REVIEW ARTICLE

An update to the toxicological profile for water-soluble and sparingly soluble tungsten substances

Ranulfo Lemus[1] and Carmen F. Venezia[2]

[1]International Tungsten Industry Association (ITIA), London, UK and [2]Global Tungsten & Powders Corp., Towanda, PA, USA

Abstract

Tungsten is a relatively rare metal with numerous applications, most notably in machine tools, catalysts, and superalloys. In 2003, tungsten was nominated for study under the National Toxicology Program, and in 2011, it was nominated for human health assessment under the US Environmental Protection Agency's (EPA) Integrated Risk Information System. In 2005, the Agency for Toxic Substances and Disease Registry (ATSDR) issued a toxicological profile for tungsten, identifying several data gaps in the hazard assessment of tungsten. By filling the data gaps identified by the ATSDR, this review serves as an update to the toxicological profile for tungsten and tungsten substances. A PubMed literature search was conducted to identify reports published during the period 2004–2014, in order to gather relevant information related to tungsten toxicity. Additional information was also obtained directly from unpublished studies from within the tungsten industry. A systematic approach to evaluate the quality of data was conducted according to published criteria. This comprehensive review has gathered new toxicokinetic information and summarizes the details of acute and repeated-exposure studies that include reproductive, developmental, neurotoxicological, and immunotoxicological endpoints. Such new evidence involves several relevant studies that must be considered when regulators estimate and propose a tungsten reference or concentration dose.

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Address for correspondence: Ranulfo Lemus, International Tungsten Industry Association, 1st Floor 454–458 Chiswick High Road, London W4 5TT, UK. Tel: +44 20 8996 2221. E-mail: rlemus@itia.info

Introduction

Tungsten, also known as wolfram, is a rare metal with the chemical symbol W, atomic number 74, and molecular weight of 183.84. The most common formal oxidation state of tungsten is $+6$, but it exhibits all oxidation states from -2 to $+6$. Its important ores include wolframite and scheelite. The free element is remarkable for its robustness, especially because it has the highest melting point of all metals

$(3422 \pm 15°C)$. Tungsten and tungsten alloys have numerous applications, most notably in hard metals used in cutting and drilling tools, X-ray tubes (as both the filament and target), electrodes in welding, and superalloys. Because of tungsten's hardness and high density (19.3 times that of water), it has military applications in penetrating projectiles. Tungsten substances are often used industrially as catalysts.

The processing of tungsten from the raw materials into tungsten substances and finished products is depicted in Figure 1. Production starts with the dissolution of the tungsten ore concentrates wolframite and scheelite by alkaline pressure digestion, using either sodium carbonate or a concentrated sodium hydroxide solution. The sodium tungstate (ST) solution obtained is purified before it is converted into ammonium paratungstate (APT) of high purity. A common secondary source of tungsten is tungsten-bearing scrap, such as used drill bits and tools, which is oxidized and then chemically digested to also form APT, which is the main tungsten substance traded in the market and the main tungsten intermediate. APT is usually calcined to tungsten trioxide (WO_3; tungsten yellow oxide) or tungsten oxides (WO_{3-x}; tungsten blue oxide), which are not defined chemical substances but rather a mixture of oxide species including WO_3, $W_{20}O_{58}$, $W_{18}O_{49}$, tungsten bronze $[(NH_4)_nWO_3 (0.06 < n < 0.33)]$, and an amorphous material undefined by X-ray diffraction analysis. The oxygen index (molar ratio between oxygen and tungsten) depends on calcination parameters such as temperature, heating time, composition, atmospheric pressure, mass of APT flow with time, gas flow, layer height in the furnace, and furnace slope and rotation rate (Lassner and Schubert 1999).

WO_3 or WO_{3-x} can be reduced at 700–1000°C to tungsten metal (W-metal) powder by hydrogen. By reaction with pure carbon powder in a process called carburization, most of the W-metal powder is converted to tungsten monocarbide, which

is commonly known as tungsten carbide (WC). By mass, quantitatively, WC is the most important tungsten substance. Because of its hardness, it is the main constituent, together with cobalt, in cemented carbide (also known as hardmetal), which is a composite of tungsten carbide embedded in a matrix of metallic cobalt. By melting together W-metal and WC, a eutectic composition is formed of WC and ditungsten carbide (W_2C), also known as fused tungsten carbide or casted tungsten carbide.

The occurrence of a cluster of cases of acute childhood lymphocytic leukemia in Fallon, Nevada, prompted a wide investigation that involved several local, state, and federal agencies led by the Centers of Disease Control and Prevention (CDC) (CDC 2003). Groundwater is the source of drinking water in this community, and during this investigation, tungsten was detected in drinking water and in biological samples (Rubin et al. 2007). Principal sources of tungsten in ground water in this area of Nevada are natural and include erosion of tungsten-bearing mineral deposits (Seiler et al. 2005). Although no direct link between tungsten and the incidence of leukemia was found (CDC 2003, Rubin et al. 2007), in 2003, tungsten was selected for study under the National Toxicology Program (NTP), and in 2011, it was nominated by the US EPA for risk assessment under the Integrated Risk Information System (IRIS) program.

In 2005, the ATSDR issued its toxicological profile for tungsten (Keith et al. 2005, 2007), identifying several data gaps in areas such as acute inhalation, oral and dermal toxicities, skin and eye irritation, as well as dermal sensitization, toxicity due to repeated subchronic and chronic oral and inhalation exposure, genotoxicity, reproductive and developmental toxicity, neurotoxicity, and immunotoxicity. In addition, for some of the endpoints in the health-effect assessment (e.g. inhalation toxicity), the ATDSR included health effects that arise pre-
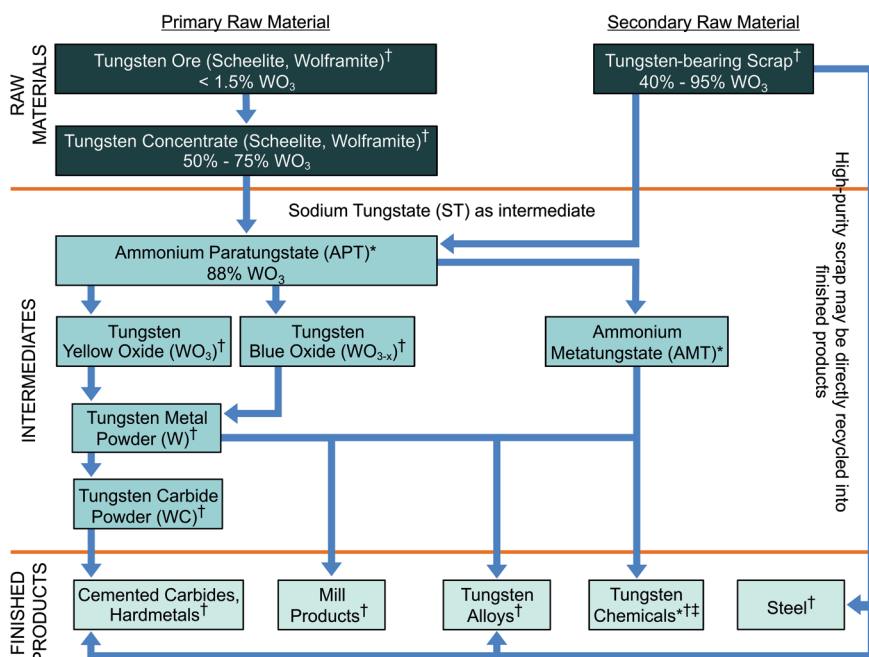
Figure 1. Flow chart depicting the processing from tungsten concentrates (raw materials) to tungsten intermediates or final products, each with different water solubility characteristics, ranging from (*) soluble (sodium tungstate, ammonium paratungstate, and ammonium metatungstate) to (†) sparingly soluble (tungsten yellow oxide, tungsten blue oxide, tungsten metal, and tungsten carbide). (‡) Tungsten chemicals include tungsten disulfide (WS_2), which is synthesized from tungsten metal and sulfur.

dominantly from occupational exposure to dust that includes particles of tungsten and other metals (particularly cobalt), at facilities in which hardmetal is produced.

To fill the data gaps identified in the 2005 ATSDR toxicological profile for tungsten (Keith et al. 2005, 2007), we reviewed the scientific research conducted since 2004 on water-soluble [e.g. ST, ammonium metatungstate (AMT), sodium metatungstate (SMT), and APT] and sparingly water-soluble [e.g. W-metal, WC, W_2C, WO_3, WO_{3-x}, and tungsten disulfide (WS_2)] tungsten substances. In addition, studies sponsored by the government and by the tungsten industry that are dated pre- and post-2005, which were not included in the ATSDR's toxicological assessment, were also reviewed, as they also help fill the data gaps identified in the ATSDR profile. Unlike the ATSDR profile, our review mainly considers relevant research on tungsten and tungsten substances, and it does not include research on alloys containing tungsten, such as hardmetal, or studies of exposure to tungsten with co-exposures to other metals.

To gather relevant information from the period of time between 2004 and 2014, a literature search was conducted. In addition, information relevant to fill gaps in the ATSDR profile was also directly obtained from the International Tungsten Industry Association (ITIA), as well as from US agencies. As recommended by the Health Risk Assessment Guidance for Metals (HERAG) (ICMM 2007), to assess study reliability, relevance, and adequacy for inclusion/exclusion, and as endorsed by the Organisation for Economic Co-operation and Development (OECD) in its Manual for Investigation of HPV Chemicals (OECD 2014) and the European Chemicals Agency's Registration, Evaluation, and Authorisation of Chemicals (REACH) program, the unpublished and peer-reviewed published research was evaluated according to the criteria established by Klimisch et al. (1997). In addition, this review is organized according to the toxicity endpoints delineated in Chapter 4 of HERAG (ICMM 2007).

Methodology

Literature search strategy

PubMed® (http://www.ncbi.nlm.nih.gov/pubmed) literature searches were conducted on tungsten and tungstate for the publication period of January 1, 2004 through May 19, 2014. In addition, endpoint gaps were filled with unpublished studies (dated pre- and post-2005) that were sponsored by the tungsten industry, and were conducted following OECD guidelines and using good laboratory practice (GLP). These studies were performed for hazard assessment at independent contract research laboratories, with

the objective of fulfilling global regulatory requirements. Several unpublished government-sponsored studies were also reviewed.

Assessment of reliability, relevance, and adequacy

A systematic approach to the evaluation of the quality of data and their use in hazard and risk assessment was described by Klimisch et al. (1997). In general, the more the details on methodology, test procedures, and analytics are documented, the easier an evaluation of their Klimisch reliability should be. The amount of information presented in the peer-reviewed publication or in the report of the study sponsored by the government or tungsten industry, provided the basis for deciding the reliability of the reported data according to the four scientific reliability categories (K1–K4), outlined in Table 1.

Regulatory status

Occupational exposure guidelines

The American Conference of Governmental Industrial Hygienists (ACGIH), the CDC's National Institute of Occupational Safety and Health (NIOSH), and the US Department of Labor's Occupational Safety and Health Administration (OSHA), have established occupational exposure limits for tungsten and tungsten substances. OSHA's current permissible exposure levels (PEL) and ACGIH's 8-hour threshold limit value (TLV)-time-weighted average (TWA) are 1 and 5 mg/m^3 for water-soluble and insoluble tungsten substances (as W), respectively (OSHA 2014a, OSHA 2014b). NIOSH's recommended exposure level (REL) 10-h TWA values for soluble and insoluble tungsten substances (as W) are 1 and 5 mg/m^3 respectively, with a 15-min short-term exposure level (STEL) of 3 and 10 mg/m^3, respectively (CDC 2010a, 2010b). OSHA PELs are intended to protect against the accumulation of insoluble tungsten substances in the lung and the acute effects of soluble substances on the central nervous system (metabolic poison) (OSHA 2014a, 2014b).

Drinking water standards

To date, the World Health Organization (WHO) has not issued any drinking water guidelines, whereas Russia has a limit of 0.05 mg/L for drinking water (Koutsospyros et al. 2006), and in the USA, the Commonwealth of Massachusetts has established action levels of 15 mg/L (ASTWSWMO 2008). The ATSDR toxicological profile (Keith et al. 2005, 2007) did not derive an oral minimum risk level for tungsten or tungsten substances, stating that adequate data were not available to derive this guidance.

Table 1. Categories for ranking the reliability of studies or data from the literature or reports, based on the approach described by Klimisch et al. (1997).

