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Polyester monomers lack ability to bind and activate both androgenic and estrogenic receptors as determined by *In Vitro* and *In Silico* methods

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ABSTRACT

The paper presents results from the screening of seven monomers used by Eastman Chemical to make various polymers. Ethylene glycol, diethylene glycol, polytetramethylene glycol, isophthalic acid, monosodium-5-sulfoisophthalic acid, 1,4-cyclohexanedicarboxylic acid, and dimethylcyclohexanedicarboxylate were screened for potential androgenicity or estrogenicity. The following studies were conducted: QSAR for binding to the AR and ER, *in vitro* Androgen Receptor Binding Assay, *in vitro* Estrogen Receptor Binding Assays (alpha and beta isoforms), *in vitro* Androgen Receptor Transactivation Assay in human cells, and *in vitro* Estrogen Receptor Transactivation Assay in human cells. None of the QSAR models predicted that any of the monomers possessed appreciable binding affinity for either AR or ER. Binding assays showed no evidence of interaction with either the AR or the alpha or beta ER receptors. Similarly, the AR and ER transactivation assays were negative. Moreover, six of the seven monomers have been subjected to 13-week and developmental toxicity studies in rats with no androgen- or estrogen-related effects being noted. Given the negative results of the *in vitro* screening assays (except PMG which demonstrated cytotoxicity) as well as available repeated dose and developmental and reproductive studies, the data suggest that none of the monomers tested exhibit androgenic or estrogenic hazards.

Abbreviations: A, agonist; ANN, Artificial Neural Network; AR, androgen receptor; CART, Classification and Regression Tree; CASRN, Chemical Abstracts Service Registry Number; CHDA, 1,4-cyclohexanedicarboxylic acid; CHDM, 1,4-cyclohexanedimethanol; DEG, diethylene glycol; DHT, dihydrotestosterone; DMCD, cis and trans dimethylcyclohexanedicarboxylate; DMSO, dimethyl sulfoxide; DMT, dimethyl terephthalate; E2, 17β-estradiol; ECHA, European Chemicals Agency; EDSP, Endocrine Disruption Screening Program; EDSTAC, Endocrine Disruptor Screening and Testing Advisory Committee; EG, ethylene glycol; EFSA, European Food Safety Authority; ER, estrogen receptor; GR, glucocorticoid receptor; mP, milli-polarization; HPV, High Production Volume; IPA, isophthalic acid; LBD, ligand binding domain; MVLN, MCF-7-derived cell line; MOE, Molecular Operating Environment; N, number; NIL, nilutamide; NRC, National Research Council; OECD, Organisation for Economic Co-operation and Development; PI, propidium iodide; OEHHA, California Office of Environmental Health Hazard Assessment; PMG, polytetramethylene glycol; QSAR, Quantitative Structure–Activity Relationship; RBA, relative binding affinity; 5-SSIPA, monosodium-5- sulfoisophthalic acid; SPMX, supramaximal response; SVM, Support Vector Machine; TMCD, 2,2,4,4-tetramethyl-1, 3-cyclobutanediol; TPA, terephthalic acid; TSCA, Toxic Substances Control Act; USEPA, United States Environmental Protection Agency; USFDA, United States Food and Drug Administration.

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1. Introduction

The topic of endocrine effects linked to chemical exposure to plastics and their components continues to receive much attention by the news media as well as the scientific community (Casals-Casas and Desvergne, 2011; Diamanti-Kandarakis et al., 2009). Numerous reports have appeared over the last few years presenting data on endocrine activity from various components of plastics or on leachates from plastic (Chung et al., 2013; Guart et al., 2013; Halden, 2010; Muncke, 2011; Ohashi et al., 2005; Ohno et al., 2001, 2003; Talsness et al., 2009; Wagner and Oehlmann, 2009; Yang et al., 2011).

Previously we reported on the absence of androgenicity and estrogenicity of the three monomers used to make Eastman's Tritan™ copolyester: dimethyl terephthalate (DMT), 1,4-cyclohexanedimethanol (CHDM), and 2,2,4,4-tetramethyl-1,3-cyclobutanediol (TMCD) (Osimitz et al., 2012). QSAR models for binding to the androgen receptor and estrogen receptors (*alpha* and *beta*) as well as a battery of *in vitro* and *in vivo* assays were conducted to determine their potential androgenicity or estrogenicity.

