Macrophage Solubilization and Cytotoxicity of Indium-Containing Particles as *in vitro* Correlates to Pulmonary Toxicity *in vivo*

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ABSTRACT

Macrophage-solubilized indium-containing particles (ICPs) were previously shown *in vitro* to be cytotoxic. In this study, we compared macrophage solubilization and cytotoxicity of indium phosphide (InP) and indium-tin oxide (ITO) with similar particle diameters ($\sim 1.5 \mu\text{m}$) and then determined if relative differences in these *in vitro* parameters correlated with pulmonary toxicity *in vivo*. RAW 264.7 macrophages were treated with InP or ITO particles and cytotoxicity was assayed at 24 h. Ionic indium was measured in 24 h culture supernatants. Macrophage cytotoxicity and particle solubilization *in vitro* were much greater for InP compared with ITO. To correlate changes *in vivo*, B6C3F1 mice were treated with InP or ITO by oropharyngeal aspiration. On Days 14 and 28, bronchoalveolar lavage (BAL) and pleural lavage (PL) fluids were collected and assayed for total leukocytes. Cell differentials, lactate dehydrogenase activity, and protein levels were also measured in BAL. All lavage parameters were greatly increased in mice treated with InP compared with ITO. These data suggest that macrophage solubilization and cytotoxicity of some ICPs *in vitro* are capable of predicting pulmonary toxicity *in vivo*. In addition, these differences in toxicity were observed despite the two particulate compounds containing similar amounts of indium suggesting that solubilization, not total indium content, better reflects the toxic potential of some ICPs. Soluble InCl_3 was shown to be more cytotoxic than InP to macrophages and lung epithelial cells *in vitro* further suggesting that ionic indium is the primary cytotoxic component of InP.

Key words: indium; InP; sintered ITO; solubilization; macrophage cytotoxicity; pulmonary toxicity

Indium is widely used in the semiconductor and microelectronics industries often in particulate form with examples being indium phosphide (InP) and indium-tin oxide (ITO). ITO, which typically consists of approximately 90% indium oxide (In_2O_3) and 10% tin oxide (SnO_2) by weight, is used in the manufacture of flat panel displays (Guo *et al.*, 2011) and accounts for a large percentage of the total indium demand in Japan (Tanaka *et al.*, 2010). ITO can be sintered or non-sintered; although the sintered form is most commonly used for industrial applications. Concerns have been raised over the safety and health of

industry workers exposed to particulate aerosols when working with materials containing these compounds. Inhalation exposure to indium-containing particles (ICPs) within an occupational setting, mostly ITO, has been associated with increased concentrations of indium measured in the blood, serum, and urine of workers and in the development of interstitial lung disease (Chonan *et al.*, 2007; Hamaguchi *et al.*, 2008; Homma *et al.*, 2005; Liu *et al.*, 2012; Miyaki *et al.*, 2003; Nakano *et al.*, 2009) as well as pulmonary alveolar proteinosis (Cummings *et al.*, 2010; Xiao *et al.*, 2010). In fact, inhalation exposure to ITO in an

occupational setting has resulted in two fatalities (Cummings *et al.*, 2010; Homma *et al.*, 2003). The American Conference of Governmental Industrial Hygienists (ACGIH) has recommended an occupational exposure limit of 0.1 mg/m^3 (threshold limit value-time weighted average) for all indium compounds (ACGIH, 2007). A critical issue to be addressed is whether the toxicity of ICPs can be predicted based on the total amount of indium present in the compound, the other constituents (eg, tin in ITO), and/or the solubilization potential. Indium ions released from the parent compound by solubilization are likely the main toxic constituents of some ICPs.

Subchronic and chronic inhalation exposure of rats and mice to InP and ITO particles resulted in pulmonary toxicity including severe alveolar proteinosis, chronic inflammation, interstitial fibrosis, and alveolar epithelial hyperplasia/metaplasia (Gottschling *et al.*, 2001; Nagano *et al.*, 2011a,b,c; NTP, 2001). In addition, carcinogenicity was observed after chronic inhalation exposure to InP and ITO in rodents and included increased incidences of alveolar/bronchiolar adenomas and carcinomas as well as squamous cell carcinoma of the lung (Gottschling *et al.*, 2001; Nagano *et al.*, 2011c; NTP, 2001). Administration of InP or ITO particles via oropharyngeal aspiration (Kirby *et al.*, 2009; Lison *et al.*, 2009) or intratracheal instillation (Tanaka *et al.*, 2002, 2010; Yamazaki *et al.*, 2000) also induced pulmonary injury, inflammation, and pleural effects. Upon deposition within the lung, alveolar macrophages (AMs) and likely epithelial cells initially phagocytose the InP and ITO particles which are insoluble at physiological pH; however, the mechanism(s) responsible for pulmonary toxicity is unclear. We recently showed *in vitro* that uptake of InP and ITO by macrophages (RAW 264.7 cells) and solubilization through the phagolysosomal acidification pathway after 24 h of treatment resulted in particle-induced cytotoxicity and the extracellular release of ionic indium (Gwinn *et al.*, 2013), presumably the primary cytotoxic constituent of InP and ITO. In this previous study, we developed a cell-based assay using graphite furnace-atomic absorption spectroscopy (GF-AAS) to measure the solubilization of ICPs and release of “free” ionic indium by macrophages. Lung epithelial (LA-4) cells were also shown to phagocytose InP and ITO; however, unlike macrophages, these cells did not solubilize the particles and were resistant to particle-induced cytotoxicity after 24 h of treatment (Gwinn *et al.*, 2013). The InP and ITO used in this previous study were of very different particle diameters and thus a valid direct comparison proved problematic with regards to relative particle solubilization and cytotoxicity.

In the current study, we compared the macrophage solubilization (release of ionic indium) and cytotoxicity of InP and ITO with similar particle diameters ($\sim 1.5 \mu\text{m}$) and then determined if relative differences in these *in vitro* parameters correlated with pulmonary toxicity *in vivo*. Our results showed that macrophage cytotoxicity and release of ionic indium *in vitro* as well as pulmonary toxicity *in vivo* were much greater for InP compared with ITO. This suggests that macrophage solubilization and particle-induced cytotoxicity of some ICPs *in vitro* are capable of predicting pulmonary toxicity *in vivo*. Furthermore, these differences in particle toxicity were observed despite the two compounds having similar indium content suggesting that particle solubilization, not total indium content, better reflects the toxic potential of some ICPs. Finally, we also demonstrated that soluble InCl_3 containing equivalent amounts of indium to that of insoluble particulate InP was more cytotoxic than InP to macrophages and lung epithelial cells *in vitro* suggesting that ionic indium is the primary cytotoxic constituent of InP.

