1.1.2.3. Occupational

In the 1800s, arsenic was used as a pigment in a number of products, including toys, candles, fabric and wallpaper [26]. It has also been used as a pesticide, wood preservative, semi-conductor, in addition to being used in glass production and copper smelting [26]. Investigations into urinary arsenic levels from workers from industries with possible occupational arsenic exposure revealed 11-fold, 18-fold, and 56-fold higher mean urinary arsenic concentrations than the control group, for workers from the timber treatment industry (handling arsenic wood preservatives), glassworkers (handling arsenic trioxide) and chemical workers (handling inorganic arsenic) respectively [27].

1.1.3. Symptoms of Arsenic Exposure

The spectrum of symptoms in response to acute exposures include gastro-intestinal symptoms and liver toxicity [28,29]. High levels of arsenic ingestion can affect membrane integrity and lead to increased permeability of blood vessels, as well as, inflammation and necrosis of the stomach and intestinal mucosa, affecting the integrity of the gut wall [15]. These can result in watery diarrhoea containing blood [15].

Chronic exposures to arsenic affect more than 200 million people worldwide [14]. There is increasing evidence to suggest the effects of arsenic exposure are systemic, as opposed to being localized to specific organs [15]. Long-term arsenic effects include cardiovascular, neurological, renal, dermal effects, with the most serious consequence being the development of malignancies [15,20,30–32]. The

clinical presentation of arsenic-induced symptoms varies among populations and individuals, with studies showing that a small percentage of the exposed population develop arsenic-induced skin lesions [33–35].

1.1.4. Regulations on Arsenic Use

The U.S. Agency for Toxic Substances and Disease Registry (ATSDR) classified arsenic as No. 1 on their Substance Priority List in 2017, based on its toxicity and potential for human exposure [36].

The International Agency for Research on Cancer (IARC) classifies arsenic in Group 1, meaning it is a proven human carcinogen [37]. The U.S. Environmental Protection Agency (EPA) also classifies arsenic as a confirmed human carcinogen [38].

The World Health Organization (WHO) has set a maximum acceptable limit of 10 μg/L on arsenic in drinking water [39]. The same standard is followed in Canada, where municipal water is well below this regulated value [40]. However, approximately 30% of Canadians rely on ground water for their daily use [41]. If ground water is from a private well there is no requirement for arsenic contamination testing, and more importantly, even when they are, residents may not have the knowledge or the means to implement the correct interventions [42].

Regulation standards on arsenic consumption have been based primarily on arsenic contamination of drinking water. While this is the predominant route of exposure, exposures from food products such as rice, rice products and fruit juices can also amount to significant values [22]. As such, the current regulations do not factor in the complete scope of arsenic exposure.

In addition, the current guideline of maximum arsenic levels in drinking water being at 10 μg/L is based on a cost-benefit analysis. Based on arsenic exposure and hazard, it has been suggested that the regulatory standards ought to be as close to zero as possible, or at least at 3 μg/L for a 1 in 10,000 estimate of cancer risk [25].

1.2. Arsenic Mechanism of Toxicity

1.2.1. Mechanism of Carcinogenesis

Despite the fact that arsenic has been established by IARC as a Group I, proven human carcinogen [37], the mechanism of arsenic-induced carcinogenicity remains inadequately understood. This is partly due to the lack of animal models of arsenic-induced carcinogenesis [43]. Moreover, the biotransformation process of arsenic, which yields multiple arsenic metabolites, with varying degrees of toxicities. Arsenic metabolism and speciation play a central role in arsenic-induced carcinogenesis. This multi-faceted pathway includes a network of genotoxic and epigenetic mechanisms that are discussed below, each of which are not independent of the other.

1.2.1.1. Arsenic-Induced Oxidative Stress

Arsenic has high affinity for cellular thiols; it can bind to free sulfhydryl groups of proteins and enzymes leading to macromolecule oxidation and the inactivation of many enzymes [44]. In this manner, arsenic can also compromise the mitochondrial membrane integrity, leading to the release of ROS from the mitochondria to the cytoplasm [45]. In addition, arsenic biotransformation consumes the cell's antioxidant reserves by depleting glutathione in phase II metabolism through the formation of arsenic-glutathione conjugates [46,47].

Excessive ROS formation, and depletion of the cell's antioxidant capacity, lead to oxidative damage [48]. The effects of arsenic-induced ROS formation are seen in the form of DNA damage, lipid and protein damage and effects on cell signalling pathways; all of which are discussed below [48].

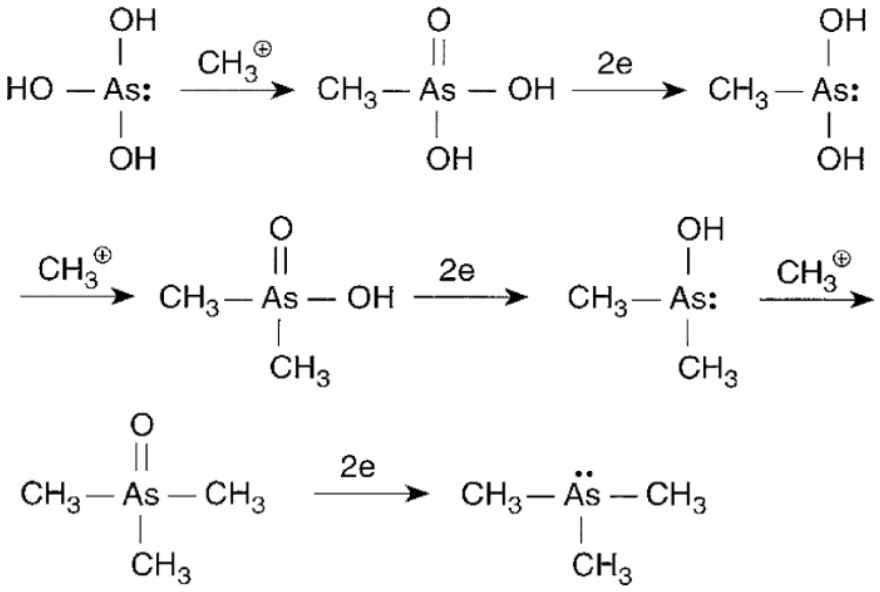

Figure 1-2. Challenger pathway for arsenic metabolism [49,50]. Taken from [49], with permission.

1.2.1.2. Genotoxic Potential of Arsenic

Although arsenic is a well-established carcinogen, it does not act as a direct mutagen. Its genotoxic potential is largely a product of increased oxidative stress and inhibition of DNA repair pathways [51]. As such, arsenic has the capacity to induce micronuclei, DNA strand breaks, sister chromatid exchanges, chromosomal aberrations and aneuploidy [51] which can then lead to genetic mutations .

Arsenic biotransformation involves a series of reduction followed by oxidative methylation steps, leading to trivalent and pentavalent methylated species of arsenic [49]. Trivalent arsenic species are orders of magnitude more toxic than the pentavalent forms. Methylated trivalents, in turn, are even more toxic than inorganic arsenite (As^{III}) [52]. When testing arsenate (As^{V}), As^{III}, monomethylarsonic acid (MMA^{V}), monomethylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^{V}), and dimethylarsinous acid (DMA^{III}) for the effect on electrophoretic migration of the plasmid, øX174 RF I DNA, Mass et al. found that MMA^{III} and DMA^{III} were the only species that had altered the

conformation of øX174 RF I DNA [53]. DNA nicking was seen at concentrations of 0.15 mM for DMA^{III} and 30 mM for MMA^{III}, while complete DNA degradation was seen at higher concentrations (10 mM for DMA^{III}, 60 mM for MMA^{III}) [53]. This provides evidence for the direct genotoxic potential of DMA^{III} and MMA^{III} at high concentrations, however As^{III} and As^{V} had no effect even at higher concentrations.

Epidemiological data also provides insight into genotoxic mechanisms of arsenic, as well as, possible biomarkers that can be used to evaluate genotoxicity in biomonitoring studies. In a West Bengal study, with 163 exposed individuals and 154 unexposed individuals, increased micronuclei prevalence was found in lymphocytes, oral mucosa cells and urothelial cells of the exposed group [54]. Similar results have been observed in individuals from Chilean populations [55]. Other studies coming from the West Bengal population, have also shown an increase in sister chromatid exchanges and chromosomal aberrations [56].

1.2.1.3. Inhibition of DNA Repair

Arsenic down regulates the expression of the DNA repair enzyme, DNA polymerase β [51]. In addition, the expression and activity of genes involved in the nucleotide excision repair and base excision repair pathways are also altered [57]. These can lead to disruption of protein coding and non-coding RNA genes, thus affecting apoptosis [57].

1.2.1.4. Inhibition of Apoptosis and Effect on Cell Signalling Pathways

Apoptosis is an active and gene directed mechanism of programmed cell death [58]. The generation of ROS, reduces the mitochondrial membrane potential, leading to cytochrome C release and the activation of caspases which result in apoptosis [59].

Increased levels of the transcription factors, activation protein 1 (AP-1) and nuclear factor κB (NF-κB) lead to upregulation of stress proteins and mitogen-activated protein kinase (MAPK) pathways, which regulate heat shock protein synthesis, cell transformation and apoptosis [31,57,60]. It has been shown that DMA^{III} and MMA^{III} are more potent than As^{III} in the activation of AP-1 [60].

1.2.1.5. Epigenetic Modulation

Epigenetic modulation refers to changes in transcription that can alter gene expression without any effect on the DNA sequence [61]. These changes can be due to diet and environmental factors. Examples of epigenetic modulation include DNA methylation, histone modifications and small noncoding microRNAs [62–65].

1.2.1.5.1. Changes in DNA Methylation

Arsenic metabolism occurs through a series of reduction followed by oxidative methylation steps (Fig 1-1) [49,50]. During methylation, S-adenosyl methionine (SAM) acts as the methyl group donor, and the reaction is catalysed by arsenite methyltransferase (AS3MT) [51,66]. SAM is required for arsenic biotransformation, but it is also a methyl donor during DNA methylation [67]. The rapid depletion of SAM results in global hypomethylation, which is often seen in cancer [28,61,68]. SAM synthesis requires homo-cysteine, folate, vitamin B12 and other vitamins and cofactors [51]. Dietary deficiencies in these can compromise SAM synthesis, offering an explanation for why arsenic induced toxicities are correlated with nutritional deficiencies [69]. However, arsenic induced global hypomethylation occurs irrespective of nutritional status. Hypomethylation can result in promoter activation, leading to aberrant gene expression including increased expression of oncogenes [68,70]. Arsenic-induced changes in DNA hypermethylation can also result in carcinogenesis. It has been shown that exposure to inorganic arsenic results in increased levels of cytosine methyltransferase in cells, while upregulation

of DNA methyltransferase 3a was observed in mice [71]. Arsenic-induced hypermethylation of promoter regions which affect conserved CpG islands of tumour suppressor genes can lead to their inhibition [68]. It is likely that early exposure to arsenic leads to DNA hypermethylation, however, with increasing arsenic exposures leading to increased methylation and depletion of SAM, arsenic-induced hypomethylation results [71]. The specific genes targeted by the altered methylation profiles likely hold the key to understanding arsenic-induced carcinogenesis.

1.2.1.5.2. Histone Modifications

Histone modifications in the form of methylation, acetylation, phosphorylation, ubiquitination, sumoylation, proline isomerization or ADP-ribosylation, can restructure the packaging of chromatin, leading to increased or decreased transcriptional activity [61,66,68]. An ex vivo study of 63 steel workers examined the effect of metal and metalloid-rich particulate matter, including arsenic, on histone modifications [72]. The results showed a positive association between histone 3 lysine demethylation and histone 3 lysine deacetylation in blood leukocytes and arsenic exposure [72]. *In vitro* studies using T-lymphocytes showed modifications in the histone deacetylase 2 (HDAC2) and histone 3 (acetyl K9) [73].

1.2.1.5.3. Small Noncoding MicroRNAs

Until recently, the regulatory function of non-coding RNAs had been overlooked. MicroRNAs (miRNAs) are a class of short non-coding RNAs, ranging from 19-25 nucleotides in length, that are involved in gene silencing [48]. They achieve this through the inhibition of mRNA translation or by inducing mRNA degradation [48]. *In vitro* studies have shown that arsenic can upregulate the expression of the miRNAs, miR-190 and miR-21 [74]. In turn, miR-190 can suppress the translation

of the pH domain and leucine rich repeat phosphatases (PHLPP) protein and miR-21 can suppress the translation of phosphatase and tensin homolog (PTEN), both of which are negative regulators of protein kinase B (PKB) [74]. The activation of PKB mediates cell survival, growth, proliferation, cell migration and angiogenesis [75]. Overactivation of PKB is common in cancer cells and likely plays a role in arsenic-induced carcinogenesis [75–77].

1.2.2. Mechanism of Other Toxicities

The immunological response to arsenic exposure depends on the dose and length of exposures. Arsenic can lead to both suppression and activation of the immune system [78]. Immunosuppression leads to increased susceptibility to infectious diseases, while immunological activation can lead to hypersensitivity disorder [78]. In infants, arsenic can impair thymic development, in addition to increased morbidity which may be due to immunosuppression upon arsenic exposure [78].

In addition to immunological effects, arsenic can also interfere with glucose homeostasis. Epidemiological data shows that chronic arsenic exposure is correlated with the incidence of diabetes in Bangladesh, Mexico and Taiwan [79–81]. This is likely linked to effects of arsenite on insulin signalling cascades [82,83]. It has been shown in murine pancreatic islet cells that glucose-stimulated insulin secretion is inhibited by methylated trivalent arsenic species [84]. Arsenite has also been shown to lead to the downregulation of glucose transporters and impaired functioning of enzymes involved in glucose metabolism, such as pyruvate dehydrogenase [85–87]. Occupational exposures to arsenic in copper smelters and glass industries are also correlated with arsenic-induced diabetes [88–90].

Epidemiological data has also established a correlation between arsenic exposure and the risk of atherosclerosis [91–93]. The apolipoprotein E (ApoE) protein removes plasma lipoproteins from blood [94]. In ApoE deficient mice, arsenic exposure in drinking water for 13 weeks led to the increases in plaque size in the aortic sinus, from as low as 10 μg/L arsenic [95]. Possible molecular

mechanism for arsenic-induced atherosclerosis is arsenic-induced oxidative stress and its binding to sulfhydryl groups of proteins, which in turn can lead to endothelial dysfunction causing inflammation, platelet aggregation and loss of vasodilation, leading to the development of arsenic-induced atherosclerosis [91,96]. Consistent with methylated trivalent arsenicals being more toxic than pentavalent species, it has also been shown in ApoE deficient and AS3MT double knockout mice, that AS3MT is required for arsenic-enhanced atherosclerosis [32].

1.2.3. Differences in Susceptibility to Arsenic-Induced Toxicities

Differences in diet and nutrition can lead to differences in arsenic induced toxicities. As discussed above, folate is necessary for SAM which is required for arsenic methylation. Lifestyle factors such as smoking can also lead to increased risk of arsenic induced carcinogenesis [31,97]. Genetic components also likely play a significant role. Polymorphisms in various proteins involved in arsenic metabolism and transport could potentially lead to differences in the toxicity and body burden of arsenic:

1.2.3.1. Polymorphisms in Arsenic Metabolism

The major genetic players of arsenic metabolism and detoxification that have been associated with interindividual variability are AS3MT, proteins from the glutathione *S*-transferase (GST) family, and DNA repair proteins [98]. GSTs are important phase II metabolism enzymes, catalyzing the conjugation of substrates with glutathione. Purine nucleoside phosphorylase (PNP) has been shown to reduce arsenate in cells. Polymorphisms in *GSTO1, GSTM1, GSTP1, GSTT1* and *PNP* have been associated with altered arsenic urinary excretion profiles [99–103].

AS3MT was first isolated from rat liver cytosol and determined to be the enzyme responsible for the majority of arsenic methylation [104]. Arsenic biotransformation can lead to the presence of more toxic intermediate metabolites, as such, AS3MT plays an integral role in arsenic metabolism and toxicity. Studies of the influence of *AS3MT* polymorphisms on arsenic metabolism have relied on

urinary arsenic excretion profiles as indicators of methylation status. Engstrom et al., also investigated the urinary arsenic profiles in indigenous women in Argentina exposed to high levels of arsenic in drinking water; polymorphisms in *AS3MT, GSTT1* and *GSTM1* were shown to alter the urinary elimination profile of arsenic [105]. Antonelli et al., screened 360 publications with a focus on *AS3MT, GSTO1* and *PNP* polymorphisms in arsenic metabolism [100]. While they concluded that there was insufficient data on the role of PNP in arsenic metabolism, and *GSTO* polymorphisms did not exhibit statistically significant associations with arsenic methylation profiles, three polymorphisms in *AS3MT* were identified that altered the urinary MMA percentage (rs3740390, rs11191439, and rs11191453) [100]. Ratio of DMA:MMA in urine reflects As metabolism efficiency; a high MMA:DMA ratio is associated with increased toxicity [106].

1.2.3.2. Polymorphisms in Arsenic Efflux

In addition to metabolism, efflux transporters also play a role in the modulation of arsenic toxicity. Efflux of arsenic metabolites has been shown to be mediated by the ATP-binding cassette (ABC) transporters multidrug resistance protein 1 (MRP1, gene *ABCC1*), MRP2 (*ABCC2*) and MRP4 (*ABCC4*) [46,107,108]. MRP4 is localized to the basolateral surface of hepatocytes in the liver and to the apical surface of the proximal tubule cells in the kidney, ideally positioned for urinary elimination of hepatic metabolites. Banerjee et al., characterized the localization and transport function of 8 single nucleotide polymorphisms (SNPs) of *ABCC4* [109]. Two SNP variants (MRP4-V776I and MRP4-C956S) did not correctly localize to the plasma membrane, while one variant (MRP4-K304N) reduced transport activity for both arsenic substrates (monomethylarsenic diglutathione, $MMA(GS)_2$ and dimethylarsinic acid, DMA^V) of MRP4 [109]. Individuals carrying these SNPs could have reduced efflux ability of arsenic species, leading to their increased body burden and toxicity.

1.3. Arsenic Toxicokinetics

1.3.1. Arsenic Absorption

As discussed, the primary route of exposure to arsenic is through diet and drinking water. The predominant species of arsenic that the intestinal epithelium first encounters are As^{III} and As^{V}. At physiological pH, As^{III} exists in solution as the neutral molecule, H_3AsO_3 with a pKa of 9.2 [110]. Arsenate exists predominantly as $H_2AsO_4^-$ with pKa values of 2.3, 6.8 and 11.3 [110].

1.3.1.1. Bioavailability of Arsenic

Bioavailability of soluble forms of arsenic is rather high, reaching 90% when it is ingested in drinking water [111]. Arsenic bioavailability is lower in a more complex matrix, such as soil [112], and in the presence of insoluble forms of arsenicals, such as arsenic triselenide [111]. Other factors that can influence arsenic bioavailability include pH, diet composition and gut microbiota.

1.3.1.2. Effect of the Gut Microbiome

The gut microbiome can be thought of as the "gate keeper" to the traditional absorption, distribution, metabolism and excretion (ADME) model [113,114]. The interplay between xenobiotics and the gut microbiome can result in pre-systemic metabolism of xenobiotics, and potential toxicity of the microbiota; leading to changes in absorption dynamics [69]. A 2010 study from Van de Wiele et al., 2010, showed that the human colon microbes, supplemented with a methyl donor, could metabolize As^{III} and As^{V} into monomethylated and thiolated forms [115]. Thiolated forms of arsenic are those where the oxygen atom bound to arsenic is replaced by a sulfur [116]. The influence of the gut microbiome on arsenic absorption kinetics is likely through transformation of arsenic by microbes, as well as, the likelihood of bacteria to sequester arsenic [117].

1.3.1.3. Arsenic Uptake Transporters

Since As^{III} exists as a small, neutral molecule at physiological pH, it can substitute for other small, neutral molecules such as glycerol and water, during uptake. Aquaglyceroporins (AQP3, AQP7 and possibly, AQP10) are involved in As^{III} uptake in enterocytes [118–121]. Proteins from the solute carrier group (SLC family) of membrane proteins have been implicated in arsenic uptake. Rat and human glucose transporters 1 (GLUT1), when expressed in *Xenopus laevis* oocytes have been shown to increase As^{III} and MMA^{III} uptake [122]. However, GLUT1 is not localized to enterocytes, while GLUT5 is. There is some evidence for GLUT5 involvement in As^{III} uptake; in Caco-2 cells, knockdown of GLUT5 decreased As^{III} accumulation, however, corroboration with heterologous expression studies is necessary [120]. As^{V} is very similar chemically and structurally to phosphate, explaining its cellular uptake through the sodium/phosphate co-transporter type IIb protein (*SLC34A2*) [123]. The organic anion transporting polypeptides family also has members that are potentially involved in the uptake of As^{III} (OATP1B1 & OATP2B1) [120,121]. As discussed above, pre-systemic metabolism by the gut microbiota can result in methylated species of As. In addition to transcellular uptake of arsenicals, arsenite, arsenate and methylated arsenic species can cross the intestinal epithelium through paracellular transport reviewed in [121].

1.3.2. Cellular Metabolism of Arsenic

Arsenic biotransformation occurs predominantly in the liver through a series of oxidative methylation, followed by, reduction steps, resulting in monomethylated and dimethylated trivalent and pentavalent species (Figure 1-2) [50]. Electrons for the reduction of arsenic are likely provided by thioredoxin or glutaredoxin, where two thiol groups are oxidized to a disulfide [49]. Arsenic methylation is catalyzed by AS3MT, with SAM as the methyl donor in the reaction [124]. Methylation of arsenic can lead to the formation of more toxic intermediates; methylated trivalent arsenicals are more reactive and toxic than methylated pentavalent and inorganic arsenicals[116].

High affinity of trivalent arsenicals for sulphur leads to thiolation (protein bound arsenic through a thiol group) [47,125]. The presence of thiolated arsenicals have been identified *in vivo*; wild-type and AS3MT knockout mice were administered a single oral dose of 0.5 mg/kg body weight arsenic and urine was analyzed for arsenic speciation. Urinary analysis of wild-type mice indicated the presence of methylated arsenicals and the thiolated arsenic species, monomethylmonothioarsonic acid ($MMMTA^V$) and dimethylmonothioarsinic acid ($DMMTA^V$), while the AS3MT knockout mice only showed the presence of inorganic arsenic species in urine [126]. Urinary analysis of populations of Bangladeshi women chronically exposed to arsenic, and Japanese men (on a diet high in arsenosugars), also revealed the presence of $DMMTA^V$ [127,128].

Trivalent arsenic species can also undergo glutathionylation to form arsenic-glutathione conjugates, which are transported out of cells by members of the ATP-binding cassette transporter (ABC) subfamily C [46,107,108,129,130].

1.3.3. Cellular Efflux of Arsenic by the ATP-Binding Cassette Transporter Family

The ABC transporter family, subfamily C; are transmembrane proteins that play a role in the transport of physiological, pharmacological, and toxicological compounds, as well as drug resistance. MRP1/*ABCC1*, MRP2/*ABCC2* and MRP4/A*BCC4* are established transporters of arsenic metabolites [46,107,108,131,132].

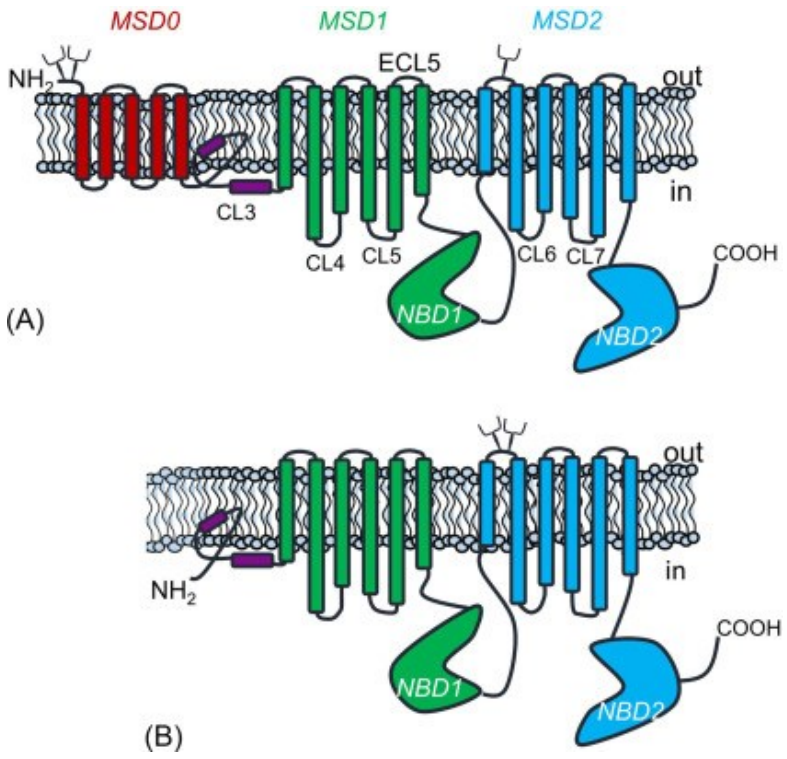

Figure 1-3. General predicted topology of the ABCC family "long" (A) and "short" (B) members. CL, cytoplasmic loop; ECL, extracellular loop; MSD, membrane spanning domain; NBD, nucleotide binding domain. Taken from [133], with permission.

ABCC transport proteins are localized to the plasma membrane and are involved in the primary active transport of their substrates. Transport can occur against a chemical gradient and requires ATP hydrolysis as the driving force [134,135]. ABCC transport proteins are unidirectional and carry several structural and sequence similarities [136]. Typical ABCC proteins have a minimum of four functional domains; two cytosolic nucleotide binding domains (NBDs) and two polytopic membrane spanning domains (MSDs) (Figure 1-3) [133,136–138]. Several ABCC family members, such as MRP1 and MRP2 have an additional amino-terminal MSD (Figure 1-3) (MSD0) [133,139]. There are highly conserved regions in the NBDs that are common among all ATP-binding proteins; these are referred to as the Walker A and Walker B sequence motifs. The Walker A motif, which is characterized by the sequence: GXXGXGK(S/T), where X can be any amino acid [140,141]. The Walker B motif is

characterized by the sequence: φφφφD, where φ can be any hydrophobic amino acid [141]. The conserved aspartic acid residue in this sequence chelates the magnesium ion that is bound to the nucleotide [142]. The aspartate residue is often followed by a glutamate residue, which facilitates the nucleophilic attack of ATP by water [142]. All ABC proteins have a highly conserved signature motif (LSGGQ), located between Walker A and Walker B, known as the C-motif [143]. The glycine residues in this motif have been shown to be involved in nucleotide binding [143,144]. Crystal structures show that the NBDs of the ABC transport proteins dimerize to allow for the binding of two ATP molecules at the interface of the Walker A motif of one NBD and the C-motif of the other NBD [145]. Two transmembrane domains can then alternate between an inward facing conformation and an outward facing conformation. This allows for substrate binding from the cytosol and release into extracellular space [134,144–146].

1.3.3.1. The Multidrug Resistance Protein 2

MRP2 is localized to the apical surface of polarized cells. Apical localization in enterocytes allows MRP2 to transport substrates into the intestinal lumen, limiting the bioavailability of xenobiotics and/or their metabolites [147]. The liver and the kidney are the major organs responsible for the elimination of xenobiotics and their metabolites. In hepatocytes and in proximal tubule cells, MRP2 is strategically positioned to play a crucial role in the biliary excretion and urinary elimination of both endogenous and exogenous compounds [148,149]. It is the only ABC subfamily C member to be found on the apical hepatocyte surface, transporting its substrates from the hepatocyte into bile. Arsenite has been shown to upregulate MRP2 and MRP4 levels in human hepatocytes [150]. *In vivo* studies have shown majority of the arsenic in bile to be present as $As(GS)_3$ and $MMA(GS)_2$ (Figure 1-4) [129]. Studies with Mrp2-deficient Wistar rats have shown all arsenic transport into bile to be Mrp2-

dependent [129]. Direct transport studies have shown $As(GS)_3$ and $[(GS)_2AsSe]^-$ transport to be MRP2-mediated (Figure 1-4) [108].

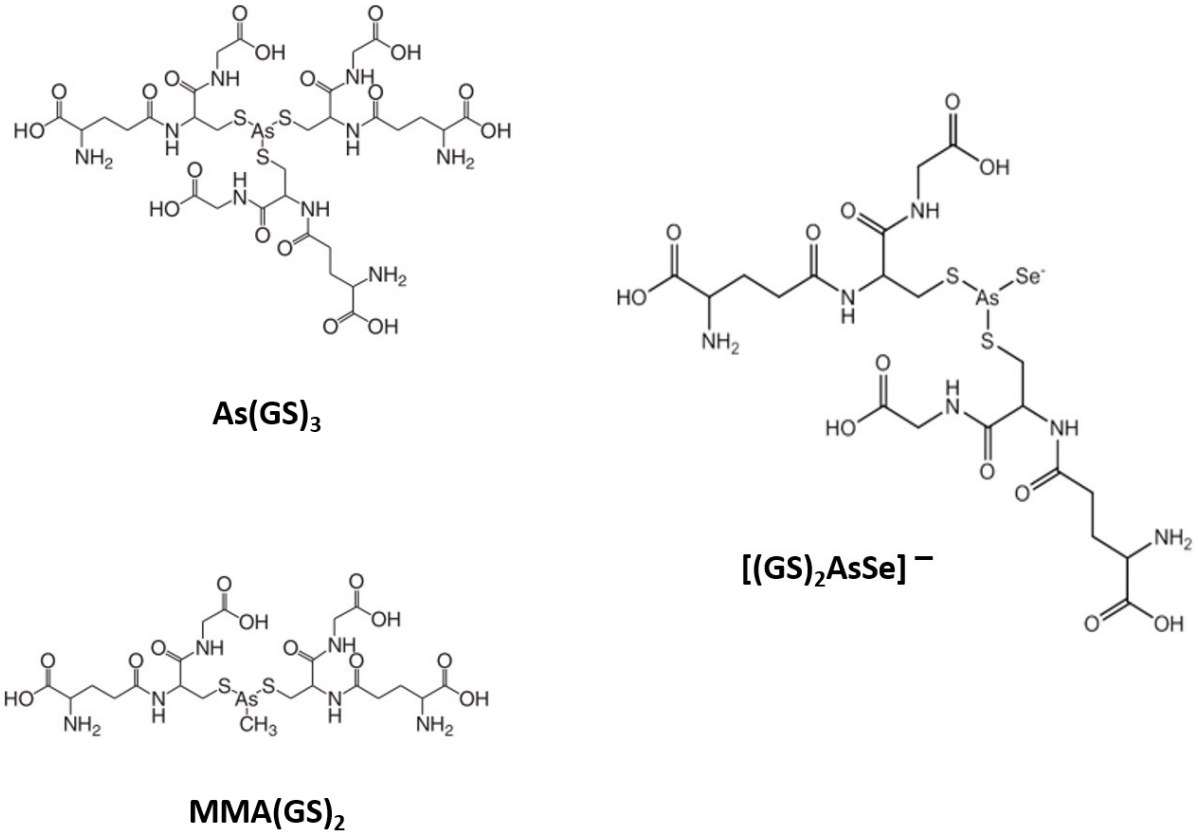

Figure 1-4. Chemical structures of arsenic conjugates found in bile. $As(GS)_3$, arsenic triglutathione; $MMA(GS)_2$, monomethylarsenic diglutathione; $[(GS)_2AsSe]^-$, seleno-bis(*S*-glutathionyl) arsinium ion.

1.3.3.1.2. SNPs (& Dubin Johnson Syndrome)

There are more than 200 known naturally occurring MRP2 variants [149]. A large number of these are silent mutations that do not result in amino acid changes and are hence likely to have no functional consequences. Variants that result in the loss of function or absence of MRP2 carry deletions, nonsense mutations, splice variants, premature stop codons or frameshift mutations [149,151,152]. These MRP2 variants that alter MRP2 function cause the Dubin-Johnson syndrome (DJS), which is an autosomal, recessively inherited disorder which is characterized by conjugated hyperbilirubinemia,

caused by the impaired efflux of bilirubin glucuronide [153–158]. DJS is rare, with prevalence in the Japanese population of 1 in 300,000 and in the Iranian Jewish population of 1 in 1,300. [149,157].

1.3.4. Arsenic Excretion

As mentioned above, MRP2, the only MRP located on the biliary surface of the hepatocyte, can transport arsenic-glutathione complexes into bile [108,129]. However, these complexes are not stable at the alkaline pH of bile and dissociate, thereby undergo enterohepatic circulation through the intestine and back to the liver, where they can be transported across the basolateral surface of the hepatocyte into sinusoidal blood for elimination via the urine [147]. In concordance with this, the majority of arsenic (60-80%) is eliminated via the urine [159]. The speciation profile of arsenic in human urine is 10-30% as inorganic As, 10-20% as monomethylated species of arsenic and the remaining 60-80% as dimethylated species [159–161]. A high MMA:iAs and high MMA:DMA ratio has been associated with increased prevalence of arsenic-induced cancers in human populations [162]. The presence of thiolated arsenicals has also been detected in human urine [163]. In a Mrp2-deficient rat model, Bu et al., found very low levels of thiolated arsenicals in urine, whereas, wild-type rats showed high levels of thiolated arsenical in urine, likely due to transformations in the gut during entero-hepatic recirculation [164].

1.4. Arsenic-Selenium Interactions

1.4.1. Selenium Background

Selenium was first identified as an environmental toxin from observation studies in animals grazing on selenium rich soil. It was later found to be an essential nutrient with a narrow therapeutic window [165]. Selenium imbalance in the environment is a problem in many parts of the world [166]. Selenium toxicity is known as selenosis, characterized by brittle nails, garlic breath and hair loss [167]. Selenium deficiency is known as the Keshan Disease, characterized by juvenile cardiomyopathy and as the

Kashin-Beck Disease, characterized by osteoarthropathy [168]. It is also associated with immunosuppression, cancer and male infertility [169]. Selenium has similar physicochemical properties to sulphur and can replace sulphur in amino acids to form the selenoaminoacids, selenomethionine, selenocysteine and selenocystathionine [170]. To date, there are 25 known selenoproteins involved in antioxidant action, maintenance of immunity and regulation of thyroid hormones [168].

1.4.2. Selenium Toxicokinetics

1.4.2.1. Selenium Absorption

Selenium bioavailability is dependent upon the species of selenium that are present in the diet. Although organic forms of selenium are more bioavailable than the inorganic forms, general absorption of all forms of selenium is relatively high at 70-95% [170].

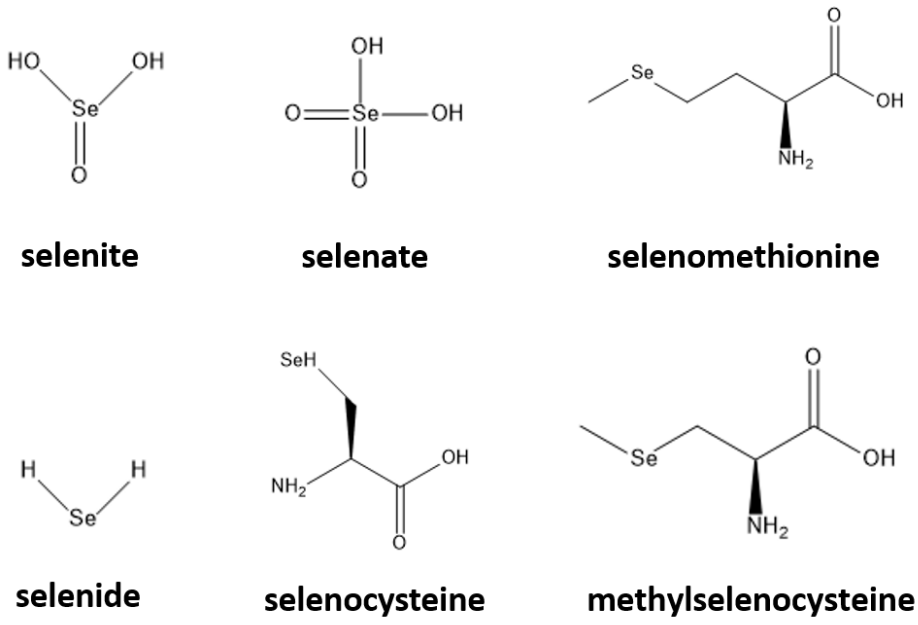

Figure 1-5. Chemical structures of dietary forms of selenium and the metabolite, selenide.

Dietary forms of selenium are predominantly selenomethionine, selenocysteine, selenite and selenate, all of which are readily absorbed (Figure 1-5) [171]. Selenocysteine and selenomethionine uptake occurs through the amino acid transport system [172,173]. Selenate uptake occurs paracellularly and it is reduced to selenite within the cell (Figure 1-6) [165]. Most *in vitro* studies involving inorganic selenium have been performed with selenite. At physiological pH, selenite exists predominantly as $HSeO_3^-$, H_2SeO_3 has pKa values of 2.6 and 8.3 [174]. Selenite uptake is likely mediated by the Cl^-/bicarbonate (HCO_3^-) anion exchanger 1 (AE1 or Band 3, gene *SLC4A1*) in red blood cells [175]. Other transport proteins that have been implicated in selenite uptake include the zinc import family transport protein, ZIP8, which transports substrates in a zinc and bicarbonate dependent manner. ZIP8 protein levels are associated with selenite uptake in human prostate cancer DU145 cells and *Xenopus laevis* oocytes [176]. Aquaglyceroporin 9 (AQP9) transports the selenium metabolite, monomethylselilinic acid at physiological pH and can also transport selenite at acidic pH [177]. This is consistent with the plant aquaglyceroporin protein, NIP III, being involved in selenite uptake [178].

1.4.2.2. Selenium Metabolism

Total selenium in the body exists essentially in two different pools. The first is the selenomethionine pool; this pool of selenium-containing proteins is not regulated as the translation machinery in the body cannot differentiate between methionine and selenomethionine. Selenoproteins, characterized by the presence of selenocysteine, and selenium metabolites form the second pool of selenium in the body [171].

