Time- and Concentration-Dependent Genomic Responses of the Rat Airway to Inhaled Nickel Sulfate

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While insoluble nickel subsulfide (Ni3S2) was carcinogenic in the lung in a 2-year rat bioassay, soluble nickel sulfate hexahydrate (NiSO4·6H2O) was not. To investigate whether differences in the cellular responses to these two nickel compounds could underlie their differential activities, we conducted parallel studies to determine the gene expression changes in micro-dissected lung distal airway cells from Fischer 344 rats following inhalation of the two compounds for one and four weeks (6 hr per day, 5 days per week). The results of the Ni3S2 study have been reported previously; this paper reports the results for NiSO4 and provides a comparative analysis. The cellular responses to NiSO4 were highly similar to those previously reported for Ni3S2, and a set of genes was identified whose expression could be used as biomarkers for comparing cellular nickel effects from in vitro or in vivo studies with soluble NiSO4 and particulate Ni3S2. Evaluation of the genomic concentration-responses for the two compounds suggests that the highest inhaled concentration in the tumor bioassay for NiSO4, which was limited by toxicity, may not have achieved the Ni concentrations at which tumors were observed in the Ni3S2 bioassay. However, several key differences in the immune responses to NiSO4 and Ni3S2 were identified that may result from the differential intracellular disposition of Ni from NiSO4 entering the cell as an ion rather than as a slowly soluble Ni3S2 particle. These differences may also contribute to the observation of tumors in the bioassay for Ni3S2 but not NiSO4. Environ. Mol. Mutagen. 58:607–618, 2017. © 2017 The Authors Environmental and Molecular Mutagenesis published by Wiley Periodicals, Inc. on behalf of Environmental Mutagen Society

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INTRODUCTION

Inhaled nickel (Ni) compounds have been associated with increased nasal and lung tumors in workers (Doll et al., 1990). Nickel compounds are only weakly genotoxic (Coogan et al., 1989; Arrouijal et al., 1990; Snow, 1992), and their carcinogenicity is suspected to involve an indirect mode of action such as oxidative stress (Huang et al., 1994a,b; Dally and Hartwig, 1997) or changes in chromatin condensation (Huang et al., 1995; Klein and Costa, 1997; Zoroddu et al., 2002; Ellen et al., 2009). While soluble and insoluble nickel compounds have been associated with increased risk of lung tumors in epidemiological studies, only insoluble compounds (nickel oxide, crystalline nickel subsulfide) demonstrated induction of lung tumors in inhalation carcinogenicity studies in rats; inhalation of 0.1 mg Ni/m3 Ni3S2 increased the incidence of lung tumors in rats while inhalation of 0.1 mg Ni/m3 NiSO4 (equal “nickel” exposure levels) did not (Dunnick et al., 1995; NTP, 1996a,b,c).

Ni sulfate hexahydrate (referred here as NiSO4) is an example of highly soluble nickel salt that easily releases
Ni(II) ions upon inhalation while nickel subsulfide (referred here as Ni$_3$S$_2$) is insoluble in water and poorly soluble in interstitial lung fluid. The different physico-chemical properties of these two compounds affect their lung clearance (more rapid for NiSO$_4$ than for Ni$_3$S$_2$), their toxicity (higher toxicity for NiSO$_4$ than for Ni$_3$S$_2$, maximum tolerated doses (MTD) of 0.5 mg/m$^3$ (0.1 mg Ni/m$^3$) for NiSO$_4$ and 1 mg/m$^3$ (0.7 mg Ni/m$^3$) for Ni$_3$S$_2$), and also their intracellular uptake and localization. More efficient uptake and delivery of Ni(II) ion to the cell nucleus from Ni$_3$S$_2$ than from NiSO$_4$ has been observed in vitro (e.g., Ke et al., 2007).

