Assessment of Anti-Androgenic Potential of Candidate "Green" Dibenzoate Plasticizers in Mammalian MA-10 cells

Marie Vanlian
M. Eng. Candidate

DEPARTMENT OF CHEMICAL ENGINEERING

MCGILL UNIVERSITY

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ABSTRACT

Plasticizers are additives to plastics that impart flexibility and the ability to process polymers. Unfortunately, due to their leaching potential and widespread use, they have become ubiquitous contaminants in the environment. Studies have correlated the rise of many health issues to the chronic exposure to these compounds and have suggested them as potential carcinogens and endocrine-disruptors. In particular, available evidence indicates that they disturb steroid production such as testosterone production and therefore, behave as anti-androgens. The seriousness of this issue has prompted researchers to develop "green" plasticizers to avoid adverse effects.

In this thesis, a series of potential alternative plasticizers were screened for their anti-androgenic potential *in vitro* using a mouse Leydig tumor cell line, the MA-10 cells. Optimal solvent and plasticizer concentrations and time frame conditions were determined to ensure viability of the cells for the duration of the experiments. Using these optimized testing conditions, potential green plasticizers, including the 1,3-propanediol (C3), 1,4-butanediol (C4), 1,5-pentanediol (C5) and 1,6-hexanediol (C6) dibenzoates, were studied by monitoring their effects on progesterone synthesis. Based on these results and those of others from our group, the candidature of C4 is supported as a potential green plasticizer.

"Cooperation between chemists, engineers and health and safety community will be critical to ensure the adoption of safe and sustainable technologies." – *Anastas, et al.* [1]

SOMMAIRE

Les plastifiants sont des adjuvants ajoutés aux formulations de plastiques pour les rendre plus flexibles et plus faciles à manipuler. Malheureusement, étant donné leur tendance à migrer de la matrice polymère et leur utilisation répandue, ils sont devenus des contaminants environnementaux omniprésents. Des études ont corrélé la hausse de certaines maladies à l'exposition chronique à ces composés et évoquent leurs risques en tant que potentiels carcinogènes et perturbateurs endocriniens. Notamment, il y a évidence qu'ils perturbent la formation de certains stéroïdes dont la testostérone et ainsi, agissent comme des anti-androgènes. L'importance de ce sujet a incité les chercheurs à développer des plastifiants 'verts' pour éviter les effets négatifs associés à ces composés.

Dans cette thèse, des composés alternatifs ont été testés pour leur effet anti-androgène *in vitro* avec la lignée cellulaire tumorale interstitielle du testicule de souris, les cellules MA-10. Des concentrations optimales de solvant et plastifiants ainsi qu'une durée optimale d'exposition ont été déterminées afin d'assurer la viabilité des cellules au cours des expériences. En employant les conditions optimales établies, des composés sélectionnés en tant que potentiels plastifiants verts, incluant les dibenzoates de 1,3-propanediol (C3), de 1,4-butanediol (C4), de 1,5-pentanediol (C5) et de 1,6-hexanediol (C6), ont été étudiés en mesurant l'impact sur la synthèse de progestérone. Les résultats de cette étude, appuyés par d'autres résultats de notre groupe, démontrent le potentiel du C4 en tant que potentiel plastifiant vert.

« La coopération entre chimistes, ingénieurs et la communauté de la santé et sécurité sera cruciale pour assurer l'adoption de technologies sécuritaires et viables. » – *Anastas, et al.* [1]

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This journey would not have been the same without the constant support of all my friends, in particular Nanor and Hovig. Their understanding and love throughout the duration of my studies pushed me every step of the way to persist in hard times and try my best to excel when occasion arrived.

Behind every accomplishment I will ever achieve, my family will be entitled to it. They are the reason of the person that I am today and the foundation of every realization.

I dedicate this work to my dad.

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NOMENCLATURE

BCA.....Bicinchoninic acid protein assay
C3.....1,3-Propanediol dibenzoate
C4.....1,4-Butanediol dibenzoate
C5.....1,5-Pentanediol dibenzoate
C6.....1,6-Hexanediol dibenzoate
DMSO.....Dimethyl sulfoxide
DEHP .....Di-(2-ethylhexyl) phthalate
D(EG)DB.....Diethylene glycol dibenzoate
D(PG)DB.....Dipropylene glycol dibenzoate
ELISA.....Enzyme-linked immunosorbent assay
hCG .....Human chorionic gonadotropin
MEHP.....Mono-(2-ethylhexyl) phthalate
MTT .....(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
PBS .....Phosphate buffered saline
TDS.....Testicular dysgenesis syndrome

1. INTRODUCTION

On a daily basis, individuals come in contact with plastic compounds from multiple sources ranging from gardening to undergoing surgical interventions. The industry of polymers has evolved enormously to such an extent that plastics have become indispensable and the material of choice to a wide spectrum of industrial applications. Their high demand stems from their low manufacturing cost, flexibility, imperviousness to water and their versatility, their most appealing asset.

The continuous growth and success of plastics relies on the development of their unique and dependable properties. It is through the use of additives such as fungicides, flame retardants, stabilizers and antioxidants that application-specific properties of plastics are achieved. One category of low molecular weight additives is termed plasticizers and is used to enhance the polymer's flexibility, elongation properties and processability. On a global scale, the consumption of plasticizers is accountable for more than one half of all plastic additives by volume [2]. The family of phthalate esters are the most prevalent with 80% of all plasticizer production, amongst which the most widely used plasticizer used in poly-vinyl chloride (PVC) formulations, the di-(2-ethylhexyl) phthalate (DEHP) accounts for over 50% of worldwide phthalate production [3].

As the plastics industry grows with an average annual consumption rate of 5.09%/year [4], the field of additives expands correspondingly. In 2000, the global production of additives had reached 8.3 million tons, increased to 11.1 million tons in 2009 and is expected to reach 13.2 million tons by 2015 [2]. Over time, additives like plasticizers tend to diffuse down the concentration gradient to the interface between the polymer surface and external medium when

they are in contact with a stationary or flowing fluid, or in contact with another solid material [5]. Since they are not covalently bonded to the polymers, they leach out of the polymer matrices, migrate and enter the environment [5, 6].

As a result of their widespread use and tendency to leach out, plasticizers are a ubiquitous pollutant in the environment [7] and have been detected in significant concentrations in soil, air, water and even in the urine of the general public [8, 9]. This constant exposure has raised concerns that plasticizers, in particular DEHP, can cause serious health effects in humans. In fact, liver failure and male reproductive tract disorders have been linked to the ubiquitous presence of plasticizers [10]. Further studies have also reported adverse effects manifested in the kidneys, lungs and heart [11] yet the liver and testis remain the main organs of concern. In particular, phthalates have been linked to the testicular dysgenesis syndrome (TDS) which includes cryptochordism and hypospadias, testicular cancer and reduced semen quality. It is widely accepted that some plasticizers act as endocrine disrupting chemicals that behave as anti-androgens and disturb testosterone production [12]. Massive amounts of research support the hypothesis that certain classes of plasticizers are the cause of TDS symptoms [13-16] and tract malformations in animal models [17-21].

Due to their inability to biodegrade or their biodegradation to toxic metabolites, plasticizers have become a significant environmental concern [22]. These negative outcomes have prompted researchers to attempt developing benign plasticizers that will be less likely to leach out of the plastic formulations and essentially be biodegradable to non-toxic metabolites. In an effort to meet this demand, the McGill group has focused on altering existing structures of certain commercial plasticizers by redesigning the chemical structures that are known to lead to toxic outcomes. Biodegradation studies with soil microbes have helped to identify toxic

biodegradation products of commercial plasticizers [7, 23]. Based on this work, series of novel plasticizers have been synthesized at McGill and are currently being studied as potential green replacements to DEHP. It is, however, of fundamental value to carefully scrutinize for any of their possible adverse effects before considering commercialization. In particular, this research evaluates the acute toxicity and anti-androgenic potential of a new series of candidate green dibenzoate plasticizers with varying size in chain length: 1,3-propanediol dibenzoate (C3), 1,4-butanediol dibenzoate (C4), 1,5-pentanediol dibenzoate (C5) and 1,6-hexanediol dibenzoate (C6).

2. OBJECTIVES

Substantial work has already been conducted on the synthesized series of candidate "green" dibenzoate plasticizers. Their mechanical properties have been assessed [24], their biodegradation has been studied in the presence of soil microbes [25-27] and their aqueous leaching tendency has been evaluated [28]. The ultimate **goal** of this thesis was to determine their potential to disturb the testosterone steroidogenic pathway and therefore, their anti-androgenic effects *in vitro* using a mammalian cell line. A well established model for androgenicity testing, the mouse Leydig MA-10 cell line, was selected to assess acute testicular toxicity of the selected potential green plasticizers of the dibenzoate series, including 1,3-propanediol dibenzoate (C3), 1,4-butanediol dibenzoate (C4), 1,5-pentanediol dibenzoate (C5) and 1,6-hexanediol dibenzoate (C6). For reference purposes, the acute testicular toxicity of the common plasticizer di-(2-ethylhexyl) phthalate (DEHP) and its monoester, mono-(2-ethylhexyl) phthalate (MEHP) alongside that of the commercial analog plasticizer, diethylene glycol dibenzoate (D(EG)DB) were also measured. Progesterone production was used as a metric to evaluate steroidogenesis.

The achievement of this goal required the successful completion of the following objectives:

1. Identify the effect of solvent on MA-10 cell acute toxicity and select a concentration that suitably solubilized the compound without significant toxic effects.
2. Identify optimal testing conditions to assure MA-10 cell viability during toxicity testing.
    a. Identify appropriate compound concentrations non-toxic to the cells.
    b. Identify suitable compound exposure timeframe to cells.
3. Quantify the effects of the plasticizer concentration on progesterone production in MA-10 cells.

3. LITERATURE REVIEW

3.1.Plasticizers

3.1.1. Mechanism of Action and Classification

In order to impart desirable properties such as flexibility and processability, plasticizers must lower the polymer formulation stiffness. The glass transition temperature (T_g) is a measure of the change of the material's hard, non crystalline, glass-like state to its elastic, rubbery form [5], which is lowered by the addition of plasticizers. One theory attempting to explain this mechanism is the free volume theory. It proposes that the plasticizer molecules insert themselves between the polymeric structure and form secondary bonds to the polymer chains. This embedment spaces out the chains and the resulting increase in free volume (internal space in polymer) allows for more mobility of the macromolecules. Consequently, the glass temperature of the plastic is significantly reduced and the resulting polymer is softer and more easily deformable [3].

Plasticizers are categorized in two classes: internal or external. Internal plasticizers are inherently part of the basic polymer chain as they are incorporated in the plastic during the polymerization process. Thus, they are permanently linked and do not dissociate from the product. In contrast, external plasticizers are additives mixed within the polymeric material and are not attached to the polymer chains by primary bonds. As a consequence, they are apt to evaporation, migration or leaching. It should be noted that internal plasticization is less common due to its high cost and extensive processing and that in most cases, external additives are necessary to incorporate as well to achieve the desired material properties [29].

The degree of plasticization is heavily dependent on the chemical structure of the plasticizers. In general, plasticizers with a low molecular weight and small polar groups tend to attribute higher flexibility and workability to the polymer. In fact, a bulky structure of the plasticizer tends to make the polymer's chain backbone stiffer and thereby reduce its flexibility whereas lower molecular weight plasticizers will introduce a greater number of chain ends hence a larger free volume and consequent greater flexibility is imparted. Nonetheless, high molecular weight plasticizers yield better mechanical properties. As well, the presence of polar groups (which increases intermolecular interactions such as hydrogen bonding) raise the energy needed for chain rotations, resulting in a T_g increase [30].

3.1.2. Current Industrial Plasticizers

The current market holds a multitude of plasticizers with a vast range of select attributes, covering the needs of almost any desired application. This unlimited array contains more than 300 different types of plasticizers of which between 50 and 100 are commercially used [31]. The various types include abietates, **adipates**, alkyl sulfonates, azelates, benzoates, chlorinated paraffins, **citrates**, energetic plasticizers, **epoxides**, glycol ethers and their esters, glutarates, hydrocarbon oils, isobutyrates, oleates, pentaerythritol derivatives, **phosphates**, **phthalates**, polymeric plasticizers, ricinoleates, sulfonamides, superplasticizers and plasticizers for concrete, **tri- and pyromellitates** and others [3] out of which the bolded categories are the most popular. Pertinent categories will be briefly presented below.

Adipates Esters

The esters of adipic acids (hexanedicarboxylic) have the chemical structure shown in Figure 1. They are manufactured via the esterification of alcohols with adipic acid. For example,

the esterified 2-ethylhexanol produces the common di(2-ethylhexyl) adipate (DEHA) also referred to as dioctyl adipate (DOA), Figure 2. They stand out for their improved low temperature performance relative to phthalates and the lower plastisol viscosities they induce in PVC plastisol applications due to their solvating strength. However, adipates have higher volatilities and migration rates and are typically more costly in comparison to phthalates [7, 8].

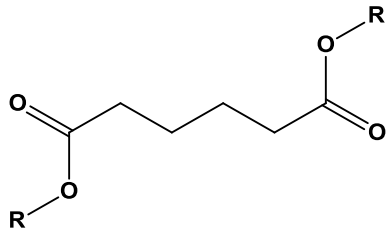


Figure 1: Esters of adipic acid chemical structure

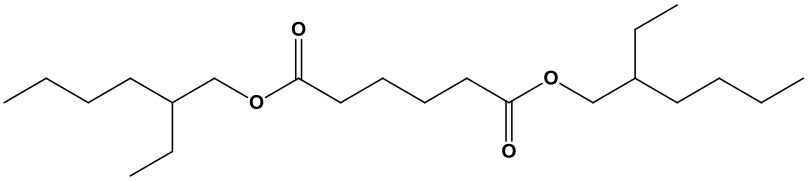


Figure 2: Di(2-ethylhexyl) adipate (DEHA)

Phthalate Esters

The family of phthalates (phthalic acid esters) is the most prominent category of plasticizers used worldwide. In fact, they are estimated to constitute 90% of all plasticizers used globally, with a consumption rate increasing of 3.7% annually [8]. Phthalic acids and corresponding phthalates have three isomeric forms presented in Figure 3 from which phthalates, esters of ortho-phthalic acid are the most common of all. The esters of the two other acids are seldom used as they are more expensive [3].

