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Improving in vitro to in vivo extrapolation by incorporating toxicokinetic measurements: A case study of lindane-induced neurotoxicity

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ABSTRACT

Approaches for extrapolating in vitro toxicity testing results for prediction of human in vivo outcomes are needed. The purpose of this case study was to employ in vitro toxicokinetics and PBPK modeling to perform in vitro to in vivo extrapolation (IVIVE) of lindane neurotoxicity. Lindane cell and media concentrations in vitro, together with in vitro concentration-response data for lindane effects on neuronal network firing rates, were compared to in vivo data and model simulations as an exercise in extrapolation for chemical-induced neurotoxicity in rodents and humans. Time- and concentration-dependent lindane dosimetry was determined in primary cultures of rat cortical neurons in vitro using "faux" (without electrodes) microelectrode arrays (MEAs). In vivo data were derived from literature values, and physiologically based pharmacokinetic (PBPK) modeling was used to extrapolate from rat to human. The previously determined EC_{50} for increased firing rates in primary cultures of cortical neurons was 0.6 μg/ml. Media and cell lindane concentrations at the EC_{50} were 0.4 μg/ml and 7.1 μg/ml, respectively, and cellular lindane accumulation was time- and concentration-dependent. Rat blood and brain lindane levels during seizures were 1.7–1.9 μg/ml and 5–11 μg/ml, respectively. Brain lindane levels associated with seizures in rats and those predicted for humans (average = 7 μg/ml) by PBPK modeling were very similar to in vitro concentrations detected in cortical cells at the EC_{50} dose. PBPK model predictions matched literature data and timing. These findings indicate that in vitro MEA results are predictive of in vivo responses to lindane and demonstrate a successful modeling approach for IVIVE of rat and human neurotoxicity.

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Introduction

As in vitro screening assays and toxicity studies become increasingly important in chemical risk assessment, accurate in vitro to in vivo extrapolation (IVIVE) will be required to interpret these data (Krewski et al., 2010; Thomas et al., 2013). Translating in vitro results to in vivo exposures and effects presents a number of challenges. Among these is an accurate assessment of in vitro concentrations of test chemicals in the media and biological matrix. In the vast majority of cases involving cell-based in vitro assays, the concentration of the test chemical is not measured in either the media or the cells. This information, as part of a more complete understanding of the in vitro toxicokinetics of a given test system, is critical in order to properly interpret in vitro data, select appropriate in vitro dose metrics for IVIVE and correctly apply IVIVE, which is ultimately used to predict chemical concentrations and effects at target sites.

Comparisons of in vitro toxicity data to in vivo results for environmental chemicals are frequently hindered by a lack of in vivo data, especially from humans. However, PBPK modeling can be used to estimate in vivo chemical dosimetry in blood and target tissues that are associated with adverse responses in humans, significantly facilitating IVIVE for humans. In vitro dose metrics can be used in these models to improve predictions of chemical exposure levels that will cause toxicity in humans.

Lindane neurotoxicity as a case study for IVIVE

Ideal chemical candidates for a human IVIVE case study should meet certain criteria which follow from the recommendations of the National Research Council report, Toxicity Testing in the 21st Century (Krewski et al., 2010). Foremost, both human exposure and health effects data and rodent dose–response data should be available, and an

Abbreviations: MEA, microelectrode array; PBPK, physiologically based pharmacokinetic; IVIVE, in vitro to in vivo extrapolation; GC-μECD, gas chromatograph micro electron capture detector; GABA, γ-aminobutyric acid; EC_{50}, effective concentration 50%; RSD, relative standard deviation.

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exceptionally large amount of human and animal in vivo data (addressing both toxicity and kinetics) exists for lindane. The animal data includes pigs (Surgeoner and Flannigan, 1986; Davey and Johnson, 1974), guinea pigs (Franz et al., 1996), dogs (Litterst and Miller, 1975), mice (Tusell et al., 1992; Hulth et al., 1978), and rats (Vucević et al., 2008; Zisterer et al., 1995; Suñol et al., 1989; Tusell et al., 1987; Eichler et al., 1983; Stein et al., 1980). Human exposures to lindane have resulted from its continued use in treating lice and scabies infestations and its use for decades as an insecticide. In 2009, the production and agricultural use of lindane was banned under the Stockholm Convention on persistent organic pollutants, but a specific exemption allows its continued use as a second-line pharmaceutical treatment for lice and scabies (UNEP/POPS/COP.4/38, 2009). Although the U.S. registration for the agricultural use of lindane was canceled in 2006, the U.S. FDA continues to permit the pharmaceutical use of lindane. The human data includes clinical trial data (Dick et al., 1997; Ginsburg et al., 1977) as well as poisoning reports (Nordt and Chew, 2000; Aks et al., 1995; Kurt et al., 1986; Davies et al., 1983; Telch and Jarvis, 1982) and occupational reports (Drummond et al., 1988; Czeglédi-Jankó and Avar, 1970). These documented lindane exposures resulting in seizures and even deaths have permitted estimation of the exposure levels and dose levels associated with adverse outcomes (Hayes, 1982). Of course, an in vitro assay system for a relevant toxic response is essential, and in this case, microelectrode arrays (MEAs) provide a means to assess firing rates of networks formed from primary neuronal cultures (Johnstone et al., 2010). Lindane has a long history of being used as a model neurotoxicant due in part to its rapid onset of toxicity. Lindane is a $GABA_A$ receptor antagonist, resulting in neuronal excitation causing seizures in vivo due to increased neuronal firing rates (Vucević et al., 2008). Lindane metabolism occurs primarily in the liver (brain or neuron metabolism is likely to be negligible) (Fitzloff et al., 1982; Copeland et al., 1986; DeJongh and Blaauboer, 1997), and unlike many other neurotoxic insecticides, lindane metabolites have no neurotoxic activity (Hulth et al., 1978) which facilitates the modeling and interpretation of results needed for IVIVE. Lindane's chemical properties are also desirable: its six chlorines allow for its detection and quantitation at low concentrations using GC-ECD; it is stable in light and water; its molecular weight (<300) and lack of nitrogens and oxygens make it unlikely to be a transporter substrate; and it is lipophilic and readily extracted by organic solvents. Finally, lindane is an EPA ToxCast Phase I chemical (Dix et al., 2007), and our goal was to select one or more chemicals from this list with an endpoint that could be extrapolated from in vitro to in vivo and from animal to human and identify critical factors for successful extrapolation. The ToxCast program is designed to determine effects of a library of environmental chemicals in in vitro assays covering a broad variety of biological endpoints and assess the utility of these data to prioritize chemicals for further testing and regulatory action.

