The Mechanisms of Toxicity of Dibutyl Phthalate (DBP) in Cultured Cells

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Abstract

The ubiquitous environmental pollutant Dibutyl Phthalate (DBP) is a known to have anti-androgenic like properties. DBP exposure has been linked to a range of developmental disorders in males such as hypospadias (defect of the penis) and cryptorchidism (undescended testes). Humans are exposed to DBP daily; therefore, it is essential to fully understand the role DBP in the pathogenesis of these developmental disorders. The mechanism of toxicity has yet to be fully established; however, DBP has been shown to reduce testosterone biosynthesis and is thought to disrupt the embryological androgen:estrogen ratio. The present study developed and characterized a LC-540 Leydig cell line model system. This model system was used to investigate the effects of DBP at physiologically relevant concentrations on key genes of testosterone biosynthesis via the use of Nanostring® nCounter technology. The metabolism of DBP was investigated using HPLC to identify if any potentially estrogenic metabolites are produced. The estrogenicity of DBP was investigated using a MCF-7 proliferation assay. The LC-540 model system is able to produce detectable quantities of testosterone with and without external stimulation in culture. The metabolism of DBP did not produce any potentially estrogenic metabolites; however, the LC-540 Phase I and Phase II processes were identified. DBP appears to be slightly estrogenic and promotes growth of MCF-7 cells. Key genes in testosterone biosynthesis are changed following exposure to DBP. The gene that codes for testosterone biosynthesis (*hsd17b3*) is down-regulated at normal daily exposure concentrations and occupational exposure concentrations. The gene that codes for 17β-estradiol synthesis (*cyp19a1*) is up-regulated at occupational exposure concentrations. The likely result of these gene changes is the feminization of males which explains the role of DBP in the pathogenesis of hypospadias and cryptorchidism. The present study is the first evidence of dual gene regulatory changes following DBP exposure at physiologically relevant exposure levels. The MCF-7 proliferation assay is the first evidence of DBPs potential estrogenic properties.

Abbreviations

Dibutyl phthalate	DBP
European Union	EU
Dioctyl terephthalate	DOTP
Polyvinyl chloride	PVC
Glass transition	T_g
Endocrine disrupting chemical	EDC
Body weight	bw
No observable adverse effect level	NOAEL
Lowest observable adverse effect level	LOAEL
Gestation day	Gd
Minimal risk level	MRL
Monobutyl phthalate	MBP
Androgen receptor	AR
17β-estradiol	E2
The anogenital distance	AGD
dihydrotestoserone	DHT
Testicular dysgenesis syndrome	TDS
Bisphenol-A	BPA
Yeast estrogen screen	YES
Chlorophenol red-β-D-galactopyranoside	CPRG
Steroidogenic acute regulatory protein	StAR
11β-hydroxysteroid dehydrogenase	CYP11
steroid 21-hydroxylase	CYP21
3β-Hydroxysteroid dehydrogenase	3β-HSD
17β-Hydroxysteroid dehydrogenase	17β-HSD
Aromatase	CYP19
Glucocorticoid receptor	Gr
Cholesterol side-cleavage enzyme	$P450_{scc}$
17α-hydroxylase	CYP17
Insulin-like factor 3	INSL3
Fetal Leydig cells	FLCs
Adult Leydig cells	ALCs
Smooth endoplasmic reticulum	SER
Rough endoplasmic reticulum	REM
Cranial suspensory ligament	CSL
Leucine-rich Leucine-Rich Repeat-Containing G Protein-Coupled Receptor 8	LGR8
Neonatal Leydig cells	NNLCs
Immature Leydig cells	ILCs
Cytochrome P450	CYP
Insulin-like growth factor	IGF
Human epidermal growth factor receptor 2	HER2
Platelet-derived growth factor	PDGF

Human chorionic gonadotropin	hCG
Tumour necrosis factor alpha	TNFα
histone deacetylases 7	HDAC7
Lutenizing hormone	LH
cyclic adenosine monophosphate	cAMP
G-coupled protein receptor	GPCR
protein kinase A	PKA
Phosphate buffered saline	PBS
Fetal bovine serum	FBS
Minimum Essential Medium	MEM
Extracellular matrix	ECM
Minimal risk level	MRL
Polyethylene	PE
Polyethylene terephthalate	PET
Poly aromatic hydrocarbons	PAHs
Uridine diphosphate	UDP
Uridine diphosphate glucuronyltransferases	UGTs
Uridine-5′-diphospho-α-D-glucuronic acid	UDPGA
Sulfotransferases	SULTs
Phosphoadenosine 5'-phosphosulphate	PAPS
Glutathione-s-transferases	GSTs
Reactive oxygen species	ROS
Membrane-associated proteins in eicosanoid and glutathione metabolism	MAPEGs
Adenosine triphosphate	ATP
adenosine triphosphate binding cassette	ABC
Nucleotide binding folds	NBF
Transmembrane domains	TMDs
Multidrug binding proteins	MDBPs
3-hydroxy-monobut-1-ol phthalate	3-OH-MBP
4-hydroxy-monobut-1-ol phthalate	4-OH-MBP
2-hydroxy-monobut-1-ol phthalate	2-OH-MBP
3-carboxy-monopropyl phthalate	MCPP
mono-4-butanol-4-hydroxyphthlate	M4B-4HP
High Performance Liquid Chromatography	HPLC
Diode array detector	DAD
Formic acid	FA
Coefficient of determination	R^2
4-nitrophenol-D-β-glucuronide	4NPDG
4-nitrophenol	4NP
Ethinyl estradiol	EE2
Liquid chromatography-mass spectrometry	LC-MS
Lutenizing hormone receptors	LHR
Gonadotropin-releasing hormone	GnRH
follicle-stimulating hormone	FSH
Guanine triphosphate	GTP

Guanine diphosphate	GDP
StAR-related lipid transfer	START
Steroidogenic factor 1	SF1
Gamma-aminobutyric acid	GABA
Dehydroepiandronstenedione	DEHA
Cytochrome P450 11	CYP11
Canterbury Health Laboratories	CHL
No-gene expression	NE
Real time polymerase chain reaction	RT-PCR

Chapter 1 – Introduction

1.1 A Brief History of Dibutyl Phthalate (DBP)

The phthalate esters, including DBP (Fig. 1.1), were first used in the 1930s[1,2] as plasticizers to replace the widely used camphor which had a tendency to produce an unpleasant odour.[3] Phthalate esters were consumer friendly plasticizers as they were seen at the time to be colourless, odourless, non-volatile and potentially non-toxic. These properties likely contributed to the mass production and proliferation of plastic products seen from the 1930s onwards. The toxicity of DBP was first demonstrated in 1970 when chick embryos exposed to high levels of DBP produced a significant increase in teratogenicity (birth defects).[4] A key study in rats linked embryological exposure of DBP and testicular atrophy in male pups born to pregnant dams exposed to DBP orally.[5] Research into the toxicity of DBP continued into the 1990s[6,7] ensuring legislative bodies in key jurisdictions (e.g. USA) undertook their own investigations. The USA[1] and the European Union (EU)[8] both legislated against use of phthalates in children's toys in 2009 and 1999 respectively.[9]

In the 21st century the general population has become increasingly aware of the potential toxic risks of plasticizers, particularly bisphenol A (BPA) and the phthalates esters.[10] The ensuing condemnation and consumer pressure has led to the plastics industries moving toward products containing additives with significantly less toxicity (e.g. dioctyl terephthalate, DOTP).[11]

1.2 DBP Production, Function and Use

DBP is synthesized commercially by reacting phthalic acid with excess butan-1-ol and sulphuric acid as a catalyst (Fig. 1.1). The worldwide production of DBP in 2007 was approximately 9 million tons.[1] DBP is produced primarily in China and the United States; however, both governments are

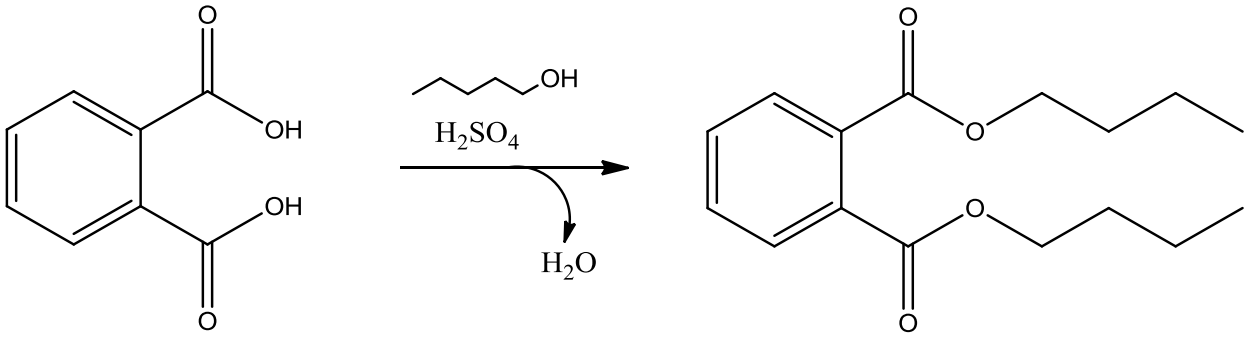


Figure 1.1: The commercial synthesis of DBP. The sulphuric acid catalyst protonates the carboxylic acid. The butanol is deprotonated and attacks the carbonyl and the water acts as the leaving group in the S_n2 reaction.

reluctant to publish data on production volumes. The volume productions at the present time are unknown. EU trends suggest a significant decrease in production over the last 15 years (Fig. 1.2), with over 50,000 tonnes in 1994 compared to less than 10,000 tonnes in 2007.[12] The decline in DBP production can almost certainly be attributed to consumer pressure relating to potentially toxic plastic additives and the subsequent replacement of DBP with less toxic phthalate alternatives (e.g. DOTP).[11] Presently, the total production of DBP is likely in the low millions of tonnes annually.

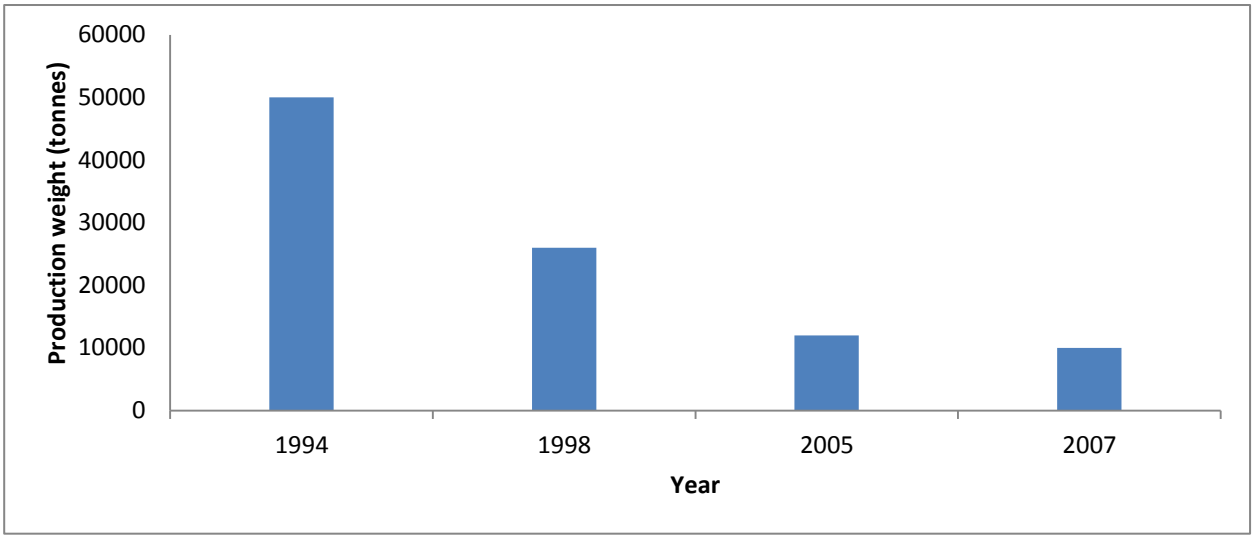


Figure 1.2: The production of DBP in the European Union from 1994-2007. The production has been steadily decreasing, likely due to legislative restrictions and public awareness of potential toxic effects.[1]

1.2.1 Uses of DBP

DBP is used primarily as a plasticizer to increase the pliability and strength of commercial plastic products; most commonly in polyvinyl chloride (PVC) and nitrocellulose polymers. PVC is the world's second most produced and used commercial polymer with an annual global production of 31 million tonnes,[13] with 40% of its market dedicated to pipes and fittings.[14] Commercial PVC production has three key steps: synthesis of vinyl chloride monomers, polymerization of the monomer units and the addition of plasticizers (most commonly phthalates).[15] Pure PVC polymer is a non-thermally stable compound that is highly brittle and prone to fractures and decomposition.[15] The decomposition of pure PVC occurs when the branching chains of the polymers react internally to release HCl. This significantly reduces the polymer strength and leads to rapid degradation of the polymer.[16] Therefore, treatment with phthalates is essential before PVC can be used in most commercial applications. PVC is plasticized by heating the pure PVC resin in the plasticizer solution until the PVC has dissolved. This ensures thorough incorporation of the plasticizer into the polymer matrix.[16]

The mechanism of action of DBP as a plasticizer has been a key area of polymer research. There are several theories; most prominent of which is the lubrication theory.[16] This theory states: during the heating and co-polymerization processes, the plasticizers (i.e. phthalates) diffuse into the polymer matrix, preventing branch chain interactions; thereby, acting as a shield to prevent the loss of HCl. This results in a significant reduction in polymer degradation. The addition of phthalates lowers the glass transition (T_g); the temperature at which a rigid, solid polymer enters a rubber-like semisolid state which is flexible, extendible and soft. The DBP-treated PVC is then able to be used in a variety of products, ranging from children's toys, water piping, plastic packaging, medical products and equipment, and carpeting.

The cosmetics and personal care products industry also use DBP extensively.[17] DBP has a duel role in many cosmetic products; e.g. DBP in nail varnish acts as both a solvent for the dyes and as a plasticizer

for the polish itself, ensuring that the applied product is not brittle. Body sprays have also been known to use DBP as a solvent, but the prevalence of its use is unknown as many of the formulations are proprietary blends that do not require disclosure of their composition on the product label.

DBP has also been used in a military setting; e.g. conflicts in Asia during World War Two up until the Vietnam War,[18,19] especially during the Malayan Emergency of 1948-1960.[20] The primary use of DBP during these conflicts was as an acaricide and sealant for the seams of military uniforms of troops involved in jungle operations to prevent infections from trombiculid mites (e.g. *Leptotrombidium delicense*) which are the vectors for bush typhus (*Orientia tsutsugamushi*). This rickettsial infection causes severe muscle soreness, gastrointestinal pain and diarrhoea which are potentially fatal. The use of DBP was essential at the time to maintain jungle operations as any troops infected with bush typhus were unable to undertake military operations.

1.3 DBP Toxicology

DBP has been classified as an endocrine disrupting chemical (EDC); particularly affecting the synthesis and regulation of the androgenic (male sex) hormones essential for male growth and development.[1] The mechanism of toxicity has yet to be fully established but there is a body of evidence that suggest DBP has a major disruptive effect on the embryological synthesis of testosterone during male sexual development.[1]

DBP has very low toxicity: LD_{50} [rat oral] = 20,000 mg/kg body weight (bw). This does not pose a significant risk of death. Indeed, the likely human LD_{50} based on rat data suggests the lethal toxic dose would be in far excess of a feasible human daily exposure concentration; i.e. rat LD_{50} = 20,000 mg/kg bw, based on a 70 kg human bw this means a dose of 1.4×10^6 mg (1.4 kg) would be needed to kill a human. However, DBP is more toxic to embryos, with the incidence of still births' increasing significantly following DBP exposure to pregnant rats at doses ranging from 630-3000 mg/kg bw.[6,21]

There are few toxicological data available for humans but there has been a significant body of work in animals. The data are highly variable among individual experiments and experimental conditions. The observable effects and the sensitivity vary between embryological and postnatal exposure.

The postnatal exposure studies demonstrate that the toxic effects of DBP primarily affect the sexual development of males (e.g. reductions in testes weight, testicular atrophy).[22] The no observable adverse effect level (NOAEL, the highest dose at which there are no observable toxic effects), ranges from 500-2000 mg/kg bw/day.[22,23] Whereas, the lowest observable adverse effect level (LOEAL, the lowest dose at which significant toxic effects are observed), ranges from 2000-2400 mg/kg bw/day (table 2.1).[23,24]

There does not appear to be a clear dose response relationship in postnatal exposures to DBP in rats with serious morphological effects observable at a range of doses (e.g. from 500-2000 mg/kg bw/day), whereas some studies report no adverse effects at doses of 2000 mg/kg bw/day. [23,24]

Embryological DBP exposure (Table 2.2), however, shows a far more sensitive response at lower doses of DBP, with significant adverse effects observable at levels well below the postnatal NOEAL.[21] Decreases in testes weight were observed at doses as low as 100 mg/kg bw/day and feminizing effects (e.g reductions in anogenital distance, AGD), were observable between 500-750 mg/kg bw/day.[25] A dose response relationship occurs between gestational day exposure and increasing toxicity of DBP. Serious developmental toxicity (e.g. permanent morphological changes) was readily observed when exposure to DBP occurred later in gestation; e.g. from gestation day (Gd) 12 onward. Exposure from Gd 0-8 produced developmental toxicity at 500 mg/kg bw/day with severe effects at 750 mg/kg bw/day.[26] Conversely, exposure from Gd 12-21 produced toxic effects at 100 mg/kg bw/day with the more severe morphological effects observed as low as 250 mg/kg bw/day (the same dose at which no toxic effects were observed from Gd 0-8).[27] The early stages of male gestation do not require the androgenic

hormones (e.g. testosterone), whereas at Gd 12-21, testosterone is responsible for myriad developmental processes. Therefore, if DBP toxicity occurs through a mechanism of diminishing testosterone production, it is unsurprising that DBP's toxic effects are far more potent during the key sexual development period.

Due to the developmental toxicity of DBP and the greatest NOEAL of 50 mg/kg bw/day, the minimal risk level (MRL) has been set at 0.5 mg/kg bw/day.[1] The MRL is an estimate of the acceptable daily exposure level that would not likely result in adverse effects.

The toxicological data provide insights into the levels of exposure that can cause developmental disorders in rats. However, it is essential to understand the pathogenesis of the disorders to fully understand the mechanism of DBP toxicity.

			LOAEL (mg/kg bw/day)			
Animal (Strain)	**Exposure Duration**	**NOAEL (mg/kg bw/day)**	**Less Serious[a]**	**Serious[b]**	**Observations**	**Reference**
Rat (Sprague-Dawley)	4 d	500		1000	Decreased testes weight	Cater et al. 1977[22]
Rat (Sprague-Dawley)	6 d			500	Decreased testes weight	Cater et al. 1977[22]
Rat (Wistar)	7 d			2400	Testicular atrophy	Fukuoka et al. 1989[28]
Rat (Wistar)	1 d			2400	Testicular atrophy	Fukuoka et al. 1989[28]
Rat (Sprague-Dawley)	9 d			2000	Sever testicular atrophy, decreased testes weight	Gray et al. 1982[23]
Rat (Wistar)	7 d			2100	Decreased testes weight, reductions in spermatocytes and spermatoginia	Oishi and Hiraga 1980[29]
Rat (unknown strain)	7 d			2400	Decreased testes weight	Tanino et al. 1987[24]
Mouse (unknown strain)	9 d		2000		Moderate testicular atrophy	Gray et al. 1982[23]
Guinea Pig (Dunkin-Harley)	7 d			2000	Severe testicular atrophy, decreased teste weight	Gray et al. 1982[23]
Syrian Hamster	9 d	2000				Gray et al .1982[23]

[a]Less serious = minor growth and development effects that would not affect overall health and fertility.

[b] Serious = severe developmental or morphological defects that would affect general health and fertility.

Table 1.1: The Toxicological data of oral postnatal exposure to DBP. There is strong agreement in the LOAEL of the studies.

			LOAEL (mg/kg bw/day)			
Animal (Strain)	**Exposure Duration**	**NOAEL (mg/kg bw/day)**	**Less Serious**	**Serious**	**Observations**	**Reference**
Rat (Wistar)	Gd 7-15	500		630	Decreased fetal body weight, post implantation loss	Ema et al. 1993[6]
Rat (Wistar)	Gd 7-9			750	Decreased fetal body weight, post implantation loss, skeletal malformations, decreased live births per litter	Ema et al. 1994[21]
Rat (Wistar)	Gd 10-12			750	post implantation loss	Ema et al. 1994[21]
Rat (Wistar)	Gd 11-21	331		555	Internal malformations	Ema et al. 1996[30]
Rat (Wistar)	Gd 0-8	250	500	750	Decreased fetal body weight, post implantation loss, altered sex ratios, decreased live births per litter	Ema et al. 2000a[26]
Rat (Wistar)	Gd 15-17			500	Increased incidence of undescended testes, reductions in AGD	Ema et al. 2000b[31]
Rat (Long Evans)	Gd 16-19			500	Increased incidence of retained nipples, reductions in AGD, decrease in volume of androgen dependant tissues	Gray et al. 1999[32]
Rat (Sprague-dawly)	Gd 12-21	50	100	250	Decreased AGD, small sex accessory glands, decreased testes weight, malformations of the reproductive tract.	Mylecreest et al. 1999[27]
Rat (Sprague-dawly)	Gd 12-21		100	500	Malformations of the epididymis, decreased AGD, retained nipples	Mylecreest et al. 2000[25]
Rat (Sprague-Dawley)	Gd 14-Ld 3			500	Increased incidence of retained nipples, reductions in AGD, decrease in volume of androgen dependant tissues	Gray et al. 1999[32]

Gd = Gestational day

Ld = Lactation day

Table 2.1: The Toxicological data of oral prenatal exposure to DBP. The response to DBP is far more sensitive with serious LOAELs well below the NOEAL of postnatal exposure.