Reliability category	Description	Criteria for studies or data
K1	Reliable without restriction	Performed according to valid and/or nationally or internationally accepted testing guidelines, preferably conducted according to the principles of GLP
K2	Reliable with restrictions	Mostly not performed according to GLP, the test parameters do not totally comply with the specific testing guidelines, but are sufficient to accept the data. Such studies are well-documented and scientifically acceptable, and include positive and negative controls, well-described methodology, animal information, test substance purity, data on dose/concentrations, detailed results, etc.
K3	Not reliable	There are interferences between the measuring system and the test substance, or cases in which the test systems were not relevant in relation to the exposure, or were performed using an unacceptable testing method; documentation is not sufficient and not convincing.
K4	Not assignable	Does not provide sufficient experimental detail and was presented as an abstract or was listed in secondary literature

GLP = good laboratory practice

Toxicological studies

2005 ATSDR toxicological profile for tungsten data gap findings

Toxicological profiles were prepared in accordance with guidelines developed by the ATSDR and EPA and provide information briefly characterizing the toxic and adverse health effects of a hazardous substance. The ATSDR profile is not intended to be an exhaustive document; however, more comprehensive sources of specialty information are referenced.

The 2005 ATSDR toxicological profile for tungsten (Keith et al. 2005, 2007) indicated that relatively few reports were located regarding health effects in animals following acute-, intermediate-, or chronic-duration exposure to tungsten or tungsten substances. Several early Russian reports were located, mainly originating from a single group of investigators. The reports predominantly assessed acute lethality or reproductive or developmental effects following oral exposure to soluble tungsten substances. In addition, for some of the assessment endpoints (e.g. inhalation toxicity), the ATDSR included health effects that resulted mainly from occupational exposure to dusts that include particles of tungsten and other metals (particularly cobalt) at facilities in which hardmetal is produced.

The ATSDR's literature search comprised tungsten toxicity studies published between the years 1924 and 2004. Hence, our literature searches were conducted on tungsten and tungstate for the publication period from January 1, 2004 through May 19, 2014, with the objectives of updating and filling the knowledge gaps identified in the 2005 ATSDR toxicological profile, and not of repeating the review of the studies already considered by ATSDR. The tungsten toxicity information included in the ASTDR's profile that pre-dated our literature search is widely available for review, and therefore, will not be discussed herein. The ATSDR had recognized that the available information was not sufficient to establish minimal risk levels, and instead, identified several data gaps in the following areas:

– Acute inhalation, oral and dermal toxicities; skin and eye irritation; dermal sensitization
– Repeated subchronic and chronic oral and inhalation exposure toxicity; reproductive and developmental toxicity; neuro- and immuno-toxicities
– Carcinogenicity and genotoxicity

Several of these data gaps have subsequently been filled with recently published studies. Other gaps can be filled using the results of unpublished studies. All of these studies are discussed in the sections below.

Literature search results

Search strings, PubMed MeSH query translations, and returned results for tungsten and tungstate for the publication period from January 1, 2004 through May 19, 2014, are shown in Table 2. Several searches at the chemical and endpoint levels were conducted to gather a complete set of toxicity endpoints for sparingly water-soluble substances (represented by tungsten) and water-soluble substances (represented by tungstate). The various tungsten substances, their CAS Registry^{SM} numbers, and solubility of the substances in water are listed in Table 3.

Although a large number of studies were found from our PubMed search, only 95 peer-reviewed studies have been included herein, as they were considered relevant and reliable, with or without restriction (Klimisch reliability category K2 or K1, respectively, as outlined in Table 1).

A standard toxicity testing summary for water-soluble and sparingly water-soluble tungsten substances is presented in Table 4. Some of the endpoints presented in Table 4 were filled with unpublished studies (dated pre- and post-2005) that were sponsored by the tungsten industry or various government agencies. See the supplementary material for a summary of the details of the unpublished, industry- and government-sponsored studies that were reviewed, with hyperlinks to online reports (Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444. 2014.1003422), and the Klimisch reliability categories assigned to all 95 peer-reviewed and unpublished studies (Supplementary Table 2 to be found online at http://informa healthcare.com/doi/abs/10.3109/10408444.2014.1003422).

Toxicokinetics

The *in vivo* data show that in the case of oral exposure to water-soluble tungsten substances (e.g. ST), and inhalation exposure to sparingly soluble tungsten substances (e.g. WO_{3-x}), the substances can be absorbed, systemically distributed, and excreted (via the urine). *In vitro* studies suggest that sparingly water-soluble tungsten substances such as W-metal, WC, and W_2C are largely eliminated via the feces, following oral exposure. *In vitro* studies using artificial sweat fluid suggest that dermal exposure (or intact skin absorption) to water-soluble (ST, SMT, and APT) and sparingly soluble tungsten substances does not contribute appreciably to the amount present in humans.

It is not known whether the tungsten ion (tungstate; WO_4^{2-}) is metabolized as such in the body; however, a recent *in vitro* study has shown that WO_4^{2-} binds human albumin and other unknown protein (MW > 300 kDa) (Rodríguez-Fariñas et al. 2008).

Table 2. Search strings, PubMed® MeSH query translations, and returned results for tungsten and tungstate publications from January 1, 2004 to May 19, 2014.

Level of search	Search term	MeSh query translation	Findings
Chemical	Tungsten	tungsten[Title/Abstract] AND ("2004/01/01"[PDAT]: "2013/05/15"[PDAT])	2909
Chemical	Tungstate	tungstate[Title] AND ("2005/01/01"[PDAT]: "3000"[PDAT])	463
Chemical and endpoint	Tungsten	(("pharmacokinetics"[Subheading] OR "pharmacokinetics"[All Fields] OR "toxicokinetics"[All Fields] OR "pharmacokinetics"[MeSH Terms] OR "toxicokinetics"[All Fields]) AND ("tungsten"[MeSH Terms] OR "tungsten"[All Fields])) AND ("2005/01/01"[PDAT]: "3000"[PDAT])	29
Chemical and endpoint	Tungsten or Tungstate and Toxicity	(((tungsten OR tungstate)) AND toxicity) AND ("2004/01/01"[Date - Publication]: "3000"[Date - Publication])	163

Table 3. Tungsten substance nomenclature and solubility in water.

Tungsten substance	Abbreviation used in this review	Chemical formula	CAS Registry[SM] number	Water-solubility (reference)
Water-soluble substances				
Sodium tungstate	ST	Na_2WO_4	13472-45-2	732 g/L at 21°C (Weast 1973)
Ammonium metatungstate	AMT	$(NH_4)_6(H_2W_{12}O_{40})\ 3H_2O$	12028-48-7	1635 g/L at 22°C (Lassner and Schubert 1999)
Sodium metatungstate	SMT	$Na_6O_{39}W_{12}$	314075-43-9	> 1000 g/L at 20°C (RCC NOTOX BV 1992)
Ammonium paratungstate	APT	$(NH_4)_{10}(H_2W_{12}O_{42})\ 4H_2O$	11120-25-5	270.2 g/L (Lassner and Schubert 1999)
Sparingly water-soluble substances				
Tungsten metal	W-metal	W	7440-33-7	Insoluble (CDC 2010a)
Tungsten carbide	WC	WC	12070-12-1	< 0.1 mg/L at 20°C (OECD SIDS 2005)
Ditungsten carbide	W_2C	W_2C	12070-13-2	Insoluble (Weast 1973)
Tungsten trioxide (tungsten yellow oxide)	WO_3	WO_3	1314-35-8	Insoluble (Budavari 1996)
Tungsten oxides (tungsten blue oxide)	WO_{3-x}	$WO_{2.91}$; $WO_{2.81}$; $WO_{2.66}$; $WO_{2.51}$	39318-18-8	Insoluble (American Elements 2014)
Tungsten disulfide	WS_2	WS_2	12138-09-9	Insoluble (Bureau of Reclamation 2011)

CAS = Chemical Abstracts Service

This finding was confirmed in rats, where approximately 80% of serum tungsten was bound to proteins, and most of the protein-bound tungsten was due to a complex with albumin, which is not highly stable. No binding to transferrin was detected. An unknown protein with a molecular weight > 100 kDa was also found to bind a small amount of tungsten (approximately 2%). The interaction between reduced glutathione (GSH) and WO_4^{2-} was also evaluated; however, the formation of complexes was not observed (Gómez-Gómez et al. 2011).

After systemic distribution, tungsten (as WO_4^{2-}) may be deposited in bone, by displacing phosphate (Weber et al. 2008). Ang et al. (2013) investigated the uptake and speciation of tungsten in bones from adult and newborn mice exposed to WO_4^{2-} at levels up to 200 mg/kg/d for 120 d via drinking water. The results suggested that exposure to tungsten results in increased bone mineralization and a resultant increase in bone strength. Whether this is due to the chemical speciation of tungsten or the alteration of cellular processes, needs to be further elucidated.

Our search identified four *in vitro* dissolution studies that estimated the bioavailability of tungsten substances in artificial human fluids. In addition, six new relevant *in vivo* studies on toxicokinetics of WO_{3-x} (a sparingly water-soluble tungsten substance) and ST (a water-soluble substance), published after 2005, were identified.

In vitro

For metal substances, it is important to understand that simple exposure of an organism to the metal or a compound does not necessarily impart the biological properties of the metal ion. It is the bioavailability, defined as the degree of the metal ion (or a redox form of this ion) that enters systemic circulation, thereby accessing target sites, that determines the potential adverse effects. However, the distinction between bioavailability and solubility needs to be mentioned, as solubility is defined as the equilibrium distribution of a tungsten substance between water and the solute phase at a given temperature and pressure.

Several *in vitro* methods have been developed to determine metal dissolution in artificial fluids, as a surrogate model for the estimation of bioavailability. Four studies were investigated to elucidate the bioavailability of tungsten associated with ingestion, dermal (Stefaniak et al. 2010a, 2010b, IITRI 2010b), or inhalation exposure (Stefaniak 2010, IITRI 2010b). These data may be useful for understanding the inhalation, dermal, or oral dosimetry of tungsten substances.

Calcination of APT at a relatively low growth temperature (~850°C) may result in the formation of WO_{3-x} whiskers. Stefaniak (2010) determined that whiskers (6–9 μm in length) containing tungsten oxide powders ($WO_{2.81}$, $WO_{2.66}$, and $WO_{2.51}$) dissolved more slowly than W-metal and WO_3 powders in artificial airway epithelial lining fluid (SUF), and more slowly than W-metal in macrophage phagolysosomal simulant fluid (PSF). Overall, these results suggest that whisker-containing, less-oxidized tungsten oxide deposited in the alveolar region of the lung would have similar biopersistence to non-fiber-shaped, more-oxidized tungsten oxides (WO_3 and WO_{3-x}), but it would be more biopersistent than W-metal.

The dissolution of WC and W-metal in three types of gastric juice was assessed by Stefaniak et al. (2010b). Their results suggest that oral exposure to WC and W-metal do not contribute appreciably to total body burden, given the short residence time of the material in the stomach, and its relatively long dissolution half-life that ranges from 60 to 380 d. Likewise, Stefaniak et al. (2010a) measured the dissolution kinetics of W-metal and WC using artificial sweat. The dissolution of WC powder was independent of solvent pH (range tested 5.3–6.5); however, W-metal dissolution increased as the pH of the artificial sweat was increased from 5.3 to 5.9. Overall, the dissolution of W and WC was not influenced by solvent composition.

The findings of Stefaniak et al. (2010a, 2010b) were confirmed by an unpublished study (IITRI 2010b) conducted under GLP guidelines. This industry-sponsored study used several artificial human fluids, such as gastric (pH = 2), alveolar (pH = 7.4), interstitial (pH = 7.4), lysosomal (pH = 4.5), and sweat (pH = 1.5), to test W-metal, WO_3, $WO_{2.91}$, WC, and

Table 4. Standard toxicity testing summary for water-soluble and sparingly water-soluble tungsten substances.