Upon absorption, selenium is carried to the liver in the portal circulation. Here, through the transsulfuration pathway, selenium from selenomethionine can join the selenoproteins pool (pathway (e) in Figure 1-6) [179,180]. Methylselenocysteine and other selenium-cysteine conjugates form methylselenol upon cleavage of the cysteine group (pathway (j) in Figure 1-6) [180]. Methylation of

selenide (shown as HSe^- in Figure 1-6) also results in the formation of methylselenol (shown as MSe in Figure 1-6). Selenide is the main metabolic intermediate, at which metabolism of selenomethionine, selenocysteine and inorganic selenium species converges (pathways (c), (b) and (g) in Figure 1-6) [165,180]. Selenide can be used for the synthesis of selenoproteins, as required (pathways (k) and (l) in Figure 1-6). Once this step reaches saturation, selenide is further metabolised into excretory metabolites of selenium [171]. Trimethylselenide (TMSe) and selenosugars (Se-sugars) are the species of selenium that have been identified in human urine to date (pathway (o) and (n) in Figure 1-6).

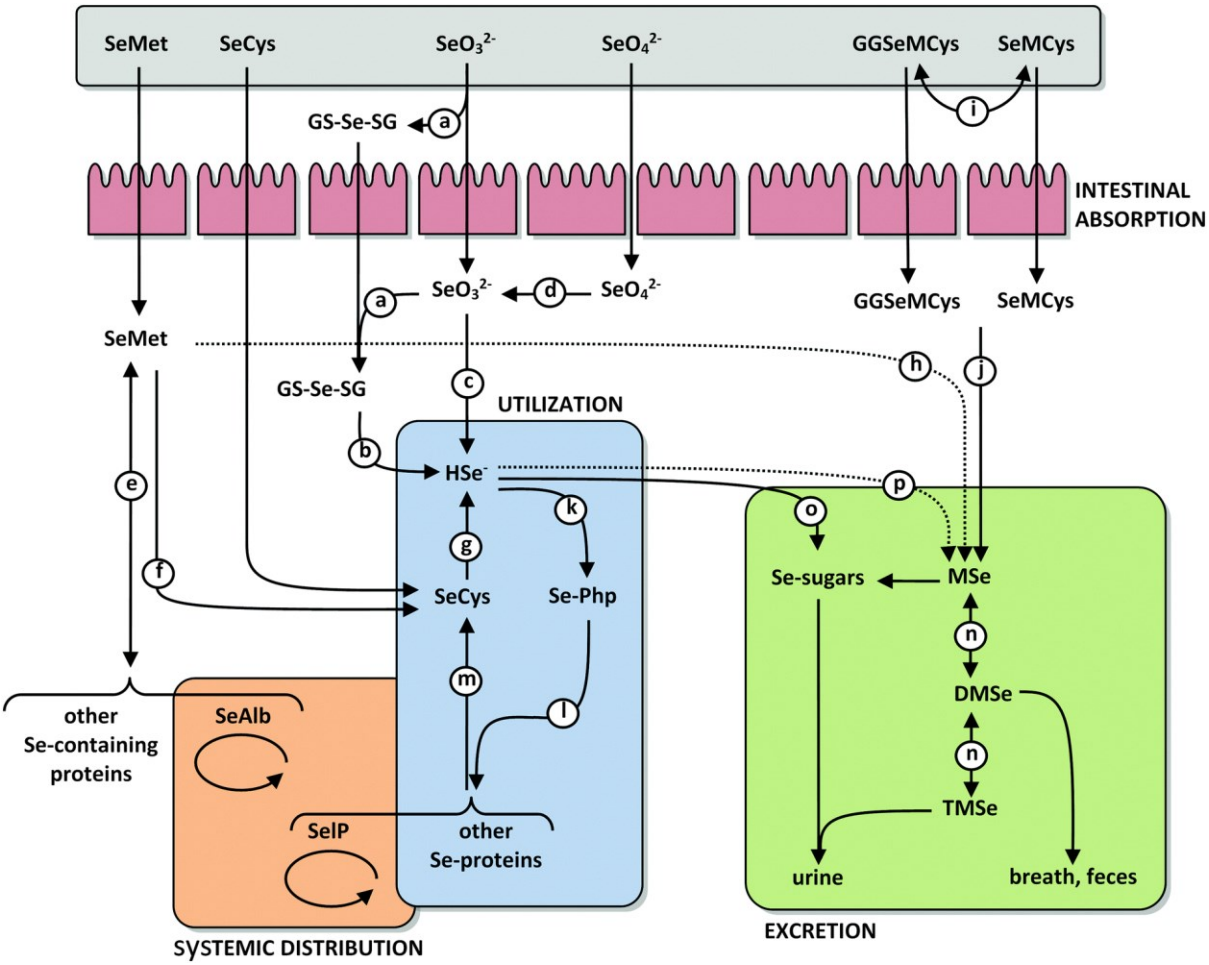

Figure 1-6. Generally accepted selenium uptake and metabolism pathway scheme. Taken from [165], with permission. Selenomethionine (SeMet); Selenocysteine (SeCys); Selenite ($HSeO_3^{2-}$); Selenate ($HSeO_4^{2-}$); Selenodiglutathione (GS-Se-SG); γ-glutamyl-Selenomethylcysteine (GGSeMCys); Selenomethylcysteine (SeMCys); Hydrogen selenide (HSe^-); Methylselenol (MSe); Dimethylselenide (DMSe); Trimethylselenide (TMSe); Selenophosphate (Se-Php); Se-albumin (SeAlb); Selenoprotein P (SelP).

1.4.2.3. Selenium Excretion

50-60% of selenium excretion occurs through the urine. Urinary elimination of selenium maintains selenium homeostasis in the body [170]. Selenium excretion does not go up when selenium intake is increased during selenium-deficient conditions [181]. It is hypothesized that once selenoprotein synthesis is saturated, the remaining selenium is then metabolized for excretion via the urine. The ratio of urinary:fecal excretion of selenium is increased, when selenium levels in the diet are high [182].

Plasma and erythrocyte selenium levels are good biomarkers for selenium intake. As with arsenic, short-term selenium exposure can also be measured through the urine, and long-term selenium exposure can be measured from hair and nails [183,184].

1.4.3. Selenium Mechanism of Protection Against Arsenic-Induced Toxicity

The antagonism between arsenic and selenium was first observed in the 1930s when selenium toxicity in rats, from being fed seleniferous grains, was reduced with the addition of arsenic to drinking water [185]. Arsenic and selenium sit as neighbours on the periodic table with atomic numbers 33 and 34, respectively. As such, arsenite and selenite have a similar electronic structure. Since their chemical and physical properties can be similar, arsenic and selenium can act in an antagonistic manner in biological systems [60].

1.4.3.1. Protection Against Oxidative DNA Damage

One of the main mechanisms of arsenic toxicity is through the generation of reactive oxygen species. It has been shown *in vitro* and in animal models that exposure to arsenite reduces the activity of glutathione peroxidase and thioredoxin reductase [186–188]. Since both GPx and TrxR are selenoproteins, co-exposure of selenite and arsenite has been shown to restore the activity of these proteins and neutralize arsenite-induced oxidative damage [187]. Selenium can thus reduce the mutagenic effects of arsenic by inhibiting oxidative damage caused to DNA, lipids and proteins [189].

1.4.3.2. Inhibition of Methylation Pathways

Selenium metabolism, as for arsenic, involves methylation [171,180]. Its methylated metabolites, trimethylselenide and selenosugars, are also excreted in urine. As such, selenium inhibits arsenic metabolism by competing for the methyl donor, SAM [168,186,190].

Glutathione is also required in both arsenic and selenium metabolism. Glutathione is involved in the reduction of pentavalent arsenic species to trivalent [125,191]. In turn, selenate and selenite are reduced by glutathione to the metabolite, selenodiglutathione, which is further metabolized to hydrogen selenide by glutathione reductase [168,192]. The parallels in arsenic and selenium metabolism mean that the reductive methylation of both species, results in competition for glutathione and SAM [60].

1.4.3.3. Formation of the Seleno-bis(S-glutathionyl) arsinium Ion in Liver

In the 1970s, Levander showed that arsenic and selenium lead to the decreased retention of the other in rat liver and biliary excretion of both species was increased [193]. The molecular basis for this interaction was later identified in rabbit bile to be the seleno-bis(*S*-glutathionyl) arsinium ion, $[(GS)_2AsSe]^-$ [194,195]. The Leslie lab determined that the human multidrug resistance protein 2 (MRP2) can transport both the glutathione-conjugated arsenic metabolites, arsenic triglutathione ($As(GS)_3$) and $[(GS)_2AsSe]^-$ (at least *in vitro*) providing potential mechanistic insight into the increased biliary efflux of both arsenic and selenium [108].

$As(GS)_3$ is not stable at alkaline biliary pH and thereby dissociates in bile and undergoes enterohepatic recirculation, preventing fecal arsenic elimination. $[(GS)_2AsSe]^-$, on the other hand, is relatively stable

at biliary pH; high levels of glutathione protect it from dissociation, thereby allowing excretion via feces [131,194].

1.4.3.4. Selenium and Arsenic Sequestration in Red Blood Cells

The presence of arsenite in rat blood has been shown to delay selenite clearance from blood and vice versa [196,197]. Glutathione levels in erythrocytes are approximately 3 mM, allowing for the *in vivo* formation of $[(GS)_2AsSe]^-$. The formation of this conjugate has been shown in rabbit erythrocyte lysate; 95% of RBC selenium was in the form of $[(GS)_2AsSe]^-$, 30 minutes after treatment [198]. Efflux studies with human RBC membrane ghosts did not exhibit any ATP-dependent efflux of $[(GS)_2AsSe]^-$, providing further evidence for the formation and sequestration of this conjugate [108]. Not only does selenium influence arsenic metabolism and efflux but also plays a significant role in arsenic sequestration in blood, influencing the organ-level distribution and toxicity of arsenic.

1.4.4. Selenium Supplementation as a Therapeutic for As-Induced Toxicities

The socio-economic status of arsenic-endemic regions is often poor, and arsenic removal from water is costly, and not often feasible [199]. Alternatively, selenium supplementation is being investigated as an inexpensive dietary palliative for the efficient elimination of arsenic. Selenium intake varies globally and is largely dependent upon selenium content in agricultural soil [180]. In most arsenic-endemic regions, low levels of selenium in soil may contribute to the high incidence of arsenic-induced toxicities [184,200,201]. Chen et al., have shown that the risk of arsenic-induced skin lesions is greater among individuals with lower blood selenium levels [202].

Epidemiological studies have investigated the effects of selenium on arsenic induced toxicities. A supplementation trial using selenium-enriched yeast with a rural community in Inner Mongolia that had been exposed to elevated levels of arsenic in their drinking water showed reduced arsenic concentrations in blood and hair [203]. Evaluation of skin lesions also showed marked improvement

in the selenium supplementation group vs the placebo control group but interestingly, selenium supplementation was most effective in ameliorating symptoms of adults that were most severely affected by arsenic toxicity [203]. Kibriya et al., investigated changes in gene expression profiles in individuals with pre-malignant arsenic-induced skin lesion supplemented with selenomethionine for 6 months daily [204]. Genes found to be up-regulated by selenium supplementation were previously found to be down-regulated in the presence of arsenic by the same group, in particular the oxidative stress related gene, superoxide dismutase 2 (SOD2) [204]. A 6-month selenomethionine supplementation trial in Bangladesh also found slight improvement in arsenic-induced skin lesions [205]. A case-control study in preschool children in Taiwan has shown that plasma selenium levels are correlated with increased dimethylated arsenic species in urine [206]. Smits et al., also reported increased urinary elimination of dimethylarsinic acid in individuals on a diet of selenium-enriched lentils, in comparison to the control group [106].

Despite all the advances in understanding made with epidemiological studies and with *in vivo* animal studies, little is known about the cellular handling and interactions between arsenic and selenium in humans or human cell lines. The focus of this thesis is the influence of selenium on arsenic transport and accumulation in human RBCs and hepatocytes.

1.5. Models of Hepatic Transport

Models of transport utilized in this thesis research are described below, providing a perspective on the limitations and advantages of each model.

1.5.1. Primary Hepatocytes

Since the liver is the largest organ in the body involved in detoxification, primary human hepatocytes are the gold standard for *in vitro* xenobiotic toxicity studies. However, in conventional culture, hepatocytes lose their morphology and other characteristics after 24-48 hours in culture, making them

of limited use in longer-term metabolism studies [207]. Further to this, correct localization of membrane proteins, cell polarization, cell-cell interactions and cell membrane integrity, especially the bile canaliculi, results in disparate results from what may be occurring *in vivo* [207]. In contrast, when hepatocytes are cultured between two layers of collagen, aptly termed sandwich-cultured hepatocytes (SCH), they retain their *in vivo*-like features [208]. Sandwich-cultured hepatocytes retain the correct liver functions, cell morphology and display the formation of intact biliary networks and excretory function [209–211]. Most notably, transport function across the basolateral and apical surfaces of hepatocytes can be elucidated using the sandwich-cultured model. As demonstrated by Liu et al., calcium can be used to modulate the integrity of tight junctions [212]. Tights junctions act as barriers between the canalicular lumen and extracellular space. Tight junctions are disrupted when calcium is depleted, allowing for the release of canalicular network contents and the design of experimental studies to investigate biliary efflux. The biliary excretion index (BEI) is a measure of the percent of total efflux that is going into bile. It is determined using the following equation:

$$BEI = \frac{Efflux_{(sinusoidal + canalicular)} - Efflux_{(sinusoidal)}}{Efflux_{(sinusoidal + canalicular)}} \times 100\%$$

1.5.2. Immortalized Liver Cell Lines

The shortage of primary human hepatocytes leads to the use of immortalized cell lines in investigations of liver function. The human hepatoma cell line, HepG2 is the most commonly used in research [213]. However, genetic expression of proteins involved in metabolism can vary over cell passages [214]. Not only do HepG2 cells lack the formation of canalicular networks, but protein profiles of membrane transporters and metabolic enzymes are different in HepG2 cells than human hepatocytes [215]. Despite that, liver-specific cell function can be maintained in these cell lines, providing a readily available model for high throughput optimization work [207].

More recently, HepaRG cells have been developed as a surrogate for human hepatocytes. Like HepG2 cells, the HepaRG cell line is a human hepatoma cell line, however, it can differentiate into both cholangiocyte-like and hepatocyte-like cells, retaining features of human hepatocytes [216,217]. Since the HepaRG cells represent the phenotype from one donor, human variability cannot be represented using this cell line [207]. However, having a high proliferation capacity, allows for data replication and reproducibility, making this cell line useful for xenobiotic metabolism and transport studies [216,217]. HepaRG cells also maintain cell polarity and expression of hepatocyte transport proteins [218]. The correct localization and efflux function of MRP2 has been shown in HepaRG cells [218].

1.5.3. Vesicular Transport Assays

Characterization of ABC-transporter function is commonly done using vesicular transport assays. Membrane vesicles can be isolated upon heterologous overexpression of a transporter of interest in immortalized cell lines [209]. MRP2 is unidirectional transport protein, effluxing substrates out of the cell [219]. Functional characterization of ABC proteins can be carried out using inside-out membrane vesicles [220,221]. Inside-out vesicles have the ATP-binding site and substrate-binding sites of proteins facing out. Their benefits are two-fold; (i) the need for prior accumulation of substrate within cells prior to the commencement of efflux studies is eliminated, and (ii) substrates transported into the vesicles will be 'trapped' for quantification.

1.6. Objectives, Hypothesis and Rationale.

Despite advances in selenium supplementation trials in efforts to alleviate arsenic-induced toxicities, the cellular handling and interactions of arsenic and selenium in humans is poorly understood. Animal studies have shown the protective mechanism of selenium to potentially be two-fold; through the increased sequestration of arsenic in RBCs and secondly, through the increased biliary efflux of arsenic. The objective of this thesis work was to characterize the effects of selenium on the uptake,

metabolism and efflux of arsenic using human RBCs and relevant human hepatic models. The main hypothesis was that selenium would alter arsenic metabolism and transport through the formation of $[(GS)_2AsSe]^-$. To address this hypothesis, specific objectives were as follows:

Objective 1: Characterization of the uptake and metabolism of arsenic and selenium in human red blood cells

The first objective was to elucidate the mechanism of uptake of arsenic and selenium in human RBCs upon crossing the intestinal epithelia following the most common route of exposure, ingestion. Presented in Chapter 2 uptake transporters of arsenite and selenite in human RBCs were characterized using inhibition studies, as well as, heterologous overexpression systems. In addition, the effect of selenite on arsenite uptake, and vice versa was investigated. Lastly, in order to elucidate the effect of selenite on arsenite metabolism, x-ray absorption spectroscopy was used to determine the speciation of arsenic and selenium in red blood cells treated with both compounds.

Objective 2: Characterization of the effects of selenium on the hepatobiliary transport of arsenic in SCHH

Interactions between arsenic and selenium likely begin in the portal circulation. First-pass metabolism of arsenic and selenium occurs in the liver. As such, the second objective of my work was to use the SCHH model to investigate the influence of selenium species on the biliary and sinusoidal efflux of arsenic. SCHH preparations from 8 different donors were used to characterize arsenic efflux. Preliminary experiments investigating the effect of arsenic on selenium accumulation and efflux were also performed. In addition to efflux and accumulation studies, the effect of selenium on arsenic-induced cytotoxicity in primary human hepatocytes was also investigated.

Objective 3: Characterization of arsenic and selenium uptake and cytotoxicity in HepG2 cells

To further extend our observations from the SCHH model and to optimize treatment condition combinations for arsenic and selenium, studies were completed using HepG2 cells, as presented in

Chapter 4. The focus of this chapter was on uptake differences in HepG2 cells between the selenium species, selenite and selenide. Cytotoxicity experiments were carried out for both selenium species and in combination with arsenic to determine treatment combinations that resulted in antagonism of toxicity between arsenic and selenium. Lastly, synchrotron-based techniques were used to investigate accumulation differences of arsenic in the presence and absence of both selenite and selenide. The results of this chapter provide important information for the better design of *in vitro* arsenic-selenium interaction studies.

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2. Human Red Blood Cell Uptake and Sequestration of Arsenite and Selenite: Evidence of Seleno-bis(*S*-glutathionyl) Arsinium Ion Formation in Human Cells

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2.1. Abstract

Over 200 million people worldwide are exposed to the Group 1 human carcinogen, arsenic, in contaminated drinking water. In laboratory animals, arsenic and the essential trace element, selenium, can undergo mutual detoxification through the formation of the seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$, which undergoes biliary and fecal elimination. $[(GS)_2AsSe]^-$, formed in animal red blood cells (RBCs), sequesters arsenic and selenium, and slows the distribution of both compounds to peripheral tissues susceptible to toxic effects. In human RBCs, the influence of arsenic on selenium accumulation, and vice versa, is largely unknown. The aims of this study were to characterize arsenite (As^{III}) and selenite (Se^{IV}) uptake by human RBCs, to determine if Se^{IV} and As^{III} increase the respective accumulation of the other in human RBCs, and ultimately to determine if this occurs through the formation and sequestration of $[(GS)_2AsSe]^-$. $^{75}Se^{IV}$ accumulation was inhibited by 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid (H_2DIDS) (IC_{50} 1 ± 0.2 μM), suggesting uptake is mediated by the erythrocyte anion-exchanger 1 (AE1 or Band 3, gene *SLC4A1*). HEK293 cells overexpressing AE1 showed concentration-dependent $^{75}Se^{IV}$ uptake. $^{73}As^{III}$ uptake by human RBCs was temperature-dependent and partly reduced by aquaglyceroporin 3 inhibitors. As^{III} increased $^{75}Se^{IV}$ accumulation (in the presence of albumin) and Se^{IV} increased $^{73}As^{III}$ accumulation in human RBCs. Near-edge X-ray absorption spectroscopy revealed the formation of $[(GS)_2AsSe]^-$ in human RBCs exposed to both As^{III} and Se^{IV}. The sequestration of $[(GS)_2AsSe]^-$ in human RBCs potentially slows arsenic distribution to susceptible tissues and could reduce arsenic-induced disease.

2.2. Introduction

An estimated 200 million people world-wide are exposed to inorganic arsenic (As) in their drinking water at levels above the World Health Organization acceptable limit of 10 μg/L [1]. This is a massive global public health issue due to the fact that As is a Group 1 (proven) human carcinogen [2], and chronic exposure is also associated with numerous other adverse health outcomes [3,4]. Decreasing exposure to As should be a major priority, however, evidence suggests that humans are susceptible to As-induced disease for decades following cessation of chronic exposure [5,6]. Furthermore, in many As-endemic regions, removing exposure is not feasible for socioeconomic and geographical reasons. Thus, strategies for the treatment and prevention of As-induced disease, in addition to exposure reduction, are necessary.

Selenium (Se) is an essential trace element with a recommended dietary allowance of 55 μg/day [7,8]. In excess, Se is toxic with a tolerable upper intake level of 400 μg/day [8]. A surprising mutually protective relationship was discovered between As and Se when As (in the form of arsenite, which is $As(OH)_3$ at physiological pH, and abbreviated in the present chapter as As^{III}) protected rats against Se-induced liver damage [9]. This mutual detoxification has also been observed in other animal species [10]. In laboratory animals, Se and As enhanced the fecal elimination of each other through increased biliary excretion, resulting in a decreased retention of both compounds in the liver [11,12]. More recently, the seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ was identified as the major As metabolite in the bile of rabbits and rats co-injected with selenite and As^{III}, providing a molecular basis for the antagonistic interaction between these two compounds [13–15]. Selenite is ~90% $[Se(OH)O_2]^-$ at physiological pH, and selenite is abbreviated in the present chapter as Se^{IV}.

Further to the biliary excretion of $[(GS)_2AsSe]^-$, sequestration of this conjugate in RBCs may be the first line of defence against As and Se toxicity [16]. Co-treatment of rats with As^{III} and Se^{IV} resulted in a dramatically increased accumulation of both species in RBCs [17–19]. The loss of As and Se from

RBCs was closely followed by the accumulation of Se in liver [19], suggesting that RBCs slow or even prevent the distribution of these toxicants to other tissues. In rabbit RBC lysate, ~70% of As^{III} and Se^{IV} were converted to $[(GS)_2AsSe]^-$ in two min [16]. Further, membrane ghosts prepared from human RBCs had no ATP-dependent export of $[(GS)_2AsSe]^-$ [20]. Taken together, these data suggest that $[(GS)_2AsSe]^-$ is the sequestered form of As and Se in RBCs. Evidence supports that both rapid formation of $[(GS)_2AsSe]^-$ in RBCs and $[(GS)_2AsSe]^-$ biliary excretion prevent the extensive methylation of As in the liver (and the resulting production of toxic methylated trivalent species) and distribution of As and Se to other organs [15,21–23].

Consistent with animal studies, Se-deficiency in people living in As-endemic regions is associated with increased risk of As-induced skin lesions [24,25]. Together with the laboratory animal data, these observations have led to human Se-supplementation trials in As-endemic regions where human exposure to As is high. Preliminary reports and small population studies show promising results, suggesting that supplementation with L-selenomethionine or selenized yeast (at ≤ 200 μg/day) reduces the symptoms of chronic As exposure [26,27]. Recently, consumption of high-Se lentils (55 μg Se/day) in a Bangladeshi population increased urinary elimination of total As [28]. Other Se-supplementation trials are underway or have yet to report results [29–31]. Despite the use of Se-supplementation in human trials, the influence of Se on As metabolism and elimination in humans remains largely unknown. In particular, it is not known if $[(GS)_2AsSe]^-$ is formed in human tissues.

The first objective of this study was to characterize the human RBC uptake pathways for Se^{IV} and As^{III} individually. Understanding these pathways lays the foundation for recognizing genetic variation that may dictate which As-exposed individuals benefit from Se-supplementation. For Se^{IV}, previous studies have concluded that uptake by human and rat RBCs is through the Cl^-/bicarbonate (HCO_3^-) anion exchanger 1 (AE1 or Band 3, gene *SLC4A1*). The evidence for AE1 involvement is inhibition of Se^{IV} uptake into RBCs by the AE1-inhibitors, 4,4'-diisothiocyano-2,2'-stilbene sisulfonic acid (DIDS) or

4,4'-dinitrosostilbene-2,2'-disulfonate (DNDS) [32,33]. The HCO_3^--dependent Zn^{2+} transporter, ZIP8 (gene *SLC39A8*), takes up Se^{IV} in different cell models (including mouse RBCs) in a Zn^{2+}- and HCO_3^--dependent manner [34]. One report suggests that ZIP8 is present in the plasma membrane of human RBCs, therefore ZIP8 must also be considered a candidate Se^{IV} transporter in human RBCs [35].

For As uptake, multiple pathways have been identified using over-expression systems and knock-down experiments in different cell lines (reviewed in [36]), however, very little has been done directly with human RBCs to characterize As uptake. That said, aquaglyceroporin 3 (AQP3) and glucose transporter 1 (GLUT1, gene *SLC2A1*) are candidates because they have been shown to promote the cellular uptake of As^{III} using heterologous expression systems, and are also expressed in human RBCs [37–39].

The second objective of this study was to characterize the influence of As^{III} on Se^{IV} accumulation, and vice versa, in human RBCs. The As and Se species present in human RBCs after As^{III} and Se^{IV} treatment (individually and in combination) were also investigated.

2.3. Materials and Methods

2.3.1. Chemicals and Reagents

$^{73}As^{V}$ was purchased from Los Alamos Meson Production Facility (Los Alamos, NM). $^{75}Se^{IV}$ was obtained from the University of Missouri Research Reactor (MURR, Columbia, MO). Sodium selenite, sodium (meta)arsenite, bovine serum albumin (BSA), BAY-876, glucose, sodium chloride, potassium chloride, potassium gluconate, calcium gluconate, magnesium sulfate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), NaH_2PO_4, and $NaHCO_3$ were purchased from MilliporeSigma (Oakville, Ontario). 3-(N-morpholino) propanesulfonic acid (MOPS) was purchased from BioShop Canada (Burlington, ON). 4,4'-Diisothiocyanatodihydrostilbene-2,2'-disulfonic acid

(H_2DIDS) was purchased from Invitrogen. Auphen ([Au (1,10-phenanthroline)Cl_2]Cl) and [Au(PbImMe)Cl_2]PF_6 (PbImMe = 1-methyl-2-(pyridine-2-yl)-benzimidazole) were synthesized as previously described [40]. Bicinchoninic acid assay reagents were purchased from Thermo Scientific (Rockford, IL).

2.3.2. Blood Collection

Human blood was obtained with the approval of the University of Alberta Human Ethics Review Board (protocol number 5614) with donor consent. Blood (5-10 mL) was drawn from healthy human volunteers into K2 EDTA Vacutainer® blood collection tubes (BD, Franklin Lanes, NJ). Blood was centrifuged at 950 *g* for 10 min, plasma and buffy coat were removed and RBCs were washed three times with isotonic saline solution (140 mM sodium chloride, 5 mM potassium chloride, 10 mM MOPS buffer, 5 mM glucose, pH 7.4). RBCs were stored at 4ºC in isotonic saline for a maximum of 8 days and diluted to 10% hematocrit before use in accumulation experiments, in the indicated buffer.

2.3.3. $^{75}Se^{IV}$ Accumulation Assay in RBCs†

The H_2DIDS-dependence of $^{75}Se^{IV}$ (1 μM, 1.85 kBq) accumulation was tested by incubating human RBCs at a 10% hematocrit in 100 or 200 μl of isotonic saline at 37°C in the presence and absence of H_2DIDS (50 μM) for the indicated time points. H_2DIDS was freshly prepared prior to each experiment in DMSO. Concentration dependence of H_2DIDS (0.1 to 30 μM) inhibition of $^{75}Se^{IV}$ (1 μM, 1.85 kBq) uptake was determined as described above at a 1 min time point. IC_{50} values were determined by fitting the data with the log[inhibitor] vs response curve using GraphPad Prism 6 software. All other $^{75}Se^{IV}$ accumulation by RBCs was done in standard Ringer's solution (5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM magnesium sulfate, 10 mM HEPES, 2.5 mM NaH_2PO_4, 5 mM glucose, 140 mM NaCl, 24 mM $NaHCO_3$ and pH of 7.4). To test the Cl^- -dependence of Se^{IV} accumulation, Ringer's solution was prepared with 140 mM sodium gluconate in place of NaCl. To test the

† Warda Javed completed all ^{75}Se experiments, with technical assistance from Diane Swanlund and Kamran Shekh Angela Casini (with assistance from Margot Wenzel) provided AQP3 inhibitors.

dependence on HCO_3^-, the standard Ringer's buffer was prepared without the addition of $NaHCO_3$. The influence of $ZnCl_2$ (10, 20, and 100 μM) was tested in standard Ringer's solution. The influence of As^{III} (1 μM) on $^{75}Se^{IV}$ (1 μM, 1.85 kBq) uptake was investigated in isotonic saline or Ringer's solution in the presence and absence of physiological levels of BSA (35 mg/ml). Prior to use in accumulation assays RBCs were washed twice in the appropriate ice-cold buffer.

At each time point, uptake was stopped by transferring the reaction to ice then centrifuging at 6800 *g* for 1 min at 4°C. Supernatant was removed and RBC pellets were washed twice with 1 ml of the appropriate 4°C buffer. RBC pellets were resuspended in 140 μl Ringer's buffer and transferred to 4.5 ml polypropylene gamma counter tubes (Sarstedt, Numbrecht, Germany). Radioactivity was quantified using a Packard Cobra II gamma counter. Untreated human RBCs were run as background controls and a known volume of the $^{75}Se^{IV}$ solution used to treat RBCs was quantified.

2.3.4. $^{75}Se^{IV}$ Accumulation by AE1 Transfected HEK293 Cells

HEK293 cells were obtained from the American Type Culture Collection (ATCC), and were maintained in Dulbecco's modified Eagle's medium containing 7.5% fetal bovine serum, for less than three months. HEK293 cells were negative for *Mycoplasma* contamination (ATCC Universal Mycoplasma Testing Kit). pcDNA3.1-AE1 with a hemagglutinin (HA)-tag at position 557 in extracellular loop 3 was constructed as previously described [41]. For transient transfections, HEK293 cells were seeded at 4 to 5 x 10^6 cells in 150 mm dishes and 24 h later transfected with pcDNA3.1-HA-AE1 or empty pcDNA3.1 using the calcium phosphate method as described previously [20]. Cells were harvested for transport assays at 48 h post-transfection.

Transport assays were completed as described for the human RBCs with the following modifications. Empty pcDNA3.1 and pcDNA3.1-HA-AE1 transfected cells were washed and resuspended at 1 x 10^6 cells/200 μl in 4°C Cl^--free Ringer's solution without the addition of HCO_3^-. $^{75}Se^{IV}$ (0.3, 1, and 3 μM, 1.85 kBq) was incubated with the cells for 10 min at 37°C and then uptake stopped by transfer to ice,

cell suspensions were then centrifuged at 1000 *g* for 2 min, supernatant was discarded, and cell pellets washed twice with 4°C Cl^-- and HCO_3^-- free Ringer's solution. Transport activity was normalized to total protein of cell lysates, determined using a bicinchoninic acid protein assay.

2.3.5. ^{73}As Accumulation Assay in Human RBCs†

Reduction of $^{73}As^{V}$ into $^{73}As^{III}$ was performed as described previously [42]. Human RBCs at a 10% hematocrit in 200 µl of isotonic saline were incubated at 37ºC with $^{73}As^{III}$ (1 µM, 1.85 kBq) for the indicated time points.

To test the influence of transport inhibitors on the accumulation of $^{73}As^{III}$ (1 µM, 1.85 kBq) human RBCs were pre-incubated with the GLUT1 inhibitor BAY-876 (1 to100 µM), or AQP3 inhibitors Auphen and $[Au(PbImMe)Cl_2]PF_6$ (1 to100 µM) or vehicle (DMSO) control for 30 min at room temperature. Inhibitor stocks were prepared in DMSO, aliquoted and stored at -20ºC. To test the influence of Se^{IV} on $^{73}As^{III}$ (1 µM, 1.85 kBq) accumulation, Se^{IV} (1 µM) was added to human RBCs at the same time as $^{73}As^{III}$. The dose dependence of Se^{IV} (0.1 to 10 µM) on $^{73}As^{III}$ accumulation was determined with $^{73}As^{III}$ at 0.3 µM (1.85 kBq/data point). H_2DIDS (1 to 10 µM) was added to human RBCs 1 minute before the simultaneous addition of Se^{IV} (1 µM) and $^{73}As^{III}$ (1 µM, 1.85 kBq).

After the appropriate time period of accumulation, RBC suspensions were placed on ice then centrifuged at 6800 *g* for 1 min at 4ºC. Pelleted RBCs were then washed twice with 1 ml of ice cold isotonic saline. $^{73}As^{III}$ accumulated was then quantified by counting samples on a Packard Cobra II gamma counter, as described for $^{75}Se^{IV}$ accumulation.

2.3.6. ^{75}Se Efflux Assay

Efflux assays were modelled after a previous study [43]. Human RBCs at a 10% hematocrit were preloaded with $^{75}Se^{IV}$ (1 µM, 1.85 kBq/100 µl RBCs) for 20 min at 37ºC. Cells were then centrifuged at 6800 *g* for 3 minutes at 4ºC and washed twice with 4ºC Ringer's solution (1 ml of wash buffer/ml

† Gurnit Kaur initiated all ^{73}As experiments, with technical replicates completed by Diane Swanlund.

of RBC suspension). Cells were then resuspended at a 10% hematocrit in 37ºC Ringer's ± BSA, incubated at 37ºC, and 100 µl removed at the indicated time points. RBCs were then processed and $^{75}Se^{IV}$ quantified as described for accumulation assays.

2.3.7. Arsenic and Selenium Near-Edge X-ray Absorption Spectroscopy (XAS)†

Human RBCs at a 5% haematocrit in isotonic saline were incubated at 37°C with As^{III} (30 µM) or Se^{IV} (30 µM) or the combination of As^{III} (30 µM) plus Se^{IV} (30 µM) for 45 minutes. Accumulation was stopped by diluting the reaction in 800 µl of ice-cold isotonic saline solution. Cells were then centrifuged at 2000 *g* for 10 min at 4°C and washed twice with isotonic saline solution. The RBCs were then transferred to 2 mm-thick Lucite cuvettes, sealed with metal- and bromine-free Mylar adhesive tape, frozen in an isopropanol bath in liquid nitrogen and then stored in liquid nitrogen until analysis. Bulk As and Se K near-edge XAS was measured on the structural molecular biology beamline 7-3 at the Stanford Synchrotron Radiation Lightsource (SSRL) using the data acquisition program XAS Collect. An incident cutoff energy of 15 keV was obtained using a Si(220) double-crystal monochromator with harmonic rejection from an upstream Rh-coated mirror.

Incident X-ray intensities were monitored using nitrogen-filled gas ionization chambers. Near-edge XAS data were measured as the primary fluorescence excitation spectrum using a 30-element germanium array detector (Canberra Ltd, Meriden, CT, USA). Soller slits and (Z-1) X-ray filters were employed to reject spurious scattered radiation contributing to background and to limit detector count rates to linear regime. The samples were kept at ~ 10 K temperature in a liquid helium flow cryostat CF1204 (Oxford Instruments, Concord, MA) in order to minimize photodamage, and resultant change of speciation, in particular ensuring maximum stability of redox-active As and Se thiol-bound compounds

† Gurnit Kaur was responsible for x-ray absorption spectroscopy (XAS) sample preparation. XAS analysis was performed by Olena Ponomarenko and Kelly Summers.

X-ray energy calibration used the lowest energy K-edge inflection point of the downstream standard foils of elemental As and hexagonal elemental Se which were assumed to be 11,867.0 and 12,658.0 eV for As and Se, respectively. Primary data reduction and the least-square fitting of the samples' near-edge XAS spectra were conducted using the EXAFSPAK suite of programs [44]. The library of standard spectra was collected previously on the 7-3 beamline using reference Se and As compounds. The details of least square fitting and rejection criteria in the refinements are discussed in [15] and the references therein.

2.4. Results

2.4.1. Uptake of Se^{IV} by Human RBCs.

Previous studies have concluded that Se^{IV} is taken up by human and rat RBCs through AE1 [32,33]. Consistent with this, Se^{IV} accumulation by human RBCs was inhibited by the AE1 antagonist H_2DIDS (Figure 2-1A and B). In Figure 1A, RBCs were treated with either 50 μM of H_2DIDS or the vehicle control (DMSO) and accumulation of 1 μM $^{75}Se^{IV}$ in the RBCs was followed for 3 min. In the absence of H_2DIDS, $^{75}Se^{IV}$ accumulation was eight-fold higher than in the presence of H_2DIDS at the 3 min time point. Investigation of this inhibition was continued by treating RBCs with increasing concentrations of H_2DIDS (0.1 to 30 μM), and 1 μM $^{75}Se^{IV}$ accumulation was measured at a 1 min time point (Figure 2-1B). The IC_{50} of H_2DIDS on $^{75}Se^{IV}$ accumulation was 1.0 ± 0.2 μM, consistent with previous H_2DIDS IC_{50} values reported for AE1 for non-Se^{IV} permeants (0.8 μM in [45]).

To further characterize Se^{IV} uptake by human RBCs, Cl^-, temperature, and HCO_3^- dependence were evaluated. AE1 exchanges HCO_3^- for Cl^-. Se^{IV} is predominantly $HSeO_3^-$ at physiological pH and is likely exchanged for Cl^- in place of HCO_3^- [32]. Se^{IV} uptake was measured in the presence and absence of Cl^-. Cl^--depleted conditions (-Cl^-) resulted in a stimulation of Se^{IV} uptake (Figure 2-1C), as would be expected if Se^{IV} uptake is mediated through the exchange of Cl^- for Se^{IV} by AE1. The -Cl^- condition

results in a large downhill Cl^--concentration gradient from inside to outside of the RBC combined with the large inward Se^{IV} concentration gradient. Se^{IV} uptake was temperature dependent with an average uptake of 1.3 pmol/min at 4ºC and 14 pmol/min at 37ºC (Figure 2-1C), providing further evidence that Se^{IV} enters RBCs through a transporter-mediated process. Se^{IV} uptake was measured in buffer with and without the addition of HCO_3^-. An increased Se^{IV} uptake trend was observed without HCO_3^- (Figure 2-1C), however, this difference did not reach statistical significance.