1.4 Developmental Toxicity

Developmental toxicity is defined as the teratogenic effects following *in utero* exposure to developmental toxins. Research into the developmental toxicity of DBP has primarily been focused postnatal physical effects following embryological exposure of rats and mice.

The male reproductive system is particularly sensitive to DBP and its primary metabolite monobutyl phthalate (MBP,[33] see Chapter 4). DBP appears to exhibit toxicity during key stages of male sexual differentiation (e.g. during decent of the testes and external genital formation) in rats, producing a range of diverse reproductive malformations in a dose dependant manner.[32] Such effects are likely extrapolatable to other species, including humans. The most common developmental effects are decreases in AGD,[32] hypospadias (defect of the penis)[34] and cryptorchidism (undescended testes,[35] see below). These developmental disorders are highly typical of the observations following embryological exposure to androgen receptor (AR) antagonists (e.g. flutamide),[36-38] 17β-estradiol (E2) and xenoestrogens (e.g. BPA).[39,40]

1.4.1 AGD

The AGD (the distance from the anus to the base of the scrotum or vagina) is sexually dimorphic, with healthy males exhibiting an AGD twice that of a healthy female.[41] AGD is a good marker of feminization and reproductive health as is particularly sensitive to the effects of anti-androgenic compounds and xenoestrogens. Exposure to DBP above the NOAEL (250 mg/kg bw/day) during the late stages of the rat prenatal period (when sexual differentiation occurs) has been shown to cause a dose-dependent decrease in AGD.[32] Higher doses (500 mg/kg/day) cause a considerably larger dose response, with AGDs 24% shorter than non-exposure control groups.[32]

The consensus among the many studies of the effects of DBP on AGD is a dose-dependent decrease comparable to effects seen following exposure to anti-androgenic compounds or xenoestrogens (see Section 1.5.1). The effects of DBP on the embryological development of the external genitalia, including

the anogenital region is unsurprising as the development of these tissues is regulated by the androgenic hormones (e.g. testosterone and dihydrotestoserone, DHT).[42]

1.4.2 Hypospadias and Cryptorchidism

Hypospadias and cryptorchidism are developmental disorders of the external genitalia. These disorders are a commonly observed in animals following embryological exposure to EDCs including DBP. Hypospadias is a malformation of the penis where the opening of the urethra is misplaced.[34] Cryptorchidism is where one or both testes fail to descend into the scrotum.[35] The pathogenesis of these disorders is not completely understood, however, the formation of the external genitalia is dependent testosterone (see Chapter 2). Therefore, exposure to EDCs such as DBP (i.e. reductions in gestational testosterone synthesis) may play a role in disease pathology of hypospadias and cryptorchidism[43] as the incidences of these disorders has increased over recent years,[44] coinciding with proliferation of environmental EDCs.[45]

High embryological exposure to DBP (500 mg/kg bw/day) in rats has been shown to increase the incidence of hypospadias. At 500 mg/kg bw/day the incidence was reported at approximately 7% across 10 individual litters.[34] There is a dose response relationship; exposure levels of 750 mg/kg bw/day produced litters with incidences of hypospadias ranging from 41-46%.[34,35] Serum testosterone levels of the rats in these exposure groups follow a similar dose response. As exposure levels increase, serum testosterone concentrations decrease linearly; particularly in the offspring with hypospadias which all had a significantly lower level of testosterone.[35] The natural incidence of hypospadias in rats is not known but in humans it has been established at 1 in 125 live births or 0.8%.[43] Therefore, if it is assumed that the incidence of hypospadias is the same between rats and humans, exposure to 500 mg DBP/kg bw/day increased the incidence 9-fold to 9 in 125 live rat births. At 750 mg/kg bw/day the incidence is 51-58 in 125 live births, over a 50-fold increase. The decline in serum testosterone coupled with

significant increases in male sexual development again point to mechanism by which DBP is diminishing testosterone production.

Cryptorchidism follows a similar trend to hypospadias but appears to exhibit a more sensitive dose response to DBP at lower exposure levels. Cryptorchidism was observed in rats following orally administered DBP at 250, 500 and 750 mg/kg bw/day, with increased incidences of 3.1%, 10.2% and 45.7% respectively.[35] This is again demonstrates the anti-androgenic effects of DBP during embryonic development. One United Kingdom study has the rate of incidence of humans at 1 in 125 live births.[46] Again, if we assume the incidences of rats and humans are the same, DBP exposure increased the incidences 4, 13, 57-fold at exposures levels of 250, 500 and 750 mg/kg bw/day respectively.

There is a clear association between increases in incidence of hypospadias and cryptorchidism with reductions in testosterone following gestational exposure to high levels of DBP. To date, there are no published data on the effect of DBP on hypospadias, cryptorchidism or testosterone levels at normal population exposure levels of humans. However, as DBP exhibits a dose-dependent reduction in testosterone, it can be assumed that at human daily exposure levels there could be slight decreases in testosterone synthesis. Therefore, there is potential for malformations such as hypospadias and cryptorchidism to occur from DBP exposure in the normal population. Hypospadias and cryptorchidism are easily treatable with surgery and hence are considered the less damaging effects of DBP exposure. There are more severe effects which can lead to permanent and destructive reproductive malformations (e.g. testicular dysgenesis syndrome, TDS).

1.4.3 TDS

TDS is the abnormal growth and development of the two key reproductively active cells of the testes, Sertoli (gamete producing) and Leydig (androgen hormone producing) cells.[47] The clinical manifestations occur during gestation and include: decreased testes weight, abnormal cell hyperplasia and poorly

developed seminiferous tubules (the region where meiosis occurs).[47] While the malformations occur during gestation, the damaging effects are more observable during puberty and adult life; particularly the localized and permanent damage to the testes and infertility.[47]

Rats exposed to 500 mg DBP/kg bw/day[47] and 750 mg DBP/kg bw/day[34] produced offspring with a 20% reduction in testes weight at birth compared to controls. Infertility was also prevalent among the DBP-exposed cohort, with 80% of the F1 males unable to reproduce.[34,47] The reductions in testes weight became more apparent over time. After 4 days the exposed rats had 45% decreased weight compared with control groups.[47] Accompanying the reduced testes weight was Leydig cell hyperplasia and abnormal seminiferous tubule structure.[6] Histological analysis of the testes in DBP-exposed males demonstrated Leydig cell clumping and abnormal distribution. As Leydig cells are responsible for healthy development of the seminiferous tubules (see Chapter 2) *in utero,* the abnormal Leydig cell activity likely disrupts the formation of the tubules.[47] The disruption in seminiferous tubule formation directly leads to reduced Sertoli and germ cell maturation (testosterone is essential for gamete production in males). This likely accounts for the reduction in testes weight and infertility following DBP exposure in rats.[48]

1.4.4 Effects in Humans

The effects of DBP *in utero* are ethically challenging to measure in humans and so perhaps the only way forward is to extrapolate from animal studies and rely on isolated human case studies. One such case relates to the New Zealand Malayan veterans (1950-60) who used DBP to paint the seams of their uniforms to prevent infections from trombiculid mites[20] (see Section 1.2.1). Estimations based on dermal absorption through the skin determined they were exposed to DBP at 64 mg/kg bw/day[20] (above the MRL and LOAEL). The offspring of the exposed soldiers have a higher incidence of hypospadias and cryptorchidism compared to the average age match control in New Zealand, 5.1% and 2.4%

respectively.[20] The authors of the study postulated that the high DBP exposure of the fathers induced a transgenerational toxicological effect via an epigenetic mechanism (see section 1.5.3).[20]

A link between clinical manifestations of DBP (and other anti-androgenic compounds) and levels of urinary phthalate metabolites has been postulated.[49] In a cohort of 134 boys aged 2-36 months, 10 boys had high levels of phthalate metabolites, 9 of whom had reductions in AGD. Conversely, 11 boys had no phthalate metabolites detected in their urine, with only one exhibiting any reductions in AGD.[49] The reliability of AGD as a measurement of phthalate induced feminisation in humans remains a contentious issue. However, considering the dose-dependent manner in which DBP reduces AGD in animals, there is a possibility that DBP does play a role in the feminization of humans.

There has been a significant rise of precocious (early) puberty among girls in the developed world[50] with exposure to EDCs being considered to play a significant role.[51] The literature on the role of phthalates in precocious puberty is conflicting. One study linked phthalates and premature breast development. High phthalate levels were detected in 68% of the girls with premature breast development.[52] However, subsequent studies found no association between phthalates and any signs of precocious puberty (e.g. early breast development and pubic hair growth),[53] and conversely linked the delay onset of pubic hair growth to urinary phthalate metabolite levels in some girls.[53]

There is a consensus that DBP toxicity has anti-androgenic like toxicity *in utero.* While these effects are well known and studied, the mechanism of toxicity is still an area of conjecture.

1.5 Potential Mechanisms of Toxicity of DBP

The mechanism of toxicity of DBP in humans has yet to be fully established. Several different mechanisms have been proposed to explain the anti-androgenic effects; particularly reduced testosterone synthesis, which is likely to be responsible for the observed developmental toxicity in

animals. The developmental disorders (e.g. hypospadias, cryptorchidism, TDS) are common observations following exposure to AR antagonists and xenoestrogens. However, reductions in testosterone could occur through a variety of mechanisms that do not affect the hormonal nuclear receptors; namely gene regulation or epigenome modification.

1.5.1 AR Antagonism Xenoestrogenic Activity

During the 20th century, a plethora of man-made chemicals were introduced and began leeching into the environment. These compounds, when absorbed into a higher order organism (including humans) have the potential to affect the sexual growth through a mechanism of molecular mimicry (Fig. 1.3). Structural analogies to natural steroid hormones leads to uptake of these compounds into cells and binding to nuclear receptors. This can change the expression of key genes in human sexual development. This often leads to a disruption of the androgen:estrogen ratio. There are two distinct classes of these hormone mimics that can effect male sexual development:

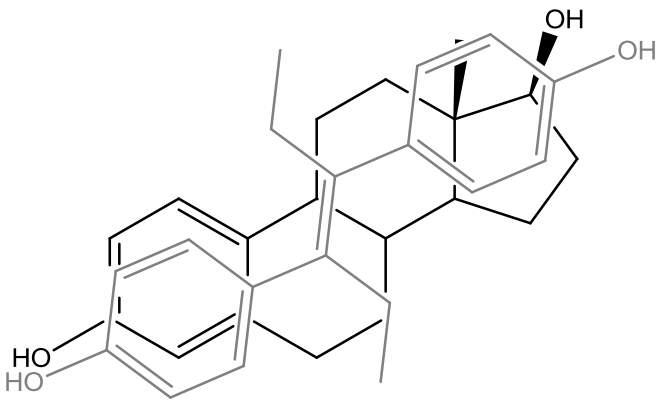


Figure 1.3: The molecular mimicry of E2 (black) and Diethylstilbestrol (grey), a known xenoestrogen. There is strong structural homology with Diethylstilbestrol having the hydrophobic backbone and hydroxyl functional groups that likely facilitate binding in the ER.

xenoestrogens, compounds that mimic the female sex hormone E2 and bind to and activate the estrogen receptor (ER) and AR antagonists which competitively bind to the AR.

1.5.1.2 Xenoestrogenicity

Compounds that mimic the structure of E2 in humans are known as xenostrogens (xeno = foreign). Xenoestrogens are commonly produced by plants (i.e. phytoestrogens, e.g. 8-prenylnarigenin) or man-made (e.g. BPA). These compounds share structural similarities with E2 which allows them to fit into the hydrophobic binding pocket of the receptor, bind to the key residues and activate the estrogenic response.[55,56] The exposure of multiple xenoestrogens has been linked to precocious puberty in females,[57] reduced sperm counts[58] and feminizing developmental effects in men (e.g. hypospadias and cryptorchidism).[43,59] All of these effects have been linked to DBP exposure. An increase expression of estrogenic growth factors via activity of DBP could explain the observed developmental effects in males. However, there is conflicting evidence for the estrogenic activity of DBP. As mentioned previously, there are some data that demonstrate the role of DBP in inducing developmental effects in females (e.g. precocious puberty) which could not be explained by anti-testosterone activity alone, but rather an increase in estrogenic activity. The estrogenic potential of DBP has been measured using the yeast estrogen screen (YES) assay which utilizes genetically modified yeast. The yeast expresses a human ER which, upon activation upregulates the expression of β-galactosidase. This enzyme cleaves the yellow substrate chlorophenol red-β-D-galactopyranoside (CPRG) which gives a strong red colour. Therefore, the estrogenicity of a compound is directly related to the intensity of the red colour in the assay.[60] DBP has been shown to be weakly estrogenic in this assay with an estrogenic potential 10^{-5}-10^{-6} less than that of E2.[61] The YES assay does not account for metabolism of DBP (the yeast lack key xenobiotic metabolism enzymes) and hence the estrogenic potential might be higher if the metabolites of DBP have more estrogenic potential than DBP itself.

1.5.1.2 Anti-androgenicity

The developmental toxicity of DBP has strong similarities to AR antagonists. These compounds bind to and inhibit the biochemical functionality of the AR. The AR, much like the ER is a nuclear hormone receptor. Upon ligand binding and activation, the AR enters the nucleus and promotes the expression of male developmental growth factors.[62] AR antagonists competitively bind to the active site of the receptor, preventing binding of the natural androgenic steroid hormones. This inhibits activation of the receptor and hence prevents the development of healthy androgenic tissues. The effect of exposure to these compounds (e.g. vinclozolin) is remarkably similar to DBP exposure. Therefore, anti-androgenicity is a possible mechanism of toxicity.

The evidence, however, does not support DBP being an AR antagonist. While the effects of DBP exposure closely resemble the effects of exposure to an AR antagonists there are some key differences. The effects of DBP exposure appear to affect tissues not directly related to AR activity. This can be observed in the morphological differences in cryptorchidism. DBP appears only to affect the early stages of testes decent, (see Chapter 2 for a detailed explanation) which leads to transabdominal testes. AR antagonists affect the later developmental stages; particularly the AR-dependant stage inguinal (i.e. in the teste duct) descent of testes.[27] Prostate development is completely dependent on AR activation and embryological exposure to AR antagonists often leads to an absence of or poorly developed prostate in the male offspring.[27] However, the prostate is insensitive to DBP exposure, even at high exposure levels.[27] This is likely due to DBP having little binding affinity for the AR.[63,64] A recombinant AR binding affinity assay found DBP to have a 10,000-fold lower dissociation constant (the molarity at which there is equal amounts of bound and unbound receptor) of 8.2×10^{-4} M compared to 1.8×10^{-8} M for DHT (the natural substrate).[64] This demonstrates that DBP only very weakly binds to the AR. However, the assay does not account for the metabolism of DBP; therefore, there is the possibility of *in vivo* metabolites

having stronger binding affinity to the AR than DBP. Overall, the evidence is strongly against DBP being an AR antagonist.

1.5.2 Gene regulation

The nature of the toxic effects of DBP suggest that reductions in the biosynthesis of testosterone plays a major role; i.e. all the toxic effects are known to be caused by reductions in testosterone levels *in utero*. The biosynthetic pathway responsible for the synthesis of the steroid hormones is known as steroidogenesis.[65] The pathway has multiple branches (Fig. 1.4) and produces the estrogens, androgens and glucocorticoids (stress hormones). Steroidogenesis is under strict genetic control, with the flux changing in response to external stimuli and possibly through effects of EDCs including DBP (for a detailed explanation of steroidogenesis, see chapter 5).

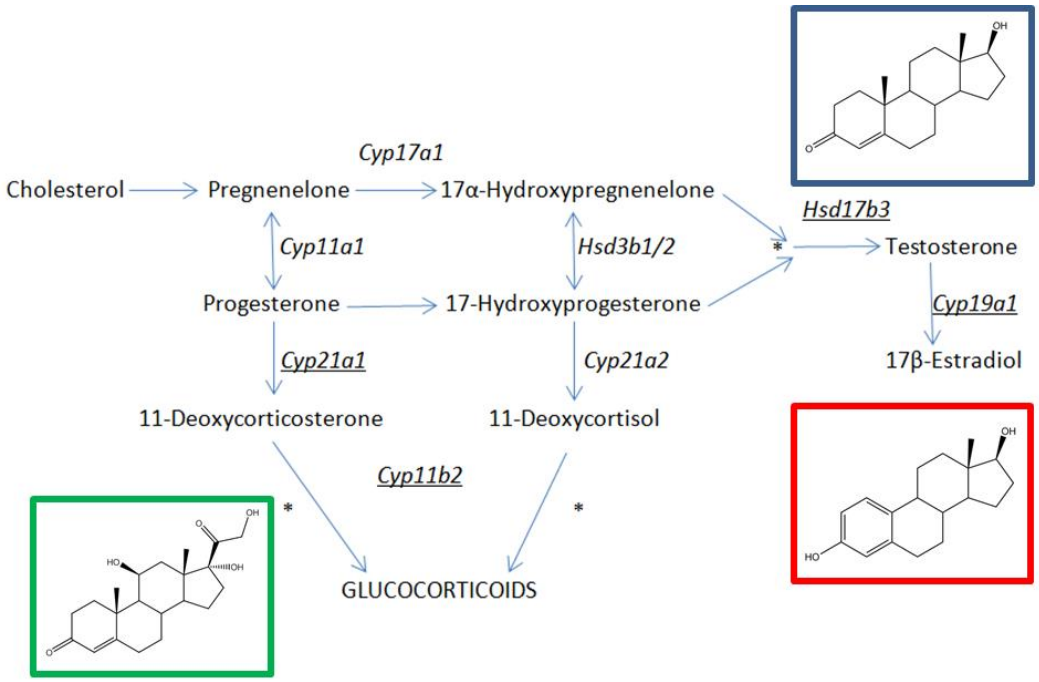


Figure 1.4: The steroidogenesis pathway (asterisks denote multiple steps). There are three main products: the glucocorticoids (e.g. cortisol, green) regulated by the expression of *cyp21a1* and *cyp11b2*, the androgens (e.g. testosterone, blue) regulated by *hsdb3* and *hsd17b3* and the estrogens (e.g. E2, red) metabolised from testosterone under control of *cyp19a1*.

Steroidogenesis begins with cholesterol uptake into the cell via the transporter, steroidogenic acute regulatory protein (StAR).[66] Cholesterol is then converted into different products under the control of several key genes which regulate the flux of the pathway such as: 11β-hydroxysteroid dehydrogenase (*cyp11b1*) and steroid 21-hydroxylase (*cyp21a1*) for the glucocorticoids, 3β-Hydroxysteroid dehydrogenase (*hsdb3*) and 17β-Hydroxysteroid dehydrogenase (*hsd17b3*) for the androgens and aromatase (*cyp19a1*) for the estrogens.[65] Changes in expression of these genes could lead to major changes in the flux of the pathway, leading to a disruption of the steroids metabolism. Therefore, it is highly possible that a change in gene expression could occur following DBP exposure, leading to diminished testosterone production.

Exposure to DBP at normal population daily exposure levels has been shown to increase cortisol production in R2C cells (Leydig cell line). R2C cells have limited expression of the glucocorticoid genes.[67] Therefore, the irregular cortisol production is indicative of a change in flux of the steroidogenesis pathway whereby glucocorticoid synthesis is favoured over androgen production. This could occur through a down-regulation of *hsd17b3* and/or the up-regulation of *cyp11b1*. An *in vivo* postnatal rat study found linear increases of *cyp11b1* in homogenates of the testes in relation to increasing DBP exposure correlating with a linear decrease in serum testosterone levels and increases of serum glucocorticoids.[68] Western blot analysis found that the glucocorticoid receptor (Gr) protein also had a significant increase in expression, demonstrating that exposure to high levels of DBP affects the genomics, proteomics and metabolomics of glucocorticoid synthesis. A significant decrease in the expression of the gene that codes for StAR was observable following exposures of 1000-2000 mg DBP/kg bw/day (the typical LOAEL).[68] Continuous StAR expression is required for the steroidogenesis pathway to function effectively. Reductions in StAR protein expression could significantly reduce the capacity of the pathway to produce steroids.

Glucocorticoids do not have a role in sex differentiation and so an overexpression of the glucocorticoids at the expense of testosterone could account for the anti-androgenic like effects of DBP exposure. If testosterone and DHT (which control sexual differentiation *in utero*) levels are diminished there could potentially be a disruption of the sensitive androgen:estrogen balance.

Changes in the expression of the intermediate genes could play a role in the reductions in testosterone by limiting the overall steroidogenic capacity of the pathway. Reductions in the expression of the genes that code for the cholesterol side-cleavage enzyme ($P450_{scc)}$), 17α-hydroxylase (CYP17) and StAR were observed along with an increase of *AR* expression and large reductions of serum testosterone levels following embryological exposure at 500 mg DBP/kg bw/day,.[69] $P450_{scc}$ and CYP17 are important initial intermediates, although they do not regulate the flux of the pathway.[69] Increases in *AR* expression is likely not in response to AR stimulation but rather a scavenger mechanism in response to drastically lower testosterone levels. This has been demonstrated in prostate cells following exposure to the xenoestrogen, BPA.[70]

The final steps in androgen biosynthesis are regulated by three genes, *hsd3b, hsd17b3* and *5α-reductase*. The last step in androgen synthesis is the production of DHT from testosterone by 5α-reductase. Embryological exposure to 500 mg DBP/kg bw/day has been shown to induce a 2-fold decrease in the gene expression of *hsd3b.* Embryonic exposures of 500 and 700 mg DBP/kg bw/day leads to significant decreases in the gene expression and protein synthesis 5α-reductase.[71] Male sexual development is dependent on the expression of testosterone and DHT *in utero.* Reductions in the expression of *hsd3b* and *5α-reductase* could reduce the capacity of Leydig cells to produce adequate testosterone.

