

Dose Response Modeling with Monoalkylphthalates: Assessing Cellular Targets for Mechanism-Based Risk Assessments

Rebecca A. Clewell*, Susan M. Ross, Kevin W. Gaido, & Mel E. Andersen
*University of North Carolina, Chapel Hill & CIIT Centers for Health Research

Phthalate esters are widely used plasticizers and ubiquitous drinking water contaminants. U.S. epidemiological studies have found phthalate metabolites in urine of all age groups, with highest levels in women of child-bearing age. At high doses, some phthalates, e.g., di-(2-ethylhexyl)phthalate (DEHP) and di-n-butylphthalate (DBP), are developmental toxicants in male rats. The monoester metabolites (MEHP and MBP) are most prevalent in blood and are considered the biologically active metabolite. They exert their effect by inhibiting testosterone (T) synthesis in the fetal rat Leydig cell. Yet, the molecular targets remain unknown. The focus of this project was to conduct dose-response assessments for phthalate effects on the basis of internal rather than administered dose and, in concert with structure activity analysis of these phthalates and other inhibitors of T-synthesis, to develop and test a hypothesis for the mechanism of action (MOA) of phthalate esters that will aid in risk assessments. T synthesis is stimulated through a cyclic AMP (c-AMP) dependent pathway activated by binding of luteinizing hormone (LH) to its receptor. This pathway does not appear to be affected by the phthalates, however. Recently, a c-AMP independent pathway was identified that uses arachidonic acid (AA) as a second messenger. Based on the similarity of molecular structures on the monophthalates and known AA inhibitors and phosphatidyl choline (PC), which is metabolized to AA by cytosolic phospholipase A2 (cPLA2), we believe the phthalates may disrupt AA signaling. These studies evaluate this hypothesis more quantitatively.

APPROACH: Since target organ dosimetry should correlate with physiological effect, we undertook a series of dose-response modeling exercises based on internal dose. Using pharmacokinetic models ranging from one-compartment to more complex physiologically based pharmacokinetic (PBPK) models, we compared the potency of the phthalates for different endpoints in the T pathway. The purpose of this analysis was to help pin-point the molecular target.

Inhibition of T Synthesis In-Vivo: Maternal DEHP and DBP exposure reduces T in male fetal rat testes. This was the target effect to which precursor endpoints were compared. To estimate external dose required to yield 50% inhibition (ED50), we evaluated published dose-response data using our fetal rat T PBPK model and an empirical description of inhibition. The model successfully describes T-inhibition across doses and yields predicted ED50s of 30 and 300 mg/kg-day for DBP and DEHP. To convert these values to IC50s, we ran our phthalate gestation model at the estimated ED50s to calculate corresponding values for the area under the curve (AUC) of the monophthalate in fetal blood. Estimated IC50s for male rat pup responses were 9 and 30 μ M for MBP and MEHP. These values served as points of comparison for the precursor effects.

Inhibition of Progesterone (PG) Synthesis In-Vitro: In vivo T is formed from cholesterol with PG as a precursor. We evaluated the ability of the monophthalates to disrupt PG synthesis in a mouse Leydig tumor cell line (MA-10). Cells were incubated with MBP or MEHP for 24 hrs prior to stimulation with LH and PG was analyzed using an ELISA assay. IC50s calculated using a one-site competition curve were 17 and 40 μ M for MBP and MEHP.

Inhibition of AA Release In-Vitro: PLA2 enzymes catalyze fatty acid release from phospholipids at the sn-2 position. cPLA2 is most specific for arachidonyl-containing phospholipids (PC) and is the primary source of intracellular AA. We used a radiolabel assay to measure the effect of monophthalates on AA release in the MA-10 cell. After incorporation of 3H-AA into cellular membranes, the cells were treated with MEHP or MBP for 30 min, followed by stimulation with LH. LH causes a rapid release of AA into the media. At 1 min, the total radioactivity in the media from LH stimulated cells was approximately 150% that of untreated cells. Pre-treatment with MEHP and MBP caused

a dose-dependent decrease in AA released 1 min after LH stimulation. At 50 μM , both phthalates abolished the LH-stimulated AA surge. Measured changes in radioactivity modeled using one-site competition curves gave estimated IC_{50} 's of 5 and 7 μM for MBP and MEHP. These IC_{50} s were similar to those obtained from the dose-response models for T inhibition in vivo and PG inhibition in this same cell model.

PROPOSED MOA: The similarity in potency for these responses is intriguing. Together with our earlier analysis of the 2-D structures of (1) the phthalate monoesters, (2) several known PLA2 inhibitors, and (3) PC, as well as molecular docking exercises we performed using the crystal structure of cPLA2 and 3-D ligand models, these results indicate that cPLA2 may be a molecular target for the phthalates in relation to male rat developmental responses. Longer chain monoalkylphthalates may mimic the shape of the phospholipid head group, bind to cPLA2, and prevent AA release from PC. Several mechanistic studies are planned to test specific aspects of this hypothesis, including binding studies using purified C2 domain of cPLA2 and observations of the cellular localization of a GFP-cPLA2 fusion protein in response to LH stimulation with and without phthalate treatment.

IMPLICATIONS FOR RISK ASSESSMENT: Differences in dependence of the adult and fetal Leydig cells on the two T signaling pathways (c-AMP v. AA) may be the cause of the apparent sensitivity of the fetus to phthalate-induced T inhibition. To test this, we will perform T and AA inhibition assays in primary Leydig cells taken from immature and adult male rats. With a better understanding of the MOA for these compounds, we can begin to reduce the uncertainty in estimating human risk. A comparison of the dependence of the rodent and human on AA signaling for T synthesis should illuminate species differences in susceptibility. We evaluated published data for the dose-dependent decrease in AA release from human platelets, which gave an approximate IC_{50} of 10 μM . This value is very similar to that measured for MEHP in mouse Leydig cell. This similarity may suggest that species differences in susceptibility are a result of differences in pharmacokinetics (phthalate distribution) or pharmacodynamics (primary T pathway) rather than differences in the effect of monophthalates on AA release.

OBJECTIVES: My primary interest is in the use of mathematical models to encourage targeted experiments and ultimately reduce uncertainty in the risk assessment process. I hope to use my previous experience in modeling and the skills I am currently gaining in the laboratory in an effective combination that will improve the accuracy of biological models and reduce the time and expense of the laboratory. *SRA Role:* The Society of Risk Analysis is an important forum to present my work and receive critical feedback which may help to refine my current research and my future fields of specialization. As my interest is in using modeling and targeted laboratory studies to improve risk assessment, this group of professionals should be a rich source of information. Furthermore, the interaction with scientists who work in this field will allow me to become familiar with the types of jobs available in the field and to build relationships with future collaborators.