Failure of Manganese to Protect from Shiga Toxin

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Abstract

Shiga toxin (Stx), the main virulence factor of Shiga toxin producing Escherichia coli, is a major public health threat, causing hemorrhagic colitis and hemorrhagic uremic syndrome. Currently, there are no approved therapeutics for these infections; however manganese has been reported to provide protection from the Stx1 variant isolated from Shigella dysenteriae (Stx1-S) both in vitro and in vivo. We investigated the efficacy of manganese protection from Stx1-S and the more potent Stx2a isoform, using experimental systems well-established for studying Stx in vitro responses of Vero monkey kidney cells, and in vivo toxicity to CD-1 outbred mice. Manganese treatment at the reported therapeutic concentration was toxic to Vero cells in culture and to CD-1 mice. At lower manganese concentrations that were better tolerated, we observed no protection from Stx1-S or Stx2a toxicity. The ability of manganese to prevent the effects of Stx may be particular to certain cell lines, mouse strains, or may only be manifested at high, potentially toxic manganese concentrations.

Introduction

Each year in the US, Shiga toxin (Stx) producing Escherichia coli (STEC) are responsible for over 100,000 cases of infectious diarrhea. Of these infected individuals, about 10% develop more severe sequelae such as life-threatening hemorrhagic uremic syndrome (HUS) [1]. The primary virulence factor, Stx, is responsible for disease symptoms. Stx is an AB$_2$ toxin, comprised of a receptor binding pentameric B-subunit and an enzymatically active monomeric A-subunit that inhibits protein synthesis. There are two major antigenic forms, Stx1 and Stx2. These forms share greater than 50% amino acid identity, but do not generate cross-neutralizing antibodies. In the past, the original toxin isolated from Shigella dysenteriae has been referred to as Stx, the highly related form isolated from E. coli has been referred to Stx1, and Stx2 has been used to refer to the highly potent form isolated from E. coli. However, numerous polymorphic forms of Stx2 have now been described which can share over 90% amino acid identity, but vary in potency by several orders of magnitude [2]. As more variants have been sequenced, the historic nomenclature has become extremely ambiguous. To avoid confusion, we will refer to the family members as Stx1 and Stx2, and variants used in this study as Stx1-S (the variant isolated from S. dysenteriae) and Stx2a (the highly potent variant first isolated from EDL933). STEC can express one, or both forms of toxin. The reduced potency of Stx1 compared to Stx2a is well documented in mice [2,3] and primates [4,5]. Furthermore, Stx2a is more commonly associated with life-threatening human disease; the majority of cases of HUS are associated with strains that produce Stx2a [6].

Other than supportive treatment, there are currently no therapeutics for STEC infections. However, past studies have shown that pre-treatment with certain ions, including Mn$^{2+}$, can play a protective role against Stx intoxication [7,8]. Sandvig and Brown previously reported protection from Stx1-S in Vero (African green monkey kidney) cells and HeLa cells when incubated in the presence of high concentrations of certain ions [7]. Using protein synthesis as an assessment of Stx1-S toxicity, Sandvig and Brown show that HeLa cells and Vero cells were protected from Stx1-S when incubated in the presence of 2 mM MnCl$_2$, CoCl$_2$, or BaCl$_2$. MgCl$_2$ at 2 mM also provided protection for Vero cells but not HeLa cells. Additionally, the presence of calcium ionophores and high concentrations (140 mM) of anions SCN$^{-}$ and SO$_4^{2-}$ also protected these cell lines from Stx1-S. It was thus hypothesized that inhibitors of Ca$^{2+}$ and Cl$^{-}$ transport could protect cells from Stx1-S. However, the toxicity of the treatments themselves was not assessed.