Interestingly, the gene and protein expression of ERα has been shown to increase following exposure to DBP.[71] Increases in expression of ERs are usually in response to estrogenic activity. Therefore, DBP could

be acting as a xenoestrogen. The increased synthesis of E2 via an increase in expression of *cyp19a1* could account for an increase in ERα synthesis (i.e. Testosterone is being converted to E2). To date there are no major studies published regarding *cyp19a1* up-regualtion in response to DBP exposure.

The consensus among the gene expression studies is that the steroidogenic pathway is particularly sensitive to DBP exposure *in utero* with multiple changes in expression of key genes. Overall, the gene expression changes likely affect the steroidogenic potential of Leydig cells; particularly through the reductions in expression of the key genes responsible for testosterone and DHT synthesis.

1.5.3 Epigenome modification

The epigenome is superimposed on the coding regions of DNA and contains regions of heritable genetic information, sensitive to behavioural and environmental influence.[72] The epigenetic modifications occur through chemical changes to cytosine bases and to histone proteins (proteins that package and order DNA in the nucleus). These modifications can alter the overall accessibility and structure of DNA, allowing for a wide array of genomic states that differ among tissues, developmental and disease states.[73-75] Cytosine modifications typically occur as methylations on the accessible backbone of DNA via action by DNA methyl transferase. These modifications are thought to be a mechanism of heritable promotor silences in response to the environment.[73] Histone modifications are more complex and typically occur at the tail of the protein, which is more unstructured and accessible. There are other common chemical modifications; e.g. phosphorylation (serine and threonine),[76] acetylation (lysine)[77,78] and methylation (lysine and arginine).[79] Epigenetic changes are non-permanent, non-coding modifications of DNA that are inherited by several generations via the modification of the parents' gametes.[80]

The toxicology of DBP suggests a transgenerational mechanism whereby individuals exhibit the toxic effects of DBP without having been exposed.[20] This may occur through modifications of the promotor

regions of the AR or, via changes in the region that expresses male growth factors (e.g. the androgen tissue dependant protein, Insulin-like factor 3, INSL3).

The transgenerational toxicity of DBP has been observed in the fourth generation of rats (F3).[81] The first generation (F_0) had *in utero* exposure with expected developmental effects (e.g. TDS, hypospadias). These effects continued into the F_1-F_3 generations in much higher incidence compared with controls.[81] Analysis of DNA epimutations (methylations) found 197 unique modifications compared with controls.[81] Epigenetic transgenerational effects of DBP have been linked to high level human exposure of the F_0 generation.[20] The male offspring of the New Zealand Malayan Veterans (see Section 1.4.4) appear to have inherited epigenetic toxicity as the rate of incidence of DBP-like toxic effects in their male offspring is nearly 5-fold higher than the general population.[20] However, no epimutational studies have been conducted on the veterans to date.

The epigenetic toxicology of DBP is clearly demonstrated in animals and is thought to have occurred in one human case example. The overall picture of the mechanism of toxicity appears to occur through a range of different mechanisms that combine to reduce potential of Leydig cells to synthesize adequate testosterone during key stages in embryonic development. A combination of epigenetic, genomic and estrogenic effects could facilitate anti-androgenicity through a mechanism which does not act upon the AR.

1.6 Research Aims

The aim of the present study is to examine two potential mechanisms of toxicity of DBP; namely gene regulation and estrogenicity utilizing a rat Leydig cell line (LC-540). This will be achieved via the following research goals:

- Develop the LC-540 culture procedure to maintain robust, long term cultures.
- Evaluate the suitability of the LC-540 cell line as a toxicological model (e.g. testosterone production).
- Determine the appropriate levels of DBP exposure to best evaluate the effects that could feasibly occur in normal human populations.
- Investigate the effects of these exposure levels on the Leydig cell growth and appearance.
- Identify any possible estrogenic metabolites and model their analogy with E2 in the ER using ChemBio 3D.
- Determine the metabolism of DBP in LC-540 cells over 24 hours using HPLC.
- Investigate the potential estrogenicity of DBP in a MCF-7 proliferation assay.
- Investigate the gene regulatory effects of DBP using the NanoString nCounter assay.
- Evaluate the effects of the gene regulation on the production of testosterone using a testosterone ELIZA.

1.7 Materials

1.7.1 Chemicals

- Dibutyl phthalate (Sigma-Aldrich New Zealand Ltd, Manukau City, New Zealand)
- Analytical Grade Ethanol (ECP ltd, Auckland, New Zealand)
- Analytical Grade Acetonitrile (ECP ltd, Auckland, New Zealand)
- Analytical Grade Diethyl ether (ECP ltd, Auckland, New Zealand)
- Milli Q water
- Benzyl penicillin (Sigma-Aldrich New Zealand Ltd)
- Streptomycin sulphate (Sigma-Aldrich New Zealand Ltd)
- Sodium Bicarbonate ($NaHCO_3$, (ECP ltd, Auckland, New Zealand)
- Sodium Sulphate ($NaSO_4$, ECP ltd)
- L-glutamine (Sigma-Aldrich New Zealand Ltd)
- Ethylenediaminetetraacetic acid (EDTA, ECP ltd)
- Ground dextran coated charcoal (Sigma-Aldrich New Zealand Ltd)
- Magnesium Chloride Hexahydrate ($MgCl_2.(H_2O)_6$, ECP ltd)
- Sucrose (ECP ltd)
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ECP ltd)
- Trypan blue (Sigma-Aldrich New Zealand Ltd)
- B-Mercaptoethanol (ECP ltd)
- Sodium Dihydrogen Phosphate Dihydrate (NaH2PO4.2H2O, ECP ltd)
- Disodium Hydrogen Phosphate (Na2HPO4, ECP ltd)
- Sodium Chloride (NaCl, ECP ltd)
- Thiomersal (ECP ltd)
- Inert N_2 Gas

1.7.2 Biological products

- Phenol red-free MEM powder (Sigma-Aldrich New Zealand Ltd
- Phosphate buffered saline (Sigma-Aldrich New Zealand Ltd)
- Fetal bovine serum (Life Technologies, Auckland, New Zealand; Catalogue number 10091-148)
- Trypsin powder (Becton Dickinson, Auckland, New Zealand)
- TrpLE® express (Life technologies)
- LC-540 rat Leydig cells (American Type Culture Collection (ATCC), Manassas, USA; ATCC number CCL-43)
- MCF-7 human breast cancer cells (American Type Culture Collection (ATCC), Manassas, USA; ATCC number HTB22)
- RNeasy Lysis Buffer (RTL)
- Bovine Serum Albumin (BSA)
- Testosterone Antibody (Canterbury Health Laboratories, CHL)

1.7.3 Equipment

- T-75 sterile culture flask (Sigma-Aldrich New Zealand Ltd)
- 10 µL Hamilton Syringe ((Sigma-Aldrich New Zealand Ltd))
- Glass Vials (4 mL, 7 mL, 20 mL)
- Eppendorf tubes (100 µL, 600 µL, 1.6 mL)
- 50 mL Centrifuge tubes
- Automatic pipette and Tips (10 µL, 100 µL, 1 mL)
- Sterile filter (Steritop-GP, 0.22 µm, polyethersulfone, 500 mL 45 mm Merck Millipore)
- Autoclave
- Laminar flow cabinet (Cytoguard CG2000 series, model CGA-180, Clyde Apac, Sydney, Australia)
- Inverted microscope (CKX41, Olympus, Melbourne, Australia)

- Microscope camera (Toup ltd)
- Toup camera software (Toup ltd)
- Haemocytometer
- Periplast plastic counting chamber (Thermo Fisher Scientific, Melbourne, Australia)
- Non sterile syringe filter (7.5mm non-sterile PTFE hydrophobic filter, Thermo Fisher Scientific, Melbourne, Australia)
- Centrifuge (multifuge 1 S-R, Heraeus, Hanau, Germany)
- Glassware (Separation funnel, Round bottom Flask, conical flask)
- Hot-plate stirrer
- Micro well plates (24, 96 wells)
- Schott bottles (100 mL, 500 mL, 1000 mL)
- HPLC (Dionex)
- C18 reverse Phase HPLC column (Phenomex, North Shore City, New Zealand)
- gDNA removal column
- nCounter cartridge (NanoString Technologies®, Seattle, WA)
- Custom nCounter CodeSet (reporter and capture probes, (NanoString Technologies®, Seattle, WA)
- GEN2 Digital Analyzer (NanoString Technologies®, Seattle, WA)
- ELISA (CHL, Christchurch, New Zealand)

Chapter 2 – The LC-540 Model System

2.1 Introduction

2.1.1 The Testes

The role of the testes in male fertility and physical development has been known since ancient times; particularly by Aristotle who noted the effects of male castration in 300 BC.[82] The testes are the reproductive organs of male mammals, responsible for the production of the androgenic hormones via steroidogenesis in Leydig cells and production of gametes through spermatogenesis in Sertoli cells.[83] The testes are paired organs structurally surrounded by two layers of connective tissue; namely the tunicia vaginalis and tunica albuginea, the latter of which evaginates to divide the testes into several hundred lobules. Within these lobules, spermatogenesis occurs inside the densely coiled seminiferous tubules which comprise the bulk of the testes. The immature sperm cells that develope in the seminiferous tubules are transported to the epididymis where they mature into reproductively active gametes. During ejaculation, the gametes are pushed by muscle contraction through the epididymis and transported out of the testes via the ductus deferens. The production of androgenic hormones occurs in the testes independently of spermatogenesis in Leydig cells through a complex multiproduct biochemical pathway (see Section 5.1.3).

2.1.2 A brief history of Leydig Cells

Franz von Leydig first characterized Leydig cells in the testes in 1850.[84] Leydig extensively studied the testes of many species, observing that small clusters of cells were consistently collected between the seminiferous tubules.[84] Leydig postulated the role of these cells as simply connective tissue. Other prominent researchers incorrectly believed Leydig cells to either be immature Sertoli cells or associated with lymphatic vessels and embryonic epithelial cells.[86] The consensus in the late 19th century was that Leydig cells aid spermatogenesis by providing energy to seminiferous tubules via metabolism of plasma nutrients (e.g. glucose).[86] Unsurprisingly, the prominent researchers at the time did not associate Leydig cells with endocrine functionality as the endocrine system as a whole was poorly understood. However,

between 1897 and 1905 Pol Bouin and Paul Ancel published four key articles explicitly linking Leydig cells and their endocrine functionality to controlling secondary sexual characteristics in males.[87] The key findings of their research were:

1. Leydig cells have the appearance of secretory cells (i.e. features of endocrine functionality).
2. Leydig cells are present in large numbers during key stages of embryonic development (i.e. likely to play a role in fetal development).
3. Leydig cells do not supply nutrients to the seminiferous tubules.

The first biochemical evidence for testosterone production in Leydig cells was published by Christensen and Mason.[88] Their research demonstrated the bioconversion of [^{14}C]-progesterone into [^{14}C]-androstenedione and [^{14}C]-testosterone in interstitial tissue isolated from rat testes. This proved unequivocally that Leydig cells, not Sertoli cells are responsible for the production of testosterone and are the primary factor that influence sexual growth and development of the male phenotype.

2.1.3. Fetal Leydig cells (FLCs)

Leydig cells are comprised of two distinct morphologies; namely fetal Leydig cells (FLCs) and adult Leydig cells (ALCs, see Section 2.1.4). The morphologies have differing functionalities which account for their diverse ultrastructural, topographic and biochemical properties. However, ALCs and FLCs are both essential in healthy male sexual growth and development from embryo and throughout adulthood. Sections 2.1.3-4 will describe the structure, ultrastructure and endocrine functionality of both morphologies.

FLCs develop *in utero* during weeks 7-8 of human pregnancies directly after the development of the testis cord. FLCs are essential during male embryonic differentiation; particularly in regulating the development of the Wolffian duct (the structures that form the male internal genitalia), external genitalia and testis descent.

2.1.3.1 Morphology of FLCs

Histological analysis has shown that FLCs typically form large clusters of oval shaped cells between seminiferous tubules. These clusters are in turn surrounded by a thin layer of fibryocytes and collagen between the individual FLCs of a cluster.[89] Human FLCs vary in size and shape, with some cells being small and circular with enclosed organelles, while others are larger, polyhedral shaped with less delimited nuclei and cytoplasm (i.e. less defined organelle boundaries).[90]

The ultrastructural features of FLCs have been elucidated by electron microscopy.[89] FLCs have round or oval shaped nuclei dense with chromatin and a central nucleolus. The cytoplasm contains an abundance of smooth endoplasmic reticulum (SER), mitochondria, membrane bound lysosomes and large lipid droplets (average diameter 0.9 µm).[89] There is a lack of rough endoplasmic reticulum (REM) and the Golgi apparatus is small. The function of SEM in part is the metabolism of lipids.[92] Therefore, the abundance of SER and the lack of REM are unsurprising as the key function of FLCs is to produce the steroid hormones (i.e. they are lipids). Therefore, an abundance of SEM is necessary for FLC function. The small size of the Golgi apparatus may be due to the lack of REM as the two organelles are closely related.[92] The surfaces of FLCs have very characteristic flat, polyhedral cytoskeletal protrusions. The clusters of FLCs are completely surrounded by the basal lamina which acts as connective tissue to fix the cell clusters between the seminiferous tubules.

2.1.3.2 Function of FLCs

FLCs produce a wide range of biologically active molecules and peptide hormones. The primary products (common to all Leydig cells) are the androgenic hormones, which are essential for healthy male sexual development during embryonic development; particularly the development of the Wollfian duct, external genitalia and the descent of the testis.[93]

The Wolffian ducts are the embryonic structures that develop *in utero* to form the male internal genitalia. The production of testosterone and DHT by FLCs plays a major role in the healthy development of Wolffian ducts. In humans, at approximately 7 weeks gestation, the Wolffian ducts form independently of FLCs and androgen hormones.[94] The FLCs develop shortly afterwards and begin excreting androgen hormones.[95] These hormones travel to the newly developed Wolffian ducts. The androgens bind to the highly expressed ARs in the cells of the Wolffian duct tissue, upregulating specific growth factor proteins which grow and differentiate the Wolffian ducts into several adjacent structures of the internal reproductive system; namely the epididymis, vas deferens and seminal vesicles (see below for a detailed explanation).[95] The external genitalia, the penis and urethra develop between weeks 11-24 of gestation[96] and their development is completely dependent of androgen hormones. Expression of *AR* is increased in the penile glans and the urethral epithelial cells of the embryonic genitalia. Testosterone and DHT bind to ARs to up-regulate the production of growth factor proteins, developing the penis by week 24 of gestation.[96]

FLCs also produce INSL3, a small peptide hormone which regulates testes descent. This process is essential for male fertility (i.e. spermatogenesis requires a lower temperature than the internal temperature of the human body). Testes descent is a two-phase process beginning approximately 10 weeks gestation in humans. The transabdominal phase and inguino-scrotal phase are regulated by INSL3 and androgen hormones respectively.[97]

During early embryonic development, the pre-differentiated gonads develop inside the urogenital ridge (a pair of dorsolateral ridges comprised early embryonic germ cells). The gonads are fixed in position by two structures: the gubernaculum and the cranial suspensory ligament (CSL).[97] The growth or recession of these ligaments is sexually dimorphic and regulated by different hormones in males and females. In males, the CSL must regress to initiate testes decent. The transabominal phase of testes descent begins shortly after Wolffian duct differentiation. This phase involves the regression of the CSL and elongation of the gubernaculum (a cord made of fibrous tissue that connects the fetal testes to the base of the scrotum) to penetrate the scrotum, forming the inguinal canal (the path that the testes descend into the scrotum). This process is regulated by INSL3 binding to and activating to the leucine-rich Leucine-Rich Repeat-Containing G Protein-Coupled Receptor 8 (LGR8), expressed on the surface of the intra-abdominal gubernacular mesenchyme cells.[97] The process is cyclic adenosine monophosphate (cAMP)-mediated with the activation of kinases inducing hyperplasia and tissue growth of the gubernaculum. The growth of the CSL is simultaneously inhibited. This prevents feminization of the gonads (i.e. development of the CSL helps fix the ovaries during female growth and development).[98] The transabdominal phase ends with the testes held close to the newly developed inguinal canal and scrotum. The inguinoscrotal phase begins after the completion of the transabdominal phase, beginning with the regression of the gubernaculum.

The inguinoscrotal phase begins at approximately 26 weeks gestation and finishes at 35 weeks after the testes enter the scrotum. The entire process is mediated by the androgenic hormones, testosterone and DHT.[95] The gubernaculum contains high expressed ARs which regulate the entire inguinoscrotal phase.[99] The androgens act as paracrine (cell-cell signalling hormones) factors in the gubernaculum cells and initiate regression of the extracellular matrices of the cells. This leads to degradation of the structural supporting materials of the cells (e.g. the fibrous proteins).[100] This alters the viscoelasticity (the property

of both viscosity and elasticity when under pressure or stress) of the gubernaculum such that the growth development of the other organs in the body cause intra-abdominal pressure, pushing the testes into the inguinal canal.[101]

2.1.4 ALCs

2.1.4.1 ALC morphology

The development of ALCs is triphasic with three distinct morphologies: neonatal Leydig cells (NNLCs), immature Leydig cells (ILCs) and mature ALCs.[102] The first four months of the male neonatal period is associated with high levels of testosterone produced by NNLCs which are present in high numbers.[89] The NNLCs have a very similar morphology to FLCs (i.e. organelles associated with steroid-producing cells); namely an abundance of SER and lipid droplets, localised RER, abundance and variability in size and shape of mitochondria.[103] The abundance of testosterone-producing Leydig cells after birth is likely due to continuing post-natal sexual differentiation of the central nervous system which develops at approximately four months post-gestation.[104] The presence of testosterone in the serum of males at this development period is essential in permanently masculinizing the male phenotype. Testosterone action prevents any reversal of sexual differentiation during the first 4 months of the postnatal period.[104]

After four months post-gestation, the NNLCs begin to regress into ILCs, the non-androgen hormone producing morphology of Leydig cells. ILCs are abundant from four months post-gestation until the late stages of puberty.[105] ILCs are small round cells that are morphologically distinct from FLCs and NNLCs. They have spherical nuclei with irregular membrane shape, more RER, few lipid droplets and a more developed Golgi apparatus.[105] Immunostaining studies found that ILCs have limited activity of 17β-HSD (a key enzyme in testosterone biosynthesis).[105] ILCs have a high activity of testosterone-metabolizing enzymes (e.g. 3-oxo-5α-steroid 4-dehydrogenases and 3α-hydroxysteroid dehydrogenase). Therefore, ILCs have steroidogenic capacity but can only producing limited amounts of testosterone. The

testosterone is typically converted into reduced forms of the androgen hormones (likely to prevent precocious puberty). The biology of ILCs is perfectly suited to their role of maintain low levels of testosterone required for prepubescent boys.

Fully mature ALCs are responsible for the majority of testosterone production during puberty. ALCs are directly responsible for the sexual maturity of males (e.g. maturation of the genitals).

2.1.4.2 ALC Function

The primary function of ALCs is to ensure the adequate production of both testosterone and DHT to regulate the sexual maturity during puberty (e.g. bone, tissue and hair growth).[106] ALCs fully develop at puberty and begin to produce and excrete large amounts of testosterone.[107] The high concentrations of testosterone act on the androgenic tissues throughout the body, initiating growth of the musculoskeletal system and development of the secondary sexual characteristics (e.g. deepening voice, hair growth). The increase in testosterone levels also affects the testes themselves; particularly the maturation of Sertoli cells (i.e. developing the machinery for spermatogenesis). Testosterone continuously acts upon the Sertoli cells to produce the gametes. ALCs are essential throughout adult life to maintain and regulate the male tissues and processes (e.g. spermatogenesis).

2.1.5 Use of Cell Culture to Study Cell function and Biochemistry

2.1.5.1 The Advantages of Cultured Tumour Cell Lines

The culture of primary cells (i.e. healthy cells isolated from tissue) is typically difficult to maintain in long term culture as they do not readily divide *in vitro* due to senescence (cellular ageing).[108] Therefore, primary cells have finite numbers of divisions after which, the cells begin to regress (i.e. lose their biochemical functionality) and undergo apoptosis. This is problematic when using cell cultures as a model system (a practise common in a variety of fields, from immunology to toxicology).[109] Therefore, the need to preserve the cells in culture is essential to modern science and so immortalized (cancer) cell

lines have been developed for a range of cell types. Immortalization can occur through several dysregulations; namely, cell cycle pathway breakdown (e.g. impairment of the signalling of p53/p16/pRb tumor suppression proteins), up-regulation of telomerase activity (i.e. elongation of the telomeres increases the stability of the chromosome, allowing for greater number of divisions) or by up-regulation of oncogenes and oncoproteins.[110] Many immortalization develop naturally in an organism (i.e. oncogenesis) and can be extracted and purified.[111] A notable example of this is HeLA cells (a cervical cancer cell line extracted from a patient named **HE**nrietta **La**cks) or Jurkat (T lymphocyte cell line isolated from a 14 year old boy suffering from T cell leukemia).[112] Isolated cancer cells can be further modified through the insertion or deletion of specific genes to develop specific pathways or impairments (e.g. the D1.1 derivative of Jurkat cells that does not contain surface receptor proteins of the parent cell line).[113]

Alternatively, viral vectors are often used to artificially induce immortality in cell lines of interest. Oncoviruses can be programmed to introduce genes that upregulate telomerase activity or inhibit normal cell cycle function.[109] Typically, the dysgenesis of cellular signalling or telomere functionality initiates a cell crisis; a signalling cascade similar to the cellular responses to DNA damage, resulting in apoptosis.[114] However, some cells can prevent the cell crisis through silencing of the DNA damage pathway (e.g. silencing of tumour suppression genes), producing a cell that escapes cellular senescence.[115,116] The resulting cells are immortal and can be cloned in culture over long periods without regression of growth. It is important to note that *in vitro* cultured cells can change over time as the cell machinery favours division over other biochemistry, potentially creating a clone that scarcely resembles the parent cell phenotype.