To further test the involvement of AE1 in Se^{IV} uptake (without the use of a pharmacological inhibitor), the ability of AE1 to transport Se^{IV} in a heterologous expression system was determined. HEK293 cells transiently transfected with AE1 displayed a dose-dependent increase in Se^{IV} accumulation relative to cells transfected with empty pcDNA3.1 (Figure 2-1D). AE1 expressing cells accumulated 3-, 4-, and 6-fold more Se^{IV} at 0.3, 1, and 3 μM, respectively, than cells transfected with empty pcDNA3.1.

In mouse RBCs the transporter, ZIP8, was reported to take up Se^{IV} through a HCO_3^- and Zn^{2+} stimulated pathway [34]. This led us to determine the influence of $ZnCl_2$ (10, 20, and 100 μM) on Se^{IV} (1 μM) uptake by human RBCs in the presence of HCO_3^- (24 mM). No significant difference was observed between Se^{IV} uptake alone or in the presence of $ZnCl_2$ (Figure 2-1E), suggesting that ZIP8 is not involved in human RBC uptake of Se^{IV}.

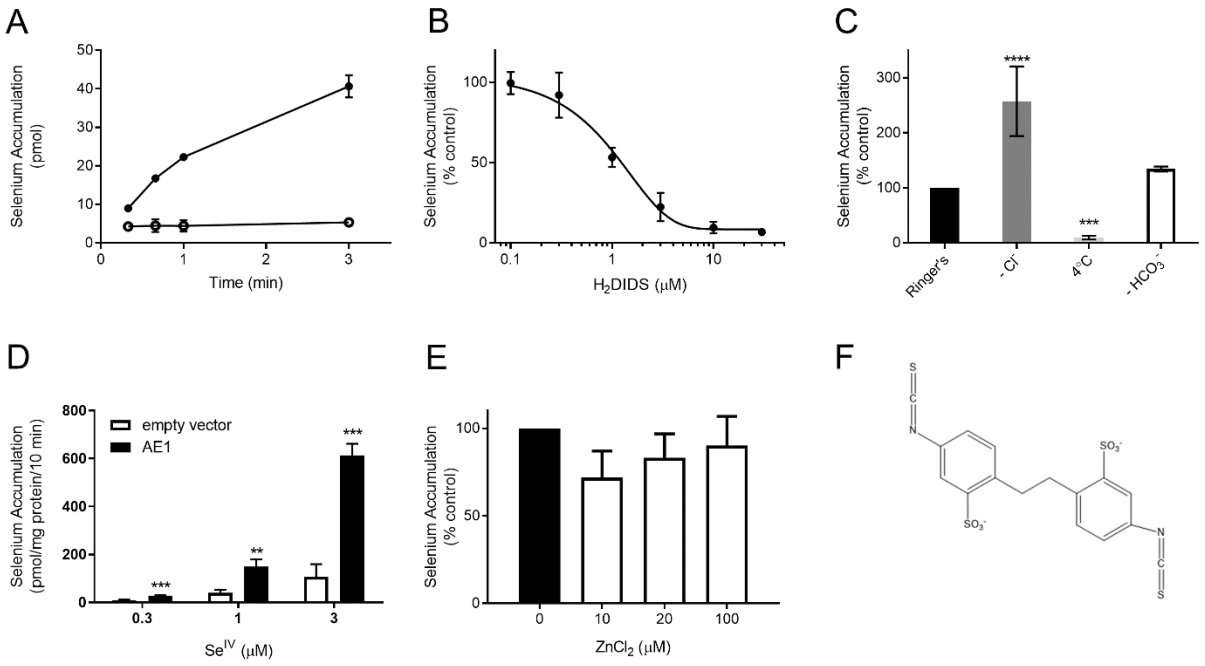

Figure 2-1. Se^{IV} accumulation by human RBCs and transfected HEK293 cells. (A-C, E) Human RBCs at a 10% hematocrit in 200 µl of the indicated buffer were incubated at 37ºC (or the temperature indicated) with $^{75}Se^{IV}$ (1 µM, 1.85 kBq) for the indicated time point. RBC suspensions were then centrifuged at 6800 *g* for 1 min at 4ºC, pelleted RBCs were then washed twice with 1 ml of ice-cold isotonic saline. $^{75}Se^{IV}$ accumulation was quantified by counting samples on a gamma counter. (**A**) Time course of Se^{IV} uptake in the presence (○) and absence (●) of the AE1 inhibitor H_2DIDS (50 µM) in isotonic saline. Symbols represent means of triplicate determinations (± S.D.) in a single experiment. Similar results were obtained in three additional experiments. (**B**) Dose dependence of H_2DIDS inhibition of $^{75}Se^{IV}$ accumulation by human RBCs in isotonic saline at a 1 min time point. Symbols represent means of three independent experiments (± S.D.). (**C**) Temperature-, HCO_3^--, and Cl^--dependence of $^{75}Se^{IV}$ accumulation were tested at 1 min time points in Ringer's solution. Bars represent mean of at least four independent experiments (± S.D.), *** P < 0.001, **** P < 0.0001, (ANOVA with Dunnett's multiple comparisons test). (**D**) HEK293 cells were transfected with either empty pcDNA3.1 (open bars) or pcDNA3.1-HA-AE1 (black bars). Cells in suspension were washed twice with Ringer's solution without Cl^- or HCO_3^- and suspended at 5 x 10^6 cells/ml. $^{75}Se^{IV}$ (0.3, 1, or 3 µM, 1.85 kBq) was incubated with the cells for 10 min at 37˚C and then uptake stopped by transfer to ice, cell suspensions were then centrifuged at 1000 *g* for 2 min, supernatant was discarded, and cell pellets washed twice with Cl^-- and HCO_3^-- free Ringer's solution. $^{75}Se^{IV}$ accumulation was then quantified by counting samples on a gamma counter. Bars represent means of three independent experiments (± S.D.), AE1 expressing HEK293 cells were significantly different from empty vector at each concentration indicated with ** P < 0.01, *** P < 0.001 (t-test with Holm-Sidak correction for multiple comparisons). (**E**) Influence of increasing concentrations of $ZnCl_2$ (10, 20, and 100 µM) on $^{75}Se^{IV}$ (1 µM, 1.85 kBq) uptake at a 1 min time point in Ringer's solution. Bars represent means of three independent experiments (± S.D.). There was no significant difference between conditions. **(F)** Chemical structure of AE1-inhibitor, H_2DIDS used in panel A and B.

2.4.2. Uptake of As^{III} by Human RBCs

To begin characterization of As^{III} transport into human RBCs, $^{73}As^{III}$ (1 μM, 1.85 kBq) uptake time courses were performed and transport was linear up to 15 min and then plateaued (Figure 2-2A). Transport was also temperature dependent with an activity of 0.62 pmol/min at 4ºC and 2.6 pmol/min at 37ºC (Figure 2-2B), suggesting that As^{III} does not enter RBCs purely through simple diffusion.

The transporter and channel proteins, GLUT1 and AQP3, respectively, are candidates for human RBC As^{III} uptake pathways [37,38]. Thus, the influences of GLUT1 and AQP3 inhibitors on $^{73}As^{III}$ uptake by human RBCs were investigated. BAY-876 has been reported as a highly selective and potent GLUT1 inhibitor [46] (structure in Figure 2-2C). The IC_{50} value of BAY-876 for GLUT1, determined using an indirect measure of glucose uptake, was 2 nM [46]. At concentrations of BAY-876 exceeding the glucose IC_{50} by at least two orders of magnitude, no inhibition of As^{III} (1 μM) uptake by human RBCs was observed (Figure 2-2D).

Auphen and $[Au(PbImMe)Cl_2]PF_6$, Au^{III} complexes developed as AQP3 inhibitors, inhibit the uptake of glycerol by human RBCs with IC_{50} values of 0.8 μM and 0.6 μM, respectively [40,47] (structures in Figure 2-2C). Auphen inhibited As^{III} (1 μM) uptake by human RBCs by 24, 33, and 37% at 10, 30, and 100 μM, respectively (Figure 2-2E). Similarly, $[Au(PbImMe)Cl_2]PF_6$ inhibited As^{III} (1 μM) uptake by 20, 36, and 43% at 10, 30, and 100 μM, respectively (Figure 2-2F). Thus, As^{III} uptake by human RBCs was reduced by these AQP3 inhibitors, suggesting that AQP3 may be important for some RBC uptake of As^{III}.

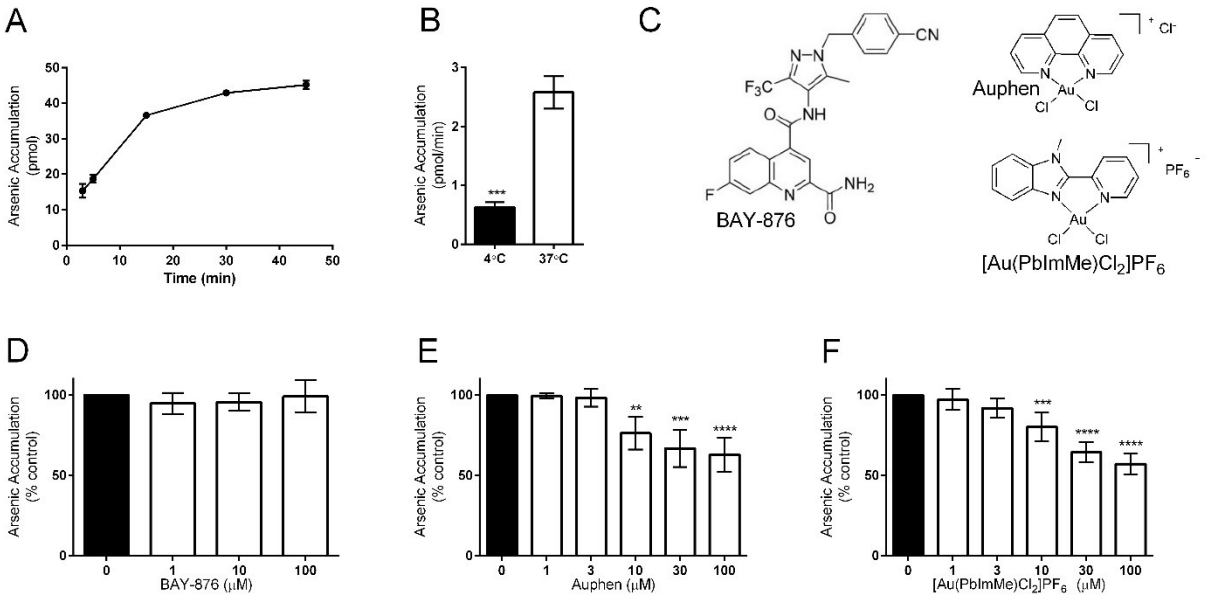

Figure 2-2. As^{III} accumulation by human RBCs. Human RBCs at a 10% hematocrit in 200 µl of isotonic saline were incubated at 37ºC (or the temperature indicated) with $^{73}As^{III}$ (1 µM, 1.85 kBq) for the indicated time point. RBC suspensions were then centrifuged at 1000 *g* for 10 min at 4ºC, pelleted RBCs were then washed twice with 1 ml of ice cold isotonic saline. $^{73}As^{III}$ accumulation was then quantified by counting samples on a gamma counter. (**A**) Time course of As^{III} uptake. Symbols represent means of triplicate determinations (± S.D.) in a single experiment. Similar results were obtained in two additional experiments. (**B**) Temperature dependence of $^{73}As^{III}$ accumulation was tested at a 15 min time point. Bars represent means of three independent experiments (± S.D.). A significant difference between the two temperatures was indicated with *** $P < 0.001$ (Student's t-test). (**C**) Structures of inhibitors used in panels (D-F)**.** (**D-F**) Human RBCs were pre-incubated with the indicated concentrations of the (**D**) GLUT1 inhibitor BAY-876, or AQP3 inhibitors (**E**) Auphen and (**F**) $[Au(PbImMe)Cl_2]PF_6$ or vehicle control for 30 min at room temperature. Accumulation of $^{73}As^{III}$ was then measured for 15 min at 37ºC. Bars represent means of 3-5 independent experiments (± S.D.). Significant differences between ± inhibitor are indicated with ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (ANOVA with Dunnett's multiple comparisons test).

2.4.3. The Influence of As^{III} on the Accumulation of $^{75}Se^{IV}$ by Human RBCs

One mechanism for the mutual protection of As and Se observed in laboratory animals, is sequestration of both compounds in RBCs as $[(GS)_2AsSe]^-$ [16]. To our knowledge this sequestration has never been reported in human RBCs. To begin to investigate this, $^{75}Se^{IV}$ accumulation was measured under different conditions in the presence and absence of As^{III} (Figure 2-3). The influence of As^{III} (1 µM) on the accumulation of $^{75}Se^{IV}$ (1.85 kBq, 1 µM) over time was measured (Figure 2-3A).

Unexpectedly, the presence of As^{III} resulted in no difference in $^{75}Se^{IV}$ accumulation, when compared to $^{75}Se^{IV}$ alone.

In blood, Se^{IV} is effluxed from RBCs and bound to plasma proteins (initially albumin and then globular proteins) [48,49]. Plasma proteins likely prevent the rapid re-uptake of Se by the RBCs. To determine the influence of physiological concentrations of albumin on the sequestration of Se^{IV} and As^{III}, $^{75}Se^{IV}$ (1.85 kBq, 1 μM) accumulation in the presence and absence of As^{III} (1 μM) with BSA (35 mg/ml) was measured (Figure 2-3B). At 10, 20, and 40 minutes, there was 1.5-, 2.3-, and 2.9-fold higher $^{75}Se^{IV}$ accumulation, respectively, in the presence of As^{III} compared to As^{III}-free conditions. These data suggest that to detect a difference between ± As^{III} conditions, the reuptake of ^{75}Se by RBCs must be prevented by the inclusion of albumin in the assay. This suggestion is supported by the fact that efflux of $^{75}Se^{IV}$ from preloaded human RBCs was not detected in the absence of BSA (Figure 2-3C).

The concentration dependence of As^{III} on $^{75}Se^{IV}$ accumulation was next evaluated using a single concentration of $^{75}Se^{IV}$ (1.85 kBq, 0.3 μM) and increasing concentrations of As^{III} (0.1-10 μM) for a 20 min time point in the presence of BSA (35 mg/ml) (Figure 2-3D). A significant increase in $^{75}Se^{IV}$ accumulation was observed with increasing concentrations of As^{III} starting at 1 μM (Figure 2-3D). This suggests that the presence of As^{III} can result in the sequestration of Se in RBCs even at Se^{IV} levels as low as 0.3 μM.

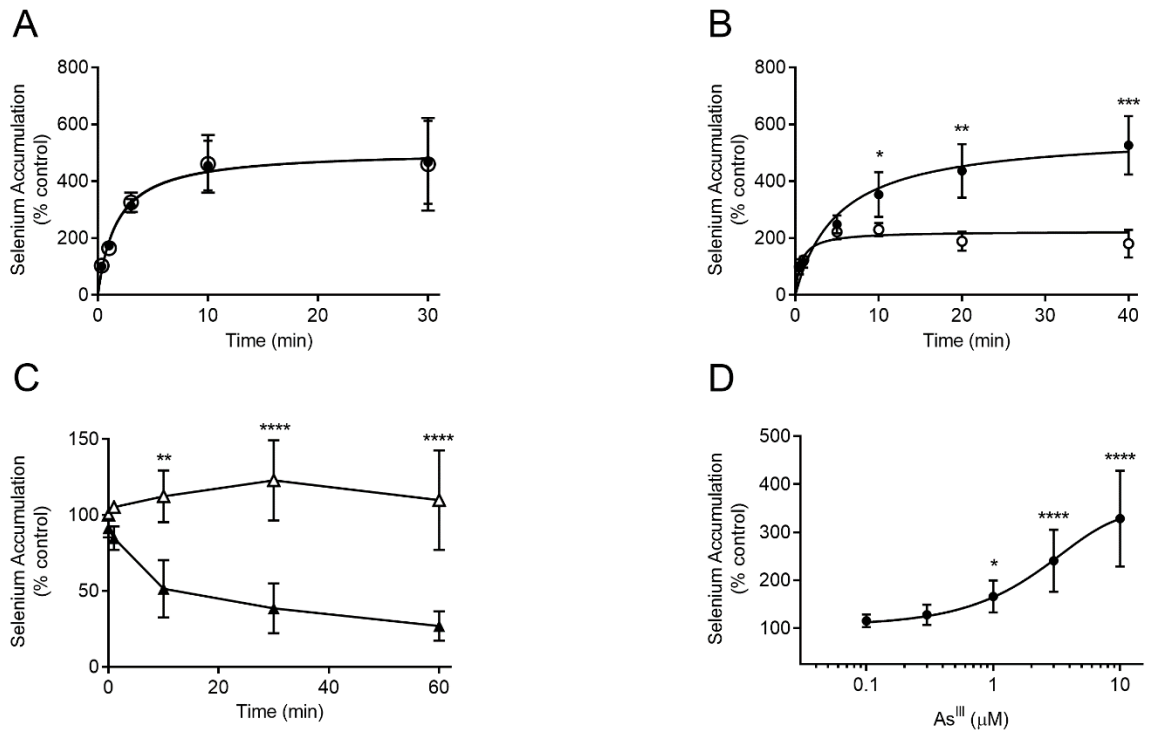

Figure 2-3. The influence of As^{III} on $^{75}Se^{IV}$ accumulation by human RBCs. Unless otherwise indicated, human RBCs at a 10% hematocrit in 200 µl of the indicated buffer were incubated at 37ºC with $^{75}Se^{IV}$ (1 µM, 1.85 kBq) for the indicated time point. RBC suspensions were then centrifuged at 6800 *g* for 1 min at 4ºC, pelleted RBCs were then washed twice with 1 ml of 4ºC isotonic saline. $^{75}Se^{IV}$ accumulated was then quantified by counting samples on a gamma counter. (**A**) Time course of $^{75}Se^{IV}$ (1 µM, 1.85 kBq) uptake in the presence (●) and absence (○) of As^{III} (1 µM) in isotonic saline. Symbols represent mean of three independent experiments (± S.D.). (**B**) Time course of $^{75}Se^{IV}$ (1 µM, 1.85 kBq) uptake in the presence (●) and absence (○) of As^{III} (1 µM) in Ringer's solution plus BSA (35 mg/ml). Symbols represent means of five independent experiments (± S.D.). Significant differences between ± As^{III} conditions are indicated with * P < 0.05, ** P < 0.01, *** P < 0.001 (ANOVA with Sidak's multiple comparisons test). (**C**) Human RBCs were incubated with $^{75}Se^{IV}$ (1 µM, 1.85 kBq/data point) for 20 min at 37ºC, then washed and resuspended at a 10% haematocrit in ice cold Ringer's buffer with (▲) and without (Δ) BSA (35 mg/ml). RBCs were then incubated at 37ºC for the indicated time points and the $^{75}Se^{IV}$ remaining quantified. Symbols represent means of three independent experiments (± S.D.). Significant differences between ± BSA conditions are indicated with ** P < 0.01, **** P < 0.0001, (ANOVA with Sidak's multiple comparisons test). (**D**) Dose dependence of As^{III} (0.1-10 µM) on $^{75}Se^{IV}$ (0.3 µM, 1.85 kBq) accumulation in human RBCs was measured in Ringer's buffer containing BSA (35 mg/ml) at a 20 min time point. Data are expressed as a % of the 0 As^{III} control and symbols represent means of four independent experiments (± S.D.). Significant differences between the ± As^{III} conditions are indicated with * P < 0.05, **** P < 0.0001, (ANOVA with Dunnett's multiple comparisons test).

2.4.4. The Influence of Se^{IV} on the Accumulation of $^{73}As^{III}$ by Human RBCs

After finding that As^{III} increases the accumulation of Se^{IV}, the ability of Se^{IV} to increase the accumulation of $^{73}As^{III}$ (1.85 kBq, 1 μM) was measured (Figure 2-4). At 15, 30 and 45 minutes, there was 1.8-, 2.3-, and 2.0-fold higher $^{73}As^{III}$ accumulation, respectively, in the presence of Se^{IV} compared to Se^{IV}-free conditions (Figure 2-4A). Unlike As^{III}-stimulated $^{75}Se^{IV}$ accumulation, there was no requirement for BSA for Se^{IV}-stimulated $^{73}As^{III}$ accumulation.

The concentration dependence of Se^{IV} on $^{73}As^{III}$ accumulation was next evaluated using a single concentration of $^{73}As^{III}$ (1.85 kBq, 0.3 μM) and increasing concentrations of Se^{IV} (0.1-10 μM) over 15 min (Figure 2-4B). Consistent with $^{75}Se^{IV}$ accumulation shown in Figure 2-3D, a significant increase in $^{73}As^{III}$ accumulation was observed with increasing concentrations of Se^{IV} starting at 1 μM (Figure 4B). This suggests that Se^{IV} can result in the sequestration of As^{III} in RBC even at As^{III} levels as low as 0.3 μM.

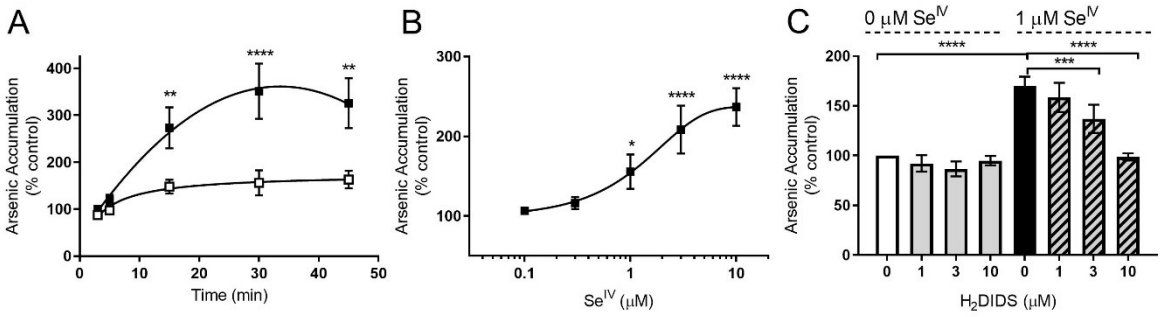

Figure 2-4. The influence of Se^{IV} on $^{73}As^{III}$ accumulation by human RBCs. Human RBCs at 10% hematocrit in 200 μl of the indicated buffer were incubated at 37ºC with $^{73}As^{III}$ (1 μM, 1.85 kBq) for the indicated time point. RBC suspensions were then cenrifuged at 950 *g* for 10 min at 4ºC, pelleted RBCs were then washed twice with 1 ml of 4ºC isotonic saline. $^{73}As^{III}$ accumulated was then quantified by counting samples on a gamma counter. (**A**) Time course of $^{73}As^{III}$ (1 μM, 1.85 kBq) uptake in the presence (■) and absence (□) of Se^{IV} (1 μM) in isotonic saline. Symbols represent means of three independent experiments (± S.D.). Significant differences between the ± Se^{IV} conditions are indicated with ** P < 0.01, and **** P < 0.0001 (ANOVA with Sidak's multiple comparisons test). (**B**) Dose dependence of Se^{IV} (0.1-10 μM) on $^{73}As^{III}$ (0.3 μM, 1.85 kBq) accumulation in human RBCs was measured in isotonic saline at a 15 min time point. Symbols represent means of three independent experiments (± S.D.). Significant differences between the concentrations, compared with 0.1 μM Se^{IV}, are indicated with * P < 0.05, and **** P < 0.0001 (ANOVA with Dunnett's multiple comparisons test). (**C**) Human RBCs at 10% hematocrit in 200 μl of isotonic saline

were incubated at 37ºC with $^{73}As^{III}$ (1 μM, 1.85 kBq) for 15 min either untreated (open bar), treated with H_2DIDS only (1, 3 , or 10 μM, gray bars), treated with Se^{IV} only (1 μM, black bar), or Se^{IV} (1 μM) plus H_2DIDS (1, 3 , or 10 μM, hatched gray bars). Bars represent means of four independent experiments (± S.D.). Treatment with Se^{IV} significantly increased $^{73}As^{III}$ accumulation (****P < 0.0001) compared to untreated control and Se^{IV}-dependent $^{73}As^{III}$ accumulation was significantly inhibited by H_2DIDS at 3 μM (***P < 0.001) and 10 μM (****P < 0.0001) (ANOVA with Sidak's multiple comparisons test).

2.4.5. H_2DIDS Inhibits Se^{IV}-dependent As^{III} Accumulation

The influence of H_2DIDS on Se^{IV}-dependent $^{73}As^{III}$ accumulation was next investigated. H_2DIDS (1, 3, and 10 μM) had no inhibitory effect on $^{73}As^{III}$ (1 μM) accumulation by human RBCs (Figure 2-4C, open bar versus light gray bars). Consistent with data in Figure 2-4A and B, Se^{IV} increased the accumulation of $^{73}As^{III}$ by 1.7-fold (Figure 2-4C open bar versus black bar). An H_2DIDS dose-dependent reduction in Se^{IV}-dependent $^{73}As^{III}$ accumulation was observed and at 10 μM H_2DIDS there was no significant difference between the $-Se^{IV}$ condition and the $+Se^{IV}$, suggesting that inhibition of Se^{IV} uptake completely prevented the Se-dependent accumulation of ^{73}As (Figure 2-4C).

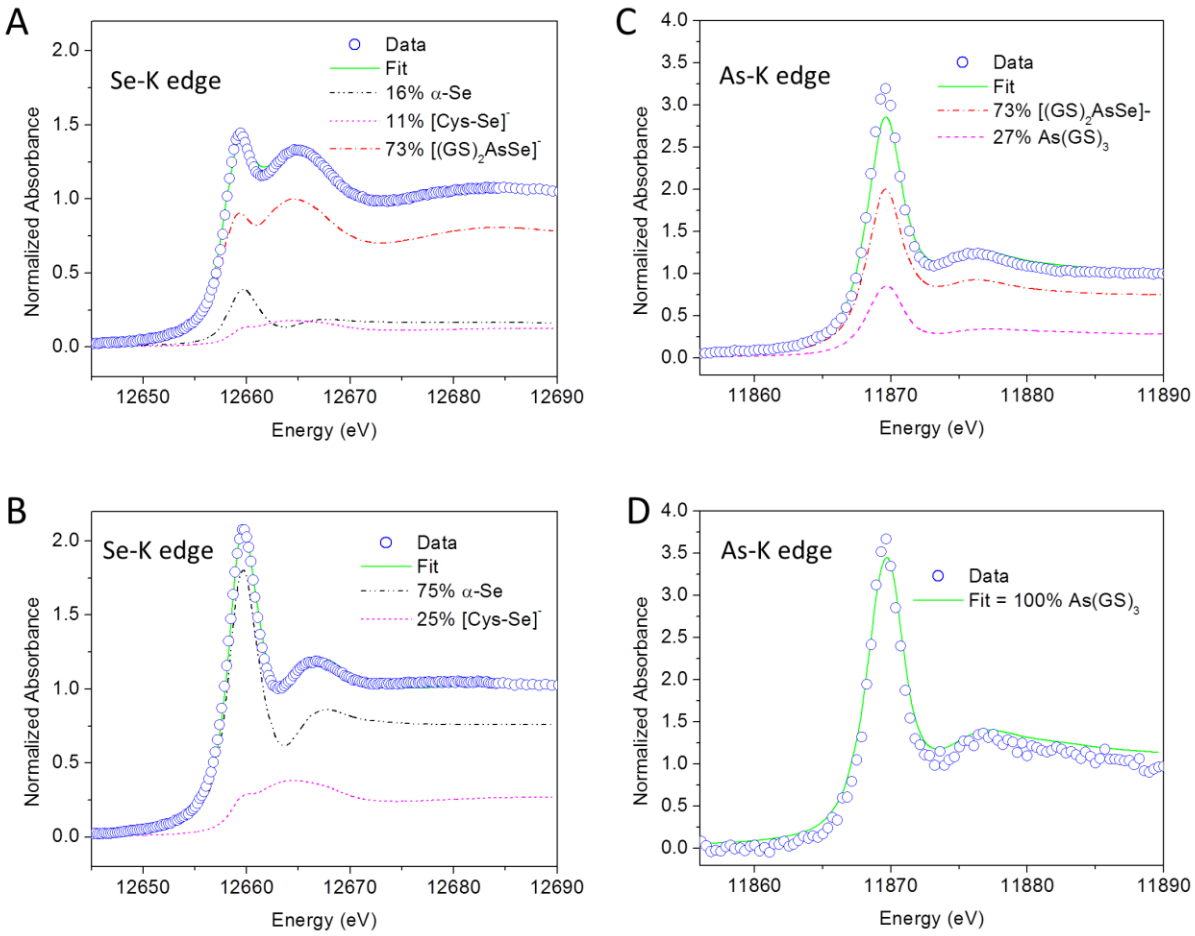

Figure 2-5. Near edge XAS of human RBCs exposed to As^{III} and Se^{IV}. Human RBCs were exposed to either 30 µM As^{III} and 30 µM Se^{IV} or to their combination for 45 min. Se K near-edge XAS of human RBCs exposed to **(A)** combination of As^{III} and Se^{IV} and, **(B)** Se^{IV} alone. Arsenic K near edge XAS of human RBCs exposed to **(C)** combination of As^{III} and Se^{IV} and **(D)** As^{III} alone. The data are represented by the open circles, the linear combination fit is denoted by a green solid line and the components of the fit are scaled according to their percentage contribution to the fit as identified in the labels. α-Se, alpha phase of elemental selenium; $[Cys\text{-}Se]^-$, selenocysteinate; $As(GS)_3$, tris-glutathione As^{III}; $[(GS)_2AsSe]^-$, seleno-bis(*S*-glutathionyl) arsinium ion. $[Cys\text{-}Se]^-$ standard fit can be interpreted as selenium in the form of a selenide $[R\text{-}Se]^-$ or a selenopersulfide $[R\text{-}S\text{-}Se]^-$.

2.4.6. Arsenic and Selenium Near-edge XAS of Human RBCs Treated with As^{III} and/or Se^{IV}

Bulk near-edge XAS was used to determine Se and As speciation in human RBC samples exposed either to As^{III} and Se^{IV} in combination (30 μM each) or individually As^{III} (30 μM) or Se^{IV} (30 μM), followed by flash freezing in an isopropanol bath in liquid nitrogen.

Analysis of the Se K near-edge XAS spectrum collected from the dual treatment scenario using least square fitting by linear combination of standard spectra suggested that ~73% of all Se present in the human RBCs treated with As^{III} and Se^{IV}, was in the form of $[(GS)_2AsSe]^-$ (Figure 2-5A). The remaining 27% was fit by the combination of 16% alpha phase of elemental selenium (α-Se) and 11% selenocysteinate $[Cys\text{-}Se]^-$ standards. Contrastingly, the Se K near-edge spectrum collected from the human RBC exposed to Se^{IV} as a single treatment, was best fit by the combination of 75% α-Se and 25% $[Cys\text{-}Se]^-$ (Figure 2-5B). XAS is sensitive to the type of bonding environment of the absorbing atom, therefore the data generated from the $[Cys\text{-}Se]^-$ standard fit can be interpreted as selenium in the form of a selenide $[R\text{-}Se]^-$ or a selenopersulfide $[R\text{-}S\text{-}Se]^-$.

Analysis of the As K near-edge XAS linear combination fit of the spectrum collected from the sample treated with both As^{III} and Se^{IV} showed that ~73% of all As present in the human RBCs was in the form of $[(GS)_2AsSe]^-$, with remaining 27% in form of *tris*-S-glutathionyl As^{III} ($As(GS)_3$) (Figure 2-5C). The sample of human RBC treated with As^{III} alone was well represented by 100% $As(GS)_3$ standard spectrum (Figure 2-5D). Consistent with previous reports that human RBCs do not methylate As [50], trivalent and pentavalent methylated As species were not detected in XAS measurements.

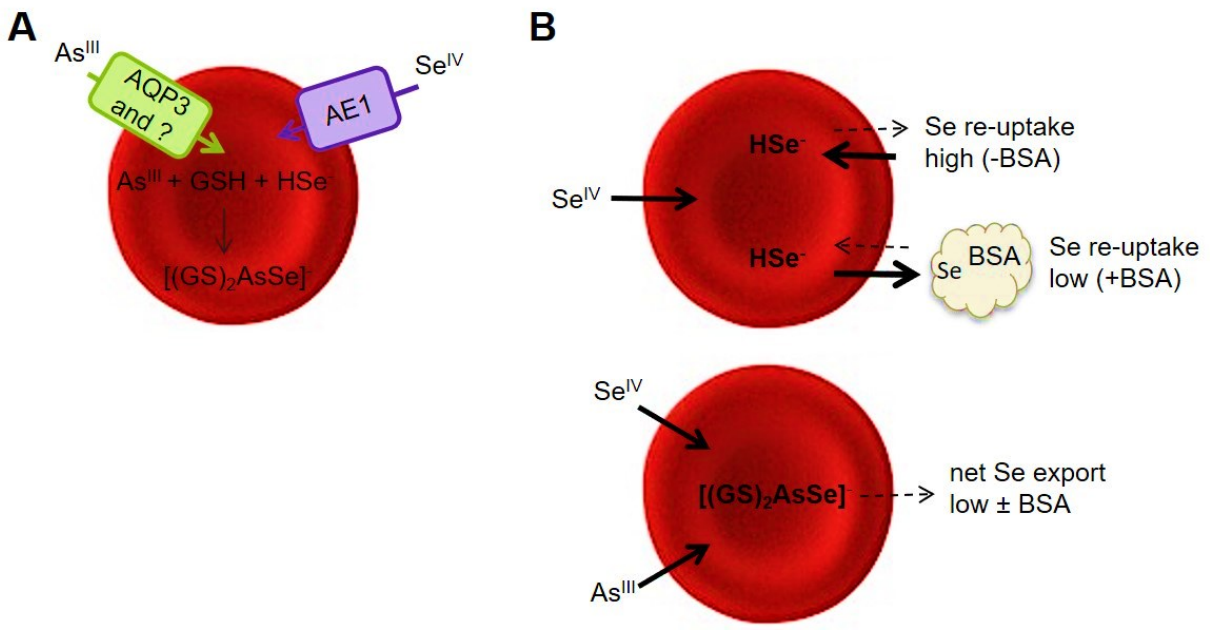

Figure 2-6. Summary of human RBC (A) As^{III} and Se^{IV} uptake pathways and (B) mechanism of As-dependent $^{75}Se^{IV}$ accumulation in the presence and absence of bovine serum albumin (BSA). (A) Data from this and other studies strongly support that the anion exchanger 1 (AE1/SLC4A1) is the major Se^{IV} uptake transport pathway in human RBCs [32,33]. AQP3 inhibitors Auphen and $[Au(PbImMe)Cl_2]PF_6$ partially reduced the accumulation of As^{III} while the GLUT1 inhibitor BAY-876 had no effect. These data suggest that AQP3 is likely involved in As^{III} accumulation by human RBCs, but additional transport pathways (or simple diffusion) are likely involved. Once inside the human RBC, Se^{IV} is rapidly converted to hydrogen selenide (HSe^-) and in the presence of As^{III} and reduced GSH (present at mM levels in RBCs) the seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ is formed. For simplicity, the chemical reactions involved in the formation of HSe^- and $[(GS)_2AsSe]^-$ have been omitted, and the reader is referred to [16]. **(B)** (top panel) Se^{IV} is accumulated by human RBCs and rapidly converted to HSe^- which is subsequently exported from RBCs where it is bound to plasma albumin [33,48,49,51]. In the absence of BSA the net export of Se is low due to rapid re-uptake of Se by the human RBCs. When physiological concentrations of BSA were included, a net loss of Se from human RBCs was observed, likely due to a reduced Se re-uptake after binding BSA (for simplicity, transport pathways for As^{III} and Se^{IV} have been left off). (bottom panel) In the presence of As, the accumulation of Se was high due to the formation of $[(GS)_2AsSe]^-$, this occurred in the presence and absence of physiological concentrations of BSA.

2.5. Discussion

Chronic exposure to As is a global health concern of massive proportions, affecting millions of people world-wide [1]. In humans, As has been proven to cause skin, lung, and bladder cancers and is associated with increased incidences of kidney, liver and prostate tumours [2]. Animal and human studies suggest that Se supplementation is a promising therapy to prevent and treat adverse health

effects of chronic As exposure [9,10,22,24–27,52]. In animal models, the formation of $[(GS)_2AsSe]^-$ is thought to be the molecular basis for As and Se mutual detoxification. $[(GS)_2AsSe]^-$ formation is protective through two major mechanisms; first through its biliary excretion, and second, through its sequestration in RBCs [16]. To begin to address the paucity of knowledge with regard to the influence of Se on As (and vice versa) handling in humans, the present study focused on As and Se transport and metabolism in human RBCs. The human RBC accumulation of Se^{IV} and As^{III} individually and in combination was characterized. Near-edge XAS provided the first evidence of $[(GS)_2AsSe]^-$ and $As(GS)_3$ formation in human cells. No detectable methylated arsenicals were observed using the XAS method. The major findings in this chapter are summarized in Figure 2-6A.

Se^{IV} uptake by human RBCs was H_2DIDS sensitive and stimulated by an outward Cl^- gradient providing evidence that AE1 is important for human RBC Se^{IV} uptake. Compared with empty vector controls, AE1-overexpressing HEK293 cells showed increased concentration dependent Se^{IV} accumulation, at physiologically relevant concentrations. In combination, these results provide strong support that AE1 is the major pathway for Se^{IV} uptake by human RBCs (Figure 2-6A).

Recently it has been reported that ZIP8 plays a role in Se^{IV} uptake, experiments in mammalian cell lines, *X. laevis* oocytes and mouse models showed Se^{IV} uptake to be associated with ZIP8, HCO_3^- and Zn^{2+} levels [34]. Human RBCs showed a lack of Zn^{2+}-dependent Se^{IV} uptake (Figure 2-1E), and the exclusion of HCO_3^- from the Ringer's solution had no effect on Se^{IV} uptake (Figure 2-1C), indicating ZIP8 is not involved in human RBC Se^{IV} uptake.