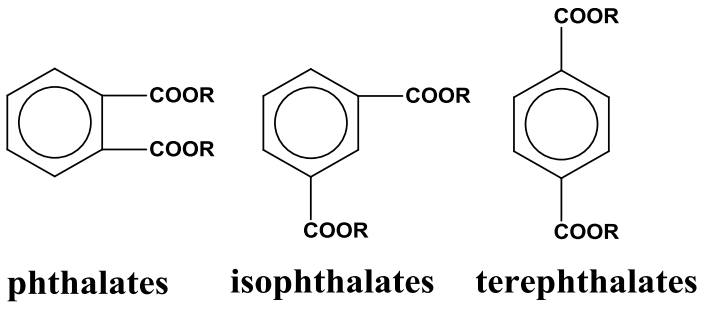


Figure 3: Isomeric forms of phthalic acids

The most predominant phthalate on the market is the di-2-ethylhexyl phthalate (DEHP), also known as di-octyl phthalate (DOP), representing 51% of all used phthalates, followed by the di-isodecyl phthalate (DIDP) (21%) and di-isononyl phthalate (DINP) (11%) [4]. The structures of DEHP and other pertinent phthalate plasticizers are shown in Figure 4. In general, phthalates display most of the desirable properties of a plasticizer such as minimal interaction with resins at room temperature, good fusion properties, relatively nonvolatile at ambient conditions and low cost. These plasticizers produce highly elastic compounds with reasonable cold strength [5]. DEHP stands out with its all around plasticizing performance and its provision of adequate properties for many cost-effective, general purpose products [4]. In general, phthalate esters have been broadly used in thermoplastic cellulose ester molding compounds, PVC and other vinyl chloride copolymers. They are present in medical tubing and bags, footwear, electrical cables, packaging, toys, paints, rubber products, adhesives and certain cosmetics.

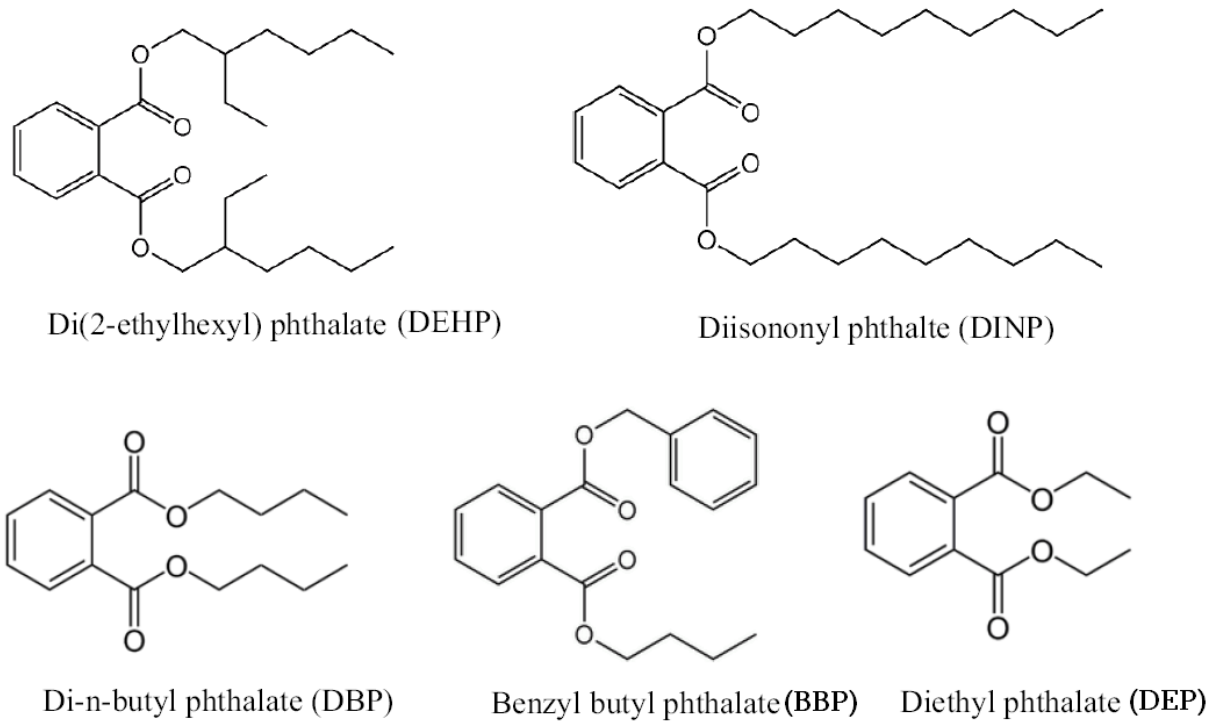


Figure 4: Structure of various phthalate plasticizers

Phthalates have been thoroughly studied and are amongst the best understood of all compounds for their plasticizing ability. They are widely used as they offer unique properties that have helped many fields such as the medical one to create superior performing materials yet affordable. Indeed, they have led many industries to advances in technology though have also been the center of great controversy in particular to critical applications such as infant care and teething products. Health hazards have been associated with their extensive use and stringent regulations concerning their applications have consequently been developed.

3.1.3. Human Exposure

Human exposure to plasticizers is inevitable [32]. The human body cannot escape close contact particularly with DEHP as it is present in many commercial and industrial products. DEHP and its metabolites have been found in air, water and food. Air is contaminated from off-gasses of PVC products escaping through flooring, fragrances, hair sprays, cleaning products and so on; drinking water is tainted with leached DEHP particles from various sources as well [5]. Moreover, besides respiratory and oral administration, humans may be exposed to plasticizers by dermal contact to toys, sunscreens, insecticides and intravenously through PVC based blood bags and other medical equipment.

It is estimated that the daily intake of DEHP is 8 μg/kg body weight for adults and 25 μg/kg body weight for infants. In certain cases, exposure to DEHP is heightened, in particular for individuals involved in occupations with constant contact to plastic such as nursing or plastics manufacturing, reaching outrageous numbers of 700 μg/kg/day, for patients with certain medical conditions, namely for hemodialysis patients, reaching 1.5 mg/kg/day, or as high as 10-20 mg/kg/day for neonates requiring intensive care [33]. On average, the DEHP exposure in the

general public ranges 3-30 μg/kg/day [34] which raises concerns as the higher end of this range exceeds the reference exposure limit recommended by the EPA of 20 μg/kg/day and nearly reaches the tolerable daily intake recommended by the EU of 37 μg/kg/day [35].

3.1.4. Adverse Effects

Several bans and regulations have been issued by the European parliament, US government, Department of Health of Canada following toxicity and health concerns exerted by plasticizers, mainly concerning phthalates. Phthalate plasticizers have been scrutinized for their possible carcinogenicity and potential endocrine-disruption activity. Studies performed on rodents exposed to common phthalates such as DEHP, discerned several related harmful effects [36, 37]. High doses impair the liver, kidney and reproductive system and also engender tumor formations. Low concentrations, comparable to environmental conditions, have been shown to interfere with the endocrine system [38]. Though broad research has been conducted on the related effects and mechanisms of these compounds, our understanding is still very limited.

Several experimental studies have demonstrated that exposure to phthalates to pregnant female rodents induce abnormal development in the male prodigy. A number of linked defects in the progeny were related to the testicular dysgenesis syndrome (TDS) concerning epididymal and gubernacular agenesis, decreased Leydig cell function, cryptorchidism, hypospadias, and impaired spermatogenesis with reduced fertility [13, 18, 20, 39]. In particular, DEHP has been shown to cause testicular damage and stimulate disorganization of seminiferous tubules, detachment of the spermatogonial cells from the basal membrane, and the lack of spermatocytes [40]. Though it has been proven that phthalates do not bind to the androgen or estrogen receptors, they still behave as anti-androgens [36, 41, 42]. Instead, pathways such as the nuclear

peroxisome proliferator-activated receptor α (PPARα) found in Leydig cells and transforming growth factor β (TGF-β) superfamily signaling may pertain to testicular dysgenesis [43, 44].

3.1.4.1. Steroidogenesis

Steroidogenesis is the process wherein hormonal steroids are produced to regulate physiological homeostasis for salt, sugar, sex differentiation and many other responses. They implement their regulating functions by binding to targeted nuclear receptors to activate them and promote the transcription of the respective genes [45]. Products of steroidogenesis include androgens, testosterone, estrogens, progesterone, corticoids and aldosterone which are all derivatives of a common precursor, cholesterol and are synthesized by a variety of tissues. Their biosynthesis is the result of the chain activity of oxidative enzymes located in both mitochondria and endoplasmic reticulum. The initial step is the rate-limiting step where cholesterol is transported from cytoplasm to be first converted to pregnenolone with an enzymatic process localized to the inner mitochondrial membrane, catalyzed by cytochrome P450 with cholesterol side-chain cleavage activity (CYP11A1) [46]. Pregnenolone then becomes the immediate precursor to any of the steroid hormones to be formed in the smooth endoplasmic reticulum [45].

In particular to the biosynthesis of testosterone, the luteinizing hormone LH which is the primary pituitary hormone, stimulates steroidogenic activity within mammalian testis by binding to a specific membrane-bound receptor. This results in the activation of adenylyl cyclase, thereby increasing the production of cyclic adenosine monophosphate (cAMP), an intracellular signaling molecule which in turn stimulates protein kinase A (PKA) synthesis for the transport of cholesterol into the membrane [47]. The steroidogenesis pathway is outlined in Figure 5 where the Δ5 pathway is active in humans whereas the Δ4 is active in rodents.

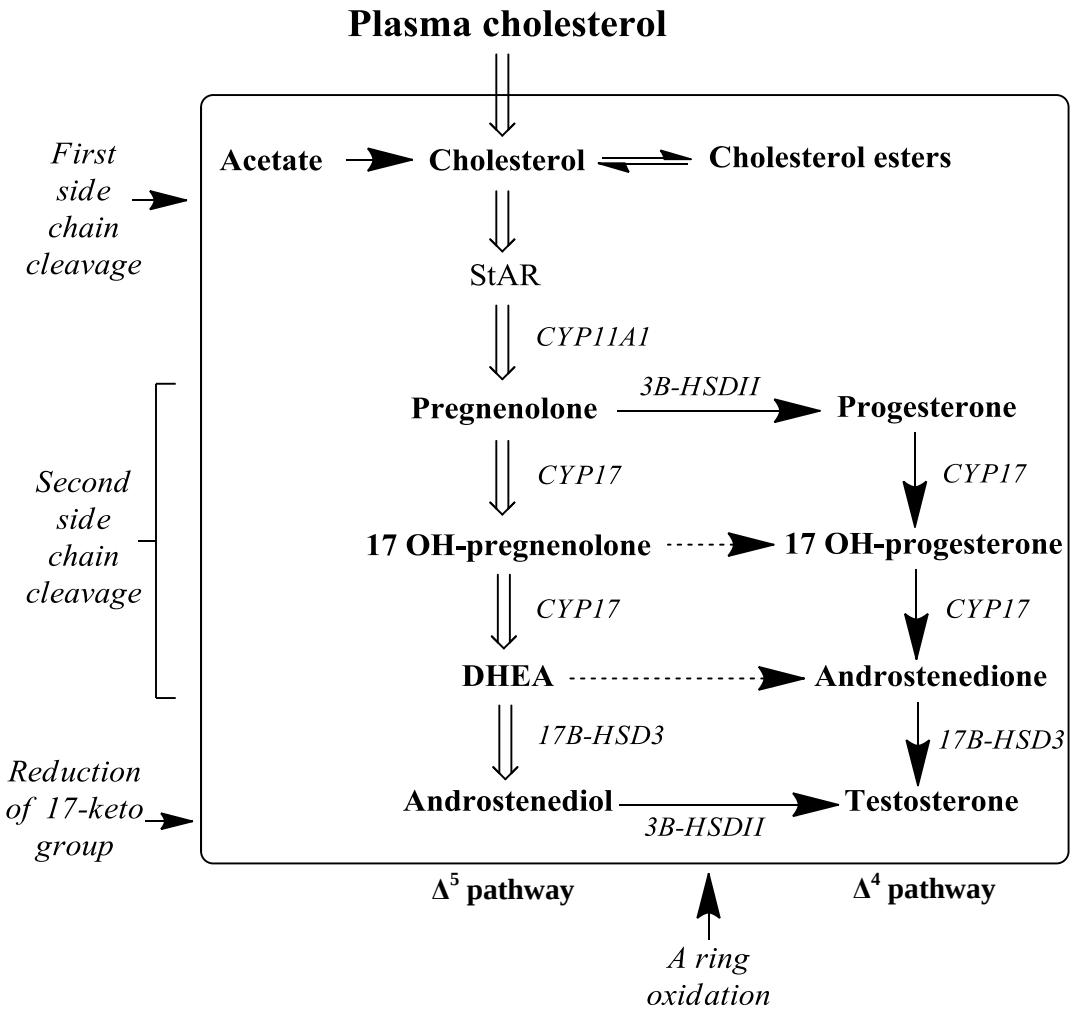


Figure 5: Steroidogenesis pathway adapted from [48]

The endocrine disrupting effects of the phthalate plasticizers stem from the disruption of the steroidogenic pathway, which is believed to be a likely cause behind certain male reproductive tract abnormalities reported in animal models [19, 21, 49]. Acute steroidogenesis was studied in the present study in mouse Leydig cells that necessitated stimulation by the human gonadotropin hormone (hCG) to initiate steroidogenic response [47].

3.2. "Green" Plasticizers

Due to the documented environmental and health effects of commercial plasticizers, new "green" plasticizers have been sought. An ideal plasticizer is characterized as highly compatible with polymers, stable in environmental temperature ranges, resistant to ultraviolet radiation, impervious to leaching and migration and cheap [5]. "Green" plasticizers must also be nontoxic and readily biodegradable to prevent accumulation in soil and environment in general [50]. The plastics' industry has put great effort in developing safer alternatives, each offering different advantages. However, the toxicological background for most has not been verified. As an example, the candidate Citroflex B-6® produced by the company Morflex, Inc. proved to exert the same exemplary properties of DEHP in terms of rendering flexibility to polymers [51] and exhibiting valuable characteristics such as their considerable low leaching rate [9], high efficiency and good solvating power for PVC and cellulose acetate [5]. Though at first, this replacement seemed to be a less toxic alternative, it was deemed later to bring forth respiratory, nervous and cardiovascular system troubles [52] and appeared to have leaching rates higher than DEHP [53].

Other promising alternatives include Mesamoll® from Bayer which has a reduced tendency to leach out [7], Benzoflex® from Eastman Chemical Company which has been found to yield low toxicity and rapid biodegradation and Hexamoll® DINCH from BASF [6]. Moreover, there are the fully biodegradable isosorbide diesters and epoxidized vegetable oils which are also examined as possible alternatives. In general, the substitutes to commercial plasticizers are uncommon and their uses are limited as they display a lack of durability or workability or are just high priced compared to conventional plasticizers.

3.2.1. Significance of Ether Functions

The benzoates category displays main characteristics of high solvating, low moisture sensitivity, excellent resistance to organic extraction, excellent stain and UV resistance and desirable environmental, health and safety profiles [5]. As a matter of fact, the commercial dibenzoates namely diethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB) shown in Figure 6 have been promoted for their less toxic nature.