2.1.5.2 Tumour Cell Line Phenotype Changes in Culture

Tumour cells in culture are known to have genetic instability (i.e. can be affected by numerous genetic and epigenetic factors) which can give rise to distinct variants (e.g. karyotypically distinct cells) with a

selective advantage (e.g. superior immunological or metabolomic factors).[117] A variant with an advantage will typically outgrow the parent cell *in vitro,* a process known as clonal evolution.[117] The unstable genetic nature of cell lines *in vitro* allows for spontaneous epigenetic changes that can result in biochemical changes of specific cell signalling and functionality (e.g. endocrine cells which require external hormones for cellular growth). Such modifications can persist if they are favourable for continuous growth. The result of clonal evolution is usually the degradation of cell specific processes and direction of metabolic energy towards maximum proliferation efficacy.

The genomic changes in cell lines has been characterized in hepatic cell lines (BRL3A and NRL clone 9).[118] Hepatic cells are essential in xenobiotic metabolism and utilize a variety of CYP isozymes and conjugation enzymes (e.g. glucuronosyltransferases) in Phase I and II metabolism (see Chapter 4 for a detailed discussion of xenobiotic metabolism). Microarray analysis of 3984 genes in both cultured hepatic cell lines and whole liver found significant (i.e. $P > 0.05$ and fold change ≤ 10) changes in expression of 781 and 714 genes in BRL 3A and NRL clone 9 cells, respectively.[118] The genes up-regulated in the cell lines were those of structural proteins (e.g. proliferin, elastin, crystallin, and collagen), proteins involved in growth and differentiation (e.g., galectine, insulin-like growth factor (IGF) binding protein and IGF receptor). Conversely, the genes down-regulated were typically enzymes involved in Phase I and Phase II metabolism (i.e. CYP isoenzymes. sulfotransferases, and glucuronosyltransferases). Western Blot analysis of the two cell lines found no detectable protein levels of some CYP isozymes (e.g. 2B, 4A, and 2C11). The down-regulation of xenobiotic metabolism genes in the BRL 3A and NRL clone 9 cell lines illustrates the problematic nature of *in vitro* cultures; i.e. the downregulation of specific cellular functionality (BRL 3A and NRL clone 9 cells would be a poor model for hepatic degradation of xenobiotics *in vitro*, as they move away from normal cell function).

The surface and nuclear receptors of tumour cells is also highly susceptible to morphological changes *in vitro;* particularly, cell surface receptors involved in suppression of tumours. An analysis of 51 cell lines derived from human breast cancers found a wide array of modifications to key nuclear and surface receptors.[119] Gene expression of ER was negative in 32 of the 51 cell analysed. Western Blot analysis found 27 of the cell lines having reduced synthesis of ER. Similarly, the expression of the progesterone receptor was also transcriptionally negative in 46 of the 51 cell lines.[119] The surface protein human epidermal growth factor receptor 2 (HER2, an oncoprotein associated with breast cancer *in vivo*) was found to be expressed in 11 of the 51 cell lines.[119] The variety of different expressions of these receptors illustrates the differences between cancer cell and the parent cell from which they were derived.

MCF-7 is a breast cancer cell line commonly used as a model of estrogenecity of xenoestrogens as it is known to be ER positive (i.e. positive expression of ER). However, spontaneous epigenetic alterations can lead to loss of ER expression *in vitro* if there is loss of ER signalling. This is a direct result of CpG methylation of the ER promotor region of MCF-7 cells.[120] The loss of ER transcription likely results in a clone which is unresponsive to E2 in culture. The typical culture conditions of MCF-7 cells have limited sources of E2. This could lead to a loss of ER signalling and produce a non-ER responsive clone. This again illustrates the problematic nature of tumour cell lines; i.e. cell lines are sensitive to factors typically only found in culture conditions, potentially altering their biochemical framework in such a way that limits their efficacy as model system. These alterations are not always immediately obvious (e.g. the loss of ER expression in MCF-7 cells). Therefore, it is essential to ensure that the model system cell line ideally has fully intact biochemical machinery.

2.1.6 Leydig Tumour Cell Lines

There are several commercial Leydig cell lines available on the market today; particular of rodent origin (e.g. R2C, LC-540, MA-10).[121] These cell lines are typically naturally occurring neoplastic cells found in epithelial tumours of inbred rodents. They are isolated, purified and frozen to be sold for use in

scientific research. Human Leydig cell lines have yet to commercially available at the time of publishing this thesis. In humans the epithelial neoplasms are rare and slow growing.[122] Rodent Leydig cell tumours express platelet-derived growth factor (PDGF) in much higher levels than human Leydig cancer cells.[123] Therefore, it can be assumed that the poor growth characteristics of human Leydig cancer cells contributes to the lack of commercial availability.

2.1.6.1 Biochemical Changes in Leydig Cell Lines

As previously discussed in sections 2.1.2-4, the primary function of Leydig cells is the biosynthesis of testosterone to regulate male sexual development. Therefore, in order for a Leydig cell line to be a viable model system it must have two key biochemical factors intact: the steroidogenic machinery and the endocrine signalling system intact (i.e. the cells steroidogenic regulation). There are many cell lines available that have impairments in a one or both of these key systems (e.g. R2C cells).

A murine cell line, M5480 has been shown to produce detectable quantities of testosterone *in vitro* under basal conditions (e.g. without external activation by a gonadal stimulating hormone such as human chorionic gonadotropin, hCG).[124] This suggests that M5480 cells have an intact steroidogenesis pathway. When cultured in medium containing hCG there is a significant increase in testosterone production by M5480 cells. However, over time the M5480 cells begin to form clones which produce less androgen hormones in favour of progesterone. These derivatives (M5480A and M5480P) have distinct steroidogenic potential; e.g. M5480A produces equal amounts of testosterone and progesterone, while M5480P produces far more progesterone that testosterone.[124] Similarly, M5480A has a far greater binding affinity to hCG than M5480P. Analysis of 5 further derivatives: MA 10, MA 12, MA 14, MA 16 and MA 18 found that for three derivatives were unable to bind significant amounts of hCG and had limited testosterone production.[124] Incidentally, 2 clones were able to be stimulated by

hCG, with modest increases in testosterone production. However, these cells tend to produce 20a-dihydroprogesterone instead of testosterone.

These data illustrate the potential for Leydig cell lines have reduced expression of the regulatory and steroidogenic machinery in long term cultures.

2.1.6.2 LC-540 cell biochemistry

LC-540 cells are a Fischer rat Leydig tumour cell line that has been used as a model system for investigating the effects of endocrine disruption; particularly the anti-androgenic effects of environmental pollutants on steroidogenesis.[67,125,126] Unlike other Leydig cell line variants, LC-540 cells have minimally impaired steroidogenesis pathway and have been shown to produce testosterone at high levels compared with other Leydig cell lines.[127] This is in contrast to other Leydig cell lines; namely, R2C cells which produce glucocorticoids instead of testosterone.[67]

LC-540 cells have been shown to be transplantable into castrated rats *in situ* and produce detectable levels of testosterone. LC-540 cells have an intact steroidogenic pathway that has been demonstrated to produce testosterone *in vitro* for up to 52 passages.[128] The amount of testosterone produced without external stimulation is approximately 60 pg/10^6 cells, a 30-fold decrease compared to primary Leydig cell cultures.[127] Stimulation of LC-540 cells by hCG increases testosterone production slightly over at 48-72 h.[127] Interestingly, compared to primary cultures the response to hCG stimulation is approximately 100-fold less. Primary cultures can exert the maximum effect of hCG stimulation when only 1% of their receptors are occupied by gonadotropins. In contrast, tumour Leydig cells require 60-80% occupancy of the gonadotropin receptors.[129,130] This coupled with a reduction (80-90%) of the surface gonadotropin receptors,[129,130] likely contributes to the significant reduction in steroidogenic potential following hCG stimulation. As mentioned above, many Leydig tumour cells produce progesterone instead testosterone. This has been attributed to a reduction in CYP17 activity.[131] There are no published data

on the production of other steroids by LC-540 cells; therefore, it is unknown if they have the same deficiency in CYP17 activity.

2.1.7 The Use of LC-540 Cells as a Toxicological Model

LC-540s have been used as a model system for investigating the effects of many compounds on steroidogenesis and testicular apoptosis.[125-127,132-134] The wide array of applications of LC-540s supports their acceptance as a model system.

LC-540 cells have been used to investigate the effects of the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα).[125,126] The intact steroidogenic pathway allowed for the identification of the steroidogenic inhibitory effects of TNFα on the gonads. TNFα was demonstrated to reduce the gene and protein synthesis of 17B-HSD, 3β-Hydroxysteroid dehydrogenase (3β-HSD) and StAR.[126] Futhermore, the inhibitory effects of TNFα on steroidogenic gene regulatory factors; namely histone deacetylases 7 (HDAC7) were identified.[125] Therefore, it can be concluded that LC-540 cells are a good model system when investigating the expression of key genes and enzymes involved in steroid biosynthesis as changes in these genes following the administration of anti-androgenic compounds are quantifiable.

The mechanism of luteinizing hormone (LH) action is a cAMP-dependant G-coupled protein receptor (GPCR) signalling cascade (see Section 5.1.2). The activity of LH increases cellular cAMP which, in turn, increases the activity of cAMP-dependent protein kinase (PKA). Theses kinases activate promotors to upregulate the expression of steroidogenic genes. LC-540 cells have been used to characterize the effects of compounds on adenylate cyclase activity. Exposure to tributyltin (an anti-fouling paint used on ships) and Gossypol (a terpenoid aldehyde found in plants) was found to inhibit adenylate cyclase activity in LC-540 cells *in vitro.*[133,134] These studies demonstrate the viability of LC-540 cells as a model system for measuring effects of compounds on steroidogenesis and hormonal stimulation of Leydig cells.

The characterization and previous use of LC-540 cells as toxicological models strongly suggest they are appropriate for investigating the effects of anti-androgenic like compounds (e.g. DBP) *in vitro.*

2.2 Research Aims

The aim of the research described in this chapter is to develop and evaluate the LC-540 Leydig cell line as a model system to investigate the potential mechanisms of toxicity.

This will be achieved via the following research objectives:

- Develop a robust cell culture protocol that allows the culture of cells of many generations.
- Characterize the appearance and growth characteristics off LC-540 cells in culture.
- Analyse the steroidogenic functionality of LC-540 cells with and without hormonal stimulation and evaluate the suitability of LC-540 cells as a model system.

2.3 Methods

2.3.1 Sterilisation

2.3.1.1 Glassware and Consumables

All glassware and consumables including Schott bottles, glass pipettes, sample vials, micropipette tips and Eppendorf tubes were autoclaved at 120°C, 15 psi for 80 min. All autoclaved equipment was then dried at 75°C for 1 h prior to use.

2.3.1.2 Maintaining an Aseptic work surface

All cell culture procedures were conducted in a laminar flow cabinet with an internal work surface pre-sterilised by UV radiation (254 nM, 2 h). Immediately prior to any cell culture work, the internal surface was sterilised with 70% v/v ethanol aerosol. All equipment and reagents were sprayed with ethanol aerosol and immediately transferred inside the laminar flow cabinet.

2.3.2 Preparation of Cell Culture Media and Related Reagents

2.2.2.1 Preparation of Antibiotics

Benzyl penicillin (3.0 g) and streptomycin sulfate (2.8 g) were added to 100 mL of sterile MilliQ water and stirred using a magnetic stirrer for 24 h. The solution was stored at 4°C for up to six months.

2.3.2.2 Preparation of Phosphate Buffered Saline (PBS)

A packet of PBS powder was added to a 1 L Schott bottle. Milli Q (900 mL) was added and the PBS dissolved via vigorous shaking. The pH was adjusted to 7.4 using 1 M and 0.1 M HCL or NaOH as appropriate. The pH adjusted solution was topped up to 1 L with miili Q water. The solution was autoclaved and stored at 4°C.

2.3.2.3 Preparation of Trypsin Protease

NaCl (8.5 g) was dissolved in MilliQ water (1 L). Trypsin powder (25 g) was added to the 0.85% (w/v aq) NaCl (1 L) and stirred (using a magnetic stirrer) at room temperature for 1 h. The trypsin solution was sterilised by filtration and dispensed into 10-20 mL aliquots and stored at -20 C. EDTA (3.72 g) was dissolved in PBS (1 L) and sterilised by autoclaving. This PBS/EDTA (PE) solution was diluted 10-fold with

PBS and 90 mL of the diluted PE was mixed trypsin solution (10 mL) to produce the final trypsin solution (2.5% aq). This solution was stored at 4°C for up to 3 weeks.

2.3.2.4 Heat inactivation of Fetal Bovine serum (FBS)

FBS (500 mL) was thawed at 4°C overnight. The thawed serum was gently warmed in a 37°C water bath for 30 min with gentle inversion every 10 min to ensure even temperature distribution. After the serum reached 37°C it was placed in a 56°C water bath for 60 min with gentle inversion every 10 min. The serum was left to rest at room temperature for 30 min. Aliquots (100 mL) were transferred to Schott bottles and stored at -20°C.

2.2.3.5 Preparation of Charcoal-dextran Stripped FBS

$MgCl_2(H_20)_6$ (0.3 g), sucrose (85.6 g), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 2.4 g) was added to a 1 L Schott bottle. Milli Q water (1 L) was and the bottle was shaken vigorously. The solution was divided into 50 mL aliquots. Two aliquots were added to two 50 mL plastic centrifuge tubes each containing dextran coated charcoal (0.137 g). The centrifuge tubes were inverted 10 times to ensure even distribution of charcoal-dextran. The tubes were incubated for 24 h at 4°C. Following incubation, the charcoal-dextran solution was centrifuged at 500 xg for 10 min and the supernatant was discarded. FBS (50 mL) was added to each pellet. The tubes were inverted 10 times and incubated for a further 24 h. The FBS-charcoal solution was then centrifuged at 1700 xg for 10 min and the stripped FBS decanted into a 100 mL Schott bottle and stored at -20°C.

2.3.2.6 Preparation of Phenol Red Free Eagles' Minimum Essential Medium (MEM) Containing 10% v/v Stripped FBS

Heat Inactivated and charcoal-dextran stripped FBS (100 mL) was thawed at 4°C overnight. Phenol red free MEM powder (9.6 g), $NaHC0_3$ (2.2 g), L-glutamine (0.292 g) was added to a 1 L Schott bottle. Milli Q (800 mL) was added and the powder dissolved via vigorous shaking. The pH was adjusted to 7.4 (see section 2.2.2.2) and milliQ (100 mL) was added. FBS (100 mL) and antibiotics (5 mL) were added to the

solution. The culture media was then sterilised via ultra-filtration through a 0.22 µM filter and stored at 4°C.

2.3.3 Cell Maintenance and Passage

2.3.3.1 LC-540 Cell Seeding

A 1 mL cyro-vial containing frozen LC-540 cells was removed from storage in liquid nitrogen (-196°C) and thawed at room temperature for 15 min. MEM (20 mL) was added to a sterile 75 cm^2 culture flask using a flame sterilised 10 mL pipette. The defrosted cell suspension (100 µL) was added to the MEM using a micropipette and sterile tip. The cells were incubated at 37°C in 5% v/v CO_2 until they reached confluence.

2.3.3.2 Maintenance Cell Culture

LC-540 cells were routinely passaged when the cultures has reached confluence (approx. 10^7 cells). Spent MEM was vacuum aspirated using a flame sterilised Pasteur pipette. PBS (4 mL) was added to inactivate the any residual MEM. TrpLE® express (a proprietary trypsin solution, 3 mL) was added to detach the cellular monolayer. The culture flasks were incubated at 37°C with 5% v/v CO_2 until cells were seen to be fully detached under an inverted microscope. MEM (10 mL) was added to inactivate the TrpLE® express and the cell suspension was transferred to a 50 mL centrifuge tube. The suspension was centrifuged at 4000 xg for 5 min and the supernatant was vacuum aspirated. The pellet was suspended in fresh MEM (10 mL for each new culture) and vortex mixed for 10 s to produce a homogenous suspension. Cell suspension (10 mL) was added to a 75 cm^2 culture flask containing 10 mL fresh MEM to give a total volume of 20 mL. The cells cultures were incubated at 37°C (see 2.3.3.1)

2.3.4 Cell Counting

2.3.4.1 Hemocytometer

A 100 µL sample of cell suspension was collected from a known total volume of cell suspension. 10 µL of this suspension was mixed in a 1:1 ratio of trypan blue. A cover slip was placed horizontally across the

hemocytometer and 10 µL of the trypan blue-cell suspension was added to each chamber. Each chamber was filled with approximately 900 nL of cell suspension. The cells contained in the four 100 nL corner grids of both chamber (n=8) were counted using an inverted microscope at 100x magnification.

The total cell count was calculated using the formulae:

$$C_{mL} = \frac{T}{k} x\ D\ x\ 10^4 \qquad C_{Total} = C_{mL}\, x\ V$$

- C_{mL} = *Cells per mL*
- C_{Total} = *Total number of cells in the cell suspension*
- *T = total number of cells counted in 8 grids*
- *k = 8*
- *D = dilution factor*
- *V = total volume of cell suspension*

2.3.4.2 Periplast® Plastic Counting Chambers

Periplast® Plastic Counting Chambers were used to count the cells with large number of samples at any given time. A 100 µL aliquot from each sample of cell suspension was collected from a known total volume and mixed 1:1 with trypan blue. A 9 µL aliquot of each sample was added to the 9 x 9 grids (10 per chamber). The same 5 squares were counted for each of the 10 samples under an inverted microscope at 100x magnification. The total cell count was calculated from the formulae:

$$C_{mL} = \frac{T\ x\ 10^3}{k\ \ x\ N} \qquad C_{Total} = C_{mL}\, x\ V$$

- C_{mL} = *Cells per mL*
- C_{Total} = *Total number of cells in the cell suspension*
- *T = total number of cells counted*
- *k = 0.111*

- *N = 5*
- *V = total volume of cell suspension*

2.3.5 LC-540 Growth Curves

Growth curve measurements were done in triplicate over 10 days. Confluent cells (3 flasks) were removed from incubation and the cells detached using 4 mL of 2.5% trypsin and incubated for 5-10 min. Fresh MEM (26 mL) was added. The cells were divided into 1 mL aliquots and added to 1.6 mL Eppendorf tubes (n=24). 20 µL was taken from each Eppendorf and the cells counted (see section 2.3.4).

To normalise the number of cells in each well, the cells were seeded into a 24 well plate and diluted to ensure a concentration of 10^5 cells in each well. Fresh media was added to give a final volume of 2 mL of media/well. The plates were incubated at 37°C with 5% v/v CO_2. The 24 well wells were counted in triplicate on day 1, day 3, and days 5-10.

2.3.6 Cell Appearance

LC-540 cells were photographed at 400X magnification using a microscope camera.

2.3.7 Steroid Analyses

2.3.7.1 Preparation of hCG MEM

hCG (10 mg, 100 IU) was dissolved in 1 mL of fresh MEM. The 1 mL of hCG medium was diluted 10-fold 3 times to give a final concentration of 10 µg/mL. hCG media was added to fresh MEM (199.6 mL) to give a final concentration of 20 ng hCG /mL.

2.3.7.2 ELISA Buffer Preparation

$NaH_2PO_4.2H_2O$ (6.24 g), Na_2HPO_4 (8.66 g), NaCl (9.0 g) were dissolved in milli Q water (800 mL). BSA (1 g) and thiomersal (0.1) were added and the pH adjusted to 7.0 with HCl or NaOH as required. The buffer was made up to 1 L and stored at 4°C until required.

2.3.7.3 Cell seeding and incubation

LC-540 cells (18 flasks) were seeded in MEM (20 mL) with approximately 3 x 10^6 cells and incubated overnight. Following incubation, the MEM was vacuum aspirated and replaced with either control MEM (i.e. no hCG, n=9) or hCG-containing MEM (n=9). Every 24 hours, three samples of spent control and hCG MEM were collected. The media was centrifuged at 4000 xg/5 min and filtered through a 0.22µm syringe filter. The cells were detached using 2.5% trypsin and counted (see section 2.3.4).