The GLUT1 inhibitor, BAY-876, did not inhibit As^{III} uptake by human RBCs even at concentrations 500 to 50,000-times higher than the IC_{50} reported for inhibition of glucose uptake [46]. This result was somewhat surprising because rat GLUT1, overexpressed in both yeast and *X. laevis* oocytes, increases the efficiency of As^{III} accumulation, and GLUT1 is found at high levels in human RBCs [53,54]. As^{III} may use a different GLUT1 translocation pathway than glucose, and therefore GLUT1 could allow

permeation of As^{III} even in the presence of BAY-876 [55]. Thus, our data cannot rule out the involvement of GLUT1. Firm conclusions about GLUT1 involvement in As^{III} uptake by human RBCs await the development of different tools to study GLUT1 As^{III} transport *in situ*. As^{III} uptake by human RBCs was partially inhibited by the AQP3 inhibitors Auphen and $[Au(PbImMe)Cl_2]PF_6$, suggesting that AQP3 may be important for uptake of As^{III}. The high concentrations required, and incomplete inhibition observed suggest that additional uptake pathways are likely involved. The low temperature sensitivity combined with incomplete AQP3 inhibition might suggest a simple diffusion component or a different transport pathway (Figure 2-6A).

In earlier studies, As^{III} delayed the clearance of Se^{IV} from rat blood by ~20 hours [19]. Subsequent animal and *in vitro* studies have shown the presence of $[(GS)_2AsSe]^-$ in RBCs to be the molecular basis of As and Se sequestration [16,20]. To investigate the influence of As^{III} on Se^{IV} accumulation in human RBCs, $^{75}Se^{IV}$ uptake assays were conducted in the presence and absence of As^{III}. Surprisingly, there was no effect on Se^{IV} uptake in the presence of As^{III} (Figure 2-3A). In blood, Se^{IV} is rapidly taken up by RBCs and reduced to hydrogen selenide. Hydrogen selenide is unstable at physiological pH but stabilized by excess GSH levels in cells. Selenide is then effluxed from RBCs and bound to plasma albumin, likely preventing the immediate reuptake of Se by the RBCs [33,48,49,51] (Figure 2-6B). The increased accumulation of $^{75}Se^{IV}$ observed in the presence of As^{III} with a physiologically relevant concentration of albumin present in the extracellular media supports the theory that albumin present in the extracellular matrix would halt the recycling process of Se, resulting in the increased accumulation of Se in the presence of As to be evident (Figures 2-3B and 2-6B). Consistent with published literature [43], further characterization of the influence of albumin on $^{75}Se^{IV}$ efflux showed that $^{75}Se^{IV}$ efflux is albumin-dependent (Figure 2-3C). This is likely due to the concentration gradient of free Se being maintained as most effluxed Se is rapidly albumin-bound (Figure 2-6B).

Consistent with the As^{III} influence on $^{75}Se^{IV}$ accumulation, Se^{IV} also increased $^{73}As^{III}$ accumulation in human RBCs. This supports the theory that sequestration of As and Se in RBCs occurs through the formation of the As-Se complex, $[(GS)_2AsSe]^-$. Moreover, $^{73}As^{III}$ accumulation, at an environmentally relevant dose (0.3 μM), increased as Se^{IV} concentration increased; consistent with previously discussed As^{III}-dependent $^{75}Se^{IV}$ accumulation. As expected, the AE1-inhibitor, H_2DIDS did not affect $^{73}As^{III}$ accumulation. However, H_2DIDS diminished Se^{IV}-dependent $^{73}As^{III}$ accumulation in a concentration-dependent manner. This provides further evidence for the role of AE1 in Se^{IV} uptake and sequestration with As^{III} in human RBCs.

While the mutually increased accumulation of As^{III} and Se^{IV} provide support for the reciprocal detoxification by reducing the levels of free As^{III} present in blood and tissues, the molecular basis for this sequestration in human RBCs had not been investigated. Near-edge XAS data (Figure 2-5) showed that the majority of As and Se present in human RBCs in the dual treatment scenario were in the form of the single ion complex, $[(GS)_2AsSe]^-$. This provides the first evidence for the formation of the $[(GS)_2AsSe]^-$ complex in human tissue.

Numerous animal studies have investigated As and Se interactions. However, due to their toxicokinetic differences, humans are more sensitive to arsenic toxicity than rodents [56,57], resulting in a lack of good animal models for arsenic-induced carcinogenesis [58,59]. This work provides valuable information on the uptake pathways and mutual sequestration of As and Se in human RBCs. Selenite and As^{III} increased the accumulation of each other from doses as low as 0.3 μM, mimicking environmental exposures. Uptake of As^{III} was found to be partially AQP3 mediated, while uptake of Se^{IV} was found to be AE1-mediated. While inorganic As is the predominant form of As in drinking water, humans chronically exposed to As in drinking water can have multiple forms of inorganic and methylated As in their blood. Our future work will consider the influence of methylated As species on human RBC sequestration of these species. Understanding how human RBCs sequester

$[(GS)_2AsSe]^-$ and the different pathways involved, ultimately provides valuable information for influencing these pathways to prevent and treat As-induced disease.

2.6. Bibliography

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3. Selenium Influence on the Hepatobiliary Efflux of Arsenic in Sandwich Cultured Human Hepatocytes

3.1. Abstract

Arsenic is a proven human carcinogen, causing cancers of the skin, lung and bladder. More than two hundred million people worldwide are exposed to levels of arsenic above the World Health Organization guideline of 10 μg/L. Yet, the cellular handling of arsenic remains inadequately understood. Selenium is an essential trace element that is known to interact with As, to form the the seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$. In contrast to other arsenic-glutathione metabolites, $[(GS)_2AsSe]^-$ is stable at biliary pH and has the potential to break the enterohepatic recirculation loop of As and promote its fecal elimination. Sandwich cultured human hepatocytes (SCHH) were used as a physiological model to characterize the influence of selenium on human arsenic hepatobiliary transport. Six out of eight SCHH preparations showed both canalicular and sinusoidal efflux of ^{73}As-arsenic at 10 min, while two out of six SCHH preparations showed only sinusoidal efflux. In the presence of selenite, sinusoidal efflux of ^{73}As was decreased or unchanged, while biliary efflux was decreased. Selenide, the reduced form of selenite, also decreased or did not change the sinusoidal efflux of ^{73}As but biliary efflux was unchanged or increased. Preliminary data from two independent SCHH preparations showed that selenide accumulation in hepatocytes was at least 59% higher than selenite after 24 hrs; and when treated with ^{75}Se-selenide ± arsenite, efflux of ^{75}Se was increased in the presence of As. Selenite did not protect hepatocytes against arsenite cytotoxicity except when biliary efflux of ^{73}As-arsenic was observed from as early as 3 min. In one SCHH preparation tested, selenide protected cells against arsenite toxicity, while selenite did not. These results suggest that selenide is likely more efficiently taken up by SCHH than selenite, and selenide is the species of selenium responsible for the mutual antagonism with arsenic.

3.2. Introduction

Contamination of drinking water sources with inorganic arsenic (As) is a global health concern [1]. More than 200 million people worldwide are exposed to levels of this Group 1 carcinogen, greater than the World Health Organization limit of 10 μg/L [2]. The predominant forms of As in drinking water are arsenite and arsenate [3]. Upon uptake into cells, these arsenicals undergo a series of reduction followed by oxidative methylation reactions to yield monomethylated and dimethylated pentavalent and trivalent As species, namely: monomethylarsonic acid (MMA^{V}), monomethylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^{V}), and dimethylarsinous acid (DMA^{III}) [4,5]. The trivalent metabolites are toxic intermediaries which can react with glutathione (GSH) to form As-GSH complexes which undergo cellular efflux through the multidrug resistance proteins (MRPs) [6]. Approximately 60-80% of As consumed undergoes urinary elimination, of which 60-70% is dimethylated forms of As, 10-20% monomethylated and 10-30% inorganic arsenic [7–9].

The majority of As metabolism takes place in the liver [10], where MRP4 located on the sinusoidal surface of hepatocytes, and MRP2, located on the apical surface, efflux As-GSH conjugates out of hepatocytes into the sinusoid and the canalicular lumen, respectively [11–13]. Arsenic-GSH conjugates are not stable at biliary pH, and dissociation is thought to occur [14,15]. This leads to the reuptake of As in the gut and enterohepatic recirculation until it is eliminated via urine [16,17].

Selenium (Se) is an essential trace element that is known to interact with As, to form the the seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ [18]. $[(GS)_2AsSe]^-$ is more stable at biliary pH than As-GSH conjugates, and has the potential to break the enterohepatic recirculation loop of As and promote its fecal elimination [6,15]. Animals treated with arsenite and selenite show the presence of $[(GS)_2AsSe]^-$ in bile and the Leslie lab has identified $[(GS)_2AsSe]^-$ efflux to be human MRP2-mediated, at least *in vitro* [13,15,19]. In animal models, it is also seen that co-treatment with arsenite and selenite

resulted in increased levels of As and Se in bile and lower amounts of As and Se being retained in the liver [20,21]. However, little work has been done in human cell lines. Despite this, Se supplementation trials are in place in As-endemic regions to investigate the effects of Se supplementation [22–24].

Organic forms of Se, such as selenomethionine (SeMet) and methylselenocysteine (MeSeCys) are found in the diet in grains, vegetables and legumes [25]. Selenomethionine is a major component of selenized yeast and enriched lentils, which are commonly used in Se-supplementation trials [24,26,27]. Methylselenocysteine is dominant in cruciferous vegetables and is also used in dietary selenium supplement tablets [28,29]. Clinical trials using purified L-SeMet have shown that it improves arsenic-induced skin lesions, and may alter genetic expression profiles [30,31]. Studies with selenized yeast have shown decreased As content in hair, nails and urine in As-exposed individuals in comparison to controls [22]. Studies with selenium enriched lentils have shown an increased urinary elimination of DMA^V [32].

Since human trials are in place to study the effects of Se on As-induced toxicities, this makes it crucial to develop physiologically relevant *in vitro* human models for As and Se studies to better understand the cellular handling of these compounds. Since hepatocytes are the major site of As methylation and subsequent export to the systemic circulation, the sandwich-cultured hepatocyte (SCH) model was used in this study to evaluate As and Se interactions [11,33,34]. Hepatocytes cultured between two layers of collagen retain cell polarity, and establish extensive canalicular networks [34]. Biliary efflux in the sandwich culture model is strongly correlated with *in vivo* biliary clearance in humans and rats [35,36].

The first aim of this study was to use sandwich cultured human hepatocytes (SCHH) to investigate the effects of selenite on As efflux. The second aim was to determine the effects of different Se species on As cytotoxicity and efflux. Our hypothesis was that Se would protect against As cytotoxicity

through increasing its biliary efflux. The results presented in this study suggest that different chemical species of Se influence the cytotoxicity and efflux of As differently, possibly due to accumulation differences. Our results add to the increasing body of evidence suggesting that selenide, in comparison with selenite, is more efficiently taken up by hepatocytes, as it is likely the form presented to the liver after metabolism in enterocytes and RBCs (36-38) (Chapter 4). This has important implications for future *in vitro* studies involving As and suggests that the form of Se used in both *in vitro* experimental studies and supplemental form needs to be carefully considered.

3.3. Materials and Methods

3.3.1. Chemicals and Reagents

[^{3}H(G)]-Taurocholic acid was purchased from PerkinElmer Life and Analytical Sciences (Woodbridge, Ontario, Canada). ^{73}As-arsenate was purchased from Los Alamos Meson Production Facility (Los Alamos, New Mexico). ^{75}Se-selenite (25.7 mCi/ml) was obtained from the University of Missouri Research Reactor (MURR, Columbia, MO).

ITS+ (insulin, transferrin and selenium), BioCoat culture plates and Matrigel were purchased from Fisher/BD Biosciences (Franklin Lakes, New Jersey). Phenol red-free Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, human recombinant insulin, modified essential media nonessential amino acids, L-glutamine were purchased from Life Technologies (Carlsbad, California).

Sodium selenite, sodium (meta)arsenite, fetal bovine serum, taurocholate, Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), Hanks' balanced salt solution (HBSS), dexamethasone, bovine serum albumin, glutathione (GSH), buthionine sulfoximine (BSO), sodium bicarbonate (HCO_3^-), 5(6)-Carboxy-2',7'-dichlorofluorescein (CDF) diacetate, and methyl selenocysteine (MeSeCys) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Sodium

selenide was purchased from Alfa Aesar (Ward Hill, MA). Triton X-100 was purchased from Fisher Scientific (Fair Lawn, NJ). Selenomethionine (SeMet) was purchased from BioVision (Miltipas, CA).

The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemicals (Rockford, Illinois). The lactate dehydrogenase (LDH) cytotoxicity detection kit was purchased from Roche, Applied Sciences (Indianapolis, Indiana).

3.3.2. Human Hepatocyte Culture[†]

With the approval of the University of Alberta human ethics review board (protocol number 00001646), human hepatocytes were isolated from the liver of patients undergoing hepatic resection at the University of Alberta Hospital. These patients did not have any significant liver nor infectious disease (HepB/HepC/HIV). Hepatocytes were counted and cell viability was determined using trypan blue exclusion. Hepatocytes with viability greater than 85% were seeded in 12-well Biocoat culture plates at 6.25 x 10^5 cells/well in seeding media (phenol red-free DMEM supplemented with 5% FBS, 0.1 mM modified essential medium nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 μg/ml human recombinant insulin, 1 μM dexamethasone). Cells were maintained at 37ºC in a humidified incubator with 95% air/5% CO_2. Cells were allowed to attach for 1- 3 hrs, after which, seeding media was replaced with growth media (phenol red-free DMEM supplemented as above with dexamethasone at 0.1 μM and the addition of ITS+ (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium) and no FBS for remaining time in culture. On day 2 of culture (12-16 hrs later), cells were overlaid with 0.25 mg/ml Matrigel in ice-cold medium. Growth media was changed daily for the remaining time in culture. SCHH were cultured for 6 or 7 days prior

[†] Gurnit Kaur completed all experiments with sandwich-cultured human hepatocyte (SCHH) (preparations 26-30 and 34-36). Drs. Norman M. Kneteman and Donna D. Douglas provided hepatocytes for all preparations.

to experiments, to allow for the development of canalicular networks and to limit variability due to donor health and lifestyle.

Cryopreserved human hepatocytes were used for SCHH preparations 35 and 36. Hepatocytes stored in liquid nitrogen were quickly transferred to a 37ºC water bath for 30 sec with loosened cap, followed by gentle shaking of tube with tightened cap in water bath for 1 min. Cells were then transferred to a 50 ml falcon tube. Seeding media (40 ml) was then added drop-wise. Cells were centrifuged at 100*g* for 4 min at room temperature and resuspended in 3 ml of seeding media for viability assessment, counting and seeding was completed as described above.

3.3.3. Fluorescence Microscopy

On day 7 of culture, 5(and 6)-carboxy 2',7'dichlorofluorescein (CDF) accumulation was measured as previously described [11]. Briefly, cells were washed three times with 1 ml HBSS buffer (37ºC) and incubated with 2 μM of CDF-diacetate for 10 min at 37ºC. Non-accumulated CDF was removed by washing cells three times with 1 ml HBSS at 37ºC and viewed under a fluorescence microscope (Olympus, 1X81, Olympus Canada, Richmond Hill, Ontario).

3.3.4. SCHH efflux studies

^{73}As-arsenite was prepared from ^{73}As-arsenate using metabisulfite-thiosulfate reduction, as previously described [40]. ^{75}Se-selenide was prepared by reducing ^{75}Se-selenite in the presence of GSH, as previously described [41]. Briefly, ^{75}Se-selenite was combined with GSH at a molar ratio of at least 1:550 and incubated at room temperature for 20 min, this was supplemented with the indicated concentration of non-radioactive sodium selenide. Excess GSH reduces selenite to selenide in a multistep reaction process resulting in the formation of Se-GSH conjugates as intermediaries; however, the final product has been shown to be predominantly selenide [41–43]. Going forward, this solution with reduced ^{75}Se-selenite and non-radioactive sodium selenide, will be referred to as ^{75}Se-selenide throughout the chapter.

On day 5, SCHH were incubated in media two, with 1 μM ^{73}As-arsenite ± 1 μM selenite/selenide/SeMet/MeSeCys or 1 μM ^{75}Se-selenite/^{75}Se-selenide ± 1 μM arsenite for 24 hours to allow for accumulation and metabolism, prior to initiating efflux. In order to distinguish between sinusoidal and canalicular efflux, efflux was measured either in the presence or absence of calcium using B-CLEAR® technology, as described [11]. In brief, SCHH were washed twice with ice cold HBSS buffer. To initiate efflux, HBSS buffer with 4 mM HCO_3^- (Ca^{2+} free HBSS buffer with 1 mM EGTA and 4.2 mM HCO_3^-) was added to wells in triplicate. Calcium depletion disrupts the canalicular tight junctions, allowing the As or Se contained in the canalicular networks to be released into the media for measurement [33]. Efflux ± Ca was measured at 1, 3, 5, 10 and 15 min by removing 100 μl of the efflux media for scintillation counting. The biliary excretion index (BEI) is a measure of the percent of total efflux that is going into bile. It is determined using the following equation:

$$BEI = \frac{Efflux_{(sinusoidal + canalicular)} - Efflux_{(sinusoidal)}}{Efflux_{(sinusoidal + canalicular)}} \times 100\%$$

As a positive control for hepatocyte canalicular network formation and function, the bile salt [^{3}H(G)]-taurocholate (1 μM, 100 nCi/well) was used in efflux studies as described above, with the exception that accumulation was allowed for 15 min, instead of 24 hr. As an additional control, cytotoxicity assays were performed on each transport well to ensure that observed efflux was not due to cell leakiness and to ensure that there was no toxicity from treatment conditions, 5 μl of media was collected at each efflux time point to measure LDH release. Media from untreated cells was used as a low release control and media from cells treated with 0.1% Triton X-100 was used as a high release control. Cytotoxicity (percentage LDH release) was calculated using the following equation:

$$Cytotoxicity = \frac{LDH_{sample} - LDH_{blank}}{LDH_{Triton\ X\text{-}100} - LDH_{blank}} \times 100\%$$

Efflux data from cells with LDH release greater than 5% were not included, with the exception of SCHH preparation 29, efflux data from cells with LDH release greater than 10% were not included. Upon completion of efflux experiments, cells were washed three times with ice-cold HBSS and lysed in 500 µl of 0.1% Triton X-100 and cell lysate collected for protein determination using a BCA assay.

3.3.5. Selenium Accumulation Studies

The accumulation of ^{75}Se-selenite or ^{75}Se-selenide in the presence and absence of arsenite was measured using SCHH. SCHH were cultured in 12-well plates as described above. Cells were then treated with ^{75}Se-selenite (1 µM, 100 nCi), ^{75}Se-selenide or in combination with arsenite (1 µM) in supplemented culture media for 24 hours. Cells were then washed three times with 1 ml of ice-cold standard HBSS, lysed in 500 µl of 0.5% Triton X-100 and the lysate (250 µl) collected for quantification. Radioactivity was measured using the Packard Cobra II gamma counter. Accumulation was normalized for total protein, determined using a BCA assay.

3.3.6. Cytotoxicity Studies

The viability of SCHH in the presence of arsenite with and without Se species was measured using the tetrazolium based CellTiter96® Aqueous Non-Radioactive Cell Proliferation Assay from Promega (Madison, MI), as previously described [44]. Briefly, cells were seeded in 96-well plates in quadruplicate at the same cell density as used in efflux studies, of 40,000 cells/well and grown in culture as described above. On day 6 of culture, cells were treated with arsenite (0.01-300 µM), selenite (0.01-300 µM), selenide (0.01-300 µM), arsenite + selenite (0.01-300 µM), arsenite + selenide (0.01-300 µM) for 24 hrs. All treatment solutions were prepared in supplemented media. Data analyses were completed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA) and IC_{50} values were determined using the sigmoidal dose response fit.

3.4. Results

3.4.1. Canalicular Networks are Formed and Functional in SCHH

When grown using conventional cell culture techniques, hepatocytes do not retain their morphological properties and lack polarity [45]. The sandwich configuration, where hepatocytes are grown between two layers of collagen, helps these cells maintain their cuboidal shape, correct polarization, form canalicular networks and allows for the correct expression and localization of transport proteins [34]. In order to test canalicular network formation, accumulation of CDF in canalicular networks was investigated. CDF is a multidrug resistance protein 2 (MRP2/*ABCC2*) substrate that is effluxed into canalicular networks [45,46]. Figure 3-1 shows a light image of SCHH and a fluorescent image of CDF accumulation in networks, suggesting (a) the formation of networks in SCHH and (b) the presence of functional MRP2.

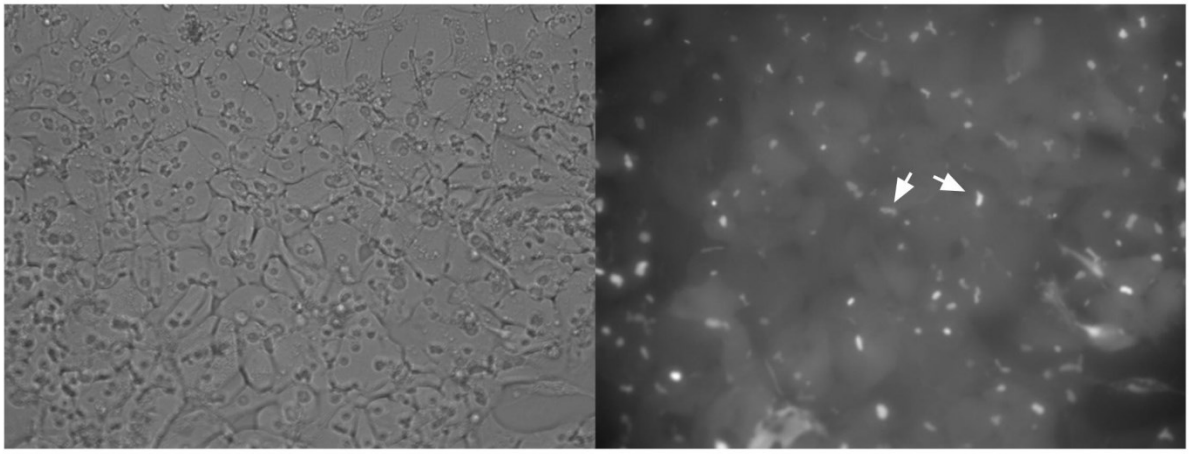

Figure 3-1. Light and fluorescent images of SCHH treated with CDF-diacetate. (Left) Light and (right) fluorescence microscopy images of SCHH#28 on day 7 of culture, incubated with CDF-diacetate (2 µM) in HBSS for 10 min at 37ºC. CDF accumulation (right) in canalicular networks is indicated with arrows. SCHH were viewed with a fluorescence microscope (Olympus 1X81, Olympus Canada). CDF accumulation was also imaged for SCHH#29 and SCHH#30 with similar results.

As an additional measure of canalicular network formation and SCHH transport function, hepatobiliary transport of the bile acid taurocholate was measured for each SCHH preparation. Tight junctions between the canalicular and sinusoidal membranes seal the canalicular networks. Integrity

of these tight junctions depends on the availability of extracellular calcium. Depletion of extracellular calcium disrupts tight junctions, allowing for taurocholate efflux to be measured across both sinusoidal and canalicular surfaces [33]. Taking taurocholate efflux from both surfaces and subtracting sinusoidal only efflux from it, allows measurement of biliary efflux. Figure 3-2 shows taurocholate efflux profiles for each SCHH preparations used in arsenic efflux experiments. Sinusoidal efflux of taurocholate ranged from 3 to 16 pmol mg^{-1} 10 min^{-1}. Taurocholate BEI values were calculated for all preparations and are listed in Table 3-1. The mean taurocholate BEI was 56 ± 14% (range 40 - 74 %) at 10 min, consistent with published literature [11,35].

Table 3-1. Summary of the BEI of taurocholate and arsenic in SCHH and viability of freshly isolated hepatocytes

	10 min BEI (%)	% Viability*	Type

SCHH preparation no.	Taurocholate	Arsenic	Arsenic + Selenite		
26	70	17	15	89	Freshly isolated
27	74	17	NS	>90	Freshly isolated
28	52	37	28	>90	Freshly isolated
29	40	NS	NS	>90	Freshly isolated
30	49	11	NS	100	Freshly isolated
34	69	17	NS	90	Freshly isolated
35	45	25	21	85	Cryopreserved, purchased from Triangle Research Labs
36	68	NS	NS	92	Cryopreserved, purchased from Cellz Direct

NS, not significant.

*Determined using trypan blue exclusion

BEI values were only calculated when the $-Ca^{2+}$ condition was significantly higher ($P<0.05$, student's t-test) than the $+Ca^{2+}$ condition for triplicate determinations from a single experiment.

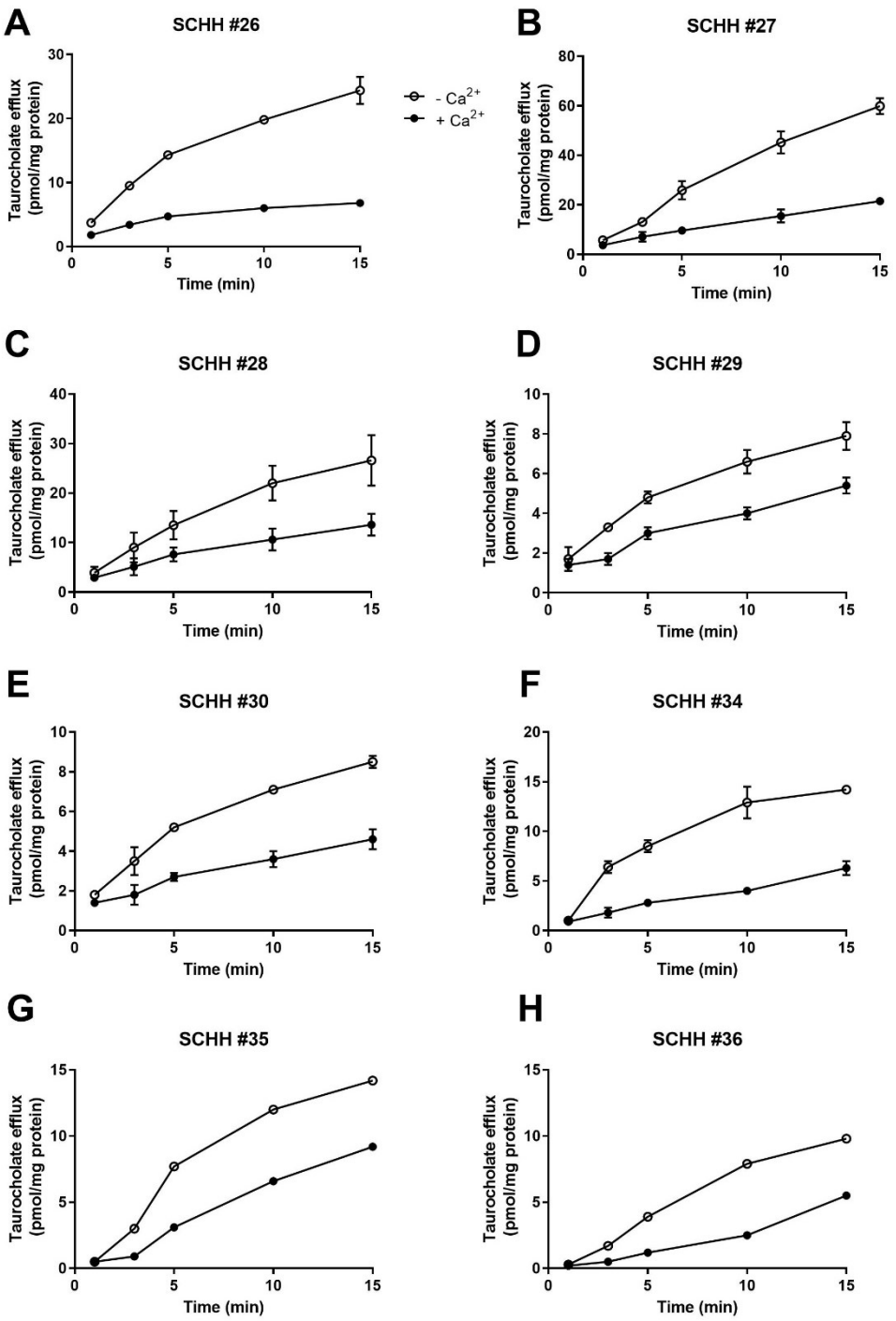

Figure 3-2. Taurocholate efflux from eight different SCHH preparations. SCHH were incubated at 37ºC with [^{3}H(G)]-taurocholate at 1 µM, 100 nCi for 15 min, incubation media was then replaced with HBSS containing Ca^{2+} (sinusoidal efflux ●) or without Ca^{2+} (sinusoidal + canalicular efflux ○), as described in methods. The difference between the closed and open circles represents taurocholate efflux into canalicular networks. Points represent means ± S.D. for triplicate determinations within a single experiment.

3.4.2. Arsenic Efflux is Unchanged or Reduced in the Presence of Selenite

To investigate the hepatobiliary efflux of As, SCHH were treated with 1 μM ^{73}As-arsenite for 24 hrs to allow for accumulation within hepatocytes and canalicular networks. Sinusoidal efflux of As ranged from 5 to 42 pmol mg^{-1} 10 min^{-1} (Figure 3-3). At 10 min, 6 out of 8 SCHH preparations showed differences between As efflux conditions in the presence and absence of Ca^{2+}, indicating there was biliary efflux (Figure 3A, B, C, E, F, G). The BEI values ranged from 11-25% in SCHH preparations 26, 27, 30, 34, 35 (Table 3-1). SCHH preparation 28 showed significant biliary excretion from as early as 3 min, with a BEI value of 35%. Consistently, Roggenbeck et al., reported predominant sinusoidal efflux of As in hepatocyte preparations with only 8 out of 14 preparations displaying biliary excretion at 10 min [11].

Based on the mutually protective effects of arsenite and selenite in animal models, we expected to see increased efflux of As in the presence of Se. However, our investigation on the influence of selenite on As efflux showed at 10 min, SCHH biliary efflux of As in the presence of selenite was reduced by 10-100% across 6 preparations (Figure 3-3A, B, C, E, F, G), whereas sinusoidal efflux was reduced by 20 to 47% across all preparations, with the exception of SCHH preparation 34, where there was no change in efflux when Se was present (Figure 3-3A to E, G and H versus Fig 3-3F). SCHH preparation 28 showed significant biliary excretion of As in the presence of selenite (BEI 28%) at 10 min, however, this BEI was reduced relative to As alone treatment (BEI 37%) (Figure 3-3C and Table 3-1).

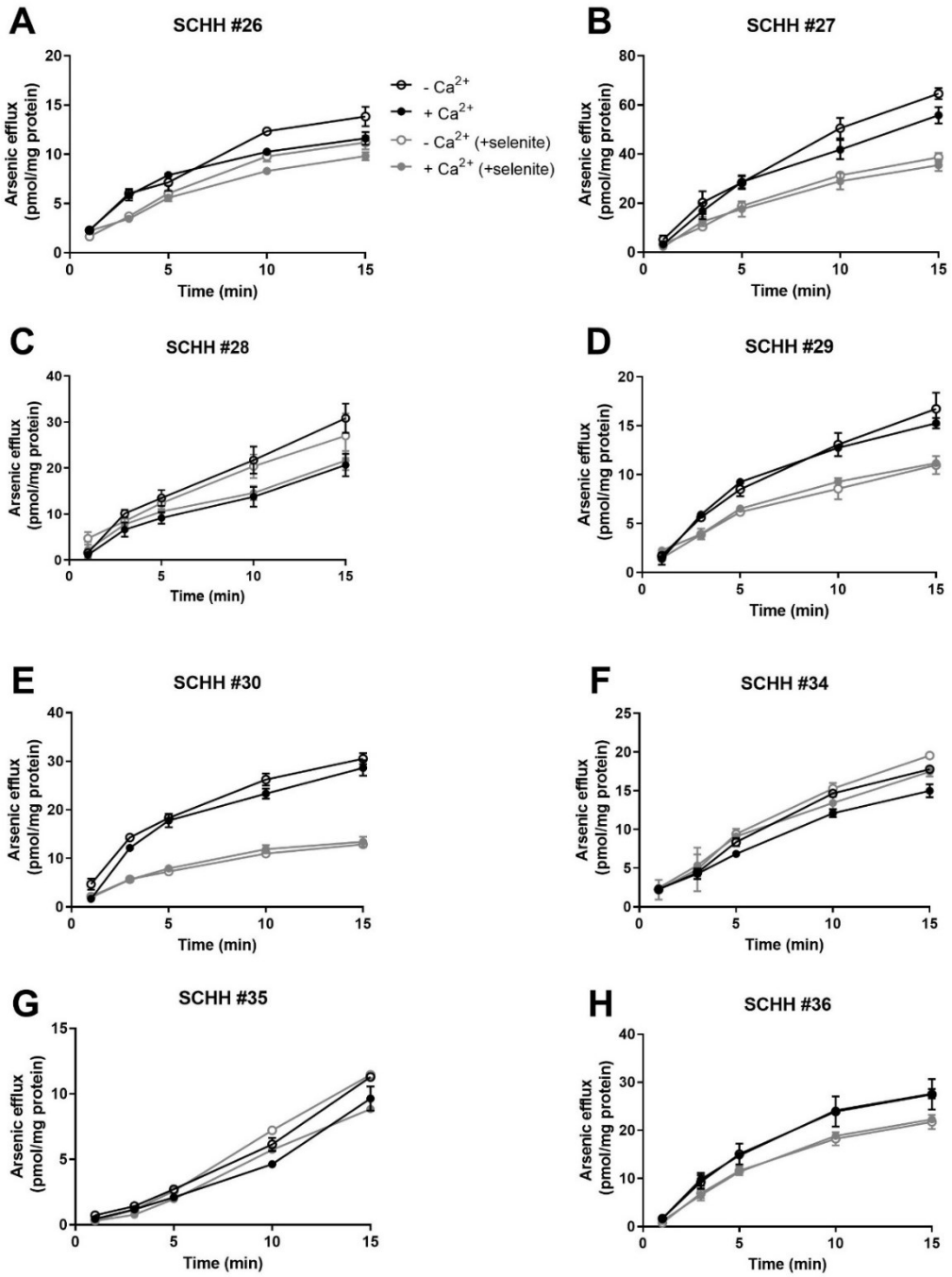

Figure 3-3. Arsenic efflux from eight different SCHH preparations. SCHH were incubated at 37ºC with ^{73}As-arsenite at 1 µM, 100 nCi for 24 hrs (●/○) or ^{73}As-arsenite + selenite at 1 µM, 100 nCi (●/○), incubation media was then replaced with HBSS containing Ca^{2+} (sinusoidal efflux ●/●) or without Ca^{2+} (sinusoidal + canalicular efflux ○/○), as described in methods. The difference between the closed and open circles represents arsenic efflux into canalicular networks. Points represent means ± S.D. for triplicate determinations within a single experiment, while the mean of duplicate values is plotted for the ^{73}As-arsenite + selenite condition of SCHH preparation 35.

3.4.3. Arsenic Biliary Efflux is GSH-dependent

Since inorganic As and methylated metabolites can form GSH conjugates and be effluxed from cells by the MRPs; GSH-dependence of As efflux in the presence of selenite was examined. Consistent with Figure 3-3, where As efflux is inhibited in the presence of selenite, and this inhibition is further pronounced in the presence of BSO, an inhibitor of the rate-limiting step of GSH synthesis. In SCHH preparation 28, which shows biliary efflux of As in the presence and absence of selenite, GSH depletion eliminates all efflux into bile. Sinusoidal efflux of As was also reduced in the presence of BSO with a mean 61 ± 6 % (mean ± SD, n=4) reduction in efflux at 10 min in the presence of Ca^{2+} in the ^{73}As-arsenite only condition, and mean 64 ± 7 % (mean ± SD, n=4) reduction in efflux in the ^{73}As-arsenite + selenite condition, across 4 preparations (SCHH preparations 27-30) (Figure 3-4). These results suggest that As efflux, in the presence and absence of selenite, across the sinusoidal and canalicular membranes is GSH-dependent.