Endpoint (Test method)	Test substance	Species	Results	References
Toxicokinetics (OECD TG 417)	Sodium tungstate*	Rat/Mouse	– Substance readily absorbed after oral exposure – Rapidly distributed to various organs (intestine, kidney, femur) – Brain relatively protected from oral exposure – Substance crosses placenta – Excreted through urine	McDonald et al. 2007, Weber et al. 2008, Guandalini et al. 2011, Kelly et al. 2013
	Tungsten oxide ($WO_{2.91}$)†	Rat	– Substance absorbed systemically – Lung, femur, and kidney showed increased levels (lung at least an order of magnitude greater than kidney or femur) – Concentration in tissues reached steady-state by exposure day-14	Rajendran et al. 2012
Skin irritation (OECD TG 404)	Sodium tungstate, ammonium paratungstate, sodium metatungstate*	Rabbit	– Substances deemed to be non-irritating – Caused no-irritation or slight erythema that was reversible within 24 h	RCC NOTOX BV 1991e, Huntingdon Life Sciences Ltd., 1999e, 1999j
	Tungsten metal, tungsten carbide, tungsten trioxide†	Rabbit	– Substances deemed to be non-irritating	Huntingdon Life Sciences Ltd., 1999p, 2000b, ARC Seibersdorf Research GmbH 2002a
Eye irritation (OECD TG 405)	Sodium tungstate, ammonium paratungstate, sodium metatungstate*	Rabbit	– ST and APT showed minimal irritation, which was fully reversible within 21 d in conjunctiva, cornea, or iris – SMT considered an eye irritant	RCC NOTOX BV 1991a, Huntingdon Life Sciences Ltd., 1999d, 1999i
	Tungsten metal, tungsten carbide, tungsten trioxide, tungsten oxide ($WO_{2.91}$)†	Rabbit	– Substances caused only transient, very-slight to well-defined conjunctival irritation, or elicited no irritation at all	Huntingdon Life Sciences Ltd., 1999o, 2000a, ARC Seibersdorf Research GmbH 2002b, 2003a
Skin sensitization (OECD TG 406)	Sodium tungstate, ammonium paratungstate, sodium metatungstate*	Guinea pig	– Substances elicited no sensitization reaction	RCC NOTOX BV 1991d, Huntingdon Life Sciences Ltd., 1998, 1999k
	Tungsten metal, tungsten carbide†	Guinea pig	– Substances elicited no sensitization reaction	Huntingdon Life Sciences Ltd., 1999q, 1999u
Repeated-dose toxicity (40 CFR 798.2650; OECD TG 412)	Sodium tungstate*	Rat	– Treatment caused progressive nephropathy at 125 and 200 mg/kg/d – LOAEL = 125 mg/kg/d – NOAEL = 75 mg/kg/d	McCain et al. 2010
	Tungsten oxide ($WO_{2.91}$)†	Rat	– Treatment induced macrophage influx (e.g. pigmented macrophages, alveolar and foamy macrophages) – No toxicologically significant effects – NOAEL > 0.65 mg/L air	Rajendran et al. 2012
Carcinogenicity‡ (OECD TG 451/453)	Sodium tungstate*	Rat/Mouse	– Ongoing, two-year drinking water carcinogenicity study of ST – Estimated exposure doses for rats = 25, 50, 100 mg/kg/d – Estimated exposure doses for mice = 100, 250, 500 mg/kg/d	NTP 2014a
Reproductive toxicity‡ (US EPA OPPTS 870.3650)	Sodium tungstate*	Rat	– Substance did not have any apparent effect on reproductive functions or offspring's physical development	McInturf et al. 2008, 2011
Developmental toxicity‡ (US EPA OPPTS 870.3650)	Sodium tungstate*	Rat	– Substance may produce subtle neurobehavioral effects related to motor activity and emotionality in offspring	McInturf et al. 2008, 2011
Immunotoxicity‡ (FDA 2002/ICH S8 2006)	Sodium tungstate*	Mouse	– Substance did not adversely affect innate immunity, humoral immunity, or cell-mediated immunity in female B6C3F1/N mice exposed via drinking water	NTP 2012

US EPA OPPTS = United States Environmental Protection Agency Office of Prevention, Pesticides and Toxic Substances; FDA, United States Food and Drug Administration; ICH, International Conference on Harmonization; NOAEL = no observable adverse effect level; OECD = Organisation for Economic Co-operation and Development; TG = Testing Guideline.
*Substances are soluble in water.
†Substances are sparingly soluble in water.
‡No data were available for this endpoint for sparingly water-soluble substances.

W_2C. Overall, the results showed that $WO_{2.91}$ has a greater bioavailability in artificial lung fluids compared with the other tungsten substances tested. None of the tungsten substances were bioavailable in artificial gastric fluid and sweat (IITRI 2010b).

Based on *in vitro* dissolution studies, a comparison of bioavailability after different exposure routes to tungsten substances can be estimated. Overall, the dissolution studies described in this section suggest that the sparingly water-soluble substances, WC, W_2C, WO_3, $WO_{2.91}$, and W-metal, are not bioavailable via the oral or dermal routes compared with water-soluble substances (such as ST), and they potentially do not contribute appreciably to total body burden. Whereas, in lung fluids (e.g. alveolar, lysosomal, interstitial, SUF, or PSF), it seems that WO_3 and $WO_{2.91}$ are more systemically available by inhalation than are W-metal, WC, and W_2C. For less bioavailable particles, toxicity may also depend on the lung's clearance, retention, and translocation to different compartments (Oberdorster et al. 1994).

In vivo

The ATSDR's toxicological profile (Keith et al. 2005, 2007) did not identify data on the quantitative absorption or distribution of tungsten by the inhalation, oral, or dermal routes in humans, and our search did not identify any recent studies that could fill this gap. Instead, our search found seven new toxicokinetic studies conducted after 2005, using rats and mice (McDonald et al. 2007, Weber et al. 2008, Radcliffe et al. 2009, 2010, Guandalini et al. 2011, Rajendran et al. 2012, Kelly et al. 2013). One study was on WO_{3-x} (a sparingly water-soluble tungsten substance), and six studies were on ST (a water-soluble substance).

Inhalation exposure. Three rat inhalation toxicokinetic studies were published after 2005: two on ST (Radcliffe et al. 2009, 2010), and one on WO_{3-x} (Rajendran et al. 2012). The study designs and results are briefly described below.

Radcliffe et al. (2009) exposed male rats, nose-only, to a 90-min single exposure of 0.256 mg ^{188}W/L (as $Na_2^{188}WO_4$; with a mass median equivalent aerodynamic diameter (MMAD) of 1.48–1.51 μm), equivalent to approximately 12 mg/kg/d (with one nostril occluded, to prevent deposition). After 0, 1, 3, 7, and 21 d post-exposure, the left and right sides of the nose, brain, olfactory pathway, and striatum, were sampled. Respiratory and olfactory epithelial samples from the side with the occluded nostril had significantly lower end-of-exposure ^{188}W levels, confirming the occlusion procedure. The ^{188}W levels of the olfactory bulb, olfactory tract/tubercle, striatum, cerebellum, and rest of brain, paralleled approximately 2–3% of measured blood ^{188}W levels. Brain ^{188}W concentrations were highest immediately following exposure, and returned to near-background concentrations within 3 d. The authors suggested that as ^{188}W in the olfactory bulb remained low throughout the experiment (approximately 1–3% of the amount of tungsten seen in the olfactory epithelium), olfactory transport plays a minimal role in delivering tungsten to the brain in rats, without indicating the primary transport.

Radcliffe et al. (2010) subjected male rats to a single exposure to tungsten for 90 min at 0.256 mg/L (as $Na_2^{188}WO_4$; with a MMAD of 1.5 μm), which is equivalent to approximately 12 mg/kg/d. After 0, 1, 3, 7, and 21 d post-exposure, ^{188}W tissue concentrations were assessed. The thyroid and urine had the highest ^{188}W levels post-exposure, and urine was the primary route of ^{188}W excretion. The pharmacokinetics of tungsten in most tissues followed a two-compartment pharmacokinetic model, with initial half-lives ($t_{1/2}$) of approximately 4–6 h, and a longer terminal phase, with a $t_{1/2}$ of approximately 6–67 d. The kidney, adrenal glands, spleen, femur, lymph nodes, and brain continued to accumulate small amounts of tungsten, as reflected by the tissue/blood activity ratios that increased throughout the 21-day period. At day-21, all tissues, except the thyroid, urine, lung, femur, and spleen, had only trace levels of ^{188}W.

Rajendran et al. (2012) exposed rats to $WO_{2.91}$ at 0.08, 0.325, and 0.65 mg/L of air, with MMAD ranging from 2.63 to 2.87 μm (estimated to be 15, 62, and 124 mg/kg/d, respectively) for 6 h/d for 28 consecutive days, followed by a 14-day recovery period. Inhaled $WO_{2.91}$ was absorbed systemically, and blood levels of tungsten increased as inhaled concentration increased. Among the tissues analyzed for tungsten levels, the lung, femur, and kidney showed increased levels, with lung tissue having at least an order of magnitude greater than the tissues of the kidney or femur. By exposure day-14, tungsten concentration in tissues had reached steady-state.

The most recent inhalation studies on water-soluble and sparingly soluble tungsten forms confirm that both forms are readily absorbed and rapidly distributed to various organs (e.g. thyroid, spleen, lung, femur, liver), and the primary route of elimination is via urinary excretion (Radcliffe et al. 2010, Rajendran et al. 2012). An important new finding from the ST studies is that olfactory transport plays a minimal role in delivering tungsten to the brain in rats (Radcliffe et al. 2009).

Oral exposure. Reports of four relevant oral toxicokinetic studies were peer-reviewed and published after 2005; all of them were on ST. A summary of these studies is presented below.

McDonald et al. (2007) subjected rats and mice to a single exposure of ST at 1, 10, or 100 mg/kg by oral gavage, or by intravenous administration of 1 mg/kg. Plasma and tissue (liver, kidney, uterus, femur, and intestine) were collected at 1, 2, 4, or 24 h after exposure. The results showed tungsten in plasma and all tissues after both gavage and intravenous administration. Plasma concentrations peaked at 4 h in rats and 1 h in mice, with amounts decreasing significantly by 24 h.

Weber et al. (2008) repeatedly exposed rats and mice to ST by gavage (10 mg/d) or drinking water (560 mg/L; estimated to be 85 mg/kg/d for rats and 148 mg/kg/d for mice) for 14 consecutive days. Plasma and tissue (intestine, liver, kidney, femur, uterus, and fetus) were collected at 1, 2, 4, or 24 h after exposure. Tungsten was detectable in plasma and all tissues and fetuses of both rats and mice. Accumulation occurred in the intestine, kidney, and femur, with urine being the primary route of elimination.

Guandalini et al. (2011) exposed mice to ST at 0, 62.5, 125, and 200 mg/kg/d for 28 d, and after 1 d, the tissues of the kidney, liver, colon, bone, brain, and spleen were collected. The results showed increasing tungsten levels in all organs as exposure dose increased, with the highest concentration

found in the bones and the lowest concentration found in brain tissue. Gender differences were seen only in the spleen (higher tungsten levels were found in female mice).

Kelly et al. (2013) exposed mice to tungsten as ST in their drinking water, at 15, 200, or 1000 mg/L (oral doses estimated to be in the order of 4, 50, and 250 mg/kg/d), for up to 16 weeks. Tungsten concentration in bone was analyzed by inductively coupled plasma mass spectrometry. Exposure resulted in a rapid deposition in the bone following 1 week, and tungsten continued to accumulate thereafter, although at a decreased rate.

These latest oral studies on water-soluble forms of tungsten have shown that it is readily absorbed, rapidly distributed to various organs (e.g. intestine, kidney, and femur), and excreted via the urine. These findings confirm most of what had been published on tungsten tissue distribution and excretion, and they also show that the brain is relatively protected when tungsten exposure occurs via oral administration.

Overall, this new toxicokinetic information on inhaled or ingested water-soluble and sparingly soluble tungsten substances in rats and mice could be used to improve the existing Legget's Physiologically-Based Pharmacokinetic model, included in the ATSDR's toxicological profile (Keith et al. 2005, 2007) regarding the biokinetics of tungsten. However, data for rats were assigned low weightage in the selection of most parameter values in the Leggett (1997) model, because of known qualitative differences in membrane transport between rats and humans in the handling of molybdate (MoO_4^{2-}; which is more easily reduced in biological systems), a chemical and physiological analog of WO_4^{2-}. These species differences recognized for molybdenum may apply to tungsten as well, as suggested by the faster excretion of tungsten by rats than by other species studied, and the apparent species differences in the distribution of retained tungsten. However, some aspects of the toxicokinetics of WO_4^{2-} or MoO_4^{2-}, including long-term bone retention due to displacement of WO_4^{2-} or MoO_4^{2-} for phosphate in bone, are expected to be independent of species (Keith et al. 2005, 2007).

Mechanistic toxicity

In vitro

The ATSDR toxicological profile did not locate information on well-designed mechanistic studies that might provide valuable information to elucidate toxicity and reduce tungsten body burden (Keith et al. 2005, 2007). Our search found several mechanistic *in vitro* and *in vivo* studies that attempted to elucidate how water-soluble forms of tungsten can treat diabetes, obesity, and hypertension, that deserve some consideration.

Mechanistic *in vitro* studies have shown that WO_4^{2-} at the cellular level may disrupt the cell cycle (Liu et al. 2012) by affecting Ca^{2+} homeostasis, cell signaling, bioenergetics (Johnson et al. 2010, Colovic et al. 2011, Fraqueza et al. 2012a, 2012b, Fernández-Mariño et al. 2014), and/or glucose uptake. Studies on different cell lines (hepatocytes, myoblasts, adipocytes, and human embryonic kidney cells) have demonstrated that WO_4^{2-} activates glucose uptake by an increase in the content and translocation of glucose transport. Other studies suggest that WO_4^{2-} reduces blood pressure by vasodilatory action on Ca^{2+}-potassium-dependent (BK) channels of arter-

ies with β1 subunit (Fernández-Mariño et al. 2012). Based on the potential *in vitro* ability to affect glucose transport in cells and the arterial vasodilatory effects, WO_4^{2-} has been proposed as an anti-diabetic and anti-hypertensive agent. Also, based on a study using hypothalamic N29/4 cells, WO_4^{2-} has been shown to be a suitable candidate to manage obesity, as a leptin-mimetic compound, by activating (in an independent way) several kinases involved in the leptin signaling system (Amigó-Correig et al. 2011).