2.3.7.4 Steroid extraction

The filtered, spent MEM (5 mL) was poured into a 50 mL separation funnel. Diethyl ether (15 mL) was added and the funnel was capped and shaken vigorously. The cap was removed and the layers were left to separate for approximately 2 min. The aqueous and organic layers were collected separately. The aqueous layer was returned to the separation funnel and the process was repeated 2 more times. In total the steroids were extracted using 3 x 15 mL of ether. The ether was dried with sodium sulphate (approximately 3 g) for 10 min. The ether was filtered into a fresh 250 mL RBF and evaporated under N_2. Fresh ether (6 mL) was added to the RBF and swirled to dissolve the extracted. The ether was then carefully poured into a 7 mL glass vial. Ether (1 mL) was added to the RBF and swirled before being added to the same 7 mL vial. The ether was evaporated under nitrogen and the extract was dissolved in ELISA buffer (500 µL). The samples were stored at 4°C until analysis.

2.3.7.4 ELISA Analysis

See Section 5.3.8

2.3.7.5 Statistical Analysis

The testosterone production was normalised against the cell count to produce the amount of testosterone per 10^6 cells. The standard deviation and standard error of the mean were calculated. A students' t-test determine the P values of the hCG-stimulated samples.

2.3.8 Cryopreservation

A flask of confluent LC-540 cells was removed from incubation. The monolayer was detached and pelleted (see 2.2.3.2). The pellet was suspended in MEM (500 µL) containing 17% v/v DMSO (100 µL). Aliquots (100 µL) were added to sterile 1 mL cryovials. Each cryovial was wrapped in cotton wool and placed at -80°C for 24 h before being stored in liquid nitrogen (-198°C).

2.4 Results

2.4.1 Growth Characteristics

The LC-540 cells exhibit a typical growth pattern consisting of an initial lag phase, a rapid log phase and finally a plateau phase (Fig. 2.1). The cells had a lag phase of approximately 4 days where the cells had a doubling time of 2.5 days. At day 5 the cells enter the log phase where the growth is at the maximum rate with a doubling time of 1 day. At day 7, the cells enter the plateau phase and the rate declined to a doubling time of 22 days. The growth curve indicates that the cells can be grown long term in culture.

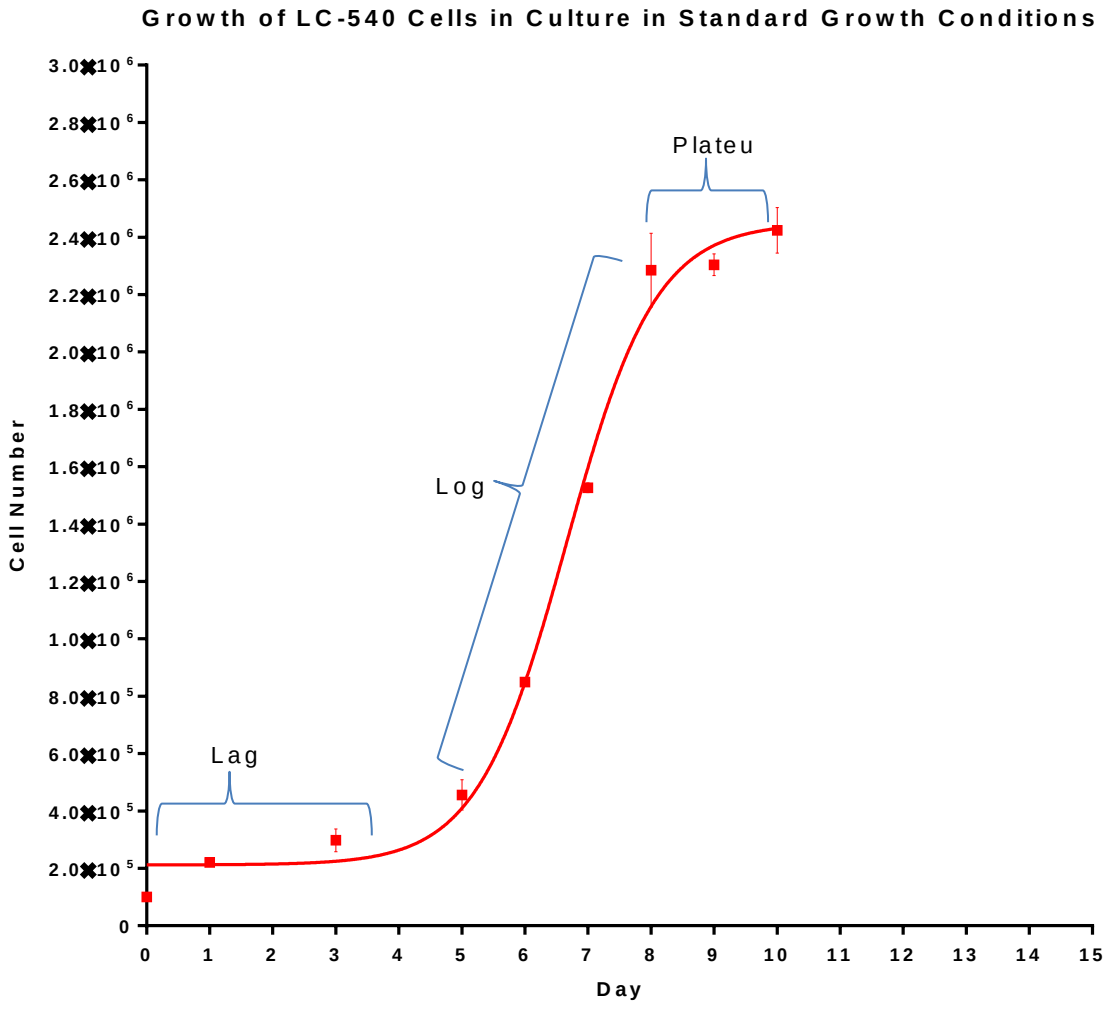
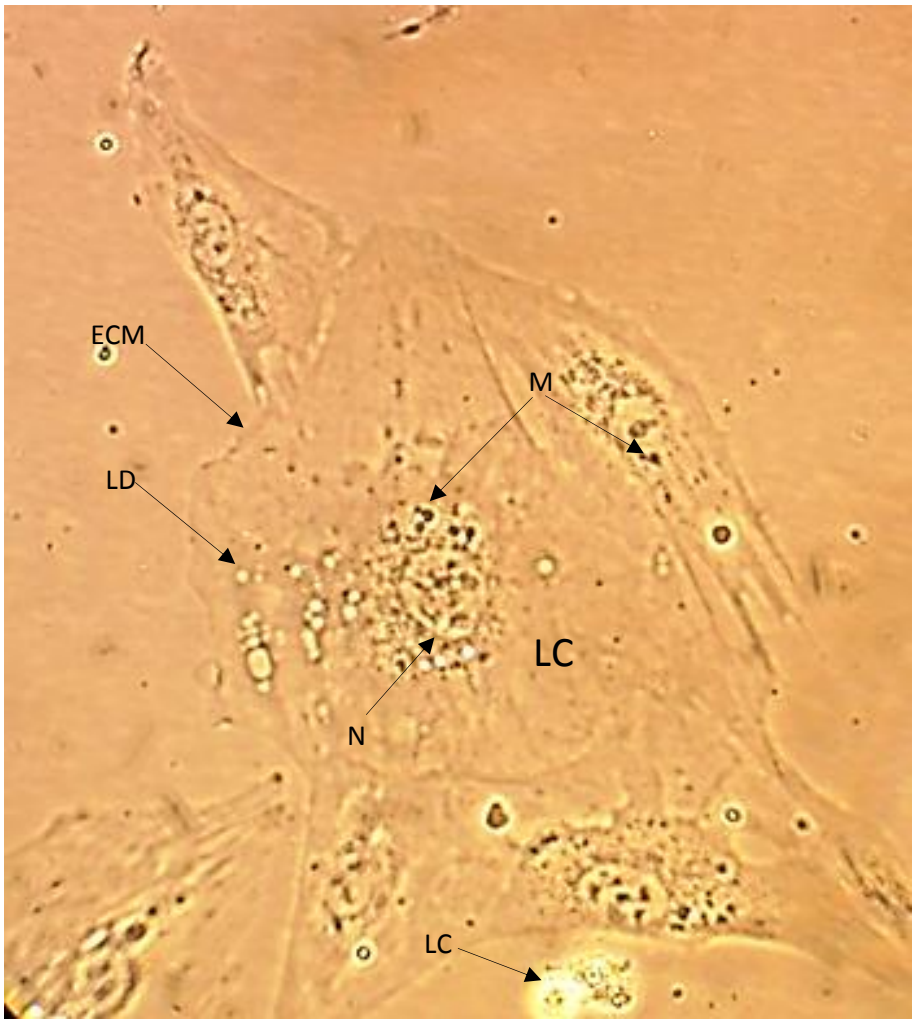


Figure 2.1: the growth of LC-540 cells in culture. The cells have typical three-phase growth pattern: lag, log and plateau phases

2.4.2 Cell Description

The LC-540 cells from a very thin, flat monolayer. The monolayer consists of wide spherical or oval shaped cells with a large extracellular matrices (ECMs) which overlap (Fig. 2.2). Like other Leydig cells, lipid droplets and what appears to be mitochondria can been seen throughout the cytoplasm. When detached from the flask surface the cells peel off in a single sheet of spherical cells before breaking up into individual cells.



ECM = Extracellular matrix
LC = Leydig Cell
N = Nucleus
LD = Lipid droplet
M = Mitochondria

Figure 2.2: LC-540 cells under 400X magnification. The tumour cell line appears to maintain some key features of primary Leydig cells such as lipid droplets.

2.4.3 LC-540 Steroid Production

LC-540 cells have the capacity to produce testosterone with and without hCG stimulation in culture over 72 h (Fig. 2.3). The levels of basal and hCG stimulated production is highest in the first 24 h. The testosterone produced by LC-540 cells over 24 h is 255 ± 47 pg/10^6 cells and 395 ± 26 pg/10^6 cells for the basal and hCG-treated respectively. The levels drop significantly at 48 h to 108 ± 29 pg/10^6 cells and 88 ± 35 pg/10^6 cells for the basal and hCG stimulated respectively. The levels do not vary much at 72 h with levels of 98 ± 32 pg/10^6 cells and 116 ± 24 pg/10^6 cells for the basal and hCG stimulated respectively.

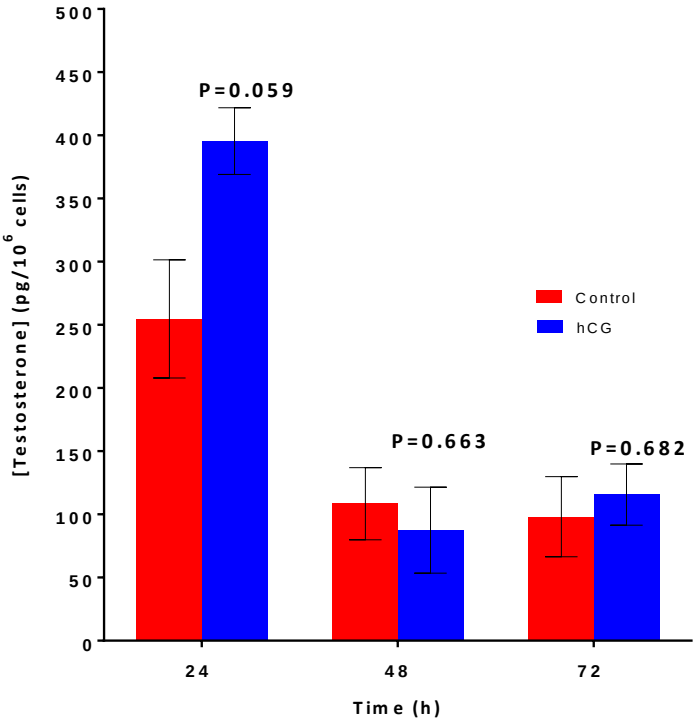


Figure 2.3: The basal and hCG stimulated production of testosterone by LC-540 cells over 72 h. The levels of testosterone are significantly higher in the first 24 h before dropping at both 48 and 72 h.

2.5 Discussion

The key aims of the LC-540 models system were to evaluate the growth, appearance and steroidogenic potential of LC-540 cells in culture and access their suitability as a model for DBP toxicity.

2.5.1 LC-540 growth and Appearance

The results of the LC-540 growth curve (Fig. 2.1) clearly illustates that LC-540 cells can be maintained long term in culture. This demonstrates that the culture procedure is robust and provides the required conditions and growth factors for long term cell maintenance. The long term maintenance of cells ensures the cellular biology and biochemistry remains intact. The appearance of the cells (Fig. 2.2) demonstrates that the cells are viable in culture and the biology remains intact. There have been has been one published study on the growth rates of LC-540 cells.[67] This study demonstrated that LC-540 cells have longer than typical lag phases. In comparison to most cell lines, LC-540 cells have a nearly 2-fold longer lag phase. This is perhaps due to the culture procedure which utilizes trypsin proteases to breakdown the extracellular matrix. It has been suggested that may cell lines only enter the rapid log phase in culture after ECMs begin to overlap. Therefore, it is possible that LC-540 cells are highly dependent on this phenomenon and require longer in culture to reach their log phase. The long term maintenance of LC-540 cells is essential in the present study as it is the basis for most of toxicological experiments. If the cells had limited growth in culture, they would not be a viable model system as the subsequent experiments relied on the constant expression of genes that code for enzymes that produce and metabolise DBP and synthesize testosterone. If the cells had not rapidly divided in culture is highly likely that the cells would have regressed and their biochemistry diminished. There have been no published studies on the appearance of LC-540 cells in culture; however, Leydig cells isolated from human and animal testes have been well characterized. The appearance of LC-540 cells in the present study demonstrate that the specific biology and biochemistry of Leydig cells (e.g. steroid producing organelles) are largely intact and that overall the cells are highly functional. There are key limitations in

both the growth curve and cell appearance methods. Firstly, the growth curves rely on small aliquots of cells and extrapolating to give a total count for a total volume of cell suspension. This process can have high error, and even in triplicate, can still be inaccurate. LC-540 cells tend to clump, even with the use of trypsin protease, clumps still persist. This can reduce errors in the extrapolated counts. A more robust procedure to improve the growth curve experiment and determine the total number of cells would be to extract and quantify the total DNA of the cells in the suspension. This would prevent the need to extrapolate from a smaller volume. The cell appearance in the present study was limited as only simple microscopy was used (i.e. 400x magnification). This only allows the visualization of some organelles. The identification of these organelles is done solely on appearance rather than histochemical reactions which can be more definitive. A technique to improve the overall microscopy would be cyro-electron microscopy. This would provide much more rich cellular detail and give a more clear indication of the biology and biochemistry of LC-540 cells.

A key aim of the present study was to characterize the LC-540 cells in culture and to assess their viability as a model system. It is clear based upon the results of the growth and appearance that LC-540 cells are indeed a viable in culture. The most important consideration is the steroidogenic potential of LC-540 cells in culture.

2.5.2 The Steroidogenic Potential of LC-540 Cells in Culture

The present study clearly demonstrated the potential of LC-540 cells to produce testosterone in culture with and without stimulation by hCG. Therefore, LC-540 cells are a viable model system or evaluating the toxicity of DBP. The stimulus by hCG is indicative of intact LH receptor (LHR) on the surface of the cells and a functional adenylate cyclase pathway. This demonstrates that expression of LHR on the surface of the LC-540 cells is maintained in culture. Research into other Leydig cell lines have demonstrated that the expression of LHR is diminished or completely absent. In those cell lines there is likely clonal evolution that selects clones that have reduced LHR expression in favour of other factors to

increase growth. The levels of testosterone are approximately 10-fold lower than isolated primary Leydig cells,[127] This is likely due to a reduction in expression of key genes in testosterone biosynthesis. Research into other Leydig cell lines have identified a significant reduction of CYP17 protein synthesis.[131] This is likely the case with LC-540 cells and explains the reduced testosterone production. There has been a study on the levels of testosterone produced by LC-540 cells with and without stimulation by hCG. In that study the levels of testosterone reached a maximum level of approximately 70 pg/10^6 cells at 72 h.[127] This is in contrast to the present study which reaches a maximum level of testosterone at 24 h. This could be due to the LC-540 cells in the present study having more steroidogenic potential compared with the LC-540 cells in the other study by Santucci et al.[127] The increased steroidogenic potential could be due to the LC-540 cells in the present study not undergoing clonal evolution and maintaining more steroidogenic potential. This is likely as the cells used were a low passage number (i.e. lower time in culture). The reduction over the subsequent 48 and 72 h is likely an artefact of the experiment. The testosterone excreted by the cells after 24 h is almost certainly re-entering the cell and interfering with steroidogenesis. Testosterone has been shown to be an inhibitor of both 17β-HSD and 3β-HSD. Therefore, if any testosterone re-enters the cell it could disrupt testosterone synthesis. The cell would likely respond by forming testosterone glucuronides the cell or by binding testosterone to steroid binding globulins. The study by Santucci et al[127] also demonstrated that LC-540 cells were able to be stimulated over 72 h by hCG. This is in contrast to the present study which has no significant changes after 24 h. This could be due to an increase in LHR in the present study. The steroidogenic capacity is increased and it is highly likely that the expression of LHR is also increased compared to the cells in Santucci et al.[127] Therefore, the hCG would undergo degradation much faster and so the levels of hCG at 48 and 72 h would be too low to have an effect.

There are some limitations in the present study. The testosterone levels are much lower than in primary Leydig cells. Therefore, to analyse the levels, the spent MEM needed to be concentrated from large

volumes. This extraction procedure is time consuming and would almost certainly have involved the loss of testosterone (up to 10%). This is typically overcome in other analyses (e.g. high performance liquid chromatography, HPLC) by the use of an internal standard. However, the use of an immunoassay prevents the addition of an internal standard. Therefore, there is no data on the recoveries following ether extraction. The levels of testosterone are normalised against cell count which can be erroneous (discussed above). Therefore, normalising the testosterone levels against quantities of DNA could be a more accurate and reliable method. The present study only measured the levels of free testosterone in spent MEM. More data is required on the levels of steroid hormone binding globulin levels as it is highly probable that a majority of the testosterone was bound to a globulin after 24 h. A glucuronidase digest could also be used to determine the levels of testosterone glucuronides.

The key aim of the present study was to evaluate the suitability of LC-540 cells as a viable model; particularly evaluating the steroidogenic potential of the cells with and without stimulation by hCG. It is clear that LC-540 cells are a viable model for DBP toxicity as they have an intact steroidogenic pathway capable of producing testosterone. The observable stimulation by hCG demonstrates the functional cell signalling pathway is intact and the expression of cell surface receptors is maintained. The growth, appearance and biochemistry of LC-540 cells in culture further suggest their viability as a model system for DBP toxicity.

Chapter 3 – DBP Exposure

3.1 Introduction

DBP is still widely used in many consumer, food and civil industry products, owing to its effectiveness in increasing both the plasticity and fluidity of plastics and its relatively low production costs.[1] Despite restrictive legislation in some industrialized countries (e.g. limits on acceptable food levels, banned in children's toys)[1], bio-monitoring data suggest ubiquitous and high daily DBP exposure to the general population.[135] Various studies[136,137] have linked the indirect exposure via DBP-containing food and consumer goods as the potential source of high daily exposure in adults.[130,131] Continued exposure to DBP (and other phthalates) could be having a sustained impact on the reproductive health of humans and other higher organisms receiving environmental exposure.

DBP production is still in the millions of tonnes annually and the many DBP-containing products are often discarded into the environment. The estimated environmental release in the USA was 177 tonnes into the water, air and soil in 1999.[138] Therefore, there is a risk of DBP leeching into the environment. This is of particular concern when phthalates are being leeched into the human water supply (e.g. into streams and water treatment facilities). The metabolism of DBP by microorganisms (Fig. 3.1) has been shown to produce MBP (i.e. a toxic metabolite of DBP) which could leech into the water supply, exposing humans to higher levels of MBP compared from DBP exposure alone. Chemical degradation also occurs through the de-esterification of DBP to phthalic acid by water.

Exposure to DBP (and other environmental pollutants) occurs through two distinct exposure routes: direct and indirect. Direct exposure is uncommon and involves the direct intake of these chemicals; usually during their manufacturing process.[139] Indirect exposure occurs when DBP leeches into the environment from DBP-treated products.

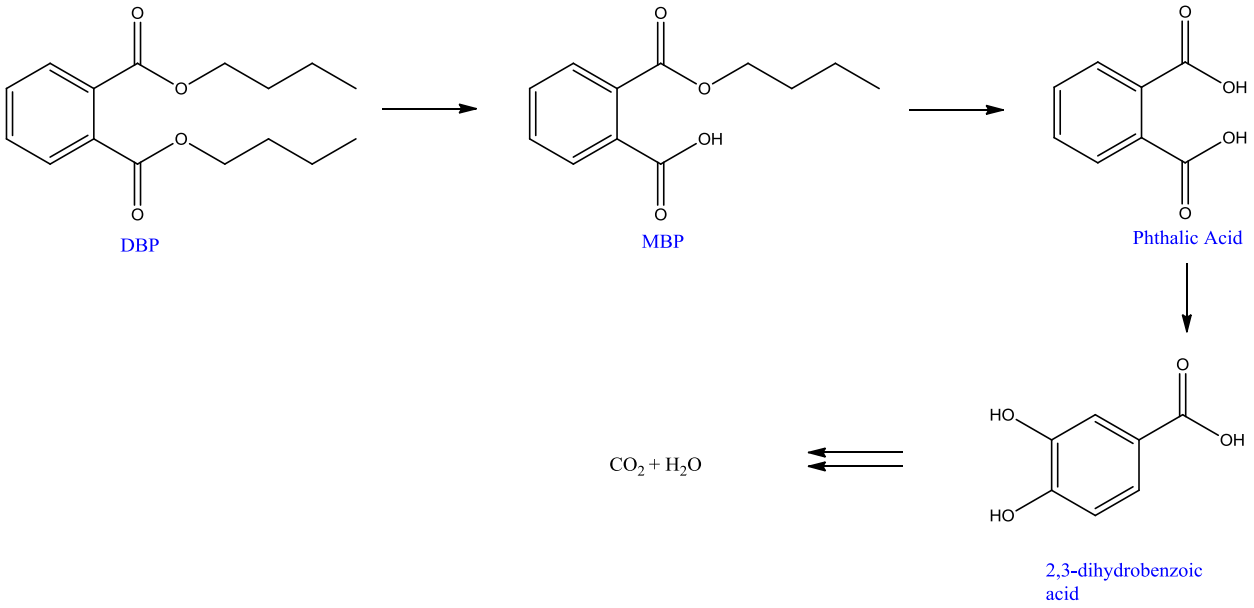


Figure 3.1: The Biodegradation of DBP in the environment

The exposure occurs through ingestion, inhalation (e.g. indoor air and dust) dermal (e.g. from cosmetics) or by leaching from PVC into water and soil.[137] As 100% of DBP is excreted by humans within 24 h,[135] there is little accumulation of DBP over time. Therefore, the daily exposure levels are a direct indication of the likely risk of posed by DBP exposure. The exposure levels are often compared to two established levels; namely the tolerable daily intake (TDI, the daily level of exposure that does not pose a risk long term risk and the MRL, the daily exposure level at which there is not likely to be any non-carcinogenic health effects.

