Effects of sulfur complexation on intestinal transport and toxicity of metalloids in cell cultures

Dissertation

zur Erlangung des Grades

Doktor der Naturwissenschaften (Dr. rer. nat.)

an der Fakultät für Biologie / Chemie / Geowissenschaften

der Universität Bayreuth

vorgelegt von

Sinikka Hinrichsen

(M.Sc. Geoökologie)

geb. am 26.03.1986 in München

Bayreuth, September 2015

Die vorliegende Arbeit wurde in der Zeit von Januar 2011 bis September 2015 in Bayreuth am Lehrstuhl Umweltgeochemie unter der Betreuung von Frau Prof. Dr. Britta Planer-Friedrich angefertigt.

Vollständiger Abdruck der von der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth genehmigten Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.).

Dissertation eingereicht am: 09.09.2015

Zulassung durch die Promotionskommission: 01.10.2015

Wissenschaftliches Kolloquium: 10.12.2015

Amtierender Dekan: Prof. Dr. Stefan Schuster

Prüfungsausschuss:

Prof. Dr. Britta Planer-Friedrich (Erstgutachterin)
Prof. Dr. Alfred Hirner (Zweitgutachter)
Prof. Dr. Egbert Matzner (Vorsitz)
Prof. Dr. Stephan Clemens

ACKNOWLEDGMENTS

First of all, I want to express my sincere gratitude to my doctoral advisor Prof. Dr. Britta Planer-Friedrich for her outstanding support. She gave me the opportunity to continue to work on the topic of arsenic and selenium cytotoxicity after my Master thesis at the Department of Environmental Geochemistry. Furthermore, she supported me in setting up a toxicological laboratory and in participating in the advanced training program *Fachtoxikologe/-in DGPT.* Thanks for all the helpful discussions and for all the enormous support during my PhD!

My sincere thank also goes to Prof. Dr. Tanja Schwerdtle (Chair of Food Chemistry, Institute of Nutritional Science, University of Potsdam, formerly Institute of Food Chemistry, University of Münster) and all her group members for kindly hosting me for four weeks in their group and teaching me methods of measuring cytotoxicity.

Thanks to my co-authors Prof. Dr. Elke Dopp and Dr. Ricarda Zdrenka (Institute of Hygiene and Occupational Health, University of Duisburg-Essen). I derived much benefit from their scientific contributions.

Many thanks go to all my colleagues of the Environmental Geochemistry Group for the pleasant working environment, especially to Regina Lohmayer, Dr. Elke Süß, Cornelia Härtig, Julia Arndt, Judith Mehlhorn, and Maria Ullrich. Thanks to my Master student, labmate, and co-author Franziska Geist, working in the laboratory together with her was such a pleasure!

Thanks to Stefan Will and Frank Thomas who supported me a lot during the AEC-ICP-MS analyses.

Finally, special thanks go to my family, especially to my mother Mathilde Hinrichsen and my sisters Katja Hantschel and Eva Hinrichsen for their support!

ABSTRACT

Arsenic is a common poison and is classified as human carcinogen. Selenium is an essential nutrient, but is highly toxic when applied in high concentrations. The cytotoxic potential of both metalloids is modified when they form sulfur-containing complexes. For arsenic compounds, only some data existed about the bioavailability and cytotoxicity of methylated thioarsenates. No data existed about inorganic thioarsenates, even though their formation during pre-systemic arsenic metabolism was already proven. For selenium compounds, a reported general higher cytotoxicity of selenosulfate compared to selenite for cancer cell lines led to the claim of replacing selenite by selenosulfate in anti-cancer therapies.

In the first two studies of the present thesis, intestinal transport (e.g. bioavailability), cellular retention, and cytotoxicity of thioarsenates were investigated. The cellular retention and the intestinal transport of synthesized standard solutions of methylated and inorganic thioarsenates were compared to those of their non-thiolated analogues by means of a model of the human small intestine (Caco-2 cell monolayer). Analyses with AEC-ICP-MS were conducted to monitor species stability during the transport experiments. Both the transcellular uptake route (by phosphate transporters) and the paracellular uptake route (through the tight junctions) were investigated for each arsenic species.

The influence of sulfide on arsenite was investigated concerning the formation of inorganic thioarsenates and an accordingly modified cytotoxicity, quantified by means of MTT assay. The cytotoxic effects of arsenite, arsenate, and inorganic thioarsenates were compared for human hepatocytes (HepG2) and urothelial cells (UROtsa). Concentrations of each arsenic species leading to 50 % cell viability (IC_{50} values) were calculated. Cellular uptake of the different inorganic arsenic compounds was quantified and linked to their cytotoxicity.

As expected, arsenite showed the highest cellular retention and intestinal transport of all tested arsenic compounds. The bioavailability of thioarsenates strongly differed from that of their non-thiolated analogues. For dimethylmonothioarsenate, the highest cellular retention and intestinal transport among all methylated arsenic compounds were measured, which is of special concern as this species is known to possess a considerably higher cytotoxicity than its non-thiolated analogue dimethylarsenate. Only low cellular retention – comparable to that of arsenate - was detected for the inorganic thioarsenates mono- and trithioarsenate, but their intestinal transport was considerably higher than that of arsenate. For trithioarsenate, the intestinal transport was even comparable to that of arsenite. Mono- and trithioarsenate were transported intact through the cell monolayer, but partial intracellular reduction to arsenite could not be excluded. Both cellular retention and intestinal transport was negligibly low for mono- and dimethylarsenate and for monomethylmonothioarsenate. The absence of phosphate increased cellular retention of all arsenic compounds indicating the importance of apical phosphate transporters. No data could be presented to interpret the importance of the

paracellular transport route as the cell monolayer was damaged during these experiments.
Addition of sulfide to arsenite-containing cell growth medium resulted in immediate formation of inorganic thioarsenates and in reduced cytotoxicity. The order of cytotoxicity of the individually applied inorganic arsenic compounds after 24 h exposure was determined as arsenite > trithioarsenate > monothioarsenate > arsenate and this corresponded to the order of cellular arsenic uptake. Considering arsenite as the original present arsenic substrate for the formation of inorganic thioarsenates, thiolation can be seen as a detoxification process due to decreased intestinal transport and cytotoxicity. In case of dimethylmonothioarsenate, which is known to form from dimethylarsenate, thiolation can be seen as an activation process due to increased intestinal transport and cytotoxicity.
In the third study, cytotoxic effects and cellular uptake of selenosulfate and selenite were compared for three different cancer cell lines (HepG2, A375, and T24) to reassess the claim of selenosulfate being generally more cytotoxic than selenite for cancer cells. Experiments in absence and presence of amino acids linked the influence of amino acids with the cytotoxicity of the selenium compounds.
Selenosulfate was comparably toxic to the three cell lines (IC_{50} 6.6-7.1 µM) and hardly influenced by incubation time and presence or absence of amino acids. Though, selenite cytotoxicity considerably differed among the three cell lines with the result that selenosulfate was more toxic than selenite for HepG2 cells (IC_{50} > 15 µM), but similar toxic to and lower toxic than selenite for A375 (IC_{50} 4.7 µM) and T24 cells (IC_{50} 3.5 µM).
In contrast to T24 cells, HepG2 cells were “routinely” cultivated with amino acids. Addition of amino acids to T24 cell growth medium led to reduced selenite uptake and toxicity, rendering it less toxic than selenosulfate. The strong effect of amino acids on selenite toxicity for T24 cells could be explained by an inhibition of the x_c^- transport system which facilitates cellular selenium uptake by secretion of cysteine and reduction of selenium compounds. Selenosulfate is less affected by the addition of amino acids as it is already a reduced species. Whether selenosulfate or selenite is more cytotoxic, does not only depend on the selenium species itself, but also on the sensitivity of the used cell line, the supplements of the cell growth medium, and the reductive state of the extracellular environment. The general claim of selenosulfate being more toxic than selenite therefore has to be reconsidered.

ZUSAMMENFASSUNG

Arsen ist ein bekannter Giftstoff und für den Menschen als kanzerogen eingestuft. Selen ist ein essentieller Nährstoff, jedoch äußerst toxisch in hoher Konzentration. Die Zytotoxizität beider Halbmetalle ändert sich, wenn sie Komplexe mit Schwefel bilden. Über die Bioverfügbarkeit und Zytotoxizität von methylierten Thioarsenaten gab es bisher nur wenige und über die von anorganischen Thioarsenaten gar keine Daten, obwohl deren Bildung während der präsystemischen Arsen-Metabolisierung bereits bewiesen wurde. Selenosulfat galt generell als zytotoxischer für Krebszellen als Selenit. Daraus entstand der Vorschlag, dass Selenosulfat Selenit in der Krebstherapie ersetzen sollte.

In den beiden ersten Studien der vorliegenden Arbeit wurden die intestinale Absorption (d.h. die Bioverfügbarkeit), die zelluläre Retention und die Zytotoxizität von Thioarsenaten untersucht. Mit Hilfe eines Modell für den menschlichen Dünndarm (Caco-2-Zellmodell) wurden die zelluläre Retention und die intestinale Absorption von synthetisierten Standardlösungen von methylierten und anorganischen Thioarsenaten mit denen ihrer nicht-thiolierten Strukturanaloge verglichen. Die Stabilität der einzelnen Verbindungen während der Transportexperimente wurde durch AEC-ICP-MS Analysen beurteilt. Sowohl der transzelluläre Aufnahmeweg (durch Phosphat-Transporter) als auch der parazelluläre Aufnahmeweg (durch die Tight Junctions) wurden für jede Arsen-Verbindung untersucht.

Der Einfluss von Schwefel auf Arsenit wurde im Hinblick auf die Bildung von anorganischen Thioarsenaten und die daraus resultierende geänderte Zytotoxizität untersucht (gemessen mit dem MTT Test). Die zytotoxischen Effekte von Arsenit, Arsenat und den anorganischen Thioarsenaten wurden für eine menschliche Leberkrebszelllinie (HepG2) und eine Blasenzelllinie (UROtsa) verglichen. Von jeder Arsen-Verbindung wurde die Konzentration berechnet, die zu einer Reduktion der Zellviabilität um 50 % führte (IC_{50} Wert). Die zelluläre Aufnahme jeder anorganischen Arsenverbindung wurde quantifiziert und mit ihrer jeweiligen Zytotoxizität in Verbindung gebracht.

Die höchste zelluläre Retention und intestinale Absorption wurde erwartungsgemäß für Arsenit gemessen. Die Bioverfügbarkeit von Thioarsenaten unterschied sich deutlich von der ihrer nicht-thiolierten Analoge. Unter allen methylierten Arsenverbindungen wurden die höchste zelluläre Retention und intestinale Absorption für Dimethylmonothioarsenat bestimmt. Dies ist besonders bemerkenswert, denn diese Verbindung besitzt eine deutlich höhere Zytotoxizität als ihr nicht-thioliertes Strukturanalog Dimethylarsenat. Die zelluläre Retention der anorganischen Thioarsenate Mono- und Trithioarsenat war vergleichbar gering mit der von Arsenat. Ihre intestinale Absorption war aber deutlich höher als die von Arsenat, für Trithioarsenat sogar vergleichbar mit der von Arsenit. Mono- und Trithioarsenat wurden intakt durch die Zellschicht transportiert, allerdings konnte eine teilweise intrazelluläre Reduktion zu Arsenit nicht ausgeschlossen werden. Die zelluläre Retention und die

intestinale Absorption von Mono- und Dimethylarsenat und von Monomethylmonothioarsenat waren vernachlässigbar gering. In Abwesenheit von Phosphat nahm die zelluläre Retention aller Arsen-Verbindungen zu, was auf die Bedeutung von apikalen Phosphat-Transportern hinweist. Es konnten keine Daten über die Bedeutung des parazellulären Transportweges erhoben werden, weil während dieser Experimente die Zellschicht beschädigt wurde.

Sulfid-Zugabe in das Zellkulturmedium, das Arsenit enthielt, führte zur sofortigen Bildung von anorganischen Thioarsenaten und zu reduzierter Zytotoxizität. Die Toxizitäts-Reihenfolge der einzelnen anorganischen Arsenverbindungen nach 24 h Inkubation war Arsenit > Trithio-arsenat > Monothioarsenat > Arsenat. Die gleiche Reihenfolge wurde auch für die zelluläre Aufnahme bestimmt. Unter der Annahme, dass anorganische Thioarsenate direkt aus Arsenit gebildet werden, kann Thiolierung als ein Prozess der Detoxifikation beschrieben werden, da dadurch die intestinale Absorption und die Zytotoxizität reduziert werden. Im Fall von Dimethylmonothioarsenat, das aus Dimethylarsenat gebildet wird, kann Thiolierung allerdings als ein Aktivierungsprozess beschrieben werden, da dadurch die intestinale Absorption und die Zytotoxizität gesteigert wird.

In der dritten Studie wurden die zytotoxischen Effekte und die zelluläre Aufnahme von Selenosulfat und Selenit für drei verschiedene Krebszelllinien (HepG2, A375 und T24) verglichen, um die Behauptung zu überprüfen, dass Selenosulfat generell zytotoxischer auf Krebszellen wirkt als Selenit. Experimente mit und ohne Aminosäuren untersuchten deren Einfluss auf die Zytotoxizität der Selenverbindungen.

Die Zytotoxizität von Selenosulfat war für alle drei Zelllinien vergleichbar (IC_{50} 6.6-7.1 µM) und größtenteils unbeeinflusst durch die Faktoren Inkubationszeit und Aminosäuren. Allerdings war die Zytotoxizität von Selenit für die drei Zelllinien sehr unterschiedlich, was dazu führte, dass Selenosulfat toxischer als Selenit für HepG2 war (IC_{50} > 15 µM), aber vergleichbar toxisch mit bzw. weniger toxisch als Selenit für A375 (IC_{50} 4.7 µM) und T24 Zellen (IC_{50} 3.5 µM). Im Gegensatz zu den T24 Zellen wurden die HepG2 Zellen routinemäßig mit Aminosäuren kultiviert. Durch die Zugabe von Aminosäuren zum T24 Zellkulturmedium wurden Selenitaufnahme und -toxizität dermaßen reduziert, dass Selenosulfat für T24 Zellen toxischer war als Selenit. Der starke Einfluss von Aminosäuren auf die Selenit-Toxizität für T24 Zellen könnte durch eine Hemmung des x_c^--Transportsystems erklärt werden, welches die zelluläre Selenaufnahme durch Exkretion von Cystein und Reduktion der Selenverbindung steuert. Selenosulfat ist durch die Aminosäuren wenig beeinflusst, da es bereits reduziert ist. Ob Selenosulfat oder Selenit zytotoxischer ist, hängt nicht nur von der Selenverbindung selbst ab, sondern auch von der Empfindlichkeit der verwendeten Zelllinie, den einzelnen Bestandteilen des Zellkulturmediums und den reduktiven Bedingungen in der extrazellulären Umgebung. Die generelle Behauptung, dass Selenosulfat toxischer als Selenit ist, muss deswegen überdacht werden.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AEC-ICP-MS	anion-exchange chromatography-inductively coupled plasma-mass spectrometry
DMA^{III}	dimethylarsenite
$DMDTA^{V}$	dimethyldithioarsenate
$DMMTA^{V}$	dimethylmonothioarsenate
DTA^{III}	dithioarsenite
DTA^{V}	dithioarsenate
EDTA	ethylenediaminetetraacetic acid
ESI-MS-MS	electrospray ionization tandem mass spectrometry
ICP-MS	inductively coupled plasma-mass spectrometry
IC_{50}	substance´s inhibitory concentration inducing 50 % cell viability
MMA^{III}	monomethylarsenite
MMA^{V}	monomethylarsenate
$MMMTA^{V}$	monomethylmonothioarsenate
MTA^{III}	monothioarsenite
MTA^{V}	monothioarsenate
$MTTMA^{V}$	monothiotrimethylarsenate
S_0	elemental sulfur
TEER	transepithelial electrical resistance
TMA^{V}	trimethylarsenate
TTA^{III}	trithioarsenite
TTA^{V}	trithioarsenate
$TetraTA^{V}$	tetrathioarsenate
XAS	X-ray absorption spectroscopy
x_c^-	cysteine/glutamate exchange system

LIST OF FIGURES

EXTENDED SUMMARY

1 INTRODUCTION

1.1 Toxicity of arsenic and selenium

Arsenic is a widespread environmental contaminant in water, soil, and food and it is classified as carcinogen. Besides numerous harmful health effects, chronic exposure to arsenic is associated with cancer of bladder, liver, skin, and other organs (reviewed in Bates et al. (1992)). In drinking water, the trivalent arsenite ($H_3As^{III}O_3$) and the pentavalent arsenate ($H_3As^{V}O_4$) are the dominant arsenic species (Smedley & Kinniburgh 2002) (Figure 1). According to laboratory studies with animals and cell cultures, trivalent arsenic species are considerably more toxic than pentavalent arsenic species (Thomas et al. 2001).

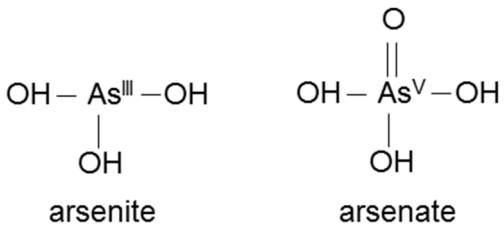


Figure 1: Chemical structures of arsenite and arsenate – the main arsenic species in water.

Ingested arsenic in form of arsenite or arsenate is metabolized into methylated arsenic compounds. The Challenger mechanism was the first widely accepted metabolism pathway for inorganic arsenic species in the body. It implicates alternating steps of reduction (catalyzed by the enzyme MMA^V reductase) and oxidative methylation leading to dimethylated arsenic compounds (Challenger 1945, Le et al. 2000, Vahter 1999). However, several other studies showed that the Challenger mechanism has to be reconsidered due to the following findings:

1) In MMA^V reductase deficient mice, arsenate metabolism did not significantly differ from arsenate metabolism in wild type mice (Chowdhury et al. 2006).
2) Pentavalent species were metabolized into trivalent species only to a considerably smaller extent than proposed by the Challenger mechanism (Cohen et al. 2006).
3) After ingestion, monomethylarsenate (MMA^V) and dimethylarsenate (DMA^V) are immediately excreted into urine and are not reduced into their trivalent analogues. The pentavalent species can therefore rather be regarded as end-products and not as intermediates during arsenic biotransformation (Marafante et al. 1987, Yamauchi & Yamamura 1984).

Subsequently, a further arsenic metabolism pathway was suggested by Hayakawa and co-workers (Hayakawa et al. 2005). These researchers suggested that ingested arsenate is

reduced to arsenite, which subsequently forms complexes with glutathione. These complexes are enzymatically methylated resulting in the formation of MMA^{III} and DMA^{III}. The end-products MMA^{V} and DMA^{V} are formed by oxidation of MMA^{III} and DMA^{III}, respectively.
Naranmandura and co-workers proposed a metabolism pathway similar to that of Hayakawa and co-workers, but suggested the presence of different arsenic-protein-complexes as substrates for methylation which are subsequently oxidized (Rehman & Naranmandura 2012).
The exact mechanisms of arsenic metabolism are not fully clarified yet. Anyway, both trivalent and pentavalent methylated species can be present in the body after arsenic ingestion (Petrick et al. 2001). Formation of pentavalent methylated arsenic species during arsenite or arsenate metabolism presents a process of detoxification, but formation of trivalent methylated arsenic species results in a substantial increase of toxicity compared to arsenite or arsenate and can therefore be regarded as activation process (Styblo et al. 2002).
Three different mechanisms of arsenic toxicity leading to the development of cancer are widely accepted (Kitchin 2001): Production of chromosomal abnormalities by induction of chromosomal breakages, alteration of growth factors and cell proliferation, and the induction of oxidative stress in cells. Induction of oxidative stress by arsenic can either arise from arsenic-induced generation of reactive oxygen species (Eblin et al. 2006) or from the depletion of antioxidant enzymes or glutathione in the cell (Radabaugh & Aposhian 2000, Tabacova et al. 1994, Zakharyan & Aposhian 1999).

In contrast to arsenic, selenium is an essential nutrient (Schwarz & Foltz 1957) and can have an anti-carcinogenic and anti-mutagenic effect in low concentrations. But in higher doses it can have toxic, carcinogenic, and mutagenic effects (Bronzetti et al. 2001). In comparison to other micronutrients, the concentration range between beneficial and toxic effects after ingestion of selenium is quite narrow (Letavayova et al. 2008). Inorganic selenium exists in the oxidation states +4 as selenite ($H_2Se^{IV}O_3$), +6 as selenate ($H_2Se^{VI}O_4$) (Figure 2), and 0 as elemental selenium. In food, selenium is mostly incorporated in selenoenzymes, e.g. in form of the amino acids selenomethionine and methylselenocysteine (Figure 2) (Letavayova et al. 2008). Numerous studies report beneficial properties of selenium such as inactivating toxic metals (Hansen 1988), neutralizing free radicals (Borek et al. 1986), and antioxidant effects of selenoenzymes (Burk 1990, Irion 1999). Most selenium-related toxicological studies were conducted with selenite (Brozmanova et al. 2010). Applied in high doses it has been shown to exert strong cytotoxic effects, e.g. on prostate (Li et al. 2007), ovarian (Park et al. 2012a), liver (Shen et al. 1999), or lung cells (Park et al. 2012b). The toxic effects of selenium compounds are not fully understood, yet. But the induction of DNA damage due to double-

strand breakages (Biswas et al. 2000, Letavayova et al. 2008), oxidative stress (Wycherly et al. 2004, Zou et al. 2007), and apoptosis (Shen et al. 1999) by selenium were reported. Based on existing toxicological data, selenium is not classified as human carcinogen (Valdiglesias et al.).

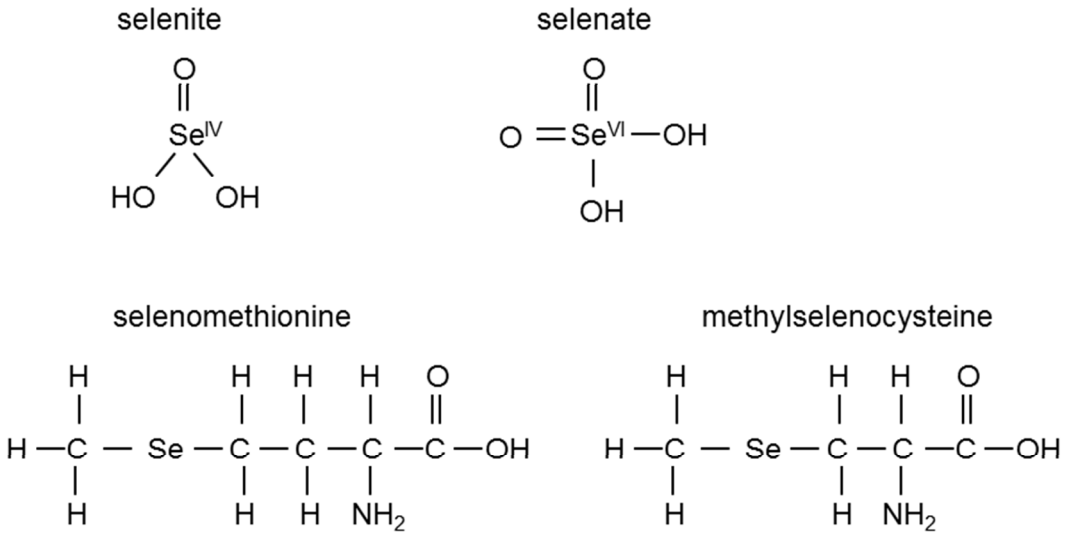


Figure 2: Chemical structures of selenite and selenate and selenomethionine and methylselenocysteine – two selenium-containing amino acids.

1.2 Arsenic-sulfur species: Thioarsenates

1.2.1 Occurrence and formation of thioarsenates in biological systems

For a long time, arsenite and arsenate were assessed to be the two main inorganic species that are present in arsenic-rich waters (Cullen & Reimer 1989). By now, it is known that in sulfidic environments, occurrence of arsenic is predominated by inorganic thioarsenic species which crucially influence arsenic chemistry (Hollibaugh et al. 2005, Planer-Friedrich et al. 2007, Stauder et al. 2005, Wilkin et al. 2003). These species were shown to form under abiotic and reducing conditions by mixing arsenite and sulfide (Wilkin et al. 2003). It was controversially discussed whether these species are trivalent thioarsenites ($As^{III}S_nO_{3-n}$ with n = 1-3) or pentavalent thioarsenates ($As^{V}SnO_{4-n}$ with n = 1-4). Based on geochemically expectations, trivalent thioarsenites were assumed to form as the species were detected under anoxic conditions (Beak et al. 2008, Bostick et al. 2005, Helz et al. 1995, Wood et al. 2002). But chromatographic analyses using anion-exchange chromatography-inductively coupled plasma-mass spectrometry (AEC-ICP-MS) and electrospray ionization tandem mass spectrometry (ESI-MS-MS) identified these species as pentavalent thioarsenates. X-ray absorption spectroscopy (XAS) data confirmed the presence of thioarsenates in these solutions and identified thioarsenites as precursors of thioarsenate formation (Planer-

Friedrich et al. 2010). Mixing sulfide and arsenite at high SH^-/OH^- ratio under anoxic conditions leads to formation of mono-, di-, and trithioarsenite (MTA^{III}, DTA^{III}, and TTA^{III}) which are immediately oxidized either directly by addition of elemental sulfur (S_0) or indirectly by O_2 addition which oxidizes sulfide to S_0, both resulting in the formation of di-, tri-, and tetrathioarsenate (DTA^V, TTA^V, and $TetraTA^V$). Monothioarsenate (MTA^V) is spontaneously formed from arsenite by addition of elemental sulfur (low SH^-/OH^- ratio) (Figure 3).

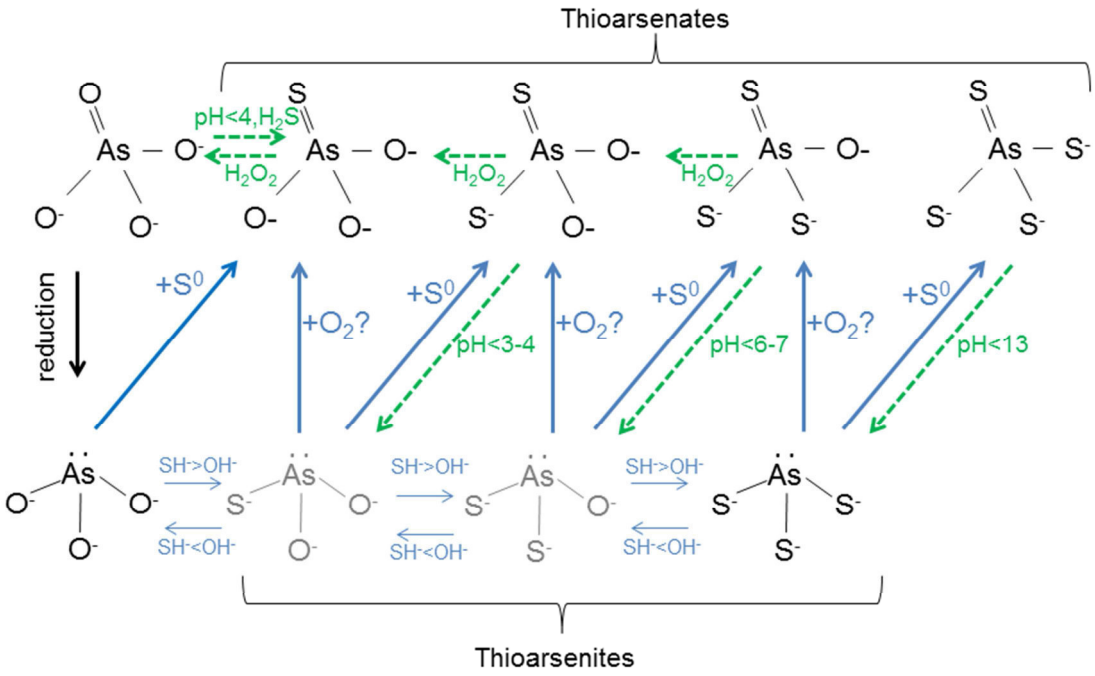


Figure 3: Reaction pathway of thioarsenite and thioarsenate formation according to Planer-Friedrich et al. (2015); the blue arrows indicate spontaneous reactions, the green dashed arrows indicate slow reactions that need an additional reactant like O_2, H_2O_2, or H^+; monothioarsenate is spontaneously formed from arsenite by addition of zerovalent sulfur (low SH^-/OH^- ratio) and is kinetically stable; the occurrence of mono- and dithioarsenite during this metabolism pathway is only postulated, whereas trithioarsenite was detected by XAS and all thioarsenates were detected by AEC-ICP-MS.

Acidification of DTA^V, TTA^V, and $TetraTA^V$ leads to arsenite formation, whereas MTA^V is stable under acidic conditions (Planer-Friedrich et al. 2010). Basically, thioarsenites cannot be detected by chromatographic methods to date as they are extremely sensitive towards the presence of oxygen. Even smallest amounts of oxygen are sufficient for immediate oxidation and high concentrations of OH^- groups in the eluent lead to immediate transformation of thioarsenites into arsenite (Planer-Friedrich et al. 2010).

Formation of thioarsenates is not only important in respect to arsenic behavior in the environment, but can crucially influence the behavior of arsenic in the body after ingestion.

Reduction and methylation during arsenic metabolism are long-known processes and it is estimated that 50-70 % of ingested inorganic arsenic - depending on the studied organism - is rapidly reduced to arsenite and subsequently methylated to dimethylated arsenic compounds which are detected in urine (Vahter 1999). High amounts of free sulfide in the

human gut and high pH (Jorgensen & Mortensen 2001) facilitate the formation of thioarsenates after arsenite intake. The presence of aerobic microbiota - even if they present the minority group of all microbiota present in the human intestine (Ramakrishna 2007) - reveals that thioarsenites do not have to be considered in terms of arsenic-sulfur speciation in the intestine due to immediate oxidation. The presence of thiolated arsenic compounds in biological samples was proven in several studies implying the need to consider these compounds in pre-systemic arsenic metabolism and arsenic risk assessments:

Yoshida and co-workers detected an unknown sulfur-containing arsenic metabolite – presumably the same metabolite that was already reported by Hughes and Kenyon (1998) - in rat urine and feces after exposure to DMA^V (Yoshida et al. 2003). This metabolite was formed by Escherichia coli strain A3-6 that was isolated from the ceca of DMA^V exposed rats (Yoshida et al. 2003). The mechanism of the arsenic-sulfur-metabolite formation was reported to be reduction of DMA^V to DMA^{III} followed by thiolation. Oxidation of the unknown metabolite resulted in DMA^V formation. Therefore, Yoshida and co-workers deduced it to be a trivalent DMA^{III} derivative. By analyses with HPLC-ICP-MS coupled simultaneously to electrospray mass spectrometry and electrospray ionization quadrupole time-of-flight mass spectrometry, Hansen and co-workers could show later that this metabolite – formed as main product during DMA^V reduction with sodium-metabisulfite ($Na_2S_2O_5$)/sodium thiosulfate ($Na_2S_2O_3$) reagent – was not a trivalent, but a pentavalent sulfur-containing DMA^V derivate, namely dimethylmonothioarsenate ($DMMTA^V$). The structure was additionally confirmed by proton nuclear magnetic resonance analyses. In the same study (Hansen et al. 2004), $DMMTA^V$ could be detected in urine and in wool extract from sheep that were naturally exposed to high concentrations of arsenosugars in their food. This study was the first that could distinctly identify thioarsenates in a biological sample.

Subsequently, several studies detected $DMMTA^V$ after arsenic exposure in urine of hamsters (Naranmandura et al. 2007b), rats (Adair et al. 2007, Naranmandura et al. 2007b), and mice (Hughes et al. 2008), in urine of DMA^V-exposed hamsters, monomethylmonothioarsenate ($MMMTA^V$) was also detected (Naranmandura et al. 2007b). Furthermore, $DMMTA^V$ was also shown to be a common arsenic metabolite in urine of women in Bangladesh who were exposed to arsenic (Raml et al. 2007).

Microbial thiolation of DMA^V leading to $DMMTA^V$ and $DMDTA^V$ formation was observed by Kubachka and co-workers (Kubachka et al. 2009): During incubation with microbiota from mouse cecum DMA^V was metabolized to $DMMTA^V$ and dimethyldithioarsenate ($DMDTA^V$), and also to monothiotrimethylarsenate ($MTTMA^V$). Based on their results, Kubachka and co-workers proposed a scheme for DMA^V biotransformation leading to sulfur-containing metabolites. Either DMA^V is directly thiolated leading to formation of $DMMTA^V$ and $DMDTA^V$

or DMA^{V} is first reduced to DMA^{III} which is subsequently methylated (formation of trimethylarsenate) and finally thiolated (formation of $MTTMA^{V}$) (Figure 4).

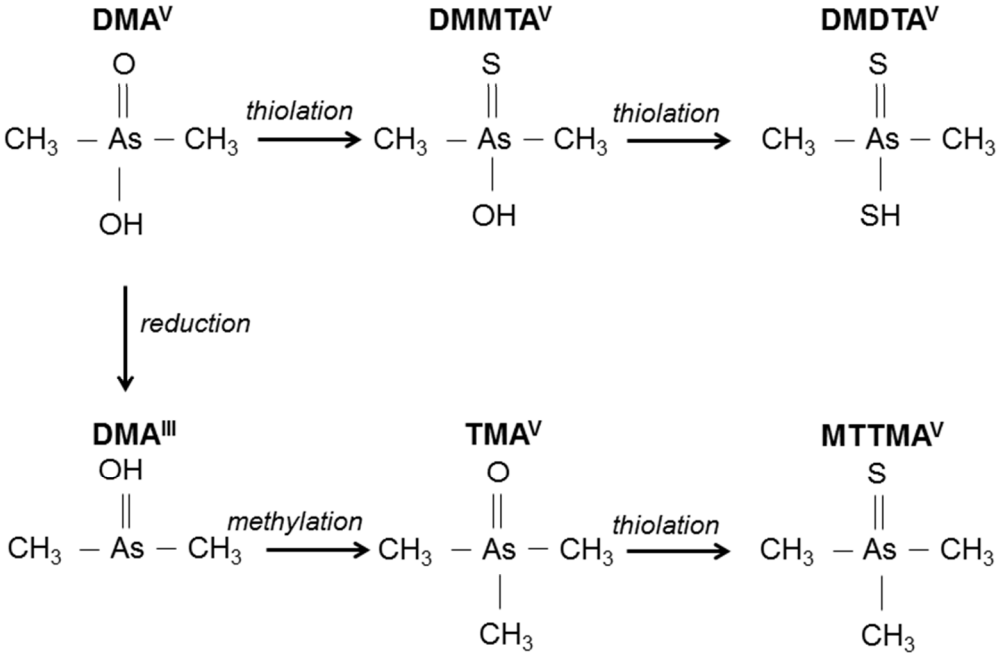


Figure 4: Formation of methylated thioarsenicals by microbiota of mouse cecum incubated with DMA^{V}. $DMMTA^{V}$ and $DMDTA^{V}$ are formed by thiolation of DMA^{V}, $MTTMA^{V}$ is formed via DMA^{V} methylation followed by thiolation; scheme proposed by Kubachka et al. (2009).

Incubating human gut microbiota with arsenate resulted in the formation of methylated arsenic compounds (MMA^{V} and MMA^{III}), methylated thioarsenic compounds ($MMMTA^{V}$) and interestingly also in one inorganic thioarsenic compound (MTA^{V}) (Van de Wiele et al. 2010).
Formation of inorganic thioarsenates by gut microbiota was confirmed in another study with microbiota from mouse ceca that were incubated with arsenate. The formation of seven different metabolites was observed: Besides the original substrate arsenate, methylated and inorganic thioarsenic species were detected in the reaction mixtures (Pinyayev et al. 2011). Pinyayev and co-workers proposed a scheme for the metabolism of arsenate: Alternating steps of reduction and oxidative methylation lead to the formation of arsenite, MMA^{V}, MMA^{III}, DMA^{V}, DMA^{III}, and trimethylarsenate (TMA^{V}). Each pentavalent species present can subsequently be reversibly thiolated leading to the formation of Mono-, Di-, Tri-, and $TetraTA^{V}$ (formed by thiolation of arsenate), $MMMTA^{V}$, $MMDTA^{V}$, and $MMTTA^{V}$ (formed by thiolation of MMA^{V}), and $DMMTA^{V}$, $DMDTA^{V}$ (formed by thiolation of DMA^{V}), and $TMMTA^{V}$ (formed by thiolation of TMA^{V}) (Figure 5). Pinyayev and co-workers also suggest the presence of trivalent intermediates (inorganic and methylated thioarsenites) in terms of their arsenate metabolism scheme. It is not clear why Pinyayev and co-workers did not detect any arsenite or $DMMTA^{V}$ in their experiments as it was shown in the mentioned study from

Kubachka et al. (2009). Other studies disagree with the presented scheme as direct thiolation of pentavalent arsenic species was shown to occur only at pH < 4 for both arsenate (Planer-Friedrich et al. 2015) and methylated pentavalent arsenic species (Rochette et al. 2000) and support the hypothesis that pentavalent arsenic species have to be reduced prior to thiolation. However, in contrast to these observations from abiotic systems, it cannot be excluded that gut microbiota are able to directly thiolate pentavalent arsenic compounds also at near-neutral pH.