MATERIALS AND METHODS

Indium compounds. InP particles (diameter $\sim 1.5 \mu\text{m}$) were obtained from Johnson-Matthey (Ward Hill, Massachusetts). This was the same InP used in the NTP 2 year chronic inhalation study (NTP, 2001). Sintered ITO particles were kindly provided by JX Nippon Mining and Metals (Tokyo, Japan) and consisted of approximately 90% indium oxide (In_2O_3) + 10% tin oxide (SnO_2) by weight (Nagano *et al.*, 2011a,b,c). The ITO particles were sonicated for 30 min in cell culture medium for the *in vitro* studies or in phosphate-buffered saline (PBS) for the *in vivo* studies based on the method as described by Levine *et al.* (2014) using a Branson 2210 ultrasonic bath (Branson Ultrasonics, Danbury, Connecticut) to reduce ITO particle agglomeration. Following sonication, the particle size for ITO was similar to that of InP (final diameter of ITO $\sim 1.7 \mu\text{m}$). Mean hydrodynamic particle diameters were measured by dynamic light scattering (DLS). We previously observed that ITO was not cytotoxic using *in vitro* assays with macrophages if it was not initially sonicated (data not shown) likely due to the presence of large particles (agglomerates) which were too big to be efficiently phagocytosed by the macrophages. InP and ITO particle suspensions were also vortexed well prior to use. The sonicated ITO particles remained finely dispersed, similar to InP, and did not re-aggregate during the cell incubation period which was visualized and confirmed by light microscopy (data not shown). On a per mass basis, InP and ITO (with $\sim 90\%$ indium oxide) contain approximately equivalent amounts of indium metal (eg, $1.37 \mu\text{moles}/158 \mu\text{g}$ indium in $200 \mu\text{g/ml}$ InP; $1.30 \mu\text{moles}/150 \mu\text{g}$ indium in $200 \mu\text{g/ml}$ ITO). Based on calculations using the particle diameters (as measured by DLS) and the theoretical densities of InP (4.8 g/cm^3) and ITO (7.2 g/cm^3), we estimated that a $200 \mu\text{g/ml}$ solution of InP or ITO contains approximately 2.4×10^7 or 1.1×10^7 particles per milliliter, respectively. InCl_3 was purchased from Sigma-Aldrich (St Louis, Missouri). The InCl_3 was combined with equimolar citrate to provide buffering to physiological pH prior to dilution in cell culture medium for *in vitro* studies.

Cell lines. The mouse adherent macrophage cell line, RAW 264.7 (RAW), and the mouse adherent lung-derived epithelial cell line, LA-4, were obtained from ATCC (Manassas, Virginia). RAW cells were cultured in DMEM (Gibco, Cat No. 11965, Life Sciences, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS) + penicillin/streptomycin at 37°C (in 5% CO_2). LA-4 cells were cultured in Ham's F12K medium (ATCC, Cat No. 30-2004) supplemented with 15% FBS + penicillin/streptomycin at 37°C (in 5% CO_2).

Cell culture. RAW cell culture with ICPs was performed as previously described (Gwinn *et al.*, 2013). Briefly, RAW cells were cultured for 24 h in 24 well plates (2×10^5 cells per well; 4–5 wells per treatment group for each experiment) followed by one rinse with medium (+3% FBS) to remove the non-adherent cells. RAW cells were then treated for 2 h with InP or ITO particles (50, 100, 200, or $400 \mu\text{g/ml}$), which contained similar amounts of indium, or medium (+3% FBS) alone in a 1 ml culture volume. After 2 h of treatment, the cells were rinsed once to wash out the remaining extracellular particles and then cultured in 1 ml of medium (+3% FBS) alone for 24 h. In some experiments, RAW cells were treated for 24 h with 1 ml of InP particles (200 or $250 \mu\text{g/ml}$), soluble InCl_3 (300 or $400 \mu\text{g/ml}$), medium alone or medium containing equivalent amounts of citrate buffer only (no indium). These concentrations were chosen so that the cells were treated with

equivalent amounts of indium for both compounds (200 µg/ml InP and 300 µg/ml InCl₃ each contain ~150 µg/ml indium; 250 µg/ml InP and 400 µg/ml InCl₃ each contain ~200 µg/ml indium). LA-4 cells were cultured for 24 h in 48 well plates (4 × 10⁴ cells per well; 4–5 wells per treatment group for each experiment) followed by 1 rinse with medium to remove the non-adherent cells. LA-4 cells were then treated for 24 or 48 h with 0.5 ml of InP particles (200 or 250 µg/ml), InCl₃ (300 or 400 µg/ml), medium alone or medium containing equivalent amounts of citrate buffer only (no indium).

Cytotoxicity assays. Lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assays were used to measure particle-induced cytotoxicity as previously described (Gwinn et al., 2013) in cell culture medium collected after 24 or 48 h of treatment. MTT and LDH absorbance values from InP- or ITO-treated cells were divided by the mean corresponding value from untreated (medium only) control cells. Absorbance values from InCl₃-treated cells were divided by the mean corresponding value from control cells treated with medium containing equivalent amounts of citrate buffer only (no indium). Mean data values for the *in vitro* studies are represented as fraction control (for MTT assay) or fold over control (for LDH assay).

Atomic absorption spectroscopy. RAW cell culture with ICPs followed by GF-AAS to detect extracellular indium was performed as previously described (Gwinn et al., 2013). Briefly, RAW cells were cultured for 24 h in 6 well plates (5 × 10⁵ cells per well; 5 wells per treatment group for each experiment) followed by 1 rinse with medium (+3% FBS) to remove the non-adherent cells. Cells were then treated for 24 h with InP or ITO particles (100 or 200 µg/ml) or medium (+3% FBS) alone. Cell culture supernatants (2 ml volume) were collected after 24 h into PYREX glass centrifuge tubes (Corning, Tewksbury, Massachusetts) and centrifuged for 3 h at 3000 × g (room temperature) to pellet any cells and/or residual particles present. Aliquots (0.5 ml) of cell and particle-free supernatants were transferred to 1.5 ml eppendorf tubes and acid-digested overnight at 70°C in 50% perchloric acid:nitric acid (2:1 [vol/vol]). This acid digestion step serves to remove any organic material contaminants, to release any free indium that may be bound to protein(s) in the culture medium (Moran and Seligman, 1989) and to concentrate the sample prior to analysis. The concentration of total ionic indium in µg/l or parts per billion (ppb) was measured by GF-AAS (AAAnalyst 600; PerkinElmer, Shelton, Connecticut). LDH activity was also measured in the supernatants. Controls for GF-AAS experiments included supernatants from 3 wells (per experiment) without cells treated with the same amount of particles in medium (background). Mean background indium concentration values were subtracted from the concentration values generated from particle-treated cells.