One of the main mechanisms of action of metal toxicity is the production of reactive oxygen species (ROS). With this in mind, Stefaniak et al. (2010c) measured the generation of hydroxyl radicals (•OH) by different particle distributions of WC and W-metal powders in artificial lung and sweat fluids. In both artificial fluids, WC and W-metal powders promoted ROS production. The concentration of ROS increased as particle size decreased. However, the *in vitro* production of ROS by WC or W-metal in artificial sweat was not consistent with the observed low dermal acute toxicity, the lack of skin irritation in rabbits, and the lack of dermal sensitization (see Sections Dermal exposure, Skin and eye irritation caused by sparingly water-soluble substances, and Skin sensitization). For some metals, ROS production may play an important role in dermal sensitization, as demonstrated by Wang et al. (2010), who showed decreasing chromium hypersensitivity in guinea pigs with the administration of N-acetylcysteine. However, for other metals such as nickel, ROS production may not be part of the sensitization mechanism, as nickel did not reduce the ratio of the reduced:oxidized forms of cellular glutathione (i.e. GSH:GSSG) (Mizuashi et al. 2005), nor did it oxidize the fluorescent dye dichlorodihydrofluorescein diacetate (Mehrotra et al. 2005) when tested *in vitro*.

Lombaert et al. (2013) measured the oxidative response in human peripheral blood mononuclear cells after exposure to WC for 15 min. Their results (confirmed by reverse transcription polymerase chain reaction and Western blotting) showed that the oxidative-stress-response *HMOX1* (the human gene that encodes for the enzyme heme oxygenase 1) was not expressed in samples treated with WC.

From these *in vitro* mechanistic studies, one may conclude the following: (1) WO_4^{2-} activates glucose uptake and the leptin signaling system; (2) WO_4^{2-} affects Ca^{2+} homeostasis, cell signaling, and bioenergetics; and (3) WC and W-metal induce the formation of ROS (without expressing *HMOX1*).

In vivo

Several *in vivo* mechanistic studies on orally administered ST have determined that tungsten can replace molybdenum as the catalytic metal in the active center of a diverse group of enzymes that, in general, catalyze oxygen atom transfer reactions (Brondino et al. 2006). The most prominent molybdenum enzymes are nitrate reductase, sulfite oxidase (SO), xanthine dehydrogenase (XDH), aldehyde oxidase (AO), and the mitochondrial amidoxime reductase (Mendel and Kruse 2012).

Rats exposed via drinking water or gavage to several concentrations of ST ranging from 28 to 100 mg/kg/d for 14 d to 6 weeks, showed a reduction in enzymatic activities of molybdenum-dependent enzymes such as xanthine oxidase (XO), XDH, and SO, in the kidney, intestine, liver, plasma,

and erythrocytes (Weber et al. 2008, Herken et al. 2009, Ozturk et al. 2010, Aydemir et al. 2012, Tutuncu et al. 2012). Mice liver AO activity was reduced by 45% when treated via drinking water for 14 d with ST at approximately 170 mg/kg/d (Swenson and Casida 2013).

According to some researchers, if WO_4^{2-} were to potentially replace MoO_4^{2-} at the binding site of the molybdenum-cofactor enzyme family, rendering the enzymes non- or partially functional, it is biologically plausible that it could cause adverse effects (Tyrrell et al. 2013). This molybdenum-enzyme inactivation appears to be due to a decrease in the redox potential, caused by tungsten (Liao 2013).

Several studies have used the tungsten inactivation of XO activity as proof of concept to confirm the toxicity mode of action mediated by ROS generation in the following events: renal apoptosis during pneumoperitoneum in rats (Khoury and Weinbroum 2012); mononuclear phagocyte-mediated acute rat lung injury (Wright et al. 2004); acute renal allograft rejection in rats (Sun et al. 2004); post-ischemic coronary endothelial injury mediated by endothelin-1-induced superoxide generation in guinea pig hearts (Duda et al. 2007); prevention of atherosclerosis in knockout mice (Schröder et al. 2006); liver necrosis and fulminant hepatic failure protection in rats (Pawa and Ali 2004, Uskokovic-Markovic et al. 2007); para-phenylenediamine-induced skin lesions in rats (Lee et al. 2008), methotrexate-induced small intestinal toxicity in rats (Miyazono et al. 2004); and joint swelling in rats (Klocke et al. 2005). Several other studies inactivated SO with tungsten to elucidate sulfite toxicity in rats (Kucukatay et al. 2006, 2007, Nacitarhan et al. 2008, Niknahad and O'Brien 2008, Herken et al. 2009, Kocamaz et al. 2012).

Contrary to the *in vitro* studies reviewed above, Ca^{2+} homeostasis was not affected in healthy rats exposed to WO_4^{2-} via gavage at 100 mg/kg/d for 6 weeks, as contractibility and intracellular calcium were not altered, nor were potassium and L-type Ca^{2+} currents of isolated ventricular myocytes (Aydemir et al. 2012).

The *in vivo* generation of ROS by water-soluble tungsten substances was not confirmed by lipid peroxidation assessed using the thiobarbituric acid reactive substances (TBARS) assay in heart tissue from rats exposed to WO_4^{2-} via gavage at 100 mg/kg/d for 6 weeks (Aydemir et al. 2012). However, when male rats were exposed to ST orally at 119 or 238 mg/kg/d or intraperitoneally at 20 or 41 mg/kg/d for 2 weeks, an increase in ROS and erythrocyte antioxidant enzyme activities (catalase and glutathione peroxidase) were observed. In addition, erythrocyte glutathione-*S*-transferase activity showed significant inhibition, while tissue ROS and TBARS levels increased, accompanied by a decrease in GSH and in the GSH/GSSG ratio. This study suggests oxidative stress as a mechanism involved in the toxic manifestations of WO_4^{2-} (Sachdeva et al. 2013).

In a follow-up study, rats orally exposed to WO_4^{2-} at 10 mg/kg/d for 3 months presented inhibited activity of blood δ-aminolevulinic acid dehydratase, decreased levels of reduced GSH in liver and blood, and increased GSSG and TBARS levels in tissues. Antioxidant supplementation post-exposure, as α-lipoic acid or *n*-acetylcysteine, resulted in increased GSH levels, and was beneficial to the recovery of altered superoxide dismutase and catalase activity. In addition, it significantly

reduced blood and tissue ROS and TBARS levels. These results confirm that WO_4^{2-} induces oxidative stress in rats (Sachdeva and Flora 2014).

An *in vivo* mechanistic study on rats exposed via intraperitoneal injection to ST at 10 mg/kg showed that WO_4^{2-} crosses the blood-brain barrier and independently increases the activity of several kinases involved in the leptin signaling system, suggesting that WO_4^{2-} could be used to treat obesity (Amigó-Correig et al. 2011). In addition, oral administration of WO_4^{2-} (40–270 mg/kg/d) for 30 d significantly decreased body weight gain and adiposity without modifying caloric intake, intestinal fat absorption, or growth rate in obese rats. These effects were mediated by an increase in whole-body energy dissipation, and by changes in the expression of the genes involved in the oxidation of fatty acids and mitochondrial uncoupling in adipose tissue (Claret et al. 2005).

Acute toxicity

The ATSDR identified a lack of information concerning adverse effects and targets of toxicity following acute-duration oral, dermal, or inhalation exposure to tungsten or tungsten substances (Keith et al. 2005, 2007). Our literature search did not find any relevant studies. However, the tungsten industry sponsored several acute toxicity studies using different exposure routes. These K1 studies were conducted under GLP and followed OECD Testing Guidelines (TG). The respective acute toxicity values are presented in Table 5.

Oral exposure

As expected, bioavailability dictated the acute oral toxicity of water-soluble tungsten substances such as ST, AMT, APT and SMT, as they are more toxic than sparingly water-soluble substances (e.g. W-metal, WC, WO_3, or WO_{3-x}). The low bioavailability found in artificial gastric fluids (Section *In vitro* under Toxicokinetics) was consistent with the low acute oral toxicity found in sparingly water-soluble tungsten substances.

Gross necropsy observations in animals that died after oral exposure to SMT at 2800–5000 mg/kg body weight and to AMT at 1373–2000 mg/kg body weight, revealed congestive changes such as dark red small intestine, dark red fluid in the stomach, red foci in the stomach, dilated urinary bladder, enlarged cervical lymph node, enlarged and red mesenteric, mandibular and bronchial lymph nodes, pale liver and kidney, and clear fluid in the pleural cavity (RCC NOTOX BV 1991c, Huntingdon Life Sciences Ltd., 1999h, IITRI 2010a).

From the oral LD50 values (adjusted by the percent tungsten composition) shown in Table 5, it cannot be concluded that polytungstates are more toxic than monotungstates, as ST is slightly less toxic than AMT, but more toxic than APT and SMT. The overall rat acute toxicity of tungsten and its substances administered orally is low, based on an LD50 value ≥ 1000 mg/kg.

Dermal exposure

Water-soluble and sparingly soluble tungsten substances are not systemically bioavailable via the dermal route, as supported by an LD50 value > 2000 mg/kg body weight in rats (Table 5). Local skin effects ranged from none to slight dermal

Table 5. Acute oral, dermal, and inhalation studies on tungsten substances.

Exposure route (Test method)	Tungsten substance tested	Acute toxicity	Reference
Oral (OECD TG 401)	Ammonium metatungstate	F – LD50: $> 1000 - < 1373$ mg/kg bw	IITRI 2010a
	Sodium tungstate	M/F – LD50: 1453 mg/kg bw	Huntingdon Life Sciences Ltd., 1999h
	Sodium metatungstate	M/F – LD50 1715 mg/kg bw	RCC NOTOX BV 1991c
	Ammonium paratungstate	M/F – LD50: > 2000 mg/kg bw	Huntingdon Life Sciences Ltd., 1999c
	Tungsten metal	M/F – LD50: > 2000 mg/kg bw	Huntingdon Life Sciences Ltd., 1999t
	Tungsten carbide	M/F – LD50: > 2000 mg/kg bw	Huntingdon Life Sciences Ltd., 1999n
	Tungsten trioxide	M/F – LD50: > 2000 mg/kg bw	ARC Seibersdorf Research GmbH 2002d
	Tungsten oxide ($WO_{2.91}$)	F – LD50 > 2000 mg/kg bw	ARC Seibersdorf Research GmbH 2003b
Dermal (OECD TG 402)	Sodium tungstate	M/F > 2000 mg/kg bw	Huntingdon Life Sciences Ltd., 1999g
	Sodium metatungstate	M/F > 2000 mg/kg bw	RCC NOTOX BV 1991b
	Ammonium paratungstate	M/F > 2000 mg/kg bw	Huntingdon Life Sciences Ltd., 1999b
	Tungsten metal	M/F > 2000 mg/kg bw	Huntingdon Life Sciences Ltd., 1999s
	Tungsten carbide	M/F > 2000 mg/kg bw	Huntingdon Life Sciences Ltd., 1999m
Inhalation (OECD TG 403)	Sodium tungstate	M/F – LC50 (4 h): > 5.01 mg/L	Huntingdon Life Sciences Ltd., 1999f
	Ammonium paratungstate	M/F – LC50 (4 h): > 5.35 mg/L	Huntingdon Life Sciences Ltd., 1999a
	Tungsten metal	M/F – LC50 (4 h): > 5.4 mg/L	Huntingdon Life Sciences Ltd., 1999r
	Tungsten carbide	M/F – LC50 (4 h): > 5.3 mg/L	Huntingdon Life Sciences Ltd., 1999l
	Tungsten trioxide	M/F – LC50 (4 h): > 5.36 mg/L	ARC Seibersdorf Research GmbH 2002c

bw = body weight; LC50 = the concentration required to kill 50% of the test animals; LD50 = the dose required to kill 50% of the test animals; F = female; M = male; OECD = Organisation for Economic Co-operation and Development; TG = Testing Guideline.

irritation (erythema) that resolved by day-4 post-exposure. No macroscopic abnormalities were observed at study termination. This low acute toxicity of sparingly water-soluble tungsten substances agrees with the low bioavailability seen in the results of the artificial sweat fluid study (Section *In vitro* under Toxicokinetics).

Inhalation exposure

Based on the rats' LC50 values > 5 mg/L, tungsten substances are not acutely toxic if inhaled (Table 5). Some acute inhalation study results showed that the mean body weight gain of male test rats were lower than that of the controls following exposure, and macroscopic pathology at day-14 showed slight lung congestion (Huntingdon Life Sciences Ltd., 1999f, 1999r). Bioavailability studies of sparingly water-soluble tungsten substances in artificial lung fluids predicted that WO_3 and $WO_{2.91}$ could be more bioavailable than W-metal and WC; however this did not translate to lower LC50 values.

Irritation and sensitization

Limited information was cited by the ATSDR concerning previous local (point of contact) toxicity observations, referencing only rabbit studies using 5% tungsten hexachloride (a water-soluble tungsten substance) that resulted in contact dermatitis and ocular irritation, as hydrolyses in an aqueous solution yielding hydrogen chloride (Keith et al. 2005, 2007). Our literature search did not find any relevant studies. However, the tungsten industry sponsored several reliable (K1) rabbit skin and eye irritation studies conducted under GLP and following OECD guidelines. The following is a discussion on local irritation toxicity based on degree of tungsten solubility in water (soluble and sparingly soluble).