In summary, the conditions in the human gut – free sulfide and high pH - definitely facilitate thioarsenate formation after arsenite or arsenate ingestion, but the exact mechanisms of their formation remain to be completely elucidated. Both methylated and inorganic thioarsenates were already detected during pre-systemic metabolism. What is primarily missing, is any information about the ability of thioarsenates to pass the gastrointestinal barrier without changing their speciation and about the subsequent relevance of their toxicity for organs.

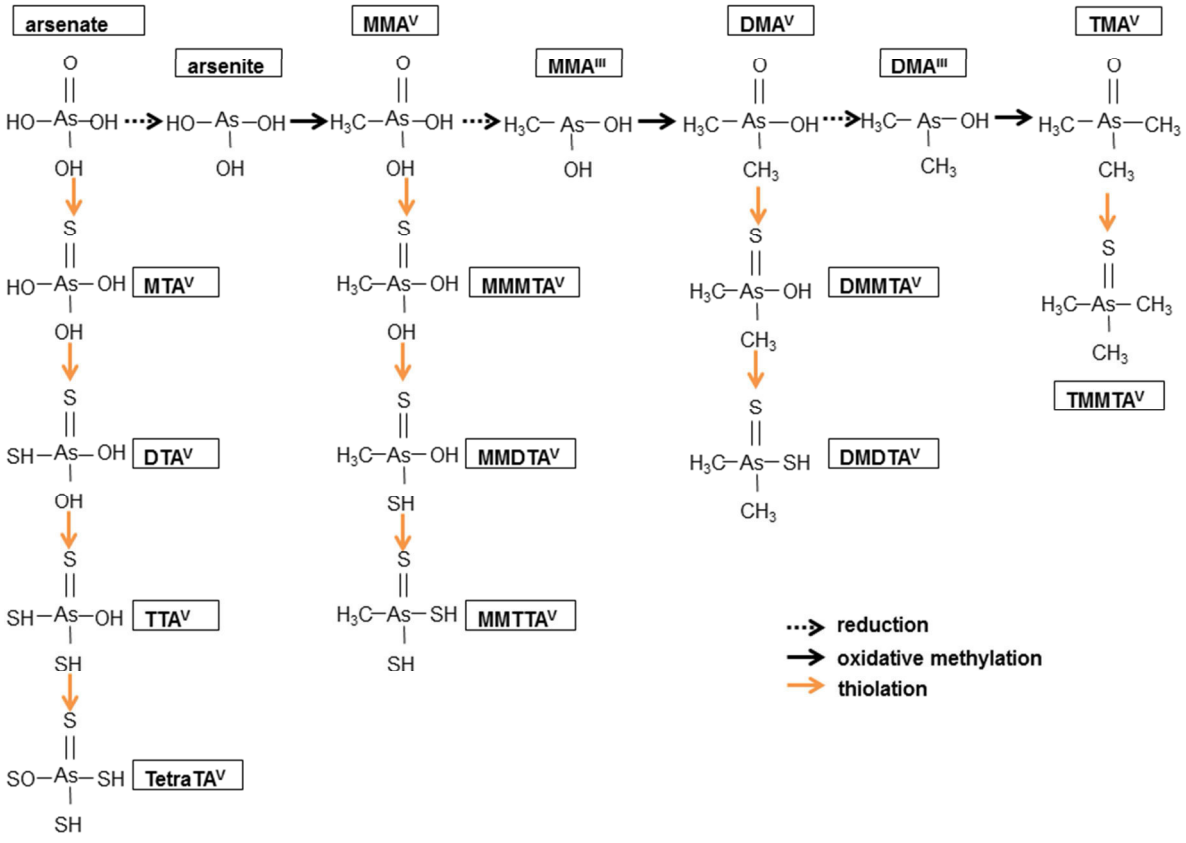


Figure 5: Arsenate metabolism by microbiota of mouse cecum; by alternating steps of reduction and oxidative methylation arsenate is metabolized firstly into arsenite and then into methylated pentavalent and trivalent arsenic compounds; each pentavalent compound can subsequently be thiolated leading to the formation of inorganic and methylated thioarsenic species. Scheme proposed by Pinyayev and co-workers (2008). The suggested presence of trivalent intermediates (inorganic and methylated thioarsenites) is not considered in this figure.

1.2.2 Bioavailability of arsenic species determined by transport through a Caco-2 cell monolayer

Bioavailability is defined as the fraction of a compound that reaches the systemic circulation after ingestion and digestion in the gastrointestinal tract. Besides their formation during pre-systemic arsenic metabolism and their cytotoxicity potential towards different cell lines, it is primarily the bioavailability of each arsenic species – i.e. the intestinal transport – which determines the toxic potential of a single arsenic species. Moreover, bioavailability is the crucial factor that makes toxicity studies of the respective compound relevant at all. Compared to toxicity studies, even less studies about the bioavailability of thioarsenates have been published so far.

The Caco-2 cell line is an immortalized cell line of heterogeneous human epithelial colorectal adenocarcinoma cells. After differentiation, their morphological and functional characteristics were shown to be very similar to those of small-intestine enterocytes (Hidalgo et al. 1989, Pinto et al. 1983). Most intestinal absorption takes places at the small intestines and so the Caco-2 cell line is suitable for laboratory studies to investigate the intestinal absorption behavior and transport mechanisms of different compounds.

In general, two mechanisms can facilitate the transport of arsenic compounds in the cells: The paracellular transport mechanism via the tight junctions of the cells and the transcellular pathway via membrane-located transport systems that are generally assumed to be phosphate transporters (Figure 6).

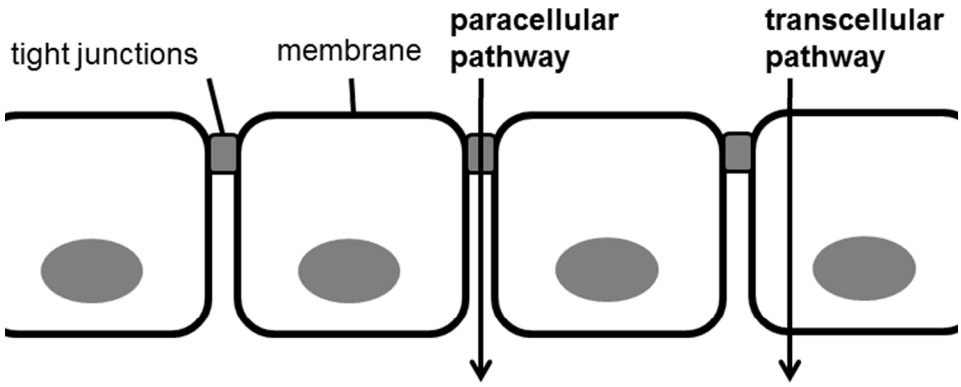


Figure 6: Paracellular and transcellular transport route in apical-basal direction through a Caco-2 cell monolayer.

Inhibited transport of a compound in presence of an excess phosphate concentration indicates the involvement of membrane-located phosphate transporters in transporting this compound. Increased transport of a compound by the addition of ethylenediaminetetraacetic acid (EDTA), which removes calcium and magnesium ions and therefore opens the tight

junctions, indicates the involvement of a paracellular transport system (Calatayud et al. 2011, Calatayud et al. 2010).

Concerning the arsenic species that are relevant for the present thesis, bioavailability studies using the Caco-2 cell line as model for the human small intestines were already published for arsenite, arsenate, MMA^{III}, MMA^{V}, DMA^{III}, DMA^{V}, and $DMMTA^{V}$ (Calatayud et al. 2011, Calatayud et al. 2010, Laparra et al. 2005).

Intestinal transport of arsenite (5.82 ± 7.71 %) was more efficient than that of arsenate (not detectable). Also cellular retention of arsenic was more pronounced for arsenite (0.87 – 2.28 %) than for arsenate (0.14-0.39 %) (Laparra et al. 2005). Further studies could show that both the cellular retention and intestinal transport of trivalent species (arsenite, MMA^{III}, DMA^{III}) were higher compared to their pentavalent analogues (arsenate, MMA^{V}, DMA^{V}) (Calatayud et al. 2011, Calatayud et al. 2010).

Comparing the intestinal transport of the pentavalent species, MMA^{V} and DMA^{V} showed lower intestinal transport than arsenate (Calatayud et al. 2010). In contrast, comparing the intestinal transport of the trivalent species, MMA^{III} and DMA^{III} showed higher intestinal transport than arsenite (Calatayud et al. 2011) and the rate of transport was proportional to the degree of methylation (DMA^{III} > MMA^{III} > arsenite). The involvement of a paracellular transport component (via the tight junctions) was shown for arsenite, arsenate, MMA^{III}, MMA^{V}, and DMA^{V}, whereas DMA^{III} was not transported via the paracellular route (Calatayud et al. 2011, Calatayud et al. 2010). Intestinal transport of arsenate was significantly inhibited by the addition of phosphate, indicating that phosphate transporters of the cellular membrane are involved in the intestinal transport of arsenate (Calatayud et al. 2010). The intestinal transport of $DMMTA^{V}$ is of special concern due to its relatively high toxicity. A recent study could show that the transport of $DMMTA^{V}$ is as high as that of arsenite (Leffers et al. 2013b). These data indicate that thiolation of arsenic compounds does not only have significant influence on their toxicity but also on their bioavailability. As studies about the intestinal transport of methylated thioarsenates are scarce and no study at all existed about the intestinal transport of inorganic thioarsenates, study 1 of the present thesis focusses on the intestinal transport of methylated and inorganic thioarsenates.

1.2.3 Toxicity of thioarsenates

For inorganic thioarsenates, the only toxicological data present until the publication of study 2, which is included in this thesis, are based on experiments with the marine luminescent bacteria *Vibrio Fischeri* (Planer-Friedrich et al. 2008, Rader et al. 2004). Even if these data are not directly transferable to arsenic toxicity to humans, they clearly indicate that thiolation has a distinct influence on arsenic toxicity. Rader and co-workers reported a significant

decrease of arsenite toxicity in the presence of sulfide. A further study repeated these experiments with concurrent arsenic speciation analyses and attributed the decrease of arsenic toxicity to the formation of thioarsenates (Planer-Friedrich et al. 2008). In this study, additional experiments with synthesized thioarsenate standards were performed. Whereas MTA^{V} and DTA^{V} were found to be significantly less toxic than arsenite, TTA^{V} showed comparable toxicity.

The toxicity of different methylated thioarsenates substantially differs from each other. Naranmandura and co-workers reported the order of toxicity of eight different arsenic compounds for the human bladder cell line EJ-1 as DMA^{III}, $DMMTA^{V}$ > arsenite, arsenate >$MMMTA^{V}$ > MMA^{V}, DMA^{V}, and $DMDTA^{V}$ (Naranmandura et al. 2011). In another study from Naranmandura and co-workers using the human epidermoid carcinoma cell line A431, DMMTA(V) was shown to exhibit slightly lower acute cytotoxicity (IC50 5.7 µM) than arsenite (Naranmandura et al. 2007a). Therefore, $MMMTA^{V}$ is slightly more toxic than the methylated arsenic compounds MMA^{V} and DMA^{V} but still less toxic compared to arsenite or arsenate and $DMDTA^{V}$ is assessed to be an arsenic species of comparably low toxicity.

An arsenic species of special concern is $DMMTA^{V}$: It was already reported to be substantially more toxic than DMA^{V} (Ochi et al. 2008, Yoshida et al. 2003) and further studies even showed that its toxicity clearly exceeded that of arsenite (Leffers et al. 2013a). The difference in toxicities between $DMMTA^{V}$ and arsenite could be traced back to different intracellular mechanisms. While $DMMTA^{V}$ exposure led to reduced expression of p21 and p53 proteins, DNA damage, reduced glutathione level, and increased level of highly reactive oxygen species, arsenite exposure resulted in increased expression of p21 and p53 proteins, but glutathione and highly reactive oxygen levels remained unaffected (Naranmandura et al. 2011). The exact mechanisms explaining the toxicities of all different arsenic compounds are not clarified, yet.

Taken together, several studies exist that compare the cytotoxicity of arsenite, arsenate, (see Paragraph 1.1), and methylated thioarsenates. Studying the cytotoxicity of inorganic thioarsenates was one focus of the present thesis (study 2).

1.3 Selenium-sulfur species: Selenosulfate

The inorganic selenium-sulfur complex selenosulfate is a structural analogue to sulfate with one oxygen atom replaced by a sulfur atom (Figure 7). It is used for the industrial preparation of nanoparticles of different selenium compounds, e.g. cadmium selenide or silver selenide (Pejova et al. 2000, Raevskaya et al. 2006). Occurrence of selenosulfate was detected in flue gas desulfurization water (Petrov et al. 2012) and in root exsudates of selenite-exposed Indian Mustard (Mounicou et al. 2006, Vonderheide et al. 2006). In general, hardly any studies exist investigating the chemical properties and toxicological potential of selenosulfate

to date. The anticarcinogenic property of selenium was first suggested in the 1960s by Shamberger and Frost (1969) who reported an inverse relationship between cancer mortality rates and the concentration of selenium in forage crop in the USA. Subsequently, several studies confirmed that selenium compounds possess anticarcinogenic properties (reviewed in Combs and Gray (1998), Rayman (2005)). Several mechanisms that account for the anticarcinogenic properties of selenium are suggested, like an antioxidative effect and the control of the cellular redox state by various selenoenzymes, reduction of DNA damage, reduction of tumor promoting inflammation, or induction of apoptosis (reviewed in Rayman (2005)).

Due to its beneficial effect in reducing side effects of commonly applied anti-cancer chemotherapy drugs like cisplatin (Baldew et al. 1989, Camargo et al. 2001, Markovic et al. 2011), selenite (Na_2SeO_3) (Figure 7) is often applied as supplement in clinical trials (Dennert & Horneber 2006). Unfortunately, at high doses, selenite is cytotoxic itself to normal tissue (Li et al. 2007, Park et al. 2012a, Park et al. 2012b, Shen et al. 1999) and as mentioned before, the concentration range between beneficial and toxic effect is quite narrow. Therefore, the use of selenite in anti-cancer therapy is discussed controversially (Micke et al. 2009, Vinceti et al. 2001, Zhang et al. 2008b).

Selenosulfate is proposed to replace selenite in anti-cancer-therapies (Zhang 2010).

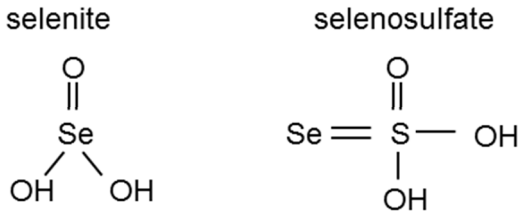


Figure 7: Structural formula of sodium selenite and selenosulfate.

Selenosulfate was shown to be more cytotoxic to cancer cells compared to selenite (Zhang et al. 2008a). Experiments with mice exposed to cisplatin combined with either selenite or selenosulfate resulted in the same toxic effect on cancer cells, but healthy tissue was less damaged in the treatment with selenosulfate (Zhang et al. 2008b). In contrast to selenite, long-term administration (55 days) of selenosulfate in the same concentration did not result in toxic symptoms in mice (Li et al. 2012). Based on the results of the conducted cell experiments using the five different cell lines HepG2, Caco-2, and three different leukemia cell lines (HL60, T lymph adenoma, and Daudi), and experiments with mice, Zhang and co-workers published a patent (Patent US 2010/0172822). This patent claims that selenosulfate is generally more toxic to cancer cells than selenite and therefore, selenite should be replaced by selenosulfate as supplement in anti-cancer chemotherapies. Study 3 of the

present thesis aims to reassess this general claim by comparing the cytotoxic effects of selenosulfate and selenite for three different cancer cell lines.

1.4 Objectives

The general aim of the present thesis was to further elucidate the influence of sulfur complexation on the cytotoxicity of the metalloids arsenic and selenium.

When arsenic or selenium is present in sulfur-containing complexes, the toxicity of these metalloids is substantially influenced. This was already shown for methylated thioarsenicals compared to arsenite and arsenate and for selenosulfate compared to selenite. However, only few studies exist dealing with the cytotoxic potential of these compounds.

For evaluating the toxic potential of thioarsenates, investigations about their bioavailability, e.g. their intestinal absorption, are crucial. Only few data exist about the intestinal transport of methylated thioarsenates and no data at all about the intestinal transport of inorganic thioarsenates. Study 1 of the present thesis therefore compares the intestinal transport of inorganic and methylated thioarsenates to the intestinal transport of arsenite and arsenate to further complete the concept of arsenic risk assessment after ingestion.

No study at all exists about the toxicity of inorganic thioarsenates, although their formation during pre-systemic arsenic metabolism was proven and their existence has to be considered in terms of arsenic risk assessment after ingestion. Based on the lack of these data, study 2 of this thesis focusses on the toxicity of inorganic thioarsenates to human bladder and liver cells in comparison to arsenite and arsenate and links the modified toxicities to the cellular uptake of the different arsenic compounds.

The reported increased toxicity of the selenium-sulfur compound selenosulfate compared to selenite to five different cancer cell lines was one crucial factor for the claim that selenosulfate is generally more potent in anti-cancer therapies than selenite. Study 3 of the present thesis aims for reassessing this general claim by comparing the toxicity of selenosulfate and selenite for three cancer cell lines.

2 METHODS

2.1 Cell lines

For testing the cytotoxicity of thioarsenates in comparison to arsenite and arsenate, two cell lines derived from target organs of arsenic toxicity were chosen: the human urothelial cell line UROtsa and the human hepatoma cell line HepG2. Just recently, several stocks of the UROtsa cell line turned out to be contaminated by another cell line and were identified as the human bladder cancer cell line T24 (Johnen et al. 2013). It is important to note that in study 2 and in study 3 actually the same bladder cell line was used, even if it was designated as "UROtsa" in study 2 and as "T24" in study 3.

As already described in section 1.2.3., the Caco-2 cell line serves as a model of the human small intestine and was therefore used in study 1 for comparing the intestinal transport of different arsenic species.

In study 3, the cytotoxicity of selenosulfate and selenite were compared for three different cancer cell lines: HepG2, T24, and the melanoma cell line A375.

The procedures of cell cultivation, cytotoxicity testing using the MTT assay, and cellular uptake of arsenic and selenium compounds are described in the respective studies.

2.2 The Caco-2 model for quantifying intestinal transport of arsenic species

The applied method for testing the bioavailability is described in detail in the Supporting Information of study 1 and is described only briefly at this point.

For simulating the intestinal barrier, Caco-2 cells were seeded on filter inlets that were placed into a six-well-microtiterplate. The well below the filter represented the basal side (basement membrane) and the filter space above the Caco-2 cells represented the apical side (intestinal lumen). The Caco-2 cells were growing on the filter inlets for 21 days. Medium was changed three times a week. After this time, a confluent cell monolayer was built. The cell monolayer integrity was monitored during the 21 days of growing and before and during the arsenic incubation by measuring the transepithelial electrical resistance (TEER). Only TEER values > 500 Ωcm^2 were assessed to present a stable and integer cell monolayer (Figure 8).

For testing the intestinal transport of an arsenic species, the respective species was applied to the apical side and the basal medium was removed after 2, 4, 6, or 8 h for analysis of arsenic content and speciation.

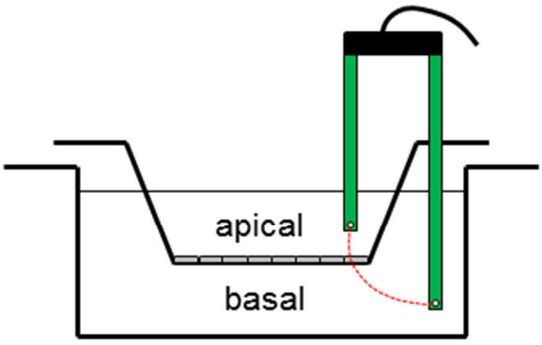


Figure 8: Scheme of the Caco-2 model and measurement of the transepithelial electrical resistance (TEER).

A method to quantify the paracellular transport of different arsenic species was applied in the context of study 1. However, the method failed and did not produce any reasonable results. The method is not considered in study 1 but is described in the following.

The impact of a paracellular transport route (transport through the tight junctions) on the intestinal transport of the different arsenic species was investigated by reducing the Ca^{2+} and Mg^{2+} concentration in the cell growth medium and therefore leading to an opening of the tight junctions.

For this purpose, the cell monolayer was pre-incubated for 5 minutes with a 5 mM EDTA solution (Applichem, Darmstadt, Germany, diluted in DPBS) just before the transport experiment according to a published method (Noach et al. 1993). The cell growth medium used in this experiment also contained 2.6 mM EDTA in order to complex excess Ca^{2+} and Mg^{2+} in the medium.

To evaluate the effect of EDTA addition on the opening of the tight junctions, 100 µM of the fluorescent dye Lucifer Yellow was added to apical control wells in each experiment. Lucifer Yellow can only be transported via the paracellular route. After 2, 4, 6, and 8 h of incubations, basal aliquots of the Lucifer Yellow-treated wells were taken (50 µL) and fluorescence was measured (Tecan F200 Prro, excitation 485 nm, emission 535 nm). The percentaged transport of Lucifer Yellow was quantified by setting cell medium containing 100 µM Lucifer Yellow to 100 % and untreated cell medium to 0 %. Additionally, the influence of EDTA addition on the cell monolayer integrity was monitored by TEER measurements during each transport experiment.

2.3 Analysis of arsenic and selenium in cell medium and cell lysates

Total content of arsenic and selenium in cell media and in cell lysates were analyzed by inductively coupled plasma – mass spectrometry (ICP-MS). The parameters are listed in the respective studies.

Thioarsenates were determined by an AEC-ICP-MS method published before by Planer-Friedrich et al. (2007). The parameters of this method are also described in studies 1 and 2. The same method was adapted for the analysis of selenite and selenosulfate (study 3).

2.4 Speciation analysis of selenosulfate in cell growth medium

For synthesizing the selenosulfate standard, selenite was mixed with glutathione and sulfide (detailed description of the selenosulfate synthesis is included in study 3). Before incubating the cells with the selenosulfate standard, each standard was diluted in ultrapure water and analyzed with AEC-ICP-MS to check the purity of the synthesized product and to detect unreacted selenite in the selenosulfate standard.

The purity of the selenosulfate standard was determined as 91 ± 9.9 % and the selenosulfate peak was clearly discriminable from the selenite peak (Figure 9).

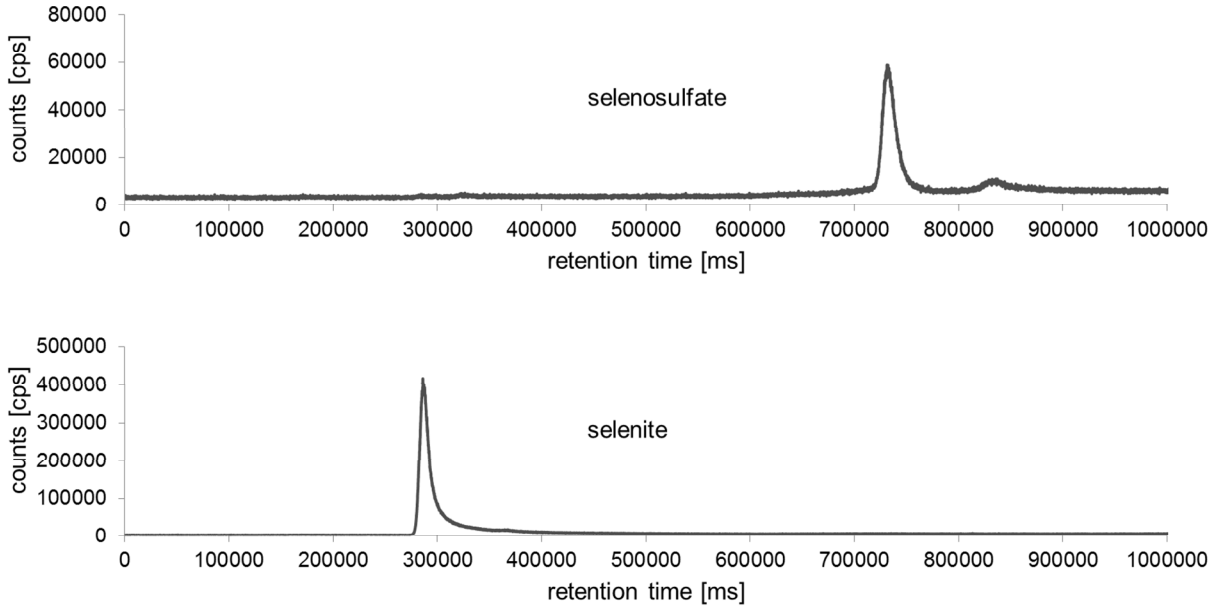


Figure 9: AEC-ICP-MS chromatograms of selenosulfate and selenite analyzed after dilution in ultrapure water.

To test whether selenosulfate was stable during the cell experiments, the media of selenosulfate experiments were analyzed for selenium species after the respective incubation times (24-72 h). Unfortunately, analysis of cell growth medium containing

selenosulfate did not result in reasonable peaks. Only when a chromatographic column had not been in contact with cell growth medium before, selenosulfate could be detected in one single sample (after 72 h incubation time). In subsequent samples, the selenosulfate peak disappeared and selenium eluted in the selenite peak only. Furthermore, the concentrations of the eluted selenium were far too low (about one third of the expected selenium concentration). Apparently, the cell growth medium changed the conditions of the stationary phase resulting in decreased elution of selenium compounds. As one single medium sample was sufficient to render the column useless for good separation of subsequent samples, frequent cleaning of the column was not sufficient to improve the method.

Due to the low selenium concentrations in the toxicity experiments, the medium samples could not be diluted in ultrapure water to diminish these negative effects and therefore, the selenium speciation could not be analyzed in medium samples.

Nevertheless, we derive sufficient stability of the selenosulfate standard for the duration of the cytotoxicity experiments from literature data (Zhang et al. 2008a), from no observed precipitation of elemental selenium, and from the result that a selenosulfate peak was detected in one medium sample after 72 h of incubation. For this analysis, a chromatographic column was used that had not been in contact with cell growth medium before.

3 RESULTS AND DISCUSSION

3.1 The influence of sulfur complexation on arsenic bioavailability and cytotoxicity

3.1.1 The intestinal transport of thioarsenates compared to their non-thiolated analogues (Study 1, Hinrichsen et al. 2015)

In this study, cellular retention and intestinal transport of two inorganic thioarsenates – MTA^V and TTA^V – and two methylated thioarsenates – $MMMTA^V$ and $DMMTA^V$ – were quantified using a Caco-2 cell monolayer model. To investigate the influence of thiolation, the intestinal transport of the thioarsenates was compared to that of the original present arsenic species after ingestion, e.g. arsenite/arsenate for all thioarsenates and additionally MMA^V and DMA^V for the methylated thioarsenates. Analyses of arsenic speciation were conducted in apical and basal media for each experiment to test whether the arsenic species were stable during the transport experiments.

To test whether thioarsenates were transported via the transcellular (phosphate transporters) or the paracellular transport route, experiments were conducted in the complete absence of phosphate and in presence of EDTA, respectively.

The different arsenic species considerably differed concerning their percentaged cellular retention in Caco-2 cells and concerning their passage through the Caco-2 cell monolayer. According to previous literature, arsenite was shown to be both retained in the cells and transported through the cell monolayer to a higher extent than all other arsenic species (Calatayud et al. 2011) and intestinal transport of arsenate, MMA^V, and DMA^V was negligibly low (Calatayud et al. 2010, Naranmandura et al. 2007a). Among the thiolated species, $MMMTA^V$ was the species of least concern, e.g. intestinal transport was only slightly higher compared to MMA^V and cellular retention was even lower than that of MMA^V. Comparing cellular retention and intestinal transport of $DMMTA^V$ and its non-thiolated analogue shows a substantially higher retention and transport for $DMMTA^V$. Moreover, among all methylated arsenic compounds tested, cellular retention and intestinal transport of $DMMTA^V$ were by far the highest.

In case of the inorganic thioarsenates, cellular uptake of MTA^V was comparable to that of arsenate. The difference between these two species was that for MTA^V, intestinal transport was more pronounced than cellular retention and for arsenate, intestinal transport was less pronounced than cellular retention. Cellular retention of TTA^V was comparably low as that of arsenate, but very interestingly, intestinal transport of TTA^V was the second highest of all tested arsenic species (only arsenite was higher).

For all methylated and inorganic thioarsenates, transport experiments in complete absence of phosphate increased cellular retention by a factor of 2 to 6 indicating the involvement of

apical phosphate transporters for cellular uptake of thioarsenates. No results can be presented concerning the experiments to investigate the involvement of paracellular transport mechanisms by the addition of the Ca^{2+} and Mg^{2+}-chelator EDTA. Unfortunately, pre-incubation with EDTA and addition of EDTA to the cell growth medium during the transport experiments considerably damaged the cell monolayer. This became obvious by monitoring the TEER values of the cell monolayers before and during the transport experiments. Before incubation, TEER values were > 500 Ωcm^2 indicating a stable monolayer. But already after 1 h of EDTA-combined incubation with MTA^V or TTA^V, TEER values were < 200 Ωcm^2 and even decreased further with time. In the absence of EDTA, the cell monolayer was not damaged by incubation with MTA^V or TTA^V alone in the same concentration (Figure 10). Percentaged transport of both Lucifer Yellow and MTA^V and TTA^V achieved values up to 50 % after 8 h of incubation (data not shown). That means that the apical arsenic-containing medium simply intermixed with the arsenic-free basal medium due to the concentration gradient as no barrier in form of an integer cell monolayer was present.

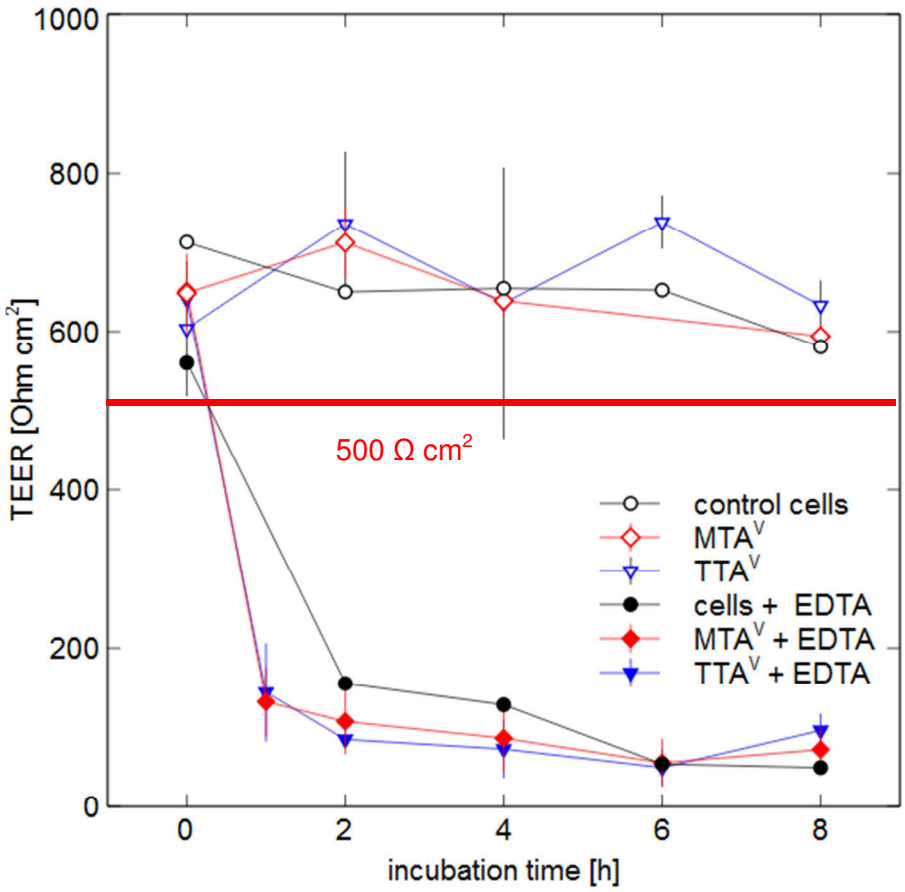


Figure 10: Transepithelial electrical resistance (TEER) values during transport experiments (2-8 h). Cells were exposed to 10 µM MTA^V or 10 µM TTA^V, each in absence and presence of EDTA. Only TEER values > 500 Ωcm^2 indicate cell monolayer integrity during arsenic exposure; treatments were conducted in triplicate; presented values are mean values ± standard deviation; control cells: cells in growth medium only; cells + EDTA: cells were treated with EDTA, but not with arsenic. TEER was also measured directly before arsenic incubation (t = 0 h) to ensure cell monolayers were intact before the transport experiment.

In transport experiments with individually applied MTA^V, TTA^V, and arsenate, arsenic speciation analyses of the basal media showed that the inorganic thioarsenates and arsenate were transported intact through the cell monolayer. As also arsenite was present in the basal media in considerable amount, intracellular reduction of the thioarsenates to some extent cannot be completely excluded.

No results about the arsenic speciation during the transport experiments with methylated thioarsenates can be presented because their basal concentrations were too low for identifying these species. Additionally, matrix interferences with components from the cell growth medium led to chromatographic problems in this case. However, based on data from previous studies (Leffers et al. 2013b), methylated thioarsenates are assumed to be transported intact through the Caco-2 cell monolayer.

The present study could show that thiolation definitely has a strong effect on the transport behavior of arsenic compounds. Still, for a general evaluation whether thiolation increases or decreases the bioavailability of the original present arsenic substrate, more research is needed to elucidate the exact mechanisms of thioarsenate formation. More precisely, it is not fully understood, yet, if thioarsenates are formed from their pentavalent analogues (arsenate for inorganic thioarsenates, MMA^V for $MMMTA^V$, and DMA^V for $DMMTA^V$) or from trivalent arsenic species with arsenite being the original present species. If thioarsenates are formed from their structural pentavalent analogues, thiolation increases intestinal transport of arsenic, but if they are formed from trivalent species, thiolation decreases intestinal transport of arsenic, whereas trithioarsenate presents an exception because it is almost as bioavailable as arsenite.

Previous studies showed that $DMMTA^V$ is considerably more cytotoxic than its non-thiolated structural analogue DMA^V (Leffers et al. 2013a, Naranmandura et al. 2011, Raml et al. 2007). Therefore, $DMMTA^V$ is an arsenic species of special concern during pre-systemic arsenic metabolism due to both increased bioavailability and toxicity.

Inorganic thioarsenates are more bioavailable than arsenate but lower bioavailable than arsenite. To further investigate their toxic potential compared to arsenite and arsenate, study 2 of the present thesis focusses on the cytotoxicity of MTA^V and TTA^V in comparison to arsenite and arsenate and links the cytotoxicity of the different arsenic compounds to cellular retention.

3.1.2 The cytotoxicity of inorganic thioarsenates compared to arsenite and arsenate (Study 2, Hinrichsen et al. 2014)

In this study, the cytotoxicity of inorganic thioarsenates was compared with that of arsenite and arsenate for human hepatocytes (HepG2) and urothelial cells (UROtsa) using the MTT assay. For this purpose, sulfide was added to an arsenite solution in cell growth medium (molar As/S ratio of 1:4) and the cytotoxic effects – e.g. the decrease of cell viabilities – of arsenite alone and the arsenite-sulfide-mixture were compared after 6 and 24 h of incubation. Thioarsenates formed in the arsenite-sulfide solutions were quantified by AEC-ICP-MS analyses. To evaluate the cytotoxicity of individual thioarsenates, synthesized standard solutions of MTA^V and TTA^V were compared to arsenite and arsenate. To assess whether simply cellular uptake determines the cytotoxicity of the respective arsenic species, cellular uptake was determined for MTA^V, TTA^V, arsenite, and arsenate and compared to the order of cytotoxicity.

For both HepG2 and UROtsa cells, the cytotoxicity of arsenite was reduced by addition of sulfide after 6 h of incubation and for HepG2 additionally after 24 h of incubation. For UROtsa cells, cell viabilities did not significantly differ after 24 h between the arsenite-sulfide treatment and the exclusive arsenite treatment. Speciation analyses showed the immediate formation of inorganic thioarsenates (36-73 % of total arsenic, dominant species was TTA^V) in the cell growth media of both cell lines that were treated with arsenite and sulfide. In general, UROtsa cells showed higher susceptibility to arsenic exposure than HepG2 cells. With time, the thioarsenates converted into arsenite in the cell growth medium and after 24 h, only 33 % of total arsenic was present as thioarsenates in cell growth medium. The general higher susceptibility of UROtsa cells towards arsenite exposure implies a faster reaction to arsenite exposure compared to HepG2 cells and thus explains the marginal influence of sulfide on arsenite cytotoxicity for UROtsa cells after 24 h as a substantial percentage of the formed thioarsenates converted to arsenite after 24 h.

The decreased cytotoxicity of arsenite in presence of sulfide could be traced back to the formation of inorganic thioarsenates, as cytotoxicity experiments with standard solutions of arsenite, arsenate, MTA^V, and TTA^V standards showed for both cell lines and both incubation times the following order of cytotoxicity: arsenite > TTA^V > MTA^V > arsenate. It is in accordance to previous literature that arsenite is considerably more cytotoxic than arsenate (Dopp et al. 2008, Styblo et al. 2000).

The order of cellular arsenic uptake after 24 h of incubation was determined for HepG2 cells as arsenite > TTA^V > MTA^V > arsenate and for UROtsa cells as arsenite ≈ TTA^V > MTA^V > arsenate. The lower uptake of arsenate compared to arsenite can be explained by different uptake mechanisms. Arsenite is taken up by cells via aquaglyceroporins or diffusion, arsenate is taken up by inorganic phosphate transporters and therefore, arsenate uptake can

be inhibited by the presence of phosphate. In the conducted experiments, phosphate was both present in the used cell growth medium and in the buffer solution used for washing the UROtsa and the HepG2 cells. Therefore, arsenate uptake was likely reduced by the presence of phosphate.

