Toxicology in Vitro 27 (2013) 1711–1718

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Evaluation of a predictive *in vitro* Leydig cell assay for anti-androgenicity of phthalate esters in the rat

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ARTICLE INFO

Article history:
Received 9 January 2013
Accepted 31 March 2013
Available online 8 April 2013

Keywords:
Phthalates
R2C
MEHP
Antiandrogenicity
MEHP metabolites
MBP

ABSTRACT

An *in vitro* assay using the rat Leydig cell line R2C was evaluated for its ability to quantitatively predict inhibition of testosterone synthesis. Results obtained for endocrine active phthalates (MEHP, MBP), and inactive phthalates (MMP and MEP) were highly consistent with *in vivo* results based on tissue and media concentrations. Statistically significant inhibition of testosterone synthesis ($p < 0.05$, 1-way ANOVA) was observed at 1 μM MBP and 3 μM MEHP, while MEP and MMP did not affect inhibition of testosterone synthesis until much higher concentrations (≫100 μM). Concentrations causing 50% inhibition of testosterone synthesis for MBP and MEHP (3 and 6 μM respectively), were similar to *in vivo* values (3 and 7 μM). The R2C assay was used to determine the relative potency of 14 structurally diverse monoesters and oxidative metabolites of MEHP. Monoesters with alkyl chains 4–5 carbons in length had the highest potency for testosterone inhibition, while 0–2 carbon alkyl chains were least potent. Phase I metabolism did not completely inactivate MEHP, underscoring the need for metabolism data when interpreting *in vitro* pharmacodynamic data. This steroid inhibition assay provides a predictive *in vitro* alternative to expensive and timeconsuming developmental rat studies for phthalate-induced antiandrogenicity.

1. Introduction

The phthalate diesters (phthalates) are a class of chemicals in which phthalic acid is esterified to two alkyl chains that may vary in carbon chain length and structure (Fig. 1). The phthalates are used to increase flexibility and longevity of plastics and are present in a variety of industrial and consumer products, such as industrial flooring, sealants, cosmetics, pharmaceuticals, children's toys and food packaging (Hernandez-Diaz et al., 2009, Christen et al., 2012). Because they are not covalently bound to the plastics, the phthalates can leach into food and consumer products from plastic containers. Upon ingestion, the diester phthalates are quickly hydrolyzed into their monoester metabolites (Rowland et al., 1977; White et al., 1980), which are generally considered the toxicologically active species (Clewell et al., 2010; Jones et al., 1993; Parks et al., 2000).

High doses of some phthalates adversely affect male rat sexual development when administered during gestation. Perinatal administration of di-n-butyl phthalate (DBP: >100 mg/kg/day) and di-2,4-ethylhexyl phthalate (DEHP: >300 mg/kg/day) cause reduced anogenital distance (AGD), increased nipple retention, hypospadias (malformed penis), cryptorchid (undescended) testes, and incomplete development or agenesis of some reproductive organs in male rats (Clewell et al., 2013a; Gray et al., 2000; Lehmann et al., 2004; Mylchreest et al., 1998, 1999). Many of these reproductive effects are thought to result from inhibition of testosterone synthesis in the fetal rat testes by the monoester phthalates (Akingbemi et al., 2004; Oishi and Hiraga, 1980; Parks et al., 2000). *In vivo* and *in vitro* studies confirm that the monoester metabolites of the endocrine active phthalates DBP (monobutyl phthalate; MBP) and DEHP (mono-2-ethylhexyl phthalate; MEHP) reduce testosterone synthesis *in vivo* and in cultured Leydig cells (Clewell et al., 2010; Jones et al., 1993; Kwack et al., 2009; Lehmann et al., 2004; Liu et al., 2005). It is generally accepted that the downstream metabolites resulting from glucuronide conjugation and oxidative metabolism are inactive (Ema and Miyawaki, 2001; Silva et al., 2003). Recently, however, it has been suggested that one or more of the oxidative metabolites of MEHP may have anti-androgenic activity (Chauvigné et al., 2009; Koch et al., 2005).