Skin and eye irritation caused by water-soluble substances

Based on rabbit skin irritation studies conducted according to OECD TG 404 (OECD 2012a), SMT, APT, and ST were found to be non-irritant, as they caused no-irritation or only slight erythema that was reversible within 24 h (RCC NOTOX BV 1991e, Huntingdon Life Sciences Ltd., 1999e, 1999j).

OECD TG 405 (OECD 2012b) studies on APT and ST showed minimal irritation that was fully reversible within 21 d, in the conjunctiva, cornea, or iris of rabbits (Huntingdon Life Sciences Ltd., 1999d, 1999i). However, SMT is an eye irritant, as it causes adverse effects in the cornea, iris, and the conjunctiva of rabbits, which in some animals, did not reverse 21 d after instillation (RCC NOTOX BV 1991a).

Skin and eye irritation caused by sparingly water-soluble substances

Rabbit skin irritation studies conducted according to OECD 404 (OECD 2012a) deemed WC, W-metal, and WO_3 to be non-irritating (Huntingdon Life Sciences Ltd., 1999p, 2000b, ARC Seibersdorf Research GmbH 2002a).

Also, sparingly water-soluble tungsten substances were found to be non-irritating, based on rabbit eye irritation studies of WC, W-metal, WO_3, and WO_{3-x}, conducted according to OECD TG 405 (OECD 2012b), causing only transient very slight to well-defined conjunctival irritation, or eliciting no irritation at all (Huntingdon Life Sciences Ltd., 1999o, 2000a, ARC Seibersdorf Research GmbH 2002b, 2003a).

Having discussed the results of non-irritation in standard *in vivo* eye and skin irritation tests, one needs to recognize that, in general, metal powders may cause mechanical eye or skin irritation.

Skin sensitization

Metal hypersensitivity is a common immune disorder, as metal ions such as Ni^{2+}, Co^{2+}, Cu^{2+}, and Cr^{3+} are haptens with a high immunogenic potential (Büdinger and Hertl 2000). Human immune systems mount allergic attacks on metal ions through skin contact, lung inhalation, and metal-containing artificial body implants.

To assess the immunogenic potential of WO_4^{2-}, the tungsten industry sponsored several studies of tungsten substances in guinea pigs. The studies followed OECD TG 406 (OECD 1992). Water-soluble (RCC NOTOX BV 1991d, Huntingdon Life Sciences Ltd., 1998, 1999k) and sparingly soluble (Huntingdon Life Sciences Ltd., 1999q, 1999u) tungsten substances elicited no positive sensitization reactions.

Repeated-dose toxicity

The ATSDR noted that the available repeated-exposure animal studies did not include critical dose-response information and other details (including methods used in statistical analysis of data), which precluded their usefulness for the purpose of risk assessment (Keith et al. 2005, 2007).

In oral repeated-exposure studies, ST has become the more studied tungsten substance because of its water solubility, and hence likely higher bioavailability, compared with other tungsten substances. Some of the ST repeated-exposure studies identified are pre-clinical investigations on the treatment of diabetes or obesity with WO_4^{2-}. To distinctly elucidate the potential adverse effects of tungsten in these pre-clinical diabetes studies, the healthy WO_4^{2-}-treated group deserves more consideration than the diabetic WO_4^{2-}-treated group.

To standardize the exposure dose as mg/kg/d in repeated-exposure studies that reported only the concentration given in the drinking water (in mg/L), the US EPA (1988) default value recommendations of body weight and water consumption per day, in conjunction with specific study details, were employed.

A common adverse effect observed in oral repeated-exposure studies in male and female rats (Ballester et al. 2007, McCain et al. 2010) or in male mice (Kelly et al. 2013) that were exposed to WO_4^{2-} was a decrease in body weight. It was suggested that this could be mediated by the activation of the hypothalamic leptin pathway (Amigó-Correig et al. 2011, 2012).

Reductions in body weight have been observed when dosing ST via drinking water at 2 mg/L (equivalent to 160 mg/kg/d) for a period of 12 weeks, or to 10–250 mg/kg/d via gavage for a period of 13 weeks. This body weight reduction was also seen in mice exposed to ST at approximately 130 mg/kg/d for 2 weeks (Amigó-Correig et al. 2012).

Statistically significant body weight reduction findings that had been reported in preclinical diabetic studies were also seen by McCain et al. (2010), who exposed male and female rats to ST for 13 weeks. Significant treatment-group differences in mean body weight compared with untreated animals were observed in males (but not in females) between days 70 and 90, from the 10, 75, and 200 mg/kg/d exposure groups. In females, glucose levels in the 75 mg/kg/d exposure group were significantly higher than those of controls. No other significant dose-related adverse alterations were observed for hematological and clinical chemistry parameters for any treatment group.

A 13-week study using rats and mice was conducted by NTP, in which the animals were exposed to ST at estimated doses of 0–200 mg/kg/d and 0–500 mg/kg/d, respectively. Preliminary results showed significant body weight reductions in male and female rats in all exposed groups after the 13-week treatment, with no mortality. However, decreases in the body weight of male and female mice were only significant at the 500 mg/kg/d dose (NTP 2014a).

Several preclinical studies have tested WO_4^{2-} as an antidiabetic or anti-obesity agent. Healthy male and/or female rats (as a healthy control group) were orally exposed for 2–12 weeks, to concentrations ranging from 100 to 190 mg/kg/d, or male mice were orally exposed for 2 weeks to 130 mg/kg/d. The exposure did not alter the biochemical parameters of the parotid salivary gland (Leite and Nicolau 2009), systemic blood pressure, or levels of serum/blood glucose and insulin, triglycerides, high density lipoprotein (HDL) cholesterol, alanine aminotransferase (ALT) activity, and/or glycogen synthase total activity (Ballester et al. 2007, Peredo et al. 2010, Nocito et al. 2012, Amigó-Correig et al. 2012, Aydemir et al. 2012). In mice, WO_4^{2-} treatment modified several groups of proteins in the hypothalamic nuclei, such as cell morphology, axonal growth, tissue remodeling, proteins related to energy metabolism, and synaptogenesis (Amigó-Correig et al. 2012).

When treating mice with WO_4^{2-} via drinking water at 5, 50 or 250 mg/kg/d, Kelly et al. (2013) did not observe liver toxicity as assessed by the levels of peripheral blood aspartate aminotransferase (AST) and ALT enzymes, but significantly lower body weight was reported in mice given the highest dose. WO_4^{2-} did not alter erythrocyte and platelet counts or hemoglobin and hematocrit levels. However, peripheral blood white blood cell counts decreased in a dose-dependent manner following 1 week of exposure, but the decrease was only significant at 12 weeks of exposure at the highest dose tested.

In the most-recent diabetes-related study, Oliveira et al. (2014) treated wild type or insulin-receptor substrate deficient mice for 3 weeks with an *ad libitum* solution of 2000 mg/L of ST (equivalent to approximately 500 mg/kg/d) in distilled water. WO_4^{2-} treatment of wild-type healthy mice had no effect on body weight, pancreas (assessed by β-cells and α-cells per mass or per pancreatic area, and β-cell survival), glucose homeostasis (evaluated by blood glucose levels and intraperitoneal glucose tolerance test), peripheral insulin sensitivity, or hepatic glucose metabolism (measured by hepatic glucogen and total or intrinsic glycogen synthase activities; active-phosphorylated glycogen phosphorylase and total glycogen phosphorylase activities). However, it seems that WO_4^{2-} slightly increased the hepatic phosphoenolpyruvate carboxykinase levels compared with the untreated control group.

Histopathological examination of the kidneys in both sexes of rats by McCain et al. (2010) revealed WO_4^{2-} treatment-related effects at 125 and 200 mg/kg/d dose groups. Kidney changes ranged from mild to severe regeneration of cortical tubules showing basophilic tubular profile and thickened basement membrane, diagnosed as progressive nephropathy. Based on these adverse kidney effects, the lowest observable adverse effect level (LOAEL) in male and female rats was 125 mg/kg/d. The no observable adverse effect level (NOAEL) in male and female rats was 75 mg/kg/d.

Sachdeva et al. (2013) also noted indicators of nephrotoxicity when exposing rats to ST orally at 119 mg or 238 mg/kg/d or intraperitoneally at 20 mg and 41 mg/kg/d for 2 weeks. An increase in the activities of plasma transaminases, creatinine, and urea levels were observed, suggesting hepatic and renal injury. These indicators of adverse effects were more promi-

nent in rats undergoing intraperitoneal administration than those that were subjected to oral administration.

As most metals are excreted through renal clearance and by gastrointestinal excretion, the findings of McCain et al. (2010) were not unexpected. Within these nephrotoxic effects, WO_4^{2-} (in addition to MoO_4^{2-}, selenate, thiosulfate, succinate, and citrate) may also specifically affect sulfate homeostasis by inhibiting the Na^+-sulfate co-transporter NaS1 (SLC13A1), which mediates the sulfate (re)absorption across the renal proximal tubule and small intestinal epithelia (Markovich 2014).

It is noteworthy that none of the preclinical studies that tested WO_4^{2-} as an anti-diabetic/obesity agent (Ballester et al. 2007, Peredo et al. 2010, Nocito et al. 2012, Aydemir et al. 2012, Amigó-Correig et al. 2012) showed any adverse histopathological findings in the kidneys, despite having exposed the animals for a period of 12 weeks (Ballester et al. 2007) to similar concentrations (i.e. 100–190 mg/kg/d or higher, in a study by Oliveira et al. (2014), who exposed mice to 500 mg/kg/d for 3 weeks) as those in the study by McCain et al. (2010).

Overall, repeated oral exposure of rats to WO_4^{2-} may result in body weight reductions and nephrotoxicity, these effects being seen with doses between 125–250 mg/kg/d. Within this dose range, it was suggested by Sachdeva et al. (2013) that oxidative stress is the mechanism involved in the toxic manifestations of WO_4^{2-}. At WO_4^{2-} doses in the range of 100–190 mg/kg/d, neither systemic blood pressure nor levels of serum/blood glucose and insulin, triglycerides, HDL, cholesterol, AST, and ALT enzyme levels or activity, and/or glycogen synthase total activity, were affected. Furthermore, no adverse effects on pancreas, glucose homeostasis, peripheral insulin sensitivity, or hepatic glucose metabolism were seen in mice exposed to WO_4^{2-} at an estimated dose of 500 mg/kg/d for 3 weeks. Surprisingly however, this high dose did not cause any body weight reduction or nephrotoxic effects, perhaps because the exposure duration was shorter (10 weeks) compared with the NTP's 13-week studies (NTP 2014a).

The ATSDR identified a gap in hazard assessment with regard to repeated-dose toxicity via the inhalation route, and described, instead, several intratracheal studies instilling W-metal and WC (Keith et al. 2005, 2007). In addition, the NTP has planned intratracheal studies on W-metal, WO_3, and WO_{3-x} (NTP 2014b, 2014c, 2014d). However, instillation is not a physiological route for human exposure. Intratracheal instillation involves invasive delivery that bypasses the upper respiratory tract. Instillation is usually done at a dose and/or dose rate substantially greater than that which would occur during inhalation. Several investigators have shown discrepancies between inhalation and instillation routes of exposure, including instilled vehicle effects on particle distribution and physicochemical nature of the test material, as well as differences in clearance and pattern of injury. Of additional concern is the potential confounding effect of anesthesia, which could influence the initial effect of the instilled material on the lung surface, as well as the retention and clearance of test material. These concerns have tempered the adoption of intratracheal instillation as an acceptable alternative to inhalation (Osier and Oberdörster 1997, Driscoll 2000).

Our literature search identified one study using the inhalation route. Female and male rats were exposed to $WO_{2.91}$ aerosol at a target air concentration of 0.08, 0.325, and 0.65 mg/L of air for 6 h/d (equivalent to 15, 62 and 124 mg/kg/d) for 28 d. Increased lung weight was noted for both terminal and recovering animals, which was attributed to deposition of $WO_{2.91}$ in the lungs, inducing a macrophage influx (e.g. pigmented macrophages, alveolar, and foamy macrophages). No toxicologically significant effects were reported, and therefore the NOAEL was > 0.65 mg/L of air (Rajendran et al. 2012).

The results of more recent repeated-exposure studies by the oral and inhalation routes may serve as a basis for calculating oral and inhalation reference doses for tungsten and tungsten substances. As dermal bioavailability is limited, as discussed in previous sections, it does not appear that repeated-exposure studies associated with dermal exposure to tungsten or tungsten substances are necessary.

Genotoxicity

The ATSDR toxicological profile indicated that the genotoxic potential of tungsten and tungsten substances has not been extensively assessed, and it cited several positive non-standard genotoxicity methods (e.g. bacterial bioluminescence, lambda prophage, gene conversion and reverse mutation in yeast, and increased recombinant frequency), and a positive result for WO_4^{2-} in the HPRT forward mutation method (Keith et al. 2005, 2007).