Similarly, a recent report by Mukhopadhayy and Linstedt presents manganese as a potential treatment for Shiga toxinsis by blocking Stx1-S trafficking [8]. Proper trafficking through the cell is essential to Stx toxicity. After endocytosis, the Stx holotoxin is trafficked from early endosomes to the Golgi apparatus and endoplasmic reticulum (ER) [9,10]. In the ER, the enzymatic A-subunit separates from the holotoxin, is processed, and released into the cytosol where it inhibits protein synthesis by cleaving a conserved adenine in 28S ribosomal RNA [11]. While the ER is the final destination of the holotoxin, trafficking through the Golgi is a required step [12]. Mukhopadhayy and Linstedt conclude that HeLa cell protection against Stx1-S toxicity in the presence of manganese is due to altered trafficking; demonstrating that pretreating HeLa cells with 500 µM MnCl$_2$ diverts trafficking of the Stx B-subunit from the Golgi to lysosomes, where it was subsequently degraded [8]. When assayed using Stx1-S holotoxin, HeLa cells were protected in the presence of manganese. Moreover, the manganese treatment is reported to protect BALB/c mice from Stx1-S toxicity.

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These prior studies were all performed using Stx1-S, the less potent form of the toxin. We set out to investigate if manganese would also provide protection from Stx2a. In our experimental systems, we observe that not only is manganese itself toxic at previously reported treatment doses, but manganese treatment at lower, less toxic MnCl₂ concentrations, offers no protection from Stx either in vitro or in vivo.

**Materials and Methods**

**Ethics Statement**

All animal work was conducted according to relevant national and international guidelines, including the requirements of the Association for the Assessment and Accreditation of Laboratory Animal Care International as described in the Guide for the Care and Use of Laboratory Animals, Eighth Edition. Animals in this study were housed in the animal facilities at University of Cincinnati and all experiments performed according to Protocol # 08-11-18-01, approved by the University of Cincinnati Institutional Animal Care and Use Committee (IACUC). Animals were weighed daily; those appearing moribund or losing more than 20% of initial body weight were euthanized in compliance

with IACUC regulations.

**Cell Lines and Materials**

Luc2P Vero cells, a Vero cell line transfected to express luc2p, a gene for destabilized luciferase, was used for all in vitro toxicity assays (strain constructed as described in [13]). Cells were cultured in Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum, 1× penicillin/streptomycin/glutamine solution, and 1× MEM vitamins solution (sMEM) (Life Technologies, Grand Island, NY). The following were obtained from the Biodefense and Emerging Infectious Diseases Research Resources Repository, NIAID, NIH: Stx1-S (Shiga Toxin Type 1, Recombinant from Escherichia coli, NR-857) and Stx2a holotoxin (Shiga Toxin Type 2, Recombinant from Escherichia coli, NR-4478).

**In vitro Toxicity Assays**

For manganese toxicity assays, a solution of concentrated MnCl₂ in Tris buffered saline, pH 7.4 (TBS) was prepared and used on the same day. This solution was diluted in sMEM in white tissue culture treated microtiter plates (BD Falcon, Falkin Lakes, NJ). As a control, a set of wells containing no MnCl₂ was included in the same plate. Luc2P Vero cells, 10⁴ per well, were added such that final MnCl₂ concentrations ranged from 1000 μM to 2.5 μM.

Cells were incubated at 37°C with 5% CO₂ for four hours. After this incubation, one half of the media was removed and replaced with an equal volume of TBS to mimic the experimental system of Mukhopadhyay and Linstedt [8]. Following a second four hour incubation, media was aspirated, cells were washed three times with phosphate buffered saline, pH 7.4 (PBS), and 25 μl of SuperLite luciferase substrate (Bioassay Systems, Hayward, CA) was added to each well. Light production was measured using Luminoskan Ascent (Thermo Labsystems, Helsinki, Finland). For the in vitro Stx toxicity assays, MnCl₂ was added to 10⁴ Luc2P Vero cells at final concentration of 250 μM as described above. Three sets of wells lacking MnCl₂ were included on the same plate, as negative controls. Cells were incubated at 37°C with 5% CO₂ for four hours. After this incubation, one half of the media was removed and replaced with purified Stx1-S or Stx2a holotoxin serially diluted in TBS. Following a second four hour incubation, cells were washed and light production was measured as described above.

Percent protein synthesis was correlated to the average light production of the control well without manganese or toxin representing 100%. A two-tailed Student’s t test (GraphPad Prism 5, La Jolla, CA) was used to calculate statistical differences.