Animals. Male B6C3F1 mice (Charles River Laboratories, Raleigh, North Carolina) were 6–7 weeks of age upon receipt and were acclimated for 7 days prior to treatment. During acclimation, mice were uniquely identified, weighed, and randomized to treatment and vehicle groups. Mice were provided with food and water *ad libitum*. All procedures were approved by the Alion Science and Technology Animal Care and Use Committee.

In vivo experiments. B6C3F1 mice were administered a single dose of InP or ITO (0.5 or 1 mg/kg) or PBS vehicle (50 µl) by oropharyngeal aspiration under isoflurane anesthesia. This

method was based on our model as previously described for InP in which B6C3F1 mice treated with a single dose of InP exhibited lung injury and inflammation, as well as a pleural effusion of leukocytes (Kirby et al., 2009). On Days 14 and 28 after dosing, pleural lavage (PL) and bronchoalveolar lavage (BAL) fluids were collected from InP or ITO-treated mice using PBS and then briefly centrifuged to pellet the cells. Following RBC lysis, total numbers of leukocytes were counted in PL and BAL fluids by trypan blue exclusion. Cytopreps were prepared for the BAL samples followed by hematoxylin and eosin (H&E) staining in order to determine cell differentials. Cell differentials were not determined for the PL samples due to the absence of a neutrophilic inflammatory response in the PL of this model. LDH activity (Units/l) and total protein (mg/ml) (using a Bradford assay) were also measured in cell-free BAL fluid. LDH activity was measured in BAL fluid with the same method as used with the cell culture samples but is reported as Units/l. LDH activity and total protein were not measured in PL due to the lack of changes as previously observed for these two parameters in the PL of this model.

Statistics. Unpaired Student's *t* test (*in vitro* data) or 1-way ANOVA with Tukey's test (*in vivo* data) were performed using Excel or GraphPad Prism 6 (La Jolla, California) software, respectively. All data are presented as mean ± SEM with a 2-sided *P* value < 0.05 considered statistically significant.

RESULTS

InP Particles Are more Cytotoxic to Macrophages Compared with ITO

To compare the cytotoxicity of InP and ITO particles with similar particle diameters, RAW cells were treated as previously described (Gwinn et al., 2013) with InP or ITO, which contained equivalent amounts of indium, for 2 h to allow for the phagocytic uptake of particles. Macrophage uptake was similar for the 2 particles (data not shown). Following treatment, particle-induced cytotoxicity was measured after 24 h of culture. Cell viability (MTT values) decreased with increasing concentrations of InP (Fig. 1A) and was lowest at the InP concentration of 400 µg/ml (0.51 ± 0.04; fraction control). Cell viability also decreased with increasing concentrations of ITO (Fig. 1A) and was lowest at the ITO concentration of 400 µg/ml (0.69 ± 0.06). Cell viability was significantly lower at all four particle concentrations for InP-treated RAW cells compared with cells treated with ITO. LDH activity in medium from InP-treated cell cultures was elevated more than 5-fold at all four concentrations of InP (Fig. 1B) but peaked at the InP concentration of 100 µg/ml (7.09 ± 1.72; fold over control). LDH activity in medium from ITO-treated cell cultures increased with increasing concentrations of ITO (Fig. 1B) and peaked at the ITO concentration of 400 µg/ml (4.39 ± 0.30). LDH activity was significantly higher at all four particle concentrations for InP-treated RAW cells compared with cells treated with ITO. The InP used throughout these studies was not sonicated as was done with ITO; however, sonicated InP was found to be slightly more cytotoxic to the macrophages *in vitro* compared with no sonication but had a smaller particle diameter (~1.2 µm) as measured by DLS (data not shown). These data indicate that InP particles, compared with ITO, are more cytotoxic to macrophages despite the fact that both compounds contain similar amounts of indium.

Particle Solubilization within Macrophages and Extracellular Release of Ionic Indium Are Greater for InP Compared with ITO

GF-AAS was used as previously described (Gwinn et al., 2013) to determine the extent to which the macrophages, which have

FIG. 1. InP particles are more cytotoxic to macrophages compared with ITO. RAW cells were treated with InP or ITO particles (50, 100, 200, or 400 µg/ml) or medium alone for 2 h, rinsed once to wash out the remaining extracellular particles and then cultured for 24 h in medium prior to measuring cell death by MTT (A) and LDH (B) assays. Absorbance values from treated cells were divided by the mean corresponding value from untreated (medium alone) control cells and expressed as fraction control (MTT) or fold over control (LDH). Data on both graphs are presented as mean $\pm$ SEM (for 2 combined experiments). Unpaired Student's t test was used to compare InP versus ITO at each concentration with

phagocytosed the InP or ITO, breakdown and solubilize the particles into ionic indium. The concentration of total (solubilized) ionic indium in culture medium from RAW cells treated with InP at 100 µg/ml (48.2 ± 8.71 ppb) or 200 µg/ml (65.4 ± 3.06 ppb) was greatly increased compared with RAW cells treated with ITO at 100 µg/ml (0 ± 0 ppb) or 200 µg/ml (4.04 ± 1.69 ppb) after 24 h (Fig. 2A). As expected, LDH activity (to confirm cytotoxicity) was also much greater in culture medium from RAW cells treated for 24 h with InP at 100 µg/ml (4.46 ± 0.12 ; fold over control) or 200 µg/ml (6.08 ± 0.07) compared with RAW cells treated with ITO at 100 µg/ml (1.53 ± 0.09) or 200 µg/ml (2.35 ± 0.08) (Fig. 2B). These data show that the InP particles are more efficiently solubilized by macrophages compared with ITO which results in a greater release of ionic indium extracellularly by the dying InP-treated cells. Furthermore, more ionic indium was released by the InP-treated macrophages compared with ITO despite the two compounds containing similar amounts of indium.

Particle-Induced Pulmonary Toxicity Is Greater for InP Compared with ITO

The next objective was to determine whether the increased macrophage solubilization (release of ionic indium) and cytotoxicity of InP observed *in vitro*, compared with ITO, correlated with particle-induced pulmonary toxicity *in vivo* (Table 1).