To understand whether the different tumor outcomes between these two compounds are driven by differences in mode of action or in dosimetry (i.e., more nickel delivered to nucleus from Ni$_3$S$_2$ than can be delivered from NiSO$_4$ at the MTD), we undertook the conduct of parallel short-term inhalation studies with Ni$_3$S$_2$ and NiSO$_4$. The design of these studies is shown in Figure 1. Briefly, we examined the changes in gene expression in lung tissue isolated from rats exposed for 1 and 4 weeks to four different concentrations Ni$_3$S$_2$ or NiSO$_4$, with exposures levels selected to match those used in the NTP studies and

Fig. 1. Experimental design for the inhalation studies with NiSO$_4$ (this study) and Ni$_3$S$_2$ (Efremenko et al., 2014) in rats. NiSO$_4$ aerosol concentrations were 0 (control), 0.03, 0.06, 0.11, and 0.22 mg Ni/m$^3$ (0, 0.125, 0.25, 0.5, and 1.0 mg NiSO$_4$·6H$_2$O/m$^3$). Ni$_3$S$_2$ aerosol concentrations were 0, 0.03, 0.06, 0.11, and 0.44 mg Ni/m$^3$ (0.04, 0.08, 0.15, 0.60 mg Ni$_3$S$_2$/m$^3$). N = 5 animals per group per timepoint for BALF and histopathology analyses and 8 animals per groups per timepoint for gene expression analysis and lung Ni burden measurement (Ni$_3$S$_2$ exposures only).
also extended twofold above the highest exposure concentration studied by NTP in the case of NiSO₄ (NTP 1996b). The already published results of the study with Ni₃S₂ (Efremenko et al., 2014), support a mode of action for Ni₃S₂ carcinogenicity driven by chronic toxicity, including immune signaling and proliferative pressure, rather than by direct genotoxicity.

This manuscript reports the complete lung histopathology, broncho-alveolar lavage fluid (BALF) evaluation and gene expression results, including benchmark dose analysis, from a 1- and 4-week inhalation study with NiSO₄. However, the primary analysis focuses on a comparison of the tumor target tissue gene expression responses to NiSO₄ and to Ni₃S₂ after 4 weeks of exposure, to elucidate factors that could contribute to the differential tumorigenicity of these two compounds. In both studies the lung distal airway was microdissected (Baker et al., 2004) to examine the tissue where lung tumors usually originate (Schuller, 1990). A number of bioinformatics approaches are used to evaluate the similarities and differences in the gene expression response to the two substances as a function of dose. Finally, the observed in vivo responses are considered with regard to informing future in vitro studies with Ni compounds, both by identifying possible hypotheses suitable for testing in vitro and by providing genomic biomarkers of internal cellular exposure that can be used to compare results from disparate studies of Ni compounds.

MATERIALS AND METHODS

The present study was intentionally conducted in a similar manner as possible to our previously reported study with Ni₃S₂ (Efremenko et al., 2014), so that direct comparisons of the effects of the two compounds could be made (Fig. 1). Since the bulk of the methods for the two studies are essentially identical, only those aspects that differ substantially are included here. However, the complete methods are included in the Supporting Information.

Test Substance

Nickel sulfate hexahydrate (NiSO₄ • 6H₂O; CAS No. 10101-97-0), 99% purity, 22% nickel content, was obtained from the NiPERA Sample Repository housed at Particle Technology Labs (PTL, Grove, Ill.). This test substance was purchased from Aldrich Chemicals (Catalog # 227676) and kept at room temperature in closed containers under nitrogen.