Although the endocrine active phthalates (DEHP, DBP, etc.) appear to act by the same mechanism (Liu et al., 2005), not all phthalates are endocrine active (Gray et al., 2000). The ability of the phthalates to inhibit testosterone synthesis and disrupt male rat reproductive tract development is determined by the structure of alkyl side chains (Gray et al., 2000; Hannas et al., 2011; Liu et al., 2005). The more potent phthalates have 3–5 carbons in their alkyl chains. Dipentyl phthalate (DPP; C5) is the most potent of the

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http://dx.doi.org/10.1016/j.tiv.2013.03.015

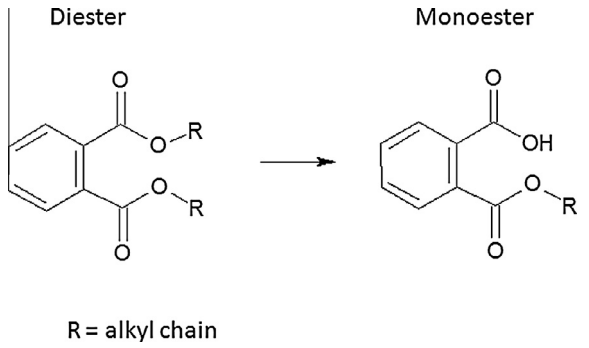

Fig. 1. Generic structure for the diester and monoester phthalates. Hydrolysis of the diester phthalates by intestinal lipases is rapid and nearly complete prior to systemic distribution (White et al., Rowland et al.). *R* indicates alkyl chains $\geqslant 1$ Carbon. Alkyl side chains of the diesters are not necessarily identical depending on the diester.

phthalates tested *in vivo* (Hannas et al., 2011; Howdeshell et al., 2008). However, since dose–response and pharmacokinetic data are only available for a few phthalates *in vivo* (Clewell et al., 2013b; Howdeshell et al., 2008; Hannas et al., 2011; Lehmann et al., 2004; Mylchreest et al., 1999), it is difficult to predict the relative potency of untested phthalates based on available animal studies.

The extensive use of phthalates in consumer products makes the study of the dose–response and the mechanism of action of the phthalates a priority. The current recommended method for identifying endocrine active phthalates is a rodent developmental study, wherein pregnant rats are administered diester phthalates during gestation and lactation and alterations in the reproductive tract of the post-pubertal male offspring are evaluated (Boberg et al., 2011; Clewell et al., 2013b; Fisher et al., 2003; Foster et al., 2001; Gray et al., 2000; Gray et al., 2009; Plummer et al., 2007). Since rats produce multiple offspring per litter, the number of animals used and the cost for these types of studies are prohibitive of in-depth dose–response and mechanistic studies. Thus, the development of *in vitro* assays to replace *in vivo* rat studies of phthalate-mediated anti-androgenicity is an important step in reducing the cost and use of animals for phthalate screening and mechanistic studies.

A mouse Leydig cell (MA-10) assay was previously shown to be a useful tool for testing anti-androgenic activity of the phthalate esters *in vitro*, showing a high degree of correlation with inhibition of testosterone synthesis in the fetal rat (Clewell et al., 2010). Although this assay proved reliable, two important differences exist between this cell line and the *in vivo* models for phthalate effects. MA-10s are derived from adult mouse testes and require luteinizing hormone (LH) receptor stimulation to induce testosterone production. In contrast, *in vivo* anti-androgenic effects are observed in the male rat, not the mouse. Furthermore, LH does not regulate testosterone in the rat fetus during development, and is therefore not required for phthalate inhibition of testosterone in the fetal testes. In this paper, we evaluated the use of a rat Leydig cell line (R2C) to predict inhibition of testosterone synthesis in fetal rats. The R2C cell line is derived from a Leydig cell tumor from a 2-month-old male Wistar-Furth rat. Like the fetal rat Leydig cell, the R2Cs do not require either hormonal stimulation or cyclic adenosine monophosphate (cAMP) induction for testosterone secretion (Freeman, 1987; Stocco and Chen, 1991), though this cell line was derived from an adult, not fetal, rat.

