Environmental Research 150 (2016) 496–512

Contents lists available at ScienceDirect

Environmental Research

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

In vitro functional screening as a means to identify new plasticizers devoid of reproductive toxicity

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ARTICLE INFO

Article history:
Received 6 March 2016
Received in revised form
19 June 2016
Accepted 20 June 2016

Keywords:
plasticizers
testis
proliferation
steroidogenesis
Leydig cells
germ cells
toxicity

ABSTRACT

Plasticizers are indispensable additives providing flexibility and malleability to plastics. Among them, several phthalates, including di (2-ethylhexyl) phthalate (DEHP), have emerged as endocrine disruptors, leading to their restriction in consumer products and creating a need for new, safer plasticizers. The goal of this project was to use in vitro functional screening tools to select novel non-toxic plasticizers suitable for further in vivo evaluation. A panel of novel compounds with satisfactory plasticizer properties and biodegradability were tested, along with several commercial plasticizers, such as diisononyl-cyclohexane-1,2-dicarboxylate (DINCH®). MEHP, the monoester metabolite of DEHP was also included as reference compound. Because phthalates target mainly testicular function, including androgen production and spermatogenesis, we used the mouse MA-10 Leydig and C18-4 spermatogonial cell lines as surrogates to examine cell survival, proliferation, steroidogenesis and mitochondrial integrity. The most promising compounds were further assessed on organ cultures of rat fetal and neonatal testes, corresponding to sensitive developmental windows. Dose-response studies revealed the toxicity of most maleates and fumarates, while identifying several dibenzoate and succinate plasticizers as innocuous on Leydig and germ cells. Interestingly, DINCH®, a plasticizer marketed as a safe alternative to phthalates, exerted a biphasic effect on steroid production in MA-10 and fetal Leydig cells. MEHP was the only plasticizer inducing the formation of multinucleated germ cells (MNG) in organ culture. Overall, organ cultures corroborated the cell line data, identifying one dibenzoate and one succinate as the most promising candidates. The adoption of such collaborative approaches for developing new chemicals should help prevent the development of compounds potentially harmful to human health.

1. Introduction

Chemists generate large amounts of new compounds yearly in response to the multiple needs of the industry. Although new regulation in Europe requires a toxicological screening for new chemicals before commercialization, this is not a universal rule, and many chemicals remain untested for potential toxicity prior to their mass production and release in the environment (Hartung, 2009). Likewise, criteria of safety to humans and the environment are not systematically included in the strategies used to design and select novel chemicals worldwide. This has led to the existence of thousands of widely used compounds for which no or little toxicology data exist.

Plasticizers are compounds used to provide adequate flexibility, elasticity and malleability to plastics. The most common plasticizers are diesters of phthalic acid, called phthalates, which are produced in the order of several million tonnes per year (Martinez-Arguelles et al., 2013). Phthalates are used mainly in poly(vinyl chloride) (PVC) applications, where the plasticizer content can reach up to 50% of the total weight of the plastic mixture, and are present in a variety of industrial and consumer products (Wypych, 2012). Because they are usually not chemically bound to the polymer they are blended with, plasticizers tend to leach from the material over time, contaminating animals, humans and the environment (Martinez-Arguelles et al., 2013; Erythropel et al., 2014). Phthalates are found in human fluids such as blood, urine and amniotic fluid (Kavlock et al., 2006). The most abundant phthalate is di-(2-ethylhexyl) phthalate (DEHP), which is metabolized to mono-(2-ethylhexyl) phthalate (MEHP) by esterases, for example in the intestine (Kavlock et al., 2006). General human