FIG. 2. Particle solubilization within macrophages and extracellular release of ionic indium are greater for InP compared with ITO. RAW cells were treated for 24 h with InP or ITO (100 or 200 µg/ml). The concentration (ppb) of total ionic indium in cell culture medium was then measured by GF-AAS (A). Control groups included supernatants from wells without cells treated with the same amount of particles in medium (background). Mean background indium concentration values were subtracted from the concentration values generated from particle-treated cells. LDH activity was also measured in the RAW cell culture medium after treatment with InP or ITO to confirm cytotoxicity (B). Data on both graphs is presented as mean $\pm$ SEM (for one representative experiment). Unpaired Student's t test was used to compare InP versus ITO at each concentration with a 2-sided P value < 0.05 considered statistically significant (**P < 0.01 , ****P < 0.0001).

B6C3F1 mice were administered a single dose of InP or ITO particles (0.5 or 1 mg/kg) by oropharyngeal aspiration. On Days 14 and 28 after dosing, BAL and PL fluids were collected from InP- or ITO-treated mice. Increased LDH activity and total protein levels in BAL are indicative of cellular injury and protein leakage into the airways and lung tissue. LDH activity (Figs. 3A and 3B) and total protein levels (Figs. 3C and 3D) were both significantly increased in the BAL of InP-treated mice on Days 14 and 28 at doses of 0.5 mg/kg and 1 mg/kg compared with mice treated with ITO at the same doses. LDH activity and total protein levels were also significantly increased in the BAL of mice administered InP, but not ITO, compared with vehicle (saline) controls at doses of 0.5 mg/kg and 1 mg/kg on both Days 14 and 28. LDH activity and total protein levels at earlier time points (Days 3 and 7 but not Day 1) were also significantly increased in the BAL of mice treated with InP (1 mg/kg) compared with the same dose of ITO (data not shown).

Total numbers of leukocytes were significantly increased in the BAL of InP-treated mice on Day 14 only at 1 mg/kg compared with mice treated with the same dose of ITO (Fig. 4A). Total numbers of leukocytes were also significantly increased in the BAL of InP-treated mice on Day 28 at doses of 0.5 mg/kg and 1 mg/kg compared with mice treated with ITO at the same doses (Fig. 4B). Total numbers of leukocytes were significantly

TABLE 1. Pulmonary Toxicity of InP and ITO

	BAL					Pleural
	LDH (Units/l)	TP (mg/ml)	Total Cells ($\times 10^5$)	Macs ($\times 10^5$)	PMNs ($\times 10^5$)	Total Cells ($\times 10^6$)
Day 14						
Vehicle	28.9 (3.69)	0.07 (0.00)	0.46 (0.08)	0.46 (0.08)	0 (0)	4.41 (0.26)
InP 0.5 mg/kg	67.9 (11.3)*	0.26 (0.03)****	1.71 (0.54)	0.97 (0.20)*	0.25 (0.08)*	6.33 (0.79)*
InP 1 mg/kg	81.2 (8.87)***	0.32 (0.03)****	3.26 (0.42)****	2.0 (0.20)****	1.27 (0.31)***	7.67 (1.06)**
ITO 0.5 mg/kg	30.8 (5.20)	0.07 (0.00)	0.49 (0.05)	0.46 (0.04)	0.02 (0.01)	4.19 (0.27)
ITO 1 mg/kg	36.4 (2.99)	0.09 (0.01)	0.77 (0.08)	0.77 (0.08)	0.01 (0.00)	4.08 (0.17)
Day 28						
Vehicle	31.2 (2.84)	0.07 (0.00)	0.59 (0.08)	0.59 (0.08)	0 (0)	3.91 (0.24)
InP 0.5 mg/kg	76.5 (11.9)**	0.27 (0.03)****	2.30 (0.52)**	1.49 (0.41)*	0.81 (0.19)***	9.73 (2.20)*
InP 1 mg/kg	167 (9.49)****	0.42 (0.03)****	4.35 (0.79)***	2.96 (0.60)**	1.39 (0.21)****	14.3 (1.49)****
ITO 0.5 mg/kg	33.0 (3.16)	0.07 (0.00)	0.61 (0.05)	0.59 (0.05)	0.01 (0.00)	3.74 (0.33)
ITO 1 mg/kg	45.6 (2.96)	0.07 (0.01)	0.97 (0.16)	0.95 (0.15)	0.02 (0.01)	5.73 (0.83)

Notes: LDH, lactate dehydrogenase; TP, total protein; Macs, macrophages; PMNs, polymorphonuclear leukocytes. Mean (SEM); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (for InP vs ITO at each dose).

FIG. 3. Lung injury and protein leakage are greater in mice following treatment with InP compared with ITO. B6C3F1 mice were administered a single dose of InP or ITO (0.5 or 1 mg/kg) or PBS vehicle (50 μ l) by oropharyngeal aspiration. On Days 14 and 28, BAL and PL were collected. LDH activity (A, B) and total protein (C, D) were measured in cell-free BAL fluid as markers of cellular injury and protein leakage. Data on graphs are presented as mean $\pm$ SEM (for one representative experiment; $n = 5$ –7 mice per group for Day 14, $n = 6$ –7 mice per group for Day 28). 1-way ANOVA with Tukey's test was used to compare all 3 treatment groups (vehicle, InP and ITO) at each dose (0.5 or 1 mg/kg) with a 2-sided P value < 0.05 considered statistically significant. Only statistics for InP versus ITO at each dose are indicated on the graphs (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

increased in the BAL of InP-treated mice compared with vehicle controls at doses of 0.5 mg/kg (Day 28 only) and 1 mg/kg (Days 14 and 28). Total numbers of leukocytes in BAL were not significantly increased for ITO-treated mice compared with vehicle controls for either dose or time point. Cell differentials were determined for the BAL samples. Total numbers of macrophages were significantly increased in the BAL of InP-treated mice on Days 14 and 28 at 0.5 mg/kg and 1 mg/kg compared with mice treated with ITO at the same doses (Figs. 4C and 4D). Total numbers of macrophages were significantly increased in the BAL of InP-treated mice compared with vehicle controls at

doses of 0.5 mg/kg (Day 14 only) and 1 mg/kg (Days 14 and 28). Total numbers of macrophages in BAL were not significantly increased for ITO-treated mice compared with vehicle controls for either dose or time point. Total numbers of neutrophils (polymorphonuclear leukocytes, PMNs) were also significantly increased in the BAL of InP-treated mice on Days 14 and 28 at 0.5 mg/kg and 1 mg/kg compared with mice treated with ITO at the same doses (Figs. 4C and 4D). Total numbers of neutrophils were significantly increased in the BAL of mice administered InP, but not ITO, compared with vehicle controls at doses of 0.5 mg/kg and 1 mg/kg on both Days 14 and 28.