The present paper presents results from the screening of several additional monomers used by Eastman Chemical to make various polymers. These include:

- Ethylene glycol and related
  - Ethylene glycol (EG; CASRN 107-21-1)
  - Diethylene glycol (DEG; CASRN 111-46-6)
  - Polytetramethylene glycol (PMG; CASRN 25190-06-1)
- Terephthalic acid (TPA) and related
  - Isophthalic acid (IPA; CASRN 121-91-5)
  - Monosodium-5-sulfoisophthalic acid (5-SSIPA; CASRN 6362-79-4)
  - Terephthalic acid (TPA; CASRN 100-21-0)
- 1,4-Cyclohexanedimethanol (CHDM) and related
  - 1,4-Cyclohexanedicarboxylic acid (CHDA; CASRN 1076-97-7)
  - Dimethylcyclohexanedicarboxylate (DMCD; CASRN 94-60-0) – cis and trans.

Please note that the test material DMCD contained circa 90% trans isomer. DMCD cis and trans isomers were modeled separately resulting in modeling data for the monomers.

The monomers discussed in this report are used to make a variety of polyesters that are approved for use in food packaging and food processing applications in the United States, the European Union, and other countries. Some of the polyesters are used for the primary food contact surface while others are used only in applications such as food packaging adhesives.

Various *in vitro* and *in vivo* assays, and *in silico* (computational) screening and related molecular modeling approaches are available to predict or evaluate endocrine activity (DeLisle et al., 2001; Meek et al., 2006; Vinggaard et al., 2008; Zauhar et al., 2003). We used a battery of *in vitro* assays that followed standardized protocols, analogous to the Tier I screen developed by the United States Environmental Protection Agency (USEPA) as part of the Endocrine Disruption Screening Program (EDSP) (USEPA, 2008). The assays were chosen to detect the ability of the monomers to bind to androgen and/or estrogen receptors and, through use of transactivation assays, to activate the androgen or estrogen receptor. Specifically, the following studies were conducted:

- QSAR for binding to the AR and ER
- *In vitro* Androgen Receptor Binding Assay
- *In vitro* Estrogen Receptor Binding Assays (*alpha* and *beta* isoforms)
- *In vitro* Androgen Receptor Transactivation Assay in Human Cells
- *In vitro* Estrogen Receptor Transactivation Assay in Human Cells

These data are considered in the context of other *in vivo* data developed to assess systemic, developmental, and reproductive toxicity regarding the potential androgenic and estrogenic hazards associated with the monomers.

2. Materials and methods

2.1. Quantitative Structure–Activity Relationship (QSAR) models

Five separate models were constructed to estimate ER binding. One of these models, the molecular docking model, provided a quantitative value that can be used to gauge relative binding affinity (RBA). The four remaining QSAR models, constructed using pre-computed molecular descriptors, were employed to classify the compounds as "active" or "inactive". Generally, classification models are more appropriate than quantitative models when the number of compounds in the training data set is too small for statistically significant numerical calculation of the RBA.

The QSAR models were constructed using well-established machine learning methods: Bayesian inference analysis, Support Vector Machine (SVM), Artificial Neural Network (ANN), and Classification and Regression Tree (CART). A brief description of each model follows:

Bayesian inference analysis: Binary QSAR is implemented in the Molecular Operation Environment molecular modeling tool (MOE) software suite. Based on a Bayesian inference technique, this method estimates the probability density classifying the compounds as *active* or *inactive*.

SVM is a non-linear model that performs pattern recognition by finding an optimal hyperplane as the decision boundary for separating two classes of patterns, which can maximize the margin between the closest data points of each class.

ANNs are designed to mimic simple biological neural networks that learn rules and relationships between stimuli (inputs) and response (outputs) through a trial-and-error process.

CART is a prediction model constructed by recursively partitioning a data set and fitting a simple model to each partition.

