Development of Alternative *In Vitro* Methods to Screen for Pulmonary Toxicity of Fine and Nanoscale Particles

Characterization of Aerosolized Zinc Oxide Exposures to Lung Epithelial- Macrophage Co-cultures at the Air-Liquid Interface (ALI) using the NACIVT System

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The development of reliable and predictive *aerosol-based*, in vitro *lung* toxicity assays for screening particulate/nanoparticulate materials could significantly limit the need for animal testing, particularly during the early phases of hazard evaluations. Additional benefits would include an enhanced capacity to screen a number of compounds during the early phases of product development using cost-effective and inexpensive *in vitro* assays. In addition, reliable hazard data developed from these tests could serve as a bridge to the subsequent implementation of longer-term particle inhalation studies, thereby obviating the need for conducting acute toxicity studies, and significantly reducing the numbers of test animals. These goals are also consistent with the vision of the National Academy of Sciences – Tox in the 21st Century Committee which advocated a transformative paradigm shift in implementing safety studies by providing greater abilities to screen chemical hazards at various life stages; to reduce testing costs and use fewer animals; and to develop a robust scientific basis for assessing health effects of environmental agents.

Unfortunately, the accuracy of predictive results from currently conducted *in vitro* lung toxicity screening studies is not reliable. In this regard, we have previously published the results of several comparative lung toxicity studies with identical particle-types using submerged *in vitro* cell culture systems vs. *in vivo* (inhalation and instillation) techniques; and concluded a lack of convergence between the test results between *in vivo* and *in vitro* effects using these cell culture methodologies. The toxicity studies used as barometer similar endpoints (inflammation and cytotoxicity biomarkers) as representative indices for comparative purposes.

The lung is a complex organ system comprised of many different cell-types (i.e., types I and II alveolar epithelial cells, macrophages, interstitial cells, vascular cells, etc.), thus the pulmonary microenvironment and the complex interactions in the lung

which occur following inhalation and subsequent deposition of particles are difficult simulate when employing *in vitro* techniques. In addition, numerous studies have reported *in vitro* toxicity results using only single cell-types (e.g., A549 lung epithelial cells – or lung or peritoneal macrophages). However, these represent simplistic models and ignore the complex interactions in the lung microenvironment that occur following exposure to aerosols of particles, bacteria, etc. Recently, we have attempted to develop a more complex system *in vitro* which can, in part, simulate particle phagocytosis, an important aspect in the simulation of lung defense responses to particles. The development of such *in vitro* techniques will better facilitate (and hopefully expedite) the transition from the current animal-based inhalation testing system to one that is based primarily on human cell lines and *in vitro* assays. Such reproducible, accurate, and validated cell-based *in vitro* screening assays for assessing pulmonary and genetic toxicity will have important benefits (i.e., screening more compounds in a faster, more reliable, and less expensive manner) to other scientific investigators and may, in addition, provide experimental designs to address mechanistic questions.

In an attempt to improve the efficacy of our *in vitro* methodological approach, we have taken a number of steps that are designed to better simulate the physiology of the distal lung microenvironment, concomitant with the implementation of an aerosol exposure system. Accordingly, we have transitioned from a traditional "submerged" cell culture system to a more physiologically relevant air-liquid interface (ALI) cell culture system, using Transwell® Permeable Support devices (microporous membranes), for providing and maintaining pulmonary cell co-cultures (of rat lung epithelial cells and alveolar macrophages) and exposing the co-cultures to particulate aerosols in a humidified atmosphere.

Many fundamental issues require consideration for optimization when facilitating a transition from an animal inhalation toxicity set-up to an *in vitro* aerosol pulmonary toxicity study. These considerations include, but are not limited to the following actions:

- Determining the cell-types to be used in a co-culture system – to better simulate the lung microenvironment
- Transitioning from primary cell types collected from animals to immortalized cells derived from cell lines and tested for biological functionality
- Determining the number of ratio of lung epithelial cells to alveolar macrophages in a co-culture plate
- Determining the aerosol generation method for particles/nanoparticles and appropriate and reproducible and quantifiable dosemetrics to be utilized for particle inhalation deposition assessments and for comparisons of results of one study to another

- Demonstrating reproducibility for particle generation studies by measuring particle deposition on filters – and choosing appropriate filter-types and measurement methods to ensure validation of results.
- Deciding on the appropriate endpoints for assessing lung toxicity parameters – which can be benchmarked against *in vivo* results on the same particle-types.