FIG. 4. Pulmonary inflammation is greater in mice following treatment with InP compared with ITO. B6C3F1 mice were administered a single dose of InP or ITO (0.5 or 1 mg/kg) or PBS vehicle (50 μ l) by oropharyngeal aspiration. On Days 14 and 28, BAL and PL were collected. Total numbers of leukocytes were counted in BAL (A, B) and PL (E, F). H&E-stained cytopreps were prepared and cell differentials were determined for the BAL samples. Macrophages (MACs) and neutrophils (PMNs) in BAL are shown (C, D). Data on graphs are presented as mean $\pm$ SEM (for one representative experiment; $n=5-7$ per group for Day 14, $n=6-7$ per group for Day 28). 1-way ANOVA with Tukey's test was used to compare all 3 treatment groups (vehicle, InP, and ITO) at each dose (0.5 or 1 mg/kg) with a 2-sided P value < 0.05 considered statistically significant. Only statistics for InP versus ITO at each dose are indicated on the graphs (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Total numbers of leukocytes were significantly increased in the PL of InP-treated mice on Days 14 and 28 at doses of 0.5 mg/kg and 1 mg/kg compared with mice treated with ITO at the same doses (Figs. 4E and 4F). Total numbers of leukocytes were significantly increased in the PL of InP-treated mice compared with vehicle controls at doses of 0.5 mg/kg (Day 28 only) and 1 mg/kg (Days 14 and 28). Total numbers of leukocytes in PL were not significantly increased for ITO-treated mice compared with vehicle controls for either dose or time point. We previously demonstrated that the recruitment of leukocytes into BAL and PL in response to InP was delayed (peaked at Day 28) (Kirby et al., 2009); thus total leukocyte numbers (and cell differentials) were not determined for earlier time points. These *in vivo* data indicate that InP particles induced greater lung injury with neutrophilic inflammation, as well as a greater pleural effusion of leukocytes, compared with ITO despite the two compounds containing similar amounts of indium.

Soluble InCl₃ Is more Cytotoxic to Macrophages and Lung Epithelial Cells Compared with InP

RAW or LA-4 cells were treated with InCl₃ or InP particles to determine whether a soluble (at physiological pH) indium

compound was more cytotoxic *in vitro* compared with an insoluble particulate one. The concentrations of InCl₃ and InP compared in these experiments (200 μ g/ml InP vs 300 μ g/ml InCl₃ and 250 μ g/ml InP vs 400 μ g/ml InCl₃) were chosen so that the cells were treated with equivalent concentrations of indium (150 or 200 μ g/ml) using both compounds. Consistent with our previous results (Gwinn et al., 2013), InP particles were cytotoxic to the RAW cells (Figs. 5A and 5B), but not the LA-4 cells (Figs. 5C and 5D), after 24 h of treatment based on both MTT and LDH assays. However, compared with InP, InCl₃ (containing similar levels of indium to that of InP) was much more cytotoxic to the RAW cells after 24 h of treatment at both indium concentrations (Figs. 5A and 5B). The viability of RAW cells treated for 24 h with InCl₃ containing 150 μ g/ml (0.03 ± 0.0004) or 200 μ g/ml (0.03 ± 0.001) indium was much lower compared with cells treated with InP particles containing 150 μ g/ml (0.50 ± 0.01) or 200 μ g/ml (0.47 ± 0.01) indium (Fig. 5A). LDH activity in culture medium from RAW cells treated for 24 h with InCl₃ containing 150 μ g/ml (15.3 ± 0.30) or 200 μ g/ml (16.5 ± 0.40) indium was also much higher compared with cells treated with InP containing 150 μ g/ml (4.96 ± 0.07) or 200 μ g/ml (4.53 ± 0.11) indium (Fig. 5B). Although statistically

FIG. 5. Soluble InCl₃ is more cytotoxic to macrophages and lung epithelial cells compared with InP. RAW (A, B) or LA-4 (C, D) cells were treated with InP particles (200 or 250 µg/ml) or InCl₃ (300 or 400 µg/ml) containing equivalent concentrations of indium (150 or 200 µg/ml) for 24 or 48 h prior to measuring cell death by MTT (A, C) and LDH (B, D) assays. Absorbance values from InP or InCl₃-treated cells were divided by the mean corresponding value from untreated (medium only) control cells or from control cells treated with medium containing equivalent levels of citrate buffer only (no indium), respectively, and expressed as fraction control (MTT) or fold over control (LDH). Data on graphs are presented as mean ± SEM (for one representative experiment at 24 h; for 2 combined experiments at 48 h). Unpaired Student's *t* test was used to compare InP versus InCl₃ at each indium concentration (at 24 h for the RAW cells in A, B; at 24 and 48 h for the LA-4 cells in C, D) with a 2-sided *P* value < 0.05 considered statistically significant (**P* < 0.05, ****P* < 0.001, *****P* < 0.0001).

significant, InCl₃ was only slightly more cytotoxic than InP to the LA-4 cells after 24 h of treatment based on MTT and LDH assays (Figs. 5C and 5D); therefore, cytotoxicity to the LA-4 cells was also measured at 48 h. InP particles began to exhibit cytotoxicity to the LA-4 cells after 48 h of treatment mainly as evidenced by the MTT values (Fig. 5C) being slightly less than those of 24 h-treated RAW cells (Fig. 5A). This suggests that the LA-4 cells over time are not resistant to InP-induced cytotoxicity but instead are less susceptible compared with the RAW cells likely due to less efficient processing of the internalized particles within the phagolysosomal pathway. InCl₃ was more cytotoxic than InP to the LA-4 cells after 48 h of treatment at both indium concentrations (Figs. 5C and 5D), although less cytotoxic compared with 24 h-treated RAW cells (Figs. 5A and 5B) especially based on measurements of LDH activity. The viability of LA-4 cells treated for 48 h with InCl₃ containing 150 µg/ml (0.18 ± 0.01) or 200 µg/ml (0.08 ± 0.01) indium was much lower compared with cells treated with InP particles containing 150 µg/ml (0.43 ± 0.02) or 200 µg/ml (0.38 ± 0.004) indium (Fig. 5C). LDH activity in culture medium from LA-4 cells treated for 48 h with InCl₃ containing 150 µg/ml (1.99 ± 0.05) or 200 µg/ml (2.55 ± 0.06) indium was also higher compared with cells treated with InP containing 150 µg/ml (1.52 ± 0.04) or 200 µg/ml (1.58 ± 0.05) indium. These data indicate that soluble InCl₃ containing equivalent amounts of indium to that of particulate InP was more cytotoxic than InP to lung epithelial cells, and especially macrophages, suggesting that solubilized (ionic) indium is the main cytotoxic component of InP.