The tungsten industry sponsored several K1 mutagenicity studies conducted under GLP, following OECD testing guidelines (Table 6). Bacterial mutagenicity tests in *Salmonella typhimurium* and *Escherichia coli* using TG 471 (OECD 1997a) on water-soluble (CCR 1989b, Covance Laboratories, Inc., 2004e, NTP 2014a) and sparingly soluble (Huntingdon Life Sciences Ltd., 2001a, ARC Seibersdorf Research GmbH 2002e, 2003c, Covance Laboratories, Inc., 2004f, NTP 2014b) tungsten substances were negative. However, according to the International Council of Mining & Metals, bacterial genotoxicity assays, in general, lack sensitivity due to either the probable mechanism of action or lack of metal uptake (ICMM 2007).

Tests that detect forward gene mutations, such as OECD TG 476 using mouse lymphoma (OECD 1997d), chromosomal aberrations (TG 473; OECD 1997b), or cytogenetic damage using the micronucleus test (TG 474; OECD 1997c) in mammalian cells, would be preferable for metal substances (ICMM 2007). Mouse lymphoma assays were negative for two tungsten substances (ST and W-metal) which bracket the water solubility extremes (Covance Laboratories, Inc., 2004c, 2004d). Chromosomal aberrations were negative in water-soluble (ST) and sparingly soluble (WO_3, WC, and W-metal) tungsten substances (Huntingdon Life Sciences Ltd., 2001b, Covance Laboratories, Inc., 2003, 2004a, 2008, IITRI 2010c). *In vivo* rodent micronucleus studies on two tungsten substances with the highest water solubility (SMT and ST) were also negative in bone marrow cells (CCR 1989a, Covance Laboratories, Inc., 2004b, NTP 2014a).

Anard et al. (1997) and Van Goethem et al. (1997) first reported the Comet assay, to detect DNA damage (strand breaks) in human cells exposed to sparingly water-soluble tungsten substances. Human peripheral lymphocytes *in vitro*

Table 6. Genotoxicity studies on tungsten substances.

Tungsten substance	Bacterial reverse mutation test (OECD TG 471)	*In vitro* mammalian cell gene mutation test (OECD TG 476)	*In vitro* mammalian chromosome aberration test (OECD TG 473)	Mammalian erythrocyte micronucleus test (OECD TG 474)
Sodium metatungstate	Negative (CCR 1989b)	–	–	Negative (CCR 1989a)
Sodium tungstate	Negative (Covance Laboratories, Inc., 2004e)	Negative (Covance Laboratories, Inc., 2004c)	Negative (Covance Laboratories, Inc., 2003)	Negative (Covance Laboratories, Inc., 2004b)
Tungsten oxide (WO$_{2.91}$)	Negative (ARC Seibersdorf Research GmbH 2003c)	–	–	–
Tungsten trioxide	Negative (ARC Seibersdorf Research GmbH 2002e)	–	Negative (IITRI 2010c)	–
Tungsten carbide	Negative (Huntingdon Life Sciences Ltd., 2001a)	–	Negative (Huntingdon Life Sciences Ltd., 2001b, Covance Laboratories Ltd., 2008)	–
Tungsten metal	Negative (Covance Laboratories, Inc., 2004f)	Negative (Covance Laboratories, Inc., 2004d)	Negative (Covance Laboratories, Inc., 2004a)	–

OECD = Organisation for Economic Co-operation and Development; TG = Testing Guideline

exposed to pure WC for 15 min did not have any more DNA breaks than those of controls at doses up to 250 µg/mL (Anard et al. 1997, Van Goethem et al. 1997). These negative Comet assay results were confirmed by De Boeck et al. (2003) using similar exposure conditions.

In a recent Comet assay study, primary murine bone marrow cell cultures were treated with vehicle control or varying concentrations of ST (5–500 µg/mL) for 6 h. In all cells tested, WO$_4^{2-}$ induced a significant increase in the Comet tail moment, indicating an increase in DNA breakage, and confirmed with Western blotting experiments using antibodies against γH2AX, a phosphorylated histone variant that is induced upon DNA damage (Guilbert et al. 2011).

As noted, the more recent Comet studies exposed cells for a longer period of time (6 h) than older studies (15 min), and this may be the reason for the conflicting results, as an exposure period of 3–6 h is recommended. In addition, neither of the Comet assays exposed cells in the presence and absence of the metabolic activation system (S9-mix) (Burlinson 2012).

To determine whether *in vivo* exposure to tungsten might induce DNA damage within the bone marrow compartment, Guilbert et al. (2011) exposed mice to ST in the drinking water (15 or 200 mg/mL, estimated to be 4 and 50 mg/kg/d, respectively) for 8 weeks. While WO$_4^{2-}$ did not significantly alter the total bone marrow cellularity at these concentrations, it was found that WO$_4^{2-}$ exposure resulted in an increase in DNA damage, as assessed by the Comet assay.

Overall, standard genotoxicity testing yielded negative results on forward mutations as well as on assays of chromosomal aberrations, and also in the *in vivo* micronucleus tests, which were all done in mammalian cell systems. These negative results support a lack of genotoxic potential of water-soluble and sparingly soluble tungsten substances. However, there are equivocal *in vitro* Comet assay results that were conducted on different cells (human peripheral lymphocytes versus mouse bone marrow cells), and equivocal *in vivo* micronucleus (CCR 1989a, Covance Laboratories, Inc., 2004b, NTP 2014a) and Comet assay (Guilbert et al. 2011) studies in bone marrow cells of mice that were orally exposed to water-soluble tungsten substances. Therefore, due to the equivocal *in vivo* results,

the genotoxicity of soluble substances in bone marrow cells merits further investigation before the genotoxic potential of soluble tungsten forms can be established. NTP (2014a) has recognized the need for a confirmatory study, and has completed Comet assays (cell type has not been identified) in rats and mice, but the results are pending.

Carcinogenicity

Information regarding the carcinogenicity of ingested tungsten in humans is restricted to a single report from CDC, in which no statistically significant association (odds ratio 0.78, *p*-value 0.57) was found between exposure to tungsten in the drinking water and cases of leukemia observed in a population of Churchill County, Nevada (CDC 2003, Rubin et al. 2007).

ATSDR information (Keith et al. 2005, 2007) concerning a carcinogenicity study of oral exposure to tungsten includes a study design in which 5 ppm of tungsten as ST (approximately 0.5 mg/kg/d) were given to rats in drinking water for a lifetime. In the study, control rats and mice exhibited similar growth patterns and incidences of gross tumors (Schroeder and Mitchener 1975). However, the ATSDR considered that the study assessed growth, gross tumor incidence, and longevity, without evaluating either dose-response data or comprehensive histopathological examinations, which precluded the results from being used in risk assessment.

An ongoing 2-year carcinogenicity study of ST using rats and mice is currently being conducted by the NTP (2014a). Based on a 13-week range-finding study (NTP 2014a) (which exposed rats and mice via drinking water to 0–2000 mg/L; the preliminary results of this study are discussed in Section *Repeated-dose toxicity*), the drinking water doses that were selected for rats ranged from 0 to 1000 mg/L (estimated exposure doses of 25, 50, and 100 mg/kg/d), and the same for mice ranged from 0 to 2000 mg/L (estimated exposure doses of 100, 250, and 500 mg/kg/d). The results of this chronic carcinogenicity study are not expected until late 2015.

To date, no carcinogenicity studies on individual tungsten substances are available. Two studies on implanted tungsten-pellets in rodents could assist in the assessment for this end-point. In the first study, Kalinich et al. (2005) reported on

tumor development in rats implanted intramuscularly with pellets comprising 91.1% tungsten, 6% nickel, and 2.9% cobalt (W/Ni/Co) or with 100% nickel (positive control) pellets, but not with 100% tantalum (negative control) pellets. However, as described below, a follow-up study in mice conducted by the same investigator concludes that not all the tungsten-based alloys are carcinogenic, and that the adverse effects are only specifically seen with the (W/Ni/Co) alloy.

In the follow-up 2-year study, Kalinich (2011) implanted pellets in mice focusing on two tungsten alloys: W/Ni/Co, with the same composition as the alloy used the first study, and an alloy composed of 91% tungsten, 7% nickel, and 2% iron (W/Ni/Fe). The rest of the study design included several treatment groups consisting of negative and positive controls (tantalum and nickel, respectively), 11 alloy combinations of tungsten (W), nickel (Ni), cobalt (Co), iron (Fe), and/or tantalum (Ta), and a toxicity reference metal (lead). The pellets were cylindrical, and measured 1 mm in diameter and 2 mm in length. The protocol for the follow-up mouse studies included serial collection of tissues, at 1, 3, 6, and 12 months post-implantation, aimed at identifying early changes relevant to the development of carcinogenic endpoints. In addition, serum and urine samples were assessed for metal content.

As in the previous study using rats, the Wi/Ni/Co alloy induced tumor formation at the implant site. However, no other tungsten alloy exhibited tumor formation or indications of pellet-induced neoplasia. Tungsten levels were detected in serum and urine of animals implanted with Wi/Ni/Co, W/Ta, W/Ni/Ta, W/Co/Ta and W/Fe/Ta pellets (Kalinich 2011). Although these studies did not focus on individual water-soluble tungsten substances *per se*, serum and urine analysis for tungsten, conducted as part of the implant study, showed that the tungsten in the implanted pellets became bioavailable *in vivo* without causing muscle tumor development.

In a separate study, Schuster et al. (2012) confirmed that the tumorigenic potential of the W/Ni/Co pellets is related to the mobilization of nickel and cobalt bioavailability, by conducting electron microscopy of pellets which had been embedded for 6 months in rats and were extracted from them. Progressive galvanic corrosion of the matrix phase of the W/Ni/Co pellet was observed, and was accompanied by high urinary concentrations of tungsten, nickel, and cobalt. The galvanic corrosion takes place because of the difference in electrode potential between the matrix phase (anode) and the tungsten phase (cathode).

Overall, evidence from these embedded pellet studies would indicate that bioavailable tungsten, in and of itself, may not be carcinogenic.

Reproductive and developmental/neurotoxicity

The ATSDR review did not identify any reproductive or developmental reports related to exposure to tungsten substances in humans, stating that "only limited animal studies were identified, and deficiencies in study details regarding exposure and quantitative results render the results of these studies of limited value for purposes of quantitative risk assessment" (Keith et al. 2005, 2007).

Four published reproductive/developmental studies of ST were found. In the first study, male rats were orally exposed for 3 months to ST with a solution of 2 mg/mL (estimated dose of 190 mg/kg/d) in 0.9% sodium chloride. All rats survived at the end of the study; however, they showed a much lower body weight gain than the untreated controls. The ST-exposed rats did not show hypoglycemia or any alteration in Leydig cell function, as shown by the absence of change in serum testosterone levels (Ballester et al. 2005).

In a related study, female rats were exposed to WO_4^{2-} at an estimated dose of 160 mg/kg/d for 12 weeks. Markers for reproductive function (progesterone, follicle-stimulating hormone, and luteinizing hormone) and the uterine expression of the progesterone and estrogen receptors were not affected by WO_4^{2-} (Ballester et al. 2007).

A one-generation study that exposed male and female rats to 5 or 125 mg/kg/d of ST for 70 d (pre- and post-natal exposure) confirmed no effect on reproductive functions (mating success) or physical development of offspring. Daily administration of ST produced no evidence of toxicity; however, in the dams, the average gestation duration was longer for the group dosed with 125 mg/kg/d than for the control and 5 mg/kg/d groups. There were no differences in the average number of pups born (McInturf et al. 2008, 2011).

Overall, the most-recent reproductive toxicity evidence confirms that WO_4^{2-} does not affect the reproductive functions or physical development of offspring.

Neurobehavioral assessment of pups born and nursed from WO_4^{2-}-exposed rats found elevation of distress vocalizations in the 125 mg/kg/d group, and the righting reflex showed unexpected sex-dependent differences (but not dose-dependent), where male pups demonstrated faster righting than females (McInturf et al. 2008, 2011). When looking closer at these results, it can be noted that the statistical difference observed in the righting reflex between male and female pups was due to a decrease in male righting reflex with increasing dose (but not statistically significant), combined with a numerical increase in the righting reflex among females (Jackson et al. 2013).

The pups' locomotor activity was affected in both groups of dams that were treated with either 5 or 125 mg/kg/d, without affecting maternal retrieval, and no apparent effects of treatment on F1 acoustic startle response or water maze navigation (McInturf et al. 2008, 2011) were seen.

McInturf et al. (2008, 2011) only used two neurobehavioral tests, and these measured only very early reflexive behavioral responses. In addition, no histopathological effects were noted that indicated effects in the brain. Based on the results, one could conclude that ST may produce subtle neurobehavioral effects in offspring related to motor activity and emotionality; however, the collection of results is insufficient to delineate a clear dose-response in either the pups or dams and deserves further investigation.