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http://dx.doi.org/10.1016/j.envres.2016.06.033
0013-9351/

exposure to DEHP ranges from 3 to 30 μg/kg/day, but can reach 1–10 mg/kg/day in specific medical conditions, due to leaching from medical tubings and bags (Kavlock et al., 2006, Erythropel, 2014). DEHP levels of 5 μM have been found in human umbilical cord blood, indicating that fetuses are exposed to relatively high levels of this compound (Latini et al., 2003). Many studies, including ours, have reported short and long term deleterious effects of phthalates on the rodent male reproductive system (Culty et al., 2008; Martinez-Arguelles et al., 2009; Sharpe and Skakkebaek, 2008; Jones et al. 2014). These findings were strengthened by epidemiological studies reporting that in humans, high phthalate levels were associated to various reproductive pathologies, classified under the term of testicular dysgenesis syndrome (Virtanen et al., 2005) including hypospadias, low testosterone levels, poor semen quality (Ormond et al., 2009; Meeker et al., 2009; Pant et al., 2008), as well as abnormal anogenital distance and hormone blood levels in fetus (Bornehag et al., 2015; Araki et al., 2014).

In light of studies reporting detrimental effects of ubiquitous environmental contaminants such as phthalates (Diamanti-Kandarakis et al., 2009), public concern regarding the safety of these chemicals has increased over the past years. As a result, the use of 6 phthalates, including DEHP, has been restricted in the production of children's toys and child care articles, since 2005 in Europe and more recently in Canada and the US (Erythropel et al., 2014). In response to these restrictions, the need has emerged to develop new compounds with good plasticizer properties, which would not lead to similar health risks as DEHP.

In the present study, we examined a panel of plasticizer candidates for their potential in vitro effects on components of the male reproductive system, in order to screen which compounds were suitable for further evaluation by in vivo studies. This screening would ensure that any putative candidate compound would not lead to effects similar as those of DEHP. Four series of compounds were selected for their adequate plasticizer effectiveness paired with acceptable biodegradation kinetics to avoid persistence in the environment. Particularly, these were n-alkyl diesters of succinic-, maleic-, and fumaric acid (Erythropel et al., 2012, 2013, 2015; 2016), as well as one series of n-alkyl dibenzoate compounds (Firlotte, 2009; Kermanshahi pour et al., 2009a, 2009b). These compounds, along with the commercial plasticizers DEHP, MEHP, DEHA and DINCH® were tested for their in vitro toxicity using Leydig and spermatogonial mouse testicular cell lines. The most promising plasticizer candidates were further assessed for their effects in organ cultures of rat fetal and neonatal testes, narrowing the choice of compounds to be further tested in vivo.

2. Materials and methods

2.1. Plasticizers and Metabolites

In all, 22 compounds were analyzed for their toxicity levels on Leydig and germ cells (Fig. 1). Based on the structure of commercial plasticizers, 15 novel plasticizer candidates were synthesized, belonging to four chemical families, the dibenzoates (Kermanshahi pour et al., 2009a; Firlotte et al., 2009), and the succinates, maleates and fumarates (Erythropel et al., 2012). The dibenzoate series varied in the length of the central alkyl chain and included 1,3-propanediol dibenzoate (PrDB), 1,4-butanediol dibenzoate (BDB), 1,5-pentanediol dibenzoate (PDB) and 1,6-hexanediol dibenzoate (HDB). In the other three series, the length and degree of branching of the side chains were varied, and the compounds included the diethyl-, dibutyl-, dihexyl-, di-*n*-octyl-, and di-(2-ethylhexyl) diesters of succinic-, maleic-, and fumaric acid (leading to DEM, DBM, DHM, DOM, and DEHM for the maleates, DEF, DBF, DHF, DOF, and DEHF for the fumarates, and DES, DHS, DOS, and DEHS for the succinates). From the above list, DEM, DBM, DEHM, DEF, and DES were available through Sigma-Aldrich (St-Louis, MO, USA), while all other compounds were synthesized, with a purity of at least 99%. The other compounds tested were di-(2-ethylhexyl) adipate (DEHA); mono (2-ethylhexyl) phthalate (MEHP), the bioactive metabolite of di-(2-ethylhexyl) phthalate (DEHP); and the inactive phthalate di-ethyl phthalate (DEP), all with a purity of at least 99% (Sigma-Aldrich). We also examined the effects of the non-aromatic plasticizer 1,2-cyclohexane dicarboxylic acid di-isononyl ester (DINCH®), a mixture of branched and possibly linear isomers, (trade name Hexamoll® DINCH®; product number 51303880, batch number BASFDE, 99% purity, BASF Canada Inc., Mississauga, ON, Canada).