No studies exist about the uptake mechanisms of thioarsenates. Based solely on the charge of MTA^{V}, its uptake mechanism should be similar to that of arsenate.

As the order of cellular uptake corresponded in general to the order of cytotoxicity for both cell lines, cellular arsenic uptake can be seen as crucial for the cytotoxic potential of different arsenic species.

Combining the results of study 1 and study 2 of the present thesis, thioarsenates possess both lower bioavailability and cytotoxicity compared to arsenite and both higher bioavailability and cytotoxicity compared to arsenate. Regarding arsenite as precursor of thioarsenate formation, thiolation presents a detoxification process. However, in comparison to arsenate as the structural analogue of thioarsenates and a potential precursor (if gut microorganisms can directly thiolate pentavalent species), thiolation presents an activation process. More research is needed about the exact formation mechanisms of thioarsenates for a better interpretation of the influence of thiolation on the cytotoxic potential of arsenic.

3.2 Comparing the cytotoxicity of selenite and the selenium-sulfur compound selenosulfate (Study 3, Hinrichsen & Planer-Friedrich submitted)

In this study, the cytotoxic effects of selenite and the selenium-sulfur-compound selenosulfate were quantified and compared by means of the MTT assay for the three different cancer cell lines HepG2 (human hepatocytes), A375 (malignant melanoma cells), and T24 (urinary bladder carcinoma cells) with the objective of verifying the general claim that selenosulfate is more cytotoxic than selenite for cancer cells (Zhang 2010). The IC_{50} values (inhibitory concentration of selenosulfate or selenite inducing 50 % cell viability) were calculated. Cellular uptake of the two selenium compounds was quantified to investigate the relation between the uptake and the cytotoxicity of selenosulfate and selenite, respectively.

The cytotoxicity of selenosulfate was similar among the three different cell lines (IC_{50} 6.6-7.1 µM), but the cytotoxicity of selenite was considerably different. The order of susceptibility towards selenite exposure among the three cell lines was T24 > A375 > HepG2 (IC_{50} values 3.5, 5.7, 15 µM).

Only HepG2 cells – the cells that were already used in the experiments from Zhang and co-workers (Zhang et al. 2008a) – showed the expected result that selenosulfate was significantly more cytotoxic than selenite after 24 h of incubation (difference in cell viabilities up to 50 %). For A375 cells, incubation with selenosulfate and selenite resulted in comparable cell viabilities and in case of T24 cells, selenosulfate was even less cytotoxic than selenite (difference in cell viabilities up to 45 %). Based on the fact that only one of three investigated cancer cell lines showed results that are consistent with the patent of Zhang and co-workers (Zhang 2010) one has to challenge the general conclusion of the patent that selenosulfate should replace selenite as supplement in anti-cancer-therapies.

Intracellular selenium concentration after 24 h incubation with 1.7 µM selenosulfate and 1 µM selenite, respectively, could be quantified in HepG2 and T24 cells. For HepG2 cells, the higher cytotoxicity of selenosulfate compared to selenite was in accordance with higher intracellular selenium concentrations after selenosulfate incubation and for T24 cells, the lower cytotoxicity of selenosulfate compared to selenite was in accordance to lower intracellular selenium concentrations after selenosulfate incubation.

Although the cytotoxicity of selenosulfate and selenite in A375 was comparable to HepG2 and T24 cells, selenium concentrations in A375 cells were not distinguishable from non-exposed control cells after exposure to 1.7 µM selenosulfate and 1 µM selenite, respectively. A potential reason could be that A375 cells were cultivated in a different growth medium (Dulbecco's modified Eagle Medium, DMEM) than HepG2 and T24 which were cultivated in minimum essential medium (MEM). The DMEM medium contained twice as much L-cystine compared to the MEM medium. The extracellular cystine likely reduced the presence of reduced glutathione (GSH) resulting in a decreased defense against oxidative stress

produced by selenium. By this, remarkable toxic effects in the A375 cells occurred even at extremely low selenium uptake.

For the 24 h experiments, HepG2 cells were routinely cultivated in non-essential amino acids (NEAA)-containing MEM and T24 cells in NEAA-free MEM. To further investigate the observed effect of selenosulfate and selenite cytotoxicity, experiments were conducted with HepG2 and T24 cells in the presence and absence of NEAA as medium supplement for three different exposure times (24, 48, and 72 h).

The cytotoxicity of selenosulfate was largely independent of exposure time, cell line, and presence or absence of NEAA. For HepG2 cells, selenosulfate was under all applied conditions more toxic than selenite, but the effect diminished with time due to a strong increase of selenite cytotoxicity with time. The large difference between selenosulfate and selenite cytotoxicity for HepG2 cells seemed to be a short-time. The relatively low selenite cytotoxicity for HepG2 cells after 24 h can be traced back to low selenite uptake. The presence of NEAA slightly decreased the cytotoxicity of selenosulfate and selenite only for longer incubation times.

In contrast, the addition of NEAA in the cytotoxicity experiments with T24 had a remarkable effect concerning the cytotoxicity of selenite: Presence of NEAA down-regulated selenite uptake leading to a significantly reduced selenite cytotoxicity. After 24 h, selenosulfate was even more toxic than selenite for T24 cells – just as observed for the HepG2 cells. At longer incubation times, cell viability curves for selenosulfate and selenite exposed T24 cells assimilated as already observed for HepG2 cells.

The strong effect of NEAA presence on T24 cells – a reversion of selenosulfate and selenite cytotoxicity - could be explained by an effect on the cellular cysteine/glutamate exchange system x_c^- which is essential for the cellular selenite uptake. This transport system regulates the extracellular reductive environment by secretion of cysteine which subsequently reduces selenium compounds. Reduced selenium species are known to be preferentially transported into cells (Conrad & Sato 2012).

The amino acid L-glutamic acid (one component in the applied NEAA mixture) is known to inhibit the x_c^- transport system resulting in decreased selenite uptake and cytotoxicity (Olm et al. 2009). The x_c^- transport system was shown to be more present in selenite-sensitive cells compared to selenite-resistent cells (Wallenberg et al. 2014) which let us conclude that it is also more expressed in T24 compared to HepG2 cells. This explains why the cytotoxicity of selenite is significantly affected by presence of NEAA in T24 cells.

The results of this study show that for evaluating the cytotoxic effects of selenosulfate and selenite for a specific cell line, one has to consider the sensitivity of the specific cell line and the composition of cell growth medium which might influence the reductive state of the extracellular environment and therefore the uptake and cytotoxicity of selenium.

4 CONCLUSION

The results of the three presented studies definitely show that cellular uptake and cytotoxicity of the metalloids arsenic and selenium change when they are sulfur-complexed.

Although formation of thioarsenates during pre-systemic arsenic metabolism had been proven before, hardly any data existed about their intestinal transport and toxicity until the publication of study 1 and 2 of the present thesis. These studies include the very first published data about the intestinal transport and cytotoxicity of inorganic thioarsenates. For methylated thioarsenates, only some data existed about their intestinal transport and their cytotoxicity and these data are considerably supplemented by the data of study 2.

Further studies are important to investigate the bioavailability and toxicity of thioarsenates, focusing on the precise mechanisms of transport and toxicity. For this purpose, a method for synthesizing a dithioarsenate standard is required to link the degree of thiolation (e.g. number of SH^- groups) to bioavailability and toxicity of the thioarsenates.

Furthermore, detailed research on the exact formation mechanisms of thioarsenates is essential for a reliable interpretation of thiolation being a process of reduced bioavailability and detoxification or increased bioavailability and activation in comparison to the originally present trivalent or pentavalent arsenic substrate.

Study 3 showed that cellular uptake and cytotoxicity of selenite is modulated by the presence of amino acids in the cell growth medium to such an extent that selenosulfate can possess higher (presence of amino acids) or lower (absence of amino acids) cytotoxicity compared to selenite in bladder cancer cells. The general claim that selenosulfate is more cytotoxic than selenite and should therefore preferentially be used in anti-cancer therapies therefore has to be rejected. For evaluating a possible role of selenosulfate in anti-cancer therapies, more toxicity studies under strictly defined conditions on the basis of different model organisms are indispensable. Again, further studies are required to elucidate the mechanisms of cellular uptake and toxicity of selenium and selenium-sulfur compounds.

REFERENCES

Adair BM, Moore T, Conklin SD, Creed JT, Wolf DC, Thomas DJ (2007): Tissue distribution and urinary excretion of dimethylated arsenic and its metabolites in dimethylarsinic acid- or arsenate-treated rats. Toxicology and Applied Pharmacology 222, 235-242

Baldew GS, Vandenhamer CJA, Los G, Vermeulen NPE, Degoeij JJM, McVie JG (1989): Selenium-induced protection against cis-diamindichloroplatinum(II) nephrotoxicity in mice and rats. Cancer Research 49, 3020-3023

Bates MN, Smith AH, Hopenhaynrich C (1992): Arsenic Ingestion and internal cancers - a review. American Journal of Epidemiology 135, 462-476

Beak DG, Wilkin RT, Ford RG, Kelly SD (2008): Examination of arsenic speciation in sulfidic solutions using X-ray absorption spectroscopy. Environmental Science & Technology 42, 1643-1650

Biswas S, Talukder G, Sharma A (2000): Chromosome damage induced by selenium salts in human peripheral lymphocytes. Toxicology in Vitro 14, 405-408

Borek C, Ong A, Mason H, Donahue L, Biaglow JE (1986): Selenium and vitamin-e inhibit radiogenic and chemically-induced transformation invitro via different mechanisms. Proceedings of the National Academy of Sciences of the United States of America 83, 1490-1494

Bostick BC, Fendorf S, Brown GE (2005): In situ analysis of thioarsenite complexes in neutral to alkaline arsenic sulphide solutions. Mineralogical Magazine 69, 781-795

Bronzetti G, Cini M, Andreoli E, Caltavuturo L, Panunzio M, Della Croce C (2001): Protective effects of vitamins and selenium compounds in yeast. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 496, 105-115

Brozmanova J, Manikova D, Vlckova V, Chovanec M (2010): Selenium: a double-edged sword for defense and offence in cancer. Archives of Toxicology 84, 919-938

Burk RF (1990): Protection against free-radical injury by selenoenzymes. Pharmacology & Therapeutics 45, 383-385

Calatayud M, Gimeno J, Velez D, Devesa V, Montoro R (2010): Characterization of the Intestinal Absorption of Arsenate, Monomethylarsonic Acid, and Dimethylarsinic Acid Using the Caco-2 Cell Line. Chemical Research in Toxicology 23, 547-556

Calatayud M, Devesa V, Montoro R, Velez D (2011): In vitro study of intestinal transport of arsenite, monomethylarsonous acid, and dimethylarsinous acid by Caco-2 cell line. Toxicology Letters 204, 127-133

Camargo SMR, Francescato HDC, Lavrador MAS, Bianchi MLP (2001): Oral administration of sodium selenite minimizes cisplatin toxicity on proximal tubules of rats. Biological Trace Element Research 83, 251-262

Challenger F (1945): Biological methylation. Chemical Reviews 36, 315-361

Chowdhury UK, Zakharyan RA, Hemandez A, Avram MD, Kopplin MJ, Aposhian HV (2006): Glutathione-S-transferase-omega MMA(V) reductase knockout mice: Enzyme and arsenic species concentrations in tissues after arsenate administration. Toxicology and Applied Pharmacology 216, 446-457

Cohen SM, Arnold LL, Eldan M, Lewis AS, Beck BD (2006): Methylated arsenicals: The implications of metabolism and carcinogenicity studies in rodents to human risk assessment. Critical Reviews in Toxicology 36, 99-133

Combs GF, Gray WP (1998): Chemopreventive agents: Selenium. Pharmacology & Therapeutics 79, 179-192

Conrad M, Sato H (2012): The oxidative stress-inducible cystine/glutamate antiporter, system x(c)(-): cystine supplier and beyond. Amino Acids 42, 231-246

Cullen WR, Reimer KJ (1989): Arsenic speciation in the environment. Chemical Reviews 89, 713-764

Dennert G, Horneber M (2006): Selenium for alleviating the side effects of chemotherapy, radiotherapy and surgery in cancer patients. The Cochrane database of systematic reviews, CD005037

Dopp E, von Recklinghausen U, Hartmann LM, Stueckradt I, Pollok I, Rabieh S, Hao L, Nussler A, Katier C, Hirner AV, Rettenmeier AW (2008): Subcellular distribution of

inorganic and methylated arsenic compounds in human urothelial cells and human hepatocytes. Drug Metabolism and Disposition 36, 971-979

Eblin KE, Bowen ME, Cromey DW, Bredfeldt TG, Mash EA, Lau SS, Gandolfi AJ (2006): Arsenite and monomethylarsonous acid generate oxidative stress response in human bladder cell culture. Toxicology and Applied Pharmacology 217, 7-14

Hansen HR, Raab A, Jaspars M, Milne BF, Feldmann J (2004): Sulfur-containing arsenical mistaken for dimethylarsinous acid DMA(III) and identified as a natural metabolite in urine: Major implications for studies on arsenic metabolism and toxicity. Chemical Research in Toxicology 17, 1086-1091

Hansen JC (1988): Has selenium a beneficial role in human exposure to inorganic mercury. Medical Hypotheses 25, 45-53

Hayakawa T, Kobayashi Y, Cui X, Hirano S (2005): A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase Cyt19. Archives of Toxicology 79, 183-191

Helz GR, Tossell JA, Charnock JM, Pattrick RAD, Vaughan DJ, Garner CD (1995): Oligomerization in As(III) sulfide solutions - theoretical constraints and spectroscopic evidence. Geochimica Et Cosmochimica Acta 59, 4591-4604

Hidalgo IJ, Raub TJ, Borchardt RT (1989): Charaterization of the human colon carcinoma cell line (Caco-2) as a model system fo intestinal epithelial permeability. Gastroenterology 96, 736-749

Hinrichsen S, Lohmayer R, Zdrenka R, Dopp E, Planer-Friedrich B (2014): Effect of sulfide on the cytotoxicity of arsenite and arsenate in human hepatocytes (HepG2) and human urothelial cells (UROtsa). Environmental Science and Pollution Research 21, 10151-10162

Hinrichsen S, Geist F, Planer-Friedrich B (2015): Inorganic and Methylated Thioarsenates Pass the Gastrointestinal Barrier. Chemical Research in Toxicology

Hinrichsen S, Planer-Friedrich B (submitted): Cytotoxic activity of selenosulfate versus selenite in tumor cells depends on cell line and presence of amino acids. Environmental Science and Pollution Research

Hollibaugh JT, Carini S, Gurleyuk H, Jellison R, Joye SB, LeCleir G, Meile C, Vasquez L, Wallschlager D (2005): Arsenic speciation in Mono lake, California: Response to seasonal stratification and anoxia. Geochimica Et Cosmochimica Acta 69, 1925-1937

Hughes MF, Kenyon EM (1998): Dose-dependent effects on the disposition of monomethylarsonic acid and dimethylarsinic acid in the mouse after intravenous administration. Journal of Toxicology and Environmental Health-Part A 53, 95-112

Hughes MF, Devesa V, Adair BM, Conklin SD, Creed JT, Styblo M, Kenyon EM, Thomas DJ (2008): Tissue dosimetry, metabolism and excretion of pentavalent and trivalent dimethylated arsenic in mice after oral administration. Toxicology and Applied Pharmacology 227, 26-35

Irion CW (1999): Growing alliums and brassicas in selenium-enriched soils increases their anticarcinogenic potentials. Medical Hypotheses 53, 232-235

Johnen G, Rozynek P, von der Gathen Y, Bryk O, Zdrenka R, Johannes C, Weber DG, Igwilo-Okuefuna OB, Raiko I, Hippler J, Bruning T, Dopp E (2013): Cross-Contamination of a UROtsa Stock with T24 Cells - Molecular Comparison of Different Cell Lines and Stocks. Plos One 8, e64139-e64139

Jorgensen J, Mortensen PB (2001): Hydrogen sulfide and colonic epithelial metabolism - Implications for ulcerative colitis. Digestive Diseases and Sciences 46, 1722-1732

Kitchin KT (2001): Recent advances in arsenic carcinogenesis: Modes of action, animal model systems, and methylated arsenic metabolites. Toxicology and Applied Pharmacology 172, 249-261

Kubachka KM, Kohan MC, Conklin SD, Herbin-Davis K, Creed JT, Thomas DJ (2009): In vitro biotransformation of dimethylarsinic acid and trimethylarsine oxide by anaerobic microflora of mouse cecum analyzed by HPLC-ICP-MS and HPLC-ESI-MS. Journal of Analytical Atomic Spectrometry 24, 1062-1068

Laparra JM, Velez D, Barbera R, Montoro R, Farre R (2005): An approach to As(III) and As(V) bioavailability studies with Caco-2 cells. Toxicology in Vitro 19, 1071-1078

Le XC, Lu XF, Ma MS, Cullen WR, Aposhian HV, Zheng BS (2000): Speciation of key arsenic metabolic intermediates in human urine. Analytical Chemistry 72, 5172-5177

Leffers L, Ebert F, Taleshi MS, Francesconi KA, Schwerdtle T (2013a): In vitro toxicological characterization of two arsenosugars and their metabolites. Molecular Nutrition & Food Research 57, 1270-82

Leffers L, Wehe CA, Huwel S, Bartel M, Ebert F, Taleshi MS, Galla H-J, Karst U, Francesconi KA, Schwerdtle T (2013b): In vitro intestinal bioavailability of arsenosugar metabolites and presystemic metabolism of thio-dimethylarsinic acid in Caco-2 cells. Metallomics: integrated biometal science 5, 1031-42

Letavayova L, Vlasakova D, Spallholz JE, Bromanova J, Chovanec M (2008): Toxicity and mutagenicity of selenium compounds in Saccharomyces cerevisiae. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 638, 1-10

Li G-X, Hu H, Jiang C, Schuster T, Lu J (2007): Differential involvement of reactive oxygen species in apoptosis induced by two classes of selenium compounds in human prostate cancer cells. International Journal of Cancer 120, 2034-2043

Li J, Sun K, Ni L, Wang X, Wang D, Zhang J (2012): Sodium selenosulfate at an innocuous dose markedly prevents cisplatin-induced gastrointestinal toxicity. Toxicology and Applied Pharmacology 258, 376-383

Marafante E, Vahter M, Norin H, Envall J, Sandstrom M, Christakopoulos A, Ryhage R (1987): Biotransformation of dimethylarsinic acid in mouse, hamster and man. Journal of Applied Toxicology 7, 111-117

Markovic SD, Djacic DS, Cvetkovic DM, Obradovic AD, Zizic JB, Ognjanovic BI, Stajn AS (2011): Effects of Acute In vivo Cisplatin and Selenium Treatment on Hematological and Oxidative Stress Parameters in Red Blood Cells of Rats. Biological Trace Element Research 142, 660-670

Micke O, Schomburg L, Buentzel J, Kisters K, Muecke R (2009): Selenium in Oncology: From Chemistry to Clinics. Molecules 14, 3975-3988

Mounicou S, Shah M, Meija J, Caruso JA, Vonderheide AP, Shann J (2006): Localization and speciation of selenium and mercury in Brassica juncea - implications for Se-Hg antagonism. Journal of Analytical Atomic Spectrometry 21, 404-412

Naranmandura H, Ibata K, Suzuki KT (2007a): Toxicity of dimethylmonothioarsinic acid toward human epidermoid carcinoma A431 cells. Chemical Research in Toxicology 20, 1120-1125

Naranmandura H, Suzuki N, Iwata K, Hirano S, Suzuki KT (2007b): Arsenic metabolism and thioarsenicals in hamsters and rats. Chemical Research in Toxicology 20, 616-624

Naranmandura H, Carew MW, Xu S, Lee J, Leslie EM, Weinfeld M, Le XC (2011): Comparative Toxicity of Arsenic Metabolites in Human Bladder Cancer EJ-1 Cells. Chemical Research in Toxicology 24, 1586-1596

Noach ABJ, Kurosaki Y, Blomroosemalen MCM, Deboer AG, Breimer DD (1993): Cell-polarity dependent effect of chelation on the paracellular permeability of confluent Caco-2 cell monolayers. International Journal of Pharmaceutics 90, 229-237

Ochi T, Kita K, Suzuki T, Rumpler A, Goessler W, Francesconi KA (2008): Cytotoxic, genotoxic and cell-cycle disruptive effects of thio-dimethylarsinate in cultured human cells and the role of glutathione. Toxicology and Applied Pharmacology 228, 59-67

Olm E, Fernandes AP, Hebert C, Rundlof AK, Larsen EH, Danielsson O, Bjornstedt M (2009): Extracellular thiol-assisted selenium uptake dependent on the x(c)(-) cystine transporter explains the cancer-specific cytotoxicity of selenite. Proceedings of the National Academy of Sciences of the United States of America 106, 11400-11405

Park JS, Ryu JY, Jeon HK, Cho YJ, Park YA, Choi JJ, Lee JW, Kim BG, Bae DS (2012a): The effects of selenium on tumor growth in epithelial ovarian carcinoma. Journal of Gynecologic Oncology 23, 190-196

Park SH, Kim JH, Chi GY, Kim GY, Chang YC, Moon SK, Nam SW, Kim WJ, Yoo YH, Choi YH (2012b): Induction of apoptosis and autophagy by sodium selenite in A549 human lung carcinoma cells through generation of reactive oxygen species. Toxicology Letters 212, 252-261

Pejova B, Najdoski M, Grozdanov I, Dey SK (2000): Chemical bath deposition of nanocrystalline (111) textured Ag2Se thin films. Materials Letters 43, 269-273
Petrick JS, Jagadish B, Mash EA, Aposhian HV (2001): Monomethylarsonous acid (MMA(III)) and arsenite: LD50 in hamsters and in vitro inhibition of pyruvate dehydrogenase. Chemical Research in Toxicology 14, 651-656
Petrov PK, Charters JW, Wallschlaeger D (2012): Identification and Determination of Selenosulfate and Selenocyanate in Flue Gas Desulfurization Waters. Environmental Science & Technology 46, 1716-1723
Pinto M, Robineleon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simonassmann P, Haffen K, Fogh J, Zweibaum A (1983): Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biology of the Cell 47, 323-330
Pinyayev TS, Kohan MJ, Herbin-Davis K, Creed JT, Thomas DJ (2011): Preabsorptive Metabolism of Sodium Arsenate by Anaerobic Microbiota of Mouse Cecum Forms a Variety of Methylated and Thiolated Arsenicals. Chemical Research in Toxicology 24, 475-477
Planer-Friedrich B, London J, McCleskey RB, Nordstrom DK, Wallschlager D (2007): Thioarsenates in geothermal waters of yellowstone national park: Determination, preservation, and geochemical importance. Environmental Science & Technology 41, 5245-5251
Planer-Friedrich B, Franke D, Merkel B, Wallschlager D (2008): Acute toxicity of thioarsenates to Vibrio fischeri. Environmental Toxicology and Chemistry 27, 2027-2035
Planer-Friedrich B, Suess E, Scheinost AC, Wallschlaeger D (2010): Arsenic Speciation in Sulfidic Waters: Reconciling Contradictory Spectroscopic and Chromatographic Evidence. Analytical Chemistry 82, 10228-10235
Planer-Friedrich B, Härtig C, Lohmayer R, Suess E, McCann SH, Oremland RS (2015): Anaerobic chemolithotrophic growth of the haloalkaliphilic bacterium strain MLMS-1 by disproportionation of monothioarsenate. Environmental Science & Technology
Radabaugh TR, Aposhian HV (2000): Enzymatic reduction of arsenic compounds in mammalian systems: Reduction of arsenate to arsenite by human liver arsenate reductase. Chemical Research in Toxicology 13, 26-30
Rader KJ, Dombrowski PM, Farley KJ, Mahony JD, Di Toro DM (2004): Effect of thioarsenite formation on arsenic(III) toxicity. Environmental Toxicology and Chemistry 23, 1649-1654
Raevskaya AE, Stroyuk AL, Kuchmiy SY (2006): Preparation of colloidal CdSe and CdS/CdSe nanoparticles from sodium selenosulfate in aqueous polymers solutions. Journal of Colloid and Interface Science 302, 133-141
Ramakrishna BS (2007): The normal bacterial flora of the human intestine and its regulation. Journal of Clinical Gastroenterology 41, S2-S6
Raml R, Rumpler A, Goessler W, Vahter M, Li L, Ochi T, Francesconi KA (2007): Thio-dimethylarsinate is a common metabolite in urine samples from arsenic-exposed women in Bangladesh. Toxicology and Applied Pharmacology 222, 374-380
Rayman MP (2005): Selenium in cancer prevention: a review of the evidence and mechanism of action. Proceedings of the Nutrition Society 64, 527-542
Rehman K, Naranmandura H (2012): Arsenic metabolism and thioarsenicals. Metallomics 4, 881-892
Rochette EA, Bostick BC, Li GC, Fendorf S (2000): Kinetics of arsenate reduction by dissolved sulfide. Environmental Science & Technology 34, 4714-4720
Schwarz K, Foltz CM (1957): Selenium as an integral part of Factor-3 against dietary necrotic liver degeneration. Journal of the American Chemical Society 79, 3292-3293
Shamberg.Rj, Frost DV (1969): Possible protective effect of selenium against human cancer. Canadian Medical Association Journal 100, 682-&
Shen HM, Yang CF, Ong CN (1999): Sodium selenite-induced oxidative stress and apoptosis in human hepatoma HepG(2) cells. International Journal of Cancer 81, 820-828

Smedley PL, Kinniburgh DG (2002): A review of the source, behaviour and distribution of arsenic in natural waters. Applied Geochemistry 17, 517-568

Stauder S, Raue B, Sacher F (2005): Thioarsenates in sulfidic waters. Environmental Science & Technology 39, 5933-5939

Styblo M, Del Razo LM, Vega L, Germolec DR, LeCluyse EL, Hamilton GA, Reed W, Wang C, Cullen WR, Thomas DJ (2000): Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. Archives of Toxicology 74, 289-299

Styblo M, Drobna Z, Jaspers I, Lin S, Thomas DJ (2002): The role of biomethylation in toxicity and carcinogenicity of arsenic: A research update. Environmental Health Perspectives 110, 767-771

Tabacova S, Baird DD, Balabaeva L, Lolova D, Petrov I (1994): Placental arsenic and cadmium in relation to lipid peroxides and glutathione levels in maternal-infant pairs from a copper smelter area. Placenta 15, 873-881

Thomas DJ, Styblo M, Lin S (2001): The cellular metabolism and systemic toxicity of arsenic. Toxicology and Applied Pharmacology 176, 127-144

Vahter M (1999): Methylation of inorganic arsenic in different mammalian species and population groups. Science Progress 82, 69-88

Valdiglesias V, Pasaro E, Mendez J, Laffon B In vitro evaluation of selenium genotoxic, cytotoxic, and protective effects: a review. Archives of Toxicology 84, 337-351

Van de Wiele T, Gallawa CM, Kubachka KM, Creed JT, Basta N, Dayton EA, Whitacre S, Du Laing G, Bradham K (2010): Arsenic Metabolism by Human Gut Microbiota upon in Vitro Digestion of Contaminated Soils. Environmental Health Perspectives 118, 1004-1009

Vinceti M, Wei ET, Malagoli C, Bergomi M, Vivoli G (2001): Adverse health effects of selenium in humans. Reviews on environmental health 16, 233-51

Vonderheide AP, Mounicou S, Meija J, Henry HF, Caruso JA, Shann JR (2006): Investigation of selenium-containing root exudates of Brassica juncea using HPLC-ICP-MS and ESI-qTOF-MS. Analyst 131, 33-40

Wallenberg M, Misra S, Wasik AM, Marzano C, Bjornstedt M, Gandin V, Fernandes AP (2014): Selenium induces a multi- targeted cell death process in addition to ROS formation. Journal of Cellular and Molecular Medicine 18, 671-684

Wilkin RT, Wallschlager D, Ford RG (2003): Speciation of arsenic in sulfidic waters. Geochemical Transactions 4, 1-7

Wood SA, Tait CD, Janecky DR (2002): A Raman spectroscopic study of arsenite and thioarsenite species in aqueous solution at 25 degrees C. Geochemical Transactions 3, 31-39

Wycherly BJ, Moak MA, Christensen MJ (2004): High dietary intake of sodium selenite induces oxidative DNA damage in rat liver. Nutrition and Cancer-an International Journal 48, 78-83

Yamauchi H, Yamamura Y (1984): Metabolism and excretion of orally-administered dimethylarsinic acid in the hamster. Toxicology and Applied Pharmacology 74, 134-140

Yoshida K, Kuroda K, Zhou X, Inoue Y, Date Y, Wanibuchi H, Fukushima S, Endo G (2003): Urinary sulfur-containing metabolite produced by intestinal bacteria following oral administration of dimethylarsinic acid to rats. Chemical Research in Toxicology 16, 1124-1129

Zakharyan RA, Aposhian HV (1999): Arsenite methylation by methylvitamin B-12 and glutathione does not require an enzyme. Toxicology and Applied Pharmacology 154, 287-291

Zhang J, Lu H, Wang X (2008a): MS07116 sodium selenosulfate synthesis and demonstration of its in vitro cytotoxic activity against HepG2, caco2, and three kinds of leukemia cells. Biological Trace Element Research 125, 13-21

Zhang J (2010): Use of sodium selenosulfate for supplementing selenium and anhancing the therapeutic efficiacy of chemotherapy agents, and a rapid process for preparing sodium sulfate.

Zhang JZ, Peng DG, Lu HJ, Liu QL (2008b): Attenuating the toxicity of cisplatin by using selenosulfate with reduced risk of selenium toxicity as compared with selenite. Toxicology and Applied Pharmacology 226, 251-259

Zou Y, Niu P, Gong Z, Yang J, Yuan J, Wu T, Chen X (2007): Relationship between reactive oxygen species and sodium-selenite-induced DNA damage in HepG2 cells. Frontiers of medicine in China 1

CONTRIBUTION TO THE STUDIES INCLUDED IN THIS THESIS

Study 1: **Inorganic and methylated thioarsenates pass the gastrointestinal barrier**

Hinrichsen, S.	80 %	concepts, laboratory experiments, data interpretation, manuscript preparation
Geist, F.	10 %	assistance with laboratory experiments, comments on manuscript
Planer-Friedrich, B.	10 %	concepts, discussion of results, comments on manuscript

Study 2: **Effect of sulfide on the cytotoxicity of arsenite and arsenate in human hepatocytes (HepG2) and human urothelial cells (UROtsa)**

Hinrichsen, S.	80 %	concepts, laboratory experiments, data interpretation, manuscript preparation
Lohmayer, R.	5 %	discussion of results, comments on manuscript
Zdrenka, R.	5 %	discussion of results, comments on manuscript
Dopp, E.	5 %	provision of cell lines, discussion of results, comments on manuscript
Planer-Friedrich, B.	5 %	concepts, discussion of results, comments on manuscript

Study 3: **Cytotoxic activity of selenosulfate versus selenite in tumor cells depends on cell line and presence of amino acids**

Hinrichsen, S.	90 %	concepts, laboratory experiments, data interpretation, manuscript preparation
Planer-Friedrich, B.	10 %	concepts, discussion of results, comments on manuscript

APPENDIX- PUBLICATIONS

Study 1

Inorganic and methylated thioarsenates pass the gastrointestinal barrier. Hinrichsen S., Geist, F., Planer-Friedrich B. (2015), *Chemical Research in Toxicology,* DOI:10.1021/acs.chemrestox.5b00268

Study 2

Effect of sulfide on the cytotoxicity of arsenite and arsenate in human hepatocytes (HepG2) and human urothelial cells (UROtsa). Hinrichsen, S., Lohmayer, R., Zdrenka, R., Dopp, E., and Planer-Friedrich, B. (2014), *Environmental Science and Pollution Research* 21, 10151–10162, DOI: 10.1021/acs.chemrestox.5b00268

Study 3

Cytotoxic activity of selenosulfate versus selenite in tumor cells depends on cell line and presence of amino acids. Hinrichsen, S. and Planer-Friedrich, B., submitted to: *Environmental Science and Pollution Research*

Study 1:

Inorganic and methylated thioarsenates pass the gastrointestinal barrier

Sinikka Hinrichsen, Franziska Geist, Britta Planer-Friedrich

Chemical Research in Toxicology

Chemical Research in Toxicology

Rapid Report

pubs.acs.org/crt

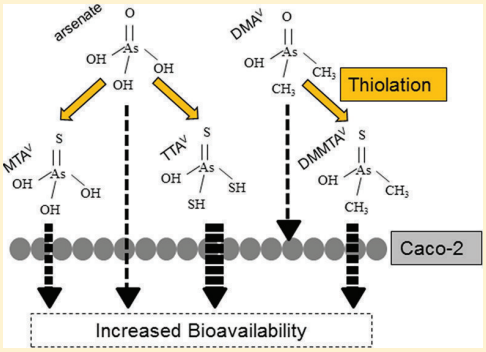
Inorganic and Methylated Thioarsenates Pass the Gastrointestinal Barrier

Sinikka Hinrichsen, Franziska Geist, and Britta Planer-Friedrich*

Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany

Supporting Information

ABSTRACT: Arsenic forms different species that are toxic for humans. Toxicity to internal organs is, however, only relevant if the respective species passes the gastrointestinal barrier. Thioarsenates were known to be produced by gut microbiota and to be toxic to bladder and liver cells, but their intestinal transport was largely unknown. Using a Caco-2 cell model, we show here that dimethylmonothioarsenate has the highest cellular retention and intestinal transport of all methylated species. Mono- and trithioarsenate show little cellular retention like arsenate, but their intestinal transport is much higher than that of arsenate; for trithioarsenate, it is almost as high as that for arsenite. The transport of all thioarsenates increases in the absence of phosphate. With the present study, we link previous reports of thioarsenate formation and toxicity by proving their bioavailability and confirm the relevance of their consideration in As risk assessments.



Arsenic (As) is a known carcinogen. Taken up mainly as arsenate, e.g., in drinking water or food, ingested As undergoes speciation changes which determine its bioavailability, systemic distribution, and toxicity. Commonly known metabolisms before absorption across the gastrointestinal barrier are reduction to arsenite or methylation. Thiolation (replacement of OH^- versus SH^- groups) is much less investigated, even though the prevalence of H_2S-producing organisms in gut microbiota and the high pH of the distal gastrointestinal tract favor thiolation. It is known that gut microbiota produce inorganic and methylated thioarsenates.[1−3] Especially dimethylmonothioarsenate (DMMTA^V, $(CH_3)_2As^VOS^-$) is a common metabolite in human urine, formed by thiolation of dimethylarsenate (DMA^V, $(CH_3)_2As^VO_2^-$).[1] For hepatocytes, DMMTA^V is 10 times more toxic than DMA^V.[4] For urothelial cells, it is 100 times more toxic than DMA^V and 4 times more toxic than arsenite.[5,6] Inorganic thioarsenates are less toxic than arsenite but more toxic than arsenate for human hepatocytes and urothelial cells.[7]

The missing link to evaluate the relevance of thiolation for human toxicity is the information whether and to what extent thioarsenates can pass the gastrointestinal barrier; tri- and pentavalent inorganic and methylated As species have been studied before; among the thiolated species, only DMMTA^V has been studied.[8−10] Here, we examined cellular retention and intestinal transport, speciation changes, and the importance of phosphate transporters on a Caco-2 cell monolayer model for the eight different As species: monothioarsenate (MTA^V, $As^VO_3S^{3-}$), trithioarsenate (TTA^V, $As^VOS_3^{3-}$), monomethylmonothioarsenate (MMMTA^V, $(CH_3)As^VO_2S^{2-}$), and DMMTA^V, in comparison to arsenate, arsenite, monomethylarsenate (MMA^V, $(CH_3)As^VO_3^{2-}$), and DMA^V. Supporting Information summarizes descriptions of cell culture, transport experiments, synthesis, and analysis of As species.