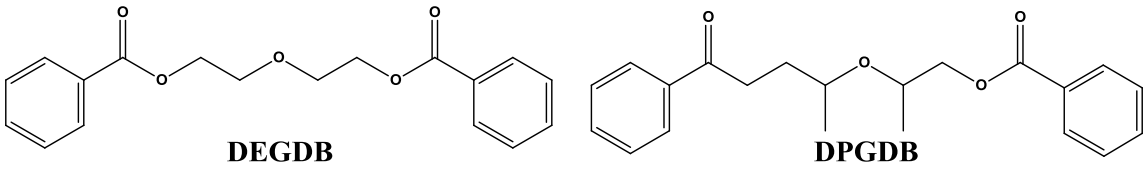


Figure 6: Structures of diethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB)

Conversely, in recent studies it was found that through microbial hydrolysis by common soil micro-organisms such as *Rhodococcus rhodochrous* and *Rhodotorula rubra*, D(EG)DB and D(PG)DB degrade to a monobenzoate metabolite that was demonstrated to be acutely toxic as measured using the Microtox^{TM} toxicity assay [25, 54]. A proposed mechanism of the bacterial biodegradation of D(EG)DB and D(PG)DB is presented in *Appendix I*. Biodegradation was triggered by hydrolysis of one ester bond liberating a monobenzoate and a benzoic acid. It was proven that the ether function present in the diols resisted the further biological transformation of the monobenzoate, consequently inducing its toxic accumulation. The underlying cause behind the blocking of further degradation of the ether linkage is based on the high energy bond of C-O relative to a C-C link [55].

3.2.2. Dibenzoate Plasticizers

In the McGill laboratories, the chemical structures of the synthesized potential replacement plasticizers were derived from the configuration of plasticizers currently on the market with alterations to improve their structure from an environmental perspective [25, 56] yet still maintaining their plasticizing properties [24, 57]. They have been designed with several replacement structures to encompass a variety of different potential plasticizers and to screen the best alternatives.

In particular, the aforementioned findings concerning the ether functional group hindering biodegradation persuaded McGill researchers to come forward with a plasticizer similar to D(EG)DB though void of the ether bond, the 1,5-pentanediol dibenzoate (C5). In fact, it was demonstrated that the C5 biodegradation of the diol fragment of its corresponding monobenzoate proceeded further down the degradation steps to be processed via the β-oxidation pathway unlike D(EG)DB in bacterial systems. In addition, the plasticizing properties of C5 were verified and deemed satisfactory. The glass transition temperature and tensile strength of PVC plasticized with C5 were comparable to those of PVC plasticized with D(EG)DB, (D(PG)DB) and DEHP [24, 57]. Hence, C5 was retained as a potential alternative to commercial dibenzoates plasticizers.

Following the same concept, plasticizers of the same structure were synthesized with varying alkyl chain lengths: 1,3-propanediol dibenzoate (C3), 1,4-butanediol dibenzoate (C4) and 1,6-hexanediol dibenzoate (C6), presented in Figure 7.

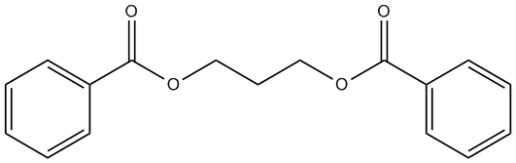
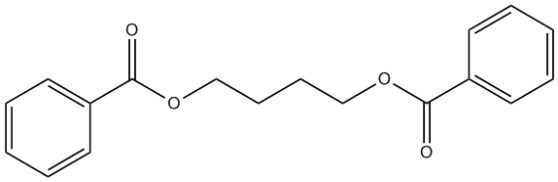
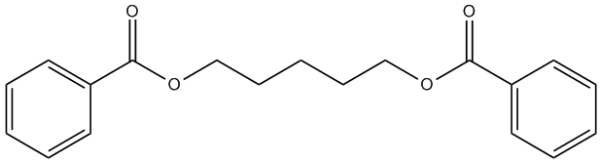
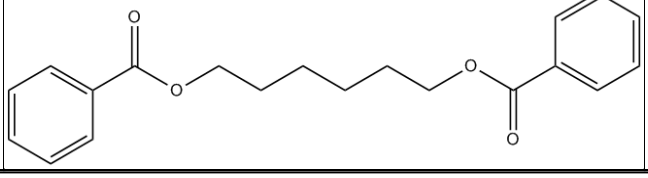
Compound Name	Chemical Structure	Molecular Weight [g/mol]
1,3-propanediol dibenzoate (C3)		284.31
1,4-butanediol dibenzoate (C4)		298.33
1,5-pentanediol dibenzoate (C5)		312.36
1,6-hexanediol dibenzoate (C6)		326.39

Figure 7: Structures of the synthesized dibenzoate plasticizers proposed as "green" plasticizers

The synthesis of these three candidate "green" plasticizers (C3, C4 and C6) follows the same mechanisms as that of C5 depicted in Figure 8 but with their corresponding diol reactants (1,3-propanediol, 1,4-butanediol and 1,6-hexanediol respectively). To not repeat previously made mistakes of introducing to the market plasticizers potentially causing harm to human health and environment, it is of critical importance to assess their toxicity beforehand. In fact, the biodegradation of C3 and C6 has also been investigated in bacterial cell cultures [25, 27] and shown to quickly reduce further down the metabolic pathway, circumventing the accumulation of their monoesters. Their proposed mechanisms of biodegradation are represented in *Appendix II* and *III*.

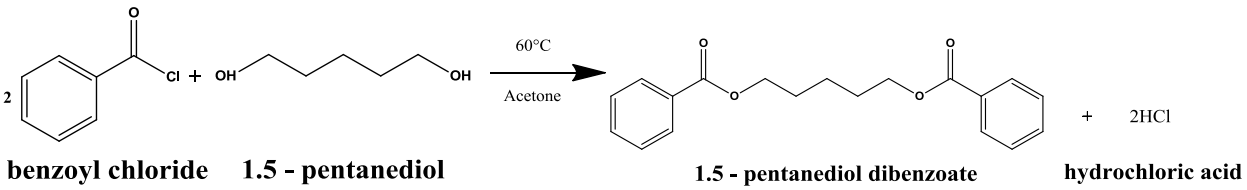


Figure 8: Synthesis of 1,5-pentanediol dibenzoate (C5)

3.3. Use of Solvents

The benefits of screening chemical entities for their toxic potencies through *in vitro* testing [58] is highly valuable. However, there is concern of its low sensitivity, and consequent inability to accurately predict behavior in *in vivo* systems. This common issue arises from a reduced bioavailability of chemicals in *in vitro* experiments [59]. Bioavailability relies on proper dissolution and distribution of the sample compound in the system and is influenced by several factors ranging from procedural steps such as dosing [60] to storage of the sample solutions and constituents in the media [59]. The matter of test sensitivity is even more significant in the case of often encountered poorly water soluble compounds. As a result, low solubility of these entities affects bioassays by causing underestimated activity, variable data, discrepancies between enzyme and cell assays and inaccurate *in vitro* testing [61]. This underlines the strong correlation between solubility and bioavailability, and hence, toxicity assessment.

Complex media additives, namely serum, have the potential to dissolve certain insoluble compounds in aqueous solutions [62] though they are not always of sufficient aid. The common procedure to deliver water insoluble test compounds to the studied culture is by the means of organic solvents. Careful review must be applied in selecting a suitable solvent; consideration must be given regarding the test organism as well as the nature of the compounds being tested. Numerous solvents such as ethanol and methanol are used in *in vitro* cultures. Dimethyl sulfoxide (DMSO) is the most frequently employed solvent to increase solubility in aqueous buffers for toxicity testing [63-65]. DMSO is a highly polar, stable, hygroscopic organic liquid and is highly regarded for its outstanding solvent properties. Miscible with water, lipoids, and organic solvents, it can solubilize a vast array of inorganic and organic chemicals [66-68]. Unfortunately, the supplementary solvent entails the probable introduction of additional toxicity.

In fact, studies done on DMSO report its toxic interaction with cells interfering with the metabolism and membrane of cells, thereby injuring the cell [69-71], interrupting their functionality [72] and proper growth [73], down regulating the expression of their genes [74] and completely inhibiting steroidal production [75] for certain cell lines. The cytotoxicity assessment of the sample compounds becomes therefore challenging to decipher due to combinatory effects and the influence of the solvent must be at all cost limited. The issue of solvent toxicity is important in cytotoxicity testing of potential "green" plasticizers. These compounds are water insoluble and the longer their alkyl chain length is, the more hydrophobic they are and the more challenging it becomes to bring them into solution. DMSO was chosen as a vehicle as it was a proven organic solvent that was effective for dissolving plasticizers.

Solvent concentration is therefore crucial to optimize in order to yield data that is significant and adequate for drawing conclusions. Strategies to limit bioassay disturbances in systems with solubility issues have been previously outlined [61] though the effects of the solvent on inaccuracy and variability in the data have not been reported. Furthermore, literature did not provide information concerning recommended solvent concentration ranges for toxicity testing with MA-10 cells and had to be, therefore, identified before proceeding to actual testing.

4. MATERIALS AND METHODS

A mouse Leydig tumour cell line (MA-10 cells) was used to evaluate the toxicity of the green plasticizers. These interstitial cells are heavily involved in the production of testosterone in men, hence are suitable for anti-androgenic effects studies. Moreover, the choice for the MA-10 cell was justified for its well established character and its ability to respond to gonadotropic stimulation. As it is a tumour cell line, the MA-10 cells have not retained their full cell specific capacity thus are not capable of undergoing all the steps involved in testosterone production. Nonetheless, they are capable of carrying out the steps required to produce progesterone. Therefore, to monitor Leydig cell steroidogenesis, progesterone was used as its marker.

4.1. Cell Culture

The mouse Leydig MA-10 cell line was a generous donation on behalf of Dr. Mario Ascoli, via Dr. Jacques J. Tremblay (Université Laval). The cells were grown in Waymouth's MB 752/1 (1X) medium, supplemented with 20 mM HEPES, 15% horse serum and 1% Penicillin-Streptomycin and incubated at $37^{o}C$ with 5% CO_2. To ensure proper adhesion of the cells, flasks and well plates were coated with 0.1% porcine skin gelatine. The media was replenished every two days and the cultures were split 1:3 every 3-4 days. To assess the exposure of the cells to the plasticizers, the MA-10 cells were cultivated in the presence of a determined plasticizer concentration solubilised with DMSO in the media with a defined initial cell count.

4.2. Plasticizer Samples

The candidate plasticizers C3, C4, C5 and C6 were synthesized by Guixin Shi and donated by Dr. Maric's lab (McGill University). To study their toxicity, aqueous solutions with various plasticizer concentrations were prepared, ranging from 10^{-3} to 10^{-7} M in the cell media.

The plasticizers exhibit poor water solubility thus had to be solubilised with the DMSO solvent at a concentration that would not introduce additional toxicity. To account for the solvent's and possibly the media's effects, a blank (only media) and a vehicle control (media with DMSO) were used for all trials.

Initially, the plasticizers were dissolved in DMSO in a concentrated form (stock DMSO solutions) after which a fixed aliquot of each stock solution of DMSO was added in cell media to create solutions containing a desired final solvent and plasticizer concentration (exposure media solutions). For example, to reach a final solvent concentration of 1% in the media within the toxicity assay, stock solutions of different plasticizer concentrations in DMSO were prepared 100-times more concentrated than the desired final media solutions. Afterwards, 10 uL of DMSO stock solution was added to 990 uL of media to obtain a final exposure media solution of 1 mL volume, 1% v/v DMSO and corresponding 100 times less plasticizer concentration. Stock solutions of DMSO were kept for a maximum of four weeks and solutions of media were freshly prepared on the day of treatment each time.

4.3. Cell Viability Assay

In order to determine optimal anti-androgenic testing conditions, cell viability had to be first assessed for cells exposed to the solvent and plasticizers to determine the maximum concentrations that would leave it unaffected. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a compound that when metabolized by living cells reduces to purple formazan crystals that could be later solubilised and quantified by spectrophotometry. As such this colorimetric assay was employed to measure the metabolic activity of the cells and permitted the evaluation of cell viability and proliferation when subjected to various concentrations of plasticizers. The experiment was conducted in a 96-well plate where wells

were pre-treated with gelatine and seeded with 5000 MA-10 cells. Controls with the same cell concentration as well as blanks with no cells were also included. A standard curve was established using seven different cell concentrations (1000, 2000, 4000, 5000, 6000, 8000, 10 000 cells/well). Each condition was prepared in triplicate and left to grow overnight in the incubator at 37ºC and 5% CO_2. To dose the compound indirectly [60], the growth media was then aspirated from the sample wells and replaced with the test solutions whereas the vehicle control and blanks (with no cells) were replenished with media containing only 1% v/v DMSO and just media for the standards and blank controls. Each media solution was prepared fresh on the day of the experiment and thoroughly mixed before use with a vortex. The plates were incubated for either 24 hours or 48 hours to analyze both exposure periods.

After incubation with the plasticizers, the medium was removed and phenol red free media with 1.1 mM MTT was added, according to the manufacturer's instructions and the plate was incubated for another 4 hours. Formazan crystals began to form which had to be solubilised and incubated with SDS-HCl detergent for an additional 18 hours. The absorbance of this colored solution could be easily quantified by a Benchmark Plus™ microplate spectrophotometer (Bio-Rad) at a 570 nm wavelength. To assess cell viability in the presence of plasticizers, the samples' absorbance had to be normalized to the vehicle control's absorbance.

4.3.1. Effect of DMSO

To study independently the effect of DMSO on cell viability (as measured using the MTT assay) and assess a suitable working range where the solvent's toxicity would not interfere, the cells seeded at a density of 5000 cells/well in a 96 well plate were treated for 24 and 48 hours with varying levels of DMSO (0.25, 0.5, 0.7, 1, 2, 3 and 4% v/v) against controls solutions consisting of only cell media.

4.3.2. Solvent Concentration Screening Test

The DMSO concentration screening test was used to determine the optimal solvent concentration that would minimize the solvent's impact on cell viability (as measured using the MTT assay) and results variability. Cells seeded at a density of 5000 cells/well in a 96 well plate were treated for 24 and 48 hours with media containing different plasticizer concentrations ($1x10^{-2}$, $1x10^{-3}$, $1x10^{-4}$, $1x10^{-5}$ and $1x10^{-6}$ M), dissolved at various DMSO concentrations (0, 0.25, 0.5, 0.7, 1, 2, 3 and 4% v/v) where the vehicle controls contained DMSO only of corresponding concentrations (0, 0.25, 0.5, 0.7, 1, 2, 3 and 4% v/v) in cell media.

4.3.3. Effect of Plasticizers

To assess the effect of the plasticizers on cell viability, MA-10 cells seeded at a density of 5000 cells/well in a 96 well plate were treated for 24 and 48 hours with media constituted of different plasticizer concentrations ($1x10^{-3}$, $1x10^{-4}$, $1x10^{-5}$, $1x10^{-6}$ M) dissolved at 1% v/v DMSO and the vehicle controls consisted of 1% v/v DMSO in cell media.