Immunotoxicity

No information was located by the ATSDR concerning tungsten-induced immunotoxicity following inhalation, oral, or dermal exposure to tungsten or tungsten substances (Keith et al. 2005, 2007).

After 2005, two *in vitro* and three *in vivo* peer-reviewed studies attempted to elucidate the immunotoxic potential of ST. In addition, NTP issued a report in 2012 on a series of

immunotoxicity studies conducted as part of the tungsten testing program (NTP 2012). The following is a discussion of these recent immunotoxicity studies.

An *in vitro* study of isolated human peripheral blood lymphocytes showed an increase in early apoptosis and reduction in the number of cells entering into the cell cycle, when the cells were treated with 0.01, 0.1, 1.0, and 10 mM ST (equivalent to 0.006, 0.063, 0.626, and 6.26 mM as tungsten). The selected concentrations were substantially higher than tungsten levels (16.2 to 39.7 nM) found in tap water from Churchill County, Nevada (CDC 2003, Rubin et al. 2007). Apoptosis and cell cycle effects were not as pronounced at lower treatment levels (Osterburg et al. 2010).

Bone marrow-derived pre-B cell lines (BU-11 cells) and BMS2 stromal cells were treated *in vitro* for 48 h with ST concentrations varying from 50, 100, 250, and 500 µg/ml (equivalent to 31.3, 62.6, 156.4, and 312.9 µg of tungsten/mL, respectively). These *in vitro* concentrations were significantly higher than tungsten levels (0.003 to 0.007 µg/mL) from tap water in Churchill County, Nevada (CDC 2003, Rubin et al. 2007). BU 11-cells exhibited a dose- and time-dependent apoptosis. As compared with BU-11 cells, BMS2 cells required higher concentrations of tungsten in order to inhibit cell growth. This suggests that the developing B-cells are more sensitive to the toxic effects of tungsten than the cells in the surrounding bone marrow microenvironment; however WO_4^{2-} induced different cell cycle changes in the stromal cells as compared with the BU-11 cells. These *in vitro* data suggest that the developing hematopoietic compartment may be sensitive to WO_4^{2-}-induced changes (Guilbert et al. 2011).

To determine if WO_4^{2-} can cause an unnatural immune response, mice were exposed *in utero* by parental inhalation or ingestion of an estimated dose of 1 and 4 mg/kg/d, respectively; and inoculated with respiratory syncytial virus (within 2 weeks of weaning). Compared with controls, WO_4^{2-}-treated animals presented significant spleen enlargement (splenomegaly); however, the nature of the hematological/immunological disease was inconclusive (Fastje et al. 2012).

Osterburg et al. (2014) tested if exposure to WO_4^{2-} can result in immune suppression. For this, parental male and female mice were orally exposed to 2, 62.5, 125, and 200 mg/kg/d for 28 d in a one-generation model, and intraperitoneally injected with *Staphylococcal* enterotoxin. Exposure to WO_4^{2-} at the highest dose resulted in a decrease of activated cytotoxic T-cells and helper T-cells. In delayed-type hypersensitivity Type IV experiments, exposure to 200 mg/kg/d of WO_4^{2-} prior to primary and secondary antigen challenge significantly reduced footpad swelling. According to the authors, these results suggest that WO_4^{2-} could cause an immune suppression that may reduce host defense against pathogens.

Kelly et al. (2013) exposed mice for 16 weeks to WO_4^{2-} via drinking water. Mice receiving 15, 200, or 1000 mg/L (oral doses estimated to be in the order of 50 and 250 mg/kg/d) had a significantly greater percentage of cells in the late pro-B/large pre-B developmental stages. An increase in DNA damage in both whole marrow and isolated B cells at the lower tungsten concentration (15 mg/L), but not at the highest concentration (1000 mg/L), was also noted.

The NTP (2012) conducted a 28-day study to establish the potential effects on the immune system of female mice receiving ST via drinking water, at concentrations of 125, 250, 500, 1000, and 2000 mg/L (estimated doses ranged from 30 to 500 mg/kg/d). Over the 28-day exposure period, WO_4^{2-} did not alter body weight, body weight gain, or the weights of the major organs of the immune system, the thymus and spleen. Total splenocyte number and both absolute values and percent values of spleen cell phenotypes were unaffected by WO_4^{2-} exposure (NTP 2012). No effects were observed on T-dependent antibody responses, as evaluated using the antibody-forming cell response, the sheep red blood cell enzyme-linked immunosorbent assay (ELISA), and the keyhole limpet hemocyanin ELISA, suggesting that humoral (antibody) immunity is not adversely affected by WO_4^{2-} exposure (NTP 2012).

The mixed leukocyte responses and the cytotoxic T-lymphocyte responses presented some significant differences, but they were not dose-responsive. Furthermore, no effects were observed on the *in vivo* delayed-type hypersensitivity response to *Candida albicans* or in the anti-CD3-mediated proliferation assay, suggesting that WO_4^{2-} does not affect cell-mediated immunity (NTP 2012).

Lastly, innate immunity was not affected by WO_4^{2-} exposure, as indicated by a lack of effect on both natural killer-cell activity and the functional activity of the mononuclear phagocytic system. With the exception of effects on bone marrow differentials at the 2000 mg/L dose level, WO_4^{2-} did not adversely alter innate, humoral, or cell-mediated immunities in mice (NTP 2012).

The latest NTP immunotoxicity evidence conflicts with the evidence obtained by Osterburg et al. (2014) and Fastje et al. (2012) with regard to potential effects of WO_4^{2-} on innate, humoral, or cell-mediated immunities. It is difficult to pinpoint the cause(s) of the discrepancy, as WO_4^{2-} doses and exposure time were similar in both studies, and it seems that the only difference was the mice strain used in the studies: Osterburg et al. (2014) and Fastje et al. (2012) used C57BL/6 mice, whereas the NTP studies were conducted using B6C3F1/N mice. A report from the International Program on Chemical Safety indicates that a lack of consistency of chemical-induced immunotoxicity in specific assays across species or strains does not necessarily represent conflicting data, and may represent species or strain differences (WHO 2012). The adverse effects on bone marrow differentials observed by Kelly et al. (2013) were also observed in NTP's immunotoxicity studies. Overall, the immunotoxicity potential of WO_4^{2-} needs to be investigated further.

Nano-tungsten toxicity

The ATSDR toxicological profile did not cover the toxicity of tungsten nanoparticles (Keith et al. 2005, 2007). However, because nano-tungsten (with a mean particle size < 100 nm) applications are being explored in various fields (e.g. catalysts, coatings, pigments, lubricants, plastics, nano-wires/fibers, textiles, films, semi-conductors, or electrodes), it was deemed appropriate to discuss the toxicity of nano-sized tungsten.

Tungsten nanoparticles have a high surface area (which can lower the sintering temperatures), low vapor pressure, unusual quantum confinement, and grain boundary effects. However, the high surface area of tungsten nanoparticles could produce

a different toxic effect than that of larger (e.g. micron-sized/bulk) tungsten particles.

Several in *in vitro* studies have been published in the last 9 years on the toxicity of W-metal, WC, WS$_2$, or WO$_3$ nanoparticles. The cytotoxicity/cell viability of nano-tungsten substances has been evaluated by assessing fluorescence indicators of cell metabolic activity and membrane integrity (Bastian et al. 2009, Kühnel et al. 2012), mitochondrial function (MTT/MTS) (Hussain et al. 2005, Lanone et al. 2009, Machado et al. 2011, Pardo et al. 2014), mitochondrial membrane potential (Hussain et al. 2005), and neutral red uptake (Lanone et al. 2009, Kühnel et al. 2012), or by measuring membrane leakage of lactate dehydrogenase (Hussain et al. 2005).

Several reports of attempts to elucidate the mode of action of nano-tungsten substances have been published, such as the recording of human keratinocyte transcription profiles by microarray analysis (Busch et al. 2010), the assessment of cellular antioxidant activity by measuring the quenching of the dye dichlorodihydrofluorescein diacetate, by quantifying GSH (Hussain et al. 2005), or antioxidant response activation (measured by the antioxidant-response element using a luciferase reporter), or by the induction Nrf2-mediated Phase II detoxification genes (Pardo et al. 2014).

The genotoxic effects of nano-WO$_3$ were assessed in bacteria using the reverse mutation assay in *S. typhimurium* (Hasegawa et al. 2012), or by looking at the direct interaction of WO$_3$ nanoplates with naked DNA plasmid, and then transferred into *E. coli* cells for mutation observation (Thongkumkoon et al. 2014). In mammalian systems, the genotoxic potential of WO$_3$ was evaluated by the transformation assay in BALB/c cells (Hasegawa et al. 2012). Nano-WC genotoxicity was assessed as a potential cause of ROS generation by micronuclei formation in HepG2 cells (Kühnel et al. 2012).

It is recognized that metal nanoparticles (e.g. silver, copper, and aluminum) can easily cross the blood-brain barrier, affecting neuronal function and possibly leading to neurotoxicity and neuropathology (Sharma et al. 2009). With this in mind, *in vitro* studies were conducted on rat neuronal and glial cells (Bastian et al. 2009) and on the hippocampal neurons of rats (Shan et al. 2012, 2013), to assess the neurotoxic potential of nano-sized WC.

A summary of the results of these studies for each nano-tungsten substance tested is presented in Table 7. Overall, from the available evidence, nano-sized W-metal, WC, and WS$_2$ were shown to be chemically inert with regard to cell viability, but not for oxidative stress in the case of WC and WS$_2$. However, equivocal WO$_3$ mutagenic results warrant further investigation, and the neurotoxicity effects by WC in *in vivo* models need to be confirmed.

Human studies

Clinical studies

A 6-week study was identified as a proof-of-concept trial on the efficacy of ST in human obesity (Hanzu et al. 2010). A prospective, randomized, placebo-controlled, and double-blind clinical trial was conducted with 30 obese non-diabetic subjects, who were randomized to receive 100 mg of ST twice per day, or placebo. The primary study endpoint was the absolute change in body weight relative to the time of randomization.

Treatment with ST was not associated with a significant weight loss compared with placebo ($p = 0.854$). Likewise, treatment with ST was not associated with significant changes in fat mass, resting energy expenditure, or caloric consumption. The data do not support ST as a pharmacological agent in the treatment of human obesity (Hanzu et al. 2010).

Tungsten levels in humans

Most background environmental exposures to tungsten are from the soluble forms such as WO_4^{2-} that may occur in drinking water. Several studies have reported tungsten levels in blood, red blood cells, and serum or urine, in a variety of human subpopulations.

A 2-year program for human biomonitoring measured tungsten and 18 other metals in the blood and serum of subjects living in two different regions of Italy. The serum tungsten level showed an arithmetic mean of 0.15 ± 0.004 µg/L, in the inhabitants of the region of Umbria ($n = 290$), and was slightly higher than that of inhabitants of the region of Calabria (0.12 ± 0.003 µg/L; $n = 218$) (Bocca et al. 2010).

Urinary tungsten in 21 athletes was reported to be 82 ± 53 µg/g-creatinine whereas levels in 26 sedentary subjects were

Table 7. Studies on nano-tungsten substances.

Nano-tungsten substance	Toxicity effects	References
Tungsten metal	– Low cytotoxicity in assays using rat liver cells and human lung epithelial cells – Low activity in oxidative stress tests using rat liver cells	Hussain et al. 2005, Machado et al. 2011
Tungsten carbide	– No acute cytotoxic effects in human pulmonary cell lines or human skin and colon cell lines, or rat neuronal and glial cells – Induced formation of ROS and micronuclei in human keratinocyte cell line – Microarray analysis of human keratinocytes showed low transcriptional responses after 3 d of exposure – *In vitro* studies on rat hippocampal neurons showed influence on potassium channels and voltage-activated sodium currents, which suggested potential for neurotoxicity	Lanone et al. 2009, Bastian et al. 2009, Busch et al. 2010, Kühnel et al. 2012, Shan et al. 2012, Shan et al. 2013
Tungsten trioxide	– Positive mutagenic response in *S. typhimurium* strains TA1537 and TA98 – No transformability in BALB/C cells – DNA contact with nano-sized WO$_3$ plates induced damage (demonstrated by mutation of DNA-transferred bacterial cells)	Hasegawa et al. 2012, Thongkumkoon et al. 2014
Tungsten disulfide (tubes)	– No acute cytotoxic effects in cells representing respiratory, immune, and metabolic systems – Did not induce pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α – Activated antioxidant response	Pardo et al. 2014

ROS = reactive oxygen species.

below the limit of detection (Llerena et al. 2012). Another investigation examined the possible relationship between metals in children and severity of autism. In a comparison between 54 case subjects and 44 controls (age and gender matched), the authors concluded that the autism group had higher urinary levels of lead, thallium, tin, and tungsten than the neurotypical control group. The mean urinary tungsten levels in the autism and control groups were 0.334 ± 0.23 and 0.232 ± 0.40 µg/g-creatinine, respectively (Adams et al. 2013).