Inhalation Exposures

Male Fischer 344 rats were exposed by whole body inhalation as described (Efremenko et al., 2014). Atmospheres of NiSO₄ aerosol targeted concentrations of 0 (control), 0.125, 0.25, 0.5, and 1.0 mg/m³ in air (0.03, 0.06, 0.11, and 0.22 mg Ni/m³). Animals were exposed to a target concentration for 6 hr/day, 5 days/week for either 1 or 4 weeks (5 or 20 exposure days). A solution of NiSO₄ was prepared in distilled water and pumped (FMI, Fluid Metering, Inc., Syosset, NY) into a Collison Nebulizer (BGI, Inc., Waltham, MA) with either a 3-Jet (Model CN-24J) or 1-Jet (Model CN-24/1J) sprayer. The pump flow rate was adjusted to maintain a standard level of solution within the Collison fluid jar. Compressed air at approximately 7–15 psi was supplied to the nebulizer and adjusted during daily exposure to maintain target exposure concentrations. The nebulizer assembly was attached to a 2-foot stainless steel sanitary tube containing a K-85 source to reduce the static charge of the particles. The sanitary tube was connected to the chamber supply airflow. The Collison nebulizer assembly was removed at the end of each exposure period and capped to avoid evaporation of the NiSO₄ solution. Dilution air was added to the two higher NiSO₄ exposure concentrations via a diluter in order to prevent aerosol concentration overload conditions. Monitoring of aerosol concentration, stability, uniformity and particles size distribution was as described in Efremenko et al. (2014).

Data Analyses

It was not possible to measure the lung Ni concentration after exposure to NiSO₄ because the solution used to preserve the RNA for genomics analysis (RNA later) chelated the Ni and removed it from the lung tissue. Therefore, the MPPD program was used to predict lung Ni burdens as described in Supporting Information, methods section.

Animal observations, necropsies, and BALF and RNA isolation procedures were identical to those described in Efremenko et al. (2014). Similarly, gene expression microarray measurements and data analysis (including Benchmark dose analysis) were conducted in the same fashion as previously described (Efremenko et al., 2014). Gene expression data was deposited in the National Center for Biotechnology Information Gene Expression Omnibus (Accession No. XXX) Note. These data will be deposited once manuscript is accepted.

RESULTS

NiSO₄ Inflammatory Responses and Predicted Ni Lung Burdens

BALF and Histopathology

The results from BALF chemistry and cytology analyses, as well as histopathology findings are reported in Supporting Information Tables S1–S3, respectively in Supporting Information). For BALF cytology and histology, only the high exposure and control groups were examined after one week.

Following one week of exposure, BALF total protein and LDH levels were increased in rats of the 0.5 and 1.0 mg/m³ NiSO₄ groups. ALP was decreased in rats from all NiSO₄ groups. Similar changes were observed following four weeks of NiSO₄ exposure, but with additional statistically significant increases in LDH in rats of the 0.25 mg/m³ group and in NAG in rats of the 1.0 mg/m³ group. A dose-dependent reduction in ALP following exposure to nickel compounds has been observed in previous nickel inhalation studies but the changes observed in this study appear to follow a U-shape dose-response pattern (Benson et al., 1995; Efremenko et al., 2014). The significance of the dose-dependent changes in ALP (observed both after one and four weeks of exposure) is unknown.

The mean numbers of BALF neutrophils and lymphocytes were significantly increased (approximately 75- and
3-fold, respectively) in the 1.0 mg/m³ group (only exposed group examined) after one week of exposure. BALF cytology results at four weeks showed significant increase in the number of neutrophils for rats of the 0.25 and 0.5 mg/m³ groups with non-significant increases in the 1.0 mg/m³ group (increases ranged from 16 to 1100-fold). Increases in the number of lymphocytes after four weeks were significant in the 0.5 and 1.0 mg/m³ groups (18- and 16-fold, respectively). Mean macrophage numbers were increased at four weeks, but were not statistically significantly different compared to the control mean due to large animal variability within groups. The increases in neutrophils following 4-weeks of exposure to NiSO₄ were consistent with those previously reported for Ni₃S₂ (Efremenko et al., 2014) when inhaled Ni concentration (mg Ni/m³) are compared (Fig. 2).