4.4.Effect on Steroidogenesis

In order to monitor the effects of the plasticizers on steroidogenesis, the testosterone production was studied using an indirect method. As aforementioned, the MA-10 Leydig cells are deprived of their testosterone producing specialized enzymes and halt at the progesterone production (section 3.1.4.1), therefore progesterone was monitored. This experiment was performed in 6-well plates seeded with a density of 200 000 cells per well. After a day of incubation, the growth media was changed to expose the cells for 24 hours to various concentrations of plasticizers, taking note that the vehicular controls had their media changed to one containing 1% v/v DMSO and no plasticizers. At the end of the exposure period, the media was removed, the cells were rinsed with phosphate buffered saline (PBS) and then subjected to

an acute stimulation with human chorionic gonadotropin (hCG) (hCG, 0.5 nM, ≥12 000 IU/mg, Calbiochem, 869031) (except for hCG controls) to trigger their progesterone production for 4 hours. The media of each condition was collected and stored at -20 ºC for progesterone analysis. The wells were washed twice with PBS after which the cells were lysed with a RIPA buffer to release their protein contents and stored at -80 ºC till analysed for total protein content.

To measure the amount of progesterone produced in the presence or absence (controls) of plasticizers, the enzyme-linked immunosorbent assay (ELISA, Fitzgerald Industries International, Inc., 55RRE52231) kit was used for the collected supernatant samples. The assay was performed following the protocol provided by the manufacturer. The progesterone is first immobilized on a solid support, a polystyrene microtiter plate, then an enzyme conjugate solution provided by the manufacturer is added which, like an antibody, forms a complex with the hormone. When fed the enzyme substrate solution, the complex changes color measurable by spectrophotometry (Benchmark Plus™ microplate spectrophotometer (Bio-Rad)) at a 450 nm wavelength. It should be noted that the plate was rinsed with a wash solution to remove any hormones not specifically bound. The measured absorbance was calibrated to the progesterone concentration of the sample via the standard curve fitted through a 4 parameter logistic (4PL) curve. Each replicate was verified for its stimulation factor corresponding to the amount of progesterone produced by cells that were stimulated by hCG compared to cells that were not (hCG controls).

Since the density of cells possibly varied between replicates, the amount of progesterone produced has to be normalized to total protein. The bicinchoninic acid protein assay (BCA™) kit (Thermo Scientific, 23227) was employed to quantify the amount of protein per well from the cell lysis samples. This assay is built around the biuret reaction where the protein's peptide

bonds reduce cupric sulphate Cu^{2+} ions (from the added highly alkaline BCA stock solution) to Cu^{1+}. The yielded Cu1+ is proportional to the amount of protein present in the solution and is detected through the formation of a purple color product resulting from the chelation between the Cu+1 ion and two bicinchoninic acid molecules. The color change was then quantified by spectrophotometry (Benchmark Plus™ microplate spectrophotometer (Bio-Rad)), at a 562 nm wavelength. For this experiment, benzonaze was added to the cell lysis samples to break apart protein clumps and was conducted as prescribed by the manufacturer. A standard curve was also built to relate the amount of proteins detected to the corresponding cell population.

4.5.Statistical Analysis

All experiments were performed in triplicates and the results of a minimum of three independent experiments are presented with error bars representing the standard error of the mean. Two-way analysis of variance (ANOVA) tests, followed by Bonferroni post-tests for multiple comparisons were used to assess statistical differences. Statistical analysis was done using GraphPad Prism 5.0 software (San Diego, California). Values were considered as statistically significantly different when $P < 0.05$.

5. RESULTS

5.1.Effect of DMSO

5.1.1. Cell Viability

The effect of DMSO on the viability of MA-10 cells was tested for an exposure period of 24 h and 48 h using the MTT assay. The results for both time points are reported in Figure 9 which displays the viability of cells exposed to varying levels of DMSO, normalized to the media control. As expected, there was an increase in cell death with increasing DMSO concentration except at the lowest points where a slight increase in cell population was observed. The results for the 48 h exposure time depicted the same behaviour as the 24 h exposure time, with an emphasized cell number decrease over the range of DMSO tested. A one-way ANOVA (repeated measures) of the 24 h time point results showed a significant effect of the solvent ($P<0.0001$); cells exposed to 4% v/v DMSO were significantly less viable than the control (Bonferroni post-test, $P<0.001$) and very close to being significantly different at 3% v/v DMSO. At 48h, the cells were significantly less viable than the control starting from 2% v/v (Bonferroni post-test, $P<0.01$) and beyond. Therefore, a concentration range between 0% and 2% v/v of DMSO was used to dissolve the plasticizers because it maintained an appropriate cell count with no apparent cytotoxicity from the solvent for an exposure time of 24 h. In addition, due to the greater cell death observed at the 48 h time point, the 24 h exposure period appeared more fitting for further testing.

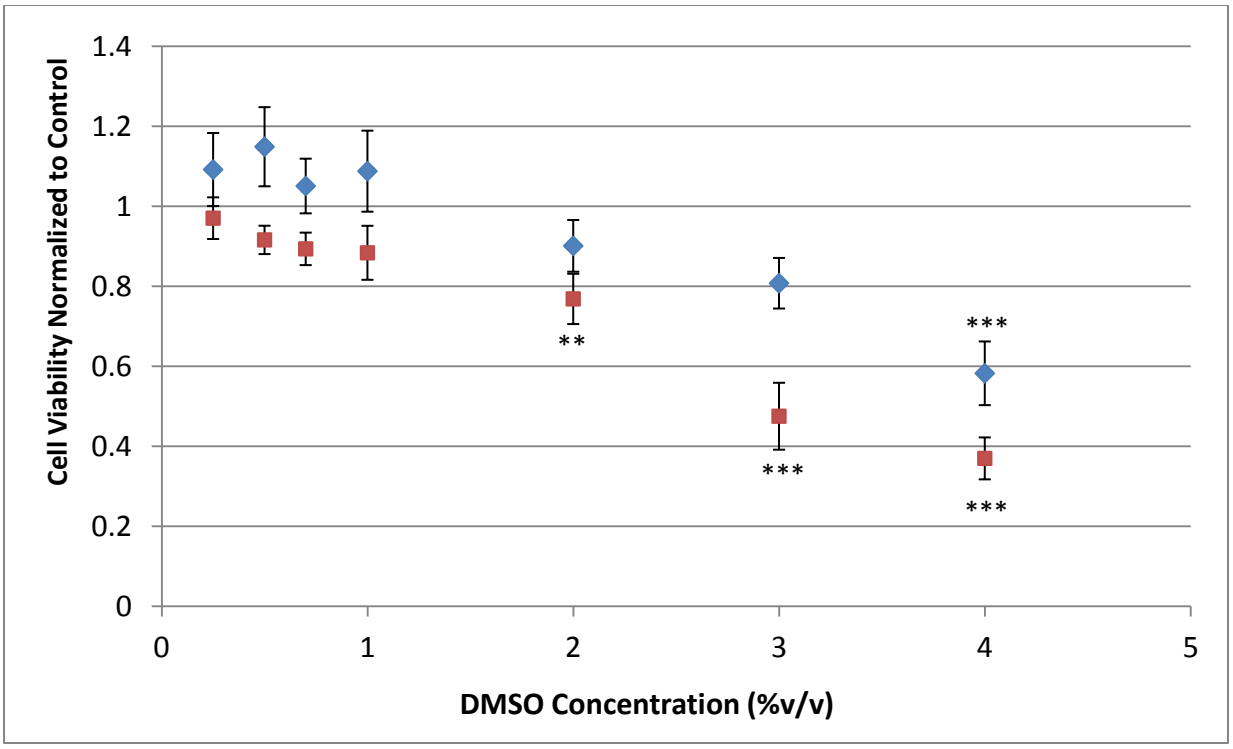


Figure 9: Effect of DMSO on viability of MA-10 cells normalized to control as a function of DMSO concentration (24 h ◆; 48 h ■) (n=6, error bar: standard errors, one-way repeated measures ANOVA Asterix indicate significant difference from control; **P<0.01, ***P<0.001, Bonferroni post-test).

5.1.2. Data Variability

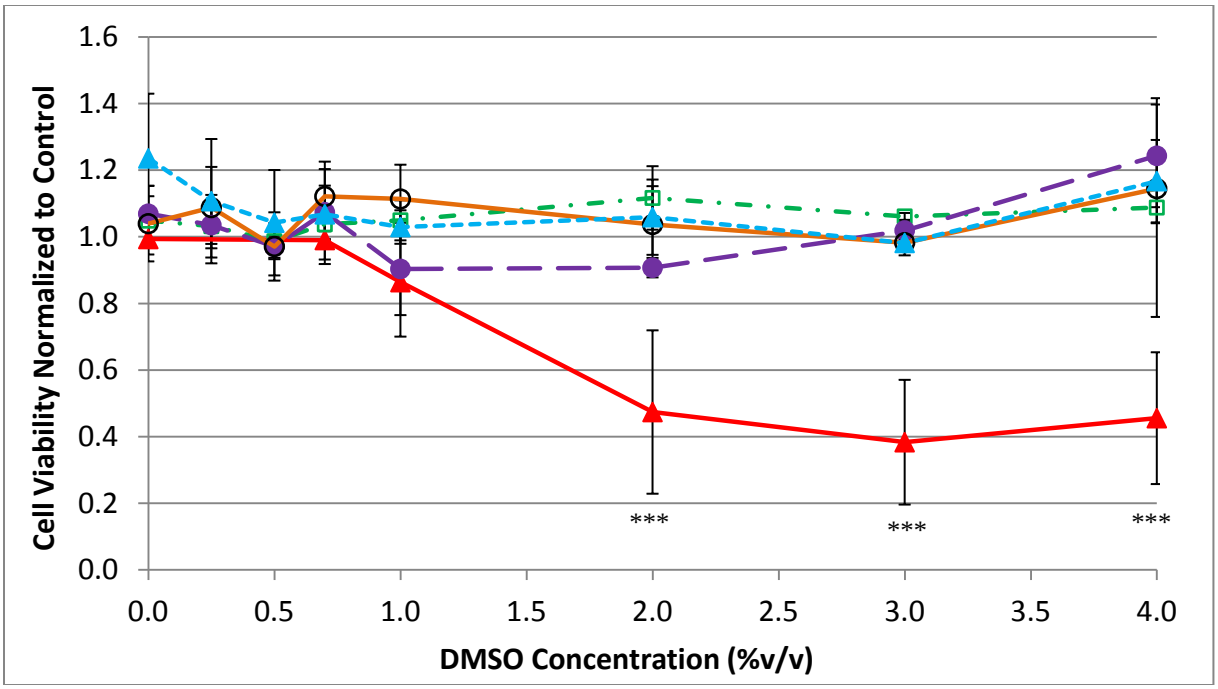
The level of DMSO needed to be sufficient enough to solubilise the plasticizer but not harm the cells. The ability to make the plasticizer available to the cells was tested by the variability in toxicity studies at different concentrations of DMSO. MTT assays were conducted on MA-10 cells exposed to C3 and C5 plasticizers with varying amounts of DMSO for 24 h. The results are reported in Figure 10 and Figure 11 as a function of increasing DMSO concentration, respectively for each plasticizer. The data presented in figures (a) represent MA-10 cell viability that have been normalized to their respective controls, which correspond to solutions with the same DMSO concentration but void of plasticizer and figures (b) report the corresponding coefficient of variation (standard deviation over average in percentage values) of the data.

A two-way ANOVA (repeated measures) showed that the concentration of the C3 plasticizer ($P<0.0001$) had a significant effect on cell viability but not the solvent. However, there was significant interaction of the data at $P<0.01$, making it difficult to decouple between the plasticizer and DMSO effect. A post-test showed that the plasticizer C3 had a significant effect on cell viability only at its highest concentration of $1x10^{-2}$ M from 2% v/v DMSO and beyond relative to the other DMSO points for a same plasticizer concentration. Figure 10*(b)* shows that there is no clear trend observed in the coefficient of variations, although there is an increase of variability towards the highest values of DMSO concentration for the highest plasticizer concentration of $1x10^{-2}$ M.

Figure 11(a) shows that C5, similar to the C3, the effect of the plasticizer was significant but not of the solvent. At 0% DMSO, the results of different plasticizer concentrations were significantly different from one another. The variability profile for C5 showed increasing

variation coefficients at lower and higher DMSO concentration, with a minimum of variability at DMSO concentrations around 2% v/v.

(a)



(b)