R2Cs have been used to assess the effects of various compounds on cell physiology and to some extent, the steroidogenic pathway (Freeman, 1987; Rao et al., 2003). Furthermore, some studies have shown that phthalates can inhibit steroid hormone production in the R2Cs, though these studies measured the testosterone precursor progesterone for a single phthalate (MEHP) and not testosterone itself (Gazouli et al., 2002). To date, no effort has been made to show whether the inhibition observed in the R2C cells is indicative of an *in vivo* response in rat.

The goal of the current study was to determine whether the R2C cell line, which is a more appropriate *in vitro* model for the rat, would provide an *in vitro* assay for phthalate inhibition that is as robust as the MA-10 cell assay, and more importantly, that will correlate with *in vivo* effects observed in the fetal rat. The R2C testosterone synthesis assay was tested with several phthalates, including known "endocrine active" phthalates (MEHP, MBP, and mono-n-octyl phthalate; MnOP), as well as known "inactive" compounds (monoethyl phthalate; MEP and monomethyl phthalate; MMP). Inhibition of testosterone synthesis in the R2C cell line was then compared to data from MA-10 cells and fetal rats *in vivo*. The R2C assay was then used for two predictive assessments. First, the potency of a battery of phthalates with different alkyl chain structures was evaluated for inhibition of testosterone synthesis, including 14 commercially available monoester phthalates. Second, the role of Phase I (P450) metabolism in detoxification of the phthalate monoesters was evaluated by testing the ability of MEHP and its oxidative metabolites to inhibit testosterone synthesis.

2. Methods

2.1. Chemicals

Mono-n-octyl phthalate (MnOP), monobenzyl phthalate (MBeP), mono-2-ethylhexyl phthalate (MEHP), monobutyl phthalate (MBP), monoethyl phthalate (MEP), and monomethyl phthalate (MMP) were purchased from AccuStandard, Inc. (New Haven, CT). DL-mono-1-methylhexyl phthalate (MMHP), mono(1,2-dimethylpropyl) phthalate (MdMPP), (+)-mono(1,2,2-trimethylpropyl) phthalate (MtMPP), mono-n-hexyl phthalate (MnHP), (+)-mono-1-tert-butyl-3-methylbutyl phthalate (MtBMBP), DL-mono-1-tert-butylpentyl phthalate (MtBPP), and (−)mono-2,2-dimethyl-1-isopropylpropyl phthalate (MdMIPP) were purchased from the rare chemical library at Sigma Aldrich Co., LLC (St. Louis, MO). Mono-(2-ethyl-5-carboxy-pentyl) phthalate (5-Cx-MEPP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (5-Hx-MEHP), mono-[(2-carboxymethyl) hexyl] phthalate (2-Cx-MMHP), mono-(2-ethyl-5-oxohexyl) phthalate (5-Oxo-MEHP), monoisodecyl phthalate (MiDP), and monopentyl phthalate (MPP) were purchased from Cambridge Isotopes, Inc. (Andover, MA). Phthalic acid (PA) was purchased from Sigma–Aldrich Co., LLC (St Louis, MO). Human luteinizing hormone (whole molecule, >95% purity) was purchased from Scripps Laboratories (San Diego, CA).

2.2. Cell culture

The mouse Leydig tumor cell line (MA-10) was generously provided by Dr. Mario Ascoli (Department of Pharmacology, University of Iowa, College of Medicine, Iowa City). MA-10 cell culture was performed as previously described (Ascoli, 1981; Clewell et al., 2010). Briefly, 150 mm × 20 mm Polystyrene tissue-culture dishes (Sigma–Aldrich) were coated with 0.1% gelatin. Cells were cultured in these dishes at a concentration of 1.5×10^6 cells per culture dish until they reached 80% confluence. Cells were grown in Waymouth's MB 752/1 cell media (Invitrogen) containing 0.002 M HEPES (Invitrogen), 50 μg/ml Gentamicin (Invitrogen), 15% horse serum (Atlanta Biologicals), and 0.4% Fungizone (Invitrogen) and kept at 37 °C with a mixture of air and 5% CO_2. When cells reached 80% confluence, they were trypsinized and reseeded in gelatin-