Canada's 2009–2011 cycle human biomonitoring program reports urinary tungsten 95th percentile geometric mean from participants aged 3 to 79 years: 0.63 µg/L (0.69 µg/g of creatinine) with 95% confidence interval (CI) of 0.50–0.76 µg/L (0.56–0.81 µg/g of creatinine), based on a representative sample of 6290 individuals (Ministry of Health Canada 2013). Urinary levels in females ($n = 3262$; 0.82 µg/g of creatinine; 95th CI: 0.57–1.1 µg/g of creatinine) were higher than in males ($n = 3028$; 0.58 µg/g of creatinine; 95th CI: 0.49–0.66 µg/g of creatinine).

Data from the National Health and Nutrition Examination Survey (NHANES) program (2009–2010), revealed the geometric mean urinary concentration in the US population to be 0.081 µg/L (0.086 µg/g of creatinine) with 95% CI 0.075–0.087 µg/L (0.080–0.093 µg/g of creatinine), based on a representative sample of 2847 individuals (CDC 2013). Over the 12 years (1999–2010) that the survey has been conducted, the geometric mean urinary concentrations ranged from 0.071 to 0.99 µg/L (0.070–0.103 µg/g of creatinine). Males, and the group comprising those less than 20 years of age, consistently show higher mean tungsten urinary concentrations than females. The groups < 20 years old, in general, show higher tungsten levels than the group ≥ 20 years or older. Over the 12-year survey, urine geometric mean range concentrations of the Mexican-Americans (0.079–0.106 µg/g of creatinine) tended to be higher than those of Non-Hispanic blacks (0.066–0.088 µg/g of creatinine) and Non-Hispanic whites (0.069–0.104 µg/g of creatinine) (CDC 2013).

The latest analysis of the NHANES data (Jain 2013) showed that the levels of urinary tungsten (together with cobalt and molybdenum) have increased over the 2003–2010 period. Smokers' adjusted geometric mean urinary concentration (0.10 µg/L; 95% CI 0.091–0.111) displayed significantly higher levels (together with lead, antimony, and uranium) than that of non-smokers (0.08 µg/L; 95% CI 0.076–0.084). Tobacco plants can uptake heavy metals such as zinc, copper, nickel, cadmium, iron, and manganese from the soil in which they grow, and tend to accumulate the metals in their leaves. When cigarettes are smoked, metals contained in the tobacco leaves can be transferred to main- and side-stream smoke and inhaled by smokers or non-smokers via environmental tobacco smoke (Golia et al. 2009). Pregnancy (0.099 µg/L; 95% CI 0.087–0.111) was found to be associated with higher levels of tungsten (jointly with barium, cesium, cobalt, molybdenum, lead, and mercury) compared with nongravid females (0.082 µg/L; 95% CI 0.078–0.086).

Several researchers have conducted statistical analyses on the NHANES data in an attempt to identify possible associations between measured parameters and health status. For example, urinary tungsten ($n = 712$; adjusted odds ratio of 2.30, 95% CI 1.72–3.07, $p < 0.001$) concentrations were associated with an increased incidence of vision disorder (Shiue

2013). Urinary cadmium and tungsten (adjusted odds ratio of 2.25 95% CI: 0.97–5.24; geometric mean 0.07 µg/L; $n = 751$) were reported to be associated with peripheral arterial disease (Navas-Acien et al. 2005), and a significant association was observed between cardiovascular disease (CVD) and cerebrovascular disease and urinary tungsten (adjusted odds ratio of 1.78), antimony, cadmium, and cobalt (Agarwal et al. 2011). In an examination of the relationship between heavy metals and various medical conditions, tungsten was found to be related to asthma (odds ratio of 1.72; 95% CI: 1.15–2.59), but not to CVDs (Mendy et al. 2012). In a study of associations of metals with total and central obesity indices based on NHANES data ($n = 3816$), no significant associations between tungsten with waist circumference and body mass index were found (Padilla et al. 2010). Yorita Christensen (2013) reported an association between urinary levels of tungsten with increased levels of thyroid-stimulating hormone.

One evaluation of the NHANES data focused specifically on tungsten. A cohort of 8614 adults with 193 reported stroke diagnoses and 428 reported diagnoses of CVD was assessed using crude and adjusted logistic regression models (Tyrrell et al. 2013). The authors concluded that elevated weighted urinary tungsten concentrations were associated (odds ratio of 1.66; 95% CI 1.17–2.34) with an increased prevalence of stroke, independent of typical risk factors even in people below 50 years of age (odds ratio of 2.17; 95% CI 1.33–3.53); but logistic regression models suggest that tungsten is not involved in the etiology of CVD.

Reported reference dose and concentration estimations

Schell and Pardus (2009) derived an oral reference dose (RfD) using the benchmark dose (BMDL10) approach, defined as the dose at the 95% lower confidence limit of a 10% response. The US EPA's Benchmark Dose Software (BMDS, Version 1.4.1) was used to model the ST data from both the 90-day oral toxicity study of McCain et al. (2010) and the rat reproductive study of McInturf et al. (2008).

The lowest (most precautionary) BMDL10 from the renal toxicity endpoint was 102 mg/kg/d; the lowest BMDL10 from the development endpoint was 93 mg/kg/d. Using the lowest of the BMDLs to estimate the tungsten RfD in the calculations, and a factor of 1000 to account for inherent uncertainty, the oral RfD for ST is 0.093 mg/kg/d, which is equivalent to approximately 0.06 mg of tungsten/kg/d or 0.08 mg WO_4^{2-}/kg/d (Schell and Pardus 2009).

Following the EPA's standard equations, Schell and Pardus (2009) developed a protective drinking water risk-based concentration for tungsten of 2.28 mg/L.

Using the studies done by McCain et al. (2010) and Rajendran et al. (2012), Jackson et al. (2013) calculated the values of derived no effect levels (DNELs) for occupational long-term inhalation, according to the REACH program's guidance on characterization of dose-response for human health. The inhalation $DNEL_{long-term}$ for ST, from which values for all other soluble tungsten substance DNELs were derived, is 3 mg/m³ (1.7 mg tungsten/m³), and the inhalation $DNEL_{long-term}$ for $WO_{2.91}$ is 7.3 mg/m³ (5.8 mg tungsten/m³). Although derived using different methodologies and supported by different studies, the

occupational inhalation $DNEL_{long-term}$ values for water-soluble and sparingly soluble tungsten substances are similar to the current NIOSH-recommended exposure level and the ACGIH 8-h TLV TWA of 1 mg tungsten/m^3 for soluble tungsten substances and 5 mg tungsten/m^3 as metal and insoluble tungsten substances (CDC 2010a, 2010b, OSHA 2014a, 2014b).

Conclusions

This review gathered new toxicokinetic information, as well information on acute and repeated-exposure studies with a variety of endpoints, including reproductive toxicity, developmental toxicity, neurotoxicity, and immunotoxicity. The recent available evidence shows that tungsten and tungsten substances are not bioavailable via the dermal route. WO_4^{2-} and WO_{3-x} can be systemically distributed and excreted via urine after oral and inhalation exposure, respectively; but sparingly water-soluble tungsten forms have a limited *in vitro* bioavailability, suggesting excretion via feces after oral exposure.

Tungsten and tungsten substances are not acute toxicants, eye and skin irritants, or dermal sensitizers. The low toxicity seen in *in vivo* studies is consistent with the low bioavailability shown in *in vitro* dissolution studies. W-metal, WC, W_2C, WO_3, and $WO_{2.91}$ are not bioavailable via the oral or dermal route, compared with soluble substances (ST, AMT, SMT, and APT), and potentially do not contribute appreciably to total body burden (e.g. mechanism of tungsten storage). As dermal bioavailability is limited, it does not appear that repeated-exposure studies associated with dermal exposure to tungsten or tungsten substances are necessary.

Standard testing supports a lack of genotoxicity of tungsten and tungsten substances; however, the *in vivo* Comet assay from one study reported positive results within the bone marrow compartment that contradicts the negative results from other *in vivo* studies of micronucleus in bone marrow cells. The contradiction may possibly arise because the studies assessed genotoxicity on different bone marrow cells, but merit further investigation as toxicokinetic studies via the inhalation or oral routes indicate that after systemic distribution, tungsten is deposited in bone by displacing phosphate. The NTP (2014a) has recognized the need for a confirmatory study and has completed Comet assays (cell type has not been identified) in rats and mice, but the results are pending.

After exposure, soluble tungsten forms are readily absorbed, rapidly distributed to different organs (e.g. thyroid, spleen, lung, femur, liver), and excreted via the urine as the primary route of elimination, causing nephrotoxicity at estimated exposure doses greater than 125 mg/kg/d. As most metals are excreted through renal clearance and gastrointestinal excretion, these findings were not unexpected. *In vivo* (Sachdeva et al. 2013) and *in vitro* (Stefaniak et al. 2010c) studies suggest that the toxic mode of action of water-soluble and sparingly soluble tungsten substances is by producing ROS, as demonstrated by an inhibition of erythrocyte glutathione S-transferase activity, and increases in catalase and glutathione peroxidase activities. These enzymatic changes are accompanied by lipid peroxidation, or by an increase in the generation of hydroxyl radicals, together with a decrease of the GSH/GSSG ratio. Possibly, this oxidative stress response is produced without expressing the *HMOX1* gene (Lombaert et al. 2013).

Alternatively to a ROS mechanism of action, toxicity could also occur if WO_4^{2-} replaces MoO_4^{-2} at the binding site of the molybdenum-cofactor enzyme family, rendering them non- or partially functional.

The healthy, treated group in diabetic and weight reduction rodent study designs deserves consideration when elucidating the potential effects of water-soluble tungsten forms. In addition to the nephrotoxic effects described above, repeated exposure to WO_4^{2-} resulted in decreased body weight (possibly mediated by the activation of the hypothalamic leptin pathway), but altered neither the systemic blood pressure nor the levels of serum/blood glucose and insulin, triglycerides, HDL cholesterol, ALT activity, and/or glycogen synthase total activity. Conversely, no adverse effects on pancreas, glucose homeostasis, peripheral insulin sensitivity, or hepatic glucose metabolism were observed.

There is no relevant information on the carcinogenic potential of tungsten and tungsten substances, and unfortunately, the results of the 2-year NTP study on ST with rodents are not expected until late 2015 (NTP 2014a). As no carcinogenic studies are available on individual tungsten substances, chronic studies on tungsten pellets (alloyed with other metals) implanted in rodents were considered to evaluate this endpoint, and the evidence suggests that water-soluble and sparingly soluble tungsten substances may not be carcinogenic.

The most recent reproductive toxicity evidence confirms that WO_4^{2-} does not have any apparent effect on the reproductive function or the physical development of offspring; however the collection of neurobehavioral results on subtle neurobehavioral effects in offspring related to motor activity and emotionality are insufficient to delineate a clear dose-response in either the pups or dams, and thus deserves further investigation.

There is some contradiction regarding the effects of WO_4^{2-} on innate, humoral, or cell-mediated immunity results reported by Osterburg et al. (2014), Fastje et al. (2012), and NTP (2012). However, there are reports of bone marrow cell changes after WO_4^{2-} exposure (NTP 2012, Kelly et al. 2013) that merit follow-up, as this is in line with evidence relating to the deposition of tungsten in bone.

With regard to nanoparticles, *in vitro* studies with nano-sized W-metal and WC have shown them to be chemically inert with regard to cell viability and oxidative stress. However, WO_3 equivocal mutagenic results warrant further investigation, and the neurotoxic effects of WC in *in vivo* models need to be confirmed.

Several studies have statistically analyzed the urinary levels of tungsten from specific cohorts or the NHANES survey. Studies have reported potential relationships of tungsten (together with other metals such as antimony, cadmium, and cobalt) with various medical conditions such as autism, asthma, vision disorder, peripheral arterial disease, CVD, cerebrovascular disease, and stroke. Future studies are warranted to clearly understand the biological mechanism along the pathway before drawing firm conclusions on the causation of a specific medical condition due to tungsten.

In 2009, when new evidence of oral toxicity of WO_4^{2-} emerged, Schell and Pardus (2009) attempted to estimate a tungsten reference dose equivalent to approximately 0.06 mg of tungsten/kg/d or 0.08 mg WO_4^{2-}/kg/d. However, a significant number of studies have been subsequently published on water-soluble and sparingly soluble tungsten substances

that filled major toxicity endpoint gaps identified by the ATSDR in the 2005 toxicological profile for tungsten (Keith et al. 2005, 2007). Such new evidence includes several recent repeated-exposure studies that need to be considered when regulators estimate and propose a tungsten reference or concentration dose.

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Declaration of interest

Details of the authors' affiliation are as shown on the title page. Mr. Carmen Venezia, Manager, Safety and Environment for Global Tungsten and Powders Corp., for more than twenty years. Dr. Ranulfo Lemus was an ITIA consultant for six years, being appointed as ITIA's Health, Safety and Environment Director in February 2013. The authors have sole responsibility for the writing and content of the paper.

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Supplementary material available online

Supplementary Tables 1–2.