DISCUSSION

We previously showed *in vitro* that InP and ITO particles, following phagocytic uptake by macrophages, were solubilized within the cell through the phagolysosomal acidification pathway which resulted in cell death and the subsequent release of indium ions extracellularly by the dying cells (Gwinn et al., 2013). This was not true for lung epithelial cells at 24 h despite uptake of the particles. These earlier studies were conducted using InP and ITO with very different hydrodynamic particle diameters (~1.5 and 0.5 µm, respectively, as measured by DLS) therefore making a direct comparison between the 2 compounds problematic since particle size in itself can influence toxicity both *in vitro* and *in vivo*. Also, non-sintered ITO was used in this previous study which is not the form of ITO most commonly used for industrial applications. In the current study, we compared InP and sintered ITO with a similar particle diameter (~1.5 µm) to determine whether the *in vitro* parameters of macrophage solubilization and particle-induced cytotoxicity correlated with pulmonary toxicity *in vivo*. We observed that InP was much more cytotoxic to macrophages *in vitro* compared with ITO containing similar amounts of indium. Macrophage particle solubilization with release of ionic indium (as measured by GF-AAS) *in vitro* was also much greater for InP compared with ITO. This suggests that the increased solubilization of InP by macrophages compared with ITO is linked to increased cytotoxicity. The percentage of InP particles solubilized after 24 h appeared very low relative to the input mass (200 µg/ml) based on GF-AAS measurements. However, one must take into consideration that not all of the particles were engulfed

(many particles adhered to the well). Also, it is likely that not all of the internalized particles were solubilized by the macrophages within 24 h and the indium measured in culture supernatants represented only that which was “leaked” or released by the cells.

When administered *in vivo* to B6C3F1 mice by oropharyngeal aspiration, particle-induced pulmonary toxicity (characterized by lung injury and inflammation as well as pleural effects) was greater for InP compared with ITO. This indicates that the *in vitro* macrophage parameters measured in this study for InP and ITO (particle solubilization, cytotoxicity, and release of ionic indium) correlated with (or predicted) particle-induced pulmonary toxicity *in vivo* and may be applicable to other ICPs. This correlation also suggests that the “free” indium ions released by AMs may play a pivotal role in the mechanism(s) driving particle-induced pulmonary toxicity. However, the mechanism(s) involved including the contribution of the solubilized (ionic) form of indium to pulmonary toxicity, either directly or indirectly, are not completely understood. We demonstrated that soluble InCl_3 containing equivalent amounts of indium and the same valence of 3 to that of particulate InP was more cytotoxic than InP to lung epithelial cells, and especially macrophages, *in vitro* suggesting that solubilized (ionic) indium is the main cytotoxic constituent of InP and perhaps other ICPs. Despite the low percentage of InP particles solubilized by the macrophages after 24 h as measured by GF-AAS, the fact that the solubilized form of indium was much more toxic than particulate InP suggests that low levels of “free” indium may still have a toxic effect. The reason(s) for the dramatically increased susceptibility of the RAW cells to InCl_3 -induced cytotoxicity compared with the LA-4 cells is not clear, but ionic indium binds with high affinity to transferrin (Moran and Seligman, 1989) and RAW cells may have increased cell surface expression of the transferrin receptor. To lend further support to the notion that ionic indium is the toxic constituent of InP, we previously showed *in vivo* that soluble InCl_3 exhibited greater pulmonary toxicity than InP particles in mice following oropharyngeal aspiration despite the InCl_3 dosing formulation containing slightly less total indium than InP (Kirby *et al.*, 2009).

Only 2 ICPs were compared in this study. It would be most informative to compare a wide variety of ICPs; however, the limiting factor and biggest challenge is obtaining particles with similar diameters in order to be able to directly compare multiple ICPs with regards to the parameters of macrophage solubilization, cytotoxicity, and *in vivo* pulmonary toxicity. Also, this model may be applicable to other metal-containing particulate compounds which are toxic to the lung and initially phagocytosed by AMs following inhalation or aspiration/instillation. Recent studies have suggested that the toxicity of some silver and zinc-containing particulate compounds *in vitro* were linked to particle dissolution and the generation of silver or zinc ions, respectively, within acidic phagolysosomes of macrophages (Cho *et al.* 2011; Hamilton *et al.*, 2014). Future studies intend to address other ICPs as well as alternative metal-containing particles. In theory, this *in vitro* macrophage model could provide a useful screening tool for predicting the relative toxicity of some ICPs (or perhaps other metal-containing particulate compounds) with a reduced need for animal toxicity testing of particulate compounds. Of course to do so, this model would have to be further validated with multiple ICPs.

InP and sintered ITO are 2 types of ICPs widely used in the microelectronics industry with ITO being the most in demand (Tanaka *et al.*, 2010). Our results demonstrated that InP was much more toxic than ITO using the same doses and endpoints

for each in both *in vitro* and *in vivo* assays despite the 2 compounds containing similar amounts of indium. Also, the generation and release of ionic indium by macrophages was much greater for InP compared with ITO despite both having similar indium content. These data suggest that the solubilization potential of some ICPs, not the total indium present in the particulate compound, determines toxicity. Tanaka *et al.* showed that systemic toxicity (as evidenced by weight loss), lung lesions, and inflammation were more severe in hamsters after long-term treatment through intratracheal instillation with multiple doses (once per week for 16 weeks) of InP compared with sintered ITO containing equivalent amounts of indium (Tanaka *et al.*, 2002). Systemic toxicity was not observed in our *in vivo* studies likely because the mice were administered lower doses (0.5 or 1 mg/kg) of InP or ITO in just a single treatment, which only caused effects locally in the airways/lung, and the animals were sacrificed much earlier (Days 14 or 28) after dosing. Sintering of In_2O_3 with SnO_2 to form ITO involves the introduction of a high density of free electrons and oxygen vacancies into the In_2O_3 crystal structure, which in turn gives ITO its unique electronic properties (Lison *et al.*, 2009). Thus, in the case of ITO, the sintering process may impart more resistance to solubilization compared with InP. In addition to solubilization potential, toxicity may also be related to the partner ion(s) in the compound, for example, the presence of tin in ITO or phosphorus in InP. However, we previously described that indium oxide (In_2O_3), not tin oxide (SnO_2), was the cytotoxic component of ITO *in vitro* (Gwinn *et al.*, 2013). Others have also reported that the toxicity of inorganic tin after inhalation is low, and phosphorus (in InP) is considered a biologically essential element (Tanaka *et al.*, 2002). In a chronic study, Yamazaki *et al.* showed that serum indium levels and systemic toxicity were greater in hamsters up to 2 years after completion of an intratracheal treatment regimen (twice per week for 8 weeks) with indium arsenide (InAs) compared with InP containing equivalent amounts of indium. Thus, the increased toxicity of InAs may have been due to increased solubilization and/or the presence of inorganic arsenic as a partner ion which is well-established as a potent lung toxicant and carcinogen (Tokar *et al.*, 2011). Lung lesions and inflammation were similar for the 2 compounds despite the difference in the concentration of serum indium; however, one must take into consideration that this study looked at the cumulative effects of multiple treatments (as opposed to just a single dose) over a much longer time period and that the presence of a partner ion as toxic to the lung as arsenic may have confounded the toxic effects of indium.