Three additional key experimental design features of this new *in vitro* system should include

- a time course protocol to assess the sustainability of any measured response;
- dose response assessments;
- and utilization of benchmark particulate control test materials, in order to develop a frame of reference for better interpretation of results

Based upon these challenges, we have successfully transitioned from a submerged cell culture system to an air-liquid interface (ALI) system, for providing co-cultures (of rat lung epithelial L2 cells and NR8383 alveolar macrophages) applicable to *in vitro* cell exposures and toxicity assays. This complex system *in vitro* can, in part, simulate particle phagocytosis and inflammation, important aspects in the assessments of lung defense responses to particles. A temperature- and humidity-controlled, *in vitro* cell exposure chamber - the Nano-Aerosol Chamber for In Vitro Toxicology (NACIVT) system - has been optimized to host and expose the epithelial-macrophage co-cultures (n=24 samples) to aerosolized particulate atmospheres at the ALI. Particle atmospheres are generated from a source chamber prior to delivery to the NACIVT system at approximately 0.6 L/min (i.e., to each cell sample at ~25 mL/min). Particle concentration (mass/volume) and aerosol size (MMAD) are sampled gravimetrically from the source chamber while particle deposition is measured by gravimetric analyses collected on filters in the NACIVT system. Cell metabolism/cytotoxicity assays (i.e., XTT/LDH assays) and cytokine (e.g., IL-6) release assay have been developed and modified for use to assess toxicity/inflammatory endpoints.

Certain cell growth conditions and exposure parameters have been optimized for the applicability of several toxicity (XTT, LDH, and cytokine release) assays to our ALI co-culture cell model. Evaluation of the system was performed with 2 cell seeding densities and with 3 epithelial cell to macrophage ratios (L2:NR8383 = 1:1, 2:1, or 5:1). Cytotoxic effects measured following 1-hr exposure to fine-sized ZnO particles (used for method development purpose) increased with L2:NR8383 ratios, revealing that both seeding density and L2:NR8383 ratio affect assay sensitivity. Co-cultures of L2 with primary macrophages recovered from rat bronchoalveolar lavage fluids following ZnO exposure *in vitro* had similar % change in metabolic activity and cytotoxicity (vs. controls) when compared to co-cultures with immortalized NR8383, suggesting that NR8383 cells are biologically relevant substitutes for animal-derived macrophage. Five types of filters with different physical properties and chemical resistance were evaluated for their capability in collecting particles. Epithelial cell-macrophage co-cultures were

also exposed to air for various time periods (10-, 30-, and 60-min) to provide baseline cell survival data for determination of optimal exposure time. Recently, in 10 separate studies, aerosols of fine-sized ZnO particles have been generated and delivered to the NACIVT system for cell exposures. The particle depositions (23-62 ug/cm^2) in the NACIVT system vary proportionally (R^2=0.8361) with the source chamber concentrations (24-140 mg/m^3) (MMAD=0.85um, GSD=2.5), with the aid of a unipolar diffusion charger and an electric field underneath the cell cultures in some cases. Metabolism data following ZnO exposure at a relatively high concentration have well correlated with cytotoxicity data obtained at multiple time-points (0, 24-, and 48-hr) post-exposure. Similar studies will be performed at mid- and low concentrations to construct concentration-response relationships for a 72-hr time-course. In conclusion, these ongoing studies are designed to simulate aerosol particulate exposures to physiologically relevant lung cell-types *in vitro,* cultured at the ALI and represents an important step in facilitating the development of a reliable testing/screening system for potential pulmonary toxicity.

The results obtained thusfar suggest that the system that we are developing has promise in the future for displacing some forms of animal acute inhalation studies. Following validation with our current model of fine zinc oxide particle aerosols, we intend pursue efforts to assess toxicity effects of other particulates, including nanoaerosols of particular interest.