**Results**

**Manganese Toxicity in Vero Cells**

A previously described cell line, Luc2P Vero African green monkey kidney epithelial cells, was used for in vitro toxicity assays [13]. These cells express Luc2P, a destabilized form of luciferase that is rapidly targeted for proteasomal degradation and does not accumulate in the cell. Thus, luciferase activity can be directly correlated to the rate of protein synthesis. In addition, the ED₅₀ for protein synthesis inhibition was shown to correlate well with traditional assays measuring cellular metabolic activity at 3 days post toxin treatment. To assess the concentrations at which MnCl₂ is toxic to Vero cells, luciferase activity was measured after Luc2P cells were incubated for four hours in the presence MnCl₂ ranging in concentration from 0 μM to 1000 μM, then for an additional four hours at half of the initial MnCl₂. This methodology was intended to be similar to that used previously to assess Mn²⁺ protection from Stx1-S toxicity in HeLa cells [8]. Cells incubated in initial MnCl₂ concentrations ranging from 250 μM to 1000 μM demonstrated significantly lower rates of protein synthesis as compared to the no manganese control (Fig. 1). At 500 μM MnCl₂, the concentration previously used with HeLa cells with no reported toxicity, Vero cells demonstrated approximately a 25% decrease in protein synthesis compared to cells with no exogenous Mn²⁺ added (P = 0.0014). Approximately a 17% decrease in protein synthesis was observed at 250 μM MnCl₂ (P = 0.0134).

**Manganese Offers No Protection from Stx Toxicity in Vero Cells**

To assess the protective effects of manganese on Luc2P Vero cells from Stx-mediated toxicity, cells were preincubated for four hours in the presence or absence of 250 μM MnCl₂, then incubated for an additional four hours in 125 μM MnCl₂ in the presence of various dilutions (ranging from 5×10⁻³ to 5×10⁻¹ μg/ml as indicated in Fig. 2) of purified Stx1-S or Stx2a. While still toxic, this manganese concentration was chosen to be high enough to see a protective effect yet be minimally toxic itself. Luciferase activity was measured at the end of this second four
hour incubation. One hundred percent protein synthesis was defined as the amount of light measured from cells incubated in sMEM and TBS without either MnCl₂ or toxin (Fig. 2). The effective dose for 50% inhibition of protein synthesis (ED₅₀) for Stx₁-S in the absence of manganese was determined to be 2.15 ng/ml, while the value for Stx₂a was determined to be 192 ng/ml. Both of these values are much higher than previously reported values for Stx₁-S (63 pg/ml) and Stx₂a (461 pg/ml) [13]. However, in the previous report, protein synthesis was measured four hours after the cells were suspended in fresh media, while in this assay, protein synthesis is measured eight hours after the cells were suspended in fresh media. Decreased metabolic activity could account for the increased resistance. Alternatively, toxin susceptibility has been shown to be influenced by cell cycle; Vero cells are most susceptible at the G₁/S boundary, and the proportion of cells at this stage in the cell cycle could be reduced in the longer assay [14]. Moreover, in the previous report cells are exposed to toxin before they adhere to the plate, whereas they are adherent in this assay at the time of toxin addition. It is possible that the increased toxicity reported previously is due to increased cell surface area of non-adhered cells for toxin binding. The ED₅₀ for Stx₁-S in the presence of MnCl₂ (4.06 ng/ml) was slightly increased compared to the cells in the absence of MnCl₂ (2.15 ng/ml) (Fig. 2A), but this difference was not statistically significant. Similarly, a manganese treatment provided no significant protection for cells exposed to Stx₂a (Fig. 2B), and the ED₅₀ values appeared to be identical.