LDH activity was found to be significantly higher in the BAL fluid of InP-treated mice on Days 3 and 7 compared with mice treated with ITO (data not shown). GF-AAS was used in an attempt to measure indium in serum as well as in (cell and particle-free) BAL fluid at these earlier time points, but indium levels were found to be below the limit of detection on both Days 3 and 7 (data not shown). Future studies are planned to use more sensitive methods of detection such as inductively coupled plasma-mass spectrometry (ICP-MS) to measure indium levels *in vivo* within the context of this model. However, in contrast to the study by Yamazaki *et al.* in which indium was detected by ICP-MS in serum over time following completion of a lengthy dosing regimen involving multiple doses of InAs (4 mg/kg) or InP (3 mg/kg) (Yamazaki *et al.*, 2000), our exposure regimen involved just a single dose of InP or ITO at a 3- to 4-fold lower dose (1 mg/kg) which may have resulted in the indium levels within serum and BAL fluid being too low to detect.

Also, it is not clear what time points are most optimal to assay *in vivo* since the kinetics of particle solubilization and ionic indium release following exposure to a single dose of InP or ITO are unknown. Furthermore, it is possible that the ionic indium (following solubilization and release by AMs) more readily enters the blood circulation and/or is scavenged by other cell type(s) in the airways and lung via high affinity binding to transferrin and the transferrin receptor pathway (Moran and Seligman, 1989), which could make detection in BAL fluid difficult.

We have previously shown that InP particles can still be seen in AMs on Day 7 following treatment in mice (Kirby et al., 2009). We believe that the delayed toxicity observed following exposure to InP may be due to the persistence of particles within AMs in the lung and/or the amount of time needed by the AMs to solubilize the particles and generate sufficient levels of ionic indium to cause pulmonary toxicity. Alternatively, solubilization of InP particles within AMs and release of ionic indium may occur relatively quickly, but the pathological effects induced downstream by ionic indium may take longer to develop. The latter would be consistent with a scenario in which the ionic indium is generated by the AMs and then rapidly cleared from the lung.

On a per mass basis (eg, in 200 µg/ml), we estimated that InP has approximately 2-fold more particles than ITO based on calculations using the hydrodynamic particle diameters and theoretical densities of the 2 compounds. However, if you compared InP and ITO at equivalent particle numbers (eg, 100 µg/ml InP vs 200 µg/ml ITO *in vitro* or 0.5 mg/kg InP vs 1 mg/kg ITO *in vivo*), InP was still significantly more toxic than ITO for the majority of the *in vitro* and *in vivo* parameters measured (data not shown). This is despite the fact that at equivalent particle numbers for the 2 compounds ITO contains approximately 2-fold more indium again suggesting that particle solubilization, not total indium content, may be most important in predicting toxicity.

In conclusion, based on our *in vitro* and *in vivo* data, our proposed model within the context of InP and ITO is as follows. AMs phagocytose the particles following lung deposition either via inhalation or aspiration/instillation; however, compared with ITO, InP particles are much more efficiently solubilized by the macrophages following uptake (via phagolysosomal acidification) into ionic indium, which is cytotoxic to the cell. Particle-induced cytotoxicity (with necrosis and/or apoptosis of the macrophage), which is much greater for InP compared with ITO, results in further release of solubilized (ionic) indium (together with other factors) extracellularly by the dying cells. This “free” ionic indium, which is much more toxic than particulate InP, may then in part drive pulmonary toxicity downstream via, for example, direct toxicity on adjacent macrophages, lung epithelial cells, and/or other cell types and/or indirectly via the induction of toxic-free radicals/reactive oxygen species causing oxidative stress (Chibbli et al., 2011; Gottschling et al., 2001; Lin et al., 2013; Lison et al., 2009; Zurita et al., 2007).

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REFERENCES

- ACGIH. (2007). *Threshold Limit Values for Chemical Substances and Physical Agents, and Biological Exposure Indices*, p. 35. American Conference of Governmental Industrial Hygienists, Cincinnati, OH.
- Chibbli, H., Carlini, L., Park, S., Dimitrijevic, N. M., and Nadeau, J. L. (2011). Cytotoxicity of InP/ZnS quantum dots related to reactive oxygen species generation. *Nanoscale* 3, 2552–2559.
- Chonan, T., Taguchi, O., and Omae, K. (2007). Interstitial pulmonary disorders in indium-processing workers. *Eur. Respir. J.* 29, 317–324.
- Cho, W. S., Duffin, R., Howie, S. E., Scotton, C. J., Wallace, W. A., Macnee, W., Bradley, M., Megson, I. L., and Donaldson, K. (2011). Progressive severe lung injury by zinc oxide nanoparticles; the role of Zn²⁺ dissolution inside lysosomes. *Part. Fibre Toxicol.* 8, 27.
- Cummings, K. J., Donat, W. E., Ettensohn, D. B., Roggli, V. L., Ingram, P., and Kreiss, K. (2010). Pulmonary alveolar proteinosis in workers at an indium processing facility. *Am. J. Respir. Crit. Care Med.* 181, 458–464.
- Gottschling, B. C., Maronpot, R. R., Hailey, J. R., Peddada, S., Moomaw, C. R., Klaunig, J. E., and Nyska, A. (2001). The role of oxidative stress in indium phosphide-induced lung carcinogenesis in rats. *Toxicol. Sci.* 64, 28–40.
- Guo, E.-J., Guo, H., Lu, H., Jin, K., He, M., and Yang, G. (2011). Structure and characteristics of ultrathin indium tin oxide films. *Appl. Phys. Lett.* 98, 011905-1–011905-3.
- Gwinn, W. M., Qu, W., Shines, C. J., Bousquet, R. W., Taylor, G. J., Waalkes, M. P., and Morgan, D. L. (2013). Macrophage solubilization and cytotoxicity of indium-containing particles *in vitro*. *Toxicol. Sci.* 135, 414–424.
- Hamaguchi, T., Omae, K., Takebayashi, T., Kikuchi, Y., Yoshioka, N., Nishiwaki, Y., Tanaka, A., Hirata, M., Taguchi, O., and Chonan, T. (2008). Exposure to hardly soluble indium compounds in ITO production and recycling plants is a new risk for interstitial lung damage. *Occup. Environ. Med.* 65, 51–55.
- Hamilton, R. F., Buckingham, S., and Holian, A. (2014). The effect of size on Ag nanosphere toxicity in macrophage cell models and lung epithelial cell lines is dependent on particle dissolution. *Int. J. Mol. Sci.* 15, 6815–6830.
- Homma, S., Miyamoto, A., Sakamoto, S., Kishi, K., Motoi, N., and Yoshimura, K. (2005). Pulmonary fibrosis in an individual occupationally exposed to inhaled indium-tin oxide. *Eur. Respir. J.* 25, 200–204.
- Homma, T., Ueno, T., Sekizawa, K., Tanaka, A., and Hirata, M. (2003). Interstitial pneumonia developed in a worker dealing with particles containing indium-tin oxide. *J. Occup. Health* 45, 137–139.
- Kirby, P. J., Shines, C. J., Taylor, G. J., Bousquet, R. W., Price, H. C., Everitt, J. I., and Morgan, D. L. (2009). Pleural effects of indium phosphide in B6C3F1 mice: nonfibrous particulate induced pleural fibrosis. *Exp. Lung Res.* 35, 858–882.