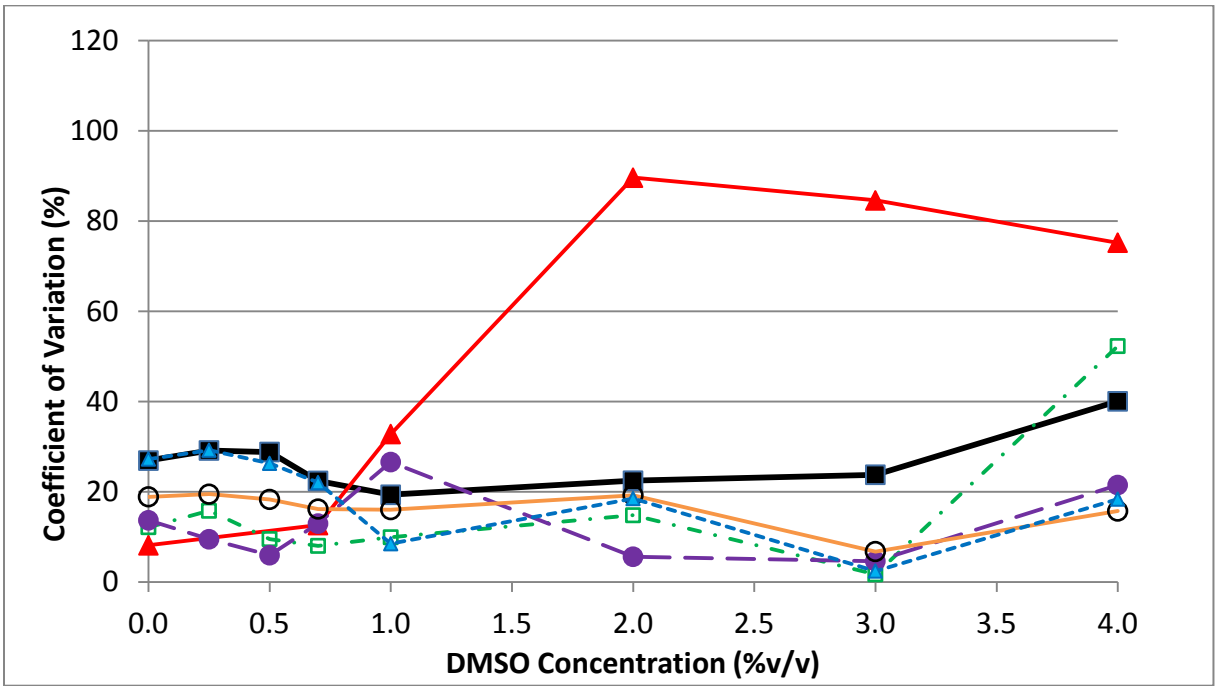
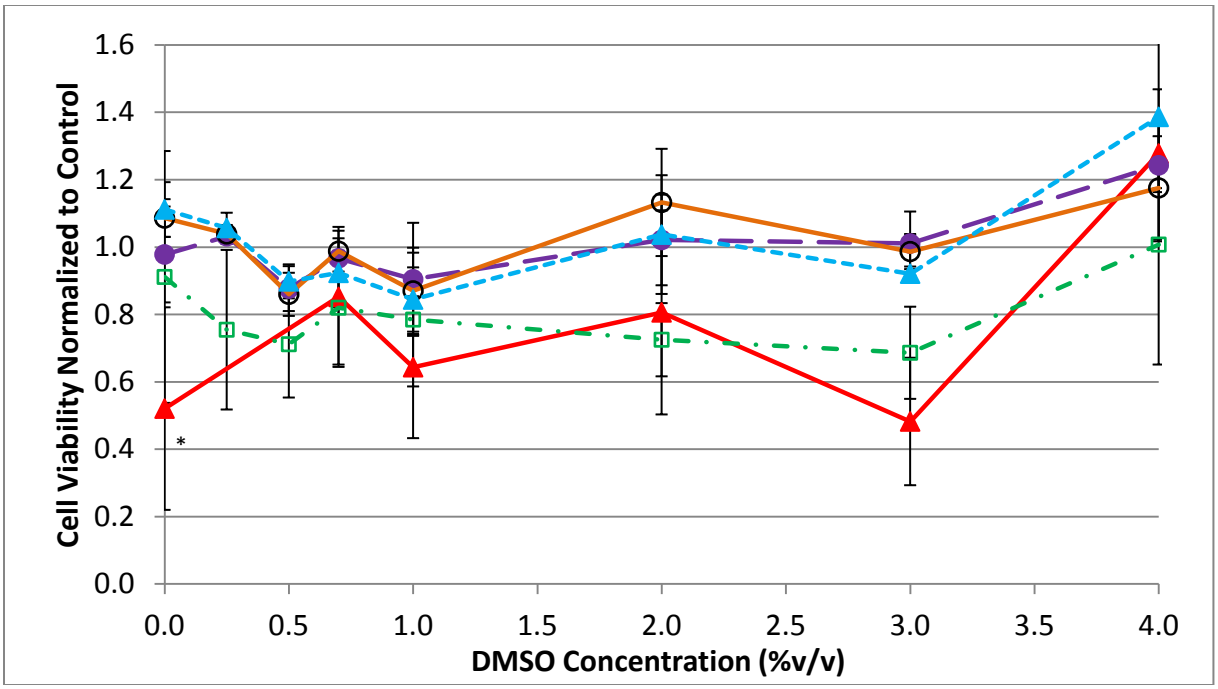


Figure 10: *(a)* Cell viability measured by MTT assay of MA-10 cells exposed for 24 hours to various concentrations of DMSO (0 to 4% v/v) and different concentrations of 1,3-propanediol dibenzoate (C3) (0M —■—; $1x10^{-2}$M —▲—; $1x10^{-3}$M - -□- - ; $1x10^{-4}$M -●-; $1x10^{-5}$M —○—; $1x10^{-6}$ M ···▲···) (n=3, error bars: two-way ANOVA (repeated measures), asterix indicate significant difference; *P<0.05, ***P<0.001, Bonferroni post-test).and *(b)* corresponding coefficient of variation (standard deviation/average*100%).

(a)



(b)

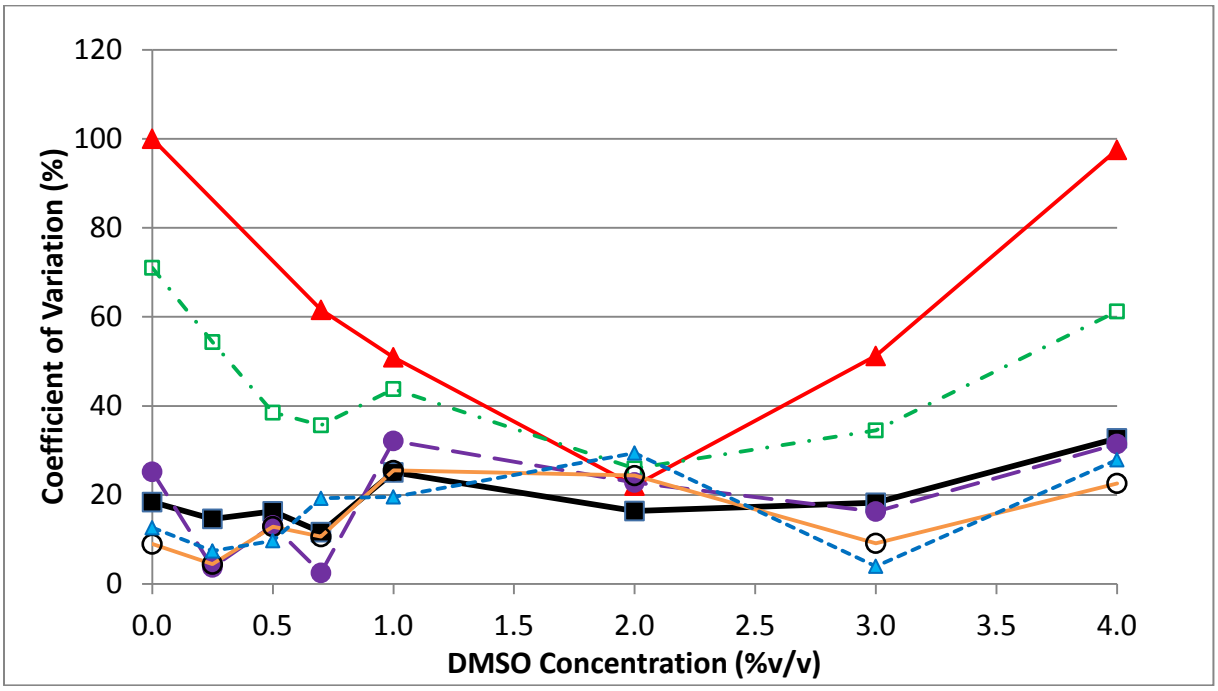


Figure 11: *(a)* Cell viability measured by MTT assay of MA-10 cells exposed for 24 hours to various concentrations of DMSO (0 to 4% v/v) and different concentrations of 1,5-pentanediol dibenzoate (C5) (0M —■—; $1x10^{-2}$M —▲—; $1x10^{-3}$M - -□- - ; $1x10^{-4}$M -●-; $1x10^{-5}$M —○—; $1x10^{-6}$ M ···▲···) (n=3, error bars: two-way ANOVA (repeated measures), asterix indicate significant difference; *P<0.05, ***P<0.001, Bonferroni post-test).and *(b)* corresponding coefficient of variation (standard deviation/average*100%).

5.2.Effect of Plasticizers

5.2.1. Cell Viability – 24 h

MA-10 cells were exposed to DEHP, MEHP, D(EG)DB and the synthesized dibenzoate plasticizer series for a period of 24 h after which their viability was assessed using an MTT assay. Figure 12 presents the cell viability of MA-10 cells subjected to concentrations varying from $1x10^{-3}$M to $1x10^{-6}$ M of the test compounds dissolved in media with 1% v/v DMSO (a concentration that did not cause toxicity but was sufficient to solubilize the plasticizers, section 5.1), normalized to the vehicle control (media with 1% v/v DMSO only). Five replicates were conducted. The effect of the solvent was not significant (P=0.1537) with a t-test (paired) performed on the cell viability of cells exposed to just media and media with 1% v/v DMSO.

A two-way ANOVA (repeated measures) indicated that neither the compound (P=0.1413) nor the concentration (P=0.2291) exhibited a significant effect on cell viability. To examine the effect of concentration for each compound separately, a one-way ANOVA (repeated measures) was performed between the concentrations for each compound individually. A significant effect (Bonferroni post-test, P<0.01) was detected for C5 at its highest concentration of $1x10^{-3}$ M.

5.2.2. Cell Viability – 48h

The same experiment as outlined in section 5.2.1 was carried out for an exposure period of 48 h for which the results are reported in Figure 13 summarizing the data of five replicates. In this case, the t-test (paired) showed that the cell viability of cells exposed to media was different than that of cells exposed to media with 1% v/v DMSO for 48h, thus the effect of the solvent was statistically significant (P=0.0233).

A two-way ANOVA (repeated measures) showed that the effect of the compounds and concentration on cell viability were significant ($P=0.0324$; $P<0.0001$, respectively). The compounds MEHP, C3 and C5 significantly reduced cell viability when added to the media in their most concentrated form ($1x10^{-3}$ M) reducing the cell population by up to 40%. Moreover, the effect of C5 was significantly different than that of DEHP at $1x10^{-3}$ M. The dibenzoates' effects were not significantly different than that of MEHP's nor D(EG)DB's.

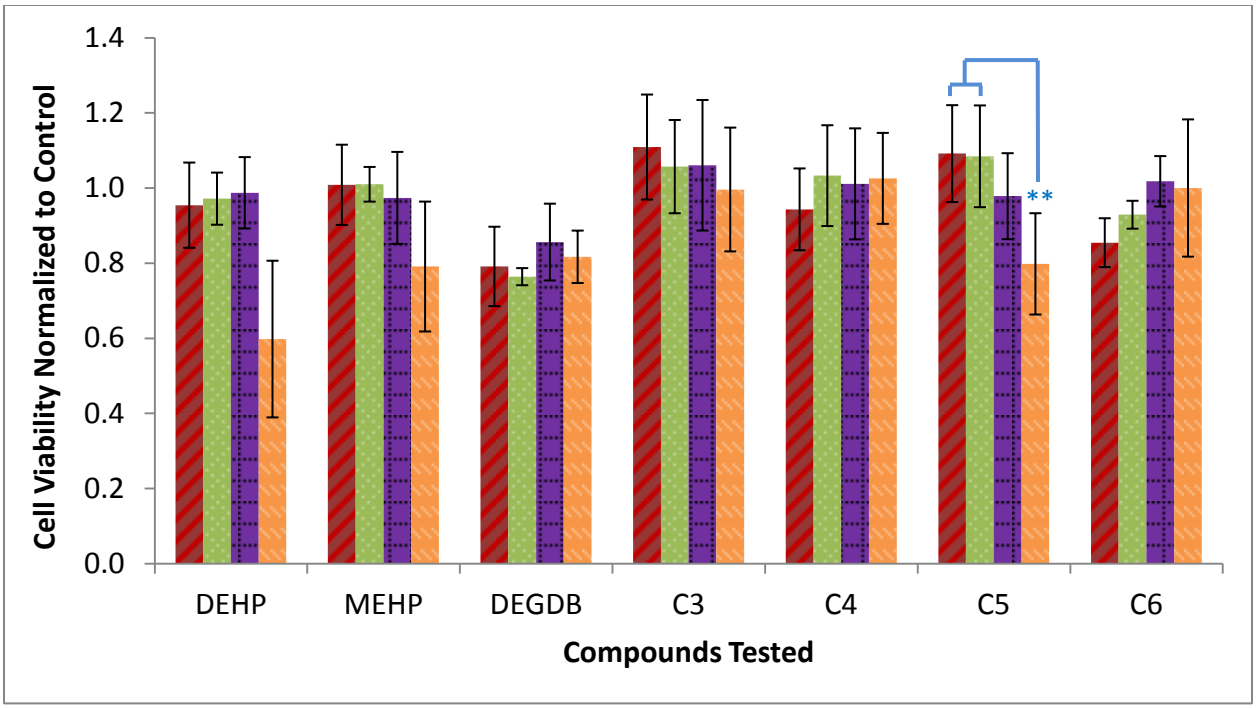


Figure 12: Cell viability of MA-10 cells exposed for 24 hours to various concentrations of plasticizers (0M ■; $1x10^{-6}$M ■; $1x10^{-5}$M ■ $1x10^{-4}$M ■ $1x10^{-3}$M ■) (n=5, error bars: one-way ANOVA (repeated measures), asterix indicate significant difference: **P<0.01, Bonferroni post-test).

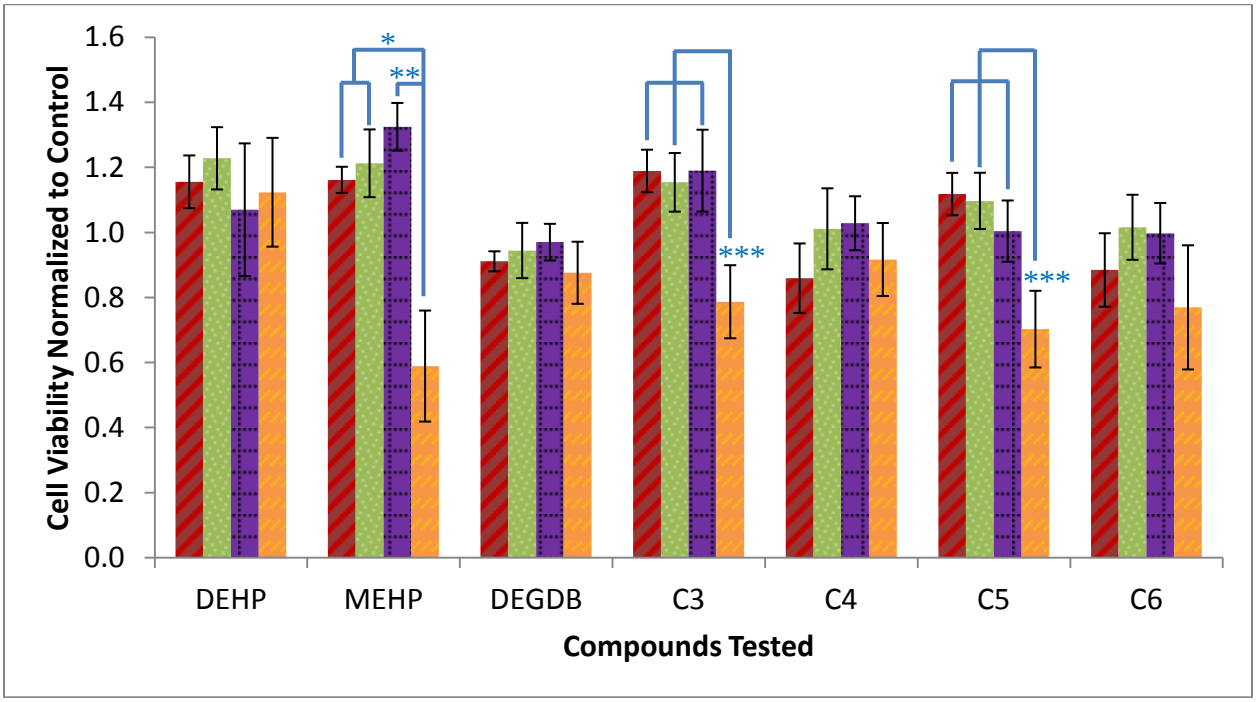


Figure 13: Cell viability of MA-10 cells exposed for 48 hours to various concentrations of plasticizers (0M ■; $1x10^{-6}$M ■; $1x10^{-5}$M ■ $1x10^{-4}$M ■ $1x10^{-3}$M ■) (n=5, error bars: one-way ANOVA (repeated measures), asterix indicate significant difference: *P<0.05, **P<0.01, ***P<0.001, Bonferroni post-test).