Total As analyses showed that cellular retention and passage through the cell monolayer varied among the tested inorganic or methylated, thiolated, or nonthiolated species (Figure 1). In accordance with previous literature, intestinal transport of arsenite clearly exceeded that of all other As species (Figure 1a,c).[9] Cellular As retention after arsenite exposure (Figure 1b) was higher than that after exposure to arsenate (reported before),[11] MMA^V, and DMA^V. Intestinal transport of arsenate (Figure 1a,c), MMA^V, and DMA^V (Figure 1d,f) was negligible as observed before.[8,12] Regarding the thiolated species, MMMTA^V was the species of least concern. Its intestinal transport was only slightly higher than that of its nonthiolated and less toxic original substrate MMA^V (Figure 1f), and cellular retention was even lower (Figure 1e).[6,11] For DMMTA^V, cellular retention (Figure 1e) and intestinal transport (Figure 1f) clearly exceeded those of the less toxic DMA^V and all other methylated As species. For inorganic thioarsenates, cellular uptake of MTA^V was comparable to that of arsenate (Figure 1a), but cellular retention was lower (Figure 1b) inducing higher intestinal transport of MTA^V (Figure 1c). Cellular retention of TTA^V was as low as that of arsenate (Figure 1b), but intestinal transport was the second highest among all tested As species (Figure 1c), and at 10 μM As incubation, it was even comparable to that of arsenite (Figure S2). In the absence of

Received: June 29, 2015

DOI: 10.1021/acs.chemrestox.5b00268
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

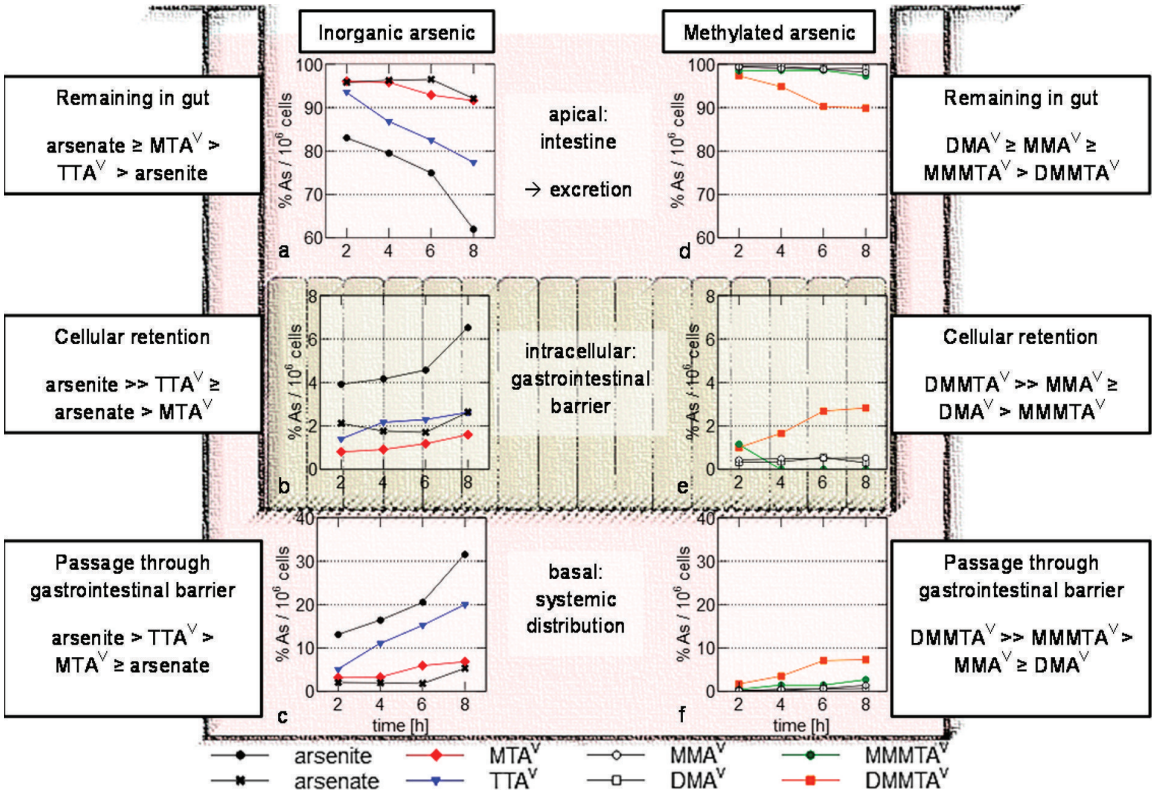


Figure 1. Total arsenic concentrations in apical (a,d), intracellular (b,e), and basal (c,f) compartments after individual exposure to inorganic and organic arsenic species. For better comparison, arsenic is presented in percent distribution between the three compartments for each species and corrected for the number of cells used in each experiment; Table S1 includes all individual values.

phosphate, cellular retention increased for all thioarsenates by a factor 2 to 6 (Table S1 and Figure S3), implying the importance of apical phosphate transporters for all thiolated species. Increased basal concentrations were only observed for the methylthiolated species (Figure S3).

Speciation analyses of As in the basal compartment confirmed that MTA^V and TTA^V were transported intact through the intestinal cells. It is not possible to answer whether this transport is quantitative, i.e., without any intracellular species transformation. Comparing relative species distributions between the apical and basal compartments, a higher share of arsenite was observed in the basal compartment (Figures S4 and S5, also for arsenate Figure S6). This could indicate that arsenate and thioarsenates were reduced intracellularly. However, all inorganic pentavalent standards contained arsenite in the applied apical solution either as small impurities in a commercial standard (<1.5% arsenite in arsenate) or as nonreacted remainders of synthesis (8% arsenite in MTA^V and 40−50% in TTA^V; see Supporting Information). Absolute concentrations of arsenite in the basal compartment were so low that the arsenite impurity in the apical compartment would be sufficient to explain the arsenite presence in the basal compartment, simply assuming preferred transport of arsenite over thioarsenates (Table S2). The thiomethylated standards contained no arsenite in the apical solution. Basal concentrations in our study were too low for identifying species above matrix interferences (see Supporting Information). However, at least DMMTA^V has already been detected in the basal compartment before.[10] The fact that thioarsenates survive gastrointestinal transport supports the need for evaluating their systemic distribution and toxicity to different organs as has been started before.[5,7] In addition, the intestinal transport of all tested As species might even be underestimated due to the strong tight junctions of Caco-2 cells compared to that of real cells of the small intestine.[13]

For a general evaluation on whether intestinal thiolation increases or decreases As bioavailability and toxicity, it is important to know the formation pathway of thioarsenates. If they form from their pentavalent structural analogues (arsenate for inorganic thioarsenates, MMA^V for MMMTA^V, and DMA^V for DMMTA^V), thiolation increases both intestinal transport (results from this study) and toxicity.[4,5,7] Some studies with mouse cecum microbiota seem to support direct thiolation of pentavalent species; no prereduction to arsenite was observed.[2] In contrast, previous abiotic studies showed that direct thiolation of inorganic and organic pentavalent As species only occurred at pH < 4, while at neutral pH, like in the human intestinal tract, thiolation of inorganic arsenates proceeded via trivalent As species.[14,15] This pathway is in line with observed presystemic microbial reduction of arsenate before thiolation and the suggestion that DMA^V reduction to DMA^{III} facilitates thiolation.[1,3] If reduced species are the original substrates for thioarsenates, thiolation means an overall decrease in toxicity. More studies are therefore required to elucidate intestinal formation mechanisms.

In summary, our study provides the link between previous reports on intestinal formation of thioarsenates and their toxicity by showing that inorganic and methylated thioarsenates can pass the gastrointestinal barrier. Compared to their pentavalent structural analogues, thioarsenates are more

DOI: 10.1021/acs.chemrestox.5b00268
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

bioavailable and more toxic. Considering that thioarsenates likely form from arsenite, thiolation is a detoxification metabolism. Trithioarsenate is an exception; it is almost as bioavailable and toxic as arsenite.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.5b00268.

Experimental procedures, analytical methods, synthesis of As standards, As content of apical, intracellular, and basal compartments during phosphate-free incubation (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +49 921 55 3999. Fax: +49 921 55 2334. E-mail: b.planer-friedrich@uni-bayreuth.de.

Funding

This project was funded by the German Research Foundation within an Emmy Noether program (grant # PL 302/3-1).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DMA^V, dimethylarsenate; $DMMTA^V$, dimethylmonothioarsenate; MMA^V, monomethylarsenate; $MMMTA^V$, monomethylmonothioarsenate; MTA^V, monothioarsenate; TTA^V, trithioarsenate

■ REFERENCES

(1) Kubachka, K. M., Kohan, M. C., Conklin, S. D., Herbin-Davis, K., Creed, J. T., and Thomas, D. J. (2009) In vitro biotransformation of dimethylarsinic acid and trimethylarsine oxide by anaerobic microflora of mouse cecum analyzed by HPLC-ICP-MS and HPLC-ESI-MS. *J. Anal. At. Spectrom. 24*, 1062−1068.

(2) Pinyayev, T. S., Kohan, M. J., Herbin-Davis, K., Creed, J. T., and Thomas, D. J. (2011) Preabsorptive Metabolism of Sodium Arsenate by Anaerobic Microbiota of Mouse Cecum Forms a Variety of Methylated and Thiolated Arsenicals. *Chem. Res. Toxicol. 24*, 475−477.

(3) Van de Wiele, T., Gallawa, C. M., Kubachka, K. M., Creed, J. T., Basta, N., Dayton, E. A., Whitacre, S., Du Laing, G., and Bradham, K. (2010) Arsenic Metabolism by Human Gut Microbiota upon in Vitro Digestion of Contaminated Soils. *Environ. Health Perspect. 118*, 1004−1009.

(4) Raml, R., Rumpler, A., Goessler, W., Vahter, M., Li, L., Ochi, T., and Francesconi, K. A. (2007) Thio-dimethylarsinate is a common metabolite in urine samples from arsenic-exposed women in Bangladesh. *Toxicol. Appl. Pharmacol. 222*, 374−380.

(5) Leffers, L., Ebert, F., Taleshi, M. S., Francesconi, K. A., and Schwerdtle, T. (2013) In vitro toxicological characterization of two arsenosugars and their metabolites. *Mol. Nutr. Food Res. 57*, 1270−1282.

(6) Naranmandura, H., Carew, M. W., Xu, S., Lee, J., Leslie, E. M., Weinfeld, M., and Le, X. C. (2011) Comparative Toxicity of Arsenic Metabolites in Human Bladder Cancer EJ-1 Cells. *Chem. Res. Toxicol. 24*, 1586−1596.

(7) Hinrichsen, S., Lohmayer, R., Zdrenka, R., Dopp, E., and Planer-Friedrich, B. (2014) Effect of sulfide on the cytotoxicity of arsenite and arsenate in human hepatocytes (HepG2) and human urothelial cells (UROtsa). *Environ. Sci. Pollut. Res. 21*, 10151−10162.

(8) Calatayud, M., Gimeno, J., Velez, D., Devesa, V., and Montoro, R. (2010) Characterization of the Intestinal Absorption of Arsenate, Monomethylarsonic Acid, and Dimethylarsinic Acid Using the Caco-2 Cell Line. *Chem. Res. Toxicol. 23*, 547−556.

(9) Calatayud, M., Devesa, V., Montoro, R., and Velez, D. (2011) In vitro study of intestinal transport of arsenite, monomethylarsonous acid, and dimethylarsinous acid by Caco-2 cell line. *Toxicol. Lett. 204*, 127−133.

(10) Leffers, L., Wehe, C. A., Huwel, S., Bartel, M., Ebert, F., Taleshi, M. S., Galla, H.-J., Karst, U., Francesconi, K. A., and Schwerdtle, T. (2013) In vitro intestinal bioavailability of arsenosugar metabolites and presystemic metabolism of thio-dimethylarsinic acid in Caco-2 cells. *Metallomics 5*, 1031−1042.

(11) Naranmandura, H., Ogra, Y., Iwata, K., Lee, J., Suzuki, K. T., Weinfeld, M., and Le, X. C. (2009) Evidence for toxicity differences between inorganic arsenite and thioarsenicals in human bladder cancer cells. *Toxicol. Appl. Pharmacol. 238*, 133−140.

(12) Naranmandura, H., Ibata, K., and Suzuki, K. T. (2007) Toxicity of dimethylmonothioarsinic acid toward human epidermoid carcinoma A431 cells. *Chem. Res. Toxicol. 20*, 1120−1125.

(13) Sun, H., Chow, E. C. Y., Liu, S., Du, Y., and Pang, K. S. (2008) The Caco-2 cell monolayer: usefulness and limitations. *Expert Opin. Drug Metab. Toxicol. 4*, 395−411.

(14) Planer-Friedrich, B., Härtig, C., Lohmayer, R., Suess, E., McCann, S. H., and Oremland, R. S. (2015) Anaerobic chemolithotrophic growth of the haloalkaliphilic bacterium strain MLMS-1 by disproportionation of monothioarsenate. *Environ. Sci. Technol. 49*, 6554−6563.

(15) Rochette, E. A., Bostick, B. C., Li, G. C., and Fendorf, S. (2000) Kinetics of arsenate reduction by dissolved sulfide. *Environ. Sci. Technol. 34*, 4714−4720.

DOI: 10.1021/acs.chemrestox.5b00268
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

Inorganic and methylated thioarsenates pass the gastrointestinal barrier

SINIKKA HINRICHSEN, FRANZISKA GEIST, BRITTA PLANER-FRIEDRICH

SUPPORTING INFORMATION

Materials and Methods

Transport through the Caco-2 monolayer was investigated and compared for the four different inorganic arsenic and arsenic-sulfur species arsenite, arsenate, monothioarsenate (MTA^V), and trithioarsenate (TTA^V), for the methylated As species monomethylarsenate (MMA^V) and dimethylarsenate (DMA^V) and for the thiomethylated As species monomethylmonothioarsenate ($MMMTA^V$) and dimethylmonothioarsenate ($DMMTA^V$). Transport of MTA^V, TTA^V, MMA^V, DMA^V, $MMMTA^V$, and $DMMTA^V$ was also investigated in complete absence of phosphate during the transport experiment.

Cell cultures

The Caco-2 cell line was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Cells were cultivated in minimum essential medium with Earle's Salts (MEM, c·c·pro, Oberdorla, Germany) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Karlsruhe, Germany), 0.5% gentamycine, 1% L-glutamine, 1% 1M HEPES, and 1% Amphotericin B (all c·c·pro, Oberdorla, Germany) and incubated in a humidified atmosphere at 37 °C and 5% CO_2 (Incubator Galaxy 170 S, New Brunswick Scientific). Cells were passaged three times weekly using 0.25% trypsin-EDTA (c·c·pro, Oberdorla, Germany).

Reagents and synthesis of thioarsenate standards

All reagents used were of the highest purity available, of at least analytical-reagent grade, and they were sterile-filtered before cell exposure. Sodium(meta)arsenite ($AsNaO_2$), sodium arsenate dibasic heptahydrate ($AsHNa_2O_4 \cdot 7\,H_2O$), and sodium cacodylate trihydrate ($C_2H_6AsNaO_2$, DMA^V) were

purchased from Sigma-Aldrich (Steinheim, Germany). Disodium methyl arsonate hexahydrate ($CH_3AsNa_2O_3 \cdot 6\ H_2O$, MMA^V) was purchased from Chem Service, West Chester, Pennsylvania.

MTA^V ($Na_3AsO_3S \cdot 7\ H_2O$) and tetrathioarsenate ($TetraTA^V$, $Na_3AsS_4 \cdot 8\ H_2O$) were synthesized based on a modification of previously published procedures with the exception that commercial arsenic pentasulfide (As_2S_5, Santa Cruz Biotechnology, Inc, Heidelberg, Germany) was used instead of self-synthesized material for $TetraTA^V$ synthesis.[1, 2] Based on speciation analysis by anion exchange chromatography coupled to an inductively coupled plasma mass spectrometry (AEC-ICP-MS), dissolving the synthesized MTA^V crystals in autoclaved water (Ampuwa; Fresenius Kabi, Bad Homburg, Germany) yielded more than 98% MTA^V. The purity of $TetraTA^V$ was greater than 90% when dissolved in 0.1 M NaOH and re-analyzed by AEC-ICP-MS. Dissolving $TetraTA^V$ in Ampuwa yielded more than 60% TTA^V (rest was mainly unreacted arsenite) due to the pH instability of $TetraTA^V$.[3] Consequently, in the experiments, cells were principally exposed to TTA^V as the $TetraTA^V$ standard was diluted in Ampuwa to ensure physiological pH.

$MMMTA^V$ and $DMMTA^V$ were synthesized under N_2/H_2 - atmosphere (Glovebox, Coy) based on a published procedure [4]: MMA^V (for $MMMTA^V$ synthesis) or DMA^V (for $DMMTA^V$ synthesis) were mixed with sodium sulfide ($Na_2S \cdot 9\ H_2O$) in a molar S:As ratio of 10. The pH value was adjusted to 3 by adding 0.1 M HCl. After a reaction time of 30 min, the pH value was increased to 12.3 by adding 1 M NaOH. In case of $MMMTA^V$ synthesis, almost complete thiolation of MMA^V was observed immediately after the reaction time. $MMMTA^V$ was synthesized one hour before the respective experiment. In case of $DMMTA^V$ synthesis, thiolation was slower. The standard was left for 7-10 days in the refrigerator to yield a maximum percentage of $DMMTA^V$ (50%; rest was unreacted DMA^V).

Transport of arsenic species through Caco-2 monolayer in apical-basal direction

A total of $7.5 \cdot 10^4$ cells in 2 mL growth medium (apical side) were seeded in 6-well Millicell® Hanging Cell Culture Inserts (Millipore, Schwalbach, Germany). The inserts were placed into 6-well plates (Omnilab, Munich, Germany) which contained 4.2 mL fresh growth medium (basal side). Medium was changed three times weekly followed by measurement of the transepithelial electrical resistance

(TEER) using the Millicell ERS-2 (Electrical Resistance System, Millipore, Schwalbach, Germany) to control the growing confluence of the Caco-2 monolayer. Transport experiments were conducted 21 days after seeding. Only cell layers with TEER values > 500 Ωcm^2 were used for the experiments as this value was assessed to indicate complete cell monolayer integrity. TEER values were measured immediately before starting the transport experiments and they were also monitored during the duration of incubation to ensure that cell monolayers were not damaged by As exposure (Figure S1). During the transport experiments, both apical and basal compartment contained 2 mL growth medium. Arsenic stock solutions were prepared in autoclaved water and were 100-fold diluted in growth medium for incubation resulting in final As concentrations of 1 and 10 µM for arsenite, arsenate, MTA^V, TTA^V and 2.7 µM for MMA^V, DMA^V, $MMMTA^V$, and $DMMTA^V$. Each treatment was conducted in triplicate. Sampling of medium was conducted after 2, 4, 6, and 8 h of incubation. Medium was removed from the apical and basal compartment, was flash-frozen and stored in liquid nitrogen until analysis of As speciation and total As content.

Arsenic retention during transport experiments

After the removal of apical and basal medium, the apical and the basal side of the cell monolayer each were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS, Gibco, Karlsruhe, Germany) to remove remaining As. The cells were trypsinized, resuspended in DPBS, counted (CASY Model TT, Roche Applied Sciences), and mechanically lysed using glass beads (Retsch GmbH, Germany).

Transport experiments in the absence of phosphate

To test whether the transport of MTA^V, TTA^V, MMA^V, DMA^V, $MMMTA^V$, and $DMMTA^V$ competes with the uptake of phosphate, transport experiments in the absence of phosphate were conducted. The As concentration added to the apical compartment was 10 µM for MTA^V and TTA^V and 2.7 µM for MMA^V, DMA^V, $MMMTA^V$, and $DMMTA^V$. For all experiments, phosphate-free MEM was used (c·c·pro, Oberdorla, Germany) and sodium dihydrogen phosphate dihydrate (Sigma-Aldrich, Steinheim, Germany) was added resulting in 10% of standard phosphate concentration. To let the cells adapt to reduced phosphate availability, they were at least passaged three times in phosphate-reduced growth medium before seeding them into the filter inlets. During the 21 day-postseeding time, cells

were also cultivated in phosphate-reduced growth medium. Morphology and amount of cells during cultivation in phosphate-reduced growth medium were not affected. For the transport experiment, phosphate-free growth medium was applied in both apical and basal compartment and sampling procedure corresponded to the experiments in standard growth medium with the exception that Ampuwa was used for rinsing the cell layer instead of DPBS.

Analysis of total As content

To quantify As cellular retention and transport in the apical-basal direction, total As concentrations of cell lysates, apical and basal medium were determined by ICP-MS (XSeries2, Thermo-Fisher). In case of As quantification in cell lysates, the results were corrected by an internal rhodium standard (rhodium standard in 5 % HCl, 1000 ppm, Ultra Scientific Analytical Solutions, No. Kingstown, RI, USA) to compensate for changes in sensitivity. Final rhodium concentration in each sample was 5 ppb. For better comparability, the percentage As concentrations in apical, intracellular, and basal compartments were always referred to a total of 10^6 cells.

Analysis of As speciation in apical and basal medium

To evaluate the stability of the As species used in the growth medium during the transport experiments and to identify changes of speciation during their transport through the Caco-2 monolayer, As species were analyzed using anion exchange chromatography (Dionex 3000) coupled to the ICP-MS (AEC-ICP-MS). For the analysis of inorganic As species, an anion suppressor was used before introducing the samples into the ICP-MS to reduce the salt load in the solutions. For the methylated As species no suppressor could be used because it also exchanges DMA^V. The instrument parameters for the separation of inorganic As species (Table S3) and methylated As species (Table S4) are listed below. Arsenic was determined as AsO^+ (m/z = 91) using the dynamic reaction cell (DRC) with O_2 as reaction gas as described before.[5] Arsenite and arsenate were quantified using standard solutions of the respective solid. Inorganic thioarsenates were quantified on the basis of the arsenate calibration curve. The validity of this approach was shown in a previous publication.[5]

The methylated thioarsenic species $MMMTA^V$ and $DMMTA^V$ could be separated nicely by chromatography when the standards were diluted in ultrapure water (Figure S7). When analyzing these

thiomethylated species in cell growth medium some chromatographic problems were encountered and, unfortunately, total As concentrations were too low to solve these matrix interferences completely by sufficient dilution in ultrapure water. In the apical solutions, the following problems occurred: For $MMMTA^V$, the MMA^V peak was not baseline-separated from $MMMTA^V$ and some As eluted in the dead volume (Figure S7 B). However, a significant transformation of $MMMTA^V$ to MMA^V would have been distinguishable and did not occur. We could also exclude transformation into arsenate or arsenite. In case of $DMMTA^V$, matrix interference in cell growth medium led to elution of both DMA^V and $DMMTA^V$ in the column's dead volume (Figure S7 C). We could thus not exclude transformation of $DMMTA^V$ into DMA^V, but we could exclude transformation into arsenite or arsenate, and significant amounts of $MMMTA^V$ and MMA^V. For the basal solutions, totals As concentrations were too low to yield good peaks above these matrix interferences. So, no speciation results are available for the basal solutions of the $MMMTA^V$ and $DMMTA^V$ experiments.

Table 1: Percentage arsenic distribution in apical, intracellular, and basal compartment [% As/10^6 cells] after incubation with the different As species; -*: sample was used for As speciation analysis; -**: lost sample; mean values and standard deviations were determined in case three samples for total arsenic analysis were available.

all values presented in %As/10^6 cells

	arsenite				arsenate				MTA^V				TTA^V			
	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
apical	85.8	79.5	72.9	64.2	95.6	96.5	96.8	93.8	95.1	-*	-*	-*	93.3	86.4	82.8	76.7
	81.1	79.8	76.7	60.2	96.6	96.5	96.6	95.2	96.6	95.8	91.8	91.8	92.6	85.7	81.7	79.1
	-*	-*	-*	-*	95.2	95.7	-*	93.8	96.4	95.8	93.8	-*	94.8	88.2	82.9	76.3
mean					**95.8**	**96.3**			**96.0**				**93.5**	**86.8**	**82.5**	**77.4**
SD					**0.7**	**0.5**			**0.8**				**1.1**	**1.3**	**0.7**	**1.5**
intracellular	3.4	3.9	5.6	6.4	1.6	1.7	1.5	4.4	0.8	0.9	1.1	-*	1.3	2.1	2.3	2.5
	3.5	4.0	3.8	6.2	1.6	1.7	1.5	1.7	0.8	0.9	1.3	1.6	1.6	2.2	2.3	2.7
	4.8	4.6	4.3	7.0	3.2	1.9	2.1	1.8	0.8	0.9	1.2	1.6	1.3	2.2	2.3	2.6
mean	**3.9**	**4.2**	**4.6**	**6.5**	**2.1**	**1.8**	**1.7**	**2.6**	**0.8**	**0.9**	**1.2**		**1.4**	**2.2**	**2.3**	**2.6**
SD	**0.8**	**0.3**	**0.9**	**0.4**	**0.9**	**0.1**	**0.3**	**1.6**	**0.0**	**0.0**	**0.1**		**0.1**	**0.1**	**0.0**	**0.1**
basal	10.8	16.6	21.5	29.5	2.8	1.8	1.7	7.5	4.1	-*	-*	7.1	5.4	11.5	14.9	20.8
	15.4	16.1	19.5	33.5	1.8	1.8	2.0	3.1	2.6	3.3	6.9	6.6	5.9	12.1	16.0	18.1
	-*	-*	-*	-*	1.6	2.3	-*	4.4	2.8	3.3	5.0	-*	3.9	9.6	14.8	21.1
mean					**2.1**	**2.0**		**5.0**	**3.2**				**5.1**	**11.1**	**15.2**	**20.0**
SD					**0.6**	**0.3**		**2.3**	**0.8**				**1.0**	**1.3**	**0.7**	**1.6**

	MMA^V				DMA^V				$MMMTA^V$				$DMMTA^V$			
	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
apical	99.4	98.8	98.7	97.8	99.5	99.4	98.8	98.9	99.4	98.7	98.6	97.2	-*	94.5	88.6	88.3
	-*	-*	-*	-*	99.5	-*	98.8	99.0	98.2	98.5	98.4	97.3	97.3	94.9	90.8	90.3
	-*	-*	98.9	98.4	-*	-*	-*	-*	-*	98.5	98.6	97.4	97.4	95.1	91.3	90.9
mean										**98.5**	**98.6**	**97.3**		**94.8**	**90.2**	**89.9**
SD										**0.1**	**0.1**	**0.1**		**0.3**	**1.4**	**1.3**
intracellular	0.4	0.5	0.6	0.5	0.3	0.4	0.7	0.3	1.1	n.d.	n.d.	n.d.	1.0	1.6	2.8	2.7
	0.5	0.5	0.5	0.6	0.3	-**	0.6	0.3	n.d.	n.d.	n.d.	n.d.	1.0	1.8	2.5	3.1
	0.4	0.5	0.5	0.5	0.3	0.4	0.5	0.3	n.d.	n.d.	n.d.	n.d.	1.0	1.6	2.7	2.8
	0.4	0.4	0.4	0.5	0.3	0.3	0.4	0.3								
mean	**0.4**	**0.5**	**0.5**	**0.5**	**0.3**	**0.3**	**0.5**	**0.3**					**1.0**	**1.7**	**2.7**	**2.8**
SD	**0.0**	**0.1**	**0.1**	**0.0**	**0.0**	**0.1**	**0.1**	**0.0**					**0.0**	**0.1**	**0.1**	**0.2**
basal	0.2	0.7	0.8	1.7	0.2	0.2	0.5	0.8	0.6	1.3	1.4	2.8	-*	3.9	8.5	9.0
	-*	-*	-*	-*	0.1	-*	0.6	0.7	0.7	1.5	1.6	2.7	1.7	3.3	6.7	6.6
	-*	-*	0.6	1.1	-*	-*	-*	-*	-*	1.5	1.4	2.6	1.7	3.3	6.0	6.3
mean										**1.5**	**1.4**	**2.7**		**3.5**	**7.1**	**7.3**
SD										**0.1**	**0.1**	**0.1**		**0.4**	**1.3**	**1.5**

all values presented in %As/10^6 cells

	10 µM MTA^V				10 µM MTA^V - phosphate				10 µM TTA^V				TTA^V - phosphate			
	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
apical	99.6	99.5	99.1	98.6	97.3	97.9	98.3	-*	-*	-*	95.2	94.6	97.6	93.6	90.2	98.5
	99.7	99.5	98.1	98.9	99.0	98.0	98.1	91.3	98.8	98.1	95.4	93.8	97.7	94.2	93.4	88.9
	99.7	99.5	-*	-*	-*	-*	-*	89.9	98.6	97.8	94.9	92.2	98.1	93.6	94.2	89.0
mean	**99.7**	**99.5**									**95.1**	**93.5**	**97.8**	**93.8**	**92.6**	**92.1**
SD	**0.1**	**0.0**									**0.2**	**1.2**	**0.3**	**0.4**	**2.1**	**5.5**
intracellular	0.1	0.1	0.1	0.1	0.4	0.9	0.7	1.1	0.2	0.1	0.3	0.2	1.2	2.1	1.5	1.5
	0.1	0.1	0.4	0.1	0.5	1.1	0.6	1.0	0.1	0.1	0.3	0.3	1.2	2.0	1.5	1.1
	0.1	0.0	0.1	0.1	0.5	1.2	0.7	1.0	0.1	0.1	0.3	0.3	0.9	1.8	1.3	1.1
mean	**0.1**	**0.1**	**0.2**	**0.1**	**0.5**	**1.1**	**0.7**	**1.1**	**0.1**	**0.1**	**0.3**	**0.3**	**1.1**	**2.0**	**1.4**	**1.2**
SD	**0.0**	**0.0**	**0.2**	**0.0**	**0.1**	**0.2**	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.2**	**0.2**	**0.1**	**0.2**
basal	0.3	0.4	0.8	1.2	2.3	1.2	1.0	-*	-*	-*	4.6	5.2	1.2	4.3	8.3	0.0
	0.2	0.4	1.5	0.9	0.4	1.0	1.3	7.7	1.0	1.8	4.4	5.9	1.1	3.8	5.1	10.0
	0.3	0.4	-*	-*	-*	-*	-*	9.1	1.3	2.1	4.8	7.5	1.0	4.6	4.5	9.9
mean	**0.3**	**0.4**									**4.6**	**6.2**	**1.1**	**4.2**	**6.0**	**6.6**
SD	**0.1**	**0.0**									**0.2**	**1.2**	**0.1**	**0.4**	**2.1**	**5.7**

	MMA^V - phosphate				DMA^V - phosphate				$MMMTA^V$ - phosphate				$DMMTA^V$ - phosphate			
	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
apical	-*	97.2	96.6	96.0	-*	98.3	98.3	97.4	-*	93.4	-*	88.9	94.6	88.8	82.8	79.3
	97.9	97.2	96.7	96.8	98.5	98.3	98.2	97.2	96.3	93.3	93.6	90.6	95.4	89.4	83.8	83.9
	97.9	99.2	97.1	96.6	98.5	98.8	98.2	97.3	96.2	93.2	94.1	90.9	95.1	89.9	83.7	83.6
mean		**97.9**	**96.8**	**96.5**		**98.5**	**98.2**	**97.3**		**93.3**		**90.1**	**95.0**	**89.3**	**83.4**	**82.2**
SD		**1.2**	**0.2**	**0.4**		**0.3**	**0.1**	**0.1**		**0.1**		**1.1**	**0.4**	**0.5**	**0.5**	**2.6**
intracellular	1.7	2.1	2.3	1.8	1.1	1.1	1.0	1.4	2.7	4.9	3.8	5.7	2.6	4.6	6.1	6.4
	1.7	2.1	2.2	1.8	1.1	1.2	1.0	1.4	2.9	4.6	3.7	5.2	2.4	5.0	6.1	5.1
	1.7	0.0	1.7	1.8	1.1	0.0	1.0	1.4	2.9	4.6	3.7	5.3	2.5	4.8	6.6	5.0
mean	**1.7**	**1.4**	**2.1**	**1.8**	**1.1**	**0.8**	**1.0**	**1.4**	**2.8**	**4.7**	**3.7**	**5.4**	**2.5**	**4.8**	**6.3**	**5.5**
SD	**0.0**	**1.2**	**0.3**	**0.0**	**0.0**	**0.7**	**0.0**	**0.0**	**0.1**	**0.2**	**0.1**	**0.2**	**0.1**	**0.2**	**0.3**	**0.8**
basal	-*	0.8	1.1	2.2	-*	0.6	0.7	1.2	-*	1.8	-*	5.4	2.8	6.6	11.1	14.3
	0.4	0.8	1.1	1.4	0.3	0.5	0.8	1.4	0.9	2.1	2.7	4.2	2.2	5.6	10.1	11.1
	0.5	0.8	1.2	1.6	0.3	1.2	0.8	1.4	0.9	2.2	2.2	3.8	2.4	5.4	9.8	11.4
mean		**0.8**	**1.1**	**1.7**		**0.8**	**0.7**	**1.3**		**2.0**		**4.5**	**2.5**	**5.9**	**10.3**	**12.3**
SD		**0.0**	**0.1**	**0.4**		**0.4**	**0.1**	**0.1**		**0.2**		**0.9**	**0.3**	**0.6**	**0.7**	**1.8**

Table S2: Total content of arsenic species [ng] that are present in apical and basal compartments after incubation with arsenate, MTA^V, and TTA^V

		1 µM arsenate				10 µM arsenate			
	time [h]	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**
apical [ng]	arsenite	0.9	1.8	1.2	1.1	6.8	5.6	5.6	7.4
	arsenate	160.8	169.2	173.8	167.5	1945.3	1527.6	1793.0	1904.3
basal [ng]	arsenite	1.7	0.8	3.4	2.6	1.4	1.6	20.3	1.2
	arsenate	1.8	0.7	6.4	8.7	5.3	3.6	86.9	70.1
		1 µM MTA^V				**10 µM MTA^V**			
	time [h]	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**
apical [ng]	arsenite	9.87	12.46	14.06	21.20	67.49	72.61	90.05	146.34
	arsenate	5.76	6.75	6.93	6.06	80.35	76.58	101.40	94.45
	MTA^V	168.61	180.58	193.55	196.17	2585.40	2314.22	2196.97	2258.08
basal [ng]	arsenite	0.44	0.82	1.80	0.99	0.66	1.37	4.72	5.43
	arsenate	0.22	0.36	0.35	0.09	0.20	0.32	1.22	0.48
	MTA^V	1.08	3.38	10.46	1.46	5.36	6.95	29.57	12.67
		1 µM TTA^V				**10 µM TTA^V**			
	time [h]	**2**	**4**	**6**	**8**	**2**	**4**	**6**	**8**
apical [ng]	arsenite	70.05	62.39	62.88	62.61	501.66	544.52	392.12	734.03
	arsenate	3.98	0.73	1.81	1.90	57.88	38.70	17.48	81.69
	MTA^V	1.58	1.63	1.69	1.43	15.19	21.84	11.05	28.65
	$DiTA^V$	32.76	22.68	25.83	22.54	119.65	115.53	158.68	164.75
	TTA^V	87.88	70.25	63.27	47.82	1079.18	1051.15	380.48	967.22
basal [ng]	arsenite	2.27	3.80	5.92	7.61	20.97	82.78	65.84	107.39
	arsenate	0.01	0.02	0.06	0.21	0.08	2.39	3.07	5.78
	MTA^V	0.01	0.01	0.05	0.01	0.15	0.96	0.15	0.80
	$DiTA^V$	0.10	0.02	0.05	0.00	1.70	13.91	1.07	6.08
	TTA^V	0.12	0.01	0.01	0.01	3.29	29.91	1.35	6.03

Table S3: Instrumental parameters for the separation of inorganic arsenic species by AEC

analytical column	IonPac AS-16 4-mm (Dionex)	
guard column	IonPac AG-16 4-mm (Dionex)	
eluent	0.1 M NaOH	
flow rate	1.2 mL/min	
gradient	[min]	[mM]
	0-7	20
	7-17	20 → 100
	17-25	100
	25-28	20
suppression	ASRS-Ultra 4-mm (Dionex), 30 mA current, 5 mL/min water (external mode)	

Table S4: Instrumental parameters for the separation of methylated arsenic species by AEC

analytical column	IonPac AS-16 4-mm (Dionex)	
guard column	IonPac AG-16 4-mm (Dionex)	
eluent	0.1 M NaOH	
flow rate	1.2 mL/min	
gradient	[min]	[mM]
	0-3	2.5
	3-5	2.5 → 20
	5-10	20
	10-20	20 → 100
	20-24	100 → 2.5
suppression	none	

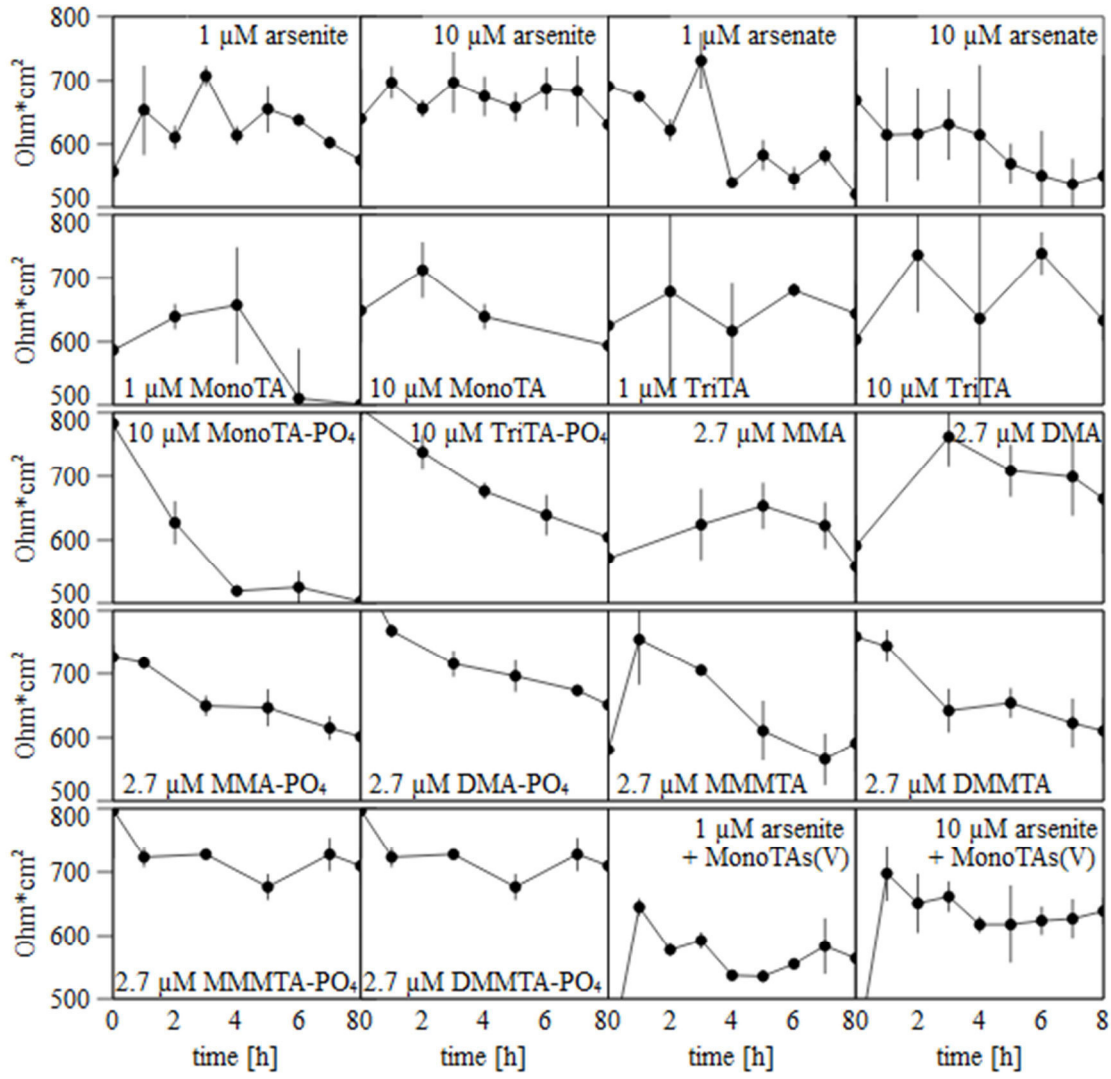


Figure S1: TEER values monitored during transport experiments (2-8 h). All TEER values were > 500 Ωcm^2 indicating cell monolayer integrity during arsenic exposure. Each treatment was conducted in triplicate; presented values are mean values ± standard deviation