Microscopic changes in the lungs of NiSO₄ exposed rats were minimal-to-mild (Supporting Information Table S3). Following one-week exposure, all five rats of the 1.0 mg/m³ group (only exposed group examined) had mild bronchiolar epithelial degeneration/hyperplasia (0.25 and 0.5 mg/m³ groups were not examined). One rat of this group also had moderate inflammation of the alveolus, characterized by an infiltration of inflammatory cells: neutrophils, macrophages, and mononuclear cells in the alveoli and interstitium. Following four weeks of inhalation exposure, minimal to mild alveolar inflammation was observed in all animals of the 0.5 and 1.0 mg/m³ groups (Supporting Information Table S3 and Fig. S1). One rat of the four-week 1.0 mg/m³ group had bronchial epithelial degeneration/hyperplasia. In some animals, including controls, minimal chronic inflammation was observed; this was considered to be consistent with the background of focal lesions observed in unexposed rats.

Predicted Lung Ni Burden

The lung burdens predicted with the MPPD model and estimates of lung clearance were consistent with total lung Ni burdens reported in previous studies of rats exposed to NiSO₄ aerosol when considering the variations in actual exposure levels, duration of exposure, and MMAD and GSD of the aerosols (Supporting Information Table S4). After 4 weeks of exposure, the predicted lung burdens in the NiSO₄ study were: 0, 0.56, 1.13, 2.21, and 4.38 μg Ni/g lung while the measured levels in the Ni₃S₂ study were: 0, 1.63, 2.33, 5.61, and 14.36 μg Ni/g lung.

The comparisons between NiSO₄ and Ni₃S₂ in this paper will be made on the basis of inhaled Ni concentration. We considered performing these comparisons on the basis of tissue Ni concentrations, but concluded that this would add uncertainty to the analyses since we were not able to measure nickel burdens in the NiSO₄ study, and we still lack information about how much Ni(II) ion is present at target sites after exposure to these very different nickel compounds. Since Ni₃S₂ is slowly dissolved in the lung, it is not possible to ascertain the concentration of nickel ion associated with a measured total lung burden of nickel for particulate exposures. Therefore, we have made all comparisons on the basis of inhaled nickel concentration only.

NiSO₄ Gene Expression Analyses

Genomic Benchmark Dose (BMD) Analysis

To identify the gene pathways that respond at the lowest concentrations of NiSO₄, BMD analysis of gene expression changes was performed for both the one week and four-week exposure durations (Supporting Information Table S5). The BMDs were adjusted for the Ni content of NiSO₄ (22%). After one week of exposure, immune responses had the lowest BMDs with a range of 0.047–0.204 mg Ni/m³ and the morphogenesis category had a BMD range of 0.067–0.147 mg Ni/m³. After four weeks of inhalation exposure the category with the lowest BMD was once again the immune responses with a range of 0.055–0.216 mg Ni/m³. Immune responses and morphogenesis were also the lowest BMDs for Ni₃S₂ (Efremenko et al., 2014). Most BMDs after four weeks of duration were slightly higher than at one week; but the BMDs for DNA damage, G-protein signaling, and signal transduction were slightly lower than after one week. The lowest BMDs in the previous study with Ni₃S₂ (Efremenko et al., 2014) were somewhat lower: 0.026 and 0.044 mg Ni/m³, respectively, at 1 and 4 weeks.

Pathway Enrichment by Dose and Time

A comparison of the responses as a function of exposure concentration and time-point was performed in order to identify changes in pathway enrichment (Supporting Information Table S6). After one week of exposure, three categories had the greatest number of enriched pathways: cell cycle (proliferation), immune responses and development (morphogenesis).
The same pathway enrichment was also found at the two highest concentrations after four weeks of exposure, but there was significantly less pathway enrichment at the two lower concentrations at week 4 as compared to week 1.