5.3.Effect on Steroidogenesis

To assess the steroidogenesis disrupting potential of the compounds, the expression profile for progesterone production was evaluated after the MA-10 cells were exposed to DEHP, MEHP, D(EG)DB and the synthesized dibenzoate plasticizer series for a period of 24 h. The amount of progesterone produced and released into the media by the cells when stimulated with hCG was quantified by an ELISA assay. The measured progesterone concentration was normalized to the total protein, which was evaluated using a Bicinchoninic Acid Protein (BCA) assay, in order to consider cell count variations between experiments. The value was in turn normalized to the control (media with 1% v/v DMSO) to account for discrepancies between ELISA assay kits. The results are reported in Figure 14 for MA-10 cells subjected to $1x10^{-4}$ M to $1x10^{-7}$ M of compounds dissolved in media with 1% v/v DMSO. The time frame of the experiment and selected concentrations were based on previously collected results (sections 5.1 and 5.2) to ensure consistent cell viability throughout experiment. Four replicates were performed and the effect of the solvent was statistically shown not to be significant (P=0.9162) with a t-test (paired) performed on the progesterone production of cells exposed to just media and media with 1% v/v DMSO. Replicates were verified for their stimulation factors and all ranged around five times the normalized amount of progesterone produced by stimulated cells versus the non-stimulated cells.

A two-way ANOVA (repeated measures) showed that the effect of the compounds and their concentration on progesterone production were significant (P=0.0002; P<0.0001 respectively). However, there was also significant interaction (P=0.0012) of the factors (nature of compound and concentration). Using a one-way ANOVA, it was shown that the compounds DEHP, C3, C5 and C6 significantly reduced the progesterone production of the cells exposed to

these plasticizers at their highest concentration ($1x10^{-4}$ M), in Figure 14. Compared to the control, DEHP reduced progesterone levels the most, by 55%, followed by C3 and C5 which caused a reduction of about 30%. The effects of C4 and C6 were significantly different than that of DEHP at $1x10^{-4}$ M (Bonferroni post-test, P<0.001, P<0.01 respectively). The effect of C6 at $1x10^{-4}$ M was significantly different from its $1x10^{-5}$ M and $1x10^{-6}$ M concentrations effects (Bonferroni post-test, P<0.01, P<0.05 respectively). Furthermore, MEHP caused an apparent but not significant 20% progesterone reduction.

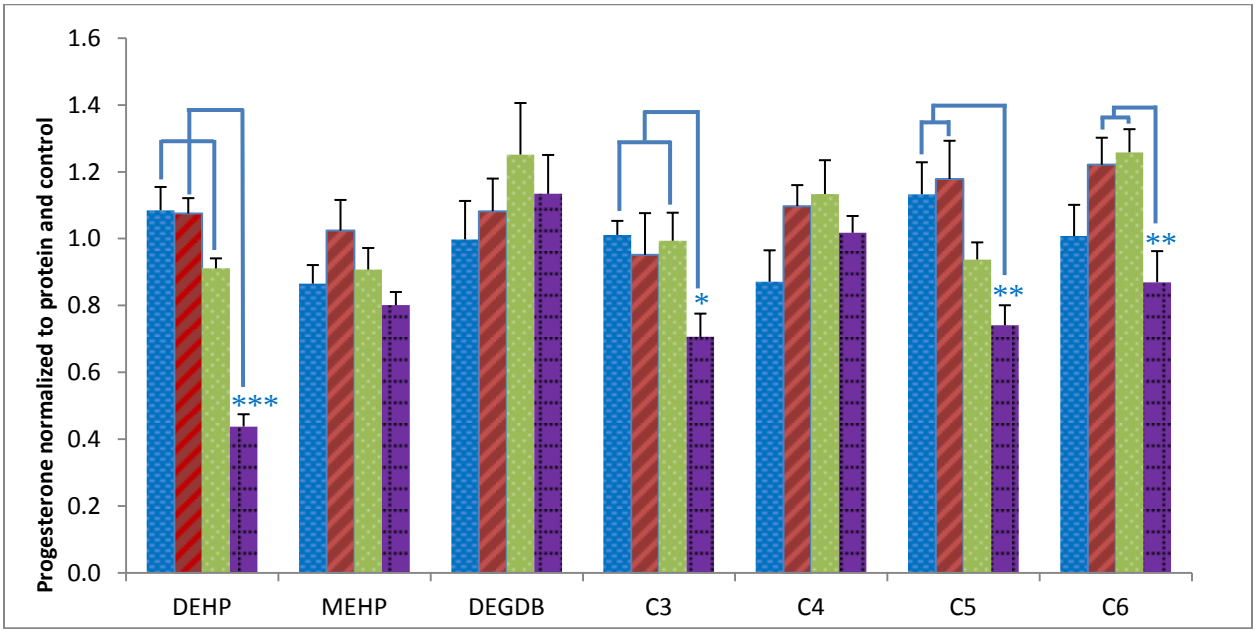


Figure 14: Progesterone production of MA-10 cells exposed for 24 hours to various concentrations of plasticizers (0M ■; 1x10-7 ■; $1x10^{-6}$M ■; $1x10^{-5}$M ■ $1x10^{-4}$M ■ (n=4, error bars: one-way ANOVA (repeated measures), standard errors. Asterix indicate significant difference; *P<0.05, **P<0.01, ***P<0.001, Bonferroni post-test).

6. DISCUSSION

Animal and *in vitro* studies have shown the toxic effects commercial plasticizers can exhibit and have motivated the development of new non-toxic plasticizers. Bearing this incentive in mind, a series of dibenzoate compounds has been synthesized by our group in an effort to meet this new market's demand. Clearly, a spectrum of studies must be carried out outlining the properties, abilities and effects of the newly synthesized compounds before labelling them as "green" plasticizers. Part of this analysis entailed the assessment of their acute toxicity to mammalian cells and their anti-androgenic potential, which was the objective of this study. Optimal solvent and plasticizer concentrations and time frame conditions were developed to ensure cell viability of the cells for the duration of the experiments.

6.1.Effect of DMSO

The use of a solvent in the *in vitro* testing of the dibenzoate plasticizers was required to solubilize the compounds and therefore enhance their bioavailability to the cells. While ensuring the availability of the test compounds to the cells was important, maintaining the cytoxicity of the vehicle to a minimum was as crucial in order to obtain consistent and meaningful data. Both these factors had to be accounted for and therefore, an optimal parameter between bioavailability and cytoxicity was screened for prior to testing for anti-androgenic activity.

The toxicity of DMSO on the MA-10 cells was tested on cell viability and a concentration of up to 3% v/v was reported to maintain a consistent cell population for a 24 h timeframe, while for a longer exposure time to DMSO of 48h, a tighter range of concentration with a maximum of 2% v/v maintained a consistent cell count (Figure 9). The increase in cell number at the lowest tested DMSO concentrations for 24 h may be attributed to the membrane

destabilization and permeability enhancing nature of DMSO which promotes the diffusion of various solutes across the cell membrane. It is believed DMSO achieves this by decreasing the lipid bilayer thickness and reducing the activation energy of passive diffusion of solutes through cell membranes [11].

The delimited ranges of DMSO concentrations maintaining a stable cell population offer some insight into how the MA-10 cells are affected by DMSO and suggest appropriate working ranges of solvent concentration though give no indication of the quantity of DMSO required to provide adequate bioavailability of the test compound to the organisms. Furthermore, the information is not sufficient to completely describe how cytotoxicity tests of particular compounds will actually respond when coupled with DMSO, as to their cumulative or not cumulative toxic nature.

To assess the combined effects of DMSO and plasticizer concentration on the repeatability of the results, two of the dibenzoates (C3 and C5) were examined at various concentrations ($1x10^{-6}$ M - $1x10^{-2}$ M) when prepared in solutions of different levels of DMSO (0 - 4%). The results of C3 (Figure 10a) showed that the concentration of the plasticizer affected cell viability; C3 at $1x10^{-2}$ M concentration exhibited more toxicity than the other concentrations though a statistically different effect wasn't observed at the lower C3 concentrations. However, due to a statistical interaction of the data uncovered by a 2 way ANOVA test between the plasticizer and the DMSO concentration, it is hard to attribute the drastic drop in cell viability of the $1x10^{-2}$ M C3 exclusively to the effect of C3. In the case of the C5 system (Figure 11a), the effect of plasticizer was noticeable earlier on, starting at lower DMSO concentrations for the $1x10^{-2}$ M and $1x10^{-3}$ M plasticizer concentrations. The underlying cause of this emphasized dose-response curve may be attributed to the liquid nature of C5 causing a more uniform distribution

of the plasticizer which remains in small droplets in the media solution when not adequately dissolved as opposed to the solid C3 which aggregates in solid masses when proper solubilisation has not been achieved.

Screening for an adequate solvent concentration requires minimum interference of the solvent. Therefore, all variability emerging from the solvent's interference with the system must be maintained at a minimum. A test chemical that has not been fully dissolved by the solvent will lead to varying degrees of bioavailability due to its non-homogeneous distribution and generate inconsistent apparent toxicity results. At low DMSO concentrations, the toxicity of the C3 plasticizer was minimal with little variability. The C3 plasticizer is the shortest amongst the plasticizers tested with its alkyl group consisting of only three carbon groups. This would suggest it is the most soluble in contrast to the plasticizers with longer chain lengths. Nonetheless, this plasticizer was a crystalline solid at room temperature and obviously not soluble at low DMSO concentrations. This is likely why the toxicity and variability (Figure 10 (b)) were limited as it was not made bioavailable to the cells. At concentrations beyond 2% v/v DMSO, the C3 plasticizer was solubilized, and the toxicity of the compound was evident at the highest concentrations. It was also expected to see high variability in the results at higher levels of DMSO as the solvent's toxicity would be greater and lead to a reduced viability of cell population, hence making it harder to interpret the data and decipher between the compound's and solvent's toxicity. This is evident for the highest concentrations of plasticizers where the enhanced toxicity of the compound is being mixed in with that of the solvent's.

The variability profile of the C5 plasticizer, the dibenzoate of longer chain length hence expected to be more hydrophobic, clearly emphasizes the trade-off between compound solubility and solvent toxicity as shown in Figure 11b. The coefficient of variation for C5 is greater at

lower DMSO concentrations, and is improved by the addition of solvent, decreasing to a minimum point as C5 is becoming more and more homogeneous and available in the system thus more consistent data is generated. As the solvent continues to contribute to an increase in toxicity with its increasing concentration, the variability in the data is once again increased. An optimal point is therefore pronounced at 2% v/v DMSO, hinting at a favorable concentration of solvent that compromises between solubility and toxicity. A colleague studying a bacterial system used for toxicity testing based on the MicroTox method observed the same trend; as the compound was more hydrophobic, a more evident minimum in data variability was exhibited.

6.1.1. Selected DMSO Concentration

To study more subtle effects such as plasticizer induced anti-androgenic response, a non-toxic level of plasticizer was chosen ($<1x10^{-3}$M). In a previous study by *Piché, et al* [76], a level of 0.3% v/v DMSO concentration was used. Based on our results and to remain close to the previous study, we selected a solvent concentration of 1% v/v DMSO. This was within the minimum variability of the $1x10^{-3}$ M C3 and C5 curves (Figure 11b). This value also considered the delimited safe regions found from the effect of DMSO on cell viability presented in Figure 9.

Even more importantly, when testing the effect of the plasticizers on the MA-10 cells progesterone production, the effect of DMSO was also monitored and it was found that the solvent did not exhibit any effect at 1% v/v concentration when compared to the progesterone production of cells exposed to media only for a period of 24 h. A study conducted on the effect of DMSO on steroidogenesis in MA-10 cells by *Stocco, et al.* [75] also showed that a level of 1% v/v DMSO did not cause an effect on progesterone production, on the cholesterol side-chain cleavage enzyme activity and overall phosphorylation of proteins. *Stocco, et al.* did find that greater DMSO concentrations led to the complete inhibition of hormone-stimulated steroid

production. They had also shown that 5% v/v DMSO, in fact, caused inhibition of the mitochondrial steroidogenic acute regulatory protein. However, it should be noted that the exposure timeframe of their study was of 2 or 6h depending on the test which is indicative of the severity of effects DMSO can have. Also, hormonal stimulation was done at the same time as the exposure to DMSO for their study whereas in this study, the cells were stimulated after being exposed to DMSO, potentially giving them the opportunity to regain their progesterone producing abilities. The MA-10 cells seem to be more sensitive to DMSO exposure comparative to other cell lines such as R2C rat Leydig tumor cell line [75] and Caco2/TC7 colon tumor cell line [77].

6.2.Effect of Plasticizers on Cell Viability

At an exposure period of 24 hours, the commercial plasticizer DEHP and its primary metabolite MEHP (recognized for its toxic effects surpassing those of its parent compound) did not show any significant effect on cell viability of MA-10 cells for concentrations ranging from $1x10^{-6}$M to $1x10^{-3}$M (Figure 12). However, for an extended exposure time of 48 hours, cell viability was reduced by 40% in the presence of $1x10^{-3}$M MEHP (Figure 13). The observed results for DEHP and MEHP are compared to previous studies summarized in Figure 15. In 2011, *Piché, et al*. [76] observed MEHP at $1x10^{-3}$M after an exposure time of 24 h to reduce cell viability by 30%. The effects of MEHP were further accentuated after a 48 h exposure causing a greater decrease in cell viability by 85% for a same concentration and a dose-response curve was developed showing 60% reduction and 40% reduction in cell viability for $1x10^{-3}$M and $1x10^{-4}$M respectively of DEHP. These results, in turn, are not as intense in comparison to the effects on cell viability measured by *Erkekoglu, et al.* in 2010 [78]. They found significant effects in cell viability of MA-10 cells at concentrations starting as low as $3x10^{-6}$M of MEHP corresponding to

a 50% decrease in viability and resulting in a complete reduction of cell population in the presence of $1x10^{-5}$M for a 24 h exposure. The effects of DEHP found on cell viability were also more severe, showing significant effect $1x10^{-5}$M of DEHP at a 24 h exposure, equivalent to 20% decrease reaching up to 40% reduction for a $1x10^{-3}$M concentration. In the end, they found that MEHP was of three orders of magnitude higher in cytoxicity than the parent compound. *Dees, et al.* in 2000 [79], found that MEHP in fact had no effect at all on MA-10 cell viability in the presence of $3x10^{-7}$M to $1x10^{-3}$M MEHP for an exposure time of 24 h. Moreover, *Fan, et al.* in 2010 [80], agreed that MEHP did not manifest cytotoxic effects in 24 h nor for an exposure period of 48h. However, their study range was limited to a maximum of $3x10^{-4}$M; therefore no conclusions can be drawn for the higher concentration of $1x10^{-3}$M where an effect was observed in this case. It should be noted that direct comparison is very difficult as the level of DMSO was different in all studies, the cell phenotype may have changed and different cell viability assays were used. Discrepancies from one study to another could also be attributed to several factors such as variations in other experimental conditions, the viability assay used, source and purity of MEHP. More specifically to the state of the cells, they could also be associated to differences in cell passages, cell density, culturing conditions, and nature and duration of preservation of the MA-10 cells. All in all, these variations in the data imply that suitable screening of the alternative plasticizers must be performed relatively to one's study.