Stx₁-S and Stx₂a are Lethal in Mice Treated with Manganese

We evaluated MnCl₂ protection from Stx₁-S and Stx₂a to outbred CD-1 mice. CD-1 mice were more sensitive to MnCl₂ than BALB/c mice. Within five minutes post-injection of MnCl₂ at the reported dose of 50 mg/kg, mice exhibited signs of distress including: hunching, isolation in the corner of cages and temporary tremor. These signs were not reported for BALB/c mice [8]. We thus used half the reported dose of MnCl₂ for our in vivo Stx toxicity assays. CD-1 mice were injected daily with MnCl₂ (25 mg/kg) five days prior to and subsequently every day post challenge (IP) with a lethal dose of Stx or sham dose of PBS. In previous studies, the LD₅₀ for Stx₁-S was determined to be greater than 1,000 ng, and the LD₅₀ for Stx₂a was determined to be about 6 ng [2]. To ensure lethality, mice were challenged IP with 2000 ng of Stx₁-S and 7 ng of Stx₂a. No increase in survival was observed compared to mice treated with water alone (Fig. 3A and B). Additionally, percent weight change can be used to assess morbidity in animals challenged with Stx. No difference in morbidity was seen between treatment groups in animals challenged with Stx, as exhibited by no significant difference in weight loss at 48 hours post challenge. Percent weight change, however, is significantly different between non-challenge and Stx challenge treatment groups (Fig. 3A and B).

Discussion

No therapeutics are available for STEC infections, and we were greatly intrigued by studies reporting that manganese could protect from Stx₁-S mediated toxicity to HeLa cells in vitro and BALB/c mice in vivo [7,8]. As these studies only investigated protection from the less potent Stx₁-S, we investigated the
potential of manganese to protect from both Stx1-S and the more potent Stx2a in experimental systems well-established for assessing Stx toxicity: \textit{in vitro}, using Vero monkey kidney epithelial cells, and \textit{in vivo}, using outbred CD-1 mice.

Mukhopadhyay and Linstedt reported that manganese protects cells \textit{in vitro} from Stx1-S [8]. However, in our studies, we did not observe manganese protection from either Stx1-S or Stx2a using an experimental system that differed from those of Mukhopadhyay and Linstedt in several respects. First, while they used HeLa cells engineered to be sensitive to Stx by up-regulating expression of the Stx receptor, we used Vero cells, which are naturally sensitive to Stx. Mukhopadhyay and Linstedt assessed cellular health by examining mitochondrial dehydrogenase activity using methylthiazolyldiphenyl-tetrazolium bromide (MTT); alternatively, we measured the rate of protein synthesis. As Stx targets protein synthesis through inactivation of the ribosome, and does not directly target mitochondrial respiration, our methodologies are a more direct assessment of protection from Stx. Finally, we assessed protection from Stx at lower concentrations of manganese because we found that the manganese treatment, in the absence of Stx, inhibited protein synthesis.

\textit{In vivo}, Mukhopadhyay and Linstedt reported that the manganese doses up to 50 mg/kg did not cause stress in the BALB/c mice [8]. However, this dose of MnCl\textsubscript{2} caused systemic symptoms within 5 minutes in the outbred CD-1 mice used in our study. We used manganese at a lower dose (25 mg/kg), which was also reported to confer protection by Mukhopadhyay and Linstedt. This dose did not appear to cause stress to the CD-1 mice, but failed to confer protection from toxin-mediated death from either Stx1-S or the more medically relevant Stx2a. While Mukhopadhyay and Linstedt reported that manganese is cleared from the mice within hours, they show protection against intoxication with a once daily injection of Mn\textsuperscript{2+} five days prior to and everyday post challenge with Stx1-S, at approximately 500 ng Stx1-S per mouse [8]. Using this same model with CD-1 mice, in our study all of the mice died on either day 2 or day 3 post-challenge. No difference in

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**Figure 3. Treatment with manganese does not protect CD-1 mice from (A) Stx1-S or (B) Stx2a.** Top panels, survival curves from mice treated with Water, MnCl\textsubscript{2}, Stx1-S + Water, or Stx1-S + MnCl\textsubscript{2} (Water, MnCl\textsubscript{2}, Stx1-S + Water, Stx1-S + MnCl\textsubscript{2}; n = 4 per group). Stx2a + Water, Stx2a + MnCl\textsubscript{2}; n = 6 per group). Bottom panels, average percentage weight change at 48 hours post challenge. Two-tailed Student’s \textit{t} test was used to assess statistical significance (n.s. no statistical significance, ** \textit{P}<0.005, *** \textit{P}<0.0005).

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body weight was seen at 48 hours after challenge, suggesting that increased time to death does not reflect protection.