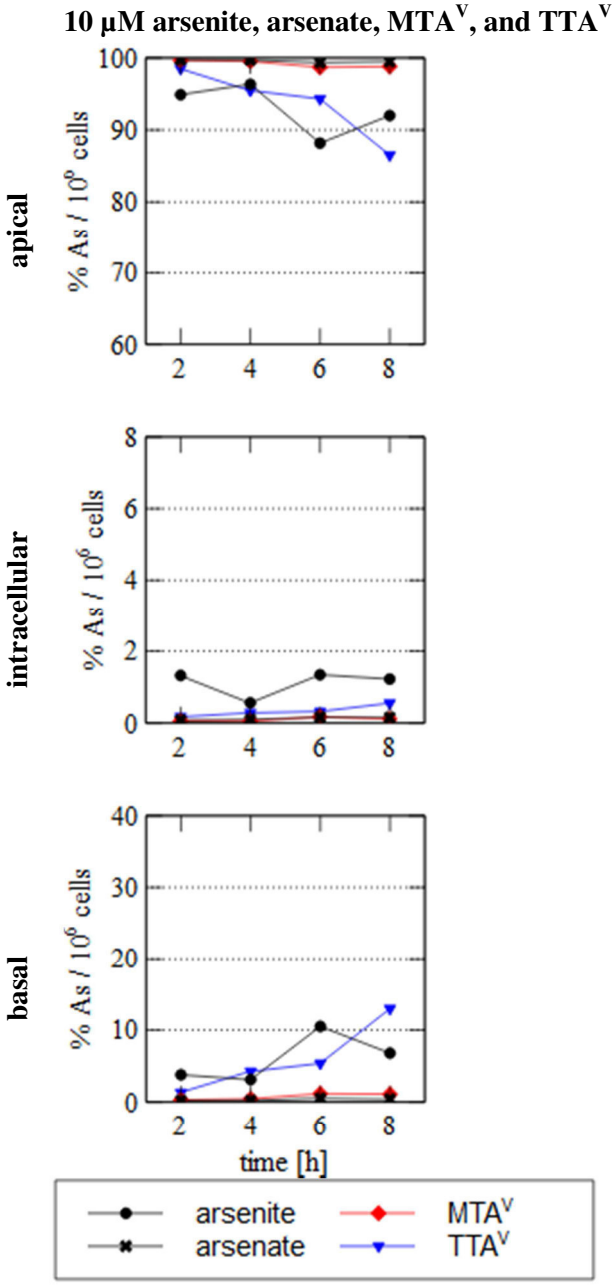


Figure S2: Apical, intracellular, and basal content of arsenic after incubation with 10 µM arsenite, arsenate, MTA^V, and TTA^V. Percentage share of arsenic in each compartment is referred to a number of 10^6 cells

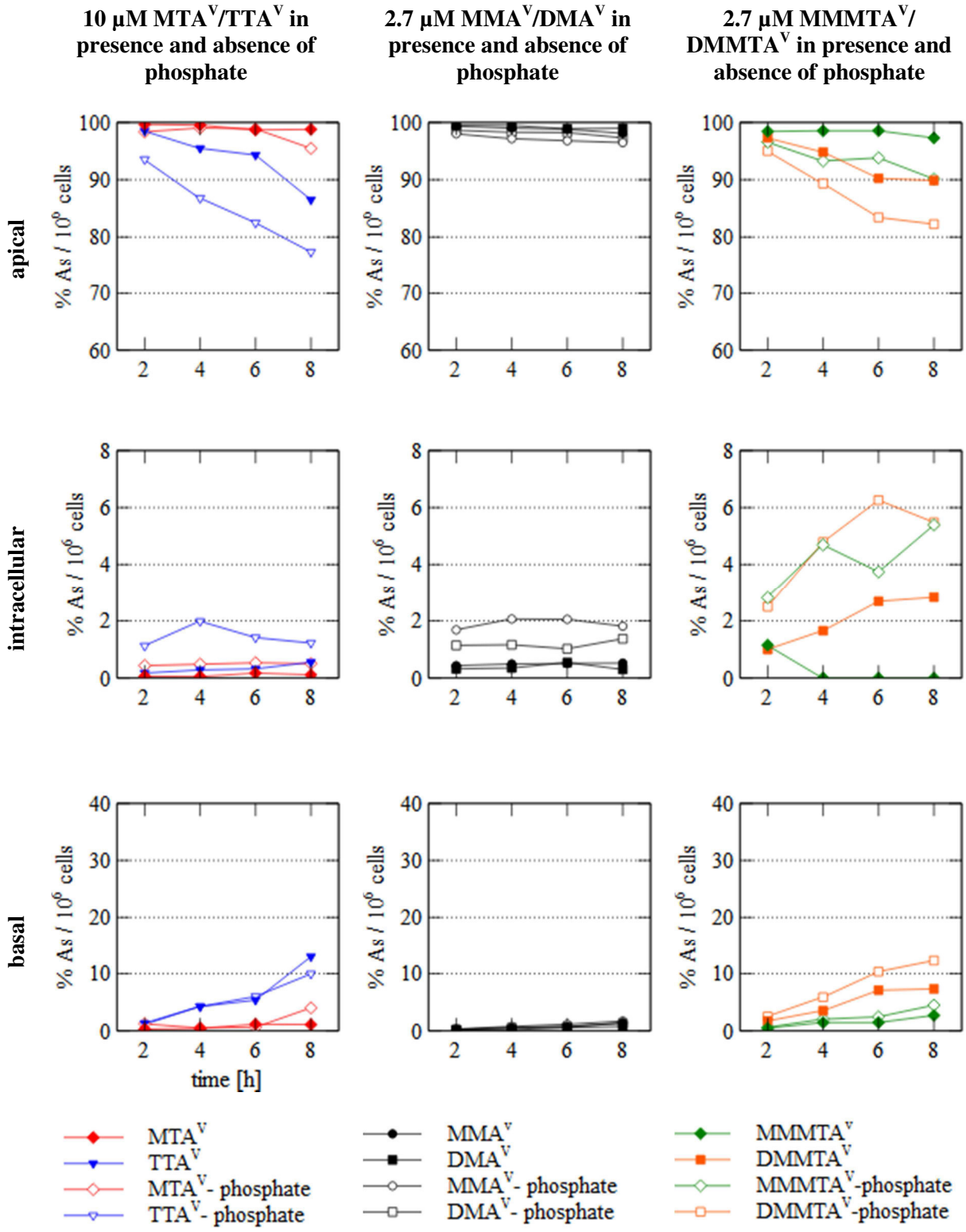


Figure S3: Apical, intracellular, and basal content of arsenic after incubation with 10 µM MTA^V and TTA^V and 2.7 µM MMA, DMA^V, $MMMTA^V$, and $DMMTA^V$. Transport of each arsenic species was quantified in the presence and in the absence of phosphate. Percentage share of arsenic in each compartment is referred to a number of 10^6 cells

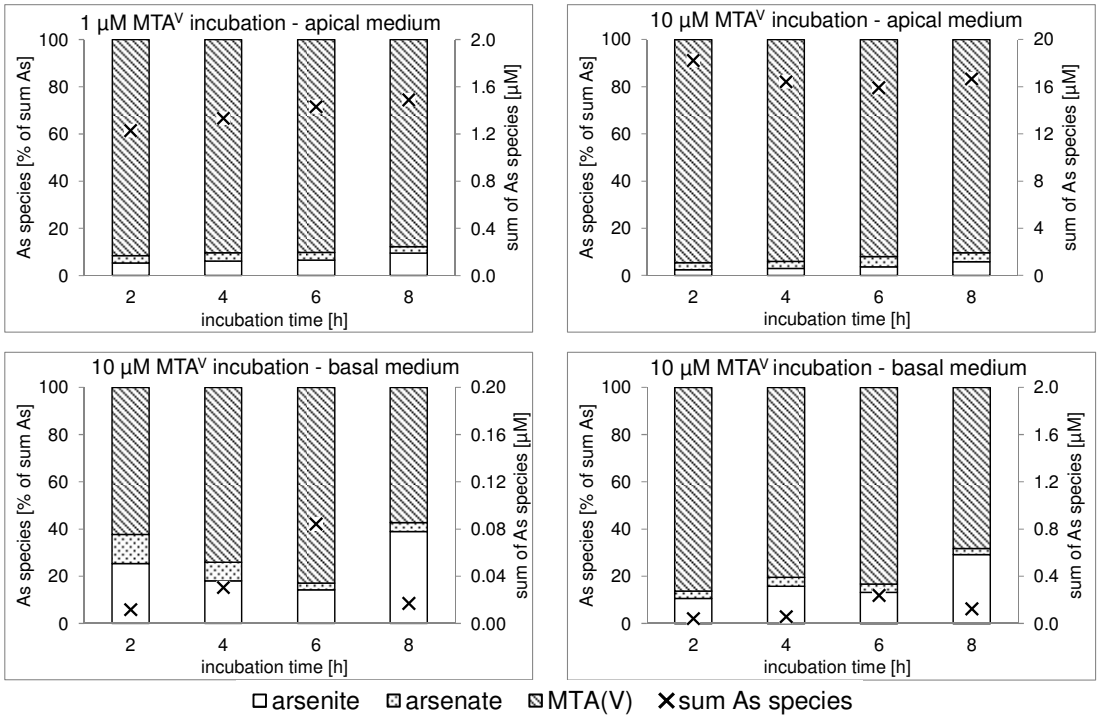


Figure S4: Arsenic speciation in apical and basal medium after incubation with 1 and 10 µM MTA^V; the crosses present the sum of arsenic species [µM]

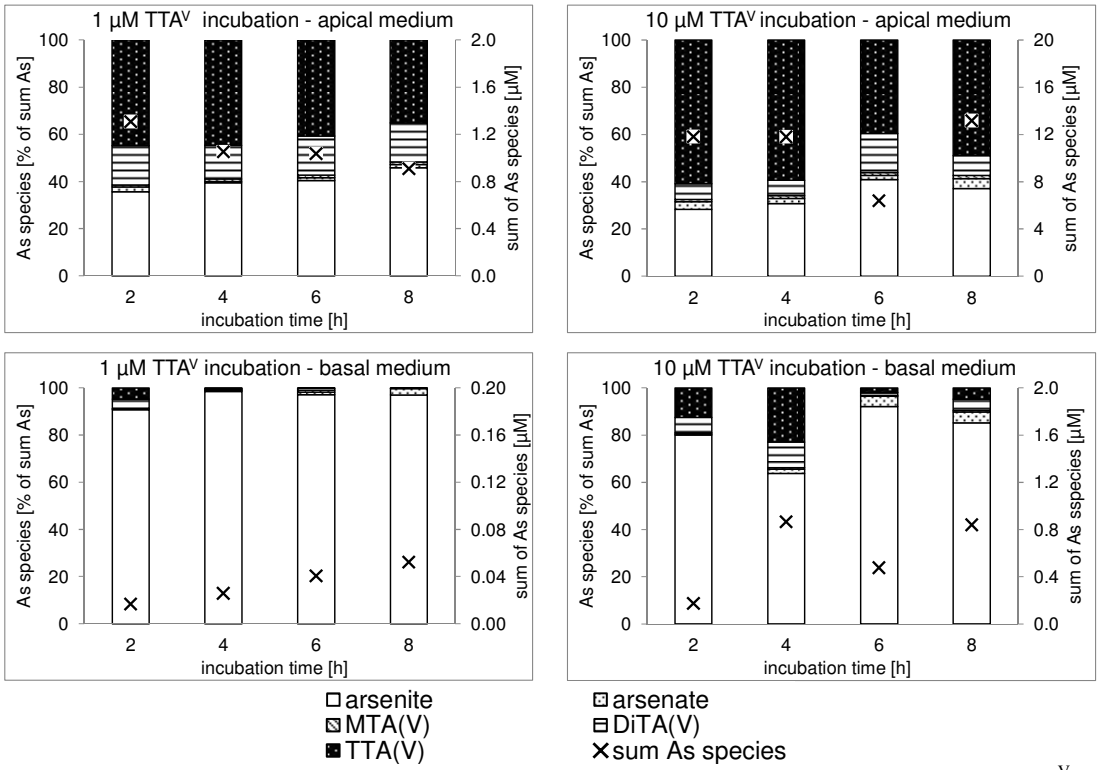


Figure S5: Arsenic speciation in apical and basal medium after incubation with 1 and 10 µM TTA^V; the crosses present the sum of arsenic species [µM]

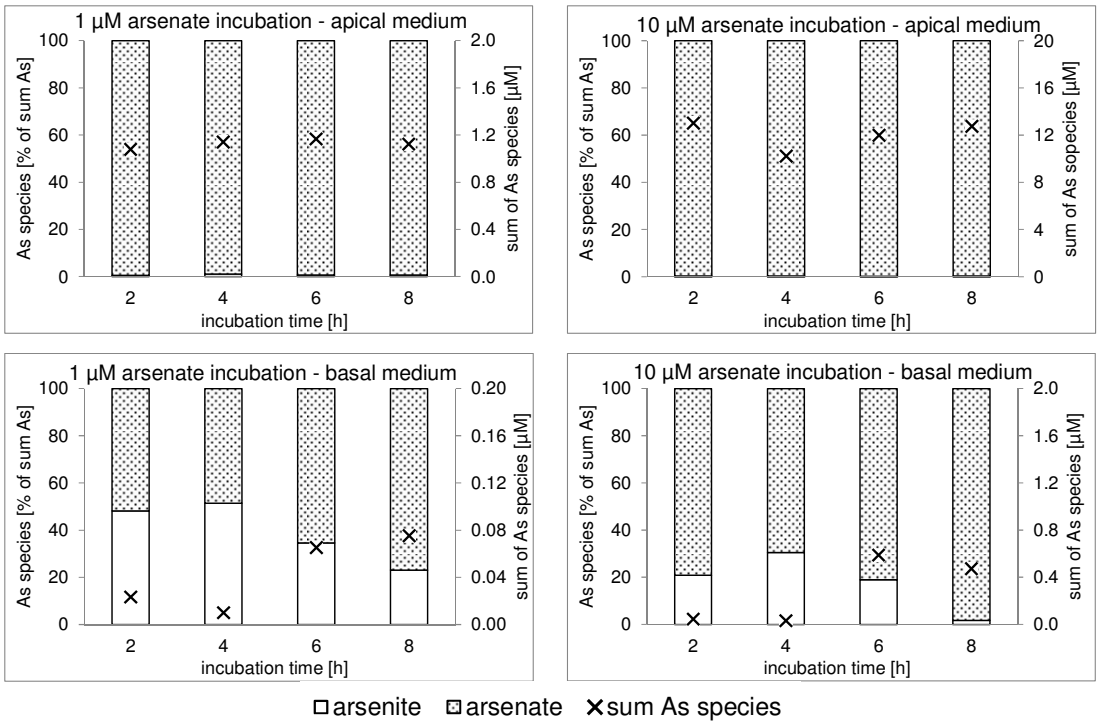


Figure S6: Arsenic speciation in apical and basal medium after incubation with 1 and 10 µM arsenate; the crosses present the sum of arsenic species [µM]

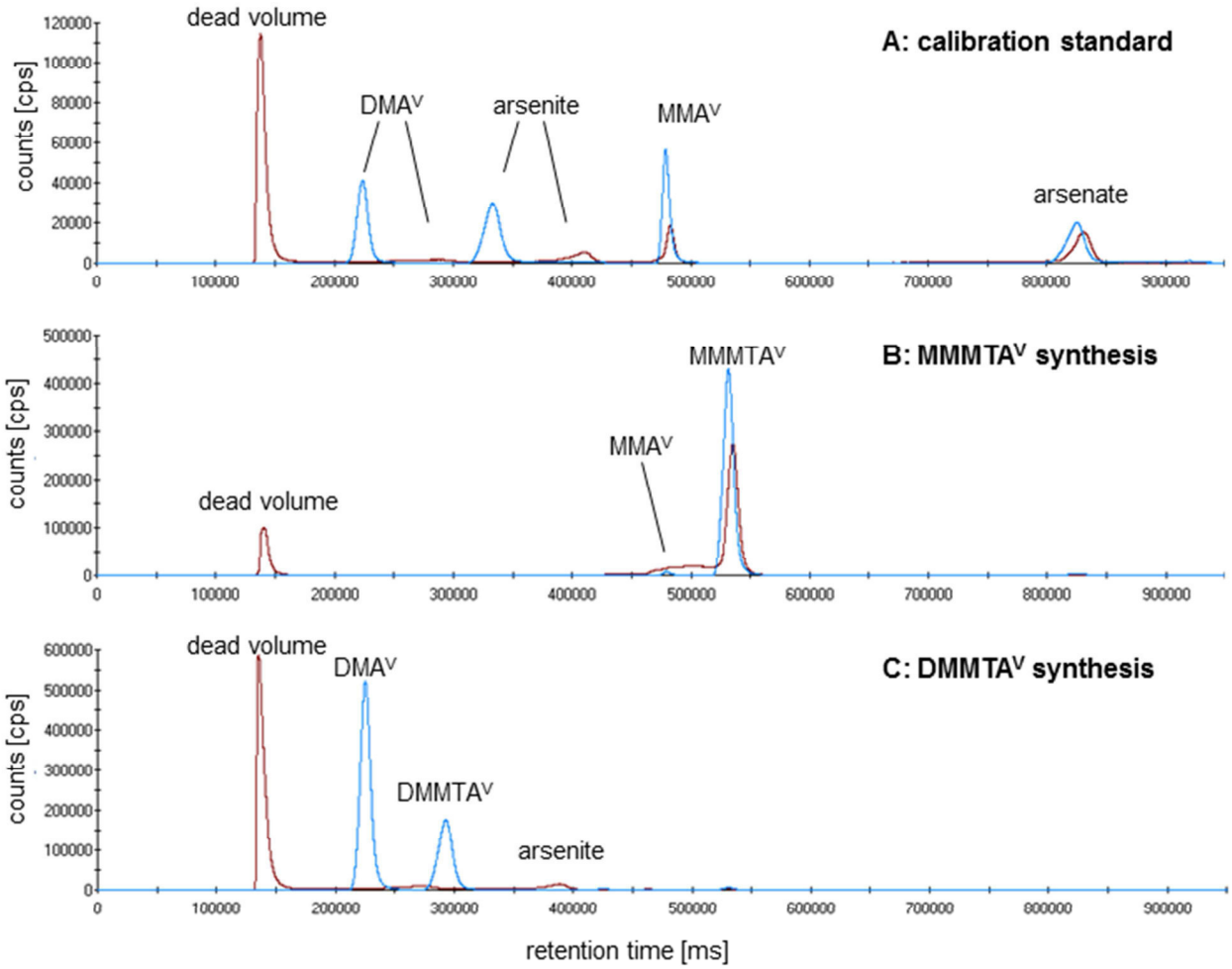


Figure S1: Separation of methylated arsenic species by AEC-ICP-MS. The blue lines represent standards diluted in ultrapure water, the red lines represent the respective standards diluted in cell growth medium. A: Calibration standard containing 10 µg/L of each arsenite, arsenate, MMA^V, and DMA^V; B: $MMMTA^V$ synthesis product with MMA^V as original substrate; C: $DMMTA^V$ synthesis product with DMA^V as original substrate

References

(1) Schwedt, G., and Rieckhoff, M. (1996) Separation of thio- and oxothioarsenates by capillary zone electrophoresis and ion chromatography. *J. Chromatogr. A 736*, 341-350.

(2) Suess, E., Scheinost, A. C., Bostick, B. C., Merkel, B. J., Wallschlaeger, D., and Planer-Friedrich, B. (2009) Discrimination of Thioarsenites and Thioarsenates by X-ray Absorption Spectroscopy. *Anal. Chem. 81*, 8318-8326.

(3) Planer-Friedrich, B., and Wallschlaeger, D. (2009) A Critical Investigation of Hydride Generation-Based Arsenic Speciation in Sulfidic Waters. *Environ. Sci. Technol. 43*, 5007-5013.

(4) Wallschlager, D., and London, J. (2008) Determination of methylated arsenic-sulfur compounds in groundwater. *Environ. Sci. Technol. 42*, 228-234.

(5) Planer-Friedrich, B., London, J., McCleskey, R. B., Nordstrom, D. K., and Wallschlager, D. (2007) Thioarsenates in geothermal waters of yellowstone national park: Determination, preservation, and geochemical importance. *Environ. Sci. Technol. 41*, 5245-5251.

Study 2:

Effect of sulfide on the cytotoxicity of arsenite and arsenate in human hepatocytes (HepG2) and human urothelial cells (UROtsa)

Sinikka Hinrichsen, Regina Lohmayer, Ricarda Zdrenka, Elke Dopp, Britta Planer-Friedrich

Environmental Science and Pollution Research 21, 10151–10162

Environ Sci Pollut Res (2014) 21:10151–10162
DOI 10.1007/s11356-014-2950-4

RESEARCH ARTICLE

Effect of sulfide on the cytotoxicity of arsenite and arsenate in human hepatocytes (HepG2) and human urothelial cells (UROtsa)

Sinikka Hinrichsen · Regina Lohmayer · Ricarda Zdrenka · Elke Dopp · Britta Planer-Friedrich

Received: 17 December 2013 / Accepted: 21 April 2014 / Published online: 1 May 2014

Abstract Arsenic, a common poison, is known to react with sulfide in vivo, forming thioarsenates. The acute toxicity of the inorganic thioarsenates is currently unknown. Our experiments showed that a fourfold sulfide excess reduced acute arsenite cytotoxicity in human hepatocytes (HepG2) and urothelial cells (UROtsa) significantly, but had little effect on arsenate toxicity. Speciation analysis showed immediate formation of thioarsenates (up to 73 % of total arsenic) in case of arsenite, but no speciation changes for arsenate. Testing acute toxicity of mono- and trithioarsenate individually, both thioarsenates were found to be more toxic than their structural analogue arsenate, but less toxic than arsenite. Toxicity increased with the number of thio groups. The amount of cellular arsenic uptake after 24 h corresponded to the order of toxicity of the four compounds tested. The dominant to almost exclusive intracellular arsenic species was arsenite. The results imply that thiolation is a detoxification process for arsenite in sulfidic milieus. The mechanism could either be that thioarsenates regulate the amount of free arsenite available for cellular uptake without entering the cells themselves, or, based on their chemical similarity to arsenate, they could be taken up by similar transporters and reduced rapidly intracellularly to arsenite.

Keywords Thioarsenates · Monothioarsenate · Trithioarsenate · UROtsa · HepG2 · Cytotoxicity

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (doi:10.1007/s11356-014-2950-4) contains supplementary material, which is available to authorized users.

S. Hinrichsen · R. Lohmayer · B. Planer-Friedrich (✉)
Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany
e-mail: b.planer-friedrich@uni-bayreuth.de

R. Zdrenka · E. Dopp
Institute of Hygiene and Occupational Medicine, University Hospital Essen, Essen, Germany

Introduction

Thioarsenates ($As^{V}S_nO_{4-n}^{3-}$ with n=1–4) are a group of inorganic arsenic species which have been known since the early nineteenth century to form in synthetic solutions of arsenite and sulfide, but have only recently been discovered to dominate in natural sulfidic environments. A number of studies have appeared about their analysis (Planer-Friedrich et al. 2010; Suess et al. 2009; Beak et al. 2008), stability in the absence (Planer-Friedrich et al. 2007; Stauder et al. 2005; Schwedt and Rieckhoff 1996) and presence of iron (Suess and Planer-Friedrich 2012; Suess et al. 2011; Burton et al. 2013; Johnston et al. 2012), and abiotic and microbial transformations (Härtig and Planer-Friedrich 2012; Planer-Friedrich et al. 2009). Still little is known about the toxicity of these species. The first to show that addition of sulfide decreases arsenite toxicity was Rader et al. (2004) using a simple test system with the marine bioluminescent bacteria *Vibrio fischeri*. Based on existing thermodynamic constants (Wilkin et al. 2003), Rader et al. (2004) predicted that thioarsenites ($As^{III}S_nO_{3-n}^{3-}$ with n=1–3) should form and cause the decrease in arsenite toxicity. Unfortunately, the thermodynamic constants used for the model turned out to be wrong (Wallschläger and Stadey 2007; Planer-Friedrich et al. 2010). The experiment was repeated later and the species were identified correctly as thioarsenates (Planer-Friedrich et al. 2008). Furthermore, the acute toxicity of mono-, di-,

and trithioarsenate (MonoTAs(V), DiTAs(V), and TriTAs(V)) for *V. fischeri* was tested with synthesized standards. While MonoTAs(V) and DiTAs(V) were in fact found to be less toxic than arsenite, TriTAs(V) turned out to be as toxic as arsenite.

For humans, direct uptake of thioarsenates is unlikely, since such sulfide-rich waters are generally non-potable. However, the presence of free sulfide in concentrations up to 60 μmol/L in the human gut lumen (Jorgensen and Mortensen 2001) can lead to thioarsenate formation in vivo after uptake of arsenite. The detection of methylated thioarsenates in urine samples of rodents (Naranmandura et al. 2007b), but also humans (Raml et al. 2007) that were exposed to arsenic, confirms an in vivo methylation/thiolation process. Another study showed that arsenate in a mouse cecum was microbially converted into methylated (thio)arsenicals (monomethylarsenate (MMA(V)), monomethyl-dithioarsenate (MMDTA(V)), dimethyldithioarsenate (DMDTA(V)), monomethyl-trithioarsenate (MMTTA(V))), but also the inorganic thioarsenates MonoTAs(V), DiTAs(V), and TriTAs(V) were found (Pinyayev et al. 2011). For human fecal microbiota, thiolation of arsenate to monomethylmonothioarsenate (MMMTA(V)) (25 % of bioaccessible arsenic) and MonoTAs(V) (in the absence of glutathione (GSH) and methylcobalamin as methylating agent) was demonstrated (Van de Wiele et al. 2010). These evidences for the occurrence of methylated and inorganic thioarsenates in vivo emphasize that thioarsenate toxicity must be studied in systems which are better suitable for predicting human toxicity than the *V. fischeri* bioluminescence tests.

Some studies do exist about methylated thioarsenicals. In MTT assays (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) with human epidermoid carcinoma cells (A431), dimethylmonothioarsenate (DMMTA(V)) was shown to exhibit slightly lower acute cytotoxicity (IC_{50} 5.7 μM) than arsenite (IC_{50} 10.7 μM) (Naranmandura et al. 2007a). Interestingly, a further study with human bladder cells (EJ-1) showed an opposite effect (IC_{50} 16.7 and 112 μM) with the additional finding of generation of reactive oxygen species (ROS) in DMMTA(V)-treated cells, which was not observed in arsenite-treated cells (Naranmandura et al. 2009). The number of thio groups was not associated to the toxicity of the methylated thioarsenicals as shown in a study with human hepatocytes (HepG2). DMMTA(V) was the most toxic compound (IC_{50} 0.026 mM), followed by dimethylarsenate (DMA(V)) (IC_{50} 0.343 mM) and DMDTA(V) (IC_{50} 3.66 mM) (Ochi et al. 2008). In another study, the toxicity of arsenic metabolites that were detected in urine was tested on EJ-1 cells and resulted in the following order of toxicity: dimethylarsenite (DMA(III)), DMMTA(V)>arsenite>arsenate>MMMTA(V)>MMA(V)>DMA(V)>DMDTA(V) (Naranmandura et al. 2011). Different intracellular mechanisms were detected when cells were exposed to DMMTA(V) and arsenite. DMMTA(V) exposure led to reduced expression of the proteins p21 and p53, increased DNA damage, reduced GSH level, and highly reactive oxygen species (hROS), indicating oxidative cell death. In contrast, arsenite exposure resulted in increased expression of p21 and p53, but no increase of GSH level, and no hROS generation. Whether thiolation increases or decreases arsenic toxicity cannot clearly be assessed, yet, but the existing studies already demonstrate different cellular mechanisms when cells are exposed to arsenite or methylated thioarsenicals.

No cell-based toxicity studies exist, yet, for inorganic thioarsenicals, which was our motivation to investigate the influence of sulfide on arsenite toxicity for HepG2 and urothelial cells (UROtsa). We added sulfide to an arsenite solution in a molar As/S ratio of 1:4 and analyzed arsenic speciation in cell growth medium after 6 and 24 h of exposure, respectively, to determine the occurrence of inorganic thioarsenates. The cytotoxicity of standard solutions of Mono- and TriTAs(V) was measured and compared to the cytotoxicity of their structural analogue arsenate and to arsenite to determine whether the formation of thioarsenates from arsenite and sulfide is a process of detoxification. To evaluate the influence of uptake on cytotoxicity, cellular uptake of arsenite, arsenate, MonoTAs(V), and TriTAs(V) was quantified and compared to the order of toxicity of the four compounds.

Materials and methods

Cell cultures

HepG2 and UROtsa cells were kindly provided by Prof. Dr. E. Dopp (Institute of Hygiene and Occupational Medicine, University Hospital Essen, Germany). UROtsa cells were cultivated in minimum essential medium with Earle's Salts (MEM, c·c·pro, Oberdorla, Germany) supplemented with 10 % Fetal Bovine Serum (FBS, Gibco), 0.5 % gentamycin (c·c·pro, Oberdorla, Germany), and 1 % L-glutamine (c·c·pro, Oberdorla, Germany). HepG2 cells were grown in MEM supplemented with 10 % FBS, 0.5 % gentamycin, 1 % L-glutamine, 1 % non-essential amino acids, and 1 % sodium pyruvate (all c·c·pro, Oberdorla, Germany). HepG2 and UROtsa cells were incubated in a humidified atmosphere at 37 °C and 5 % CO_2 (Incubator Galaxy 170 S, New Brunswick Scientific). Trypsin and trypsin–EDTA (0.25 %) were purchased from c·c·pro (Oberdorla, Germany). As a result of a recent research project, Johnen et al. (2013) have shown that the UROtsa cells used in this study are in fact T24 cells. The used cell line was intensively investigated and characterized by these authors.

Reagents

All reagents used were of the highest purity available, of at least analytical-reagent grade, and they were sterile-filtered before cell exposure. Sodium(meta)arsenite ($AsNaO_2$), sodium arsenate dibasic heptahydrate ($AsHNa_2O_4 \cdot 7H_2O$), and sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$) were purchased from Sigma-Aldrich (Steinheim, Germany). MonoTAs(V) ($Na_3AsO_3S \cdot 7H_2O$) and tetrathioarsenate (TetraTAs(V), $Na_3AsS_4 \cdot 8H_2O$) were synthesized based on a modification of previously published procedures (Schwedt and Rieckhoff 1996; Suess et al. 2009) with the exception that commercial arsenic pentasulfide (As_2S_5, Santa Cruz Biotechnology, Inc, Heidelberg, Germany) was used instead of self-synthesized material for TetraTAs(V) synthesis. Based on speciation analysis by anion exchange chromatography coupled to an inductively coupled plasma mass spectrometry (AEC-ICP-MS), dissolving the synthesized MonoTAs(V) crystals in Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO) yielded more than 98 % MonoTAs(V). Dithioarsenate (DiTAs(V)) could not be tested because it currently cannot be synthesized as solid or liquid standard in sufficient (at least >50 %) purity. The purity of TetraTAs(V) was greater than 90 % when dissolved in 0.1 M NaOH and re-analyzed by AEC-ICP-MS. Dissolving TetraTAs(V) in DPBS yielded more than 60 % TriTAs(V) but also 30 % arsenite due to the pH instability of TetraTAs(V) (Planer-Friedrich and Wallschlaeger 2009). Consequently, in the cytotoxicity assays, cells were principally exposed to TriTAs(V) as the TetraTAs(V) standard was diluted in DPBS to ensure physiological pH. For washing the cells prior to lysis, 2,3-dimercapto-1-propanesulfonic acid sodium salt monohydrate (DMPS, Alfa Aesar, Karlsruhe, Germany, purity 95 %), DPBS, and autoclaved water (Ampuwa; Fresenius Kabi, Bad Homburg, Germany) were used. Caution: The following chemicals are human carcinogens and should be handled with care: sodium(meta)arsenite, sodium arsenate dibasic heptahydrate, and arsenic pentasulfide.

MTT cytotoxicity assay

In vitro acute cytotoxicity of the arsenicals was determined using the MTT assay. The standard concentration range for all arsenic species was 1–1,000 μM. In case IC_{50} was not reached at 1,000 μM, concentrations of 5,000 and 10,000 μM were tested. The cells were seeded at a density of 5,000 cells per well into 96 well plates (Falcon, Becton Dickinson, Meylan Cedex, France), allowed to attach for 24 h, and subsequently treated with the respective concentration of the desired arsenical in fresh growth medium. For each experiment, the arsenic solutions were prepared freshly and diluted in DPBS. All experiments were at least conducted in triplicate with three equally treated wells per replicate. After the respective exposure duration, the medium was removed and the cells were incubated for 2 h with 100 μL fresh medium and 10 μL MTT (Sigma-Aldrich, USA). The formed formazan crystals were dissolved in solubilization solution, consisting of 10 g sodium dodecyl sulfate (SDS; Sigma-Aldrich, Steinheim, Germany) dissolved in 99.4 mL dimethyl sulfoxide (DMSO; Sigma-Aldrich, Steinheim, Germany) and 0.6 mL acetic acid ($C_2H_4O_2$; VWR PROLAB, Briare, France). The 96-well plate was spectrophotometrically measured at 570 nm with a reference wavelength of 630 nm (Infinite 200 PRO, TECAN). Absorbance measured in this test was finally correlated to percent cell viability.

$$\mathrm{CV}[\%] = 100 \times \frac{\text{number of living cells}}{\text{total number of cells}} \quad (1)$$

Determination of IC_{50}

Instead of the commonly applied procedure of estimating an IC_{50} value by interpolating between measured data points, we developed an independent statistical approach to obtain reproducible means for an objective IC_{50} calculation.

The mean values of the calculated cell viability (Eq. 1), including the standard deviation, were plotted as a function of the logarithmic arsenic concentration of the respective species. Since the decrease of cell viability with increasing concentrations has the form of a smoothed-out step function in these plots, the following function

$$f(c) = 50 \times erfc\left(\gamma \lg\left(\frac{c}{\mathrm{IC}_{50}}\right)\right) \quad (2)$$

was fitted to the data points, where c is the concentration, γ and IC_{50} are fit parameters, and the function $erfc(x)$ denotes the complementary error function. As the values of this function range from 0 to 2, the complementary error function was multiplied by 50 to ensure that the values of $f(c)$ were between 0 and 100 (Eq. 2). The parameter γ determines the steepness of the function $f(c)$ in the vicinity of IC_{50}, which represents the concentration at which 50 % of the cells die. Figure 1 shows the fitting on the basis of an MTT assay with HepG2 cells that were exposed to 0.1–5000 μM arsenite for 24 h. The two parameters γ and IC_{50} were determined using the Solver function in Microsoft Excel. Therefore, the squares of the deviations between the experimentally determined cell viability and the corresponding values of $f(c)$ were calculated and weighted by the variance of the experimental values. Subsequently, the sum of these single values was minimized by varying the fit parameters γ and IC_{50}. It should be noted that

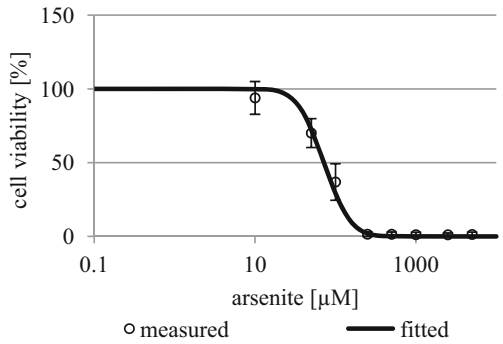


Fig. 1 Cell viability of HepG2 after 24 h exposure to arsenite (10–5,000 μM). The *circles* show the measured results of the MTT assay, the *continuous line* shows the cell viability curve fitted with the modified complementary error function (cf. Eq. (2))

the fitting only served for the determination of IC_{50} values. Diagrams that represent the results of the MTT assays only include the measured values.