Recently, the concentration-response for lindane effects on mean firing rate in networks of primary cortical neurons was determined using MEAs (Mack et al., 2014). Thus, we can now compare in vitro lindane levels associated with increased neuronal firing rates to in vivo blood and brain lindane levels associated with seizures. A key goal of the present study was to characterize the in vitro distribution of lindane in the media and cells under the conditions of these recordings using microelectrode array "chips" that did not contain electrodes ("faux" MEAs). This information, together with the concentration-response data, can then be used to conduct in vitro to in vivo extrapolations for lindane effects on nervous system function. Determinations of in vitro dosimetry (media and cell concentrations) using this system provided the basis for comparisons to in vivo data (blood and brain concentrations) and PBPK model estimates needed for dose metric evaluation and a complete exercise in IVIVE. This analysis also permits the identification of the minimal data and analytical requirements needed for successful IVIVE of lindane-induced neurotoxicity, another goal of this study.

Methods

Chemicals. All chemicals were purchased from Sigma-Aldrich Corp., St. Louis, MO, U.S.A., unless noted otherwise. The CAS number for lindane is 58-89-9, its molecular weight is 290.83, and the purity of the preparation used in this study was 97%.

Culturing cortical cells for in vitro dose–response analysis. Primary rat cortical cell cultures were prepared as previously described (Robinette et al., 2011) following EPA Institutional Animal Care and Use Committee approved protocols. Briefly, cells were prepared from 0 to 1 day-old male and female Long–Evans rats weighing 5–6 g. Pups were decapitated, and the neocortex was carefully extracted from the brain and collected in cortical buffer. Cortical cells were dissociated (trituration and centrifugation), filtered and plated onto glass MEA chips. MEAs contain delicate and expensive electrodes that can be neither scraped to harvest cells nor treated with harsh chemicals such as extraction solvents. To quantify the concentration of chemical in the cortical cells, an alternative system was developed to allow for the collection of the cells after treatment. This system ("faux MEA") was designed to replicate the number of cells, well volume, and assay conditions used in the toxicity studies so that the toxicokinetic studies could be performed without damaging the expensive MEAs. "Faux" MEAs were created by attaching the same glass rings used in making the MEAs to plain glass slides using the same adhesive (Sylgard). Prior to plating of cells, faux MEAs were precoated with poly-L-lysine, and then on the day of culture, with laminin. Cells were then plated in a 50 μl "drop" (containing 2×10^5 cells) placed in the center of the faux MEA. After plating the cells, 10–15 min were allowed for cell adhesion prior to the addition of 1 ml culture medium (Dulbecco's modified Eagle's medium w/GlutaMax (Invitrogen) containing 1 M HEPES, 10% heat inactivated horse serum (Invitrogen), 100 U/ml penicillin/streptomycin, and NeuralBasalA supplements, pH 7.4).

Effects of lindane on spontaneous activity in networks of cortical neurons. The data for lindane effects on network activity have been published previously (Mack et al., 2014). Briefly, between days 12 and 17 in vitro, MEAs were placed in a Multichannel Systems amplifier and baseline activity was recorded for approximately 30 min at 22–25 °C, followed by treatment with increasing concentrations of lindane (0.01–250 μM) in a cumulative manner. Approximately 30 min elapsed between addition of each concentration of lindane. The EC_{50} value was determined by non-linear regression analysis (log-agonist vs. response; variable slope) using GraphPad Prism v5.03.

In vitro lindane concentrations. In the faux MEA experiments, lindane was dissolved in an ethanol:DMSO (1:1) solution and added to the culture medium to yield nominal final concentrations of 0.1, 0.25, 0.5, 1.0, 2.5, 10, 25, 100 and 250 μM. Time (5, 10, 20, 30, 60, 120 min) and concentration (0.1 to 250 μM) dependent accumulation of lindane was determined at room temperature (22–25 °C). Faux MEAs were allowed to equilibrate at room temperature for 10 min. When lindane concentration was varied, media were collected from faux MEAs after a 60 min exposure. Each faux MEA was then gently rinsed twice with pharmaceutical-grade saline taking care to avoid removing cells. Cells were scraped off the faux MEAs utilizing a 200 μl pipette tip bent at 90° approximately 4 mm from the narrow end to form a scraping tool. A 250 μl volume of saline was added to the MEA wells and the remaining liquid and cells were collected by pipetting with 200 μl tips and placed in a labeled borosilicate-glass cell-culture tube. Ponceau S, a hydrophilic red dye, was added to the tube to make it easier to differentiate between the aqueous and organic layers during extraction. A final concentration of 400 nM 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47) was added to this mix as an internal standard (due to its stability and low limit of detection) to determine extraction efficiency. The tubes were sealed and stored at −20 °C prior to extraction. Faux MEAs were cleaned

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