**Dose- and Time-Dependent Gene Expression Changes**

As observed in the previous study with Ni$_3$S$_2$, the gene changes underlying the observed pathway enrichment were primarily related to morphogenesis (cytoskeleton remodeling and development), immune signaling, oxidative stress response, and cell cycle/proliferation (Supporting Information Table S7). In this table, expression (fold change) is shown only for concentration-time points at which the alteration of the gene was statistically significant; absence of a value does not necessarily imply that the gene was not altered compared to the control. Expression of a number of genes associated with morphogenesis was decreased at both low and high concentrations after 1 week of exposure, and a smaller number were decreased at the highest concentration after 4 weeks of exposure (e.g., Acta2, Actg2, Myl9, Src, Tgfbi1i1, and Wnt2). On the other hand, gene expression for the Tgf-beta receptor (Tgfbr1) and the apoptosis inhibitor Xiap was increased at both time points. Some immune signaling was down-regulated at week 1, but was not different from controls at week 4 (e.g., Igf1r, Il2rg, Il4ra, Prkcb, Nfatc4, Nfkb2, and Rela). Notable exceptions to this pattern were the key inflammatory gene Ptgs2 (COX-2), which was up-regulated at the two highest concentrations at both time-points, gene Ptgs1 (COX-1), which was down-regulated, and Interleukin receptor 7 (Il7r) gene expression, which was increased at both time-points. Gene expression associated with oxidative stress (Nfe2l2, Hmox1) and proliferation (cyclins and associated kinases) was increased at the highest concentrations at both time points. On the other hand, other genes associated with proliferation and cell cycle control, such as Mapk3 and Tp53, were down-regulated at the two highest concentrations at 1 week, but were not significantly altered at 4 weeks.

**Comparison of NiSO$_4$ and Ni$_3$S$_2$ Results**

The inhalation exposure concentrations selected for the present NiSO$_4$ study included the concentration range used in the NTP 2-year study and also extended twofold above the highest exposure concentration studied. Thus, our gene expression results are relevant to the interpretation of the tumor outcome in the NTP studies.

**Benchmark Dose Analysis**

As reported in the previous study with Ni$_3$S$_2$ (Efremenko et al., 2014), BMDs for NiSO$_4$ at both time-points were relatively uniform across pathway categories, suggesting that the cellular effects of Ni from these two compounds are not highly pathway-specific. The lowest NiSO$_4$ BMDs in terms of inhaled Ni concentration of 0.047 and 0.055 mg Ni/m$^3$ at 1 and 4 weeks, respectively, for immune responses were above the lowest exposure concentration used in the study, 0.125 mg/m$^3$ NiSO$_4$, which equates to 0.03 mg Ni/m$^3$. These results suggest that the 0.125 mg/m$^3$ concentration should be considered a No Observed Transcriptional Effect Level (NOTEL) for repeated NiSO$_4$ exposure. The lowest BMDs in the previous study with Ni$_3$S$_2$ (Efremenko et al., 2014) were 0.026 (immune signaling) and 0.044 (morphogenesis/development) mg Ni/m$^3$, respectively, at 1 and 4 weeks, within a factor of two of this study.

**Gene Expression Analysis**

In general, the gene expression changes observed following NiSO$_4$ exposure were remarkably similar to those observed with Ni$_3$S$_2$ (Efremenko et al., 2014). This close correspondence between the two compounds is illustrated in Figure 3, which shows the results of hierarchical clustering of gene expression changes following exposures to similar inhaled Ni concentrations: 0.15 mg/m$^3$ Ni$_3$S$_2$ (0.11 mg Ni/m$^3$) or 0.5 mg/m$^3$ NiSO$_4$ (0.11 mg Ni/m$^3$) for 4 weeks. Figure 4 expands this comparison to all three common inhaled Ni concentrations (0.03, 0.06, and 0.11 mg Ni/m$^3$), but focuses on the genes identified as statistically significantly altered in the previous study with Ni$_3$S$_2$ (Efremenko et al., 2014) at any of the three concentrations. In this figure, log2 fold-changes following exposure to Ni$_3$S$_2$ (vertical axis) are plotted against log2 fold changes following exposure to NiSO$_4$ (horizontal axis). The dose response for the expression change of each gene for both compounds is represented by a series of three points connected by a line. Low exposures (0.03 mg Ni/m$^3$) are red, medium exposures (0.06 mg Ni/m$^3$) are orange, and high exposures (0.11 mg Ni/m$^3$) are yellow. The resulting plot displays clear evidence of a positive correlation between the gene expression changes for the two compounds.