Mechanical lysis of the cells

To determine arsenic uptake into the cells, 5×10^6 cells were seeded 24 h before exposure to arsenite, arsenate, MonoTAs(V), or TriTAs(V) at concentrations of 1 to 75 μM in fresh growth medium. After 24 h of exposure, the medium was collected. To ensure complete removal of extracellular arsenic, the cell layer was rinsed four times using DPBS, Ampuwa, 0.1 mM DMPS, and DPBS according to a published procedure (Hippler et al. 2011). Subsequently, the cells were trypsinized (trypsin–EDTA was used for HepG2 cells, trypsin was used for UROtsa cells), collected in 2 mL DPBS, counted with the CASY Model TT (Roche Applied Sciences), and mechanically lysed using glass beads (Retsch GmbH, Germany). The extracted cell lysates were analyzed without further dilution. For each arsenic concentration, the cells were exposed to, the analyzed intracellular arsenic content was calculated for 1 Mio cells.

Analysis of total arsenic content and arsenic species

Inorganic arsenic species were analyzed using AEC-ICP-MS (XSeries2, Thermo-Fisher) without anion suppressor. The instrument parameters are listed in the supporting information. Arsenic was determined as AsO^+ ($m/z = 91$) using the dynamic reaction cell (DRC) with O_2 as reaction gas as described before (Planer-Friedrich et al. 2007). Arsenite and arsenate were quantified using standard solutions of the respective solid. Thioarsenates were quantified on the basis of the arsenate calibration curve. Samples that were prepared for speciation analysis were flash-frozen in liquid nitrogen and stored in a freezer until analysis.

Total arsenic concentrations were determined by ICP-MS. The results were corrected by an internal rhodium standard (rhodium standard in 5 % HCl, 1,000 ppm, Ultra Scientific Analytical Solutions, No. Kingstown, RI, USA) to compensate changes in sensitivity. A total of 25 μL of the rhodium standard was added per 5 mL sample. For stabilization until analysis, the samples were acidified in 0.15 M HNO_3.

Results

Cytotoxicity of arsenite and arsenate and the effect of sulfide addition

Figure 2 shows the cell viabilities of HepG2 and UROtsa cells that were incubated for 6 or 24 h with arsenite and arsenate, respectively. For both cell lines and exposure times, IC_{50} values were lower for arsenite than for arsenate indicating a higher acute toxicity of arsenite (Table 1). The IC_{50} was not reached after 6 h at the highest arsenate concentration tested (10,000 μM) in HepG2 cells. Adding sulfide at a molar As/S ratio of 1:4 positively influenced cell viabilities in case of arsenite for both cell lines in case of 6 h incubation. For UROtsa cells, no more difference between arsenite and arsenite–sulfide toxicity was observed after 24 h, while for HepG2 cells the mediating effect of sulfide on arsenite toxicity grew larger from 6 to 24 h (Fig. 2). The cell viabilities of HepG2 and UROtsa cells that were incubated with arsenate were not significantly influenced by addition of sulfide up to an arsenate concentration of 1,000 μM (750 μM for UROtsa for 6 h). Altogether, UROtsa cells were more susceptible than HepG2 cells to arsenite and arsenate exposure.

Speciation analysis showed that arsenite and sulfide (molar As/S ratio 1:4) in HepG2 growth medium instantly formed between 36 and 73 % thioarsenates, primarily TriTAs(V) (26–57 %), besides DiTAs(V) (6–15 %) and some MonoTAs(V) (1–5 %) (Fig. 3). No methylated thioarsenicals were detected. The share of inorganic thioarsenates increased with increasing total arsenic and corresponding sulfide concentrations, which has previously been described to be an effect of an increasing SH^-/OH^- ratio (Planer-Friedrich et al. 2010). Immediately after mixing (t = 0 h), the solutions with the two highest arsenic concentrations (500 and 1,000 μM) showed lower shares of thioarsenates than the one with 250 μM, which is likely a kinetic effect of retarded thioarsenate formation. After 6 and 24 h, all solutions with arsenic >250 μM showed similar shares of thioarsenates (Fig. 3). Thioarsenates have previously been shown to be susceptible to oxygen and to be transformed to arsenite over time (Planer-Friedrich et al. 2010). As thus expected, the thioarsenate content decreased to <65 % after 6 h and to <33 % after 24 h (Fig. 3). The arsenic speciation

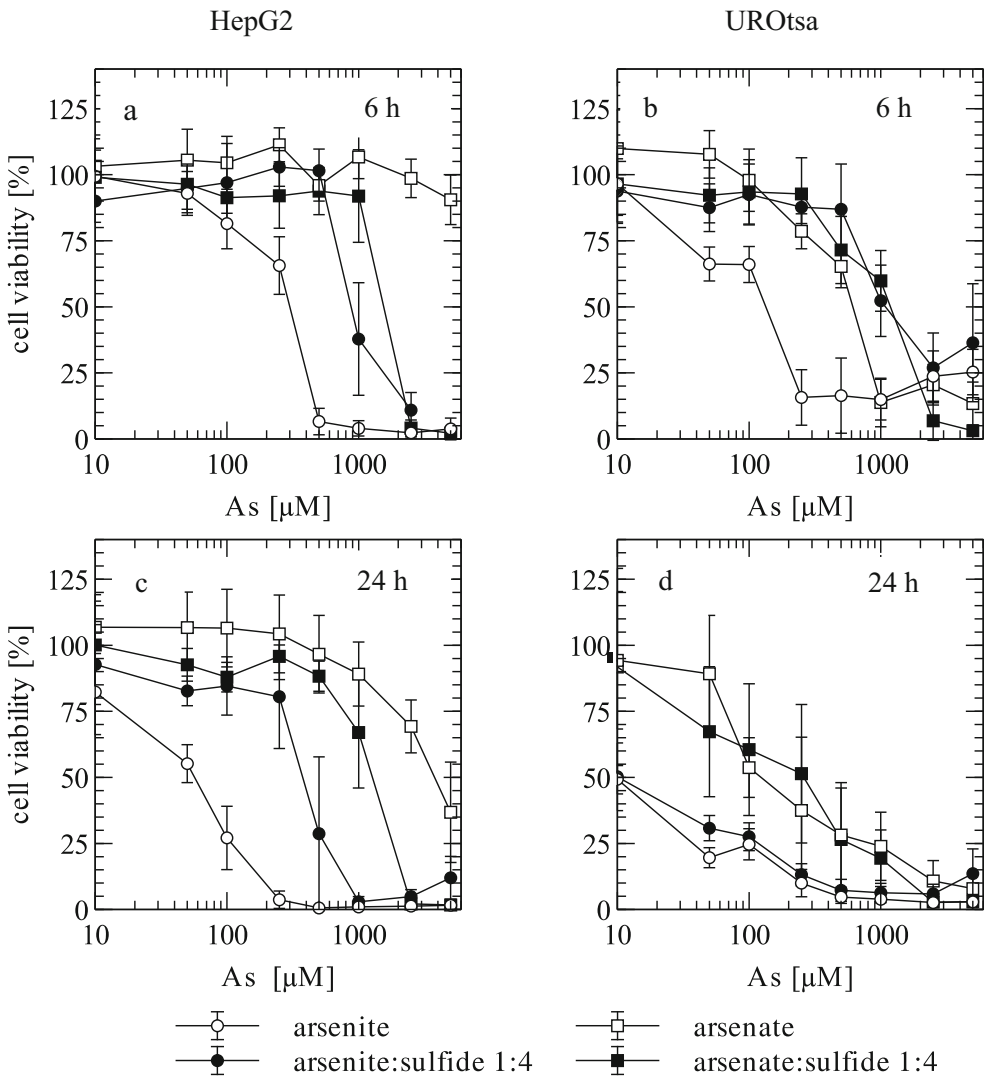
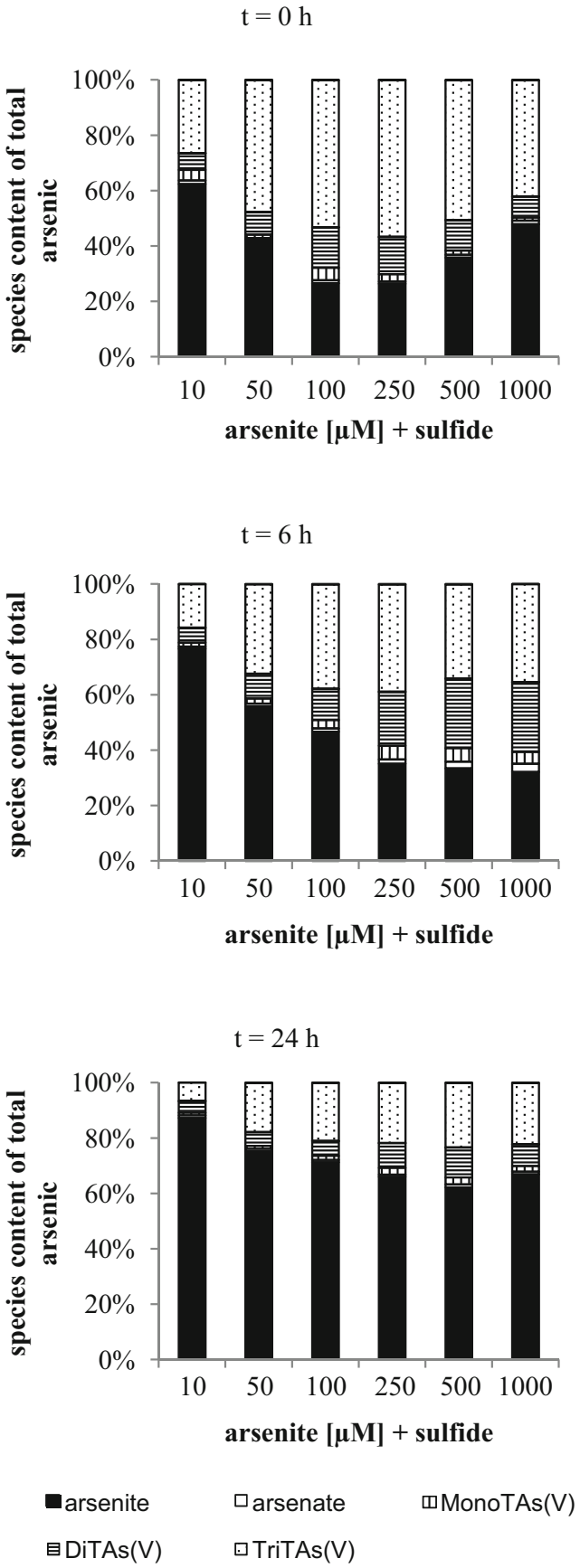


Fig. 2 Cell viabilities [% of control, $n = 3$] of HepG2 and UROtsa cells determined with MTT assay after 6 and 24 h exposure to arsenite, arsenate, and each combined with sulfide in a molar As/S ratio of 1:4; in each experiment three wells were treated with the same concentration of the arsenic species; **a**, **c**: HepG2 cells exposed for 6 and 24 h, respectively; **b**, **d**: UROtsa cells exposed for 6 and 24 h, respectively

data for UROtsa growth medium were comparable to the HepG2 growth medium data for 0, 6, and 24 h. The AEC-ICP-MS analyses of arsenate and sulfide solutions showed no change of arsenic speciation: all arsenic was recovered as arsenate, no thioarsenates were formed (data not shown).

Table 1 IC_{50} values (arsenic concentration at which the cell viability is 50 %) of arsenite, arsenate, MonoTAs(V), and TriTAs(V) for UROtsa and HepG2 cells after 24 and 6 h of exposure; standard concentration range of exposure was 10 to 1,000 μM; IC_{50} >1,000 μM could be determined by testing 5,000 and 10,000 μM in case IC_{50} was not reached at 1,000 μM. IC_{50} <10 μM could be determined by testing 0.1 and 1 μM

	HepG2		UROtsa	
	IC_{50} (6 h) [μM]	IC_{50} (24 h) [μM]	IC_{50} (6 h) [μM]	IC_{50} (24 h) [μM]
Arsenite	287	72	125	4
Arsenate	>10,000	3,914	552	166
MonoTAs(V)	1,573	371	599	48
TriTAs(V)	719	142	162	20
Arsenite/sulfide 1:4	961	186	1,186	11
Arsenate/sulfide 1:4	1,501	952	977	161



◀ **Fig. 3** Arsenic speciation in HepG2 growth medium after exposure (6 and 24 h) to arsenite and sulfide at a molar ratio of As/S 1:4; t = 0 represents the arsenic speciation in medium directly after adding arsenite and sulfide

Cytotoxicity of MonoTAs(V) and TriTAs(V) in comparison to arsenite and arsenate

Since thioarsenates were shown to form upon addition of sulfide to arsenite solutions, toxicity of the available thioarsenate standards MonoTAs(V) and TriTAs(V) was tested. As standard procedure, only concentrations up to 1,000 µM arsenic were tested and led to cell viabilities <20 % for most arsenic species. In some cases, when higher concentrations were applied, an apparent re-increase in cell viabilities was observed for MonoTAs(V) and TriTAs(V). By microscopic cell-counting, cell death at these high concentrations was confirmed. The apparent re-increase in cell viabilities is thus attributed to a reaction of the thioarsenates themselves or a sulfur component formed from the thioarsenates with MTT which could be observed for arsenic concentrations ≥1,000 µM in cell-free medium (data not shown). The respective data points were therefore removed from the following graphs. Figure 4 shows the acute cytotoxicity of MonoTAs(V) and TriTAs(V) in comparison to arsenite and arsenate. In the applied concentration range of 10–1,000 µM arsenic, UROtsa cells were more susceptible to exposure of arsenite, arsenate, MonoTAs(V), and TriTAs(V) than HepG2 cells. In both cell lines, the cytotoxicity of all arsenic species increased with time. Based on the cytotoxicity curves (Fig. 4) and the respective IC_{50} values, the order of acute cytotoxicity was determined for both cell lines as follows: arsenite > TriTAs(V) > MonoTAs(V) > arsenate (Table 1).

Figure 5 shows the stability of the thioarsenate standards during cell exposure times. Under the conditions applied (37 °C, 5 % CO_2), TriTAs(V) in growth medium was shown not to be completely stable. Directly after adding the TriTAs(V) standard to the HepG2 growth medium, 66 % of total arsenic was analyzed as TriTAs(V) (72 % in UROtsa growth medium). The content of TriTAs(V) was reduced to 52 % after 6 h and to 30 % after 24 h (45 and 28 % in UROtsa growth medium, respectively) (Fig. 5). The conversion of TriTAs(V) with time led to an assimilation of the toxicity curves of arsenite and TriTAs(V) (Fig. 4). MonoTAs(V) in growth medium was more stable than TriTAs(V). The initial MonoTAs(V) percentage in HepG2 growth medium of 87 % (90 % in UROtsa growth medium) decreased to 66 % after 6 h and to 53 % after 24 h of incubation (61 and 56 % for UROtsa growth medium, respectively) (Fig. 5).

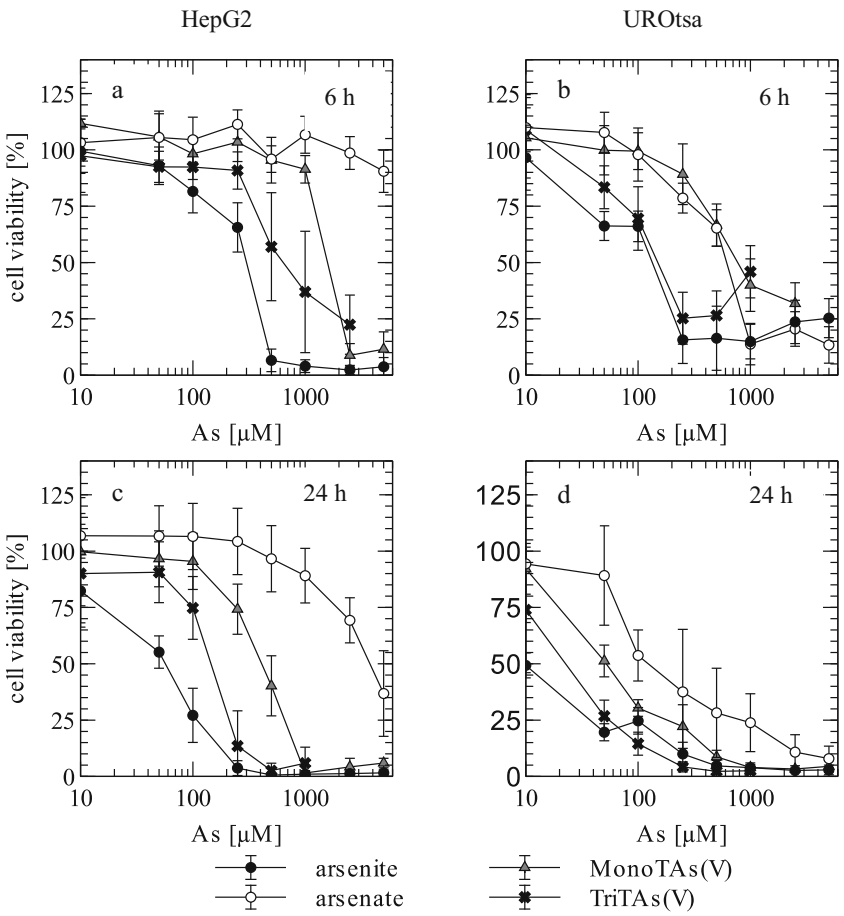


Fig. 4 Cell viabilities [% of control, $n = 3$] of HepG2 and UROtsa cells determined with MTT assay after 6 and 24 h exposure to arsenite, arsenate, MonoTAs(V), and TriTAs(V) (10–10,000 μM); in each experiment, three wells were treated with the same concentration of the arsenic species; **a**, **c**: HepG2 cells exposed to the arsenic species for 6 and 24 h, respectively; **b**, **d**: UROtsa cells exposed to the arsenic species for 6 and 24 h, respectively

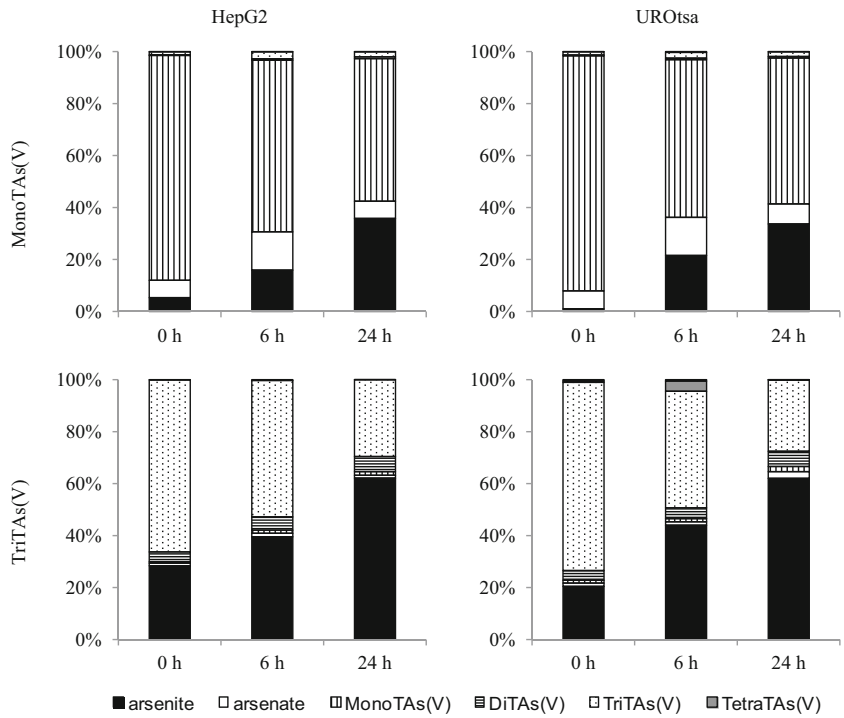


Fig. 5 Arsenic speciation in HepG2 and UROtsa growth medium after 6 and 24 h of exposure to 100 μM MonoTAs(V) and 100 μM TriTAs(V)

Cellular uptake of Mono- and TriTAs(V) in comparison to arsenite and arsenate

In order to determine whether the observed differences in toxicity between arsenite, TriTAs(V), MonoTAs(V), and arsenate can be explained simply by different concentrations in the cells, cellular uptake was determined. After 24 h of exposure to the different arsenic species (0.1–100 μM), arsenic concentrations in medium, washing solutions, and cells were quantified. Arsenic recovery rate was 105 ± 13 % for HepG2 cells and 103 ± 5 % for UROtsa cells. Since cells were considerably damaged when treated with 100 μM of any arsenic species, a reliable cell counting with the CASY TT was not practicable and arsenic uptake could only be quantified up to 75 μM. The uptake of arsenite and TriTAs(V) per single cell was higher in HepG2 cells than in UROtsa cells, whereas the uptake of MonoTAs(V) and arsenate was higher in UROtsa cells. The order of arsenic uptake was determined for HepG2 cells as arsenite > TriTAs(V) > MonoTAs(V) > arsenate and for UROtsa cells as arsenite ≈ TriTAs(V) > MonoTAs(V) > arsenate (Fig. 6).

Speciation analysis showed that after 24 h of exposure, the speciation of both arsenite and arsenate was unchanged in the cell culture media of both UROtsa and HepG2 cells (≥98 % of the original species). Incubation with TriTAs(V) yielded 6–29 % TriTAs(V), 1–10 % DiTAs(V), and 71–89 % arsenite in medium after 24 h for HepG2 cells, which was comparable to the speciation in UROtsa growth medium. Speciation of MonoTAs(V) in growth medium slightly differed after 24 h incubation among HepG2 and UROtsa growth medium. More MonoTAs(V) was transformed into arsenite in UROtsa growth medium as compared to HepG2 growth medium (HepG2: 58–69 % MonoTAs(V), 17–31 % arsenite, 9–13 % arsenate; UROtsa: 42–52 % MonoTAs(V), 34–45 % arsenite, 12–13 % arsenate) (Online Resource Fig. SI-1). Intracellularly, arsenic was determined to be mainly arsenite. In arsenite-exposed cells, arsenite represented more than 97 % in HepG2 cells and more than 87 % of total arsenic in UROtsa cells (exception at exposure to 1 μM arsenite: 57 % arsenite, the rest arsenate, which might be attributed to the greater instability of species in the more dilute solution). In arsenate-exposed cells, more than 67 % arsenate was reduced to arsenite in both cell lines, whereat in UROtsa cells generally more arsenate was found. In cells treated with MonoTAs(V), more than 98 % of arsenic was found as arsenite (exception at 1 μM MonoTAs(V): 57 % arsenite in HepG2, 82 % arsenite in UROtsa cells, the rest arsenate). TriTAs(V) was found to be transformed into more than 97 % arsenite in both cell lines (Online Resource Fig. SI-2).

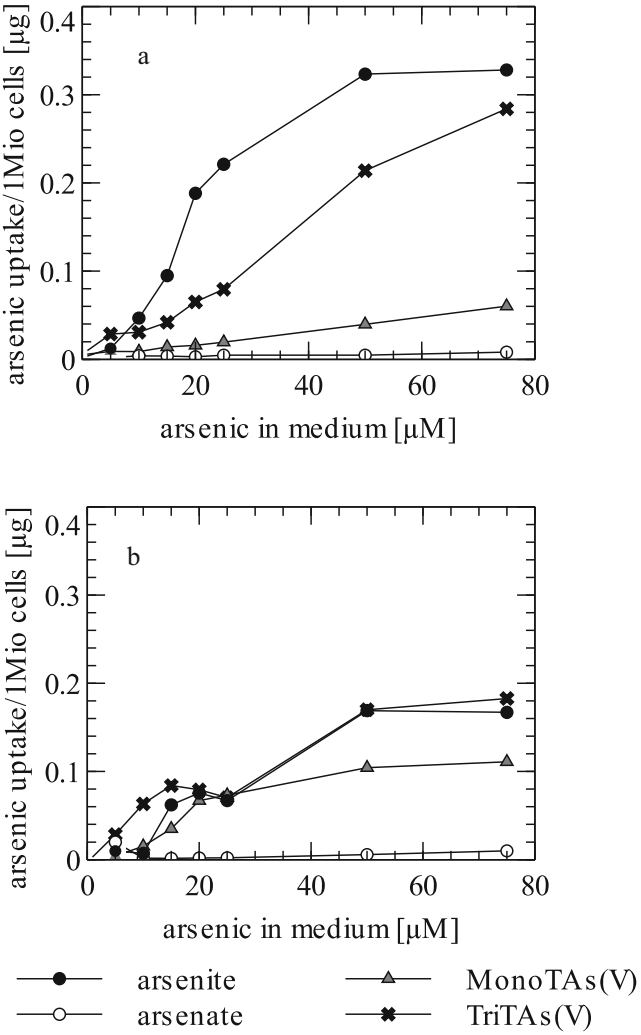


Fig. 6 Arsenic uptake in HepG2 (**a**) and UROtsa cells (**b**) after 24 h of exposure to arsenite, arsenate, MonoTAs(V), and TriTAs(V); intracellular arsenic content [μg] was calculated for 1 Mio cells

Discussion

In general, the results of our studies on arsenite and arsenate cytotoxicity are in accordance with previous literature (Dopp et al. 2008; Styblo et al. 2000) and show arsenite to be more toxic than arsenate for UROtsa and HepG2 cells. In comparison to data published by Dopp et al. (2008) using trypan blue staining to assess cytotoxicity (IC_{50} arsenite 170 μM, IC_{50} arsenate >500 μM), our results showed a higher toxicity for both arsenite and arsenate as a result of the higher sensitivity of the MTT assay compared to trypan blue staining. However, the results are in the same range as the values from Styblo et al. (2000) (IC_{50} arsenite 17.8 μM, IC_{50} arsenate >20 μM, determined with MTT assay). The determined IC_{50} value of 72 μM for HepG2 cells following arsenite exposure for 24 h agrees well with the value of 90.5 ± 6.5 μM published by Qu et al. (2009), who used the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to determine cell viabilities. Altogether, UROtsa cells were shown to be more susceptible than HepG2 cells to both arsenite and arsenate

exposure which is expressed in generally lower IC_{50} values and a stronger decrease of cell viabilities between 6 and 24 h of exposure for UROtsa cells as compared to HepG2 cells.

No data existed so far about the influence of sulfide on the acute cytotoxicity of arsenate and arsenite, or, specifically, the acute cytotoxicity of thioarsenates. In agreement with previous publications (Planer-Friedrich and Wallschlaeger 2009; Rochette et al. 2000), the presence of sulfide in arsenate solutions did not induce any speciation changes at physiological pH. Specifically, it did not lead to formation of thioarsenates. Expectedly, cell viabilities of UROtsa cells exposed to arsenate–sulfide mixtures and arsenate alone did not differ significantly. At concentrations of >1,000 μM arsenate and >4,000 μM sulfide, cell viabilities of HepG2 cells were substantially lower when cells were exposed to arsenate and sulfide as compared to arsenate alone. That sulfide exerts a cytotoxic effect at these concentrations is in line with Caro et al. (2011) who observed a decrease in cell viability of 34 ± 3 % at 200 μM NaHS.

In contrast to observations in arsenate solutions, the presence of sulfide did significantly decrease the acute cytotoxicity of arsenite solutions. Our data show a clear reduction of arsenite cytotoxicity in HepG2 cells after 6 and 24 h of exposure and in UROtsa cells after 6 h of exposure by addition of sulfide (As/S ratio 1:4). Formation of thioarsenates up to 73 % of total arsenic was observed. TriTAs(V) was the dominant species (up to 57 %). The share of thioarsenates declined with time and after 24 h of incubation only 33 % of all arsenic was present as thioarsenates, the rest was detected as arsenite. The conversion of thioarsenates to arsenite with time might account for the marginal difference of toxicity in UROtsa cells concurrently treated for 24 h with arsenite and sulfide versus arsenite only. Though exposed to the same substances with the same problem of thioarsenate conversion, this effect was not observed in HepG2 cells. We suggest that the higher susceptibility of UROtsa cells implicating a faster reaction to arsenite exposure compared to HepG2 cells accounted for the approximation of the toxicity of arsenite–sulfide mixtures versus pure arsenite solutions with time. A further explanation for the differences observed upon sulfide addition to arsenite solutions for HepG2 and UROtsa cells relates to the antioxidant properties of sulfide. One mechanism of arsenite cytotoxicity is the production of reactive oxygen species (Eblin et al. 2006; Smith et al. 2001; Scott et al. 1993). Sulfide as an antioxidant can counteract oxidative stress in cells, e.g., by increasing the intracellular GSH level (Kimura et al. 2006, 2011, 2012; Yonezawa et al. 2007; Xiao et al. 2012; Fan et al. 2013; Wen et al. 2013). Since radical formation was shown to be more pronounced in human hepatocytes than in urothelial cells (Dopp et al. 2008), this might explain the more pronounced positive effect of sulfide on HepG2 cells exposed to arsenite for 24 h in contrast to UROtsa cells, too.

Experiments with individual standards of MonoTAs(V) and TriTAs(V) showed that both thioarsenates were more toxic than their structural analogue arsenate. However, they were considerably less toxic for both cell lines and exposure times than arsenite, from which they naturally form in the presence of sulfide. The decreased toxicity as compared to arsenite was more pronounced for MonoTAs(V) than for TriTAs(V). The interpretation of the toxicity of TriTAs(V) must take into account that the initially applied TriTAs(V) standard already contained 30 % arsenite as an impurity. In our own experiments with HepG2 and UROtsa cells, we found, in accordance with previous literature on rat urothelial cells (Nascimento et al. 2008), that application of two different arsenic compounds leads to additive acute cytotoxic effects (data not shown). Even considering this additive toxic effect of a 30 % arsenite impurity in our original TriTAs(V) solution, the toxicity of a calculated "pure" TriTAs(V) solution still is significantly lower than that of arsenite and higher than that of MonoTAs(V).

Overall, thiolation can be seen as a process of detoxification for UROtsa and HepG2 cells with respect to the originally existing arsenite (not compared to the structural analogue arsenate!). The observed increase of toxicity with a higher number of thio groups is in contrast to an earlier study with HepG2 cells that did not show a connection between the number of thio groups and their toxicity for methylated thioarsenicals (Ochi et al. 2008). However, it is in accordance with a toxicity study (Planer-Friedrich et al. 2008), in which the marine bioluminescent bacterium *V. fischeri* was exposed to the same arsenic and arsenic–sulfur compounds as in the present study. To reliably assess the influence of the number of thio groups on thioarsenate toxicity, testing of Di- and TetraTAs(V) standards would be necessary. However, DiTAs(V) can currently not be synthesized in sufficient purity and TetraTAs(V) is only stable at highly alkaline pH. As observed for arsenite and arsenate, the toxicity of the thioarsenates increased with time and this effect was more pronounced in UROtsa cells than in HepG2 cells. Since the actual thioarsenic speciation in growth medium did not substantially differ between HepG2 and UROtsa cells, this effect can be attributed to the different susceptibilities of the two used cell lines.

To assess what explains the different cytotoxicity of the four investigated arsenic species, uptake after 24 h of exposure and intracellular speciation were determined. Even when exposed to arsenate, the dominant intracellular arsenic species was arsenite (up to 90 % for UROtsa, up to 93 % for HepG2 cells). Since arsenate in the cell growth medium was stable during 24 h of exposure, we assume that arsenic was actually taken up as arsenate and reduced inside the cells by GSH (Delnomdedieu et al. 1994) or in the case of HepG2 cells by As(V)reductase (Radabaugh and Aposhian 2000). The differences in arsenite and arsenate toxicity must thus be caused by

different amounts of uptake. In fact, when exposing both cell lines to the same concentrations, arsenate uptake was lower than the arsenite uptake up to a factor of 40 for UROtsa cells and 65 for HepG2 cells. These results correspond to an earlier study where HepG2 cells were exposed for 1 h to 50 μM arsenic and uptake was 1.54 ± 0.03 ng/10^6 cells for arsenate but 10.71 ± 0.07 ng/10^6 cells for arsenite (Dopp et al. 2005). The lower uptake of arsenate compared to arsenite can be explained by different uptake mechanisms: arsenite is taken up by cells via aquaglyceroporins (Shinkai et al. 2009; Liu et al. 2004) or via diffusion because it is uncharged at physiological pH (pK_{a1}, 9.23) (Lerman et al. 1983). In contrast, arsenate (pK_{a1}, 2.19; pK_{a2}, 6.94) is taken up by inorganic phosphate transporters and therefore competes with the uptake of phosphate. Arsenate uptake was shown to be reduced up to 90 % in the presence of 1 mM inorganic phosphate, which is the concentration of extracellular inorganic phosphate in mammals maintained by homeostatic mechanisms (Villa-Bellosta and Sorribas 2010). The buffer solution DBPS used in the toxicity tests to dissolve the arsenic compounds also contains inorganic phosphate in a considerable amount (1.46 mM potassium phosphate monobasic and 8.06 mM sodium phosphate dibasic). The correlation between reduced uptake and reduced toxicity was also used by Dopp et al. (2005) to explain for HepG2 cells the lower cytotoxicity of pentavalent methylated compared to inorganic and trivalent methylated species.

No information existed about the amounts or mechanisms for the cellular uptake of thioarsenates, yet. Based solely on charge, uptake of MonoTAs(V) (pK_{a2} = 7.2 (Thilo and Hertzog 1970)) should be similar to that of arsenate. At physiological pH, TriTAs(V) dominates as twofold negatively charged complex (pK_{a3}, 10.8 (Thilo and Hertzog 1970)), even though its fate is also determined by its instability with transformation to arsenite over time (Planer-Friedrich and Wallschlaeger 2009). Like for arsenate exposure, the only arsenic species observed inside the cells was arsenite when cells were exposed to Mono- or TriTAs(V). The order of cellular uptake was mainly consistent with the order of cytotoxicity for HepG2 and UROtsa cells, namely arsenite > TriTAs(V) > MonoTAs(V) > arsenate. For UROtsa cells, uptake of arsenite and TriTAs(V) did not differ significantly. As shown before, 70–90 % of the originally added trithioarsenate transformed to arsenite after 24 h in the medium. For MonoTAs(V), 17–31 % arsenite had formed in HepG2 and 34–45 % in UROtsa growth medium. Interestingly, total arsenic concentrations detected intracellularly were higher for UROtsa (0.11 μg/10^6 cells at 75 μM MonoTAs(V)) than for HepG2 cells (0.06 μg/10^6 cells), which might be related to the higher share of arsenite in medium. Taken together, it currently remains unclear whether thioarsenates are taken up as thioarsenates—which seems likely given their chemical similarity to arsenate—followed by intracellular reduction or first reduced in the medium and then taken up as arsenite exclusively. Certainly, the mediating effect of thioarsenate formation on arsenite toxicity must be considered in light of their instability under changing pH conditions and in the presence of oxygen with transformation to either arsenite or arsenate. Further studies on the precise nature of cellular uptake and intracellular reactions as well as on the chronic toxicity of thioarsenates at environmentally relevant low concentrations are currently underway in our laboratory.

Conclusion

In the present study, we showed that acute arsenite cytotoxicity was significantly reduced in HepG2 and UROtsa cells in the presence of sulfide due to formation and transformation of thioarsenates. Over time, the mediating effect of thioarsenates decreased. The order of acute cytotoxicity (arsenite > TriTAs(V) > MonoTAs(V) > arsenate) correlated with the amount of uptake but uptake alone was not sufficient to fully explain the observed differences in cytotoxicity. Intracellularly, arsenic was observed almost exclusively as arsenite. Whether sulfide merely decreases the amount of cellular available, cytotoxic free arsenite by extracellular formation of thioarsenates, or thioarsenates are actively transferred into the cell and undergo intracellular transformation to arsenite, remains subject to future investigation. However, our study clearly showed that thiolation and the subsequent toxicity of thioarsenates will have to be considered in studies about arsenic toxicity.

Acknowledgments We acknowledge generous funding by the German Research Foundation within the Emmy Noether program to B. Planer-Friedrich (grant # PL 302/3-1) and thank Stefan Will for help with AEC-ICP-MS analyses.