The use of different experimental systems could account for the failure to reproduce the reported results. In human disease, Stx is known to target three different cell types which naturally express glycophospholipid (Gb3), the manganese receptor for Stx: kidney cells, endothelial cells and neurons [1,13-17]. The female reproductive tract, where HeLa cells originated, has not been reported to be targeted by Stx. HeLa are likely susceptible to Stx because upregulation of Gb3 expression is common in cancer cells [18]. Nevertheless, HeLa cells are still more resistant to Stx than Vero cells. Mukhopadhyay and Linstedt used HeLa cells transfected to express Gb3 synthase, to increase expression of the Gb3 receptor, and demonstrated that Stx resistance is due to altered intracellular trafficking in HeLa cells [8]. However, it is known that Stx uses different pathways to enter cells [19], and it is possible that manganese does not alter Stx trafficking in its natural target cells, including kidney cells.

MnCl2 was also reported to protect BALB/c mice from Stx1-S [8]. We did not observe manganese protection from either Stx1-S or Stx2a in the outbred CD-1 mouse line. BALB/c mice are null mutants for Slc11a1 (formerly Nrampl), an H+divalent cation antiporter expressed by phagocytes with a high affinity for Mn2+ [20]. It is not clear if this genetic mutation could have been a factor in the observed protection against Stx1-S, but outbred CD-1 mice are likely to more closely reflect normal human physiologic responses to Mn2+. In addition, Stx2a, not Stx1-S, is most associated with development of fatal human disease, and the failure to observe protection form Stx2a is significant when considering treatment of human disease.

Manganese is an essential trace mineral that is used as a cofactor by enzymes that prevent oxidative stress (superoxide dismutase) [21], to detoxify byproducts of amino acid metabolism in the liver (arginase) [22], and function in collagen production (prolactinase) [23]. However, manganese overexposure can be toxic, especially to the brain, resulting in permanent neurodegenerative disorders [24]. The most notorious of the conditions caused by manganese overexposure is manganism, the symptoms of which mimic those of Parkinson’s disease. Excess manganese is also implicated in decreased fertility [25], decreased sperm count and motility [26,27], fetal skeletal development manifestations and fetal death [28,29], and liver toxicity [30,31]. The potential for toxicity of the treatment itself raises serious concerns whether manganese can be used to treat STEC infections. The current suggested daily allowances of manganese is 0.14 mg/kg/day, or about 10 mg/day for adults, as determined by the Environmental Protection Agency Reference Dose for Chronic Oral Exposure based on central nervous system effects in adults [32]. The US National Research Council Estimated Safe and Adequate Daily Dietary Intake suggests that 5 mg manganese per day for children and adults 10 years and older is sufficient daily intake [33]. Considering the manganese dose administered to BALB/c mice that conferred protection from Stx1-S, a daily therapeutic dose for an adult weighing approximately 70 kg would be approximately 3,500 mg, 350 times greater than the suggested daily allowance in adults.

In summary, currently there are no therapeutics for Stx-mediated toxicity, and our studies suggest manganese holds little promise as a therapeutic candidate.

Author Contributions
Conceived and designed the experiments: MAG CAP AAW. Performed the experiments: MAG CAP. Analyzed the data: MAG CAP AAW. Wrote the paper: MAG CAP AAW.