A review of the network maps for the enriched pathway categories in this study indicated that most of the pathway enrichment in Supporting Information Table S6 could be explained by concentration- and duration-related patterns of altered expression in three categories of genes: those related to morphogenesis (cytoskeleton remodeling and developmental pathways), immune signaling, and cell cycle/proliferation. When we looked more closely at the specific genes associated with the effects on these categories, we discovered that the patterns of altered gene expression (Supporting Information Table S7) were remarkably similar to those observed in our study of Ni$_3$S$_2$ (Efremenko et al., 2014, Table 9). Figure 5 compares the expression changes for some key genes after 4 weeks of exposure at the 2nd highest concentrations in...
each study, which correspond to equivalent inhaled Ni concentrations. Both the direction and magnitude of the gene changes are highly comparable between the two compounds. Notable exceptions to this conclusion are Xiap and Il7r, which were altered in the opposite direction for the two compounds. Xiap and Il7r are both proteins that inhibit apoptosis; down-regulation by NiSO₄ would favor programmed death of damaged cells, whereas up-regulation by Ni₃S₂ would inhibit this protective response.

Another potentially important difference in the response to the two compounds is that Cox2 (Ptgs2) expression was substantially increased (9–15 fold) at both time-points following NiSO₄ exposure, but was only moderately affected (2–3 fold) by either duration of exposure to Ni₃S₂ (Fig. 6). Induction of Cox2 is a key element of the acute inflammatory response. In the present study there was also evidence of alveolar inflammation from histopathological and BALF examinations at the two highest concentrations, 0.5 and 1.0 mg/m³.
0.22 mg Ni/m³) particularly after 4 weeks of exposure. These results are consistent with the reported evidence of inflammation in the NTP (1996b) study. The severe toxicity produced by NiSO₄ inhalation exposure limited the maximum tolerated concentrations (MTD = 0.1 mg Ni/m³) in the NTP (1996b) study. In the present study we could not increase exposure to NiSO₄ higher than 0.22 mg Ni/m³ without producing unacceptable toxicity. It is possible that lung inflammation (as a contributor to overall toxicity) is better tolerated after Ni₃S₂ exposure, as supported by the weaker induction of Cox2 expression.

This would be consistent with the higher maximum tolerated concentration (0.73 mg Ni/m³) for Ni₃S₂ in the rat NTP (1996a) study. To better capture the relationships between gene expression changes following Ni₃S₂ and NiSO₄ exposure, we used a recently developed visualization strategy (McMullen et al., 2014). Differentially expressed genes were assigned to pathways based on definitions in the Reactome database. Significantly overrepresented pathways (Fisher’s exact text, FDR-corrected, \( P < .05 \)) are plotted according to their relationships described in the Reactome ontology (Fig. 7). Here,
categories are shown if they are significantly enriched in genes perturbed in response to 4-week exposures to 0.44 mg Ni/m$^3$ Ni$_3$S$_2$ (blue), 0.24 mg Ni/m$^3$ NiSO$_4$ (yellow), or both (green). The size of the circle reflects the number of differentially expressed genes in the target pathway.