- Levine, K. L., Han, L., Gwinn, W. M., Morgan, D. L., Ross, G. T., Essader, A. S., Fernando, R., Haines, L. G., and Robinson, V. G. (2014). Development and optimization of a procedure for the determination of indium-tin oxide particle size and concentration in cellular media. *Anal. Lett.* **47**, 1614–1625.
- Lin, R.-H., Yang, M.-L., Li, Y.-C., Chang, H.-M., and Kuan, Y.-H. (2013). Indium chloride-induced micronuclei via reactive oxygen species in Chinese hamster lung fibroblast V79 cells. *Environ. Toxicol.* **28**, 595–600.
- Lison, D., Laloy, J., Corazzari, I., Muller, J., Rabolli, V., Panin, N., Huaux, F., Fenoglio, I., and Fubini, B. (2009). Sintered indium-tin-oxide (ITO) particles: a new pneumotoxic entity. *Toxicol. Sci.* **108**, 472–481.
- Liu, H. H., Chen, C. Y., Chen, G. I., Lee, L. H., and Chen, H. L. (2012). Relationship between indium exposure and oxidative damage in workers in indium tin oxide production plants. *Int. Arch. Occup. Environ. Health* **85**, 447–453.
- Miyaki, K., Hosoda, K., Hirata, M., Tanaka, A., Nishiwaki, Y., Takebayashi, T., Inoue, N., and Omae, K. (2003). Biological monitoring of indium by means of graphite furnace atomic absorption spectrophotometry in workers exposed to particles of indium compounds. *J. Occup. Health* **45**, 228–230.
- Moran, P. L., and Seligman, P. A. (1989). Effects of transferrin-indium on cellular proliferation of a human leukemia cell line. *Cancer Res.* **49**, 4237–4241.
- Nagano, K., Gotoh, K., Kasai, T., Aiso, S., Nishizawa, T., Ohnishi, M., Ikawa, N., Eitaki, Y., Yamada, K., Arito, H., and Fukushima, S. (2011a). Two- and 13-week inhalation toxicities of indium-tin oxide and indium oxide in rats. *J. Occup. Health* **53**, 51–63.
- Nagano, K., Nishizawa, T., Eitaki, Y., Ohnishi, M., Noguchi, T., Arito, H., and Fukushima, S. (2011b). Pulmonary toxicity in mice by 2- and 13-week inhalation exposures to indium-tin oxide and indium oxide aerosols. *J. Occup. Health* **53**, 234–239.
- Nagano, K., Nishizawa, T., Umeda, Y., Kasai, T., Noguchi, T., Gotoh, K., Ikawa, N., Eitaki, Y., Kawasumi, Y., Yamauchi, T., et al. (2011c). Inhalation carcinogenicity and chronic toxicity of indium-tin oxide in rats and mice. *J. Occup. Health* **53**, 175–187.
- Nakano, M., Omae, K., Tanaka, A., Hirata, M., Michikawa, T., Kikuchi, Y., Yoshioka, N., Nishiwaki, Y., and Chonan, T. (2009). Causal relationship between indium compound inhalation and effects on the lungs. *J. Occup. Health* **51**, 513–521.
- NTP. (2001). Toxicology and carcinogenesis studies of indium phosphide (CAS No. 22398-90-7) in F344/N rats and B6C3F1 mice (inhalation studies). *Natl. Toxicol. Program Tech. Rep. Ser.* **499**, 7–340.
- Tanaka, A., Hirata, M., Homma, T., and Kiyohara, Y. (2010). Chronic pulmonary toxicity study of indium-tin oxide and indium oxide following intratracheal instillations into the lungs of hamsters. *J. Occup. Health* **52**, 14–22.
- Tanaka, A., Hirata, M., Omura, M., Inoue, N., Ueno, T., Homma, T., and Sekizawa, F. (2002). Pulmonary toxicity of indium-tin oxide and indium phosphide after intratracheal instillations into the lung of hamsters (short communication). *J. Occup. Health* **44**, 99–102.
- Tokar, E. J., Benbrahim-Tallaa, L., and Waalkes, M. P. (2011). Metal ions in human cancer development. *Met. Ions Life Sci.* **8**, 375–401.
- Xiao, Y. L., Cai, H. R., Wang, Y. H., Meng, F. Q., and Zhang, D. P. (2010). Pulmonary alveolar proteinosis in an indium-processing worker. *Chin. Med. J. (Engl.)* **123**, 1347–1350.
- Yamazaki, K., Tanaka, A., Hirata, M., Omura, M., Makita, Y., Inoue, N., Sugio, K., and Sugimachi, K. (2000). Long term pulmonary toxicity of indium arsenide and indium phosphide instilled intratracheally in hamsters. *J. Occup. Health* **42**, 169–178.
- Zurita, J. L., Jos, A., del Peso, A., Salguero, M., Camean, A. M., Lopez-Artiguez, M., and Repetto, G. (2007). Toxicological assessment of indium nitrate on aquatic organisms and investigation of the effects on the PLHC-1 fish cell line. *Sci. Total Environ.* **387**, 155–165.