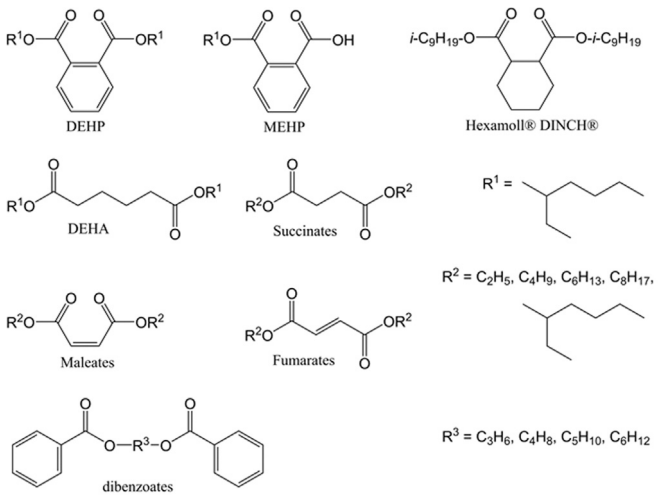

Fig. 1. Chemical structure of the various candidate and commercial plasticizers tested, and the prototype compound DEHP. Backbone structures are shown. The dibenzoate compounds are 1,3-propanediol dibenzoate (PrDB; R=$_{C3H6}$), 1,4-butanediol dibenzoate (BDB; R=C_4H_8), 1,5-pentanediol dibenzoate (PDB; R=C_5H_{10}) and 1,6-hexanediol dibenzoate (HDB; R=C_6H_{12}). For the succinate, maleate and fumarate series, the compounds included diethyl- (R=C_2H_5), dibutyl- (R=C_4H_9), dihexyl- (R=C_6H_{13}), di-*n*-octyl- (R=C_8H_{17}), and di-(2-ethylhexyl)- (R=$CH_2CH(C_2H_5)(C_4H_9)$) diesters of succinic-, maleic-, and fumaric acid.

2.2. Cell cultures and treatments

The hormone-responsive mouse Leydig MA-10 tumor cell line was a gift from Dr. Mario Ascoli (University of Iowa, Iowa city, IA, USA). Cells were plated in 96 well plates (Corning, NY, USA) in DMEM/Ham's F12 (1 × 1)+ GlutaMax supplemented with 5% fetal bovine serum (FBS), 2.5% horse serum (all from Gibco by Life Technologies, Grand Island, NY, USA), 1% penicillin/streptomycin (Cellgro, Mediatech, Manassas, VA, USA) at 37 °C, 3.5% CO_2. While MA-10 cell metabolic competency, a useful parameter for in vitro toxicological studies (Garcia-Canton, 2013), had not been thoroughly studied, this cell line has been shown through gene expression and proteomics studies to express a variety of phase 1 and 2 enzymes, and multiple oxidative stress response genes (Fan et al., 2010).

The LTAg-immortalized mouse type A spermatogonia C18-4 cell line was a gift from Marie-Claude Hofmann (The University of Texas MD Anderson Cancer Center, Houston, Texas) (Hofmann et al., 2005). Cells were grown in DMEM containing 4.5 g/L D-Glucose, L-Glutamine and 110 mg/L of sodium pyruvate (Gibco) supplemented with 10% FBS (Life Technologies, Grand Island, NY, USA) and 1% penicillin/streptomycin (Cellgro) at 34 °C, 5% CO_2. Despite its unknown metabolic competency, this cell line has been used in several toxicological studies, including studies examining