References

Beak DG, Wilkin RT, Ford RG, Kelly SD (2008) Examination of arsenic speciation in sulfidic solutions using X-ray absorption spectroscopy. Environ Sci Technol 42(5):1643–1650

Burton ED, Johnston SG, Planer-Friedrich B (2013) Coupling of arsenic mobility to sulfur transformations during microbial sulfate reduction in the presence and absence of humic acid. Chem Geol 343:12–24. doi:10.1016/j.chemgeo.2013.02.005

Caro AA, Thompson S, Tackett J (2011) Increased oxidative stress and cytotoxicity by hydrogen sulfide in HepG2 cells overexpressing cytochrome P450 2E1. Cell Biol Toxicol 27(6):439–453. doi:10.1007/s10565-011-9198-2

Delnomdedieu M, Basti MM, Otvos JD, Thomas DJ (1994) Reduction and binding of arsenate and dimethylarsinate by glutathione—a magnetic-resonance study. Chem-Biol Interact 90(2):139–155. doi:10.1016/0009-2797(94)90099-x

Dopp E, Hartmann LM, von Recklinghausen U, Florea AM, Rabieh S, Zimmermann U, Shokouhi B, Yadav S, Hirner AV, Rettenmeier AW

(2005) Forced uptake of trivalent and pentavalent methylated and inorganic arsenic and its cyto-/genotoxicity in fibroblasts and hepatoma cells. Toxicol Sci 87(1):46–56. doi:10.1093/toxsci/kfi218

Dopp E, von Recklinghausen U, Hartmann LM, Stueckradt I, Pollok I, Rabieh S, Hao L, Nussler A, Katier C, Hirner AV, Rettenmeier AW (2008) Subcellular distribution of inorganic and methylated arsenic compounds in human urothelial cells and human hepatocytes. Drug Metab Dispos 36(5):971–979. doi:10.1124/dmd.107.019034

Eblin KE, Bowen ME, Cromey DW, Bredfeldt TG, Mash EA, Lau SS, Gandolfi AJ (2006) Arsenite and monomethylarsonous acid generate oxidative stress response in human bladder cell culture. Toxicol Appl Pharmacol 217(1):7–14. doi:10.1016/j.taap.2006.07.004

Fan HN, Wang HJ, Yang-Dan CR, Ren L, Wang C, Li YF, Deng Y (2013) Protective effects of hydrogen sulfide on oxidative stress and fibrosis in hepatic stellate cells. Mol Med Rep 7(1):247–253. doi:10.3892/mmr.2012.1153

Härtig C, Planer-Friedrich B (2012) Thioarsenate transformation by filamentous microbial mats thriving in an alkaline, sulfidic hot spring. Environ Sci Technol 46(8):4348–4356. doi:10.1021/es204277j

Hippler J, Zdrenka R, Reichel RAD, Weber DG, Rozynek P, Johnen G, Dopp E, Hirner AV (2011) Intracellular, time-resolved speciation and quantification of arsenic compounds in human urothelial and hepatoma cells. J Anal At Spectrom 26(12):2396–2403. doi:10.1039/c1ja10150a

Johnston SG, Burton ED, Keene AF, Planer-Friedrich B, Voegelin A, Blackford MG, Lumpkin GR (2012) Arsenic mobilization and iron transformations during sulfidization of As(V)-bearing jarosite. Chem Geol 334:9–24. doi:10.1016/j.chemgeo.2012.09.045

Jorgensen J, Mortensen PB (2001) Hydrogen sulfide and colonic epithelial metabolism—implications for ulcerative colitis. Dig Dis Sci 46(8):1722–1732. doi:10.1023/a:1010661706385

Kimura Y, Dargusch R, Schubert D, Kimura H (2006) Hydrogen sulfide protects HT22 neuronal cells from oxidative stress. Antioxid Redox Signaling 8(3–4):661–670. doi:10.1089/ars.2006.8.661

Kimura Y, Y-i G, Kimura H (2011) Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. J Pharmacol Sci 115:77P–77P

Kimura Y, Y-i G, Kimura H (2012) Hydrogen sulfide prevents neuronal cell death by increasing glutathione production and suppressing oxidative stress in mitochondria. J Pharmacol Sci 118:104P–104P

Lerman SA, Clarkson TW, Gerson RJ (1983) Arsenic uptake and metabolism by liver-cells is dependent on arsenic oxidation-state. Chem-Biol Interact 45(3):401–406. doi:10.1016/0009-2797(83)90087-x

Liu ZJ, Carbrey JM, Agre P, Rosen BP (2004) Arsenic trioxide uptake by human and rat aquaglyceroporins. Biochem Biophys Res Commun 316(4):1178–1185. doi:10.1016/j.bbrc.2004.03.003

Naranmandura H, Ibata K, Suzuki KT (2007a) Toxicity of dimethylmonothioarsinic acid toward human epidermoid carcinoma A431 cells. Chem Res Toxicol 20(8):1120–1125. doi:10.1021/tx700103y

Naranmandura H, Suzuki N, Iwata K, Hirano S, Suzuki KT (2007b) Arsenic metabolism and thioarsenicals in hamsters and rats. Chem Res Toxicol 20(4):616–624. doi:10.1021/tx700038x

Naranmandura H, Ogra Y, Iwata K, Lee J, Suzuki KT, Weinfeld M, Le XC (2009) Evidence for toxicity differences between inorganic arsenite and thioarsenicals in human bladder cancer cells. Toxicol Appl Pharmacol 238(2):133–140. doi:10.1016/j.taap.2009.05.006

Naranmandura H, Carew MW, Xu S, Lee J, Leslie EM, Weinfeld M, Le XC (2011) Comparative toxicity of arsenic metabolites in human bladder cancer EJ-1 cells. Chem Res Toxicol 24(9):1586–1596. doi:10.1021/tx200291p

Nascimento MG, Suzuki S, Wei M, Tiwari A, Arnold LL, Lu X, Le XC, Cohen SM (2008) Cytotoxicity of combinations of arsenicals on rat urinary bladder urothelial cells in vitro. Toxicology 249(1):69–74. doi:10.1016/j.tox.2008.04.007

Ochi T, Kita K, Suzuki T, Rumpler A, Goessler W, Francesconi KA (2008) Cytotoxic, genotoxic and cell-cycle disruptive effects of thiodimethylarsinate in cultured human cells and the role of glutathione. Toxicol Appl Pharmacol 228(1):59–67. doi:10.1016/j.taap.2007.11.023

Pinyayev TS, Kohan MJ, Herbin-Davis K, Creed JT, Thomas DJ (2011) Preabsorptive metabolism of sodium arsenate by anaerobic microbiota of mouse cecum forms a variety of methylated and thiolated arsenicals. Chem Res Toxicol 24(4):475–477. doi:10.1021/tx200040w

Planer-Friedrich B, Wallschlaeger D (2009) A critical investigation of hydride generation-based arsenic speciation in sulfidic waters. Environ Sci Technol 43(13):5007–5013. doi:10.1021/es900111z

Planer-Friedrich B, London J, McCleskey RB, Nordstrom DK, Wallschläger D (2007) Thioarsenates in geothermal waters of Yellowstone National Park: determination, preservation, and geochemical role. Environ Sci Technol 41(15):5245–5251

Planer-Friedrich B, Franke D, Merkel B, Wallschlager D (2008) Acute toxicity of thioarsenates to Vibrio fischeri. Environ Toxicol Chem 27(10):2027–2035

Planer-Friedrich B, Fisher JC, Hollibaugh JT, Süß E, Wallschläger D (2009) Oxidative transformation of trithioarsenate along alkaline geothermal drainages—abiotic versus microbially mediated processes. Geomicrobiol J 26:339–350

Planer-Friedrich B, Suess E, Scheinost AC, Wallschlaeger D (2010) Arsenic speciation in sulfidic waters: reconciling contradictory spectroscopic and chromatographic evidence. Anal Chem 82:10228–10235. doi:10.1021/ac1024717

Qu W, Liu J, Dill AL, Saavedra JE, Keefer LK, Waalkes MP (2009) V-PROLI/NO, a nitric oxide donor prodrug, protects liver cells from arsenic-induced toxicity. Cancer Sci 100(3):382–388. doi:10.1111/j.1349-7006.2008.01050.x

Radabaugh TR, Aposhian HV (2000) Enzymatic reduction of arsenic compounds in mammalian systems: reduction of arsenate to arsenite by human liver arsenate reductase. Chem Res Toxicol 13(1):26–30

Rader KJ, Dombrowski PM, Farley KJ, Mahony JD, Di Toro DM (2004) Effect of thioarsenite formation on arsenic(III) toxicity. Environ Toxicol Chem 23(7):1649–1654. doi:10.1897/03-443

Raml R, Rumpler A, Goessler W, Vahter M, Li L, Ochi T, Francesconi KA (2007) Thio-dimethylarsinate is a common metabolite in urine samples from arsenic-exposed women in Bangladesh. Toxicol Appl Pharmacol 222(3):374–380. doi:10.1016/j.taap.2006.12.014

Rochette EA, Bostick BC, Li GC, Fendorf S (2000) Kinetics of arsenate reduction by dissolved sulfide. Environ Sci Technol 34(22):4714–4720. doi:10.1021/es000963y

Schwedt G, Rieckhoff M (1996) Separation of thio- and oxothioarsenates by capillary zone electrophoresis and ion chromatography. J Chromatogr A 736(1–2):341–350. doi:10.1016/0021-9673(95)01319-9

Scott N, Hatlelid KM, Mackenzie NE, Carter DE (1993) Reactions of arsenic(III) and arsenic(V) species with glutathione. Chem Res Toxicol 6(1):102–106. doi:10.1021/tx00031a016

Shinkai Y, Sumi D, Toyama T, Kaji T, Kumagai Y (2009) Role of aquaporin 9 in cellular accumulation of arsenic and its cytotoxicity in primary mouse hepatocytes. Toxicol Appl Pharmacol 237(2):232–236. doi:10.1016/j.taap.2009.03.014

Smith KR, Klei LR, Barchowsky A (2001) Arsenite stimulates plasma membrane NADPH oxidase in vascular endothelial cells. Am J Physiol-Lung Cell Mol Physiol 280(3):L442–L449

Stauder S, Raue B, Sacher F (2005) Thioarsenates in sulfidic waters. Environ Sci Technol 39(16):5933–5939

Styblo M, Del Razo LM, Vega L, Germolec DR, LeCluyse EL, Hamilton GA, Reed W, Wang C, Cullen WR, Thomas DJ

(2000) Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. Arch Toxicol 74(6):289–299

Suess E, Planer-Friedrich B (2012) Thioarsenate formation upon dissolution of orpiment and arsenopyrite. Chemosphere 89(11):1390–1398. doi:10.1016/j.chemosphere.2012.05.109

Suess E, Scheinost AC, Bostick BC, Merkel BJ, Wallschlaeger D, Planer-Friedrich B (2009) Discrimination of thioarsenites and thioarsenates by X-ray absorption spectroscopy. Anal Chem 81(20):8318–8326. doi:10.1021/ac901094b

Suess E, Wallschlaeger D, Planer-Friedrich B (2011) Stabilization of thioarsenates in iron-rich waters. Chemosphere 83:1524–1531

Thilo E, Hertzog K (1970) Processes in formation of As(V) sulfide in acidification of tetrathioarsenate solutions. Z Anorg Allg Chem 373(2):111. doi:10.1002/zaac.19703730203

Van de Wiele T, Gallawa CM, Kubachka KM, Creed JT, Basta N, Dayton EA, Whitacre S, Du Laing G, Bradham K (2010) Arsenic metabolism by human gut microbiota upon in vitro digestion of contaminated soils. Environ Health Perspect 118(7):1004–1009. doi:10.1289/ehp.0901794

Villa-Bellosta R, Sorribas V (2010) Arsenate transport by sodium/phosphate cotransporter type IIb. Toxicol Appl Pharmacol 247(1):36–40. doi:10.1016/j.taap.2010.05.012

Wallschläger D, Stadey CJ (2007) Determination of (oxy)thioarsenates in sulfidic waters. Anal Chem 79:3873–3880

Wen Y-D, Wang H, Kho S-H, Rinkiko S, Sheng X, Shen H-M, Zhu Y-Z (2013) Hydrogen sulfide protects HUVECs against hydrogen peroxide induced mitochondrial dysfunction and oxidative stress. PLoS ONE 8(2):e53147. doi:10.1371/journal.pone.0053147

Wilkin R, Wallschläger D, Ford R (2003) Speciation of arsenic in sulfidic waters. Geochem T 4(1):1–7

Xiao LC, Lan AP, Mo LQ, Xu WM, Jiang N, Hu F, Feng JQ, Zhang CR (2012) Hydrogen sulfide protects PC12 cells against reactive oxygen species and extracellular signal-regulated kinase 1/2-mediated downregulation of glutamate transporter-1 expression induced by chemical hypoxia. Int J Mol Med 30(5):1126–1132. doi:10.3892/ijmm.2012.1090

Yonezawa D, Sekiguchi F, Miyamoto M, Taniguchi E, Honjo M, Masuko T, Nishikawa H, Kawabata A (2007) A protective role of hydrogen sulfide against oxidative stress in rat gastric mucosal epithelium. Toxicology 241(1–2):11–18. doi:10.1016/j.tox.2007.07.020

Supplementary material

Article title: Effect of sulfide on the cytotoxicity of arsenite and arsenate in human hepatocytes (HepG2) and human urothelial cells (UROtsa)

Journal name: Environmental Science and Pollution Research

Authors:Sinikka Hinrichsen[†], Regina Lohmayer[†], Ricarda Zdrenka[‡], Elke Dopp[‡], and Britta Planer-Friedrich[†]*

[†] Environmental Geochemistry, Faculty of Biology, Chemistry and Earth Sciences, University of Bayreuth, Universitaetsstrasse 30, 95447 Bayreuth, Germany

[‡] Institute of Hygiene and Occupational Medicine, University Hospital Essen, Essen, Germany

* Corresponding author at: Environmental Geochemistry, Faculty of Biology, Chemistry and Earth Sciences, University of Bayreuth, Universitaetsstrasse 30, 95447 Bayreuth, Germany

Tel.: +49 921 55 3643; Fax: +49 921 55 2334.

E-Mail address: b.planer-friedrich@uni-bayreuth.de

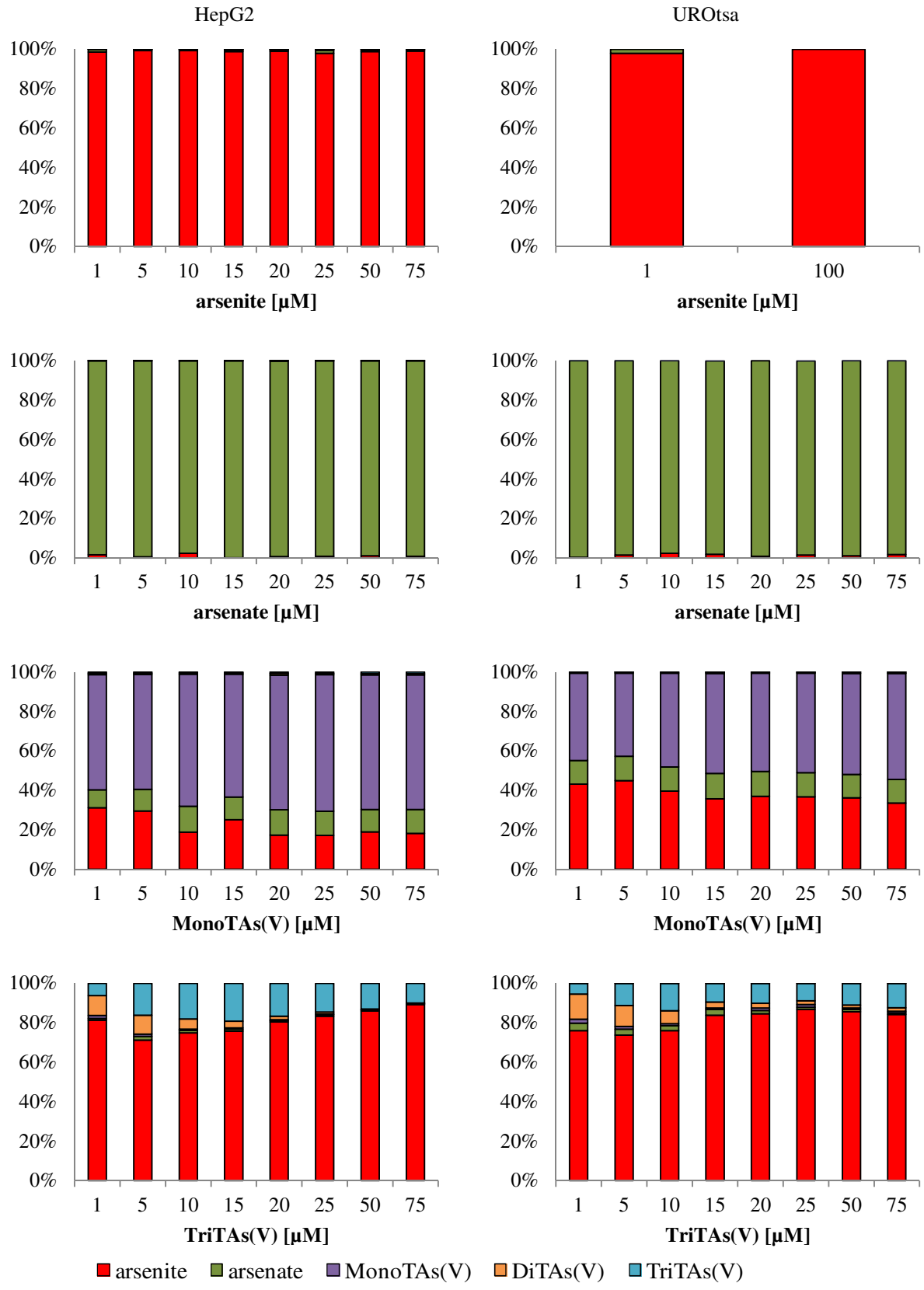


Fig. SI- 1 Arsenic species [% of total arsenic] in HepG2 and UROtsa growth medium after 24 h of exposure to arsenite, arsenate, MonoTAs(V), and TriTAs(V) (1-75 µM)

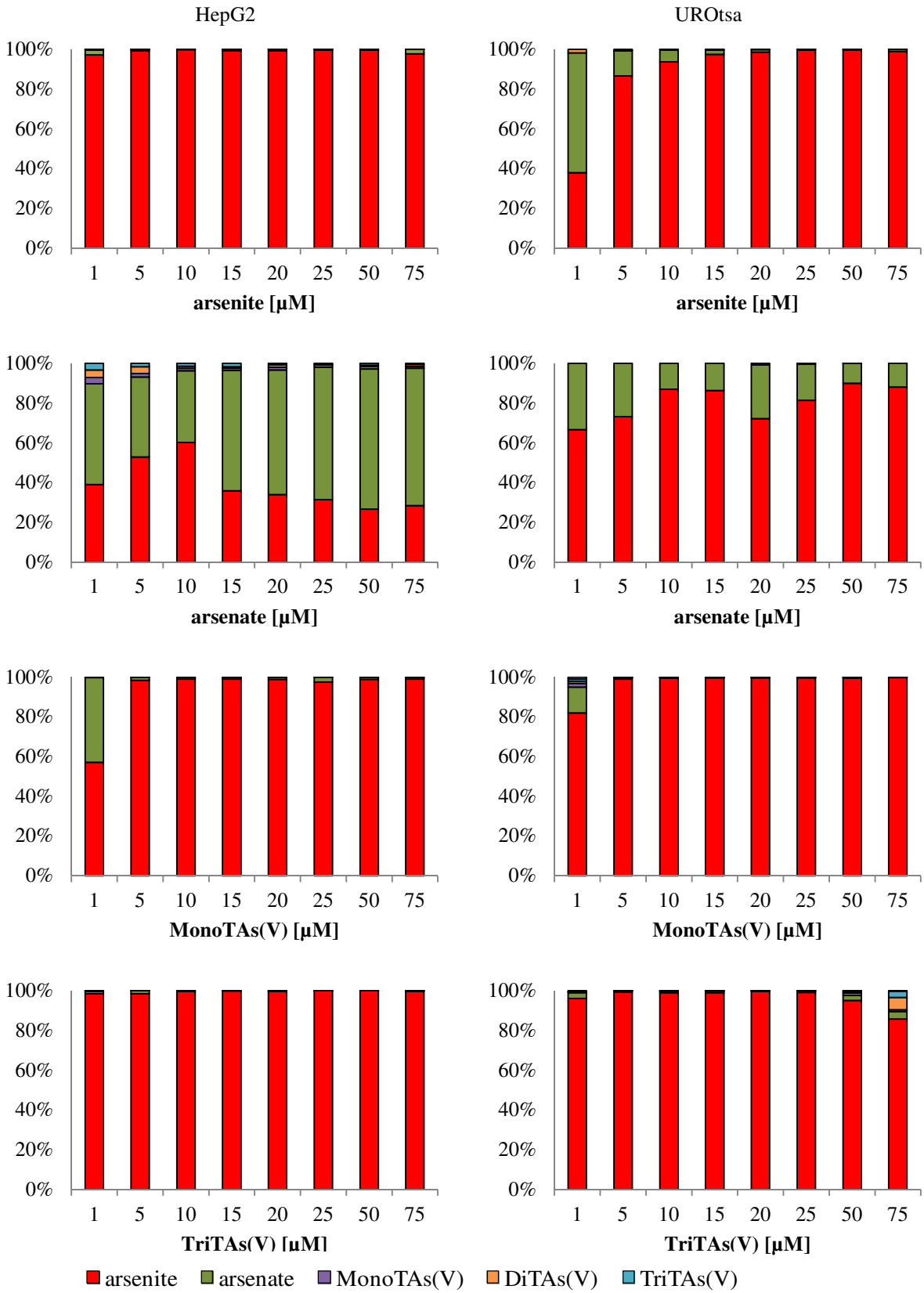


Fig. SI- 2 Intracellular arsenic species [% of total arsenic] in HepG2 and UROtsa cells after 24 h of exposure to arsenite, arsenate, MonoTAs(V), and TriTAs(V) (1-75 µM)

Tab. SI – 1 Instrumental parameters for the separation of arsenic species by anion exchange chromatography

analytical column	IonPac AS-16 4-mm (Dionex)
guard column	IonPac AG-16 4-mm (Dionex)
eluent	0.1M NaOH
gradient	[min] [mM] 0 - 7 20 7-17 20 → 100 17 - 25 100 25 - 28 20
flow rate	1.2 mL/min
suppression	none

Study 3:

Cytotoxic activity of selenosulfate versus selenite in tumor cells depends on cell line and presence of amino acids

Sinikka Hinrichsen, Britta Planer-Friedrich

submitted to:

Environmental Science and Pollution Research

Cytotoxic activity of selenosulfate versus selenite in tumor cells depends on cell line and presence of amino acids

SINIKKA HINRICHSEN†, BRITTA PLANER-FRIEDRICH† *

† Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany

* Corresponding author at: Environmental Geochemistry, Faculty of Biology, Chemistry, and Earth Sciences, University of Bayreuth, Universitaetsstrasse 30, 95447 Bayreuth, Germany. Tel.: +49 921 55 3999; Fax: +49 921 55 2334.

E-Mail address: b.planer-friedrich@uni-bayreuth.de

Keywords: HepG2, T24, A375, non-essential amino acids, cellular selenium uptake, L-glutamic acid

ABSTRACT: Based on acute cytotoxicity studies, selenosulfate ($SeSO_3^-$) has been suggested to possess a generally higher toxic activity in tumor cells than selenite. The reason for this difference in cytotoxic activity remained unclear. In the present study, cytotoxicity tests with human hepatoma (HepG2), malignant melanoma (A375), and urinary bladder carcinoma cells (T24) showed that the selenosulfate toxicity was very similar between all three tested cell lines (IC_{50} 6.6-7.1 µM after 24 h). It was largely independent of exposure time and presence or absence of amino acids. What changed, however, was the toxicity of selenite, which was lower than that of selenosulfate only for HepG2 cells (IC_{50} > 15 µM), but similar to and higher than that of selenosulfate for A375 (IC_{50} 4.7 µM) and T24 cells (IC_{50} 3.5 µM), respectively. Addition of amino acids to T24 cell growth medium down-regulated short-term selenite uptake (1.5 versus 12.9 ng Se /10^6 cells) and decreased its cytotoxicity (IC_{50} 8.4 µM), rendering it less toxic than selenosulfate. The suggested mechanism is a stronger expression of the x_c^- transport system in the more sensitive T24 compared to HepG2 cells which creates a reductive extracellular microenvironment and facilitates selenite uptake by reduction. Selenosulfate is already reduced and so less affected. The cytotoxic activity of selenosulfate and selenite to tumor cells therefore depends on the sensitivity of each cell line, supplements like amino acids as well as the reductive state of the extracellular environment.

Introduction

Selenium (Se) is an essential trace element for humans. Various selenium compounds are known to possess anticarcinogenic properties, e.g. selenite (Na_2SeO_3) is often used in clinical trials as addition to commonly applied anti-cancer chemotherapy drugs (Dennert &Horneber 2006). High concentrations of the anti-cancer drug cisplatin can result in different side effects such as leucocyte reduction, diarrhea, vomiting, edema, or nephrotoxicity. As an essential antioxidant selenite can reduce the toxicity of cisplatin without compromising its anticarcinogenic activity as shown in animal studies (Baldew et al. 1989, Camargo et al. 2001, Markovic et al. 2011). However, high doses of selenite have been shown to exert strong cytotoxic effects themselves, e.g. on prostate (Li et al. 2007), ovarian (Park et al. 2012a), liver (Shen et al. 1999), or lung cells (Park et al. 2012b). Due to the narrow concentration range between beneficial and toxic effects and a potential negative influence also on healthy tissue, application of selenite in cancer treatment is still discussed quite controversially (Micke et al. 2009, Vinceti et al. 2001, Zhang et al. 2008b).

A recent patent (Patent US 2010/0172822) claims selenosulfate (Na_2SeSO_3) to be preferable over selenite and suggests its use as complementary substance in anti-cancer therapy (Zhang 2010). Mice studies showed that selenosulfate effectively reduced gastrointestinal toxic effects induced by cisplatin from 80 to 6% (Li et al. 2012) without disturbing its therapeutic effect on tumor cells (Zhang et al. 2008b). In addition, while long-term (55 d) administration of selenite resulted in dose-dependent growth suppression and hepatoxicity in mice, selenosulfate administration in the same concentrations (12.7 and 19 µM) did not result in toxic symptoms (Li et al. 2012). That means while being as efficient as selenite in reducing negative side effects of chemotherapy drugs such as cisplatin, selenosulfate is less toxic to healthy tissue, thus, higher doses can be applied.

An interesting side effect in selenite-based cancer therapy is that cell culture studies suggest selenite to have a higher cytotoxicity in tumor cells compared to healthy cells, shown e.g. in studies on human malignant glioma (Kim et al. 2007) and osteosarcoma cells (Chen et al. 2012) versus comparable healthy cells. The differences in toxicity for tumor versus healthy cells are mainly explained by different uptake mechanisms, which in turn are governed by the extracellular reductive capacity (lm et al. 2009). The redox state of the extracellular environment largely depends on the cysteine/glutamate exchange system (x_c^-) first identified by Bannai et al. (1986) and the presence of multidrug resistance proteins (MRP) which are overexpressed in many tumor cell lines (reviewed in

Conrad &Sato 2012). The x_c^--overexpression drives the intracellular cystin/cysteine redox cycle. Cysteine is then increasingly secreted by MRP into the extracellular environment and induces increased selenium uptake by formation of more reduced selenium compounds, especially HSe^-. Both HSe^- and selenite enter the cells by anion channels, but there is a higher affinity for HSe^- (Conrad &Sato 2012). A strong relationship was found between extracellular thiol production, selenite uptake, and cellular susceptibility to selenite in different lung tumor cells (Olm et al. 2009). Selenite uptake was increased by extracellular reduction through e.g. GSH addition and decreased by extracellular oxidation (through addition of 55'-dithiobis-(2-nitrobenzoic acid)) (Olm et al. 2009). Addition of extracellular thiols also increased selenite uptake in a keratinocyte model (Ganyc &Self 2008). Amino acids might decrease selenite uptake, at least the reverse process of selenite decreasing the uptake of amino acids was confirmed previously (Hogberg &Kristoferson 1979, Vernie et al. 1974). An influence of amino acids on selenite uptake actually generates a problem when comparing cytotoxicity data of different cell culture studies. Numerous different cell growth media compositions have been used for growing the same cell line and non-essential amino acids (NEAA) have been added in some studies (McKelvey et al. 2015, Zhang et al. 2008a) but not in others (Chu &Crawford-Brown 2006, Peng et al. 2007, Zou et al. 2007) without separating pure selenite from combined selenite-NEAA effects.

For selenosulfate, patent US 2010/017282 claims that it has an even higher activity than selenite to suppress and kill specifically tumor cells (Zhang 2010) which would be another advantage of its preferred application. The claim is based on acute cytotoxicity tests in cell culture studies with human hepatocellular carcinoma (HepG2), epithelial colorectal adenocarcinoma (Caco-2), and three different kinds of leukemia cells (HL60, T lymph adenoma, Daudi) (Zhang et al. 2008a). The amount of intracellular uptake or the effect of extracellular compounds on cytotoxicity and uptake were not investigated and no reason was given as to what could have caused the difference in cytotoxic activity between selenosulfate and selenite.

The goal of the present study was therefore to repeat the previously published cytotoxicity tests (Zhang et al. 2008a) to see if this claim can really be generalized to different types of tumor cells and to elucidate why selenosulfate should be more toxic than selenite. Besides human hepatoma cells (HepG2), which were selected for comparison with the previous studies (Zhang 2010, Zhang et al. 2008a), we tested two further tumor cell lines: Malignant melanoma (A375) and urinary bladder carcinoma cells (T24). The T24 cells were selected because high selenium levels in the body have been shown to be inversely correlated with bladder cancer risk (Kellen et al. 2006) and selenium is discussed to act as chemopreventive agent against bladder cancer (Brinkman et al. 2006). The A375 cells were selected because previous experiments related the presence of different selenium compounds - selenium nanoparticles (Chen et al. 2008), selenocystine (Chen &Wong 2008), and 1,4-Diselenophene-1,4-diketone (Vinceti et al. 2014) – to oxidative stress and mitochondrial dysfunction leading to apoptosis in A375 cells. Furthermore, selenium compounds were found to be able to enhance X-ray induced growth inhibition in A375 cells (Lo et al. 2008).

Using these three different cell lines, we compared cytotoxicity of selenosulfate and selenite for three different incubation times (24, 48, and 72 h) and, for HepG2 and T24 cells, in the presence and absence of NEAA as medium supplement. We also determined intracellular selenium concentrations to investigate the relation between uptake and respective selenium cytotoxicity.

Materials and methods

Synthesis and stability of selenosulfate standards

Selenosulfate was synthesized according to a published procedure (Zhang et al. 2008a). Briefly, selenite (Fluka Analytical, Steinheim, Germany), glutathione (GSH, Applichem, Darmstadt, Germany), and sulfite (Sigma-Aldrich, Steinheim, Germany) were mixed in a molar ratio of 1:4:4 and gently homogenized until the solution was clear. The purity of the standard (91.7 ± 9.9%, rest on average 6.8% selenite and 1.5% selenate) was determined by speciation analysis using anion exchange chromatography (Dionex) with an AS16 column, a 0.02-0.1 M NaOH-eluent and no anion suppressor, coupled to an inductively coupled plasma mass spectrometry (AEC-ICP-MS, XSeries2, Thermo-Fisher). The method had previously been described for separation of thioarsenates and thioantimonates (Planer-Friedrich et al. 2007). In the experimental solutions themselves we could not determine selenium speciation chromatographically due to interferences from the applied cell growth medium. Pretests under cell cultivation conditions (37° C, 5% CO_2) showed after 72 h no precipitation of elemental red selenium which had previously been taken as an indication of selenosulfate instability (Zhang et al. 2008a). We therefore conclude that selenosulfate was stable during all our experiments.

Cell cultures

Both HepG2 and T24 cells were kindly provided by Prof. Dr. E. Dopp (Institute of Hygiene and Occupational Medicine, University Hospital Essen, Germany). The T24 cell line was primarily assumed to be the UROtsa cell line which is a *non*-tumorigenic urothelial cell line. However, several stocks of UROtsa cells were cross-contaminated and were recently identified as bladder tumor cell line T24 (Johnen et al. 2013). The A375 cells were purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany). The HepG2 cells were grown in minimum essential medium with Earle's Salts (MEM, c·c·pro, Oberdorla, Germany) supplemented with 10% FBS, 0.5% gentamycine, 1% L-glutamine, 1% NEAA, and 1% sodium pyruvate (all c·c·pro, Oberdorla, Germany). The T24 cells were cultivated in MEM supplemented with 10% Foetal Bovine Serum (FBS, Gibco), 0.5% gentamycine (c·c·pro, Oberdorla, Germany), and 1% L-glutamine (c·c·pro, Oberdorla, Germany). The A375 cells were cultivated in Dulbecco's modified Eagle Medium (DMEM, c·c·pro, Oberdorla, Germany) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin (DMEM, c·c·pro, Oberdorla, Germany). All cell lines were incubated in a humidified atmosphere

at 37 °C and 5% CO_2 (Incubator Galaxy 170 S, New Brunswick Scientific) and passaged thrice weekly using trypsin (0.25%, c·c·pro, Oberdorla, Germany) for T24 cells and trypsin-EDTA (0.25%, c·c·pro, Oberdorla, Germany) for HepG2 and A375 cells. Before conducting toxicity experiments with the three cell lines, the thawed cells (HepG2 passage number P22, T24 passage number P28, A375 passage number P25) were passaged three times. To maintain comparable conditions, no more than ten further passages were performed with one cell line.

Non-essential amino acids were only used for standard cultivation of HepG2 cells. To investigate the effect of NEAA on selenosulfate and selenite cytotoxicity and uptake, experiments with HepG2 cells were additionally conducted in MEM without NEAA addition and with T24 cells in MEM with NEAA supplementation. To let the cells adapt to the modified medium, they were passaged for at least three times before starting an experiment.

MTT assay

For cytotoxicity testing, 5000 cells/well were allowed to attach for 24 h in 96 well plates (Falcon, Becton Dickinson, Meylan Cedex, France) and subsequently exposed to selenosulfate (0.17-25 µM) or selenite (0.1-15 µM). For each experiment, selenosulfate and selenite standards were prepared freshly, sterile-filtered, and diluted in autoclaved water (Ampuwa, Fresenius Kabi, Bad Homburg, Germany). All experiments were conducted as triplicate with six equally treated wells per replicate (n = 18). For HepG2 cells, the 24 h incubation experiments were conducted twice (each setup conducted in triplicate). In each experiment, six wells were treated with the same concentration. After the respective incubation time (24, 48, and 72 h for HepG2 and T24 cells, 24 h for A375 cells), cells were incubated for 2 h with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA). Afterwards, the formazan crystals were dissolved in solubilization solution (10 g sodium dodecyl sulfate (Sigma-Aldrich, Steinheim, Germany) dissolved in 99.4 mL dimethyl sulfoxyde (Sigma-Aldrich, Steinheim, Germany) and 0.6 mL acetic acid (VWR PROLAB, Briare, France)), and the formazan product was spectrophotometrically measured at 570 nm (reference wavelength 630 nm) (Infinite 200 PRO, TECAN). Cell viability was calculated by setting the cell viabilities of non-treated cells to 100%. Calculation of the substance´s inhibitory concentration inducing 50% cell viability (IC_{50}) was conducted by fitting an erfc(x) function to the measured cell viability values as published previously (Hinrichsen et al. 2014). The lower the IC_{50} value, the more toxic the substance.

Determination of intracellular Se

A total of $5 \cdot 10^6$ cells were incubated for 24 h with fresh growth medium containing 1 µM selenite or 1.7 µM selenosulfate, respectively. Both selenium compounds were freshly prepared. These concentrations were selected because they were shown to be non-cytotoxic after 24 h incubation (CV 98.8 ± 3.7% and 94.9 ± 5.1%, respectively). The exact selenium concentrations in selenosulfate and selenite solutions were analyzed by ICP-MS. For better comparability, results are reported in ng Se taken up per 10^6 cells normalized to µM of initially applied selenium. The medium was removed after 24 h and the cells were successively rinsed with Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO), Ampuwa, and 0.1 mM 2,3-dimercapto-1-propanesulfonic acid sodium salt monohydrate (DMPS, Alfa Aesar, Karlsruhe, Germany, purity 95%) according to a published procedure (Hippler et al. 2011) to ensure the absence of extracellular selenium. Cells were trypsinized, collected in 2 mL DPBS, and mechanically lysed using glass beads (Retsch, Haan, Germany). Selenium uptake after 24 h incubation was additionally quantified in HepG2 cells grown in NEAA-free medium and in T24 cells grown in medium supplemented with NEAA. Total selenium concentrations in the cell lysates were determined without any further dilution by ICP-MS.

Statistical analyses

Separate two-way analyses of variance (ANOVA) were used to test for significant differences in the effects of incubation time and NEAA addition on cell viability curves of the two different selenium species. All data met assumptions of homogeneity of variance and normal distribution. Statistical analyses were performed with SPSS version 21.0 (IBM, Armonk, New York, USA).

Results

Comparing the three investigated cell lines - HepG2, A375, and T24 cells -, it becomes obvious that only HepG2 cells showed the expected trend of selenosulfate being more toxic than selenite (Fig. 1). The IC_{50} values were 7.1 or 7.8 µM and > 15 µM for selenosulfate and selenite, respectively, for a 24 h exposure (Table 1). The cell viability curves showed significant differences for all selenium concentrations ≥ 3 µM with maximal differences up to 50%. For A375 cells, exposure to selenosulfate and selenite resulted in comparable cell viability curves. Slight differences were observed for the calculated IC_{50} values (6.6 µM for selenosulfate and 4.7 µM for selenite, respectively, Table 1) due to large standard deviations in the mid concentration range. For T24 cells, the cytotoxicity of selenosulfate was lower than that of selenite (IC_{50} values 6.9 or 7.0 and 3.5 µM, Table 1) with maximal differences up to 45%. It is interesting to note that the cytotoxicity of selenosulfate was rather similar for all three cell lines while the cytotoxicity for selenite increased in the order HepG2 < A375 < T24 cells (Fig. 1).

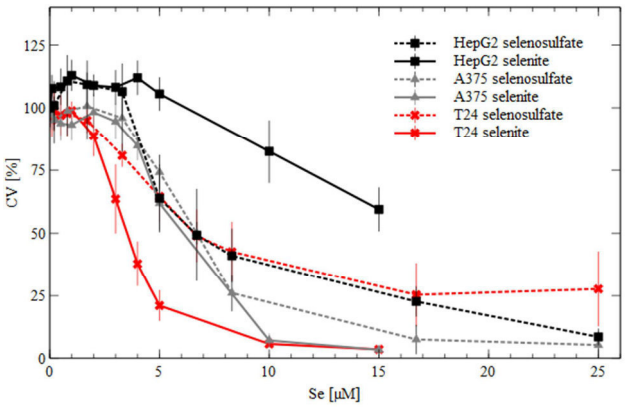


Figure 1: Cell viabilities (CVs) [%] of HepG2, A375, and T24 cells after 24 h incubation with selenosulfate (0.17-25 µM) and selenite (0.1-15 µM Se), respectively; CVs were determined by MTT assay

For the two cell lines with opposing trends of selenosulfate-selenite toxicity, HepG2 and T24 cells, more detailed investigations were done varying exposure time (24, 48, and 72 h) and in the presence or absence of NEAA. For easier comparison, Fig. 2 (species comparison), Fig. 3 (time comparison), and Fig. 4 (cell comparison) present essentially the same 24 cell viability curves derived from different combinations of these variables.