As with the previous analyses comparing the high-concentration responses to these two compounds, the predominant difference in the gene expression changes are related to immune signaling observed with Ni$_3$S$_2$ but not with NiSO$_4$. Significantly induced genes were classified in ontology categories (e.g., NFAT in immune response, leukocyte chemotaxis, T-cell activation). To investigate whether these differences were driven by compound-specific differences or could reflect a dose-dependent transition in the mode of action for Ni(II) ion, we conducted a similar analysis with the two highest concentrations of Ni$_3$S$_2$ and found a similar pattern (Fig. 8). The results of these two analyses support the view that the genomic (and toxic/carcinogenic) effects of nickel compounds may primarily result from the exposure of cells to Ni(II) ion, and that there is a dose-dependent transition in the mode of action for Ni(II) ion associated with up-regulation of immune signaling.

**DISCUSSION**

**Application of Genomic Data for in Vitro Dosimetry**

One of the secondary goals of this study, as well as the previous study with Ni$_3$S$_2$, was to provide genomic data that could be used to support comparisons either between two *in vitro* studies with different Ni compounds, or between *in vitro* and *in vivo* Ni exposures. To the extent
that the assumption can be made that the effects of Ni(II) on the cell are produced by ionic Ni delivered to nuclear sites, the genomic data can serve as a biomarker of internal (cellular) exposure. Put simply, the measure of equivalence is that if two exposures to Ni compounds produce quantitatively similar fold-changes in gene expression, then the internal (cellular) ionic Ni concentrations resulting from the two exposures are also similar. The applicability of this approach in the case of Ni compounds is supported by a comparison of the expression changes in Cdk1, a key driver of proliferation, as a function of inhaled Ni concentration (mg Ni/m³), following 4 weeks of exposure to NiSO₄ or Ni₃S₂ (Fig. 5c). When plotted against inhaled Ni concentration, the expression changes in Cdk1 after one week are roughly equivalent for both compounds (Fig. 9). The Cdk1 gene was selected for this comparison because it demonstrated the strongest dose-response behavior in both studies, but the rough equivalence is also apparent for many of the other genes in Figure 5 and Supporting Information Table S7.

This approach could be applied using either individual gene expression changes (e.g., Cdk1) or an ensemble of genes as the biomarker(s) of internal exposure. The *in vitro* assay would be conducted over a range of concentrations and the concentration that achieves roughly the same fold-change in expression as the *in vivo* study would be identified as the equivalent exposure under the conditions of the assay. This simple *in vivo* dosimetry anchoring may be particularly useful for comparing *in vitro* exposures with compounds having different properties, for example highly soluble Ni compounds that are rapidly ionized and distributed throughout the media vs. poorly soluble compounds that remain in the particulate phase and settle onto the cells, where they can be endocytosed. Of course, there are a number of challenges associated with this approach. First, it depends on the validity of the assumption that nickel ion is the only stressor producing transcriptomic alterations. If, for example, the anion in a soluble nickel compound (or the particles themselves in the case of insoluble compounds) also acted
as a stressor, then the biomarker would not be nickel specific. In addition, the approach relies on the assumption that *in vitro* transcriptomic responses would be similar to those observed *in vivo*. *In vitro* studies to test this hypothesis are currently being designed.

**Mode of Action for Ni Carcinogenicity**

The estimated Ni lung burdens for this study range from 0.4 to 4.38 mg Ni/m³ Ni₃S₂ or 0.11 mg Ni/m³ Ni₃S₂ for 4 weeks (blue: 0.44 mg Ni/m³ Ni₃S₂; yellow: 0.11 mg/m³ Ni₃S₂; green: both). Nodes represent Reactome ontology categories and edges represent the hierarchical relationships between categories. All colored nodes were statistically significant at an enrichment FDR < 0.05 and had a minimum of 10 query genes found thin the ontology category elements. Node size is proportional to the total number of query genes found in a particular ontology category’s elements.