3.1.2 Indirect Exposure

DBP is used extensively as a plasticizer in a variety of plastics such as PVC and nitrocellulose polymers (see Section 1.2). These plastics are cheap and easy to manufacture and so have become ubiquitous in modern times. Therefore, the risks of DBP (and other phthalates) leeching into the environment is a serious issue around the world. During the manufacture of plastics, DBP is incorporated into PVC and nitrocellulose. However, DBP is not covalently bonded to the polymer molecules bur rather held in place

by weak Van der Waals forces.[136] These intermolecular bonds can be easily broken at low temperatures (between 25-30°C). Therefore, it is highly likely that the DBP in some plastics will leeching over time.

3.1.2.1 Ingestion of DBP

3.1.2.1.1 Adult Dietary Exposure

Ingestion of DBP through food that comes into contact with DBP-containing products (packaging, etc.) has been demonstrated to be a major exposure route.[140] DBP-containing packaging can allow migration of DBP into the foods, particularly those that have a high fat content.[140] While modern packaging plastics typically do not contain DBP (likely due to consumer pressure), contact and contamination of DBP into food still occurs during processing; e.g. equipment used in food production is often made from or contains DBP, which can leach into food during processing.[140] The EU Scientific Committee for Food has set a guideline of 6 mg DBP/kg as the limit for acceptable DBP levels into food for human consumption.[141] A study conducted in the United Kingdom compared a high fat and a regular healthy diet for DBP intake.[142] Analysis of urinary metabolites suggested a considerable difference in high and low fat diets with levels of 31 µg DBP/kg bw/day and 13 µg DBP/kg bw/day respectively. Studies conducted in Switzerland[140] and Denmark[143] analysed daily DBP intake for an average and healthy diet. The calculated daily intake was considerably higher in the average diet; nearly a ten-fold increase compared to the reported DBP levels in the healthy diet studies. The large increase in whole diets is in accordance with European data on DBP levels present in individual foods.[140] Sugars and cereals, a large majority of the western diet, have high concentrations of 603 µg DBP/kg and 565 µg DBP/kg respectively. This represents significantly higher dietary exposure compared to levels in animal products, which are reported as approximately 100 µg DBP/kg.[142] These data suggest that if western diet trends continue (e.g. increasing daily sugar consumption) daily DBP exposure may also increase.

3.1.2.1.2 Infant Dietary exposure

Infants and toddlers appear to have the highest daily exposure to DBP of any age group.[140] Several studies have investigated the levels of DBP in the diet of children aged 0-3 years. New-borns have a primary diet of breast milk and/or infant formulae, while toddlers usually begin to eat baby foods between 6-12 months. The levels of DBP in breast milk is very low and of little concern. A study investigating DBP levels in the breast milk of German woman found a mean concentration of 0.82 µg DBP/kg, which gives a daily intake of approximately 0.6 µg DBP/kg bw/day assuming the mean consumption per day is 0.75 kg.[144] These results are in accordance with similar studies conducted in Canada[145] and Sweden[146] which reported DBP concentrations of 0.5 µg DBP/kg and 1.5 µg DBP/kg in breast milk respectively. Data from China[147] reported ten-fold higher concentrations of DBP in the milk of 40 women. The Chinese study reported mean concentrations of 54 µg DBP/kg which would indicate daily intakes for new-borns at 60 µg/kg bw/day,[147] under the MRL of 100 µg/kg bw/day.

Studies have been conducted to determine DBP concentrations in baby foods and infant formulae. An analysis of 11 different brands of baby food and infant formulae found baby food samples contained between 10-20 µg DBP/kg while the infant formula was slightly higher at 10-100 µg/kg.[148] This is consistent with an earlier study which reported 20-85 µg DBP/kg and 10-55 µg/kg for baby food and infant formula respectively.[149] With intakes of 60 µg DBP/kg bw/day via the ingestion of food diet contributes significantly to the observed high daily intake of infants. This along with the potential of other routes of exposure could pose a risk of DBP toxicity for infants.

3.1.2.1.3 Exposure in Drinking Water

DBP can migrate into drinking water from the DBP-containing plastics which holds water and other drinks that use phthalate esters as plasticizers.[144] The use and long term storage of drinking water in polyethylene (PE) and polyethylene terephthalate (PET) bottles has been shown have levels of DBP[150-152]. Analysis of water in both PE and PET bottles after 10 weeks storage found levels of DBP increased

from initially undetectable levels of DBP to 0.046 µg DBP/L for both bottle types.[151] Another study found that the levels of DBP in water stored in DBP-containing plastic bottles for 30 days was much higher at 11.33 µg DBP/L.[150] The study did not measure the DBP levels before the 30 day storage. Incidentally, the same study analysed DBP concentrations in soft drinks contained in the same type of plastic bottles and found a range of 9.00-26.75 µg DBP/L. Water stored over 5 months has been shown to increase the DBP concentration up to 20% from 2.0 µg DBP/L to 2.4 µg DBP/L.[152] Interestingly, the many studies have large ranges of DBP concentrations in drinking water. This could be due to large variations in the phthalate levels used to manufacture of plastics can be up to 50% w/w of the polymer.[153]

DBP has also been shown to contaminate drinking water when DBP-containing plastics are discarded into fresh water supplies or by PVC piping. Analysis of tap water in China[154] and Japan[155] found 1.4 and 2.4 µg DBP/L respectively. Tap water in California was found to be considerably lower, ranging from 1.44-8.34 µg DBP/L.[156] This is in agreement with tap water levels in Germany with ranges of 0.12 to 8.80 µg DBP/L.[157]

3.1.2.2 Inhalation

Since humans spend up to 80% of their time indoors, the Inhalation of compounds in indoor air and dust can represent a major exposure route.[158] Therefore, it is essential to evaluate the levels of contaminants including DBP in the household air and dust. As DBP is semi-volatile, it can be released the air or by coming into contact with dust and leaching into the particles.[159] DBP has been detected in the household dust in numerous studies.[160-165] Therefore, when humans inhale this air and dust, they are likely to be indirectly exposed to DBP.

A study of 120 homes in North Eastern USA found a range of DBP levels in household air. All the homes studied had some levels of DBP ranging from 0.052 to 1.1 µg DBP/m^3 air.[161] The levels in dust in the same study were found to be 20.1 mg DBP/kg dust. A study of 30 homes in Germany (selected by the

presence of PVC flooring, pipes, etc.) found a median level of 87.5 mg DBP/kg dust.[165] Another German study of 59 apartments and 74 kindergartens found median air levels of DBP at 1.1 and 1. 2 µg DBP/m^3 air respectively.[163] Analysis of the dust in the same apartments found 47 mg DBP/kg dust. The levels of DBP in 390 Swedish bedrooms found higher levels of DBP with a median of 150 mg DBP/kg dust.[162] The levels of DBP are relatively low in these studies compared to studies in Italy[160] and Bulgaria[164] which had DBP levels of 799 and 7,860 mg DBP/kg dust respectively.

The data demonstrates the wide range of DBP levels in air and dust worldwide. The variability in DBP levels can likely be attributed to the consumer behaviour relating to the use of DBP-containing plastics in the home. Overall the consensus of the data is that household exposure via inhalation is a major source of DBP exposure.

3.1.2.3 Dermal absorption

The application of some DBP-containing products on the skin can facilitate the indirect exposure of DBP via dermal absorption. Cosmetics, personal care and cleaning products often contain DBP and are the most likely household products to contact the skin. There is a lack of data surrounding the levels of dermal exposure to DBP. The quantification of dermal absorption is challenging as there is a wide array of DBP-containing products that could come in contact with the skin.

A comprehensive study of 253 cosmetics and personal care products commercially available in Canada found detectable levels of phthalates in a range of products.[166] DBP levels were as high as 6.6 µg DBP/g of shampoos, body sprays, cleansing wipes and body lotions. However, nail polish levels were significantly higher at 24304 µg DBP/g polish.[166] Analysis of personal care and cosmetics available in Spain found levels of DBP in the some types of products as high as 141 µg DBP/g of product, with

particularly high levels of DBP in hand creams.[167] Analysis of nail polish available in South Korea found 3901 µg DBP/mL polish.[168]

The data demonstrate the ubiquity of DBP in personal care and cosmetic products. However, the risk of dermal DBP exposure is still an area of conjecture. Dermal absorption of DBP is slow in most areas of human skin.[169] Some areas (e.g. facial skin) pose a greater risk of absorption as these areas have much thinner layers of skin. Therefore, DBP-containing facial creams and shampoos (i.e. shampoo comes into to contact with the face during washing) can pose a greater risk of DBP absorption. There is significant higher levels of DBP in nail polishes compared to other cosmetics. Therefore, those who use large volumes of it daily (e.g. manicurists) have a much higher risk of DBP exposure. This has been demonstrated by the high levels of DBP metabolites found in the urine of manicurists.[170] The levels of DBP in nail polish can lead to exposure levels well above the TDI. Long term application of DBP-containing nail polishes could potentially pose a significant risk; particularly to pregnant woman where a low doses can have significant effects on the developing embryo.

It is clear that indirect exposure is the most common route of DBP exposure for the average human. There ubiquity of DBP ensures daily human exposure is almost unavoidable regardless of geographical location and consumer choices. The effects of DBP at the normal population daily exposure levels are not fully known.

3.1.3 DBP Daily exposure levels

3.1.3.1 Typical Human Exposure

Legislative variations and consumer behaviour is likely a major contributor to the worldwide variation in daily human DBP exposure. Normal population daily exposure levels are estimated by analysis of urine concentrations of DBP metabolites[1,171]. USA data over a six year period indicates the normal population daily exposure is estimated to be in the range of 0.08-113 µg DBP/kg bw/day.[149] A study conducted in

Germany over a period of 9 years established daily exposures in the range of 0.22-116 µg DBP/kg bw/day. [171]The maximum exposure levels in these studies are well over the TDI (10 µg DBP/kg bw/day)[172] and slightly above the MRL (100 µg DBP/kg bw/day).[1] Therefore, the acute daily DBP exposure in the USA and EU could pose a risk to the reproductive health of the general population. As China, North America and Europe account for the majority of the world's DBP production and use, the exposure levels elsewhere in the world is are presumably lower in comparison. Data from two studies conducted in Asia have shown a considerable lower daily exposure to DBP for adults.[173,174] The daily exposure in China and Japan ranged from at 8.70-12.5 µg DBP/kg bw/day and 1.20-2.20 µg DBP/kg bw/day respectively. The low levels in China are counter-intuitive (China is considered to be the world leader in DBP production) but suggests differing consumer behaviour of the Chinese people; i.e. less fatty diet and less use of cosmetics, etc.

The normal population daily DBP exposure levels are perhaps the most important to quantify as they provide insights into the real risk DBP poses to humans on a daily basis. However, they are much lower compared to occupational exposure levels which are upwards of 10-fold higher.[170]

3.1.3.2 Occupational exposure levels

Occupation exposure is uncommon and specific to DBP production or manufacturing of DBP-containing products. The exposure is unique as it represents the only source of direct exposure today. However, as the exposure is specific, the data published to date is scarce. One study analysed several industries involved in either DBP production or used products containing high levels of DBP.[170] The study found large increases in the levels of DBP metabolites in the urine of workers in those industries compared to the general population. The levels of DBP exposure in workers involved in commercial DBP synthesis, rubber gasket and rubber hose manufacturing are 25, 26 and 10-fold higher respectively than the normal population.[170] Interestingly, the same study found manicurists have a 2.7-fold increase in daily

DBP exposure compared to the average person. This has been linked to the high levels of DBP found in nail polish. The maximum levels detected in all the four industries all exceeded 100 µg DBP/kg bw/day. Therefore, those involved in these industries have a significant higher risk of DBP toxicity (e.g. poor semen quality, reduced testosterone levels).[175,176]

Most people are exposed to the ubiquitous environmental pollutant DBP daily in the developed world. Therefore, it is essential that the associated risk of daily and occupational exposure is fully understood. Presently, there is a consensus that the daily exposures levels pose no risk to humans.[1] However, as the mechanism of toxicity is not fully understood, there is potential for effects to humans at the daily exposure levels. It is clear the occupational exposure of DBP is at levels that could affect the reproductive health of workers involved in the manufacture of DBP-containing products. The long term exposure to DBP at low levels may have effects that have yet to be identified. Therefore, it remains important to evaluate the risk of DBP at both normal population and occupational exposure levels.

3.2 Research Objectives

The aim the research described in this chapter is to determine the DBP levels for use in the LC-540 cell exposures and evaluate whether the cells can tolerate these exposure levels. This will be done via the following research goals:

- Selecting the most appropriate exposure concentrations to reflect the general population exposure, occupational exposure, and New Zealand Malayan Veterans' exposure and calculate the concentrations to best represent the average human.
- Develop a robust and accurate exposure methodology to exposure LC-540 cells and prevent DBP contamination.

- Determine the cell growth response to the levels of DBP to evaluate their suitability in for gene expression and metabolism experiments.

3.3 Methods

3.3.1 Calculation of Exposure levels

The daily exposure level was calculated using a range of exposure data from normal human exposure levels.[149] The range of daily exposure is between 0.08-113 µg/kg bw/day. Therefore, the TDI was chosen as the daily levels as this in within the range and is a widely accepted value. The assumptions used to calculate the exposure levels are listed in Table 3.1.

Assumption	Result
Absorption	100%
Human Body Weight[177]	70 kg
Blood level[178]	75 mL/kg bw
General population exposure[1]	TDI
Occupational exposure[170]	10 x General population exposure
New Zealand Malayan Veterans[179]	50 x General population exposure

Table 3.1: Assumptions used to determine the exposure levels used in the experiments.

3.3.2 LC-540 Exposure

The exposure standards and MEM were prepared using only glassware. No plastics were used to prevent contamination of DBP. Two exposure mediums (1X and 2X) were prepared as appropriate. The 1X exposure was used for exposures in 75 cm^2 culture flasks. The 2X exposures were used during the growth curves in 24 well plates.

3.3.2.1 Preparation of Exposure Standards

The 5 mg/mL DBP exposure standard was prepared using a 10 µL Hamilton syringe to add DBP (4.76 µL, 5 µg) to a 4 mL glass vial. The DBP was dissolved in of 1 mL HPLC grade ethanol and inverted 20x. The remaining standards were prepared using serial dilutions (Table 3.2).

DBP Standard	Dilution Standard (volume used)	Ethanol
1 mg/mL	5 mg/mL (200 µL)	800 µL
0.1 mg/mL	1 mg/mL (100 µL)	900 µL
Control	0	1000 µL

Table 3.2: Preparation of the exposure standards

3.3.2.2 Preparation of Exposure medium

The preparations of the exposure MEMs are listed in Table 3.3.

Exposure Medium concentration	DBP standard (volume used)	Volume of fresh MEM
5 µg/mL	5 mg/mL (100 µL)	100 mL
1 µg/mL	1 mg/mL (100 µL)	100 mL
0.1 µg/mL	0.1 mg/mL (100 µL)	100 mL
Control	control (100 µL)	100 mL

Table 3.3: Preparation of the 1X exposure medium

The 2X exposure media was prepared similarly, however, 200 µL of each standard was added to the culture medium to give final concentrations of 0, 0.2, 2, 10 µL/mL of culture medium.

3.3.2.3 1X LC-540 exposure

The 1X exposure of LC-540 cells always occurred when cells were approximately 50-60% confluent in 75 cm^2 culture flasks. This was done to ensure the cells were not in the lag or plateau phase. The spent MEM was vacuum aspirated and replaced with 20 mL of 1X exposure MEM. The cells were left to incubate until required.

3.3.3 Growth Curves

The cell seeding procedure was carried out as outlined in section 2.5.4 (i.e. seeding 10^5 cells/well). The 2X exposure medium was used to allow for the normalization of the cells at 10^5 cells/well. Each well was topped up with 1 mL of the 2X exposure medium to give final concentrations of 0.1, 1 or 5 µg DBP/mL MEM.

3.4 Results

3.4.1 Exposure Level Calculations

The general population exposure DBP concentrations used in the present study was calculated from the following formula:

$$E_D = \frac{Ex\ x\ BW}{BV}$$

- E_D = Daily blood level (µg/mL)
- TDI = tolerable daily intake (µg/kg bw/day)
- BW = body weight (70 kg)
- BV = blood volume (5250 mL)

The general population blood level was calculated to 0.1 µg DBP/mL blood. The occupational exposure levels are 10 and 50-fold higher than the daily respectively; therefore, they were calculated from the general population exposure concentration to give 1 µg DBP/mL blood and 5 µg DBP/mL blood respectively. The absorption was assumed to be 100% and so the final concentrations of the exposure medium were 0.1 µg DBP/mL MEM, 1 µg DBP/mL MEM and 5 µg DBP/mL MEM for the general population, occupational and NZMVs respectively (Table 3.4).

Dose	Exposure level
Daily exposure	0.1 µg/mL culture medium
Occupational exposure	1 µg/mL culture medium
NZ Malayan Veterans' exposure	5 µg/mL culture medium

Table 3.4: The calculated exposure levels used in the exposure experiments

3.4.2 Determining the LC-540 growth rates following DBP exposure

The growth of the LC-540 cells following exposure to varying concentrations of DBP is shown in Figure 3.2. None of exposure levels have any major cytotoxic effects. The 0.1 µg DBP/mL, 1 µg DBP/mL exposure levels and control groups all appear to have the same growth patterns (e.g. comparable sigmoid curves). These groups all reach a maximum number of approximately 2.4 x 10^6 cells after 10 days. The 5 µg DBP/mL exposure level has a significant effect on the growth of the cells. The maximum cell number is diminished by 30% to 1.8 x 10^6. The lag phase is also slightly extended to 5 days compared to 4 for the control, 0.1 µg DBP/mL, 1 µg DBP/mL exposure levels

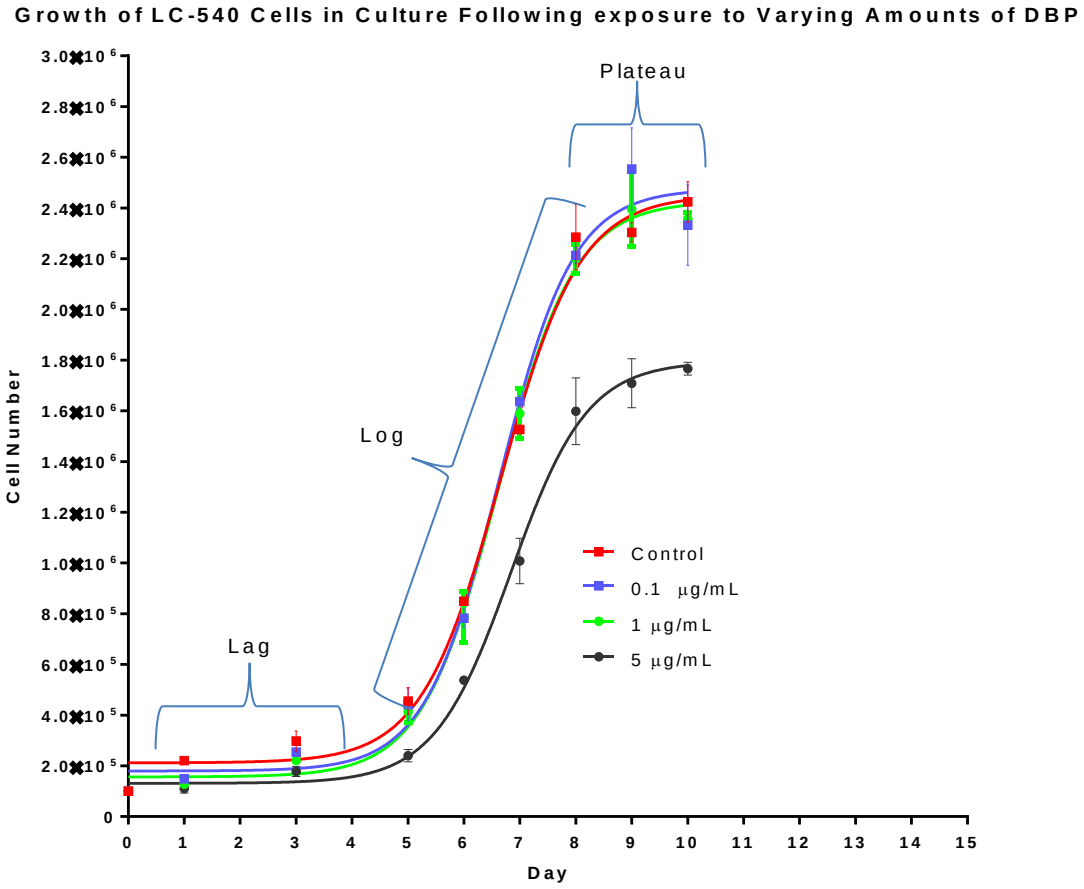


Figure 3.2: The growth of LC-540 cells following varying DBP concentrations. The growth rates are approximately the same for the controls, 0.1 and 1 µg DBP/mL. The 5 µg DBP/mL dose appears to have significant effects of the maximum growth of LC-540 cells.