When assessing the cytoxicity of the dibenzoate compounds on MA-10 cells, only the C5 had a significant effect on cell viability at its highest concentration of $1x10^{-3}$M, for an exposure time of 24 hours (Figure 12). Its effect was greater after 48 h where the C3 also began to show effect (Figure 13). It should be noted that it was the dibenzoate plasticizers with the odd number

of carbons that exhibited acute toxicity to the cells after 48 hours, similar to MEHP. The C4 and C6 are therefore likely better candidate plasticizers.

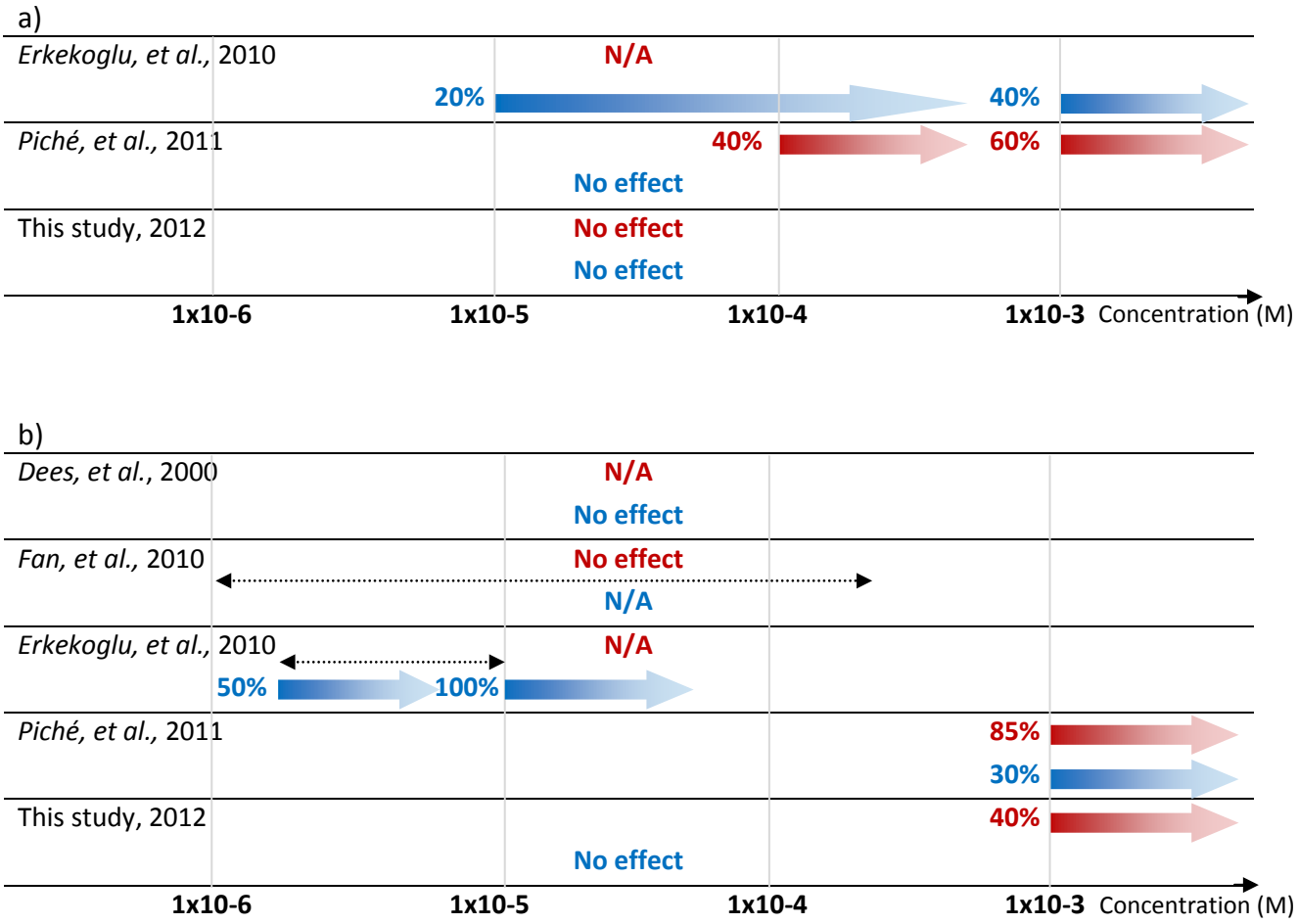


Figure 15: Effects reported on cell viability of MA-10 cells exposed to levels of a) DEHP and b) MEHP for 24 hours (blue) and 48 hours (red) for a concentration range of $1x10^{-6}$M to $1x10^{-3}$M unless delimited by ◄·····► for the corresponding tested range. Values in percentage represent population reduction with the arrow indicating the start of the reported effect and N/A notes unavailable data.

6.2.1. Selected Concentrations and Exposure Time

In order to study the potential anti-androgenic effects of the candidate compounds, cell viability had to be maintained throughout the duration of the progesterone production experiments. This allows for a constant cell population to be sustained between each condition and guarantees that any changes observed in progesterone concentration will not be skewed with

the toxicity effects of the compounds influencing the cell count. As such, the results obtained from the cell viability experiments suggested compound concentrations ranging from $1x10^{-7}$ M to $1x10^{-4}$ M that would limit the effect of cell viability. These concentrations of plasticizers are physiologically relevant to exposed adults and especially neonates requiring intensive care. Studies have shown urinary contents of DEHP and its metabolites in the ranges of $3x10^{-7}$ M (MEHP in neonates) [81] and reaching as high as $1x10^{-3}$ M (DEHP, after use of parenteral nutrition PVC infusion lines) [33, 82]. As a matter of fact, the leaching potential of the newly synthesized compounds was found to be minimized to rates reaching up to ten times below that of DEHP [28], suggesting that the dibenzoate plasticizers will see even lessened levels of exposure. Nonetheless, it was preferable to be cautious and test a wider range.

Moreover, an exposure time of 24 h was selected for further experimentation as greater cell death was observed at the 48 h time point in all conditions tested, including the control (Figure 13). Most importantly, as MTT assays assess the metabolism of cells through the activity of their mitochondrial enzymes [83], 24 h exposure at 1% v/v DMSO preserved the cells' mitochondrial integrity which is necessary for the primary steps of the steroidogenesis pathway (see section 3.1.4.1).

6.3.Effect on Progesterone Production

The ability of the compounds to disrupt steroidogenesis in MA-10 cells was characterized by the acute progesterone production of the MA-10 cells in response to hCG stimulation. Since testosterone monitoring was not possible due to the incapacity of the MA-10 cells to complete the steroidogenic pathway, the expression of progesterone was used as its marker. In an effort to normalize the amount of progesterone produced in the different conditions, the total protein was assessed to correct for variations in cell number. However, the collected samples of lysed cells

were so viscous from the elevated amount of protein that it was difficult to sample consistently and obtain accurate numbers. This was overcome by adding benzonase nuclease to alleviate the viscosity by breaking apart the DNA [84]. The quantity of progesterone produced was therefore normalized to the average total protein of the experiment set to consider for possible fluctuations in amount of cells from one replicate to another. Moreover, to account for variations between ELISA assays [85], each replicate was normalized to its control containing 1% v/v DMSO.

Amongst all the compounds tested for 24 h, the industry standard plasticizer, DEHP exhibited the greatest effect on progesterone production (Figure 14). Interestingly, its active metabolite MEHP did not manifest a significant effect though a 20% reduction of progesterone produced was noted at a $1x10^{-4}$ M concentration. Results previously reported on the effect of DEHP and MEHP on progesterone production in MA-10 cells is compiled in Figure 16. Similarly, *Piché, et al*. had observed a greater effect issuing from the parent compound DEHP though a more pronounced dose-response was developed in their case and instead found a 30% decrease also starting at $1x10^{-4}$ M MEHP. Comparatively, *Dees, et al.* observed a significant effect of MEHP at an exposure of $3x10^{-6}$ to $1x10^{-5}$ M, however not at $3x10^{-5}$ to $3x10^{-4}$ M and showed extreme effects starting only at $1x10^{-3}$ M and higher, with 90% decrease in progesterone production. In addition, these results tie in with the findings of *Gazouli, et al*. that claimed an effect of MEHP initiated at a lower concentration of $1x10^{-5}$ M which is reasonable as their timeframe was for an extended period of 48 h and consequently, where effects are more visible. All in all, the results gathered from the current study and the previous study by *Piché, et al*., agree that DEHP causes a greater reduction in progesterone production than MEHP. The differences between the levels of inhibition of MEHP at different exposures could be attributed to experimental variations stated earlier in section 6.2, an important one being the differences in

solution preparation, DMSO concentration and storage of the compounds. It is important to note that the effects observed can be directly associated to the compounds themselves and not to the products of their biodegradation as the MA-10 cells do not biodegrade DEHP and its metabolites in 24 h [76].

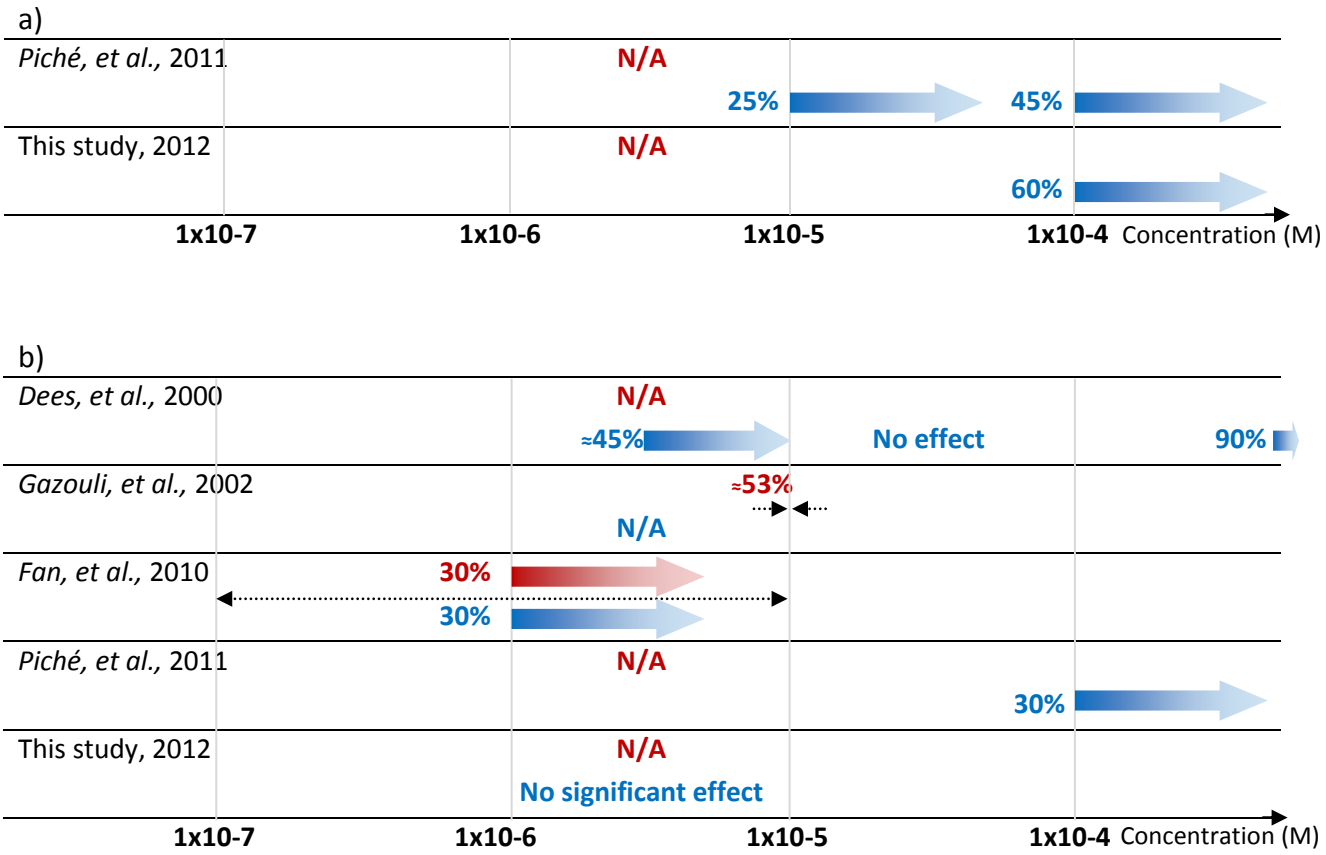


Figure 16: Effects reported on progesterone production of MA-10 cells exposed to levels of a) DEHP and b) MEHP for 24 hours (blue) and 48 hours (red) for a concentration range of $1x10^{-6}$M to $1x10^{-3}$M unless indicated otherwise by ◄·····► . Values in percentage represent progesterone production reduction with the arrow indicating the start of the reported effect and N/A notes unavailable data.

6.3.1. Effect of Dibenzoate Plasticizers

In comparison, the tested alternative plasticizers were not exempt of anti-androgenic effects. The C3, C5 and C6 exhibited effects on progesterone production of the MA-10 cells. Nonetheless, their inhibitory effects were not as intense as the one caused by DEHP at the

highest concentration tested of $1x10^{-4}$M (Figure 14). In particular, the effects of C4 and C6 were significantly different than that of DEHP. Interestingly, these are the compounds with an even number of carbons though it is not clear if this has significance. It may be that the cells are metabolizing the plasticizers and biodegrading them by beta-oxidation, which possibly degrades the compound two carbons at a time, like in the biodegradation pathway of C3 by resting cells of *R. rhodochrous* [27] and C6 by *R. rhodochrous* [25], leaving odd numbered carbon chains with end products that are foreign to cells, exhibiting higher toxicity. In order to verify if degradation by beta-oxidation actually occurs by cleaving two carbons at a time, using isotopes of carbon such as carbon 13 could be interesting. However, this toxicity trend does not agree with the findings of *Segura, et al*. [86] who found C5, C6, C4, C3 in increasing order of toxicity to *V. fischeri*, stressing more an inverse relationship between chain length and toxic effects. In contrast, there was a lack of toxicity of DEHP towards *V. fischeri*, which was also previously observed by *Nalli, et al*. [87], outlining a difference in mechanism of action between bacterial *V. fischeri* and mammalian MA-10 cells.