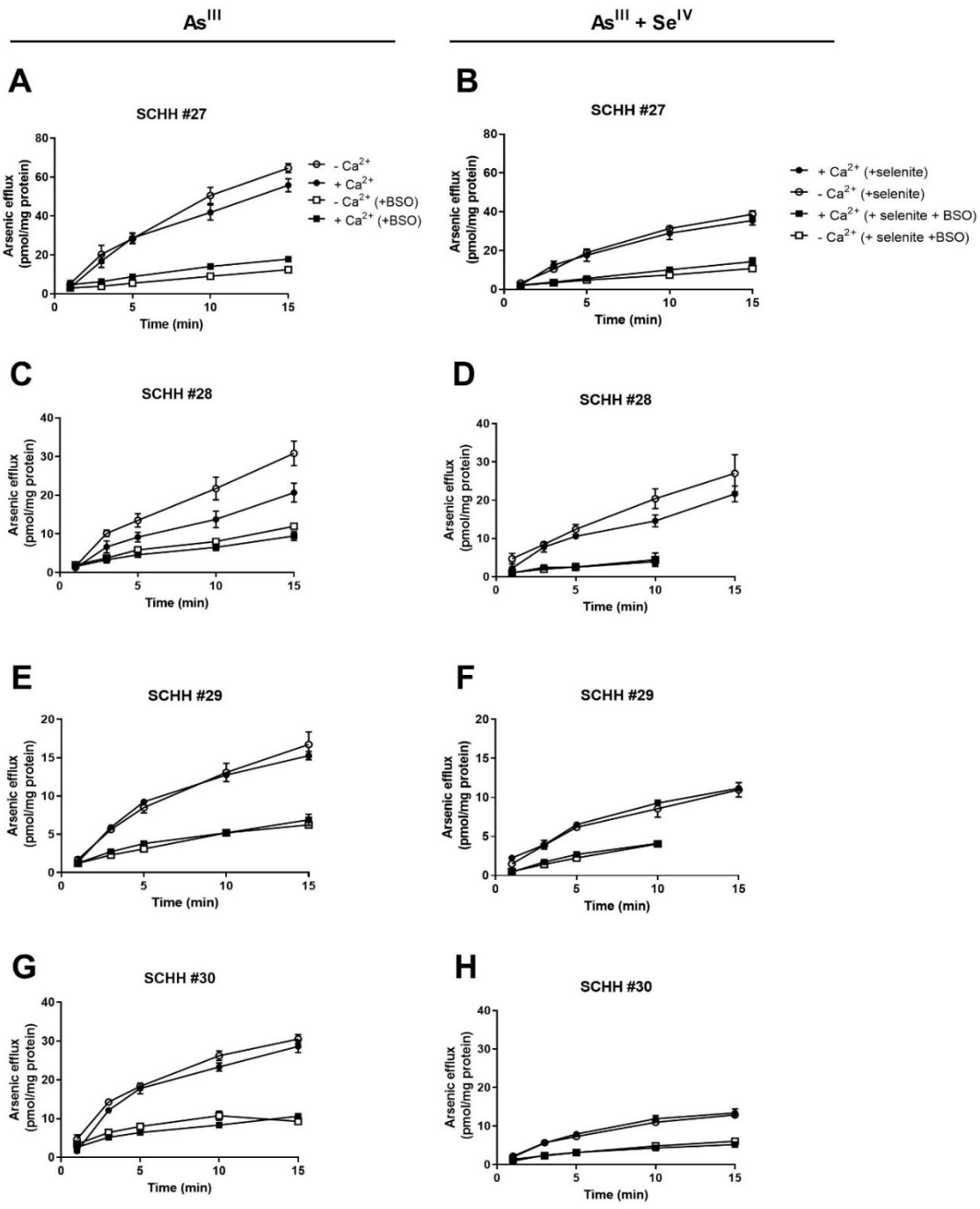

Figure 3-4. The effect of GSH-depletion on arsenic efflux was assessed from four different SCHH preparations pretreated with 0.5 mM BSO for 48 hrs. SCHH were incubated at 37ºC with **(A,C,E,G)** ^{73}As-arsenite or **(B,D,F,H)** ^{73}As-arsenite + selenite at 1 µM, 100 nCi for 24 hrs (●/○) or in the presence of BSO pretreatment (■/□), incubation media was then replaced with HBSS containing Ca^{2+} (sinusoidal efflux ●/■) or without Ca^{2+} (sinusoidal + canalicular efflux ○/□). The difference between the closed and open circles represents arsenic efflux into canalicular networks. Points represent means ± S.D. for triplicate determinations within a single experiment.

3.4.4. Arsenic Biliary Efflux is Increased or not Modified in the Presence of Selenide

Given the large body of evidence surrounding the mutual detoxification of arsenite and selenite, the surprising lack of increase in As efflux in the presence of selenite led to the investigation of different Se species and their effect on As efflux, especially the more reduced form, selenide, which may be the predominant form of Se presented to the liver [38,47].

Sinusoidal and canalicular efflux of As in the presence of selenite alongside selenide was investigated in 3 SCHH preparations (SCHH 34, 35 and 36). Consistent with SCHH preparations presented in Figure 3-3, selenite inhibited As efflux or did not alter it. In the presence of selenide, As efflux in the presence of Ca^{2+} (sinusoidal efflux), showed a similar trend to selenite, where sinusoidal As efflux was either unchanged (SCHH 34 and 35) or inhibited by 31% (SCHH 36) (Figure 3-5A to 3-5C). Unlike selenite (where biliary efflux of As was reduced), selenide treatment did not the change As biliary efflux in preparations which were able to efflux As in ^{73}As-arsenite alone treatment conditions (Figure 3-5A and 3-5C). Interestingly, upon treatment with ^{73}As-arsenite alone, SCHH preparation 36 showed no biliary efflux, whereas when treated with selenide and ^{73}As-arsenite, biliary efflux of As was observed with a BEI value of 11% (Figure 3-5B).

SeMet and MeSeCys are components of selenized yeast which has been used in As-Se studies and has shown to have beneficial effects. The influence of these Se species on As sinusoidal efflux was investigated. Preliminary data shows no difference between As and SeMet sinusoidal efflux, while MeSeCys increased As sinusoidal efflux by 12% (Figure 3-5D).

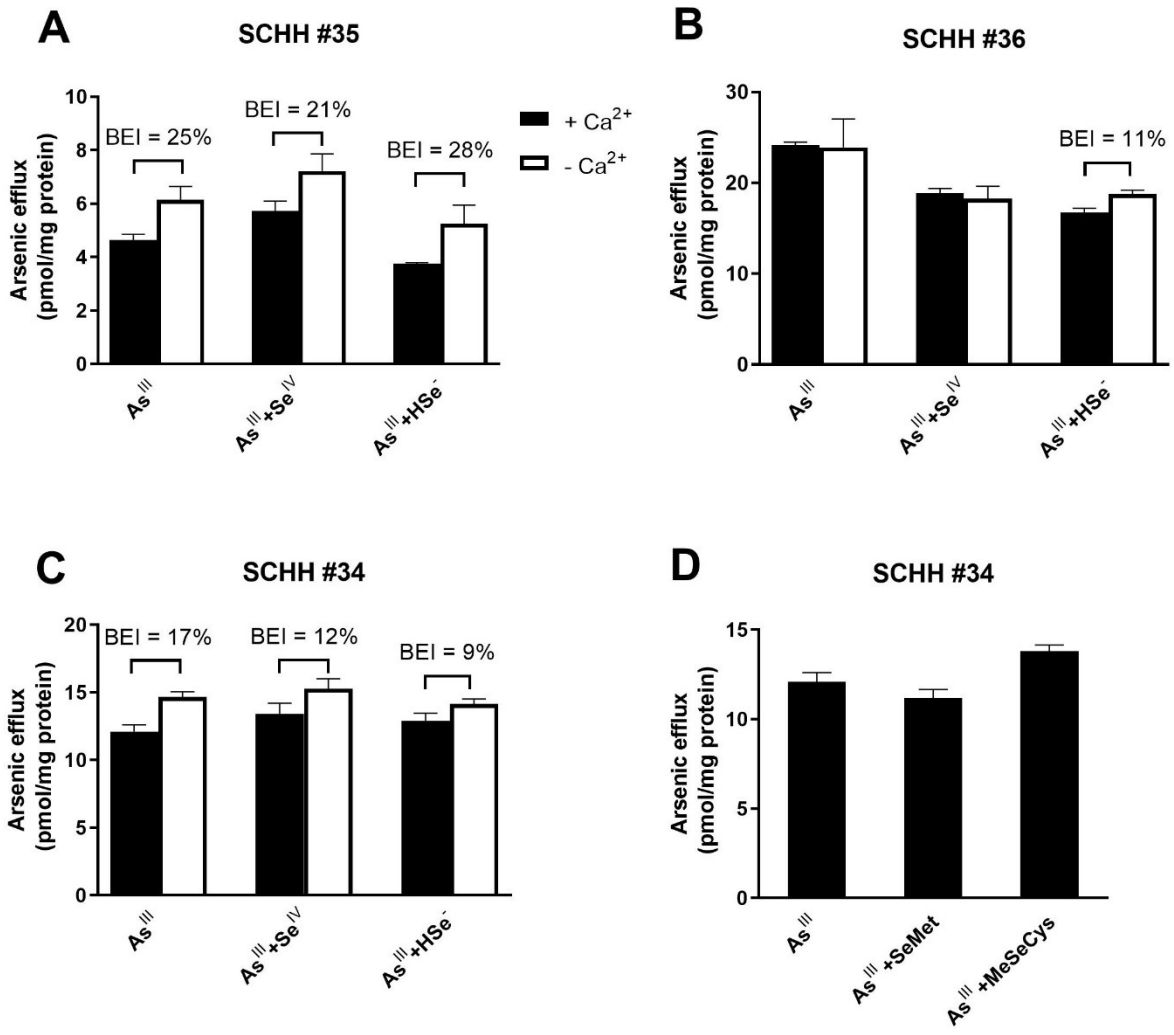

Figure 3-5. Influence of selenium species on sinusoidal and canalicular arsenic efflux from SCHH. SCHH were incubated at 37ºC with ^{73}As-arsenite ± selenium (1 µM, 100 nCi) for 24 hrs. **(A-C)** The effects of selenite and selenide on arsenic efflux from SCHH preparations 34, 35 and 36 was measured in the presence (sinusoidal efflux, closed bars) and absence (sinusoidal + canalicular efflux, open bars) of calcium. **(D)** The effects of selenomethionine (SeMet) and methyl selenocysteine (MeSeCys) on only arsenic sinusoidal efflux from SCHH preparation 34 was measured (i.e., in the presence of calcium Ca^{2+}). Bars represent means ± SD of triplicate determinations at 10 min in a single experiment. (while the mean of duplicate values are plotted for the ^{73}As-arsenite + selenite condition of SCHH preparation 35). BEI values were only calculated when the -Ca^{2+} condition was significantly higher ($P<0.05$, Student's t-test) than the +Ca^{2+} condition for triplicate determinations from a single experiment.

3.4.5. Preliminary Data Suggest Selenide Accumulation is Higher than Selenite by SCHH

In order to investigate whether poor selenite uptake resulted in the lack of stimulation of As efflux, SCHH accumulation studies were carried out to investigate uptake differences between selenite and

selenide. It has been shown that Se metabolism takes place in RBCs, resulting in selenite reduction to selenide [48]. It is likely then that selenide is predominantly the species presented to the liver for uptake into hepatocytes. Consistent with this, over a 24 hr period, our experiments showed that the amount of ^{75}Se-selenide accumulation in SCHH is at least 59% higher than ^{75}Se-selenite (Figure 3-6A and 3-6B). These results are consistent with previous reports in yeast, human keratinocytes, lung cancer cell lines and trout hepatocytes, as well as in HepG2 cells (Chapter 4), where it has been shown that selenide is more efficiently taken up into cells, in comparison with selenite [37,39,41,43,49].

Investigation into the effect of As uptake on Se accumulation showed a trend towards higher levels of Se present in cells when exposed to As in SCHH 35 (Figure 3-6A), with differences in Se accumulation between the selenite + arsenite and selenide + arsenite treatment conditions in SCHH 36 (Figure 3-6B), likely due to differences in selenite vs selenide uptake.

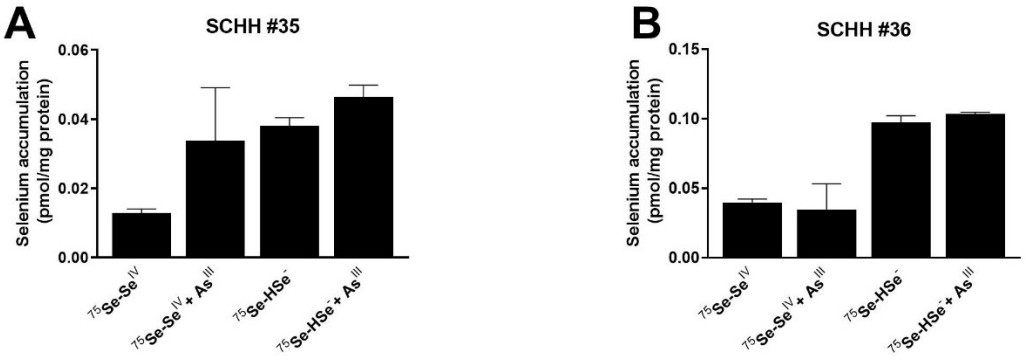

Figure 3-6. Accumulation differences between ^{75}Se-selenite and ^{75}Se-selenide, in the presence of arsenite. SCHH were incubated at 37ºC with ^{75}Se-selenite or ^{75}Se-selenide ± arsenite (1 µM, 100 nCi) for 24 hrs. Accumulation was measured from SCHH preparations 35 (**A**) and 36 (**B**). Bars represent means ± SD of triplicate determinations at 10 min in a single experiment. (while the mean of duplicate values is plotted for SCHH preparation 35).

3.4.6. Preliminary Data Suggest Selenium Efflux is Increased in the Presence of Arsenic

Based on the increased accumulation of selenium when SCHH are treated with selenide compared to selenite, and the potential for increased biliary efflux of As in the presence of selenide, Se efflux in the presence and absence of arsenite in two SCHH preparations was investigated. Total efflux of Se (± Ca^{2+}, across both the sinusoidal and canalicular surfaces) was increased in the presence of arsenite

across both preparations (28% increase in SCHH 35 and 17% increase in SCHH 36) (open bars, Figure 3-7A and 3-7B). ^{75}Se efflux experiments in SCHH preparation 35 showed a 10% increase in sinusoidal efflux of Se in the presence of arsenite (compare closed bars Figure 3-7A). Co-treatment with arsenite also resulted in the biliary efflux of ^{75}Se with a BEI value changing from 0% to 18% (Figure 3-7A).

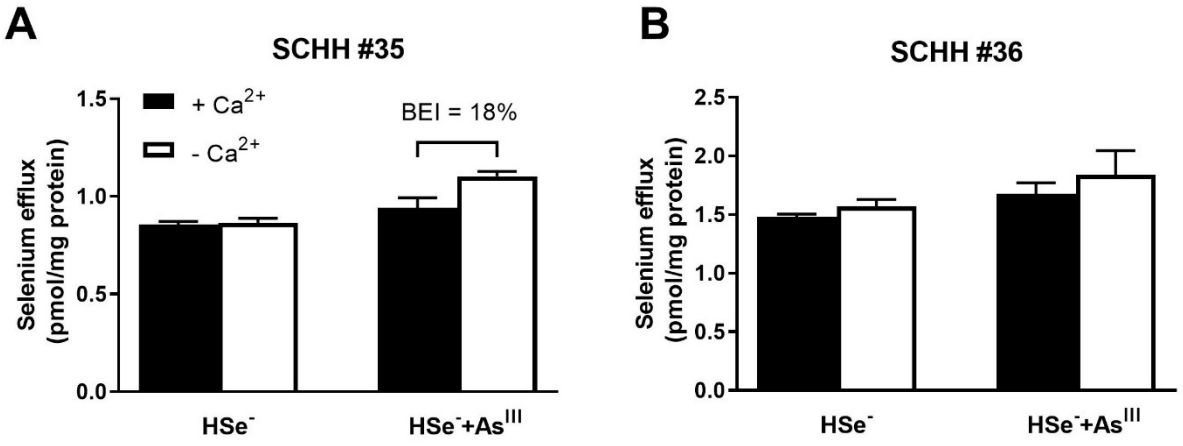

Figure 3-7. Influence of arsenite on sinusoidal and canalicular ^{75}Se-selenide efflux from SCHH. SCHH were incubated at 37ºC with ^{75}Se-selenide ± arsenite (1 µM, 100 nCi) for 24 hrs. The effects of arsenite on ^{75}Se-selenide efflux from SCHH preparations 35 **(A)** and 36 **(B)** was measured in the presence (sinusoidal efflux, closed bars) and absence (sinusoidal + canalicular efflux, open bars) of calcium. Bars represent means ± SD of triplicate determinations at 10 min in a single experiment. BEI values were only calculated when the -Ca^{2+} condition was significantly higher ($P < 0.05$, Student's t-test) than the +Ca^{2+} condition for triplicate determinations from a single experiment.

3.4.7. Preliminary Data Suggests Selenide Protects Against Arsenite Cytotoxicity Better than Selenite

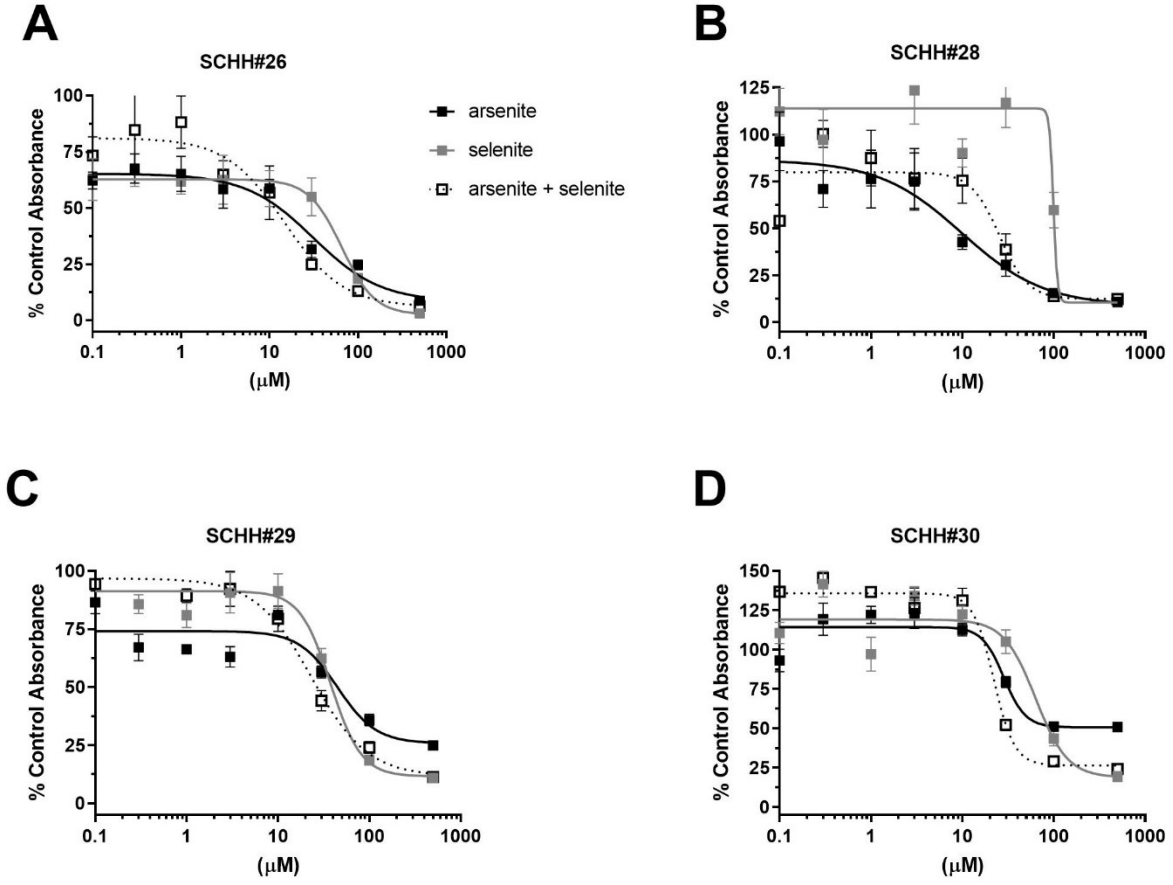

Figure 3-8. Effect of arsenite, selenite and arsenite + selenite on the viability of SCHH. SCHH from four preparations, **(A)** SCHH 26 **(B)** SCHH 28 **(C)** SCHH 29 **(D)** SCHH 30 were incubated with arsenite, selenite and arsenite + selenite for 72 hrs. Cell viability was determined using a tetrazolium-based cytotoxicity assay and IC_{50} values were determined using the sigmoidal dose response fit (GraphPad Prism 6). Data points are means (± S.D.) of quadruplicate determinations within a single experiment.

In addition to uptake and efflux, the effect of Se on As cytotoxicity was also investigated for 5 SCHH preparations (Figure 3-8 and 3-9). In almost all preparations (SCHH 26, 29, 30, 34), selenite (arsenite + selenite mean IC_{50} 22 ± 5 μM) did not protect against arsenite (mean IC_{50} 29 ± 14 μM) toxicity (Table 3-1, Figure 3-8A, C, D). Our results show that when there is biliary efflux of arsenic, from as early as 3 min, selenite protects against arsenite cytotoxicity (arsenite IC_{50} 10 μM; arsenite + selenite IC_{50} 26 μM) (SCHH 28, Figure 3-8B). This supports our hypothesis that increase in biliary efflux of As in the presence of Se is a protective mechanism. In addition, selenide protected against arsenite-

induced cytotoxicity better than selenite (arsenite + selenite IC_{50} 25 μM; arsenite + selenide IC_{50} 109 μM) (Figure 3-9, Table 3-2).

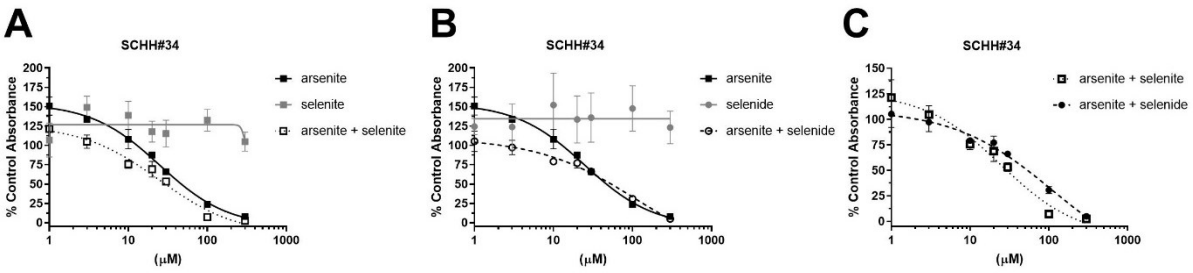

Figure 3-9. Effect of arsenite, selenite, selenide, arsenite + selenite and arsenite + selenide on the viability of SCHH. SCHH from preparation 34 were incubated with **(A)** arsenite, selenite and arsenite + selenite or **(B)** arsenite, selenide and arsenite + selenide for 72 hrs. **(C)** Combinations of selenite + arsenite and selenide + arsenite shown in A and B, respectively are plotted together. Cell viability was determined using a tetrazolium-based cytotoxicity assay and IC_{50} values were determined using the sigmoidal dose response fit (GraphPad Prism 6). Data points are means (± S.D.) of quadruplicate determinations within a single experiment.

Table 3-2. IC_{50} values for arsenite, selenite, selenide, and a combination of arsenite and selenium species in SCHH cells after 24 hrs of exposure.

SCHH preparation no.	IC_{50} (μM)				
	Arsenite	selenite	arsenite + selenite	selenide	arsenite + selenide
26	31	66	15	-	-
28	10	100	26	-	-
29	45	38	23	-	-
30	28	63	22	-	-
Mean IC_{50} ± S.D.	29 ± 14	67 ± 25*	22 ± 5		
34	25	> 300	25	> 300	109

*P < 0.05 compared with arsenite + selenite (analysis of variance followed by a Tukey's multiple comparison test).

3.5. Discussion

The molecular basis for the mutual antagonism of toxicity between As and Se is through the formation of $[(GS)_2AsSe]^-$, which is thought to increase As fecal elimination [15,18,19,50]. In the current study, it has been shown that As efflux from SCHH is predominantly sinusoidal which is consistent with previously published data [11]. Arsenic biliary efflux, in the presence and absence of selenite was found to be GSH-dependent. Unexpectedly, we found that selenite either decreased As biliary efflux or had no effect on it. In contrast, selenide, expected to be the predominant form of inorganic Se presented to the liver either increased or did not alter As biliary efflux. Further characterization of differences in selenite and selenide accumulation revealed selenide accumulation in SCHH was higher. Se efflux was increased in the presence of arsenite, when SCHH were treated with ^{75}Se-selenide and arsenite. In corroboration of selenide being the form of Se that likely interacts with As, cytotoxicity assays revealed that selenide decreased arsenite-induced toxicity, while selenite did not.

Consistent with urinary excretion being the major route for As elimination, As efflux from hepatocytes takes place predominantly across the sinusoidal surface to reach the kidneys via the systemic circulation. Methylated arsenicals comprise the majority of As species present in urine, with more than 60% being present as dimethylated arsenic [7,9]. Hepatocytes methylate inorganic As to produce DMAs and MMAs in the same proportions as found in urinary analysis [51,52].

Selenite has been shown to be an inhibitor of As methylation, in both rat and human hepatocytes [53,54]. Furthermore, previous studies have shown that rat hepatocyte exposure to selenite leads to increased accumulation of arsenite [55]. Consistent with these studies, our results show selenite leads to inhibition of sinusoidal efflux of As. This could be due in part to the inhibition of As methylation by Se. MRP4/*ABCC4* is present on the sinusoidal surface of the hepatocyte and *in vitro* transport assays have shown MRP4 to mediate the transport of the di-methylated and mono-methylated arsenic

metabolites, DMA^V and monomethylarsenic diglutathione ($MMA(GS)_2$) [12]. Roggenbeck et al., have shown through arsenic speciation studies that both methylated and inorganic As forms are effluxed across the sinusoidal surface of the hepatocyte, with the majority of As present as inorganic As [11]. Thus, inhibition of methylation only provides a potential partial explanation for the decrease of As sinusoidal efflux in the presence of selenite.

Selenium has been investigated as an important modifier of As metabolism and toxicity; protecting against As-induced toxicity by forming a glutathione complex with As, $[(GS)_2AsSe]^-$ [18,56,57]. *In vitro* efflux of $[(GS)_2AsSe]^-$ has been shown to be mediated by the human multidrug resistance protein 2 (MRP2), which is located on the canalicular surface of the hepatocyte, allowing efflux of this conjugate into bile [13]. Surprisingly, our SCHH efflux data shows that biliary efflux of As was inhibited in the presence of selenite, across all preparations that displayed the capacity for As biliary efflux. *In vivo* studies with WT and Mrp2-deficient Wistar rats showed that Mrp2 is responsible for almost all of arsenic efflux into bile in the forms of arsenic triglutathione ($As(GS)_3$) and $MMA(GS)_2$ [14]. $[(GS)_2AsSe]^-$, has been identified in rat and rabbit bile [15,19,50].

Consistent with published data in Roggenbeck et al., GSH depletion results in a complete loss of As biliary efflux from SCHH [11]. This is also the case in arsenite + selenite treatment conditions, suggesting As efflux into bile is GSH-dependent. MRP2 is highly expressed on the canalicular surface of the hepatocytes and in this location is the only known protein which transports As GSH-conjugates [14]. This supports the observed loss of biliary efflux of As in the absence of GSH, both in the presence and absence of selenite.

Depletion of glutathione does not completely inhibit As sinusoidal efflux due to the potential for efflux of non-glutathionylated metabolites of As (such as DMA^V) by transport proteins like MRP4 and aquaglyceroporin 9 (AQP9) which is a channel shown to allow permeation of neutral species of

As, such as arsenite and MMA^{III}, down their concentration gradient [12,58,59]. Rat studies have also shown a 50% decrease in urinary elimination of As when administered BSO to deplete GSH synthesis [60].

After oral ingestion of selenite, it is thought to cross the intestinal epithelium paracellularly and transcellularly [61]. During transcellular passage some reduction of selenite to selenide might occur in the enterocyte, while selenite transported paracellularly will reach the portal blood, be taken up by RBCs and rapidly reduced to selenide [48] . As such, the form of Se that reached the liver is likely selenide. Indeed, we see either an increased biliary efflux of As in the presence of selenide or at minimum, no change in the biliary efflux (Figure 3-5A to C). Investigation of accumulation differences between these two species, showed selenide uptake to be significantly higher than selenite (Figure 3-6). This is consistent with other studies demonstrating increased selenide uptake in trout hepatocytes and HepG2 cells (Chapter 4) [37,39].

Previous studies have shown that organic forms of selenium have higher bioavailability and are thus preferred for use in therapeutic applications [62–64]. Among commercially available selenium supplements, selenized yeast (major component, SeMet) and MeSeCys are common components identified on manufacturer's labels [28,65,66]. In small population studies, treatment of As-exposed individuals with selenized yeast led to lower levels of As in hair, urine and blood [22]. *In vitro* studies with human osteosarcoma cells showed SeMet and MeSeCys blocked As-induced mutagenesis [67]. All Se sources are metabolized to a selenide pool before being incorporated into selenoproteins or cellular reactions, while SeMet, as a Se analogue of methionine, is also incorporated into proteins non-specifically [25,26,68–70]. Suzuki et al., show SeMet and MeSeCys to be transported to target organs in their intact forms in rats [71]. SeMet undergoes trans-selenation to form selenocysteine, which then forms selenide in cells, while MeSeCys is first transformed into methyl selenol, followed by

demethylation to form selenide [25,42,72,73]. When undergoing transformation to selenide, not only do these seleno-amino acids not compete for methylation, but MeSeCys conversion to selenide releases a methyl group [25,26]. Preliminary data shows sinusoidal As efflux is increased in the presence of MeSeCys (Figure 3-5D), possibly due to increased levels of demethylated and monomethylated As available for efflux.

Previous studies have also investigated the effects of As on Se efflux. It was shown that Se levels in bile increased from 4% of total Se dose to 41% of total Se dose in the presence of As [21]. Consistent with these *in vivo* observations, SCHH experiments with ^{75}Se-selenide in the presence and absence of arsenite, revealed an increase in biliary efflux in SCHH preparation 35 (Figure 3-7A). Preliminary data from a limited study in Bangladesh show increased urinary and fecal Se levels in 5 participants that were exposed to arsenic in drinking water [74]. These participants were accommodated in a clinic for 10 days and fed a fixed diet with drinking water from their homes and received a dose of ^{77}Se-selenite or a placebo on the 6th day. This preliminary data is consistent with the observed increase in sinusoidal and canalicular efflux of ^{75}Se in the presence of arsenite in the present study.

While epidemiological studies and *in vivo* work with animal models show a protective effect of selenite on arsenite-induced toxicity, Styblo et al., reported that *in vitro* selenite (2 μM) increased the cytotoxicity of arsenite (10 μM) in rat hepatocytes [22,30,31,55,75,76]. Cytotoxicity assays show that in 3 out 5 SCHH preparations, selenite decreases the IC_{50} of arsenite (Figure 3-8). This is likely due to the inhibition of As efflux in the presence of selenite, leading to the increased retention of toxic As species. Preliminary results show that co-treatment of arsenite with selenide (IC_{50} 109 μM) leads to a higher IC_{50} than arsenite treatment alone (IC_{50} 25 μM), and arsenite plus selenite treatment (IC_{50} 25 μM) (Figure 3-9). This led us to explore the differences between selenide and selenite in HepG2 cells (Chapter 4) using a readily available model system.

This work shows that consideration of the physiological relevance of the species of Se for better design of experimental studies is required. The reduction of selenite to selenide, means that this is likely the form of Se presented to the liver, resulting in its increased uptake in comparison to selenite. In addition, using primary human hepatocytes, this work has shown that there are inter-individual differences in the cytotoxicity and efflux of As and Se. The evidence of direct interaction between As and Se through the $[(GS)_2AsSe]^-$ conjugate, has led to clinical trials and small population studies in arsenic-endemic regions [22,32,77]. The complex nature of interactions between As and Se require careful extrapolation of *in vivo* and *in vitro* studies.

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4. Studies of Selenium and Arsenic Mutual Detoxification in Human HepG2 Cells

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4.1. Abstract

Hundreds of millions of people worldwide are exposed to unacceptable levels of carcinogenic inorganic arsenic. Animal models have shown that selenium and arsenic are mutually protective through the formation and elimination of the seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$. Consistent with this, human selenium deficiency in arsenic-endemic regions is associated with arsenic-induced disease, leading to the initiation of human selenium supplementation trials. In contrast to the protective effect observed *in vivo*, *in vitro* studies have suggested that selenite increases arsenite cellular retention and toxicity. This difference might be explained by the rapid conversion of selenite to selenide *in vivo*. In the current study, selenite did not protect the human hepatoma (HepG2) cell line against the toxicity of arsenite at equimolar concentrations, however selenide increased the IC_{50} by 2.3-fold. Cytotoxicity assays of arsenite + selenite and arsenite + selenide at different molar ratios revealed higher overall mutual antagonism of arsenite + selenide toxicity than arsenite + selenite. Despite this protective effect, in comparison to ^{75}Se-selenite, HepG2 cells in suspension were at least 3-fold more efficient at accumulating selenium from reduced ^{75}Se-selenide, and its accumulation was further increased by arsenite. X-ray fluorescence imaging of HepG2 cells also showed that arsenic accumulation, in the presence of selenide, was higher than in the presence of selenite. These results are consistent with a greater intracellular availability of selenide relative to selenite for protection against arsenite, and the formation and retention of a less toxic product, possibly $[(GS)_2AsSe]^-$.

4.2. Introduction

Inorganic arsenic (As) is a Group I (proven) human carcinogen, found throughout the environment [1]. Arsenic contamination of drinking water is a global problem, with more than 200 million people exposed to levels higher than the World Health Organization standard of 10 μg/L [2]. In Bangladesh, a nation-wide program to evade microbial contamination in surface water, resulted in the contamination of well water through the disturbance of As-containing strata [3]. Chronic As consumption can have adverse health consequences for decades after exposure cessation [4]. Poor socioeconomic status of developing countries means cost-effective As exposure solutions are required.

Selenium (Se) is an essential trace element required in human nutrition [5]. There is an intriguing toxicity antagonism between some forms of As and Se, first observed decades ago when Se toxicity in rats (from seleniferous grains), was reduced with the addition of As in the form of arsenite to drinking water [6]. More recently, the seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ was identified in the bile of rabbits and rats [7–10]. In contrast to other As-glutathione (As-GSH) metabolites, $[(GS)_2AsSe]^-$ is more stable at alkaline biliary pH and is thought to break the enterohepatic recirculation of As-GSH complexes (reviewed in [11]). $[(GS)_2AsSe]^-$ biliary efflux is likely mediated by the ATP-binding cassette transporter multidrug resistance protein 2 (MRP2/*ABCC2*) [11,12]. Further to its biliary elimination, $[(GS)_2AsSe]^-$ is sequestered in red blood cells and, likely delays the distribution of As and Se to tissues [13–16]

Low blood Se levels have been associated with a higher incidence of As-induced skin lesions [17,18]. Due to this correlation, and the evidence from animal studies, Se supplementation trials have been initiated [19–22]. Initial research into Se supplementation with L-selenomethionine and selenized yeast suggest that Se may improve As-induced toxicities [23–25]. Following suggestions from George,

Pickering and co-workers [26], a recent study supplementing a Bangladeshi population with lentils grown in soil with a high Se content, showed higher urinary As elimination [27]. If successful, Se supplementation has the potential to be a cost-effective solution for populations that are chronically exposed to As. However, the interaction between As and Se, especially in humans, is not fully understood.

Within cells, arsenate is reduced to arsenite and undergoes a series of reduction and oxidative methylation steps. Inorganic Se biotransformation is similar to As and as such, Se competes with As for methyl donors, decreasing As methylation [28]. In addition to decreasing the formation of the highly toxic methylated trivalent arsenicals, Se also reacts with arsenite to form $[(GS)_2AsSe]^-$ [29].

Most of the characterization of As and Se interactions have been done in laboratory animals dosed with arsenite and selenite, and very little work has been done with human cell lines [30]. It was established early on in rats that the mutual detoxification of arsenite and selenite was not due to a reduced bioavailability of either compound [31]. In rats that had been dosed individually with arsenite or selenite compared with a combination of both, it was found that arsenite and selenite together resulted in lower retention levels of both compounds in the liver, and increased fecal elimination [32]. More recently, in rats injected with arsenite and selenite, all the As and Se detected in the bile was in the form of $[(GS)_2AsSe]^-$ [9].

There are inconsistencies between what happens *in vitro* and *in vivo*. For example, rats dosed with arsenite and selenite are protected through increased biliary elimination of $[(GS)_2AsSe]^-$, however, *in vitro* work with primary rat hepatocytes showed that selenite increased cellular retention of As, decreased As methylation and slightly increased As toxicity [9,15,33,34]. Selenite is known to be rapidly converted to selenide (HSe^- at physiological pH) within cells, including red blood cells [35–37]. Selenide can also be formed non-enzymatically from selenite, in the presence of reduced glutathione

(GSH), however, extracellular GSH levels might not be sufficient for this reduction to occur [38,39]. Furthermore, it has been shown that selenide uptake is more efficient than selenite in yeast, trout hepatocytes, lung cancer cells and keratinocytes [39–42]. This information has led us to hypothesize that extra-hepatic formation of selenide may be necessary for sufficient hepatocyte uptake of Se for protection against As. Thus, the aim of this study was to investigate the influence of selenite and selenide on arsenite toxicity and cellular accumulation using the human hepatoma cell line HepG2. HepG2 cells are a convenient and well characterized model, widely used in toxicology studies, and are functional for As methylation [43–46]. HepG2 cells also have functional glutathione reductase and thioredoxin reductases [47,48], enzymes important for the conversion of selenite to selenide [49,50].

4.3. Material and Methods

4.3.1. Chemicals and Reagents

^{73}As-arsenate (1.2 mCi/ml) was purchased from Los Alamos Meson Production Facility (Los Alamos, NM). ^{75}Se-selenite (25.7 mCi/ml) was obtained from the University of Missouri Research Reactor (MURR, Columbia, MO). Sodium selenite, sodium (meta)arsenite, bovine serum albumin (BSA), glucose, sodium chloride, potassium chloride, magnesium sulphate heptahydrate, Tris base, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), reduced glutathione (GSH), and poly-L-lysine solution were purchased from MilliporeSigma (Oakville, ON). Sodium selenide was purchased from Alfa Aesar (Ward Hill, MA). Triton X-100, calcium chloride and paraformaldehyde was purchased from Fisher Scientific (Fair Lawn, NJ). Silicon nitride windows (5x5 mm frame, 2x2 mm membrane, 200 nm thickness) were purchased from Norcada (Edmonton, AB). Glass fibre type A/E filters were purchased from Pall Corporation (Mississauga, ON). Pierce™ bicinchoninic acid (BCA) protein assay reagent was purchased from Thermo Scientific (Rockford, IL).

4.3.2. Cell Culture

The human liver carcinoma cell line, HepG2, was obtained from the American Type Culture Collection (ATCC catalog No. HB-8065) (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum in a humidified incubator with 5% CO_2. Routine testing for mycoplasma contamination was performed, using the ATCC Universal Mycoplasma Testing Kit (Manassas, VA).