2.1.1. Molecular docking

The molecular docking studies were performed using the crystal structures of human ER alpha, ER beta and AR. The three-dimensional X-ray crystallographic structural models of the ER *alpha*, ER *beta* and AR ligand binding domains were retrieved from the Protein Data Bank (entries 3ERD, 2J7Y, and 1XOW, respectively). Using the commercial software GOLD (Verdonk et al., 2003) each small-molecule compound in the data set, comprising >100 compounds, was computationally docked inside the ligand binding pocket of the three respective receptors. To determine the cutoff threshold for docking, the same compounds comprising the training set for QSAR modeling were docked into each of the three human protein structures 30 times independently. For each protein, the docking score with the highest value for a given compound was used in ranking the series of compounds. The mean value of these ranked scores (score = 40) was taken as a soft cutoff threshold in the molecular docking study to distinguish between binding and non-binding ligands. In this regard, the term "soft" is meant to convey that any compound scoring in the 35–45 buffer region was visually scrutinized in its receptor-docked pose and re-docked multiple times to verify the reproducibility of the compound's docking pose and score. In all cases, the docking scores of the compounds were sufficiently reproducible to justify our class assignments (i.e., binders vs. non-binders).

2.2. In Vitro *androgen receptor binding assay*

The androgen receptor (AR) binding assay (Invitrogen™, Life Technologies Corporation, Carlsbad, CA) uses a sensitive polarographic detection system based on a fluorescent labeled ligand Fluormone™ to form a receptor–ligand complex with a subsequent high polarization value that is added to varying concentrations of test compounds. A reduction in the polarization signal will occur if a test compound has the ability to displace the Fluormone ligand from the complex and to competitively bind to the receptor. The assay provides data on the absolute and relative binding affinity and potency of test compounds (Osimitz et al., 2012).

Briefly, 20 μL of 2 nM Fluormone and 50 nM AR ligand binding domain (AR-LBD) were added to test articles serially diluted with dimethylsulfoxide (DMSO) in 384 well black plates, but not to wells designated as "free ligand" controls. The free ligand control wells received 20 μL of AR Green assay buffer with 2 nM Fluormone but without receptor. Final concentrations in the test wells were 1 nM Fluormone AR Green and 25 nM AR-LBD and 0.05% DMSO and test article. The limit of solubility of the monomers and reference controls were determined by nephelometry, a light scattering procedure (Nepheloskan Ascent® by Labsystems). Each test article and control exposure concentration was run in triplicate in at least two independent experiments. The monomers were evaluated for fluorescent properties that might interfere with the fluorescent polarization assay. The monomers in assay buffer were read with non-polarized light at the same excitation and emission wavelengths used in the fluorescence polarization assay.

Following the 4 to 6 hour incubation, the assay plates were read with a PerkinElmer Envision spectrophotometer at excitation and emission wavelengths of 480 nm and 535 nm, respectively. Data were transferred into Prism 5.01 (GraphPad Software, Inc.) for plots and regression curve analysis. The response values were normalized to 100% response (highest concentration of DHT, 10 μM) and 0% response (DMSO control). Ligand displacement curves were analyzed by performing regression analysis using the equation for one site competition (Y = bottom + (top-bottom)/(1 + 10^ (X-logEC50)) with the bottom of the curve constrained to 0% millipolarization (mP).

The data were evaluated in accord with the USEPA Technical Review Document for the EDSP Proposed Tier 1 Screening Battery (USEPA, 2008). Under these guidelines, if the test article causes the binding curve to drop below 50%, it is considered evidence of a potential AR binder, between 50 and 75% is equivocal, and if the curve is above or equal to 75% it is considered to be a non-binder.

2.3. In Vitro *estrogen receptor binding assay (*alpha *and* beta*)*

The ER Binding Assays are highly specific *in vitro* screens that are used to identify chemicals that can bind to the ER (*alpha* and *beta*). These assays use the same test system (Invitrogen™, Life Technologies Corporation) and evaluation algorithm as the AR Binding Assay. Briefly, stock solutions of the monomer were prepared in DMSO. E2 was used as the positive control for estrogen binding. Vehicle controls (DMSO) were included as negative controls on each plate. Thirty microliters of 2 nM