6.4. Biodegradation of Dibenzoate Compounds

Elucidation of the possible occurrence of biodegradation of the dibenzoate compounds by the MA-10 cells was attempted though was not successful. Initially, the method employed for assessing the possibility of further degradation of DEHP and its metabolites in 24 h [76] was repeated but was deemed inadequate for the dibenzoates series. The efficiency of extraction of the plasticizers from the medium's aqueous phase to a chloroformic phase to be analyzed through gas chromatography was extremely poor (results not shown). The protein content, originating namely from the horse serum supplement, is hypothesized to interact and adhere strongly to the compound, trapping the compounds in the formed pellet during the extraction

step, thereby preventing them from entering the chloroform phase. This is believed to be the leading cause of poor extraction. Carrying the experiment in a serum free medium could be an alternative especially since the cell viability was proven to be unchanged without serum (results not shown). However, it is not recommended to alter the state of the cells as the studies on the anti-androgenicity require the use of serum and would make the interpretation difficult.

Moreover, it seemed that exposure to heat from the incubation of the cells also had an effect on the extraction efficiency, perhaps by strengthening the protein interaction bonds. Although exposure to heat was unavoidable, cooling down samples and conducting the extraction step in cold conditions was experimented in the hopes of denaturing the proteins and breaking the strong bonds but to no avail. On the contrary, extraction efficiency had decreased more due to the decreased solubility of the extraction solvent at colder temperature. In addition, the hydrophobic compounds adhere to the test plates composed of polystyrene. Once again, this effect is inevitable as cells do not grow well on untreated glassware (results not shown), making plastic irreplaceable and emphasizing the importance of rinsing of the plates when collecting the samples. Furthermore, several attempts were made in an effort to increase extraction efficiency by using different solvents, namely toluene, hexane and ethyl acetate though none were helpful. It is important to keep in mind that many of these solvents are of aggressive nature to plastic and so, it is suggested to use Teflon*FEP (fluorinated ethylene propylene) tubes instead of the regular polycarbonate ones when extracting and centrifuging the samples. Solid-phase extraction was also tried using a cartridge though was also fruitless. Although the cartridge (Oasis HLB 150 mg) used seemed to be appropriate for extracting these types of compounds, perhaps another type would be more suitable.

6.5. "Green" Potential of Dibenzoate Plasticizers

With respect to the findings of this study, interestingly the commercial plasticizer D(EG)DB maintained a status of a plasticizer exempt from toxic effects. Unlike its rival DEHP, it preserved cell viability and did not impose disturbances on the steroidogenic functions of MA-10 cells. Similarly, *Segura, et al.*, observed a lower toxicity of D(EG)DB with *Vibrio fischeri* relative to its derived compounds. As a matter of fact, in recent years, D(EG)DB has been proposed as a replacement to common plasticizers due to its fast degradation tendency in presence of common microorganisms [88, 89]. However, though its fast degradation feature and seemingly less toxic nature are appealing and encourage its selection as a substitute, *Kermanshahi, et al* [25]., have shown that its degradation in bacterial culture is in fact partial and lead to accumulation of its monoester with substantial amounts close to 70% of the initial molar concentration which, in turn exhibits significant toxicity [54]. Putting this in perspective with the synthesized dibenzoates, the analogous monoesters produced, for example from C6, were observed only in trace amounts and degraded rapidly and so, did not result in accumulation of persistent metabolites [26]. In fact, the rate of their biodegradation reaches up to 7.5 times faster than the monoester produced from D(EG)DB [27]. The lack of ether bonds in the synthesized dibenzoate compounds therefore allows them to circumvent the build-up of toxic monoesters or any other toxic metabolite and makes them better alternative plasticizers.

Overall, this thesis suggests a suitable candidate to be the C4 plasticizer from the dibenzoate plasticizers series. The results from this study showed that it does not exhibit anti-androgenic effects on the mammalian mouse Leydig MA-10 cell line and is significantly better relatively to DEHP. Furthermore, in comparison to its series' compounds, previous work done at McGill indicated that it seems to be the middle point between biodegradation and leaching

potential [25, 27, 28]. As the chain length of a structure gets longer, its hydrophobicity increases and accordingly, its aqueous leaching is diminished [28]. This is a favourable asset for plasticizers to have as by remaining in their polymer matrix, their toxic exposure to the environment will be lessened, on a health point of view. Also, the properties it imparts to the polymer will be preserved for an extended period of time, hence prolonging the lifespan of the plastic materials that are in continuous contact with moving fluids, and so, is beneficial on a commercial point of view. On the other hand, longer chain length is usually associated with slower biodegradation of the compound [56]. This could be problematic if the biodegradation yields an accumulation of toxic metabolites in the environment. As such, C4, with its four carbon alkyl chain, is a good trade-off between the benefits and disadvantages of having a long chain length. All in all, this study supports the potential use of C4 as a green plasticizer.

7. CONCLUSION

In an effort to screen potential plasticizer alternatives, the acute toxicity and anti-androgenic effects of C3, C4, C5 and C6 were assessed and compared to that of the common DEHP plasticizer and its primary metabolite MEHP and to the effects of their analog commercial plasticizer, D(EG)DB on the MA-10 cell line. Anti-androgenicity testing of the hydrophobic structures was executed at a solvent concentration of 1% v/v DMSO, a concentration which balanced solubility and cytoxicity of the cell at a 24 h time point.

This thesis found that:

1. MEHP, C3 and C5 had effects on MA-10 cell viability after a 48 h exposure at the highest test concentration of $1x10^{-3}$ M,
2. DEHP manifested the greatest decrease in progesterone production of the cells at the highest test concentration of $1x10^{-4}$ M,
3. C3, C5 and C6 exhibited effects progesterone production,
4. D(EG)DB did not cause any significant effect on cell viability and progesterone production.

Overall, C4 is promoted as a potential candidate for a green plasticizer. It did not cause significant effects on cell viability nor did it exhibit anti-androgenic effects on the mammalian MA-10 cells. Moreover, its chain length size compromises between leaching potential and biodegradation ability.

8. FUTURE WORK

Designing safer alternate compounds is the result of a collaborative work. A rigorous analysis of mechanical properties has to be made to validate their efficient use and a meticulous screening must be performed to attempt in detecting possible adverse effects. This entails the collection of several studies putting to the test the synthesized chemicals to various systems. As to what concerns their acute toxicity and anti-androgenicity potential, their effects were studied *in vitro* with the mammalian MA-10 cells. To carry a thorough examination of their possible steroidogenic interference, another mammalian cell could be used such as the R2C rat Leydig cell line or verify their acute toxicity in a bacterial system other than *V. fischeri* to see if different reactions would occur.

In particular to this work, it would also be interesting to further investigate the dibenzoates' effects on steroidogenesis by examining their effects on the expression of genes involved in testosterone production. This would allow for the elaboration of their mechanism of action and possibly help in their design. This work would also be complemented by the study of their biodegradation potential by MA-10 cells in order to attribute the observed effects to the parent compound or to the metabolites. Furthermore, the metabolites of the dibenzoates could also be examined *in vitro* for their possible toxicity.

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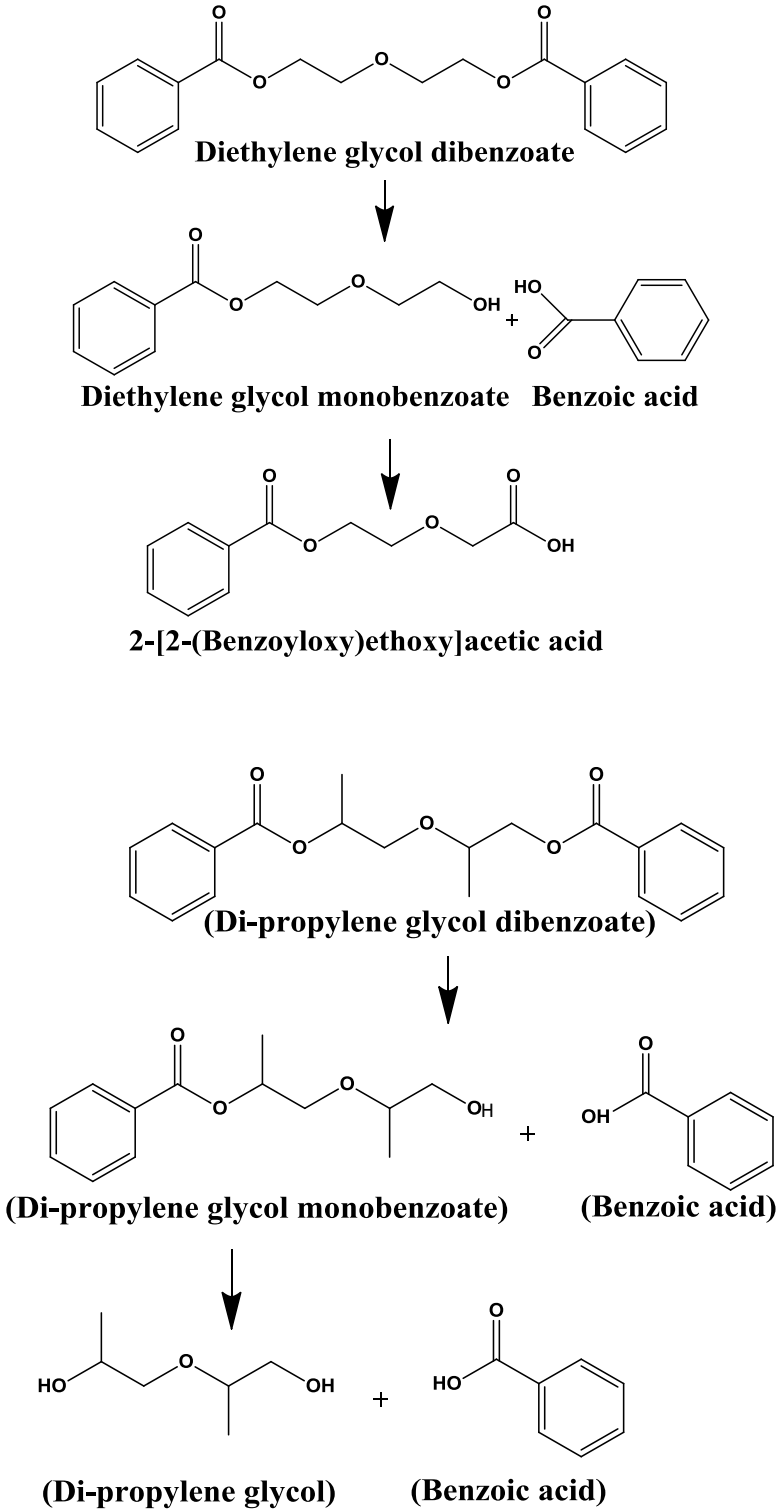
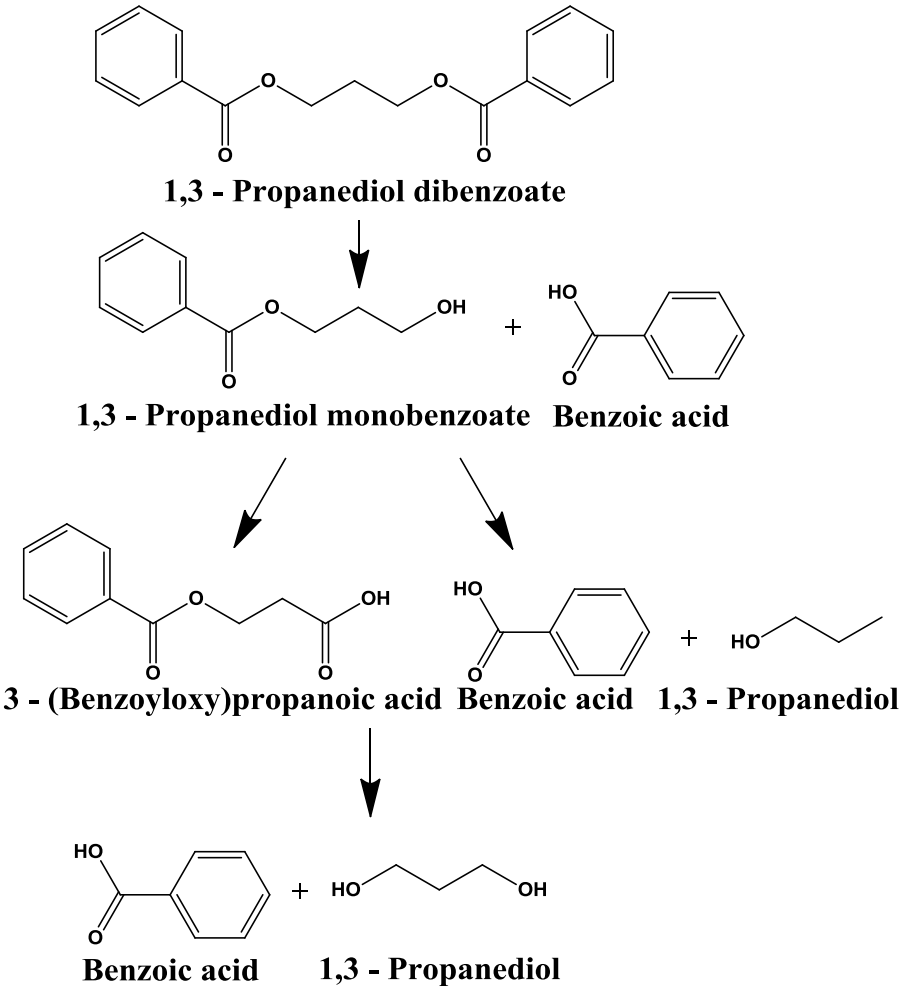
Appendix I: Proposed biodegradation pathway of diethylene glycol dibenzoate by resting cells *of Rhodococcus rhodochrous* [27] and of dipropylene glycol dibenzoate by *Rhodotorula rubra* [54] and by resting cells *of Rhodococcus rhodochrous* [27]



Appendix 1I: Proposed biodegradation pathway of 1,3-propanediol dibenzoate by resting cells *of Rhodococcus rhodochrous* [27]



Appendix III: Proposed biodegradation pathway of 1,6-hexanediol dibenzoate by *Rhodococcus rhodochrous* [25]