3.5 Discussion

There are two key questions when investigating the effects of DBP on the LC-540 cells. Firstly, what are the appropriate exposure levels? Secondly, what unintended cytotoxic effects will these exposure levels have on the cells?

3.5.1 The Calculated Exposure Levels

The exposure levels in the present study were selected to best represent the daily human exposure levels that are or have been observed and published. The exposure levels were calculated on some common assumptions. The average person was assumed to be 70 kg[177] and have 75 mL of blood/kg.[177] The absorption of DBP into the blood was assumed to be 100%.

The normal population exposure level in the present study represents the best estimate based on recent exposure data. The aim of this calculation was to demonstrate the effects of DBP on Leydig cells at the TDI. There have been few studies published that evaluate the effects of DBP at level on animals. The exposure studies in animals typically involve doses that far exceed the normal human daily exposure. The animal studies have determined the NOAEL to be 100 mg DBP/kg bw/day[1]. Therefore, those studies have not detected any observable effects lower than the 100 mg DBP/kg bw/day. However, these studies often do not focus on the effects to the cells of the animals (e.g. genomic changes). Genomic changes following normal population daily DBP exposure have been shown in R2C cells.[67] Therefore, levels in the present study were chosen to further evaluate the effects of low level DBP exposure in Leydig cells.

The occupational exposure levels have been reported to be 10-fold higher than that of daily intakes. [170] Therefore it is logical that in the present study we multiply the daily dose the cells are exposed to (i.e. 0.1 µg/mL) to give the appropriate dose. This value represents the highest possible dose (outside of extreme isolated incidents) that humans can be exposed to in the present day. This level will give insights into the more extreme effects DBP can have on humans in modern times.

The final exposure level represents the dose that the New Zealand Malayan Veterans were exposed to during the Malayan Emergency.[179] The exposure of soldiers to DBP has been linked to reproductive malformations of their male offspring in multiple generations. Therefore, at this dose it is highly likely that real toxic effects could be observed. However, this dose appears to have significant cytotoxic effects in LC-540 cells.

There are limitations with the exposure level calculations. There was the necessity for a range of assumptions based upon widely accepted values. The occupational exposure is based upon limited data. The lack of other major occupational exposure studies could reduce the accuracy of the exposure level.

3.5.2 The Cytotoxicity of DBP in LC-540 Cells

The present study demonstrates that DBP exposure at the normal population and occupational daily levels do not have any major cytotoxic effects in LC-540 cells. The cell number and growth rate was unchanged following exposure at 0.1 and 1 µg DBP/mL concentrations (Fig. 3.1). Therefore, the DBP exposure does not have any major effects on the biochemistry of the cell. This is essential for the overall viability of the LC-540 model system. If the DBP exposure levels had a much larger cytotoxic effect on the cells, the validity of gene expression results would be questionable.

Model systems can produce artefacts of the experimental conditions that would not otherwise occur in humans. This is particularly problematic when investigating the toxicity of compounds that have been reported to be cytotoxic.[67] This was observed at the 5 µg DBP/mL exposure level. The cells had poor growth throughout the experiment at this exposure level. There were large numbers of dead cells present initially. This is similar to a study investigating the effects of DBP at this concentration in R2C cells. The author postulated that high doses of DBP can interfere with membrane fluidity.[67] The cells in the present study did recover over time and the viability increased. However, the growth potential was severely impacted with the log phase ending with 30% less maximum growth. This is similar to the

reported effects of another study which demonstrated significant reductions is cell growth after 72 h in culture at high levels of DBP.[180] The cytotoxicity of high levels of DBP can not only induce apoptosis, but severely impact the biochemistry of the cell. A cell that exhibits diminished growth in culture following exposure to high levels of DBP can have broad scale alterations to gene expression that are unrelated to the mechanism of toxicity of DBP. This could lead to many false positives in a gene expression assay and therefore, severely affect the validity of the results. The possibility of clonal evolution is particularly high; i.e. as there is a large initial die off, the stronger, DBP resistant cells are selected. These clones might prevent DBP from exhibiting much of toxic effects on the cell and diminish its effects and potentially give a false negative. It is clear that the LC-540 model system does not tolerate 5 µg DBP/mL of DBP. Therefore, it can be concluded that DBP mechanisms of toxicity experiments cannot be carried out at 5 µg DBP/mL.

There are limitations to the growth curve. As discussed in Section 2.5.1, the growth curves can have high error due to the nature of cell counting. Therefore, the quantification of DNA could provide a more accurate picture of the changes in growth following DBP exposure.

The aim of the research in this chapter was to calculate the best exposure levels for the DBP mechanisms of toxicity experiments and evaluate the cytotoxicity to LC-540 at these exposure levels. The results demonstrate that 0.1 DBP/mL and 1 DBP/mL represent the best estimates for normal population and occupational daily exposure levels. These levels do not have major cytotoxic effects of LC-540 cells. Therefore, these exposure levels are appropriate for use in DBP mechanisms of toxicity experiments.

Chapter 4 – DBP metabolism and Estrogenicity

4.1 introduction

4.1.1 Xenobiotic Metabolism

The ability of organisms to break down and excrete unknown and potentially toxic compounds is essential for survival. Nearly all organisms possess the biochemical framework known as xenobiotic (from the Greek '*xeno'* meaning strange and *'bioticos'* meaning related to living) metabolism which allows for the detoxification and excretion of most compounds.[181] The process has three key steps; namely, oxidation, conjugation and excretion.[182] The xenobiotic metabolism can have a role in disease. This occurs via with the modification of compounds (with low toxicity) into metabolites that are harmful to an organism.[182] A good example is the conversion of the procarcinogen (i.e. a compound converted into a carcinogen *in vivo*) benzo[a]pyrene into the more toxic, mutagenic benzo[*a*]pyren-7,8-dihydrodiol-9,10-epoxide (Fig. 4.1).[183] However, xenobiotic metabolism is essential in the detoxification of drugs (e.g. warfarin) and other compounds that could otherwise harmful or even fatal if left unmodified[184] (i.e. most drugs are lipophilic and cannot be excreted in the body's water-based excretion system).[185] When investigating the pharmacology of compounds, it is essential to understand not only the effects of the compound on the organism but also the modifications to the compound by the organism.

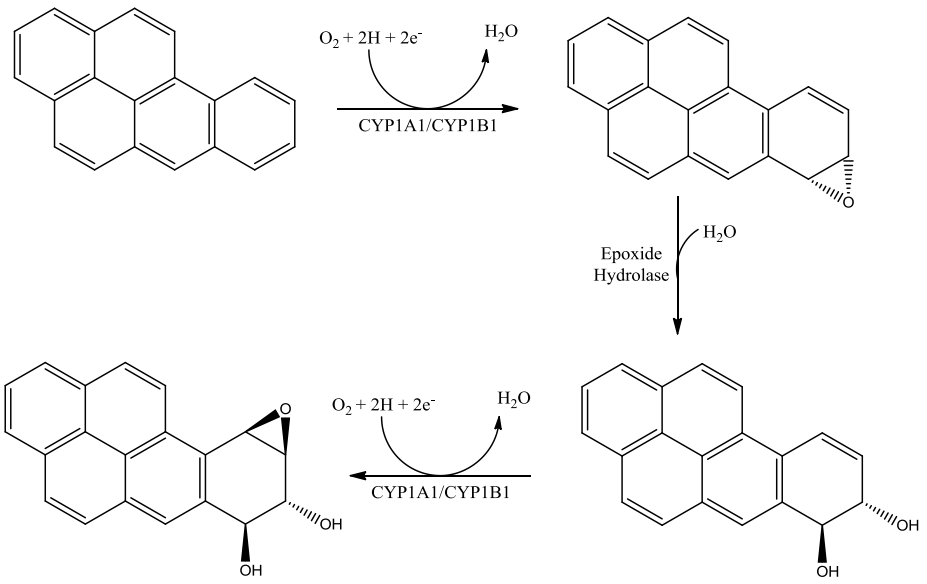


Figure 4.1: The bioconversion of benzo[a]pyrene to the carcinogen benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide. The resulting epoxide can intercalate into DNA causing irreparable damage to DNA resulting in cancer.

4.1.2 Phase I metabolism

The first phase of xenobiotic metabolism is an oxidation step whereby compounds are modified to increase their polarity. However, other reactions such as hydrolysis, reduction, cyclization or decyclization are used.[186] The primary objective of Phase I metabolism is to attach and activate nucleophilic functional groups to facilitate excretion or provide sites for conjugation (i.e. for Phase II metabolism).[186] The primary metabolising enzymes are the family of CYP monooxygenases. CYP enzymes are primarily localised the endoplasmic reticulum of hepatocytes; however, they are also present in most cell types (except the brain). The enzymes are all heme-thiolate proteins that incorporate Fe ions in catalysis.[187] The general reaction of CYP enzymes (Fig. 4.2) incorporates NADPH in a catalytic cycle (Fig. 4.3).[187]

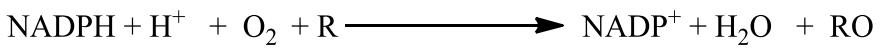


Figure 4.2: The general scheme of cytochrome P450 oxidation. The reaction uses NADPH as an oxidation/reduction cofactor. The result of the reaction is typically oxidation of xenobiotics.

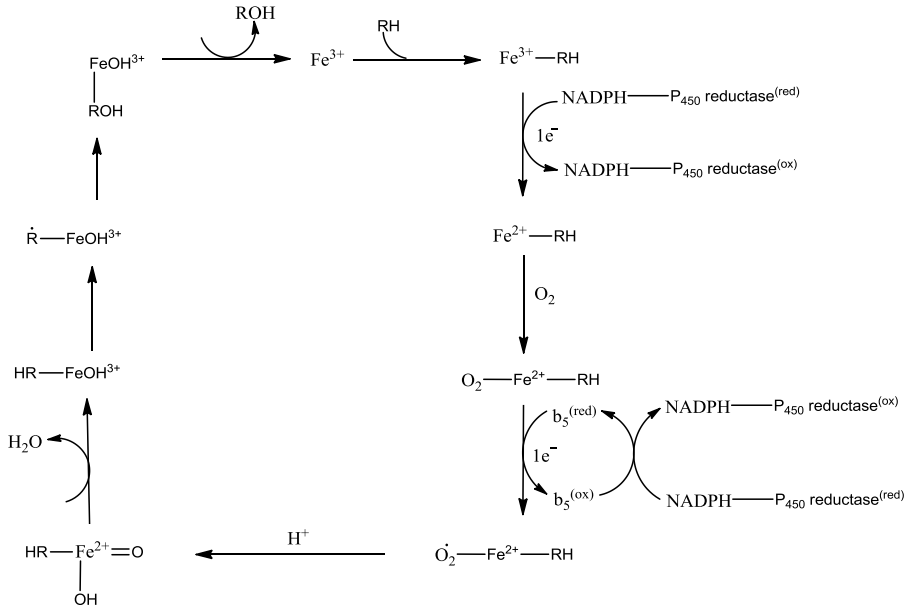


Figure 4.3: The CYP catalytic cycle. The cycle begins with a reaction between the heme Fe of the CYP and the xenobiotic. The Fe^{3+} in the active site undergoes reduction to Fe^{2+} with concomitant oxidation of the xenobiotic. The final step involves transfer of an oxygen and release of the oxidised xenobiotic.

4.1.2.1 Alkyl Hydroxylation

A common reaction of CYPs is the addition of an hydroxyl to an alkyl chain via carbon hydroxylation (Fig. 4.4).[188] The oxidation process can occur multiple times on the same site (e.g. carbon) to further convert alcohols into aldehydes and carboxylic acids. The process can also be repeated following loss of formaldehyde which reduces the length of alkyl chain to facilitate chain breakdown and reduce the lipophilicity of compounds (this is an essential part of fatty acid metabolism).[189] Carbon hydroxylation may be the final step before excretion[188] as this can produce a molecule of sufficient water

solubility.[190,191] However, most often excretion requires the addition of a conjugate to ensure the excretion in the urine or bile.

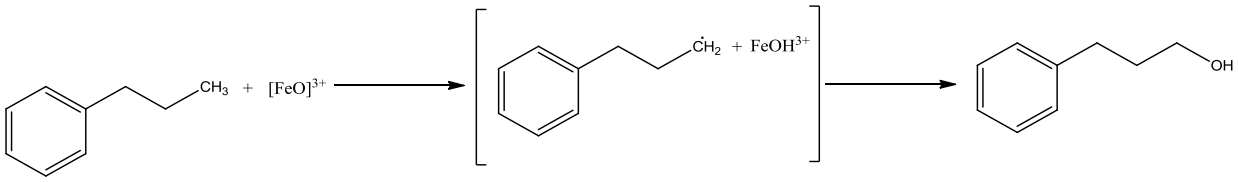


Figure 4.4: The enzyme-catalysed mechanism of an alkyl hydroxylation. The process goes through a radical transfer intermediate before forming the alcohol.

4.1.2.3 Oxidation of Unsaturated Carbons

The metabolism of unsaturated carbon bonds (e.g. alkenes, aromatic rings, etc.) is perhaps the most 'dangerous' of the Phase I metabolism processes. The formation of epoxide intermediates, particularly common among poly aromatic hydrocarbons (PAHs), potentially produces toxic compounds that can interact with DNA.[183] Epoxides are unstable and can spontaneously degrade or be hydrolysed by epoxy hydrolase to form phenols (Fig. 4.5).[192] As epoxides are unstable they have not been isolated in metabolism studies, however they're presence is inferred by the formation of the phenoxy compounds and their conjugates. The mechanism of epoxide and phenol formation is not fully understood; however, there are several possibilities such as carbocation (e.g. S_n1 reaction), radical or carbon-Fe intermediates.[192]

There are many other Phase I processes to increase the polarity of xenobiotics, however at the end of Phase I the compounds are often unable to excreted without the conjugation step of Phase II metabolism.

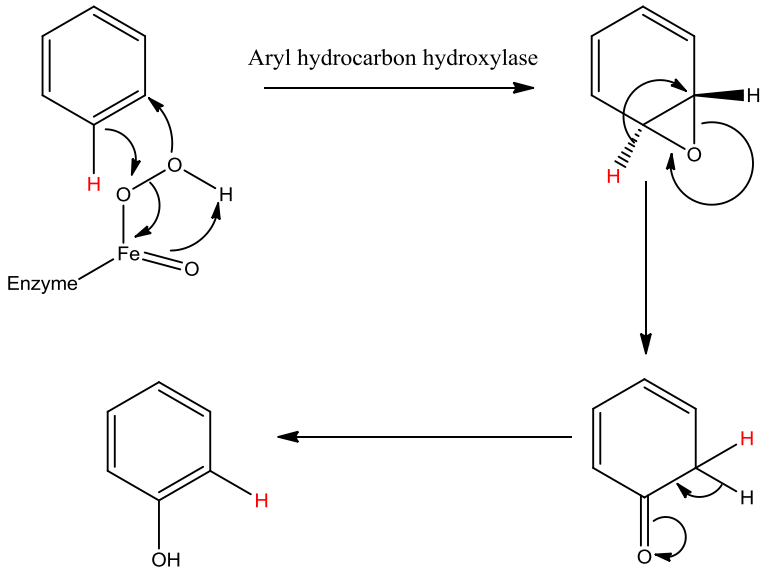


Figure 4.5: A potential scheme of unsaturated carbon epoxidation and hydroxyl formation. The epoxide forms first before being broken down in 1,2 shift of an aryl proton.

4.1.3 Phase II metabolism

The primary function of Phase II metabolism is to conjugate the xenobiotics to highly water soluble moieties that both increases water solubility and obliterates toxicity. This ensures their excretion and inactivates any pharmacological properties of the compound (i.e. Phase I metabolism leaves the molecules largely unmodified which often maintains the xenobiotic's biochemical activity).[193] The enzymatic conjugation reactions form much more water soluble products that and typically excreted via specially designed transfer proteins.[193] The conjugates are typically glucuronide, sulfide, acetyl, glutathione and amino acid (e.g. glycine) moieties. The enzymes that facilitate conjugation are transferases that utilize a variety of cofactors. The conjugation mechanisms vary between organisms, with humans primarily having glucuronide or glycine conjugation (40-70% of all drugs are glucuronidated).[194]

4.1.3.1 Glucuronidation

Glucuronidation, the most important of the Phase II metabolism processes utilizes a superfamily of Uridine diphosphate (UDP) glucuronyltransferases (UGTs).[195] The UGT superfamily has 117 mammalian isoforms (humans have four). All UGTs are membrane bound proteins that catalyse the covalent O-linkage between uridine-5′-diphospho-α-D-glucuronic acid (UDPGA) and nucleophilic carbons, alcohols, amines (primary, secondary, tertiary), thiols and carboxylic acids (Fig. 4.6).[195,196] Interestingly, the reaction involves the inversion of the stereochemistry of glucuronic acid from α (when bound to a UGT) to form β-D-glucuronides.[195,196]

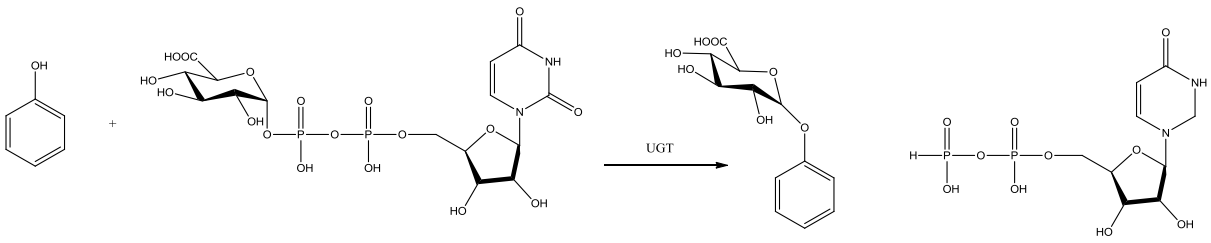


Figure 4.6: A general scheme of glucuronidation. The UDPGA transfers glucose to a xenobiotic. The reaction is characterized by an inversion of the stereochemistry.

The formation of β-D-glucuronides is only the first step of detoxification, the molecules must be transported across the endoplasmic reticulum membrane into the cytosol whereby they are excreted into urine and bile.[197]

4.1.3.2 Sulphonation

Glucuronidation is the most common form of conjugation; however some organisms have little UGT activity. Therfore, these organism utilize other conjugation reactions (e.g. sulfotransferases, SULTs). SULTs are a superfamily of enzymes that catalyse the formation of 3'-phosphoadenosine 5'-phosphosulphate (PAPS) conjugates with molecules containing nucleophllic O, S or N groups (Fig. 4.7).[198]

PAPs is a universal donor in all sulfonation reactions (its importance is highlighted by the fact that can be synthesized in all tissues of all mammals.[199]

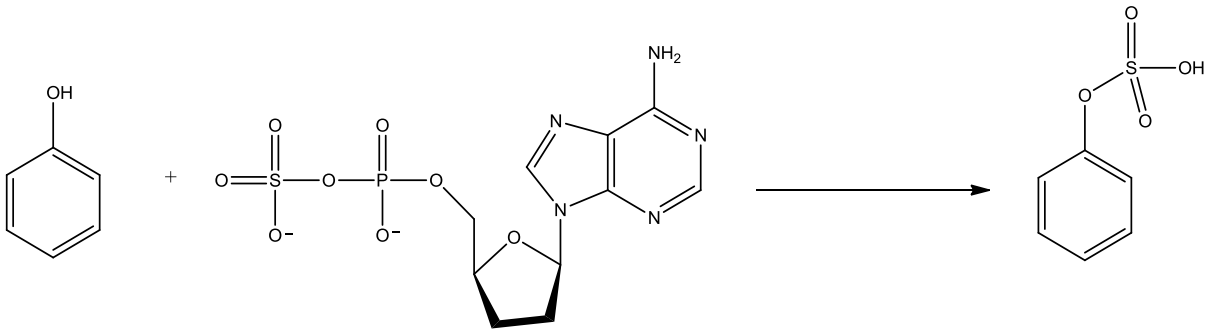


Figure 4.7: A general scheme of sulfonation. The PAPS transfers a sulphate functional group.

There are two classes of SULTs; namely, cytosolic and membrane bound.[200] The cytosolic forms are primarily involved in sulfonation of cytosolic (i.e. more polar) xenobiotics and endobiotics (i.e. cellular molecules). Membrane-bound forms are located on the Golgi apparatus and conjugate sulphates to lipids, proteins and carbohydrates.[201] Sulfonation has high biochemical functionality outside of xenobiotic metabolism and is essential is biotransforming low-molecular weight biomolecules such as steroids. SULTs are found in a plethora of tissues including brain, liver, endometrium and intestine, breast, and owing to its steroid conjugation functionality, in the testis.[200] Like all Phase II metabolism products sulphonates are excreted in the urine and bile.

