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Time- and concentration-dependent genomic responses of the rat airway to inhaled nickel subsulfide

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ABSTRACT

Objective: To provide insights into the mode of action for Ni_3S_2 lung carcinogenicity by examining gene expression changes in target cells after inhalation exposure.
Methods: Gene expression changes were determined in micro-dissected lung broncho-alveolar cells from Fischer 344 rats following inhalation of Ni_3S_2 at 0.0, 0.04, 0.08, 0.15, and 0.60 mg/m^3 (0.03, 0.06, 0.11, and 0.44 mg Ni/m^3) for one and four weeks (6 h/day, 5 days/week).
Results: Broncho-alveolar lavage fluid evaluation and lung histopathology provided evidence of inflammation only at the two highest concentrations, which were similar to those tested in the 2-year bioassay. The number of statistically significant up- and down-regulated genes decreased markedly from one to four weeks of exposure, suggesting adaptation. Cell signal pathway enrichment at both time-points primarily reflected responses to toxicity, including inflammatory and proliferative signaling. While proliferative signaling was up-regulated at both time points, some inflammatory signaling reversed from down-regulation at 1 week to up-regulation at 4 weeks.
Conclusions: These results support a mode of action for Ni_3S_2 carcinogenicity driven by chronic toxicity, inflammation and proliferation, leading to mis-replication, rather than by direct genotoxicity. Benchmark dose (BMD) analysis identified the lowest pathway transcriptional BMD exposure concentration as 0.026 mg Ni/m^3, for apoptosis/survival signaling. When conducted on the basis of lung Ni concentration the lowest pathway BMD was 0.64 μg Ni/g lung, for immune/inflammatory signaling.
Implications: These highly conservative BMDs could be used to derive a point of departure in a nonlinear risk assessment for Ni_3S_2 toxicity and carcinogenicity.

Introduction

Epidemiological studies of sulfidic ore refinery workers have demonstrated associations between inhalation exposure to a mixture of nickel (Ni) compounds (including crystalline Ni subsulfide, Ni monoxide, complex Ni oxides and water soluble Ni compounds) and cancers of the nasal sinuses and the lung (ICNCM, 1990). Lifetime inhalation studies of Ni subsulfide (Ni_3S_2) in rats and mice demonstrated clear evidence of carcinogenicity in the lung of male and female rats (alveolar/bronchiolar adenomas and carcinomas) (Dunnick et al., 1995; NTP, 1996).

A consensus has not been reached on the mode of action for nickel carcinogenesis. Most of the existing evidence comes from *in vitro* studies. The results of *in vitro* assay systems indicated that nickel is a weak mutagen at best (Arrouijal et al., 1990; Coogan et al., 1989; Snow, 1992). It has also been suggested that nickel may induce DNA damage indirectly through the formation of reactive oxygen species (Dally and Hartwig, 1997; Huang et al., 1994a,b). Many studies by M. Costa's group have indicated that nickel may bind to heterochromatin, resulting in chromosomal aberrations or deletions (Ellen et al., 2009; Huang et al., 1995; Klein and Costa, 1997; Zoroddu et al., 2002), and that nickel may silence the expression of oncogenes by inducing changes in histone methylation and acetylation and triggering DNA hypermethylation (Arita and Costa, 2009; Costa et al., 2003; Tchou-Wong et al., 2011; Zhou et al., 2009).

Moreover, several *in vitro* studies have demonstrated that nickel is capable of interfering with the DNA repair process (Hartwig et al., 1994; Schwerdtle et al., 2002) and that Ni can replace iron in oxygen sensing proteins such as HIF proline hydroxylase, leading to the induction of hypoxia inducible factors (Costa et al., 2005). Nickel can also interfere with other iron-containing proteins such as iron- and ketoglutarate-dependent histone demethylases. Because some of the affected enzymes are involved in histone demethylation and DNA repair, this may contribute to the development of tumors (Costa et al., 2005; Chen et al., 2010), provided that levels of nickel high enough to

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interfere with iron can be achieved *in vivo*. Although the precise mode of action for nickel carcinogenesis has not been elucidated, nickel ion is considered to be the ultimate carcinogen. For lung tumor induction, the *in vivo* intracellular delivery of Ni ions is expected to be dependent on toxicity-limited exposure levels, clearance, cellular uptake, and extra and intracellular particle dissolution (*e.g.*, Benson et al., 1992; Costa and Mollenhauer, 1980; Costa et al., 1981; Goodman et al., 2011; Hack et al., 2007).

A limitation of *in vitro* studies to inform the mechanisms of lung tumor formation *in vivo* relates to the relevance of *in vitro* exposure conditions (concentration and duration) to the *in vivo* situation and how comparable are the intracellular Ni levels that can be achieved *in vitro versus in vivo*. There are few instances where *in vitro* results with Ni_3S_2 have been confirmed *in vivo*. For example, the lack of mutagenicity of Ni_3S_2 was confirmed in a short-term inhalation study using transgenic rats (*lac I* F344) (Mayer et al., 1998). Down-regulation of DNA repair genes has also been observed in peripheral blood monocytes of a group of refinery workers with assumed high exposures (up to 1 mg/m^3) to nickel compounds (Arita et al., 2013).

Over the past decade, the development and application of high-throughput, broad-coverage technologies have made it possible to comprehensively examine transcriptional changes in cells and tissues following chemical exposure, both *in vitro* and *in vivo*. This approach can provide insights into potential key events leading to the observed biological responses (*e.g.*, potential mode-of-action for lung tumor induction). In addition, it has been suggested that the dose–response component of cancer risk assessment could be based on quantitation of molecular endpoints, or "bioindicators" of response, selected on the basis of their association with obligatory precursor events for tumorigenesis (Preston, 2013). Even when short (4 weeks) dose–response studies are performed, the analysis of the transcriptional alterations can be used to inform risk-based decisions on doses at which long-term cancer and non-cancer effects (apical responses) are likely to be observed (Thomas et al., 2007, 2013a,b).