References
1. Tarr PI, Gordon CA, Chandler WL (2005) Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome. Lancet 365: 1073–1086.
2. Fuller CA, Pellino CA, Flager MJ, Strasser JE, Weiss AA (2011) Shiga toxin subtypes display dramatic differences in potency. Infect Immun 79: 1329–1337.
3. Taylor VL, Norris JA, Owens JW, Gordon VM, Wardowski EA, et al. (1995) Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. Infect Immun 61: 3392–3402.
4. Sugler KL, Ohrig TG, Pyhers TJ, Tesh VL, Denkers ED, et al. (2003) Response to Shiga toxin 1 and 2 in a baboon model of hemolytic uremic syndrome. Pediatr Nephrol 18: 92–96.
5. Stearns-Kurosawa DJ, Collins V, Freeman S, Tesh VL, Kurosawa S (2010) Distinct physiologic and inflammatory responses elicited in baboons after challenge with Shiga toxin type 1 or 2 from enterohemorrhagic Escherichia coli. Infect Immun 78: 2497–2504.
6. Boerlin P, McEwen SA, Boerlin-Petzold F, Wilson JR, Johnson RP, et al. (1999) Associations between virulence factors of Shiga-toxin-producing Escherichia coli and disease in humans. J Clin Microbiol 37: 497–503.
7. Sandvig K, Brown JE (1987) Isonicotinic requirements for entry of Shiga toxin from Shigella dysenteriae type I cells. Infect Immun 55: 298–303.
8. Mukhopadhyay S, Linstedt AD (2012) Manganese blocks intracellular trafficking of Shiga toxin and protects against Shiga toxicity. Science 335: 332–335.
9. Sandvig K, van Deurs B (2000) Entry of ricin and Shiga toxin into cells: Differential association with membrane lipid raft microdomains. J Lipid Res 52: 618–634.
10. Ohata F, Tohyama K, Bonev AD, Kolling GL, Keepers TR, et al. (2008) Shiga toxin 2 affects the central nervous system through receptor glibo- triaoxacyc- amide localized to neurons. J Infect Dis 198: 1390–1406.
11. Ohrig TG, Louise CB, Langford MG, Bolin B, Garber J, et al. (1993) Endohergic heterogeneity in Shiga toxin receptors and responses. J Biol Chem 268: 15484–15488.
12. Engelstad N, Skudova T, Torgersen ML, Sandvig K (2011) Shiga toxin and its use as a novel molecular imaging probe for targeted cancer therapy and imaging. Microb Biotechnol 4: 32–46.
13. Sandvig K (2005) The Shiga toxins: Properties and action on cells. In: Alouf JE, Popoff MR, editors. The Comprehensive Sourcebook of Bacterial Protein Toxins. Academ Press. 310–322.
14. Papp-WalACE KM, Magure ME (2006) Manganese transport and the role of manganese in virulence. Annu Rev Microbiol 60: 187–209.
15. Borgstahl GE, Parge HE, Hickey MJ, Beyer KD, Haller WA, et al. (1992) The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. Cell 71: 107–118.
16. Kanyo ZF, Scodnick LR, Ash DE, Christianen DW (1996) Structure of a unique binuclear manganese cluster in arginase. Nature 383: 534–537.
17. Besin R, Baratto MC, Gioria R, Monzani E, Nicolis S, et al. (2012) A MnII-MnII center in human prolidase. Biochim Biophys Acta.
18. Hurdie HK (1999) Effects from environmental Mn exposures: A review of the evidence from non-occupational exposure studies. Neurotoxicology 20: 379–397.
19. Lauwerys R, Roels H, Genet P, Toussaint G, Bouchart A, et al. (1985) Fertility of male workers exposed to mercury vapor or to manganese dust: A questionnaire study. Am J Ind Med 7: 171–176.
20. Ponnapakkam TP, Bailey KS, Graves KA, Iszard MB (2003) Assessment of male reproductive system in the CD-1 mice following oral manganese exposure. Reprod Toxicol 17: 547–551.
21. Wirth J, Rossano MG, Daly DC, Paneth N, Puscheck E, et al. (2007) Ambient manganese exposure is negatively associated with human sperm motility and concentration. Epidemiology 18: 270–273.
22. Trettin KA, Gray TJ, Blazak WF (1995) Developmental toxicity of mangafodipir trisodium and manganese chloride in Sprague-Dawley rats. Teratology 52: 109–115.
29. Colomina MT, Domingo JL, Llobet JM, Corbella J (1996) Effect of day of exposure on the developmental toxicity of manganese in mice. Vet Hum Toxicol 38: 7–9.
30. Crossgrove J, Zheng W (2004) Manganese toxicity upon overexposure. NMR Biomed 17: 544–553.
31. Symonds HW, Hall ED (1983) Acute manganese toxicity and the absorption and biliary excretion of manganese in cattle. Res Vet Sci 35: 5–13.
32. [Anonymous] (1996) Chronic health hazard assessments for noncarcinogenic effects, Reference dose for chronic oral exposure (RfD). In: Anonymous. Integrated Risk Information System, Manganese (CASRN 7439-96-5).
33. World Health Organization (1999) Manganese and its compounds. Concise International Chemical Assessment Document 12.