Looking at the species comparison graph (Fig. 2) it becomes obvious that for HepG2 cells, selenosulfate was always more toxic than selenite. The addition of NEAA had no significant effect on selenosulfate cytotoxicity ($p > 0.19$ for all incubation times, Table SI-1) and only significant influence on selenite cytotoxicity after 48 and 72 h ($p < 0.001$, Table SI-2). For T24 cells, addition of NEAA had an important effect: While selenosulfate curves were almost identical with (IC_{50} 6.9/7.0 µM) or without NEAA (IC_{50} 6.6 µM), selenite became much less toxic in the presence of NEAA (IC_{50} 8.4 µM compared to IC_{50} 3.5 µM in the absence on NEAA). In fact, with the addition of NEAA, selenite was less toxic than selenosulfate for T24 cells just as observed for HepG2 cells. Over time (48 and 72 h versus 24 h), the cell viability curves for both species became more similar, essentially due to a stronger increase in toxicity for selenite and a less pronounced increase in toxicity for selenosulfate (Fig. 2 c-f). After 72 h, IC_{50} values were nearly identical with 4.3 µM for selenosulfate and 4.2 µM for selenite for HepG2 cells and 1.0 µM for both selenosulfate and selenite in T24 cells. The absence of NEAA increased cytotoxicity for both species in both cell lines with IC_{50} values being lower than in the presence of NEAA (Table 1) (exception for HepG2 cells after 24 h incubation).

Fig. 3 shows nicely the time effect, again. Cell viability curves for each selenium species are presented separately for HepG2 and T24 cells. While for selenosulfate the cell viability curves for 24, 48, and 72 h were quite close together for both cell lines (Fig. 3 c, d), there was a significant difference be

tween 24 and 48 h ($p < 0.001$, Table SI-2) as well as between 48 and 72 h ($p < 0.002$) independent of NEAA absence or presence for selenite in HepG2 cells (Fig. 3 a). The selenite toxicity in HepG2 cells after 24 h of exposure was remarkably low with cell viabilities of ≥ 67% even at concentrations of 15 µM. In T24 cells, selenite after 24 h exposure in the presence of NEAA was the curve most prominently distinct from all others (Fig. 3 b).

Comparing the general susceptibility of HepG2 versus T24 cells shows that HepG2 cells are more robust for both species and over all exposure times (Fig. 4). It is, however, also obvious that this difference is even more pronounced for selenite than for selenosulfate. Fig. 4 also shows again that addition of NEAA has a larger effect on selenite compared to selenosulfate, especially for short-term exposure (24 h) and the more susceptible T24 cells (Fig. 4 a).

In contrast to what was observed for HepG2 and T24 cells, no cellular Se retention could be determined for A375 cells after 24 h exposure to 1 µM selenite or 1.7 µM selenosulfate as there was no difference between intracellular selenium concentrations of non-treated A375 control cells (0.73 ng/10^6 cells) or A375 cells exposed to selenosulfate (0.65 ng/10^6 cells) or selenite (0.71 ng/10^6 cells) (Table 2). However, exposure to low Se concentrations (< 5 µM) were associated with cytotoxic effects that were even more pronounced than in HepG2 cells (Fig. 1, Table 1).

For HepG2 and T24 cells, intracellular selenium concentrations were above those of non-treated control cells and there was a significant difference for the two selenium species. For selenite, uptake was always greater in T24 cells than in HepG2 cells. The addition of NEAA enhanced the difference between the two cell lines with 8.5 ng/10^6 cells in T24 cells in the absence of NEAA compared to only 2.7 ng/10^6 cells in HepG2 cells in the presence of NEAA. The order of increasing intracellular concentrations corresponds to the increasing toxicity and decrea-sing IC_{50} values (Fig. 4 a). While differences in

intracellular concentrations were small (maximum differences 8.5-2.7 = 5.8 ng/10^6 cells), differences in IC_{50} values were large (> 11.5 µM) (Table 1). For selenosulfate, intracellular concentrations showed a wider range between the two cell lines with the lowest concentrations of 1.5 ng/10^6 cells in HepG2 cells, interestingly also in the presence of NEAA. Uptake in the absence of NEAA was surprisingly lower (3.2/2.5 ng/10^6 cells). The wider range of intracellular selenium after selenosulfate exposure (maximum difference 23.2-1.5 = 21.7 ng/10^6 cells) is not reflected in the quite comparable cell viability curves (maximum difference of IC_{50} values 13.8-6.6 = 7.2 µM) (Fig. 4 b, Table 1). If we compare the effects in the "routinely" applied media (i.e. with NEAA for HepG2 and without NEAA for T24 cells), intracellular selenite concentrations were lower (2.7 versus 8.5 ng/10^6 cells) in HepG2 than in T24 cells.

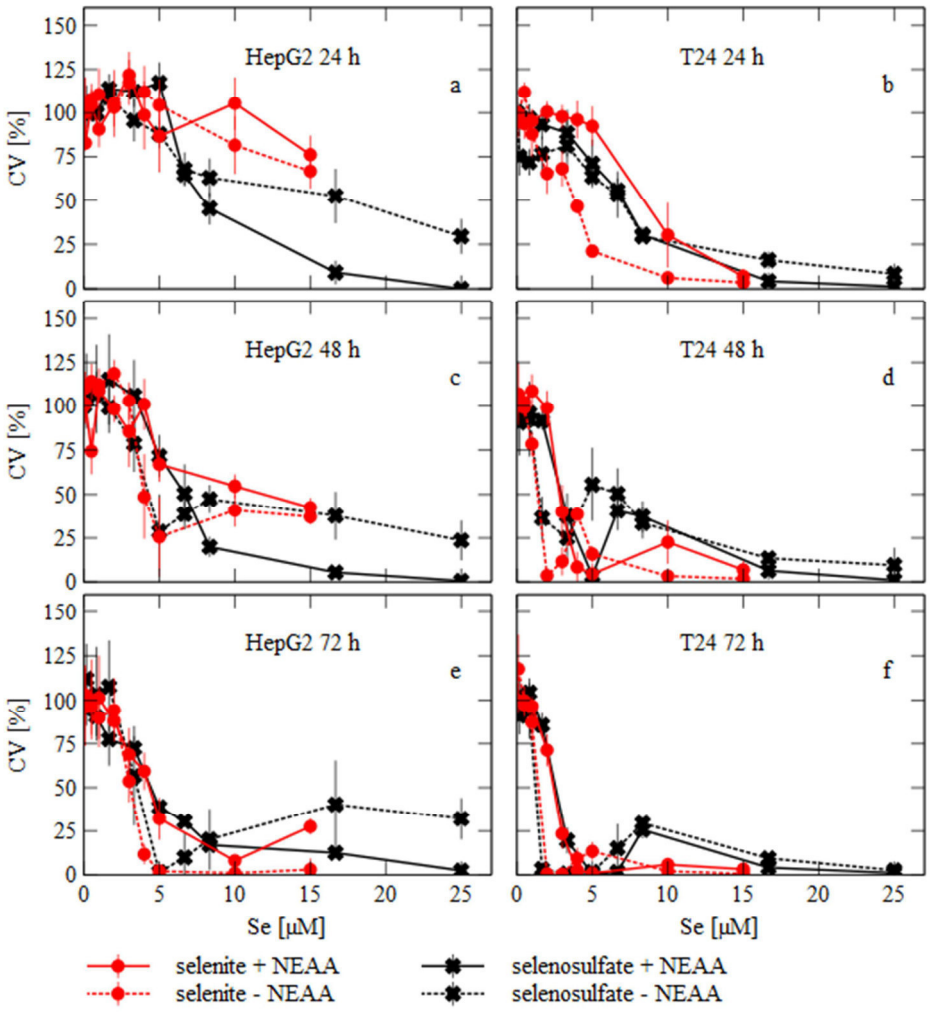


Figure 2: Comparison of selenosulfate and selenite: Cell viabilities (CVs) after selenosulfate (0.17-25 µM) or selenite incubation (0.1-15 µM) in HepG2 (a, c, e) and T24 cells (b, d, f) after 24 h (a, b), 48 h (c, d), and 72 h (e, f); in addition to treatment with standard growth media, HepG2 cells were cultivated and incubated in growth medium without NEAA, T24 cells were cultivated and incubated in growth medium with NEAA; CVs were determined by MTT assay

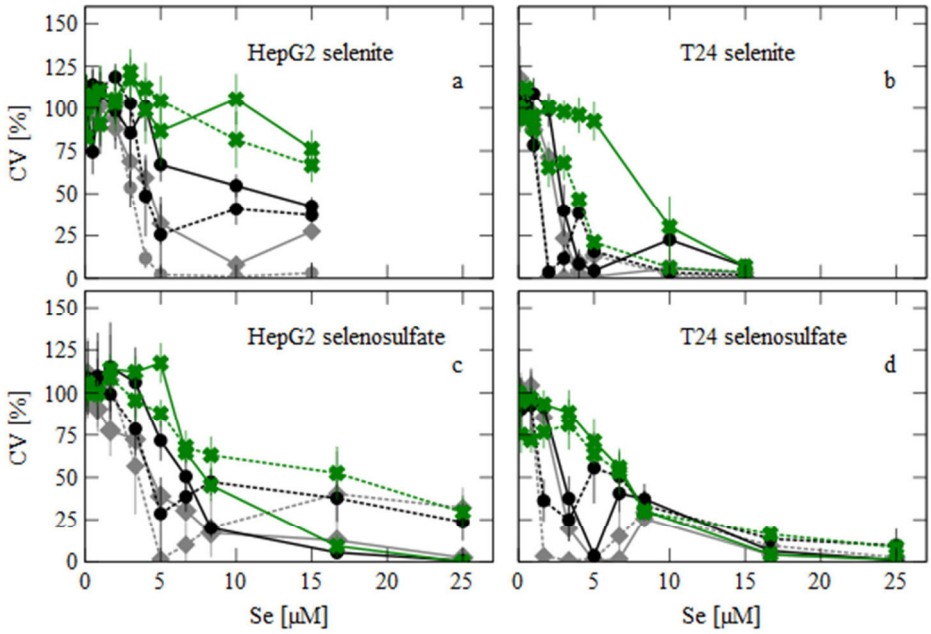


Figure 3: Comparison of incubation times: Cell viabilities (CVs) after 24 h, 48 h, and 72 h incubation with 0.17-25 µM selenosulfate or 0.1-15 µM selenite in HepG2 (a, c) and T24 cells (b, d); in addition to treatment with standard growth media, HepG2 cells were cultivated and incubated in growth medium without NEAA, T24 cells were cultivated and incubated in growth medium with NEAA; CVs were determined by MTT assay

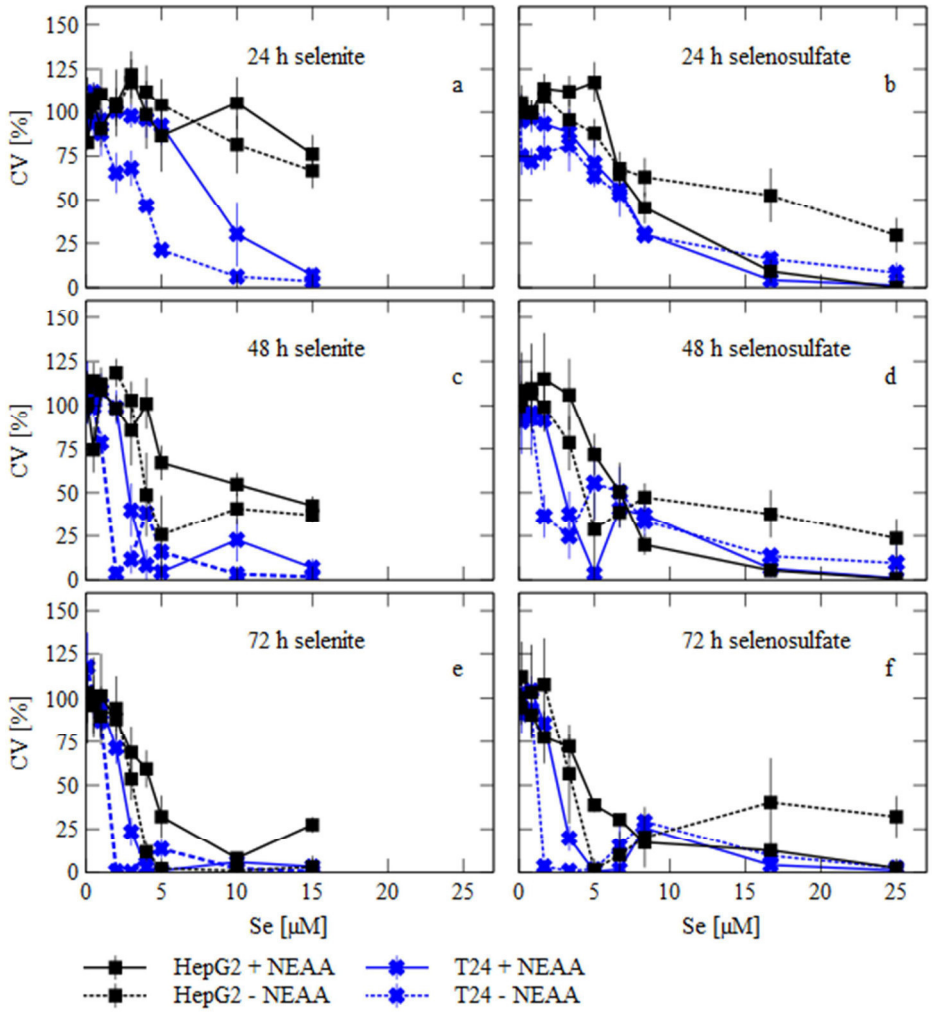


Figure 4: Comparison of HepG2 and T24 cells: Cell viabilities (CVs) after 24 h (a, b), 48 h (c, d), and 72 h (e, f) incubation with 0.17-25 µM selenosulfate (b, d, f) or 0.1-15 µM selenite (a, c, e); in addition to treatment with standard growth media, HepG2 cells were cultivated and incubated in growth medium without NEAA, T24 cells were cultivated and incubated in growth medium with NEAA; CVs were determined by MTT assay

Table 1: IC_{50} values for HepG2, A375, and T24 cells exposed to selenosulfate (0.17-25 µM) or selenite (0.1-15 µM) for 24, 48, and 72 h; 24 h experiments with HepG2 and T24 cells were conducted twice (I and II); growth conditions were changed to test the influence of non-essential amino acids (NEAA), i.e. HepG2 cells were grown without NEAA, T24 with NEAA addition

IC_{50} [µM]		24 h (I)	24 h (II)	48 h	72 h
HepG2 + NEAA	selenosulfate	7.1	7.8	6.0	4.3
	selenite	>15	>15	11.7	4.2
HepG2 - NEAA	selenosulfate		13.8	4.2	3.4
	selenite		>15	4.2	3.0
A375 (no NEAA)	selenosulfate	6.6			
	selenite	4.7			
T24 + NEAA	selenosulfate		6.6	2.8	2.4
	selenite		8.4	2.9	2.4
T24 - NEAA	selenosulfate	6.9	7.0	1.5	1.0
	selenite	3.5	3.5	1.0	1.0

Table 2: Intracellular selenium content [ng Se/10^6 cells/µM of applied Se] in HepG2, A375, and T24 cells; cells were incubated with 1.7 µM selenosulfate or 1 µM selenite for 24 h prior to mechanical lysis and analysis of total selenium content of the cell lysates by ICP-MS. Growth conditions were changed to test the influence of non-essential amino acids (NEAA), i.e. HepG2 cells were grown without NEAA, T24 with NEAA addition

intracellular Se [ng/10^6 cells/µM of applied Se]	+ NEAA	- NEAA
HepG2 selenosulfate	23.2	3.2/2.5*
HepG2 selenite	2.7	4.5/4.0*
HepG2 without selenium addition (control)	1.5	
A375 selenosulfate		0.65
A375 selenite		0.71
A375 without selenium addition (control)		0.73
T24 selenosulfate	1.7/1.5*	12.9
T24 selenite	6.2/2.8*	8.5
T24 without selenium addition (control)		0.5

*Experiments were conducted in duplicate

Discussion

Based on the results of our study we have to caution that the claim of patent US 2010/0172822 that "sodium selenosulfate had a much stronger cytotoxicity to tumor cells than sodium selenite" (Zhang 2010) cannot be generalized. While selenosulfate was more toxic than selenite in previous cytotoxicity tests with tumor cells of the liver (HepG2), the intestine (Caco-2), and the blood (HL60, T lymph adenoma, Daudi) (Zhang et al. 2008a), we showed that for tumor cells of the skin (A375) and the bladder (T24) this is not the case.

Our study showed that the selenosulfate toxicity was similar between HepG2, A375, and T24 cells (Fig. 1) and largely independent of exposure time or absence and presence of amino acids, despite great variations in intracellular concentrations. This is in contrast to a previous study (Olm et al. 2009) where selenium uptake was found to determine its extent of cytotoxicity in different cell lines. What changed among the three cell lines was the toxicity of selenite, which was lower than that of selenosulfate only for HepG2 cells, but similar to and higher than that of selenosulfate for A375 and T24 cells, respectively.

HepG2 cells were generally the least susceptible cell line among the three cell lines tested, which is in accordance with previous observations from our own studies on cytotoxicity of arsenite and thioarsenates ($AsS_{4-n}O_n^{3-}$) (Hinrichsen et al. 2014). Especially at short exposure times, selenite toxicity to HepG2 cells was remarkably low, both compared to selenosulfate in HepG2 cells but also to selenite toxicity in A375 and T24 cells. Over time, selenite toxicity increased much more than that of selenosulfate and for longer exposure times, toxicities for both species became similar. In this context it is interesting to note that the cytotoxicities that Zhang et al. (2008) measured after 72 h for HepG2 cells were more comparable to what we measured after 24 h already. In the present study 5 µM selenosulfate resulted in 117% CV and 5 µM selenite resulted in 87% CV. In the study from Zhang et al. (2008) 6 µM selenosulfate resulted in 80% CV and 6 µM selenite resulted in 90% CV after 72 h. The difference between both studies is that Zhang et al. (2008) used fresh growth medium for Se incubation whereas we already grew HepG2 cells in the medium 24 h before selenium incubation to maintain the routine of fresh medium supply as applied during the cell cultivation. It thus seems that the large difference between selenosulfate and selenite toxicity is only a short-term effect, observable under optimum conditions - short exposure (24 h in our case) or fresh medium (Zhang et al. 2008) - for this specific cell line. An obvious explanation might be the very low selenite uptake compared to selenosulfate uptake in HepG2 or to selenite uptake in T24 cells. Amino acids had little short-term effect on either selenite or selenosulfate cytotoxicity in HepG2 cells but decreased cytotoxicity slightly for longer exposure times.

For T24 cells, we observed the opposite effect as for HepG2 cells that selenite was more toxic than selenosulfate. Small increases in intracellular selenite concentrations had a much higher effect on increasing cytotoxicity both in comparison to selenite in HepG2 cells as well as to selenosulfate in T24 cells. Over time, the difference between selenite and selenosulfate decreased. The addition of NEAA actually even reversed the order of toxicity and decreased cellular uptake of selenite. The strong effect that NEAA had on T24 cells, but much less so on HepG2 cells, could be explained by an effect on the cellular transport system x_c^-. Cellular uptake of selenite was previously shown to be highly dependent of the cellular transport system x_c^-. Inhibition of the x_c^- system decreases the extracellular cysteine concentration and therefore, less selenite is reduced to HSe^- leading to decreased uptake and cytotoxicity (Olm et al. 2009). One amino acid that was contained in the NEAA mixture we applied and for which an effect had previously been shown (Olm et al. 2009) is L-glutamic acid. In the form of its salt monosodium glutamate it had been shown to prevent toxic effects of selenite in lung tumor cells by inhibiting cystine transport in the cells (dependent on the x_c^- transport system) in a competitive manner (Gout et al. 1997, Olm et al. 2009). The x_c^- transport system was reported to be more expressed in selenite-sensitive compared to selenite-resistant lung tumor cells (Olm et al. 2009). The higher susceptibility of T24 compared to HepG2 cells and the higher selenite uptake in T24 cells compared to HepG2 cells after 24 h, let us conclude that the x_c^- transport system is more expressed in T24 than in HepG2 cells and that this is the reason for the stronger effect of presence and absence of NEAA in T24 cells.

In contrast to selenite, selenosulfate cytotoxicity seemed to be less affected by changes in the x_c^- transport system. This could be explained by facilitated uptake because of its reduced state. Compared to selenite with an oxidation state of +4, selenosulfate has an oxidation state of -1. Furthermore, selenosulfate is synthesized from glutathione and sulfite and non-reacted excess of any of these two compounds creates a reductive extracellular microenvironment (Olm et al. 2009). Thiols of excess GSH could also facilitate selenium uptake (Bannai 1986) by formation of further reduced selenium forms in addition to the formed selenosulfate (Ganyc &Self 2008), e.g. selenotrisulfide, selenopersulfide, and hydrogen selenide (Bannai 1986). Transport of these reduced compounds could occur through anion channels of the plasma membrane as reported previously (Conrad &Sato 2012).

Another observation from our study where the growth medium seemed to have governed the toxicity of selenium species for the respective cell line, is A375. In contrast to the other two cell lines, intracellular concentrations after both selenite and selenosulfate incubation were not distinguishable from untreated control cells, but caused comparable toxicities as in HepG2 and T24 cells which indicated that A375 cells were especially susceptible to selenium. A potential reason could be that in contrast to HepG2 and T24 cells, A375 cells were cultivated in DMEM medium, which contained about twice as much L-cystine compared to MEM medium. We hypothesize that the presence of extracellular cystine changes the GSH/GSSG ratio, resulting in a decreased defense against oxidative stress produced by selenium. Increased toxicity of oxidative stress producing H_2O_2 in the presence of high extracellular cystine levels was already shown for *E.coli* (Smirnova et al. 2005).

Experiments with human tumor cell lines are a basic preclinic test in terms of anti-cancer drug permission. As already described, compositions of cell growth media differ between laboratories leading to limited comparability of cellular uptake and cytotoxicity values of substances. The results of our study showed that the absence or presence of only one medium supplement - NEAA - can completely change the interpretation of the cytotoxicity for a specific substance.

Conclusion

The claim that selenosulfate is more toxic than selenite for tumor cells and this could be an additional reason for its preferred application in anti-cancer therapy compared to selenite, cannot be generalized. While this has been demonstrated for HepG2 cells, our cell culture study showed that there are cells for which selenite is at least as toxic (A375 cells) or more toxic (T24 cells) than selenosulfate. Our cell culture studies further showed that the choice of growth medium significantly influences the outcome of cytotoxicity data. Uptake of selenite was shown to be down-regulated by the presence of NEAA, especially in selenium-sensitive cells, most likely because L-glutamic acid inhibited the x_c^- transport system. The uptake of selenosulfate, on the other hand, was generally facilitated by it being the more reduced species. We conclude that whether selenite or selenosulfate is more toxic to a specific tumor cell line depends largely on the sensitivity of each cell line as well as the reductive state of the extracellular environment. The study shows the importance of considering the effects supplements in cell growth media might have on uptake and cytotoxicity of different compounds for individual cell lines.

Supplemental Data

ANOVA results for selenosulfate and selenite showing which cell viability curves are significantly different from each other with regard to cell type, exposure time, and presence or absence of NEAA are available in Online Resource Table SI-1 and SI-2.

Acknowledgement

We acknowledge generous funding by the German Research Foundation within the Emmy Noether program to B. Planer-Friedrich (grant # PL 302/3-1) and thank Franziska Geist for help in the laboratory, Stefan Will for help with AEC-ICP-MS analyses, as well as Prof. Dr. Elke Dopp for providing us HepG2 and T24 cells.

References

Baldew GS, Vandenhamer CJA, Los G, Vermeulen NPE, Degoeij JJM, McVie JG (1989): Selenium-induced protection against cis-diamindichloroplatinum(II) nephrotoxicity in mice and rats. Cancer Research 49, 3020-3023

Bannai S (1986): Exchange of Cystine and Glutamate across Plasma Membrane of Human Fibroblasts. Journal of Biological Chemistry 261, 2256-2263

Brinkman M, Buntinx F, Muls E, Zeegers MP (2006): Use of selenium in chemoprevention of bladder cancer. Lancet Oncology 7, 766-774

Camargo SMR, Francescato HDC, Lavrador MAS, Bianchi MLP (2001): Oral administration of sodium selenite minimizes cisplatin toxicity on proximal tubules of rats. Biol. Trace Elem. Res. 83, 251-262

Chen T, Wong YS (2008): Selenocystine induces apoptosis of A375 human melanoma cells by activating ROS-mediated mitochondrial pathway and p53 phosphorylation. Cellular and Molecular Life Sciences 65, 2763-2775

Chen T, Wong YS, Zheng W, Bai Y, Huang L (2008): Selenium nanoparticles fabricated in Undaria pinnatifida polysaccharide solutions induce mitochondria-mediated apoptosis in A375 human melanoma cells. Colloids and Surfaces B-Biointerfaces 67, 26-31

Chen XJ, Duan FD, Zhang HH, Xiong Y, Wang J (2012): Sodium Selenite-Induced Apoptosis Mediated by ROS Attack in Human Osteosarcoma U2OS Cells. Biol. Trace Elem. Res. 145, 1-9

Chu H-A, Crawford-Brown DJ (2006): Inorganic arsenic in drinking water and bladder cancer: a meta-analysis for dose-response assessment. Int J Environ Res Public Health 3, 316-22

Conrad M, Sato H (2012): The oxidative stress-inducible cystine/glutamate antiporter, system x(c)(-): cystine supplier and beyond. Amino Acids 42, 231-246

Dennert G, Horneber M (2006): Selenium for alleviating the side effects of chemotherapy, radiotherapy and surgery in cancer patients. The Cochrane database of systematic reviews, CD005037

Ganyc D, Self WT (2008): High affinity selenium uptake in a keratinocyte model. Febs Letters 582, 299-304

Gout PW, Kang YJ, Buckley DJ, Bruchovsky N, Buckley AR (1997): Increased cystine uptake capability associated with malignant progression of Nb2 lymphoma cells. Leukemia 11, 1329-1337

Hinrichsen S, Lohmayer R, Zdrenka R, Dopp E, Planer-Friedrich B (2014): Effect of sulfide on the cytotoxicity of arsenite and arsenate in human hepatocytes (HepG2) and human urothelial cells (UROtsa). Environmental Science and Pollution Research 21, 10151-10162

Hippler J, Zdrenka R, Reichel RAD, Weber DG, Rozynek P, Johnen G, Dopp E, Hirner AV (2011): Intracellular, time-resolved speciation and quantification of arsenic compounds in human urothelial and hepatoma cells. Journal of Analytical Atomic Spectrometry 26, 2396-2403

Hogberg J, Kristoferson A (1979): Inhibition of amino-acid uptake in isolated rat hepatocytes by selenite. Febs Letters 107, 77-80

Johnen G, Rozynek P, von der Gathen Y, Bryk O, Zdrenka R, Johannes C, Weber DG, Igwilo-Okuefuna OB, Raiko I, Hippler J, Bruning T, Dopp E (2013): Cross-Contamination of a UROtsa Stock with T24 Cells - Molecular Comparison of Different Cell Lines and Stocks. Plos One 8, e64139-e64139

Kellen E, Zeegers M, Buntinx F (2006): Selenium is inversely associated with bladder cancer risk: A report from the Belgian case-control study on bladder cancer. International Journal of Urology 13, 1180-1184

Kim EH, Sohn S, Kwon HJ, Kim SU, Kim M-J, Lee S-J, Choi KS (2007): Sodium selenite induces superoxide-mediated mitochondrial damage and subsequent autophagic cell death in malignant glioma cells. Cancer Research 67, 6314-6324

Li G-X, Hu H, Jiang C, Schuster T, Lu J (2007): Differential involvement of reactive oxygen species in apoptosis induced by two classes of selenium compounds in human prostate cancer cells. International Journal of Cancer 120, 2034-2043

Li J, Sun K, Ni L, Wang X, Wang D, Zhang J (2012): Sodium selenosulfate at an innocuous dose markedly prevents cisplatin-induced gastrointestinal toxicity. Toxicol. Appl. Pharmacol. 258, 376-383

Lo M, Wang Y-Z, Gout PW (2008): The x(c)(-) cystine/glutamate antiporter: A potential target for therapy of cancer and other diseases. Journal of Cellular Physiology 215, 593-602

Markovic SD, Djacic DS, Cvetkovic DM, Obradovic AD, Zizic JB, Ognjanovic BI, Stajn AS (2011): Effects of Acute In vivo Cisplatin and

Selenium Treatment on Hematological and Oxidative Stress Parameters in Red Blood Cells of Rats. Biol. Trace Elem. Res. 142, 660-670

McKelvey SM, Horgan KA, Murphy RA (2015): Chemical form of selenium differentially influences DNA repair pathways following exposure to lead nitrate. Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements (GMS) 29

Micke O, Schomburg L, Buentzel J, Kisters K, Muecke R (2009): Selenium in Oncology: From Chemistry to Clinics. Molecules 14, 3975-3988

Olm E, Fernandes AP, Hebert C, Rundlof AK, Larsen EH, Danielsson O, Bjornstedt M (2009): Extracellular thiol-assisted selenium uptake dependent on the x(c)(-) cystine transporter explains the cancer-specific cytotoxicity of selenite. Proc. Natl. Acad. Sci. U. S. A. 106, 11400-11405

Park JS, Ryu JY, Jeon HK, Cho YJ, Park YA, Choi JJ, Lee JW, Kim BG, Bae DS (2012a): The effects of selenium on tumor growth in epithelial ovarian carcinoma. J. Gynecol. Oncol. 23, 190-196

Park SH, Kim JH, Chi GY, Kim GY, Chang YC, Moon SK, Nam SW, Kim WJ, Yoo YH, Choi YH (2012b): Induction of apoptosis and autophagy by sodium selenite in A549 human lung carcinoma cells through generation of reactive oxygen species. Toxicology Letters 212, 252-261

Peng DG, Zhang JS, Liu QL (2007): Effect of sodium selenosulfate on restoring activities of selenium-dependent enzymes and selenium retention compared with sodium selenite in vitro and in vivo. Biol. Trace Elem. Res. 117, 77-88

Planer-Friedrich B, London J, McCleskey RB, Nordstrom DK, Wallschlager D (2007): Thioarsenates in geothermal waters of yellowstone national park: Determination, preservation, and geochemical importance. Environmental Science & Technology 41, 5245-5251

Shen HM, Yang CF, Ong CN (1999): Sodium selenite-induced oxidative stress and apoptosis in human hepatoma HepG(2) cells. International Journal of Cancer 81, 820-828

Smirnova GV, Muzyka NG, Oktyabrsky ON (2005): Effects of cystine and hydrogen peroxide on glutathione status and expression of antioxidant genes in Escherichia coli. Biochemistry-Moscow 70, 926-934

Vernie LN, Bont WS, Emmelot P (1974): Inhibition of in-vitro amino-acid incorporation by sodium selenite. Biochemistry 13, 337-341

Vinceti M, Wei ET, Malagoli C, Bergomi M, Vivoli G (2001): Adverse health effects of selenium in humans. Reviews on environmental health 16, 233-51

Vinceti M, Dennert G, Crespi CM, Zwahlen M, Brinkman M, Zeegers MPA, Horneber M, D'Amico R, Del Giovane C (2014): Selenium for preventing cancer. Cochrane Database of Systematic Reviews

Zhang J, Lu H, Wang X (2008a): MS07116 sodium selenosulfate synthesis and demonstration of its in vitro cytotoxic activity against HepG2, caco2, and three kinds of leukemia cells. Biol. Trace Elem. Res. 125, 13-21

Zhang J (2010): Use of sodium selenosulfate for supplementing selenium and anhancing the therapeutic efficiacy of chemotherapy agents, and a rapid process for preparing sodium sulfate.

Zhang JZ, Peng DG, Lu HJ, Liu QL (2008b): Attenuating the toxicity of cisplatin by using selenosulfate with reduced risk of selenium toxicity as compared with selenite. Toxicol. Appl. Pharmacol. 226, 251-259

Zou Y, Niu P, Gong Z, Yang J, Yuan J, Wu T, Chen X (2007): Relationship between reactive oxygen species and sodium-selenite-induced DNA damage in HepG2 cells. Frontiers of medicine in China 1

Supplemental Data

Article title: Cytotoxic activity of selenosulfate versus selenite in tumor cells depends on cell line and presence of amino acids

Journal name: Environmental Science and Pollution Research

Authors: Sinikka Hinrichsen[†] and Britta Planer-Friedrich[†]*

[†] Environmental Geochemistry, Bayreuth Center for Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany

* Corresponding author at: Environmental Geochemistry, Faculty of Biology, Chemistry, and Earth Sciences, University of Bayreuth, Universitaetsstrasse 30, 95447 Bayreuth, Germany. Tel.: +49 921 55 3999; fax: +49 921 55 2334.

E-Mail address: b.planer-friedrich@uni-bayreuth.de

Table SI-1 ANOVA results for selenosulfate showing which cell viability curves are significantly ($p < 0.05$) different from each other with regard to cell type, exposure time, and presence or absence of NEAA

significance level p*			HepG2						T24					
			-NEAA			+NEAA			-NEAA			+NEAA		
			24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
HepG2	-NEAA	24 h		**< 0.001**		0.193			**< 0.001**					
		48 h	**< 0.001**		**0.049**		(0.322)			0.177				
		72 h		**0.049**				(0.867)			**< 0.001**			
	+NEAA	24 h	0.193				0.328					**0.006**		
		48 h		(0.322)		0.328		**0.026**					(< 0.001)	
		72 h			(0.867)		**0.026**							**0.004**
T24	-NEAA	24 h	**< 0.001**							0.170		0.495		
		48 h		0.177					0.170		**< 0.001**		**0.017**	
		72 h			**< 0.001**					**< 0.001**				0.056
	+NEAA	24 h				**0.006**			0.495				**< 0.001**	
		48 h					**(< 0.001)**			**0.017**		**< 0.001**		0.165
		72 h						**0.004**			0.056		0.165	

*Significant p values are marked in bold; p values presented in parentheses indicate significant differences between two intersected cell viability curves, which did not derive from incubation time or addition of NEAA. All experiments were conducted as triplicate with six equally treated wells per replicate, i.e. n = 18 for ANOVA analysis.

Table SI-2 ANOVA results for selenite showing which cell viability curves are significantly ($p < 0.05$) different from each other with regard to cell type, exposure time, and presence or absence of NEAA

significance level p*			HepG2						T24					
			-NEAA			+NEAA			-NEAA			+NEAA		
			24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
HepG2	-NEAA	24 h		< 0.001		**0.005**			**< 0.001**					
		48 h	**< 0.001**		**< 0.001**		**< 0.001**			**< 0.001**				
		72 h		**< 0.001**				**< 0.001**			(0.008)			
	+NEAA	24 h	**0.005**				**< 0.001**					**< 0.001**		
		48 h		**< 0.001**		**< 0.001**		**0.002**					**< 0.001**	
		72 h			**< 0.001**		**0.002**							**< 0.001**
T24	-NEAA	24 h	**< 0.001**							**< 0.001**		**< 0.001**		
		48 h		**< 0.001**					**< 0.001**		0.259		**< 0.001**	
		72 h			(0.008)					0.259				**< 0.001**
	+NEAA	24 h				**< 0.001**			**< 0.001**				**< 0.001**	
		48 h					**< 0.001**			**< 0.001**		**< 0.001**		**0.043**
		72 h						**< 0.001**			**< 0.001**		**0.043**	

*Significant p values are marked in bold; p values presented in parentheses indicate significant differences between two intersected cell viability curves, which did not derive from incubation time or addition of NEAA. All experiments were conducted as triplicate with six equally treated wells per replicate, i.e. n = 18 for ANOVA analysis.

(Eidesstattliche) Versicherungen und Erklärungen

(§ 5 Nr. 4 PromO)

Hiermit erkläre ich, dass keine Tatsachen vorliegen, die mich nach den gesetzlichen Bestimmungen über die Führung akademischer Grade zur Führung eines Doktorgrades unwürdig erscheinen lassen.

(§ 8 S. 2 Nr. 5 PromO)

Hiermit erkläre ich mich damit einverstanden, dass die elektronische Fassung meiner Dissertation unter Wahrung meiner Urheberrechte und des Datenschutzes einer gesonderten Überprüfung hinsichtlich der eigenständigen Anfertigung der Dissertation unterzogen werden kann.

(§ 8 S. 2 Nr. 7 PromO)

Hiermit erkläre ich eidesstattlich, dass ich die Dissertation selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

(§ 8 S. 2 Nr. 8 PromO)

Ich habe die Dissertation nicht bereits zur Erlangung eines akademischen Grades anderweitig eingereicht und habe auch nicht bereits diese oder eine gleichartige Doktorprüfung endgültig nicht bestanden.

(§ 8 S. 2 Nr. 9 PromO)

Hiermit erkläre ich, dass ich keine Hilfe von gewerblichen Promotionsberatern bzw. -vermittlern in Anspruch genommen habe und auch künftig nicht nehmen werde.

……

Ort, Datum, Unterschrift