In this study we determined the concentration- and time-dependent changes in gene expression in the rat lung distal airway (*i.e.*, target tissue for nickel carcinogenicity in rats) following repeated inhalation exposure to different levels of Ni_3S_2. The two highest Ni_3S_2 inhalation exposure concentrations selected for this evaluation (0.15 and 0.6 mg/m^3) were similar to those used in the cancer 2-year study (0.15 and 1.0 mg/m^3, Dunnick et al., 1995), and two lower exposure concentrations (0.04 and 0.08 mg/m^3) were added to more fully explore the dose–response for genomic changes. These exposure levels are roughly equivalent to inhalable workplace exposure levels ranging from 0.2 to 3.3 mg Ni/m^3, when differences in parameters that affect deposited daily doses per surface area of the thoracic region of the lung are considered using a dosimetric model (see Goodman et al., 2011). The exposure duration of four weeks was selected to assure that steady-state concentrations of nickel were achieved and that cellular genomic responses had stabilized. Following exposure, lung distal airway microdissection (Baker et al., 2004) was performed to isolate the distal airway tissue where lung tumors may originate. These microdissected bronchiolar and alveolar tissues were processed for the genomic analysis reported here.

The goals of this study were three-fold: 1) provide insights into the mode of action for Ni_3S_2 carcinogenicity by conducting global analyses of gene expression changes in target cells after inhalation exposure to Ni_3S_2 (including concentrations at which tumors would occur); 2) confirm the relevance of the current results to previously-conducted carcinogenicity studies by also collecting traditional toxicological data (histopathology, BALF); and 3) identify exposure levels and target-tissue concentrations of nickel at which particular cellular processes are altered. The gene expression results from this *in vivo* rat study with Ni_3S_2 constitute a unique dataset that is being made available to the research community. The gene expression data will be deposited in the National Center for Biotechnology Information Gene Expression Omnibus.

Materials and methods

Test substance. Crystalline Ni_3S_2 (CAS no. 12035-72-2) was obtained from the NiPERA's Sample Repository housed at Particle Technology Labs (PTL, Grove, IL). The Ni_3S_2 test substance was kept in tightly closed containers under nitrogen at normal ambient conditions. Documentation of the methods of synthesis, fabrication, or derivation of the test substance is retained by Vale Inco Technical Services Ltd., 2101 Hadwen Rd., Mississauga, Ontario, CA L5K 2L3.

Test animals and housing. Male Fischer-344 (CDF) rats (approximately 7–8 weeks old on receipt) were obtained from Charles River Laboratories, Kingston, NY. Group size was 13 animals per each time point-exposure level combination. Animals were acclimated in the animal facility for approximately two weeks prior to beginning the inhalation exposure of Ni_3S_2. Animals were singly housed in stainless steel wire mesh cages (Hazelton R-24 cage units, Lab Products, Seaford, DE) in a climate-controlled room. Animals were fed pelleted food (NIH-07, Zeigler Bros., Gardners, PA) and water (reverse-osmosis purified) *ad libitum*, except during the inhalation exposure when food was removed. Animals were identified by ear tags and cage assignments. One day prior to their first inhalation exposure, animals were randomly assigned, using the Provantis™ 8 protocol (Provantis, Conshohocken, PA), to study groups based on body weight.

Animals were exposed in 1 m^3 whole body exposure chambers (H1000, Lab Products, Seaford, DE) using the Hazelton R-24 cage units. One chamber was used for each concentration level including a control (air-only) chamber. Stainless steel pans were placed under each R-24 cage unit to collect urine and feces. Animals remained in the R-24 cage units during non-exposure periods (food was added to the cage units). The incoming air for the exposure chamber was filtered with 95% high-efficiency particulate absorption (HEPA) filters (95% efficiency for 0.3 μm particles). Environmental conditions in the 1 m^3 chambers during the exposure were recorded. A temperature and humidity probe (Hygromer 200 Series, Rotronic Instrument Corp., Huntington, NY) measured the temperature and humidity in each exposure chamber. Air flow in the chamber was determined by measuring the pressure drop across a calibrated orifice plate. The temperature, humidity, and air flow measurements were periodically recorded by a building automation system (Andover Continuum System, Andover Controls Corp., Andover, MA) and a report printed at the end of the exposures.

The Hamner Institutes for Health Sciences is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Currently acceptable practices of good animal husbandry were followed per National Research Council's Guide for the Care and Use of Laboratory Animals (NRC, 1996) and were in compliance with all appropriate parts of the Animal Welfare Act: Public Law 89-544 (1966). In addition, the study design and protocol were approved by The Hamner Institutes' Institutional Animal Care and Use Committee (IACUC) prior to the initiation of study.

Inhalation exposures. Atmospheres of Ni_3S_2 aerosol were generated at target concentrations of 0 (control), 0.04, 0.08, 0.15, and 0.60 mg/m^3 (approximately 0.03, 0.06, 0.11, and 0.44 mg Ni/m^3) in air. Animals were exposed to the target concentrations for 6 h/day, 5 days/week for either 1 or 4 weeks (5 or 20 exposure days). Ni_3S_2 atmospheres were generated by aerosolizing Ni_3S_2 using a dry powder generator (Wright Dust Feeder, Model WDF-II, BGI, Inc., Waltham, MA). The Ni_3S_2 test material was packed in a dry powder generation cup at a pressure of 1000 psi, using a hydraulic press (Model C, Carver Inc., Menomonee Falls, WI). The air delivery pressure through the dry powder generators was maintained between 2 and 13 psi during generation.