4.3.3. Cytotoxicity Testing

The cytotoxicity of arsenite in the presence and absence of Se species was measured using HepG2 cells, as previously described [51]. Briefly, cells were seeded in 96-well plates in quadruplicate at a cell density of 10,000 cells/well. After 24 hrs, cells were treated with arsenite (0.01-300 μM), selenite (0.01-300 μM), selenide (0.01-300 μM), arsenite + selenite (0.01-300 μM), or arsenite + selenide (0.01-300 μM) for 72 hrs. All As and Se were prepared in supplemented media, with the exception of selenide, which was weighed into BSA (33 mg/ml) in phosphate buffered saline (PBS), followed by serial dilutions in supplemented media. Cell viability was determined using the tetrazolium based CellTiter96® Aqueous Non-Radioactive Cell Proliferation Assay from Promega (Madison, MI). Data analyses were completed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA) and IC_{50} values were determined using the sigmoidal dose response fit.

4.3.4. ^{73}As Accumulation Assays with HepG2 Cells in Suspension[†]

^{73}As-arsenite was prepared from ^{73}As-arsenate using metabisulfite-thiosulfate reduction, as previously described [52]. To measure ^{73}As-arsenite accumulation, HepG2 cells were suspended at 1 x 10^6 cells/ml in Hank's balanced salt solution (HBSS) (137 mM sodium chloride, 10 mM Tris, 5 mM glucose, 5.4 mM potassium chloride, 1.8 mM calcium chloride, 0.9 mM magnesium sulphate heptahydrate, 10 mM HEPES, pH 7.4) at 37ºC. ^{73}As-arsenite (1 μM, 50 nCi/pt) was added to the cell

[†] All ^{73}As experiments were performed by Gurnit Kaur.

suspension and uptake was stopped at the indicated time points (1, 3, 5, 15, and 30 min) by diluting the reaction in 800 µl of ice-cold HBSS. Samples were filtered over type A/E glass fibre filters, washed twice with ice-cold HBSS, and radioactivity on filters was quantified using the PerkinElmer MicroBeta^2 liquid scintillation counter.

4.3.5. ^{75}Se Accumulation Assays with HepG2 Cells in Suspension[†]

Excess GSH reduces selenite to selenide in a multistep reaction process and prevents selenide from re-oxidation [38–40]. This reaction process results in the formation of Se-GSH conjugates as intermediaries; with the final product being inorganic selenide [39,40]. ^{75}Se-selenite was reduced in the presence of excess GSH, as previously described [40]. Briefly, ^{75}Se-selenite (50 nCi/ data point) was combined with GSH at a molar ratio of at least 1:550 and incubated at room temperature for 20 min; this was supplemented with the indicated concentration of non-radioactive Na_2Se (dissolved in 33 mg/ml BSA in PBS) before the accumulation assay. For simplicity, this solution of ^{75}Se-selenite + GSH and non-radioactive Na_2Se, will be referred to as ^{75}Se-selenide throughout the chapter, although it is likely that not all Se will be present as selenide (some ^{75}Se-GSH conjugates as well as ^{75}Se-selenite oxidized to elemental ^{75}Se may be present. These conditions resulted in the presence of 0.35 µM GSH and 0.075 mg/ml BSA in ^{75}Se-selenide containing transport assays.

For all assays, HepG2 cells were suspended at 1 x 10^6 cells/ml in HBSS at 37ºC and ^{75}Se-selenite or ^{75}Se-selenide (0.1 µM, 50 nCi/pt) was added to the cell suspension. At the indicated time points (1, 3, 5, 15, 30 min), uptake was stopped by diluting the reaction in 800 µl of ice-cold HBSS. Temperature dependence was measured by determining uptake of ^{75}Se-selenite or ^{75}Se-selenide (0.1 µM, 50 nCi/pt) at 4ºC and 37ºC at 1 and 30 min time points. For concentration dependence assays, ^{75}Se-selenite or ^{75}Se-selenide (50 – 75 nCi/pt) was added to the HepG2 cell suspension at the indicated concentrations

[†] All ^{75}Se accumulation experiments in suspension were initiated by Gurnit Kaur with technical replicates for Figure 4-2B completed by Diane Swanlund and Figure 4-2D completed by Janet Zhou.

(0.1 – 2.5 μM). At 1 min, uptake was stopped by diluting the reaction in 800 μl of ice-cold HBSS. Samples were filtered over type A/E glass fibre filters, washed twice with ice-cold HBSS, and radioactivity on filters was quantified using the Packard Cobra II gamma counter.

In order to investigate the influence of arsenite on Se uptake, ^{75}Se uptake assays were carried out in the presence of arsenite, as described above. ^{75}Se-selenite ± arsenite or ^{75}Se-selenide ± arsenite (0.1 μM of each and 50 nCi/pt of ^{75}Se) was added to the cell suspension and uptake measured at a 1 min time point, as described above.

4.3.6. Assessment of Toxicity of Different As and Se Concentration Combinations on HepG2 Cells[†]

To assess the toxicity of different arsenite and selenide/selenite concentration combinations on HepG2 cells, cells were seeded in 96-well plates in triplicate at a density of 10,000 cells/well. After 24 hrs, cells were treated with arsenite (0.01-300 μM) plus selenite (0.01-300 μM) and arsenite (0.01-300 μM) plus selenide (0.01-300 μM) for 72 hrs. This resulted in each concentration of As being paired with every concentration of Se, resulting in 81 different treatment combinations over 9 different concentrations. Assessment of cytotoxicity was done using the tetrazolium-based assay as described above.

Cytotoxicity data were analyzed using the ComBenefit software [53]. The software compares dose-response experimental data (for arsenite and Se combinations) to a mathematical model derived dose-response matrix (based on the As and Se single agent dose response curves), resulting in a synergy distribution output. The Loewe, Bliss and Highest Single Agent (HSA) mathematical models were used to analyze the cytotoxicity data to capture synergy/antagonism profiles [53–59]. The SUM_SYN_ANT value captures the summary between the synergistic and antagonistic effect of As

[†] All experiments were completed by Gurnit Kaur

and Se with a negative value indicating toxicity antagonism and a positive value indicating toxicity synergy.

4.3.7. ^{75}Se Accumulation Assays with Adherent HepG2 Cells[†]

The accumulation of ^{75}Se-selenide or ^{75}Se-selenite in the presence and absence of arsenite was measured using adherent cells. HepG2 cells were seeded in 6-well plates at 500,000 cells/well and allowed to reach 60-70% confluence. Cells were then treated with ^{75}Se-selenite (3 μM, 100 nCi), ^{75}Se-selenide or in combination with arsenite (30 μM) in supplemented culture media for 24 hrs. Cells were then washed three times with 1 ml of ice-cold phosphate buffered saline, lysed in 500 μl of 0.5% Triton X-100 and the lysate collected. Radioactivity was measured using the Packard Cobra II gamma counter. Accumulation was normalized for total protein determined using a BCA assay.

4.3.8. X-ray Fluorescence Imaging

4.3.8.1 Sample Preparation[‡]

HepG2 cells were seeded on 200 nm-thick low stress silicon nitride windows (Norcada ®, Edmonton, AB) with window dimensions of 1.5 × 1.5 mm or 1.75 x 1.75 mm, manually coated with 0.01% poly-L-lysine and allowed to reach 60-70% confluency. Cells were treated with selenite, selenite + arsenite, selenide or selenide + arsenite all at 60 μM in culture media for 2 hrs. These concentrations were necessary for optimal detection by X-ray fluorescence imaging (XFI). After 2 hrs, cells were washed twice with ice-cold PBS, fixed in 4% paraformaldehyde and rinsed in ammonium acetate, then allowed to dry at room temperature. Samples were stored at 4°C in the presence of desiccant until analysis.

[†] These experiments were performed by Janet Zhou.
[‡] All sample preparation for XFI was carried out by Gurnit Kaur.

To determine if the high concentrations of As and Se used for XFI were toxic, tetrazolium-based cytotoxicity assays were conducted as described above, except exposure was for 6 hrs instead of 72 hrs.

The accumulation of ^{75}Se under conditions used for XFI was also investigated using adherent cells as described above in Section 4.3.7. Cells were treated with ^{75}Se-selenite, ^{75}Se-selenite + arsenite, ^{75}Se-selenide or ^{75}Se-selenide + arsenite (at 60 μM each, with 100 nCi of ^{75}Se/well) in supplemented culture media for 2 hrs.

4.3.8.2. X-ray Fluorescence Imaging[†]

XFI was conducted at the 2-ID-E X-ray fluorescence microprobe beamline of the Advanced Photon Source (APS), a 7 GeV synchrotron light source, at Argonne National Laboratory (Lemont, IL) [60]. The 2-ID-E station is equipped with Fresnel zone plates which allow focusing of the incident beam to a sub-micron (400 nm) spot size. The HepG2 cultures prepared on Si_3N_4 windows, first were imaged using the Leica optical microscope with encoded stages in order to align the samples and find areas of interest. The Si_3N_4 window then was attached to a motorized stage and raster-scanned, with the incident beam energy set to 13.5 KeV. Initially, "fly" scans (in which the sample is continuously moved) with pixel size 2.5 μm × 2.5 μm and 100 ms effective dwell time/pixel were performed on a large area of the sample, after which higher resolution 500 nm × 500 nm or 300 nm x 300 nm "step" scans with 0.4 s or 1 s dwell time/pixel were conducted on smaller groups of cells.

The fluorescence radiation excited at each scan point was collected by an energy dispersive silicon drift detector (Vortex-ME4, SII Nanotechnology, Northridge, California, USA). Spectra for each pixel

[†] Data collection was carried out by Gurnit Kaur and Olena Ponomarenko (with assistance from Nataliya Dolgova and Kelly Summers). Olga Antipova and Olena Ponomarenko analyzed x-ray fluorescence imaging (XFI) samples shown in Figure 4-4A.

were fitted and quantified with reference to an AXO 1X thin-film standard (AXO Dresden GmbH, Germany). The fitting of two-dimensional elemental maps and analysis of the regions of interest (ROI) were performed using MAPS software [61].

4.3.9. Selection of Arsenic and Selenium Concentrations for Experiments

In humans, chronic As exposure has been reported to result in blood total As levels of 0.01-0.8 μM, generally with means in 10-100 nM range in populations exposed to 10-100 μg/L of As in water [17,19,62,63]. Mean blood total Se levels range from ~1.6 μM (typical North American diet) to 4 μM (population with high Se intake, without adverse effects) [62,64,65]. For suspended HepG2 transport experiments, As and Se were used at sub to low μM concentrations, and therefore are relevant to As exposed and Se supplemented populations. Higher concentrations (60 μM) were used for As and Se XFI due to detection limits.

4.3.10. Data Analysis and Statistics

Cell viability assays were analyzed using the GraphPad Prism 6 software and IC_{50} values were determined using the sigmoidal dose response fit. Significant differences between IC_{50} values were determined using one-way ANOVA followed by Tukey's post hoc test. Cell viability assays to assess antagonism were analyzed using the Combenefit software and synergy distribution plots were determined using the Loewe additivity synergy model [53,55]. Significant differences between uptake time courses were determined using the mixed-effects analysis followed by the Sidak post hoc test. Uptake differences between ^{75}Se-selenite and ^{75}Se-selenide at multiple concentrations and temperatures were compared with two-way ANOVA followed by the Sidak post hoc. Uptake differences between all other groups were compared using one-way ANOVA followed by Tukey's post hoc test. Differences were considered significant with $P < 0.05$.

4.4. Results

4.4.1. Selenide but Not Selenite Protects HepG2 Cells Against Arsenite Toxicity

To determine if selenite protects HepG2 cells against the toxicity of arsenite and vice versa HepG2 cells were treated with increasing concentrations of arsenite and selenite alone, and in combination (arsenite + selenite) at equimolar concentrations. IC_{50} values were determined (Table 4-1 and Figure 4-1A), and were not significantly different for any of these conditions ($P > 0.05$, ANOVA with Tukey's post-hoc test).

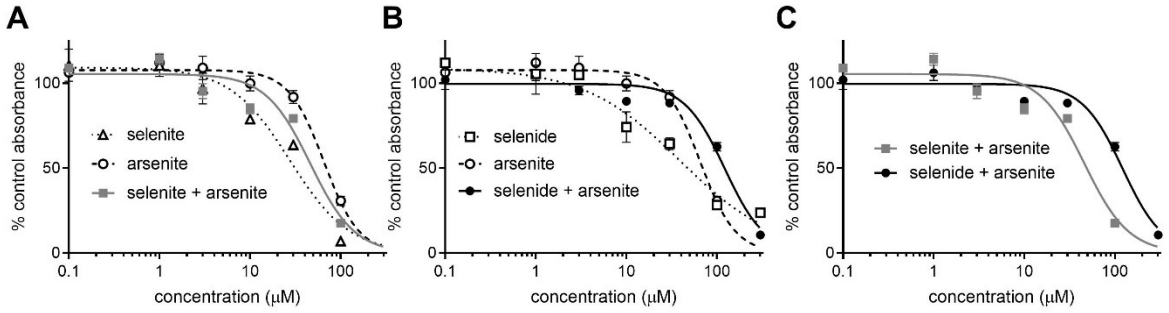

Figure 4-1. Effect of selenite and selenide on arsenite cytotoxicity in HepG2 cells. Cells were seeded in a 96-well plate at 1 x 10^4 cells/well and treated for 72 h with (**A**) selenite, arsenite and selenite + arsenite, and (**B**) selenide, arsenite, and selenide + arsenite. (**C**) Combinations of selenite + arsenite and selenide + arsenite shown in A and B, respectively are plotted together. Cell viability was determined using a tetrazolium-based cytotoxicity assay and IC_{50} values were determined using the sigmoidal dose response fit (GraphPad Prism 6). Data points are means (± S.D.) of quadruplicate determinations in a representative experiment; similar results were obtained in at least three additional experiments (see means of independent experiments in Table 4-1).

The lack of effect of selenite on arsenite toxicity was somewhat surprising due to the reported mutual antagonism of these two compounds *in vivo* [32,66]. Interestingly, upon transport across the intestinal epithelium, and subsequent uptake into RBCs, selenite is metabolized to selenide, exported, then bound to albumin for distribution to other tissues [35–37]. Thus, hepatocytes *in vivo* might be exposed to Se in the form of selenide (stabilized by albumin) rather than selenite, and be more efficient at taking up selenide than selenite. To determine if selenide was capable of protecting HepG2 cells

against the toxicity of arsenite and vice versa, cells were treated with increasing concentrations of arsenite and selenide individually and in combination (arsenite + selenide) at equimolar concentrations. IC_{50} values were then determined (Table 4-1 and Figure 4-1B). The IC_{50} of the arsenite + selenide condition was significantly increased compared to HepG2 cells treated with arsenite and selenide individually (the IC_{50} for arsenite + selenide was 2.3- and 3.5-fold higher than arsenite and selenide, respectively). Furthermore, the IC_{50} value for arsenite + selenide was 2.8- and 6-fold higher ($P < 0.05$, ANOVA with Tukey's post hoc test) than the combination of selenite + arsenite and selenite alone (Table 4-1 and Figure 4-1C).

Table 4-1: IC_{50} values for arsenite, selenite, selenide, and a combination of arsenite and selenium species (0.01 – 300 μM) in HepG2 cells after 72 h of exposure.

Arsenic/Selenium Species	IC_{50} ± S.D. (μM)
Arsenite *(n=4)*	61 ± 6*
Selenite *(n=4)*	23 ± 3**
Selenide *(n=4)*	40 ± 15**
Arsenite + Selenite *(n=4)*	50 ± 6*
Arsenite + Selenide *(n=4)*	138 ± 76

*$P < 0.05$ and **$P < 0.01$ compared with arsenite + selenide (analysis of variance followed by a Tukey's post hoc test).

4.4.2. Se is Accumulated from Selenide to a Greater Extent than from Selenite, and to a Higher Level than As is Accumulated from Arsenite, by HepG2 Cells

To determine differences in cellular accumulation, time course experiments with ^{73}As-arsenite at 1 μM, and both ^{75}Se-selenite, and ^{75}Se-selenide at 0.1 μM were performed with HepG2 cells in suspension (Figure 4-2A and B). Despite the fact that ^{73}As-arsenite accumulation studies were done at a 10-fold higher concentration than ^{75}Se experiments, it was observed that accumulation of ^{73}As from ^{73}As-

arsenite was at least 3 to 5-fold lower than accumulation of Se from ^{75}Se-selenite, and 22 to 46-fold lower than the accumulation of Se from ^{75}Se-selenide at all time points. For example, at 5 min, ^{75}Se accumulation from ^{75}Se-selenite was 4-fold higher than ^{73}As accumulation from ^{73}As-arsenite, while ^{75}Se accumulation from ^{75}Se-selenide was 46-fold higher (Figure 4-2A and B). Between the two Se species, ^{75}Se accumulation over time from ^{75}Se-selenide was higher than from ^{75}Se-selenite ($P < 0.01$) (Figure 4-2B).

Temperature sensitivity of ^{73}As-arsenite (1 μM), ^{75}Se-selenite (0.1 μM), and ^{75}Se-selenide (0.1 μM) accumulation by HepG2 cells was determined at 1 and 30 min time points (Figure 4-2C). At a 1 min time point, accumulation of ^{73}As-arsenite was not temperature dependent, however at 30 min it was, with a 1.6-fold increase in accumulation at 37°C compared with 4°C. ^{75}Se-selenite and ^{75}Se-selenide accumulation was temperature-dependent at both time points. For ^{75}Se-selenite there were 2.6-and 6.7-fold increases in accumulation at 37°C compared with 4°C, at 1 and 30 min, respectively ($P < 0.05$ and 0.0001, respectively). For ^{75}Se-selenide there were 2.2-and 4.3-fold increases in accumulation at 37°C compared with 4°C, at 1 and 30 min, respectively ($P < 0.05$ and 0.0001, respectively). This temperature dependence provides support for a carrier mediated process, at least for selenite and selenide uptake by HepG2 cells.

To further investigate the differences in accumulation of Se from selenide and selenite, concentration dependent accumulation experiments were conducted at 0.1 to 2.5 μM for 1 min (Figure 4-2D). Over the different concentrations, Se accumulation from ^{75}Se-selenide was significantly higher (by 3 to 8-fold) than Se accumulation from ^{75}Se-selenite ($P < 0.0001$).

In order to investigate the influence of As on Se accumulation, ^{75}Se accumulation studies at 0.1 μM with equimolar arsenite concentrations were completed (Figure 4-2E). Consistent with the time course studies (Figure 4-2B), ^{75}Se accumulation from ^{75}Se-selenide was significantly higher than from ^{75}Se-

selenite. Accumulation of ^{75}Se from ^{75}Se-selenide in the presence of arsenite was 23% higher than accumulation from ^{75}Se-selenide alone, while ^{75}Se-selenite accumulation from ^{75}Se-selenite remained unchanged.

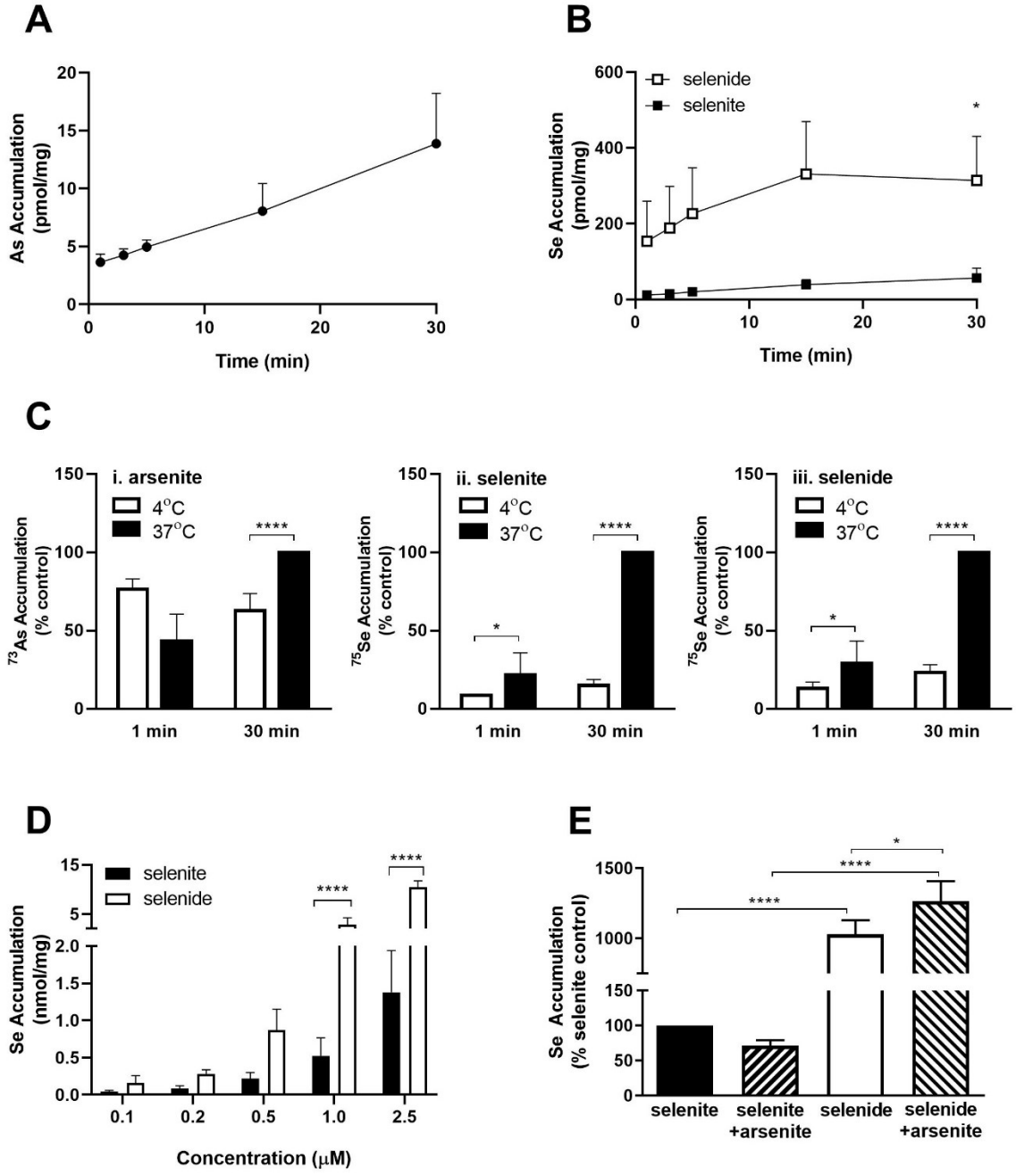

Figure 4-2. Accumulation of arsenite, selenite, and selenide by HepG2 cells in suspension. HepG2 cells at 1 x 10^6 cells/ml in HBSS buffer were incubated at 37ºC (or 4 ºC where indicated) with (**A**) ^{73}As- arsenite (1 µM, 50 nCi) for the indicated time points, (**B**) ^{75}Se-selenite or ^{75}Se-selenide (0.1 µM, 50 nCi), for the

indicated time points, (**C**) (i) ^{73}As- arsenite (1 μM, 50 nCi), (ii) ^{75}Se-selenite (0.1 μM, 50 nCi), or (iii) ^{75}Se-selenide (0.1 μM, 50 nCi), for the indicated time points (presented as % of 30 min 37°C control), (**D**) ^{75}Se-selenite or ^{75}Se-selenide (0.1 - 0.2 μM, 50 nCi and 1 - 2.5 μM, 75 nCi) for 1 min, or (**E**) ^{75}Se-selenite ± arsenite or ^{75}Se-selenide ± arsenite (0.1 μM, 50 nCi) for 1 min. Uptake was stopped by diluting the reaction in 800 μl of ice-cold HBSS. Samples were filtered over type A/E glass fibre filters and washed twice with ice-cold HBSS; radioactivity on filters was then quantified using a Packard Cobra II gamma counter. Symbols represent means (± S.D.) of at least three independent experiments. Significant differences between conditions are indicated with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ for (**B**) using mixed-effects analysis followed by Sidak post hoc test; for (**C**) using two-way ANOVA, followed by Sidak post hoc test; for (**D**) using one-way ANOVA, followed by Tukey's post hoc test.

4.4.3. Arsenite + Selenide Display a Higher Level of Toxicity Antagonism than Arsenite + Selenite in HepG2 Cells

The influence of arsenite and Se (selenite or selenide) concentration combinations on HepG2 cytotoxicity were assessed. Cells were treated with arsenite + selenite or arsenite + selenide in different combinations over nine concentrations, resulting in 81 dose-combinations, which were analyzed using the Combenefit program [53].

Arsenite and selenite matrix plots analyzed using the Loewe, BLISS and HSA models, showed a mutually antagonistic effect on cytotoxicity when both compounds were present at high concentrations (Figure 4-3A) [53–58]. Statistically significant antagonism, i.e., reduced cytotoxicity, was observed at 30 μM arsenite in combination with 0.1, 1, 3, 10, 30 and 100 μM selenite compared to untreated cells. At 30 μM selenite, statistically significant antagonism was observed with 0.01, 1, 10 and 30 μM arsenite, and at 100 μM selenite in combination with 30, 100 and 300 μM arsenite. The 1:1 combination of 30 μM arsenite and 30 μM selenite had the highest degree of mutual toxicity antagonism. The summary metric value for arsenite and selenite antagonism and synergy (SUM_SYN_ANT) was -140, the negative value indicates that these two compounds have overall mutual toxicity antagonism.

Arsenite and selenide matrix plots show significant antagonism at many more concentrations than arsenite and selenite, most notably including lower concentrations, of relevance to environmental and

dietary exposures. Antagonism was observed at concentration combinations as low as 0.01-10 μM arsenite paired with 0.1-3 μM selenide, in addition to the 10, 30 and 100 μM block observed with arsenite plus selenite. The SUM_SYN_ANT score for arsenite and selenide was -590, indicating a higher level of overall mutual toxicity antagonism between the two species in comparison with arsenite and selenite. Overall, a greater antagonism effect was observed at higher concentration combinations of arsenite and selenite, whereas with arsenite and selenide, treatment combinations at both low and high concentrations yielded antagonistic interactions.

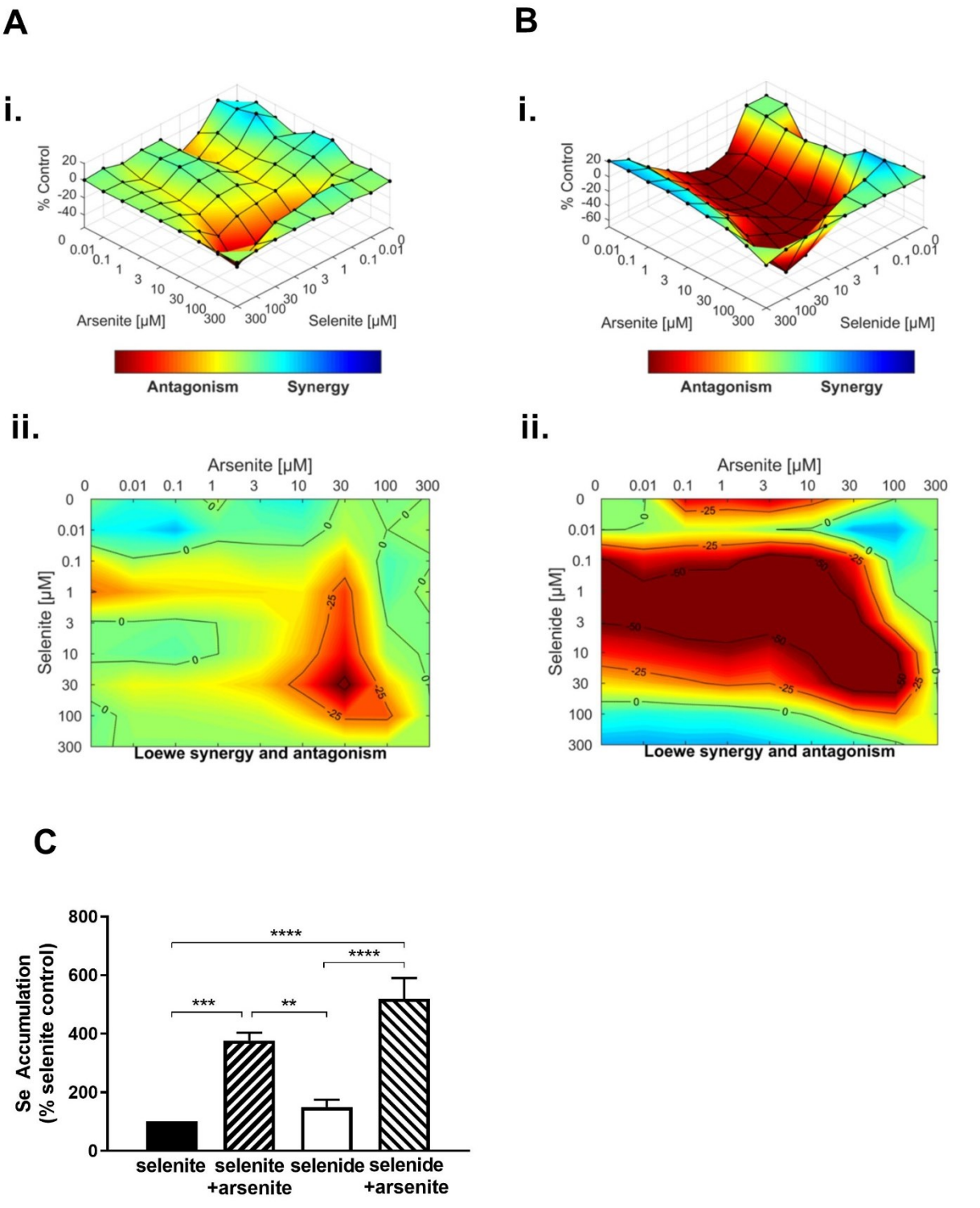

Figure 4-3. Toxicity and accumulation evaluation of arsenite + selenide and arsenite + selenite combination concentrations in HepG2 cells. Cells were seeded in a 96-well plate at 1 x 10^4 cells/well and treated with different concentration combinations of (**A**) selenite + arsenite, (**B**) selenide + arsenite for 72 h. Cell viability was determined using a tetrazolium-based cytotoxicity assay. Data used in analyses were

means of at least three independent experiments. Synergy distribution contour (i) and matrix (ii) plots were obtained using the Loewe additivity synergy model [54,55]. Statistically significant interactions ($P < 0.05$) are indicated with yellow/red for an antagonism effect, and blue for a synergistic effect. **(C)** Accumulation of ^{75}Se-selenite and ^{75}Se-selenide by adherent HepG2 cells in the presence and absence of arsenite at antagonistic concentrations was measured. HepG2 cells were seeded in a 6-well plate at 500,000 cells/well and treated with ^{75}Se-selenite (3 μM, 100 nCi), ^{75}Se-selenide (3 μM, 100 nCi) and in combination with arsenite (30 μM) for 24 h. Cells were then washed with ice-cold phosphate buffered saline, lysed in 0.5% Triton X-100 and the lysate was collected for gamma counting. Data points are means (± S.D.) of at least three independent experiments. Significant differences between the conditions are indicated with ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ (One-way ANOVA, followed by Tukey's multiple comparison post hoc test)

4.4.4. Accumulation of ^{75}Se from ^{75}Se-selenite and ^{75}Se-selenide in the Presence and Absence of Arsenite at Antagonistic Concentrations

To determine if mutual antagonism of arsenite and Se toxicity is due, at least in part, to influences on their accumulation, accumulation experiments using adherent HepG2 cells with a concentration combination that displayed mutual antagonism were completed. Arsenite (30 μM) in combination with selenite (3 μM) or selenide (3 μM) showed mutual antagonism of toxicity in dose response assays (Figure 4-3A and 4-3B). HepG2 accumulation of ^{75}Se delivered as ^{75}Se-selenite and ^{75}Se-selenide ± arsenite at the above-mentioned concentrations under similar conditions was measured. An increased accumulation of ^{75}Se from both ^{75}Se-selenite and ^{75}Se-selenide (3.8- and 3.5-fold, respectively) was observed in the presence of arsenite (Figure 4-3C).

4.4.5. X-ray Fluorescence Imaging shows Greater As and Se Accumulation in Arsenite + Selenide than Arsenite + Selenite Treated Cells

Cells were exposed to arsenite and selenite or selenide at a 1:1 ratio, at 60 μM for 2 hrs. High resolution XFI elemental maps for S, Zn, As and Se collected from HepG2 cultures are shown in Figure 4-4A. The S and Zn elemental maps allow for the definition of the whole cell. Mean and median densities (concentrations) for As and Se were calculated in different regions of interest of the cell images, and background support, are presented in Table 4-2. The background values were calculated in the areas of silicon nitride windows that were free from cells. The areal density of Se was 1.5-2 times higher in

HepG2 cells treated with arsenite + selenide, compared with that in cells treated with arsenite + selenite (Table 4-2). The areal density of As, was almost 3 times higher in cells treated with arsenite + selenide compared to similar areas of the cells treated with arsenite + selenite.

Table 4-2. The mean and median (med) areal densities of As and Se in HepG2 cells treated with arsenite in combination with selenite or selenide.

Treatment	As [ng/cm^2]			Se [ng/cm^2]		
	mean	med	S.D.	mean	med	S.D.
arsenite + **selenite**						
ROI#1 – area of high As and Se in the cell	14	13	6	22	21	9
ROI#2 – cell and its external skeleton	9	8	6	15	13	9
ROI#3 – background	2	2	2	3	3	3
arsenite + **selenide**						
ROI#1 – area of high As and Se in the cell	38	37	12	44	43	14
ROI#2 – cell and its external skeleton	29	29	14	32	31	16
ROI#3 – background	5	3	8	7	4	9

The standard deviation (denoted by S.D.) in this case is a measure of non-homogeneity of elemental distributions within the sample.

High resolution XFI elemental maps for S, Zn, As and Se collected from HepG2 cultures either untreated or treated individually with arsenite, selenite, or selenide are shown in Figure 4-5. There was no Se or As detected in untreated controls, no Se detected in the arsenite only treated control, and no As detected in the Se treated controls (Table 4-3). The level of As in the arsenite only treated control was similar to levels observed in the arsenite + selenite treatment group, but lower than the arsenite + selenide treatment group. Selenite-alone treated cells had similar levels of Se, while selenide-alone

treated cells had much lower levels of Se, than their respective + arsenite dual treatments (Figure 4-5, Table 4-3). Discrepancies in Se levels in the selenide-alone treatment sample are likely due to poor sample quality, as a result of a high number of crystals formed during the fixing and washing steps of sample preparation.

^{75}Se accumulation studies with ^{75}Se-selenite, ^{75}Se-selenide, ^{75}Se-selenite + arsenite and ^{75}Se-selenite + arsenite (with all Se and As at 60 μM) corroborated the dual treatment results, with significantly higher accumulation of ^{75}Se-selenide in the presence of arsenite compared to ^{75}Se-selenide alone (Figure 4-4B). Consistent with XFI results, accumulation of ^{75}Se-selenide + arsenite was also significantly higher than accumulation of ^{75}Se-selenite + arsenite.

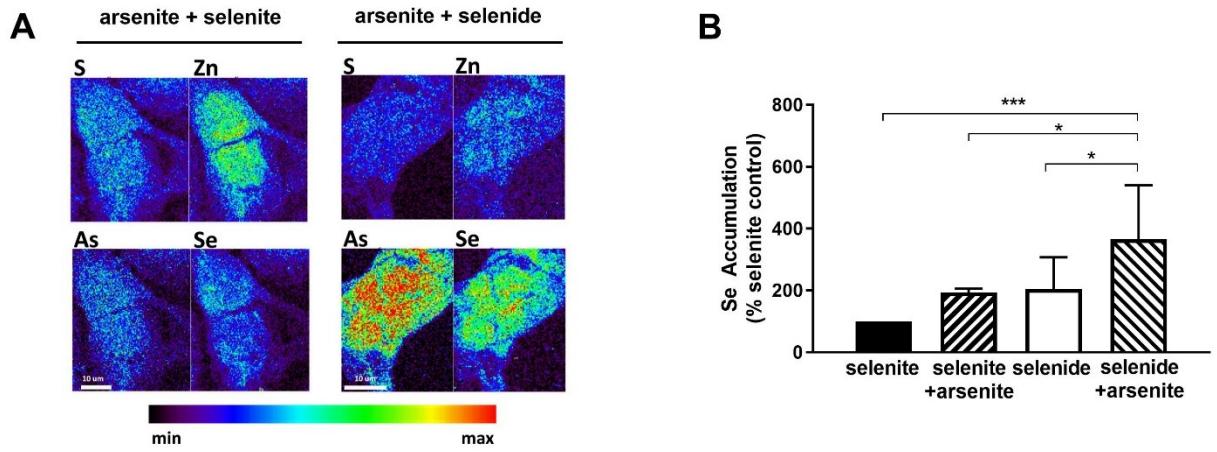

Figure 4-4. (**A**) XFI elemental maps for S, Zn, As and Se collected from HepG2 cell cultures treated with arsenite + selenite (left) or arsenite + selenide (right) for 2 h. Lower values correspond to a blue range, and higher values are denoted by the red range, as shown in the color bar. The images were collected using a step size of 300 nm, and 1 s dwell time/pixel. (**B**) ^{75}Se accumulation under XFI sample preparation conditions. HepG2 cells were seeded in a 6-well plate at 500,000 cells/well and treated with ^{75}Se-selenite, ^{75}Se-selenite + arsenite, ^{75}Se-selenide or ^{75}Se-selenide+ arsenite (60 μM, 100 nCi) for 2 h. Cells were then washed with ice-cold phosphate buffered saline, lysed in 0.5% Triton X-100 and the lysate was collected for scintillation counting to detect radioactivity. Data points are means (± S.D.) of at least three experiments. Significant differences between the conditions are indicated with * $P < 0.05$ and *** $P < 0.001$ (One-way ANOVA, followed by Tukey's multiple comparison post hoc test)

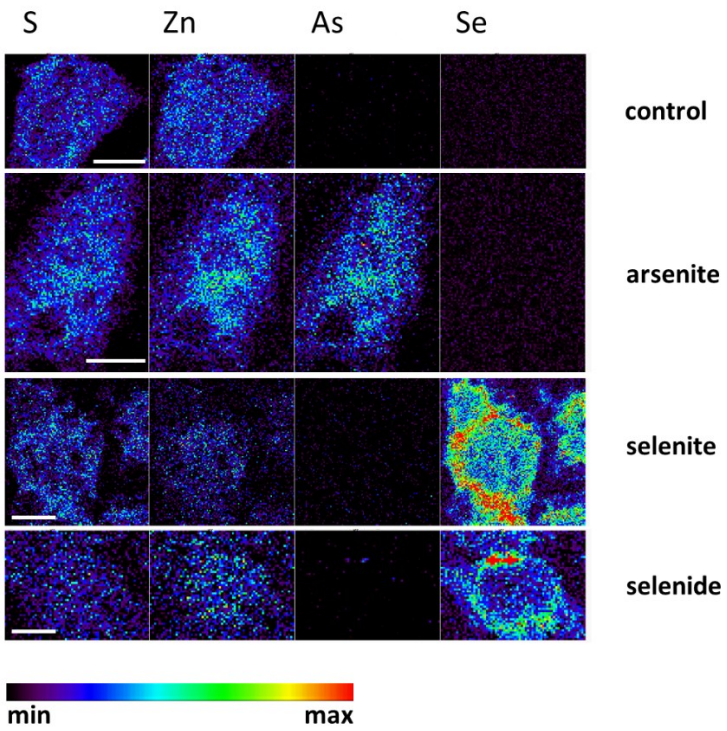

Figure 4-5. XFI elemental maps for S, Zn, As and Se collected from HepG2 cultures either untreated or treated with arsenite, selenite, or selenide. HepG2 cultures were exposed to 60 µM arsenite, selenite, or selenide for 2 h. Lower values correspond to a blue range, and higher values are denoted by the red range. The images were collected using a step size of 300 nm, and 1 s dwell time/pixel.