Fig. 8. Up-regulation of gene expression following exposure to 0.44 mg Ni/m³ Ni₃S₂ or 0.11 mg Ni/m³ Ni₃S₂ for 4 weeks (blue: 0.44 mg Ni/m³ Ni₃S₂; yellow: 0.11 mg/m³ Ni₃S₂; green: both). Nodes represent Reactome ontology categories and edges represent the hierarchical relationships between categories. All colored nodes were statistically significant.

similar inhaled Ni concentrations translate to about 2- to 3-fold higher lung burdens for Ni₂S₃ than for NiSO₄. Indeed, Ni lung burdens measured after 7 or 15 months of exposure at the Ni₃S₂ exposure concentration associated with tumors, 0.15 and 1.0 mg/m³, were on the order of 4–9 μg Ni/g lung (NTP, 1996a), which is similar to the measured concentrations in our study of the genomic responses to that compound (Efremenko et al., 2014). Therefore, the genomic data obtained in these studies are informative for the modes of action for the effects of these two compounds under bioassay conditions. It is, of course, likely that gene expression will continue to evolve with more prolonged exposure, but based on our studies with other compounds (Andersen et al., 2010, Clewell et al., 2011; Thomas et al., 2013) we believe that the 4-week exposures used in these studies should be sufficient
to identify the key cellular functions that drive the mode of action.

Overall, the genomic response of the distal airway lung epithelium of the rat to inhalation of NiSO$_4$ for up to 4 weeks is consistent with a mode of action involving relatively non-specific toxicity from Ni ion, resulting in disruption of cellular function and cell proliferation. In general, the cellular responses to NiSO$_4$ are similar to those previously reported for Ni$_3$S$_2$ (Efremenko et al., 2014), both in the nature of the cellular responses and the inhaled Ni concentrations at which they occur. However, some key differences in the genomic responses to the two compounds were identified that may reflect differential delivery of ionic and particulate Ni to intracellular sites. There is also genomic evidence for a dose-dependent transition in the mode of action for Ni ion, characterized by changes in cellular immune signaling. Therefore, the ultimate tumor outcome for a given Ni compound may depend on the extent to which the compound is able to deliver sufficient Ni ion to critical cellular targets at exposure levels that lack overt toxicity (Hack et al., 2007; Goodman et al., 2011). In vitro studies have demonstrated that the intracellular disposition of particulate and ionic Ni is very different (Costa and Mullenhauer, 1980; Costa et al., 1981). It is certainly possible that some of the differences noted between the cellular response to NiSO$_4$ and Ni$_3$S$_2$ could reflect the effects of particulate vs. ionic cellular uptake on cellular disposition and function.

CONCLUSION

Comparison of genomic benchmark doses for NiSO$_4$ and Ni$_3$S$_2$ at four weeks, coupled with reported lung burden measurements for longer exposures (as a surrogate for relative Ni bioavailability at critical cellular targets), suggests that the highest exposure concentration in the NTP bioassay for NiSO$_4$, (0.5 mg NiSO$_4$/m$^3$, 0.11 mg Ni/m$^3$), which was limited by toxicity, may not have been sufficient to achieve the lung Ni concentrations at which tumors were observed in the Ni$_3$S$_2$ bioassay. However, key differences in the cellular immune responses to NiSO$_4$ and Ni$_3$S$_2$ were noted that may also contribute to the observation of tumors in the bioassay for Ni$_3$S$_2$ but not NiSO$_4$. The differential regulation of the anti-apoptotic genes Xiap and IIL7r (up-regulated by NiSO$_4$ but down-regulated by Ni$_3$S$_2$) is of particular interest and needs to be further evaluated with in vitro studies. In designing such studies, the approach described above for in vivo genomic dosimetry anchoring should be considered for comparing in vitro exposures with highly soluble NiSO$_4$ vs. poorly soluble particulate Ni$_3$S$_2$.

AUTHOR CONTRIBUTIONS

Ms. Efremenko and Drs. Campbell, Dodd, Oller, and Clewell III all contributed to the design of the study. Dr Dodd collected the samples and conducted the BALF and histopathology evaluation. Ms. Efremenko and Drs Clewell and Campbell analyzed the genomics and prepared the figures and tables. All authors contributed to the manuscript preparation. All authors had access to the study data and all approve the final manuscript.

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