Table 4-3. The mean and median (med) areal densities of As and Se in untreated HepG2 cells or treated with arsenite, selenite, or selenide alone.

	As [ng/cm^2]			Se [ng/cm^2]		
Treatment	mean	Med	S.D.	mean	med	S.D.
Untreated	1	1	2	3	2	2
arsenite	9	8	6	3	2	2
Selenite	1	0	2	27	24	20
Selenide	1	0	2	6	5	4

The standard deviation (denoted by S.D.) in this case is a measure of non-homogeneity of elemental distributions within the sample.

The ROIs analyzed were from the total cell area.

To determine if cells were viable at concentrations used for XFI experiments, cytotoxicity assays were performed and IC_{50} values calculated for all treatment conditions at 6 hrs (Table 4-4). At this short time point, no significant cytotoxicity was detected for arsenite, selenide, arsenite + selenite and arsenite + selenide up to 300 μM, whereas, selenite had an IC_{50} of 69 μM at 6 hrs. This was in contrast with the 72-hr data (Figure 4-1 and Table 4-1), and suggests that toxicity induced by these compounds (detected with the tetrazolium-based assay), was relatively slow. Overall, the two-hour exposure to 60 μM of arsenite, selenite, or selenide either alone or in combination, for XFI, allowed us to image cells that were viable prior to fixation.

Table 4-4: IC_{50} values for arsenite, selenite, selenide, and a combination of arsenite and selenium species (0.01 – 300 μM) in HepG2 cells after 6 h of exposure.

Arsenic/Selenium Species	IC_{50} ± S.D. (μM)
Arsenite *(n=4)*	> 300
Selenite *(n=4)*	69 ± 7
Selenide *(n=4)*	> 300
Arsenite + Selenite *(n=4)*	> 300
Arsenite + Selenide *(n=4)*	> 300

4.5. Discussion

Arsenic and Se display a remarkable mutual antagonism of toxicity despite similarities in their chemical properties and metabolic pathways. Arsenite leads to the increased production of toxic reactive oxygen species (ROS) [67]. Selenium is an essential micronutrient and critical for the enzymatic activity of Se-containing enzymes such as glutathione peroxidase and thioredoxin reductase, important in reducing cellular ROS levels [68,69]. Depending upon chemical form, Se can be toxic in excess and can increase ROS production through reaction with thiol groups [70]. One direct mechanism for their mutual detoxification is through the formation of $[(GS)_2AsSe]^-$, which has been identified in the bile of laboratory animals co-treated with arsenite and selenite [7–10,71].

Small population studies with selenized yeast in Inner Mongolia have shown decreased levels of As content in blood, hair and urine in comparison to controls [19]. For these reasons, Se supplementation is being explored as a therapeutic in As-endemic regions. However, little work has been done to understand the handling of As and Se in human cell lines. The focus of this study was to investigate

the effects of exogenous Se (as selenite and selenide) on As toxicity in the human hepatoma cell line, HepG2. Different Se species (selenite vs selenide) were found to have differing effects on arsenite toxicity.

Although selenite is the form that has been used in most animal studies observing As and Se mutual antagonism, it is rapidly reduced in red blood cells and possibly enterocytes prior to efflux into plasma, where it is bound to albumin [35,37]. Thus, albumin-bound selenide is potentially the predominant form of Se presented to the liver after ingestion of selenite. Selenium accumulation in HepG2 cells was higher when treatment was with selenide rather than selenite, and selenide had a greater protective effect on As-induced cytotoxicity in HepG2 cells than selenite. Cytotoxicity assays at equimolar Se and As concentrations showed that selenite did not protect cells from arsenite toxicity, but selenide did (Figure 4-1). The reducing intracellular environment results in the conversion of selenite to selenide, likely by GSH [39]. The differences in IC_{50} between arsenite + selenite and arsenite + selenide led us to hypothesize that differences in cellular accumulation between selenite and selenide could influence their ability to antagonize arsenite toxicity.

Consistent with published literature, our results with HepG2 cells show that ^{75}Se accumulation was 6- to 13- fold higher with ^{75}Se-selenide than ^{75}Se-selenite treatment over multiple concentrations (0.1 to 2.5 μM) (Figure 4-2B and 4-2D). In human keratinocytes, selenite uptake was linear and lower than selenide uptake over a range of concentrations, while selenide uptake followed saturation kinetics with a K_m of 279 nM [40]. Furthermore, Ganyc and Self provided strong evidence that it was selenide being taken up by cells and not a conjugate [40]. Olm et al., have shown in cancer cells that extracellular cysteine increases the uptake of selenite, presumably through the formation of selenide [41]. In trout hepatocytes, uptake differences between selenite and its reduced form were investigated by conducting experiments in the presence and absence of GSH. The rate of selenite + GSH (presumably, selenide)

uptake was 8-fold higher compared to selenite [42]. Thus, consistently in multiple different model systems, selenide is taken up by cells more efficiently than selenite.

Our results show that statistically significant mutual antagonism between arsenite and selenite is observed at higher concentrations of both species (Figure 4-3A), whereas mutual antagonism between arsenite and selenide is observed at both low and high concentrations (Figure 4-3B). This difference in arsenite toxicity antagonism between selenite and selenide is likely explained by the lower amount of Se accumulation within the cell with selenite versus selenide treatment for intracellular reaction with arsenite and GSH.

This is consistent with the observed increase in ^{75}Se-selenide accumulation in the presence of arsenite, when HepG2 cells are exposed to 0.1 μM of both species (Figure 4-2E), while the presence of arsenite has no effect on selenite accumulation at this concentration. Additionally, at this same concentration combination for arsenite:selenide (0.1:0.1 μM), dose response assays show an antagonism of toxicity between these two species but no significant change in viability for arsenite:selenite (0.1:0.1 μM) (Figure 4-3A and 4-3B). However, when cells were exposed to a concentration combination (As:Se, 30:3 μM) that showed strong mutual antagonism for both arsenite + selenite and arsenite + selenide in the cytotoxicity assays (Figure 4-3A and 4-3B), we observed increased Se accumulation with both selenite and selenide in the presence of arsenite, while noting that Se accumulation for selenide was still higher (Figure 4-3C).

Both these differences favour the increased accumulation of selenide + arsenite in cells. In addition, trivalent arsenicals and selenite can bind to sulfhydryl groups of proteins in cells with high affinity [72,73]. It is interesting that arsenite and selenite binding to sulfhydryl groups of proteins is one of the reasons for their individual cytotoxicity, yet when together they are bound to GSH to form $[(GS)_2AsSe]^-$, it is also the reason for their mutual antagonism [74]. Co-localization data for S, As, and

Se (Figure 4-6) supports that $[(GS)_2AsSe]^-$ is likely formed in HepG2 cells, higher accumulation of ^{75}Se-selenide could explain the increased protective effect. Arsenic and Se also demonstrated high co-localization in brain regions of rainbow trout given arsenite and selenomethionine in diet, and in the liver, gall bladder and small intestine of hamsters injected with arsenite and selenite [75,76]. Additionally, dose response (Figure 4-3A and 4-3B) and radioactive accumulation data (Figure 4-4B) support the increased accumulation seen through XFI studies for both arsenite:selenite and arsenite:selenide at 60:60 μM (Figure 4-4A).

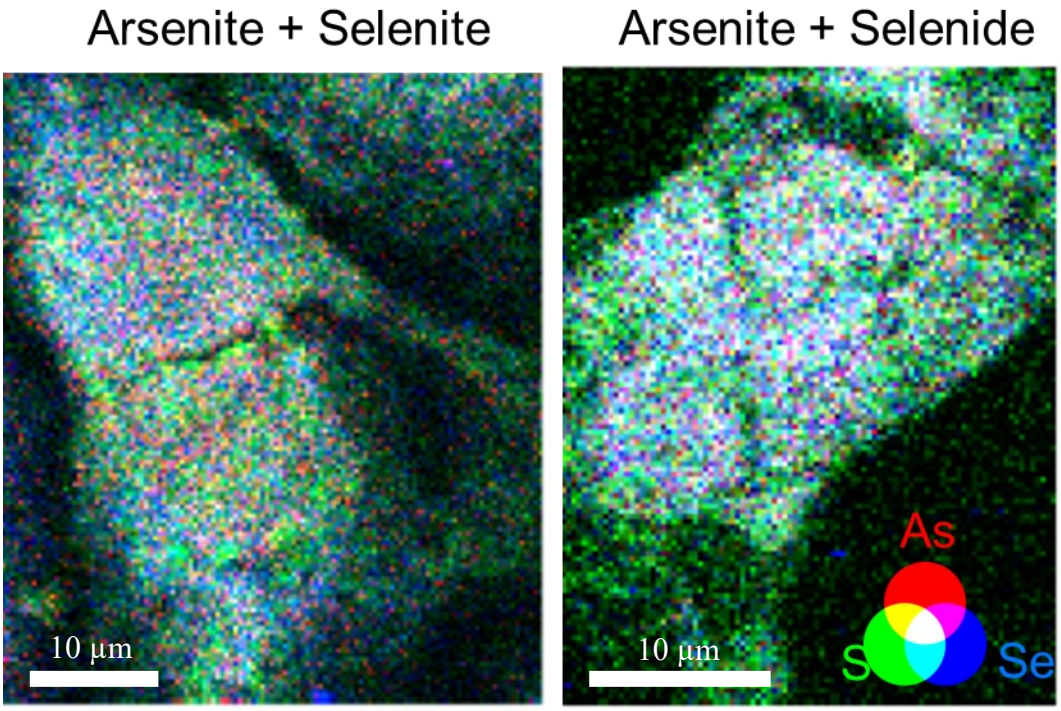

Figure 4-6. Overlay of arsenic, selenium, and sulphur XFI elemental maps collected from HepG2 cell cultures treated with arsenite + selenite or arsenite + selenide. HepG2 cell cultures were treated with (left) 60 μM arsenite + selenite or (right) 60 uM arsenite + selenide for 2 h. Sulphur is denoted by green, arsenic by red and selenium by blue color. Higher intensity of the color corresponds to the higher areal density (areal concentration) of the respective elements. The images were collected using a step size of 300 nm, and 1 s dwell time/pixel.

It was unanticipated that increased accumulation of toxicants should be indicative of protection. Indeed, it has been shown in rat and human hepatocytes that selenite prevents As biotransformation,

resulting in increased levels of As in cells [34,77]. However, *in vivo* studies have shown arsenite and selenite to increase biliary efflux of each other, leading to reduced retention in the liver [32]. Biliary efflux of $[(GS)_2AsSe]^-$, is likely mediated by the ATP-binding cassette transporter multidrug resistance protein 2 (MRP2/*ABCC2*) [12]. Thus, both the formation and subsequent efflux of $[(GS)_2AsSe]^-$ would be important in As and Se mutual antagonism. Future studies should utilize human hepatic models that are properly polarized. One of the limitations of the HepG2 cells is the absence of extensive canalicular networks and the polarized phenotype, which may impact the ability of hepatocyte canalicular proteins to transport substrates [78]. This study provides evidence for the physiological role of selenide in As detoxification and lays the groundwork for better design of future *in vitro* As-Se studies.

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5. General Discussion

In this chapter, the main findings of my thesis will be summarized, and future directions described.

5.1. Chapter 2. Human Red Blood Cell Uptake and Sequestration of Arsenite and Selenite: Evidence of Seleno-bis(*S*-glutathionyl) Arsinium Ion Formation in Human Cells

The mutually protective relationship between As and Se has been established since the early 1900s. Animal studies have shown that the first mechanism of Se protection against As-induced toxicity is likely through the sequestration of As and Se in RBCs through the formation of $[(GS)_2AsSe]^-$. This sequestration of $[(GS)_2AsSe]^-$ would likely slow the distribution of As to tissues, reducing As burden. However, $[(GS)_2AsSe]^-$ has not been identified in human cell lines, and As and Se cellular interactions are poorly understood. The objectives were to first characterize As and Se uptake pathways in RBCs, secondly to investigate the effect of Se on As accumulation in RBCs, and vice versa, and lastly to determine the species of As and Se present in RBCs when co-treated with both compounds. These studies showed that Se^{IV} uptake is AE1-mediated, and As^{III} uptake is partially AQP3-mediated, while no evidence of GLUT1-mediated As^{III} uptake was found. Se^{IV} resulted in increased accumulation of As^{III}, at doses as low as 0.3 μM. As^{III} also resulted in increased accumulation of Se^{IV}, in the presence of albumin; corroborating evidence in the literature that Se effluxed from RBCs is likely albumin bound in plasma. Speciation analysis using XAS showed that 73% of the As and Se present in RBCs co-treated with As^{III} and Se^{IV} (30 μM, 45 min) was in the form of $[(GS)_2AsSe]^-$. The remaining 26% of As was present as $As(GS)_3$, providing the first evidence of the formation of both As-GSH conjugates in human cells.

Future speciation analysis will be carried out at lower treatment concentrations of As and Se, to determine the extent of $[(GS)_2AsSe]^-$ formation at physiologically relevant concentrations mimicking blood As and Se levels of individuals with chronic As exposure. The more sensitive technique, high

energy resolution fluorescence detection (HERFD) X-ray absorption near-edge spectroscopy (XANES) will be employed.

Inter-individual variability is a common phenomenon in As-induced toxicities. This can be attributed to differences in nutrition and lifestyle habits, as well as, polymorphic variants in metabolism enzymes and transporters. The next steps of this study are also to characterize SNP variants of AE1, to investigate their influence on Se uptake.

In addition to inorganic As, methylated species of As can also be present in the blood of people chronically exposed to arsenic in drinking water. Investigations of the uptake and sequestration mechanism of methylated arsenical in RBCs may also be of importance.

5.2. Chapter 3. Selenium Influence on the Hepatobiliary Efflux of Arsenic in Sandwich Cultured Human Hepatocytes

The second mechanism of Se protection against As-induced toxicity in humans is likely through the formation and biliary efflux of $[(GS)_2AsSe]^-$. $[(GS)_2AsSe]^-$ is stable at biliary pH and can potentially break the enterohepatic recirculation cycle of As, allowing for increased fecal elimination. In this chapter, the influence of Se on the hepatobiliary efflux of As in SCHH was investigated. Consistent with published literature, all human hepatocyte preparations displayed sinusoidal efflux of As, and all As biliary efflux observed was GSH-dependent. Surprisingly, it was found that selenite resulted in the reduced or unchanged efflux of As across the sinusoidal and canalicular surfaces of SCHH. Preliminary data shows that selenide increases or does not change As biliary efflux; selenide accumulation in hepatocytes is higher than selenite; increased Se biliary efflux is observed in the presence of As; and selenide is able to protect SCHH against As-induced toxicity while, selenite only confers protection when the preparation displays a potential for As biliary efflux.

Due to the limited availability of primary human hepatocytes, future studies to investigate the effects of different Se compounds on the hepatobiliary efflux of As can be carried out in HepaRG cells. These are immortalized cells, often used as a surrogate for hepatocytes, which can differentiate and develop functional bile canalicular networks. Results from HepaRG cells can then be corroborated in human hepatocytes, when and as they become available.

MRP2 is the transporter present on the canalicular surface of hepatocytes, that has been shown to efflux $As(GS)_3$ and $[(GS)_2AsSe]^-$. MRP2 is encoded by the *ABCC2* gene, which is highly polymorphic. Inter-individual variation in As-induced toxicity has also been attributed to differences in arsenic metabolism, likely caused by arsenic methyltransferase (AS3MT). Samples from all hepatocyte preparations have been saved for future genotyping studies to correlate observed differences in cytotoxicity and efflux among SCHH preparations with possible *ABCC2* and *AS3MT* SNP variants.

Lastly, as part of optimization efforts to carry out As and Se speciation in bile canalicular network, SCHH samples treated with As and Se alone and in combination have been saved for XAS analysis. Speciation of As and Se species in canalicular networks would provide valuable information as to the interactions of As and Se in human hepatocytes.

5.3. Chapter 4. Studies of Selenium and Arsenic Mutual Detoxification in Human HepG2 Cells

Following up on unanswered questions from Chapter 4, HepG2 cells, being readily available, were utilized to characterize differences in selenite and selenide uptake and cytotoxicity. In addition, HepG2 cells were used to determine optimal treatment combinations of As and Se, which resulted in mutual antagonism of toxicity. Our results showed selenide uptake in HepG2 cells to be higher than selenite, in a concentration dependent manner. In addition, selenide displays a greater overall antagonism of toxicity with arsenic, compared to selenite which displayed antagonism with arsenic at higher concentrations only. Functional uptake assays corroborated observed results from the cytotoxicity

assays, showing increased accumulation of Se in the presence of As, at antagonistic concentrations. If $[(GS)_2AsSe]^-$ is formed within HepG2 cells, the increased accumulation would be explained by the lack of polarized phenotype and canalicular networks in HepG2 cells, resulting in limited efflux of As metabolites.

As mentioned above, HepaRG cells are a more suitable *in vitro* model for investigation of hepatocyte metabolic and transport functions in the absence of primary human hepatocytes. They would also serve as an excellent tool for the continued optimization of As and Se interactions for functional studies in primary human hepatocytes.

Since the cellular handling of both As and Se is inadequately understood, the use of genomics techniques to identify genes in HepaRG cells that are up-regulated in As and Se treatments, followed by functional assays to characterize activity would be an excellent next step to better understand As and Se interactions. In addition, the use of knockdown systems, such as CRISPR-Cas9 would provide direct evidence for the physiological role of MRP2 in As transport.

5.4. Conclusion

This thesis work provides insight into As and Se interactions in human cell lines. Figure 5-1 provides a general overview of the physiological interactions between As and Se species in human cells. Upon oral exposure and absorption across enterocytes, arsenite uptake into human RBCs is partially AQP3-mediated. Selenite uptake is mediated by AE1, followed by its reduction to selenide. High levels of GSH in RBCs favour the formation of $[(GS)_2AsSe]^-$. Sequestration of As and Se in RBCs in the form of $[(GS)_2AsSe]^-$, leads to the delayed release of As to susceptible tissues. This detoxification mechanism lowers the burden of As at target organs. Following release from RBCs, arsenite and selenide are the likely forms presented to hepatocytes. Upon uptake, arsenite and selenide possibly

interact to form the non-toxic metabolite, $[(GS)_2AsSe]^-$, which may then be effluxed into bile, likely by MRP2.

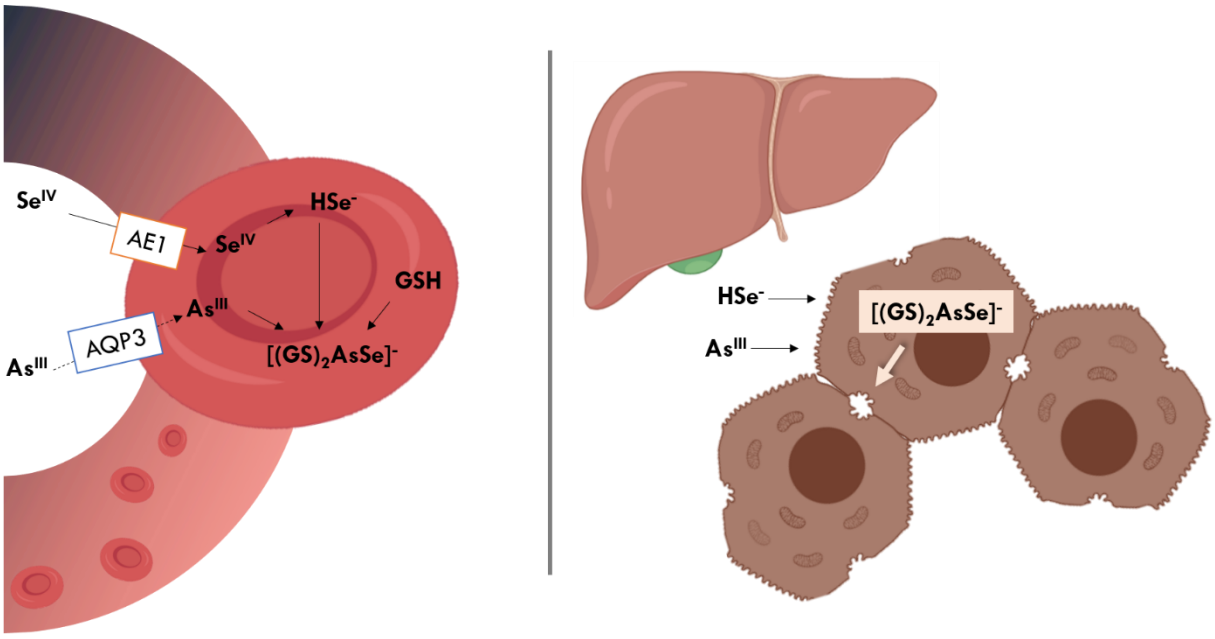

Figure 5-1. Overview of arsenic and selenium uptake and cellular handling in red blood cells and hepatocytes.

Information on arsenic and selenium interactions has mainly come from studies using animal models and human exposure studies. Although arsenic is a carcinogen in humans, it is a poor one in rodents and because of this, the focus of this thesis has been to use human cell models to understand mechanisms of arsenic and selenium detoxification. Epidemiological studies of Se-supplementation in As-endemic regions need to be supported by robust *in vitro* data in human cell lines. This thesis work lays the foundation to understand how human RBCs sequester $[(GS)_2AsSe]^-$, provides support to the physiological role of selenide and characterizes the influence of different selenium species on the hepatobiliary efflux of arsenic. A better understanding of the mechanistic basis of As and Se interactions would inform better therapeutic strategies to prevent and treat As-induced disease.

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Chapter 1

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Appendix 1

Characterization of Single Nucleotide Polymorphic Variants of the Multidrug Resistance Protein 2 (MRP2/*ABCC2*)

A.1. Introduction

Arsenic is a metalloid found ubiquitously in the earth's crust and is a Group I human carcinogen[1]. Chronic exposure to arsenic causes tumours of the skin, lung and bladder [1]. Millions of people world-wide are exposed to arsenic contaminated drinking water with concentrations higher than acceptable limits of 10 μg/L set by the World Health Organization [1,2]. In Canada, there are reported arsenic hotspots that naturally exist where well water exceeds 10 μg/L [2,3]. Thirty percent of Canadians rely on well water for their water supply [2]. Apart from this, mining is a large industry in Canada. Today, at the Giant Mine in Yellowknife, there is a quarter of a million tonnes of arsenic waste that awaits disposal[4]. These are examples of exposure scenarios that reveal arsenic remains a global public health concern, including in Canada[2,4,5]. In addition to environmental exposure, arsenic is also in use in therapeutic applications. Arsenic trioxide is used in the treatment of acute promyelocytic leukemia [6]. Treatment outcome is remarkably successful with a >90% remission rate[7].

Arsenic biotransformation occurs predominantly in the liver through a series of oxidative methylation, followed by reduction, steps, resulting in monomethylated and dimethylated trivalent and pentavalent arsenic species [8,9]. Monomethylated arsenic species, as well as inorganic arsenic, can then undergo glutathione conjugation[10,11]. The multidrug resistance proteins (MRPs) are involved in the efflux of methylated arsenic species and arsenic-glutathione conjugates across the basolateral, as well as, the apical surface of the hepatocyte[10,12,13]. They belong to the ATP-Binding Cassette (ABC) transporter family, subfamily C; which are primary active transmembrane proteins that play a role in drug resistance and xenobiotic elimination[14].

The human multidrug resistance protein 2 (MRP2, gene name *ABCC2*), located on the canalicular surface of the hepatocytes is a transporter of arsenic triglutathione ($As(GS)_3$) and seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ and, likely $MMA(GS)_2$ [13,15,16]. The *ABCC2* gene is highly

polymorphic, resulting in genetic variants of MRP2 being expressed in certain individuals[17–19]. These may alter the efflux of arsenic species by MRP2 and provide insight into inter-individual differences in arsenic-induced carcinogenesis.

MRP2 SNP variants (SNVs) have been shown to alter transport activity of other non-arsenic compounds both using *in vitro* models and in clinical settings [20]. The aims of this study were to characterize the cellular localization of selected MRP2 SNVs and to determine whether they altered MRP2 transport of $As(GS)_3$ and $[(GS)_2AsSe]^-$. SNVs were selected for characterization based on the following: (i) a non-synonymous change, (ii) location of the amino acid change on a cytosolic loop or transmembrane domain, and/or (iii) known association with clinical toxicity to a therapeutic agent in humans or disruption of function *in vitro*. Mutations in the transmembrane domain and cytosolic loop were selected because MRP2 substrates are hydrophilic and substrate binding sites were likely to be in those regions of the protein sequence [21]. Thirteen different MRP2 variants were selected to be characterized, with the following amino acid changes: R353H, R412G, V417I, S789F, R1150H, R1181L, V1188E, P1291L, N1244K, A1450T, T1477M, C1515Y, and the double mutant, V1188E/C1515Y (Fig A-1). MRP2-R353H is the only variant with a mutation in the extracellular loop, selected for its high prevalence and altered transport activity of fluorescence substrates of MRP2 [22].

A.2. Methods

A.2.1. Materials

^{73}As-arsenate (1.2 mCi/ml) was purchased from Los Alamos Meson Production Facility (Los Alamos, NM). ^{75}Se-selenite (25.7 mCi/ml) was obtained from the University of Missouri Research Reactor (MURR, Columbia, MO). [6,7-^{3}H(N)]-estradiol-17β–glucuronide (41.4 Ci mmol^{-1}) was from Perkin Elmer (Woodbridge, ON). Sodium selenite, sodium (meta)arsenite, reduced glutathione (GSH), Tris

base, ATP, AMP, $MgCl_2$, estradriol-17β-glucuronide ($E_2$17βG), N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), polyvinylidene difluoride (PVDF) membranes and sodium butyrate (NaB) were purchased from Millipore Sigma (Oakville, ON). Creatine kinase, creatine phosphate, GSH reductase, NADPH, X-tremeGENE 9 transfection reagent, PhosSTOP phosphatase inhibitor cocktail tablets and Complete, Mini, EDTA-free protease inhibitor cocktail tablets were purchased from Roche Applied Science (Laval, QC). SuperSignal™ West Pico Chemiluminescent Substrate, Lipofectamine 2000® transfection reagent, $CaCl_2$ and the anti-MRP2 monoclonal anitbody (mAb) M_2I-4 were from Thermo Scientific (Fair Lawn, NJ). The anti-Na^+/K^+ ATPase polyclonal antibody (pAb) H-300 was from Santa Cruz Biotechnology (Dallas, TX).

A.2.2. Cell lines and Expression Constructs

The HEK293T cell line was from the American Type Culture Collection (ATCC) (Manassa, VA) and was maintained in Dulbecco's modified Eagle's medium containing 7.5% fetal bovine serum. MRP2 complementary DNA was a gift from Dr. Susan P.C. Cole (Queen's university) and its construction in pcDNA3.1(-) vector has been described previously [23].

A.2.3. Generation of MRP2 Variants

Variants were generated using a QuikChange II XL site-directed mutagenesis kit (Stratagene, Agilent Technologies, Santa Clara, CA), using pcDNA3.1-MRP2 as the PCR template according to the manufacturer's instructions. Mutagenic primers used are listed in Table A-1.

Table A-1. Mutagenic primer sequences for synthesis of MRP2 SNP variants

Amino Acid Change	Nucleotide Change	Primer Sequences (5' → 3')
R353H	1058G>A	F: GCTGATCTCCTTTGCAAGTGACCATGACACATATTTGTGGATTGG R: CCAATCCACAAATATGTGTCATGGTCACTTGCAAAGGAGATCAGC
R412G	1271A>G	F: CTATCCAATTTGGCCGGGAAGGAGTACACCG R: CGGTGTACTCCTTCCCGGCCAAGTTGGATAG
V417I	1249G>A	F: GGCCAGGAAGGAGTACACCATTGGAGAAACAGTG R: CACTGTTTCTCCAATGGTGTACTCCTTCCTGGCC
S789F	2366C>T	F: GATGACCCCCTGTTTGCAGTGGATGC R: GCATCCACTGCAAACAGGGGGTCATC
R1150H	3449G>A	F: CGCCAGCTGAGGCATCTGGACTCTGTCACC R: GGTGACAGAGTCCAGATGCCTCAGCTGGCG
R1181L	3542G>T	F: CCGTGCCTTTGAGCACCAGCAGCTATTTCTGAAACACAATGAGG R: CCTCATTGTGTTTCAGAAATAGCTGCTGGTGCTCAAAGGCACGG
V1188E	3563T>A	F: CTGAAACACAATGAGGAGAGGATTGACACCAACC R: GGTTGGTGTCAATCCTCTCCTCATTGTGTTTCAG
N1244K	3732T>G	F: GGCTTTGTTCTGTCCAAGGCACTCAATATCACACAAACCC R: GGGTTTGTGTGATATTGAGTGCCTTGGACAGAACAAAGCC
P1291L	3872C>T	F: GATAAGAGGCCTCTGCCAGATTGGCCC R: GGGCCAATCTGGCAGAGGCCTCTTATC
A1450T	4348G>A	F: CTGTGCCTGGGCAGGACTCTGCTTCGGAAATCC R: GGATTTCCGAAGCAGAGTCCTGCCCAGGCACAG
T1477M	4430C>T	F: GACAACCTCATTCAGATGACCATCCAAAACGAG R: CTCGTTTTGGATGGTCATCTGAATGAGGTTGTC
C1515Y	4544G>A	F: CGGGAAGATTATAGAGTACGGCAGCCCTGAAGAACTGC R: GCAGTTCTTCAGGGCTGCCGTACTCTATAATCTTCCCG

A.2.4. MRP2 expression in HEK293T Cells

Transfections in HEK293T cells were carried out using the calcium phosphate method, as described previously, with minor modifications. Briefly, 3 x 10^6 cells were seeded onto 150 mm tissue culture plates. After 24 hrs, DNA solution was prepared with 18 μg DNA added to 250 mM $CaCl_2$, which was then added dropwise to HEPES buffer (275 mM NaCl, 1.5 mM Na_2HPO_4 and 55 mM HEPES, pH 7.0). This DNA solution was then added dropwise to cells and allowed to incubate for another 24 hrs, followed by a media change. Cells were allowed to grow for 72 hrs post-transfection. Transfections were completed by the calcium phosphate method for all data except Figure A-4B; where indicated, NaB (2, 5, 10 mM) was added to the culture medium for 24, 48 or 72 hrs prior to harvesting, in cells transfected using the calcium phosphate method, or lipofectamine 2000® or X-tremeGENE 9 transfection reagents were used as per manufacturer's instructions, after which cells were allowed to grow for 48-72 hrs. For whole cell lysate extracts, cells were washed twice with ice-cold phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4), and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 0.01 M Tris HCl, pH 7.4, 0.14 M NaCl), followed by centrifugation at 2800 *g* for 10 min. The supernatant was collected for immunoblotting.

For membrane vesicle preparations, cells were washed twice with ice-cold homogenization buffer (250 mM sucrose, 50 mM Tris, 0.25 mM $CaCl_2$, pH 7.4), scraped into homogenization buffer and centrifuged at 800 *g* for 10 min. Cell pellets were then stored at -80ºC in homogenization buffer containing protease inhibitor tablets, until ready for membrane vesicle preparation.

A.2.5. Membrane Vesicle Preparation

Plasma membrane-enriched vesicles were prepared from HEK293T cells overexpressing WT or mutant-MRP2 or empty vector control, as previously described [24]. Briefly, cell pellets were thawed on ice and resuspended in protease inhibitor-containing homogenization buffer. Nitrogen cavitation (pressurized to 200 p.s.i. and released into atmospheric pressure) was used to disrupt cells.

Ethylenediaminetetraacetic acid (1 mM) was then added to the disrupted cells and centrifuged at 800 *g* for 10 min to remove unbroken cells and nuclei. The supernatant was collected and layered onto 12 ml of 35% w/w sucrose in 50 mM Tris, pH 7.4, and centrifuged at 100 000 *g* for 1 hr at 4ºC. The interphase was collected and diluted with dilute Tris sucrose buffer (25 mM sucrose, 50 mM Tris, pH 7.4) and centrifuged at 100 000 *g* for 30 min at 4ºC. The membrane pellet was washed with 1 ml Tris-sucrose buffer, followed by resuspension in the same using a 27 gauge needle, then aliquoted and frozen at -80ºC.

A.2.6. Immunoblotting

MRP2 protein expression in the plasma membrane-enriched vesicles was confirmed by immunoblot analysis. WT- and variant-MRP2 whole cell lysate extracts (10 μg) and membrane vesicles (1 μg) were resolved by SDS-PAGE on 6% gels and transferred to a PVDF membrane. The PVDF membranes were then immunoblotted using the anti-MRP2 rat mAb M_2I-4 (1:10 000), followed by an HRP-conjugated secondary antibody. Upon the addition of SuperSignal™ West Pico Chemiluminescent substrate, blots were imaged on ChemiDoc™ (BioRad). To control for protein loading, blots were also probed with anti-Na^+/K^+ ATPase rabbit pAb H-300 (1:10 000) followed by an HRP-conjugated secondary antibody. Relative protein levels of MRP2 variants were quantified using ImageJ software, normalized for protein loading (Na^+/K^+ ATPase signal) and reported as a percent of WT MRP2.

A.2.7. ^{3}H-$E_2$17βG Vesicular Transport Assay

ATP-dependent transport of ^{3}H-$E_2$17βG by WT- and variant-MRP2 enriched, plasma membrane vesicles was measured as described previously [25,26]. Briefly, membrane vesicles (10 μg) were incubated with (^{3}H-$E_2$17βG (1 μM, 50 nCi) in transport buffer (adenosine triphosphate (ATP) or adenosine monophosphate (AMP) (4 mM), $MgCl_2$ (10 mM), creatine phosphate (10 mM), creatine kinase (100 μg/μl), Tris (50 mM, pH 7.4), sucrose (250 mM)) at 37ºC for 90 sec. Transport was stopped by diluting the reaction with 800 μl of ice-cold Tris-sucrose buffer (50 mM Tris, 250 mM

sucrose, pH 7.4), followed by filtration over glass fibre filters (type A/E) and two washes with 3.5 ml of Tris-sucrose buffer. Filters were left to air dry, placed in scintillation vials, 3 ml of liquid scintillation cocktail added and radioactivity was quantified by liquid scintillation counting using a Microbeta^2 counter (PerkinElmer Life). ATP-dependent transport was calculated by subtracting transport in the presence of AMP from transport in the presence of ATP.

A.2.8. Chemical Synthesis of $^{73}As(GS)_3$, $[(GS)_2{}^{73}AsSe]^-$ and $[(GS)_2As^{75}Se]^-$

^{73}As-arsenite ($^{73}As^{III}$) was reduced from ^{73}As-arsenate ($^{73}As^{V}$) in a metabisulfite-thiosulfate solution, as previously described [27]. $^{73}As(GS)_3$ was prepared from $^{73}As^{III}$ and GSH, as described previously [13,28]. Briefly, $^{73}As^{III}$ (50 nCi/pt), As^{III} (25 μM) and GSH (75 mM) were added under a nitrogen atmosphere in Tris-sucrose buffer and incubated at room temperature for 30 min. $[(GS)_2{}^{73}AsSe]^-$/ $[(GS)_2As^{75}Se]^-$ were prepared as described previously[13,29]. Briefly, GSH (150 mM) was dissolved in Tris-sucrose buffer and pH was adjusted to 7.5. $^{73}As^{III}$ and As^{III} and selenite (Se^{IV}) or ^{75}Se-selenite ($^{75}Se^{IV}$) (50 μM, 50 nCi/pt) were added to GSH (150 mM) and incubated at room temperature for 15 min.

A.2.9. $^{73}As(GS)_3$, $[(GS)_2{}^{73}AsSe]^-$, $[(GS)_2As^{75}Se]^-$ Vesicular Transport Assays

ATP-dependent transport of $^{73}As(GS)_3$, $[(GS)_2{}^{73}AsSe]^-$ and $[(GS)_2As^{75}Se]^-$ by WT- and variant-MRP2 enriched, inside-out plasma membrane vesicles was measured as described previously [13]. Briefly, membrane vesicles (20 μg) were incubated with $^{73}As(GS)_3$, $[(GS)_2{}^{73}AsSe]^-$ or $[(GS)_2As^{75}Se]^-$ (1 μM, 50 nCi) in transport buffer (adenosine triphosphate (ATP) or adenosine monophosphate (AMP) (4 mM), $MgCl_2$ (10 mM), creatine phosphate (10 mM), creatine kinase (100 μg/ml), GSH reductase (5 μg/ml), NADPH (0.35 mM), GSH (3 mM), Tris (50 mM, pH 7.4), sucrose (250 mM)) at 37ºC for 3 min. Transport was stopped and vesicles washed and filtered as described for the ^{3}H-$E_2$17βG vesicular transport assay, with differences in ^{75}Se quantification, where radioactivity on air-dried filters was directly quantified using the Packard Cobra II gamma counter.

A.3. Results and Discussion

Polymorphisms in the *ABCC2* gene can cause Dubin Johnson syndrome (DJS), characterized by conjugated hyperbilirubinema due to impaired hepatobiliary transport of conjugated bilirubin. *ABCC2* polymorphisms can also result in changes in phenotype that result in subtle, poorly understood changes in MRP2 function, such as an altered response to xenobiotic toxicity. For the purposes of this study, MRP2 SNVs were selected based on their localization in the transmembrane domain or cytosolic loop, as well as for their effect on MRP2 function or clinical significance. SNVs were selected based on a minor allele frequency of at least 1%. Two additional variants, R1150H and T1477M, with less than 1% allele frequency were included due to their effects on transport activity and implication in DJS (Table A-2). It is noteworthy that all selected SNVs are present in membrane spanning domains (MSD) 1 and 2 and associated extracellular and cytosolic regions (including NBDs). Mutations in MSD0 (transmembrane (TM) helices 1-5) were not selected as the MSD0 is unlikely to be important for transport function but is important for the correct apical localization of MRP2 in polarized cells [30–32].

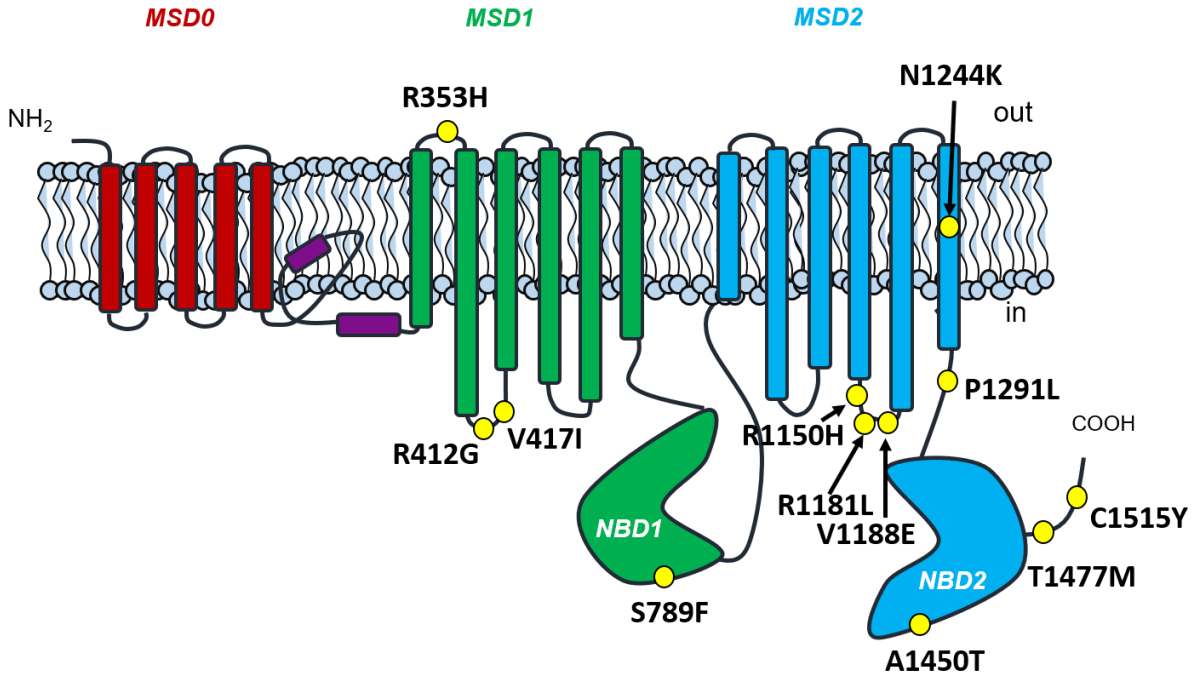

Figure A-1. Localization of the human MRP2 single nucleotide polymorphic variants in a general predicted topology of MRP2. The membrane spanning domains (MSD) and the nucleotide binding domains (NBD) are indicated. Figure modified from [33] and used with permission.

Prior to functional characterization of the 13 SNVs, the influence of the mutations on total cellular MRP2 levels was investigated. Immunoblot analysis of the whole cell lysate extracts revealed comparable protein levels for WT-MRP2 and all variants, with the exception of S789F, R1150H and A1450T, which showed a 81%, 80% and 89% decrease in cellular protein levels, respectively (Figure A-2A and A-2B). Protein levels of MRP2-V1188E, MRP2-C1515Y and the double mutant, MRP2-C1515Y/V1188E, were 2.3-, 1.9- and 1.8-fold higher than WT-MRP2 levels, respectively (Figure A-2A and A-2B).

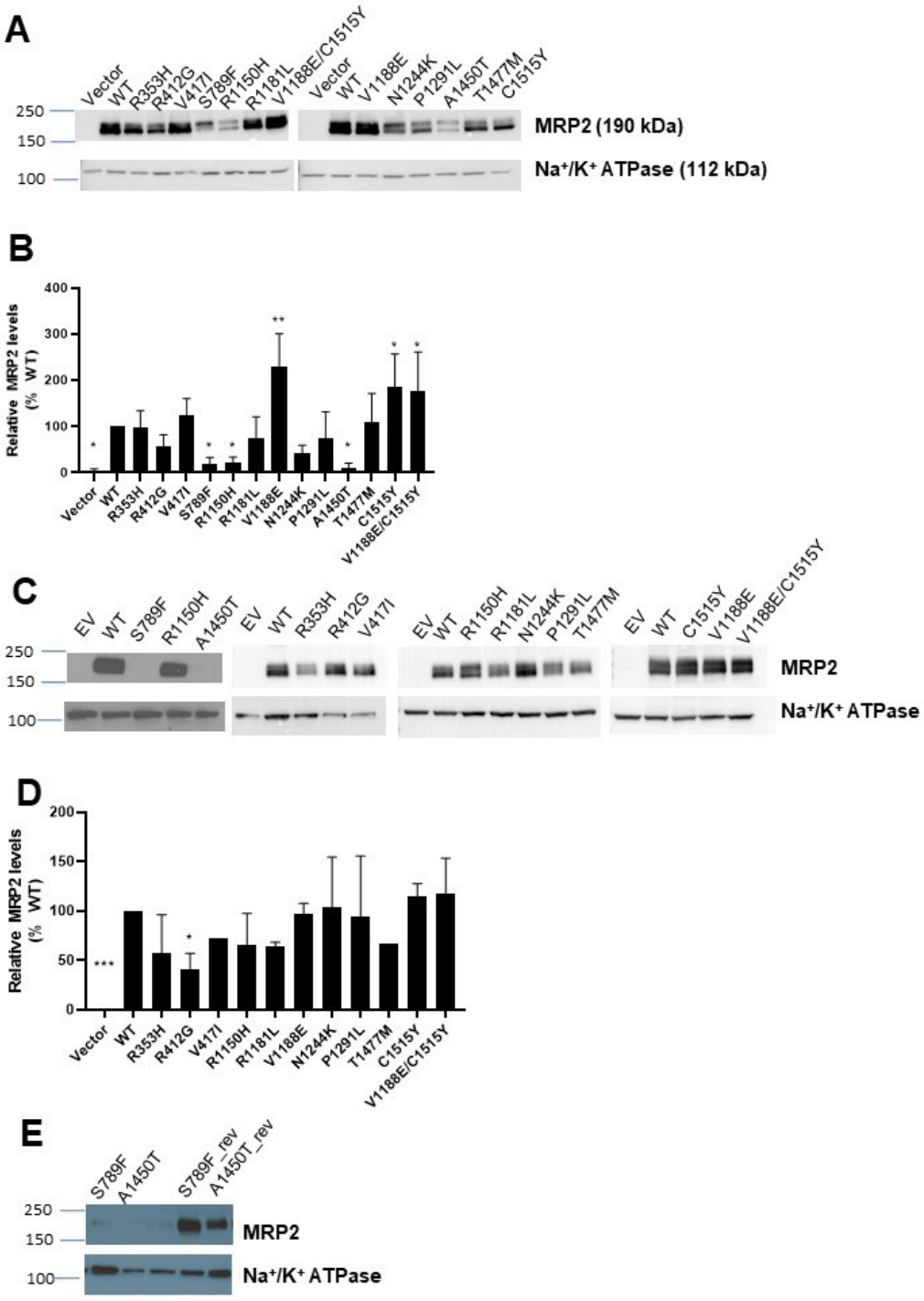

Figure A-2. WT-MRP2 and variant-MRP2 protein levels in transiently transfected HEK cells. (A) Total cellular protein levels determined by resolving lysate protein (10 µg) by SDS-PAGE (6%), followed by immunoblotting using the MRP2-specific antibody, M_2I-4. Blots were then probed with the Na^+/K^+ ATPase antibody, H-300. **(B)** densitometry analysis on cell lysates from 3 independent experiments was carried

out using the ImageJ software. WT and variant-MRP2 levels were corrected for loading using Na^+/K^+ ATPase protein levels and data are expressed as variant-MRP2 protein levels relative to WT-MRP2. *p < 0.05 (one-way ANOVA followed by a Dunnett's post hoc test). **(C)** protein levels in plasma membrane-enriched vesicles of WT-MRP2 and MRP2 variants were determined by resolving membranes vesicles (1 µg) by SDS-PAGE (6%), followed by immunoblotting as described for (A). **(D)** Densitometry analysis on plasma membrane-enriched vesicles immunoblots from 3 independent experiments, carried out as described for (B). Densitometry analysis for V417I and T1477M membrane vesicles are from 2 independent experiments. **(E)** MRP2-S789F and MRP2-A1450T cDNA was used as templates in site-directed mutagenesis with primers that reverted the mutations back to WT-MRP2. Plasma membrane vesicles (1 µg) of MRP2-S789F and MRP2-A1450T and their revertants were resolved by 6% SDS-PAGE and immunoblotted with the MRP2-specific antibody, M_2I-4. Blots were then probed with the Na^+/K^+ ATPase antibody, H-300.

Plasma membrane-enriched vesicles were isolated to characterize the plasma membrane level and localization of MRP2-variants (Figure A-2C and A-2D). MRP2-S789F and MRP2-A1450T were not detected in plasma membrane-enriched vesicles, while MRP2-R412G levels were lower than WT-MRP2. Protein levels of all other MRP2 variants were comparable to WT-MRP2.

Next, steps were taken to ensure that the absence of some mutant MRP2 in plasma membrane-enriched preparations was due to the intended mutations (S789F-, A1450T-) and not due to unintentional introduction of random mutations during site-directed mutagenesis polymerase chain reaction. Mutant plasmids were mutated back to the WT-MRP2 sequence by reverse site-directed mutagenesis, re-expressed in HEK293T cells, plasma membrane-enriched vesicles were prepared and immunoblot analysis performed. As shown in Figure A-2E, protein levels of the two revertants were higher than protein levels of the original variants, showing that the S789F and A1450T mutations were responsible for the lowered protein levels in the plasma membrane. Since, protein levels for S789F and A1450T were detected in cell lysates but not in plasma membranes, this potentially indicates impaired trafficking and increased degradation of these two variants, despite being expressed in HEK293T cells.

Predictions by the Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (https://sift.bii.a-star.edu.sg/) algorithm for MRP2-S789F and MRP2-A1450T, both of which showed low protein

levels in the whole cell lysate, were deleterious (SIFT) and probably and possibly damaging, respectively (Polyphen-2) (Table A-2). Both these variants are localized to the nucleotide binding domains of MRP2 [35]. The NBDs in ABC proteins are characterized by the Walker A region, the C Signature motif and the Walker B region. The NBDs are important in transport as they bind ATP at their interface resulting in conformation change to allow for substrate transport. MRP2-S789F is located in the D-loop of NBD1, which follows the Walker B region, while MRP2-A1450T is located in the region between the C Signature motif and Walker B in NBD2. Hirouchi et al., also observed low membrane protein levels of MRP2-S789F and MRP2-A1450T in the porcine kidney cell line, LLC-PK1 [34]. Studies in membrane vesicles from the insect cell line, *Sf9* showed reduced MRP2-S789F and unchanged MRP2-A1450T levels [35]. Discrepancies in protein levels detected at the plasma membrane may be accounted for by the different cell lines (mammalian cells vs insect cells), as insect cells have reduced quality control and therefore, are more likely to correctly traffick proteins to the membrane and maintain function [36–38].

$E_2$17βG is a well-characterized substrate of MRP2; transport assays with $E_2$17βG were used to characterize function of MRP2 variants that were present at substantial levels in plasma-membrane enriched vesicles (Figure A-3A). Preliminary experimental data revealed all MRP2 variants tested were functional, except MRP2-R1150H and MRP2-N1244K. All other MRP2 variants have comparable activity to WT-MRP2. Consistently, previous studies with MRP2-N1244K expressed in the HT1080 fibrosarcoma cell line and MRP2-R1150H expressed in HEK293T cells also revealed similar plasma membrane protein levels to WT-MRP2 with impaired transport activity[22,39]. The variant N1244K had an 80% decreased transport activity of the fluorescent substrates, glutathione-methylfluorescein and glutathione-monochlorobimane[22]. MRP2-R1150H also had considerably impaired transport activity of carboxyfluorescein, in comparison with WT-MRP2 [39].

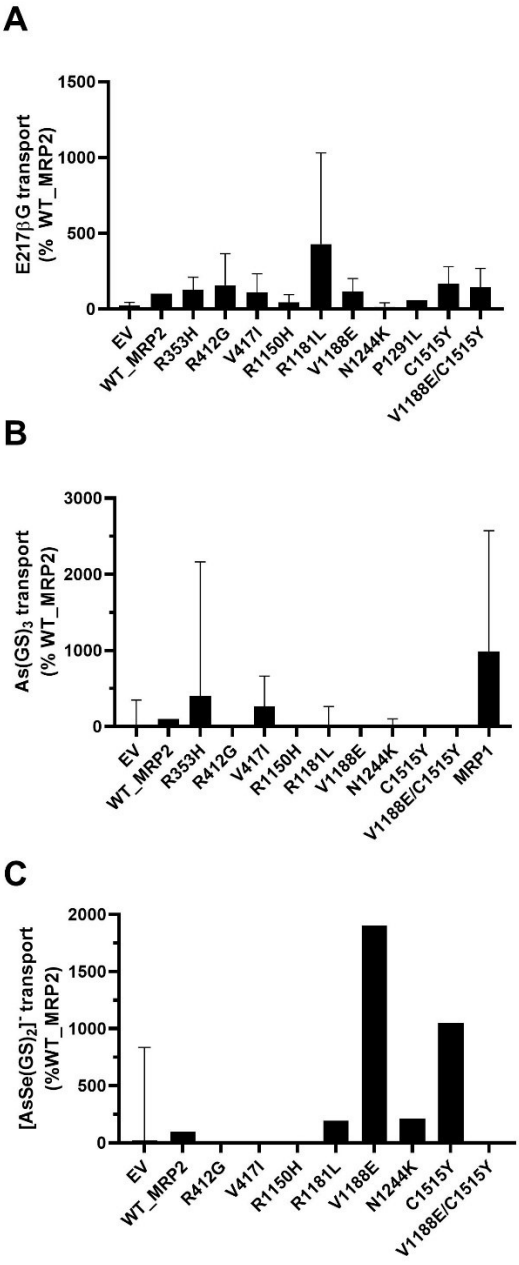

Figure A-3. Effect of MRP2 variants on ^{3}H- E$_2$17βG, ^{73}As(GS)$_3$ and [(GS)$_2$^{73}AsSe]$^-$ transport using membrane vesicles prepared from WT- and variant-MRP2 transfected HEK293T cells. (A) Vesicles were incubated with ^{3}H- E$_2$17βG (1 µM, 50 nCi) for 90 sec at 37ºC. Bars represent mean activity (± S.D.) from three independent experiments, except P1291L where activity from one independent experiment is plotted. **(B)** Vesicles were incubated with ^{73}As(GS)$_3$ (1 µM, 50 nCi) for 3 min at 37ºC. Bars represent mean activity (± S.D.) from three independent experiments, except V1188E, C1515Y and V1188E/C1515Y, where activity from one independent experiment is plotted. **(C)** Vesicles were incubated with [(GS)$_2$^{73}AsSe]$^-$ (1 µM, 50 nCi) for 3 min at 37ºC. Bars represent mean activity from two independent experiments, except V1188E, N1244K, C1515Y and V1188E/C1515Y where activity from one independent experiment is plotted.

$As(GS)_3$ and $[(GS)_2AsSe]^-$ transport activity with WT-MRP2 and its variants was investigated (Figure A-3B and A-3C), however due the large variability in arsenic transport data for WT-MRP2, no conclusive interpretations could be made from the data for WT-MRP2 nor variant-MRP2. In order to investigate whether poor expression of MRP2 was the cause of variable $As(GS)_3$ transport activity, the amount of DNA used in calcium phosphate transfections was optimized (Figure A-4A). MRP2 protein levels, corrected for Na^+/K^+ ATPase, in whole cell extracts revealed relatively high protein levels when transfected with the conventionally used 1 μg MRP2 DNA (Figure A-4A). This led to the optimization of transfection methods; comparing three commonly used methods - calcium phosphate transfection, X-tremeGENE 9 transfection and lipofectamine 2000® transfection. In addition, published reports show NaB, an inhibitor of histone deacetylase which results in increased gene transcription, and increased MRP2 protein levels [40,41]. This led to the investigation of the influence of different concentrations of NaB on MRP2 protein levels (Figure A-4B). As expected, no MRP2 protein was detected in the untransfected cell lysate extract. In comparison to the calcium phosphate transfection, the addition of 2 mM NaB to cells 48 hrs prior to harvesting increased cellular MRP2 protein levels 1.7-fold. X-tremeGENE 9 transfections at a 1.7:1 reagent:DNA ratio and lipofectamine 2000® tranfections at a 3:1 reagent:DNA ratio showed the highest increase in cellular protein levels (Figure A-4B).

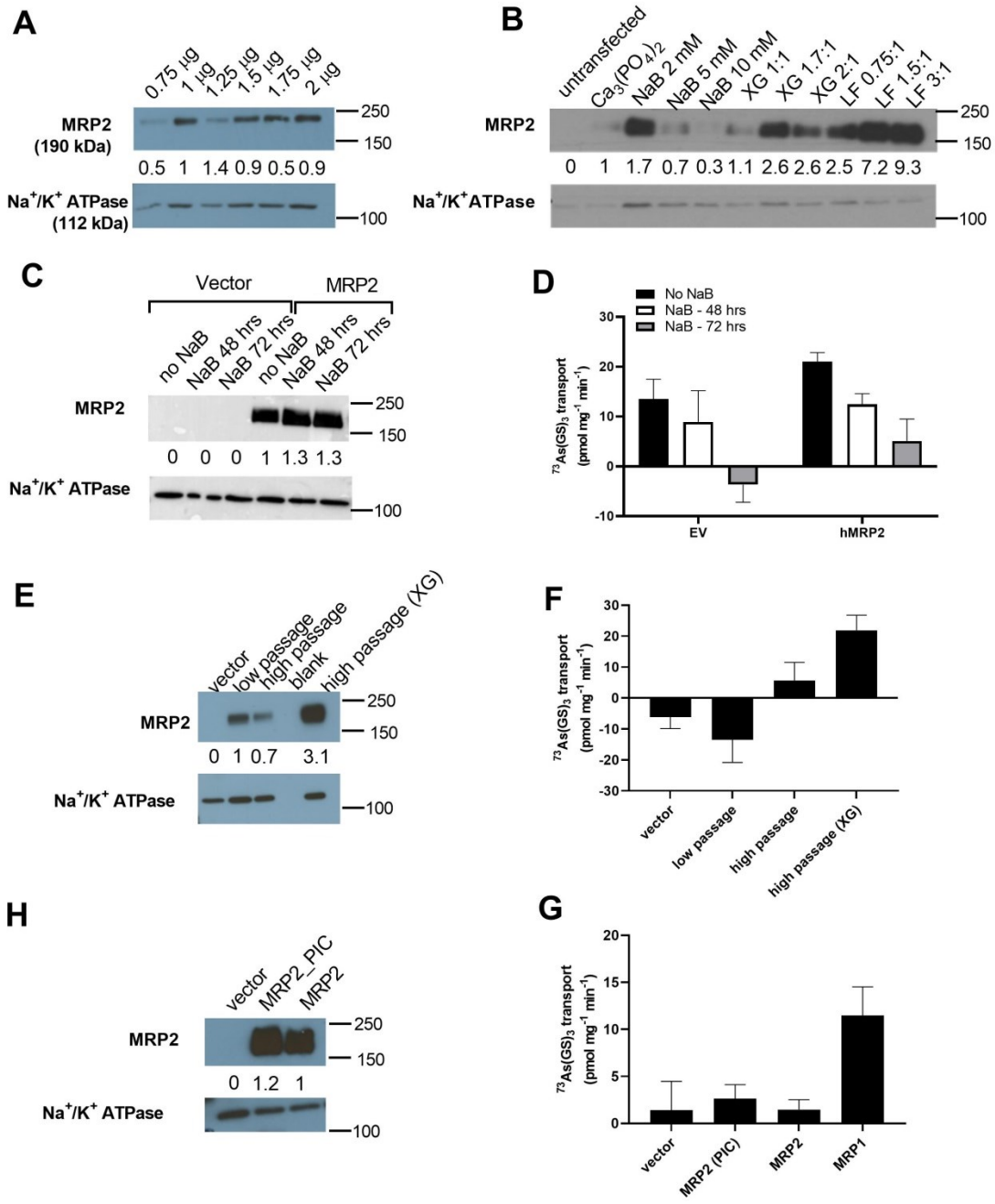

Figure A-4. Optimization of transfection methods and $^{73}As(GS)_3$ transport. (A) Total cellular MRP2 protein levels in HEK293T cells upon transfections with 0.75, 1, 1.25, 1.5, 2 µg MRP2 DNA using the calcium phosphate transfection method were determined by resolving lysate protein (10 µg) by SDS-PAGE (6%), followed by immunoblotting using the MRP2-specific antibody, M_2I-4. Blots were then probed with the Na^+/K^+ ATPase antibody, H-300. **(B)** Total cellular MRP2 protein levels in untransfected HEK293T cells (lane 1), upon transfections with the calcium phosphate method (1 µg MRP2 DNA) (lane 2), calcium phosphate method 1 µg MRP2 DNA with 2 mM NaB (lane 3), 5 mM (lane 4) or 10 mM (lane 5), transfections with X-tremeGENE 9 transfection reagent (µl): DNA ratio (µg) 1:1 (lane 6), 1.7:1 (lane 7), 2:1 (lane 8), transfections with lipofectamine 2000® transfection reagent (µl): DNA (µg) ratio 0.75:1 (lane 9),

1.5:1 (lane 10) and 3:1 (lane 11). **(C)** Immunoblot analysis of corresponding vesicles used in (D), 1 µg protein was resolved using 6% SDS-PAGE followed by immunoblotting using the MRP2-specific antibody, M_2I-4. Blots were then probed with the Na^+/K^+ ATPase antibody, H-300. **(D)** HEK293T cells were transfected with the calcium phosphate method using pcDNA3.1 vector or MRP2 DNA (1 µg) with the addition of 2 mM NaB in culture media for 24 hrs, 48 hrs or 72 hrs prior to harvesting. Plasma membrane-enriched vesicles were isolated and $^{73}As(GS)_3$ transport activity (50 nCi, 1 µM) at 37ºC over 3 min was characterized. Bars represent triplicate determinations (mean ± S.D.) from a single experiment. **(E)** Immunoblot analysis of corresponding vesicles used in (F), 1 µg protein was resolved using 6% SDS-PAGE followed by immunoblotting using the MRP2-specific antibody, M_2I-4. Blots were then probed with the Na^+/K^+ ATPase antibody, H-300. **(F)** HEK293T cells with a high passage number were transfected with the calcium phosphate method using pcDNA3.1 vector and MRP2 DNA (1 µg), or with the X-tremeGENE 9 tranfection reagent with MRP2 DNA (1 µl:1 µg) DNA. HEK293T cells with a low passage number were transfected with the calcium phosphate method using MRP2 DNA (1 µg) and $^{73}As(GS)_3$ transport activity (50 nCi, 1 µM) at 37ºC over 3 min was characterized. Bars represent triplicate determinations (mean ± S.D.) from a single experiment. **(G)** Immunoblot analysis of corresponding vesicles used in (H), 1 µg protein was resolved using 6% SDS-PAGE followed by immunoblotting using the MRP2-specific antibody, M_2I-4. Blots were then probed with the Na^+/K^+ ATPase antibody, H-300. **(H)** HEK293T cells were transfected with the calcium phosphate method using pcDNA3.1 vector, MRP2 and MRP1 DNA (1 µg). Upon harvesting, phosphatase inhibitor cocktail tablet was added to one set of MRP2-transfected cells (labelled MRP2(PIC)). Plasma membrane enriched vesicles were isolated and $^{73}As(GS)_3$ transport activity (50 nCi, 1 µM) at 37ºC over 3 min was characterized to check for differences in transport activity in the presence of phosphatase inhibitors. Bars represent triplicate determinations (mean ± S.D.) from a single experiment.

In order to check for the effect of NaB on membrane localization and $As(GS)_3$ transport function, further characterization of the effect of 2 mM NaB on protein levels of vector- and MRP2- transfected plasma membrane vesicles was carried out (Figure A-4D). *In vivo* studies have provided evidence for the transport of As-GSH conjugates into rat bile [15,42,43].

Expectedly, no MRP2 protein was detected in vector-transfected HEK293T membrane vesicles. MRP2 showed comparable protein levels when 2 mM NaB was added to culture media 48 hrs or 72 hrs prior to harvesting, and higher protein levels than cells grown without NaB in media (Figure A-4C). Growing HEK293T cells in culture media containing NaB for 72 hrs resulted in the loss of detectable protein at the plasma membrane. $As(GS)_3$ transport activity was high for empty vector, both when NaB was present (48 hrs) and absent from culture media, with the lowest activity when NaB was present in media for 72 hrs (Figure A-4D). MRP2 showed the highest transport activity when

no NaB was present in the culture media, with the lowest activity when NaB was present in media for 72 hrs (Figure A-4D).

Next, HEK293T cell passage number effects were investigated to determine whether variable transport activity was due to a high number of passage cycles (up to a maximum of 16 passages) of HEK293T cells. No protein was detected in vector transfected HEK293T membrane vesicles, while MRP2 protein was detected in membrane vesicles isolated from cells with both high and low passage cycles (Figure A-4E). Immunoblot analysis revealed slightly higher MRP2 membrane protein levels when freshly thawed cells (low passage) were used (Figure A-4E), however this does not translate to increased activity in the $As(GS)_3$ transport assay (Figure A-4F). The highest protein levels were detected for X-tremeGENE 9 transfected cells, with corresponding $As(GS)_3$ transport activity also being higher than membrane vesicles from calcium phosphate transfected cells (Figure A-4F). Future replications of this experiment should include an X-tremeGENE transfected vector control.

Since the large variability in $As(GS)_3$ is potentially due to the low transport activity, phosphorylation inhibitors were used to characterize whether this would lead to increased transport activity. Previous studies of $As(GS)_3$ transport by MRP1 using HeLa cells demonstrated increased transport of $As(GS)_3$ in the presence of phosphatase inhibitors [44]. Preliminary results from the addition of phosphorylation inhibitor cocktail during harvesting of MRP2-transfected HEK293T cells did result in increased MRP2 membrane protein levels, with no detectable protein in membranes of vector-transfected cells (Figure A-4G). $As(GS)_3$ transport activity showed comparable activity levels for MRP2 harvested in the presence and absence of phosphorylation inhibitors, with activity being no different than the vector control (Figure A-4H). Multidrug resistance protein 1- (MRP1-) enriched membrane vesicles from HEK cells served as a positive control and showed a transport activity of 11.5 ± 3 pmol mg^{-1} min^{-1} (Figure A-4G).

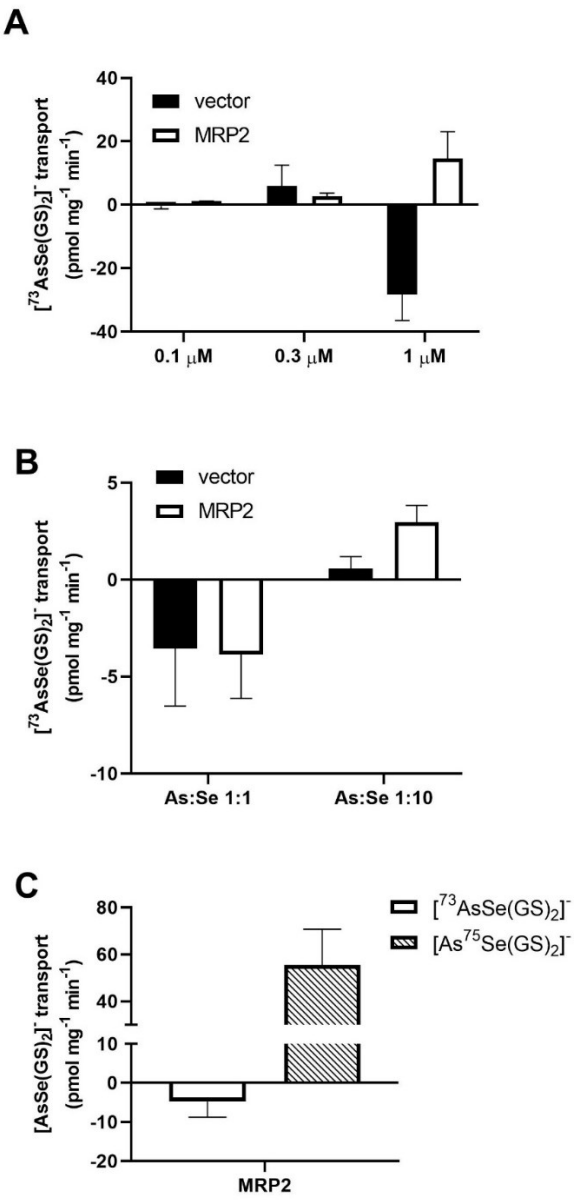

Figure A-5. Optimization of $[(GS)_2{}^{73}AsSe]^-$ transport. (A) $[(GS)_2{}^{73}AsSe]^-$ transport activity (30 nCi, 0.1 μM; 30 nCi, 0.3 μM; 50 nCi, 1 μM) at 37ºC over 3 min was characterized in plasma membrane-enriched vesicles isolated from HEK293T cells transfected with pcDNA3.1 vector or MRP2. Bars represent triplicate determinations (mean ± S.D.) from a single experiment. **(B)** $[(GS)_2{}^{73}AsSe]^-$ conjugate was synthesized, as described in the methods above, using As:Se ratio of 1:1 or 1:10. $[(GS)_2{}^{73}AsSe]^-$ transport activity (50 nCi, 1 μM) at 37ºC over 3 min was characterized in plasma membrane-enriched vesicles isolated from HEK293T cells transfected with pcDNA3.1 vector or MRP2. Bars represent triplicate determinations (mean ± S.D.) from a single experiment. **(C)** $[(GS)_2AsSe]^-$ conjugate was synthesized, as described in the methods above, using $^{73}As^{III}$ or $^{75}Se^{IV}$. $[(GS)_2AsSe]^-$ transport activity (50 nCi, 1 μM) at 37ºC over 3 min was characterized in plasma membrane-enriched vesicles isolated from HEK293T cells transfected with pcDNA3.1 vector or MRP2. Bars represent triplicate determinations (mean ± S.D.) from a single experiment. Similar results have been observed in one other independent experiment.

$[(GS)_2{}^{73}AsSe]^-$ transport activity was characterized at 0.1, 0.3 and 1 μM, and as expected, higher concentration of the conjugate resulted in higher detection of transport activity (12-fold and 5-fold increase transport activity than 0.1 μM and 0.3 μM, respectively) (Figure A-5A). Going forward, all $[(GS)_2AsSe]^-$ transport was carried out at 1 μM. Synthesis of $[(GS)_2{}^{73}AsSe]^-$ was optimized by investigating the effect of levels of selenite present in the mixture. When Se^{IV} is present at 10x higher levels than As^{III}, $[(GS)_2{}^{73}AsSe]^-$ transport is detected at 2.87 ± 0.87 pmol mg^{-1} min^{-1} (Figure A-5B). It is possibly that the increased availability of the Se^{IV}, favours a higher formation of $[(GS)_2{}^{73}AsSe]^-$.

It is possible that the low and inconsistent transport activity of MRP2 with $[(GS)_2{}^{73}AsSe]^-$ was due to the presence of reducing agents in the ^{73}As solution, leading to the inhibition of MRP2 activity [56]. Going forward, $^{75}Se^{IV}$ was used in the synthesis of the $[(GS)_2As^{75}Se]^-$ conjugate and transport activity was characterized. Preliminary data suggests that ^{75}Se is a better radioactive component to characterize $[(GS)_2AsSe]^-$ transport activity and further investigation is warranted (Figure A-5C). Future experiments should also include a vector control to characterize $[(GS)_2As^{75}Se]^-$ transport.

Future directions of this study are to further optimize and characterize the transport activity of arsenic conjugates which are substrates of human MRP2, these include $As(GS)_3$ and $[(GS)_2AsSe]^-$, and likely $MMA(GS)_2$. Kinetic characterization would be carried out for MRP2 variants that display an altered transport profile in comparison to WT-MRP2. Cell-surface biotinylation studies can be done with all variants to confirm that the mutants are reaching the plasma membrane. Cellular imaging studies can also be done for variants with lower plasma membrane protein levels to determine cellular localization. The results of this study would contribute to the understanding of inter-individual variability in arsenic-induced toxicities, as well as, understanding variability in individual responses to selenium supplementation.

Table A-2. Single nucleotide polymorphic variants of MRP2 (*ABCC2*) and their published effects in literature

Amino Acid Change	Nucleotide Change	Minor Allele Frequency (%)	PolyPhen-2 Prediction	SIFT Prediction	Effect in Literature	Cell System	NCBI SNP ID
R353H	1058G>A	1.4 (1000 genomes)[45] 6.7 (African American)[22]	Benign	0.17 (tolerated)	Lower GS-MCB and GS-MF efflux compared to WT.[22] Similar expression to WT [22]	HEK293 cells	rs7080681
R412G	1271A>G		Benign	0.01 (affect function)	Decrease in methotrexate elimination[20] Expressed at cell surface, similar to wild-type[20] DJS[46]	CHO cells	
V417I	1249G>A	18.7 (1000 genomes)[45]	Benign	0.5 (tolerated)	Reduced risk of neurotoxicity[47] Higher hepatic MRP2 expression with possibly reduced activity[48] Significant correlation with overall survival of colorectal cancer patients[49] Influence on urinary-Hg concentrations[50] Reduced transport of CDCF[31]	HEK293 cells	rs2273697
S789F	2366C>T	1 (Japanese)[17]	Probably damaging	0.00 (affect function)	Lower expression and membrane localization[34,35] Decreased transport of MRP2 substrates [34].	LLC-PK1 cells Sf9 cells	
R1150H	3449G>A	0.9 (Moroccan Jews)[39]	Probably damaging	0.00 (affect function)	Impaired transport activity (carboxyfluorescein)	HEK cells	

					Normal expression and localization DJS[39]		
R1181L	3542G>T	3.3 (1000 Genomes)[45]	Possibly damaging	0.1 (tolerated)	Lower expression[22] Mislocalization to cytoplasm (ER-like distribution) [22] Reduced only GS-MCB transport by 50% & increased GS-MF efflux by 137% [22] Increased transport of calcein AM[31]	HT1080 cells Flp-In HEK293 cells	rs8187692
V1188E	3563T>A	1 (Japanese) [51] 5.8 (African American)[22]	Benign	0.94 (tolerated)	Associated with non-alcoholic fatty liver disease[18] Associated with acute anthracycline induced cardiotoxicity[52] Increased transport of calcein AM[31]	Flp-In HEK293 cells	rs8187694
N1244K	3732T>G	1 (Japanese)[51]	Possibly damaging	0.41 (tolerated)	Similar expression to WT. [22] Reduced transport of glutathione-methylfluorescein (GS-MF) and glutathione-monochlorobimane (BS-MCB) [22]	HT1080 cells	
P1291L	3872C>T	1 (1000 genomes)[45]	Possibly damaging	0.01 (affect function)	Similar expression to WT[22] Reduced only GS-MCB transport by 50%, GS-MF similar to WT [22]	HEK HT1080	rs17216317
A1450T	4348G>A	1 (Japanese)[17]	Possibly damaging	0.00 (affect function)	Lower expression and membrane localization in transfected cells[35]	LLC-PK1 cells Sf9 cells	[19,34,35]

					Decreased transport of MRP2 substrates[53]		
T1477M	4430C>T	0.6 [14]	Benign	0.04 (affect function)	Lower expression in Sf9-plasma derived membrane vesicles[35] Altered transport activity[35]	Sf9 cells	
C1515Y	4544G>A	6.7 (1000 genomes)[45] 13 (African American)[22]	Benign	1.00 (tolerated)	Associated with non-alcoholic fatty liver disease[18] Associated with acute anthracycline induced cardiotoxicity[52] Associated with reduced MRP2 activity[48] Higher accumulation of LPV in peripheral blood mononuclear cells of HIV treated patients[54] Higher accumulation of MRP2 substrates, impairs ATP-ase activity[55] Increased transport of calcein AM[31]	HEK293 cells Flp-In HEK293 cells	rs8187710
V1188E/C1515Y	3563T>A and 4544G>A				Correlated with nonalcoholic fatty liver disease[18] Higher protein expression. [22]	HT1080 cells	

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