4.1.3.3 Glutathione Conjugation

Glutathione-s-transferases (GSTs) represent another major Phase II metabolism process that is essential in protecting cells from oxidative stress and potentially toxic electrophiles (e.g. reactive oxygen species, ROS); many of which are formed by the activity of monooxygenases (e.g. CYPs).[202] GSTs are utilized to

breakdown ROS species (e.g. epoxides and peroxides). GSTs are often found conjugated to PAHs following Phase I metabolism.[203] The GST family of enzymes form O-linked thioether conjugations between the tripeptide glutathione (containing a glutamine, cysteine and glycine) (Fig. 4.8).[203]

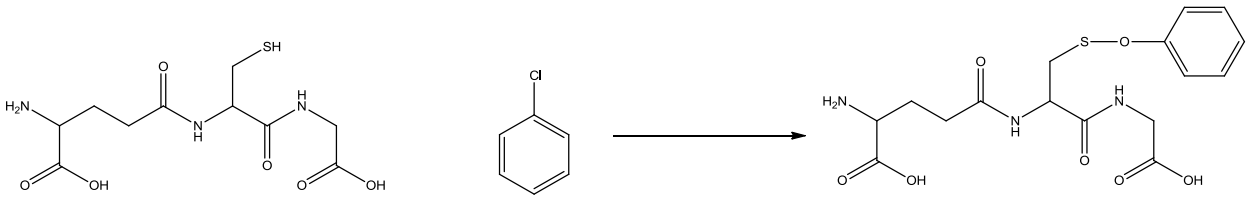


Figure 4.8: The conjugation of glutathione to a xenobiotic via GST.

There are two distinct forms of GSTs; namely, soluble GSTs and membrane bound GSTs (membrane-associated proteins in eicosanoid and glutathione metabolism, MAPEGs).[203,204] The soluble isoforms are dimeric enzymes responsible for detoxification of xenobiotics and endobiotics.[203] Interestingly, while these enzymes are primarily found in the cytosol, they have been detected in the nucleus, mitochondria and peroxisomes.[204] Conversely, MAPEGs are thought to be trimeric enzymes responsible for arachidonic acid metabolism.[205] The two forms of GST are widely distributed in mammals. GSTs have been detected in the brain, liver, kidney lung, intestine, muscle and testis among others.[204]

4.1.4 Transport Proteins and Excretion

The final step in xenobiotic metabolism is the excretion of the detoxified compound; typically in bile or urine.[193] In most cases, cell transport proteins are essential in removing xenobiotics from within cells.[206] The efflux proteins, adenosine triphosphate (ATP)-binding cassette (ABC) transporters represent a

diverse range of proteins that facilitate the removal of compounds from cells (using ATP as an energy source).[207] Naturally, these proteins have a diverse range of substrates, including both conjugated and unconjugated xenobiotics.[208] There are 49 known genes in the ABC family[209] which code for seven isoforms ABCA to ABCG; each distinguished by domain and amino acid homology.[210]

The ABC proteins contain two ATP-binding folds (nucleotide binding folds, NBF), two sets of transmembrane domains (TMDs, each containing six membrane-spanning α-helices).[211] The NBF folds are cytoplasmic and bind ATP to push the xenobiotic through the membrane[211]. As the NBF domains are only found in the cytoplasm, the ABC transporters act only as efflux proteins. The TMDs alternate between in-ward and out-ward conformations while NBFs alternate between open and closed states.[211]

The mechanism of efflux is not fully understood however the contemporary thinking is that multidrug binding proteins (MDBPs) initially transport the detoxified xenobiotics to the NBFs.[212] Subsequent ATP binding and hydrolysis in a region between the NBFs closes and brings the two folds 10-15 Å closer together. This flips the TMDs outwards, pushing the xenobiotic through the membrane and outside the cell.[212]

4.1.5 DBP metabolism

The metabolism of DBP into MBP (and other metabolites) is an important consideration when investigating mechanisms of toxicity. The MBP has been demonstrated to be more toxic than DBP at the same concentration *in vivo*.[213] This suggests that MBP metabolism is the initial step of DBP toxicity. There has been considerable work done on DBP metabolism in rats.[214-218] The proposed metabolism in rats (Fig. 4.9) consists of the major metabolite, MBP and its glucuronide conjugate (approximately 90-95%) with other minor products being phthalic acid (up to 2%) and CYP oxidised metabolites (up to 10%).[203,204] N-butanol is often not measured in DBP metabolism studies; however, it is almost certainly the produced along with MBP in the first metabolism step (i.e. the hydrolysis of DBP to form MBP). Both

glucuronidation and CYP oxidation are thought to occur in the endoplasmic reticulum of hepatocytes; however, as these enzymes are found in many tissues, it is likely these metabolism processes occur in other locations (e.g. skin, Leydig cells). The metabolites of DBP (including glucuronides) have been found in the blood and urine.

Interestingly, analysis of metabolites in rat serum has found MBP to be in predominantly the free form (80-90%). However, in humans the inverse is true with only 25-30% being free MBP while the remainder is glucuronidated.[219]

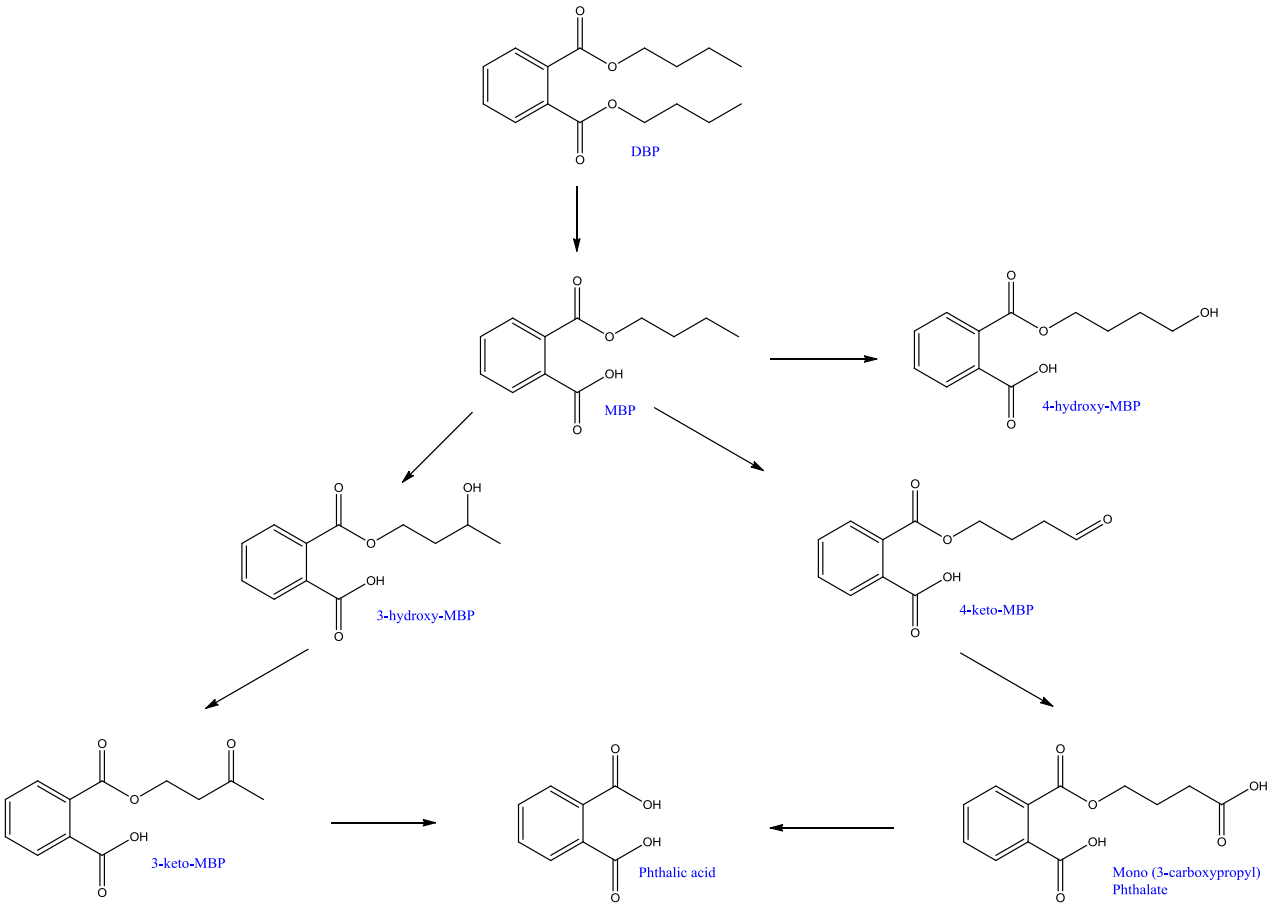


Figure 4.9: The proposed metabolic pathway of DBP in rats. The primary metabolite is MBP. Other hydroxyl metabolites are also common[1].

The DBP metabolic pathway in humans is difficult to measure as it is ethically challenging to dose humans. However, one such study has measured the DBP metabolites in human urine following a single oral dose (Fig. 4.10).[135] The primary metabolite detected was MBP (90%). The oxidation products of MBP were also detected; 3-hydroxy-monobut-1-ol phthalate (3-OH-MBP), 4-hydroxy-monobut-1-ol phthalate (4-OH-MBP), 2-hydroxy-monobut-1-ol phthalate (2-OH-MBP) and 3-carboxy-monopropyl phthalate (MCPP) accounted for 6%, 0.7%, 0.1% and 0.3% respectively.[135] The ratios of glucuronide products were not analysed as all samples were treated with glucuronidase before analysis.[135] However, as previously mentioned the ratio in humans is typically 7:3 free MBP: glucronidated MBP.

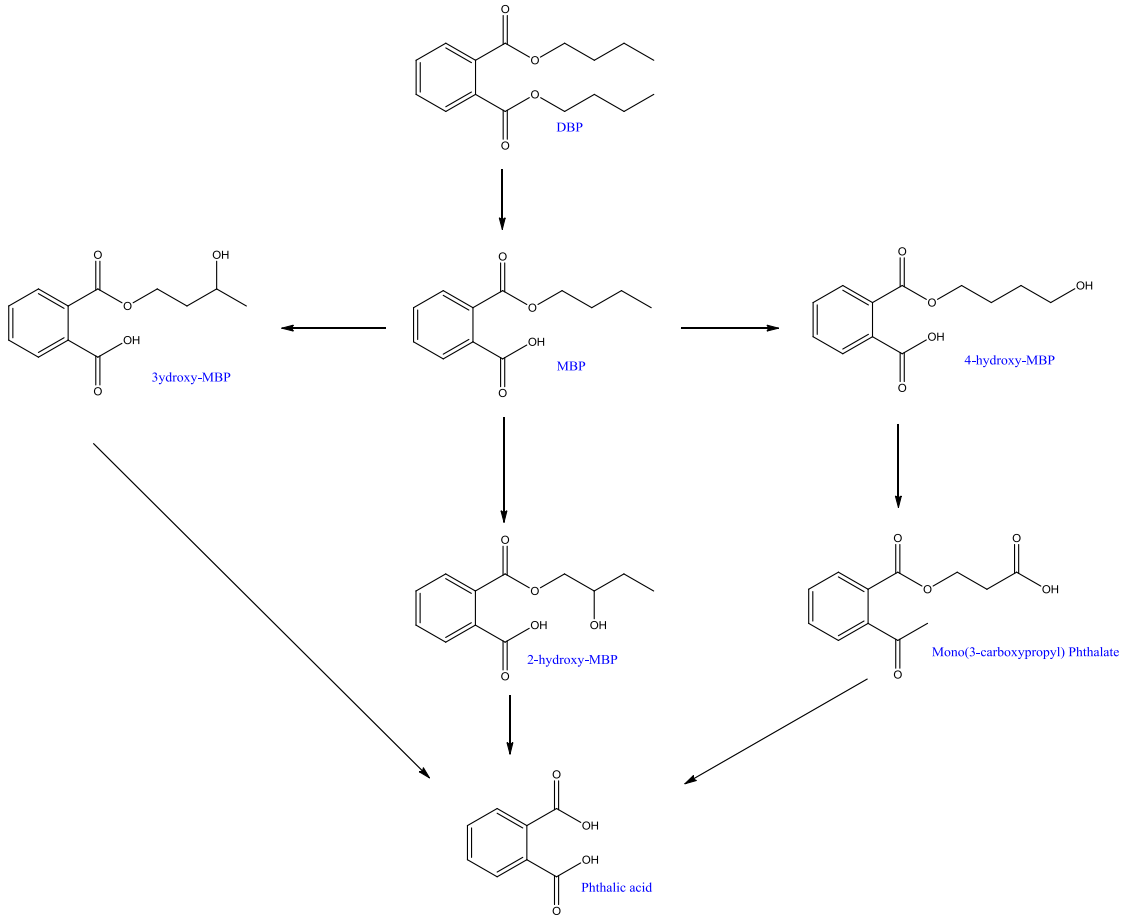


Figure 4.10: The proposed metabolic pathway of DBP in humans. The primary metabolite is MBP. Other hydroxylated metabolites are also common (e.g. 2-hydroxy-MBP).[129]

4.1.6 Potential Estrogenicy of DBP

DBP has the potential to act as a xenoestrogen *in vivo* (previously discussed in section 1.5.1.2). The metabolism of DBP *in vivo* may in fact contribute the estrogenicity via oxidation at key sites (Fig. 4.11). This potential metabolite, mono(4-butanol)-4-hydroxyphthlate (M4B-4HP) has a possible structural analogy with E2. If this molecule could occupy the ER, there is the possibility of an estrogenic effect.

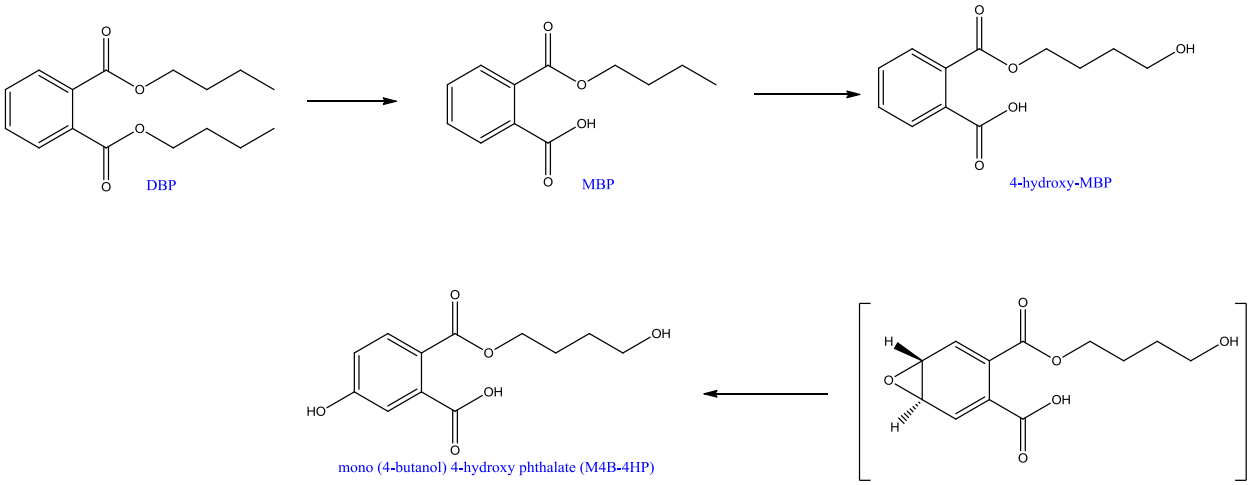


Figure 4.11: the potential metabolism to an estrogenic DBP metabolite.

4.2 Research Aims

The aim of this research described in this chapter is to evaluate potential estrogenicity of a theoretical DBP metabolite, analyse the metabolism of DBP in cultured LC-540 cells and determine if the estrogenic metabolites are produced and evaluate the estrogenicity of DBP exposure at physiologically relevant exposure levels. This will be done via the following research goals:

- Conduct *in silico* modelling studies to evaluate the structural analogy of M4B-4HP and E2 in the ER.
- Investigate the Phase II metabolism of DBP using a glucuronidase enzymatic digest.
- Evaluate the major DBP metabolite/s produced in LC-540 cells following exposure 24 h using HPLC.
- Investigate the estrogenic potential of DBP using an MCF-7 proliferation assay.

4.3 Methods

4.3.1 in silico modelling studies

The modelling was done using ChemBio 3D software. M4B-4HP was drawn using ChemBio 3D. The ER coordinates were download from the PDB into ChemBio 3D and M4B-4HP was overlayed with E2 in bound in the active site using the fast overlay function. The key binding residues were identified and distances between them and the overlayed M4B-4HP were measured.

4.3.2 Preparation of Bufffers, standards and enzymes

4.3.2.1 Preparation of exposure standards

The Exposure standards were prepared as outlined in section 3.3.2.1

4.3.2.2 Preparation of Internal Standards

Ethinyl estradiol (EE2, 0.25 mg) and 4-nitrophenol-D-β-glucuronide (4NPG, 0.5 mg) were dissolved in HPLC grade ethanol (10 mL).

4.3.3.3 Preparation of Glucuronidase Buffer

The buffer was prepared by dissolving sodium acetate trihydrate (13.6 g) in milli Q water (80 mL). The solution was adjusted to pH 5.0 using glacial acetic acid and topped up to 100 mL using miili Q water. This gave a final concentration of 100 mM.

4.3.3.4 Preparation of Glucuronidase

Glucuronidase powder (20 mg, 38400 U) was dissolved in milli Q water (5 mL) to produce a 7610 U/mL solution.

4.3.3 DBP metabolism, Glucuronidase Digest and Extraction

4.3.3.1 Preparation of Exposure Medium

The 1 µg/mL, 1X exposure medium was prepared as outlined in section 3.3.2.2

4.3.3.2 DBP Exposure

LC-540 cells (10 flasks) were grown to semi-confluence. The flasks were divided into two groups: exposure group (n=5) and control (n=5). The exposure group was exposed to 1 µg/mL DBP (see section 3.3.2.3). Both cell groups were left to incubate.

4.3.3.3 Culture Medium Collection and Glucuronidation

The spent MEM was collected at 1 h, 8 h, 12 h and 24 h. The spent MEM was centrifuged to remove any cells. The MEM was divided evenly into two 10 mL aliquots. EE2 (0.25 µg/mL) EE2 (0.5 µg/mL) 4NPG (0.5 µg/mL) was added to each aliquot. Glucuronidase (735 µL, 10000 U) was added to one 10 mL aliquot giving a concentration of 1 U/ µL MEM. The MEM was added 1:1 in glucuronidase buffer. The glucuronidase medium (pH 5.0) was left to incubate at 37°C for 2 h.

4.3.3.4 Culture Medium Extraction

The 10 mL aliquots were extracted in diethyl ether (3 x 30 mL). The ether was collected in a 250 mL RBF. The ether was dried using sodium sulphate (approximately 9 g). The ether was dried under N_2 for 15 min. The extract was dissolved in ether (5 mL) and the RBF was swirled gently for 10 s. The ether was poured into a 7 mL glass vial. A further 2 mL of ether was added to the RBF and swirled before being poured into the vial. The ether was again dried under N_2 and the extract was dissolved in HPLC grade ethanol (500 µL). The samples were filtered through a 0.22 µm filter into 2mL vials and stored at 4°C until analysis.

4.3.4 HPLC

All samples were analysed using a C18 reverse phase column and a step gradient. Each run included a solvent only and ethanol only blank. The UV absorbance was measured using a photo diode array detector (DAD) at 210, 235, 280 and 330 nm. Each sample had 20 µL injected per analysis.

4.3.4.1 HPLC preparation

A Dionex brand HPLC system was used for all analyses. The HPLC needle was washed using 10% (v/v aq) MeOH and primed 5 times with 10% (v/v aq) MeOH. The HPLC system was purged (5 mins, 3

mL/min) with a solvent mixture comprising 10% Acetonitrile containing 0.1% v/v formic acid (FA), 90% MilliQ water containing 0.01% v/v FA.

4.3.4.2 Mobile Phase

Two elution buffers were used: Buffer A; 99.9% Milli Q water containing 0.01% v/v FA, Buffer B; 99.9% Acetonitrile containing 0.1% v/v FA

A single gradient was used for all analyses:

90%A 10%B-50%A 50%B (0-20 min), 50%A 50%B (20-25 min), 50%A 50%B-10%A 90%B (25-45 min), 1%A 90%B (45-55 min), 10%A 90%B-90%A 10%B (55-60 min), 90%A 10%B (60-65 min).

4.3.4.3 Identification of Metabolites

DBP, MBP, EE2 and 4-ntrophenol (4NP) analytical standards were run before injection of sample batches. The peaks that co-chromatographed with the standards peaks were identified as either MBP or DBP. The fractions of any unknown peaks were collected as identified by mass spectrometry.

4.3.4.4 Calibration Graphs

A stock solution of DBP, MBP and EE2 (300 mg/L ethanol) was prepared in a single volumetric flask. The stock solution was shaken vigorously and inverted 20 times to fully dissolve the compounds. The stock solution was diluted to produce standards of 0.5, 1, 2.5, 5, 10, 15, and 20 mg/L. The standards were filtered through a 0.22 µm filter into a HPLC vial. Each standard had three injection replicates. The peak areas were correlated with concentration. Linear regression analysis was performed to give the coefficient of determination (R_2). This was used to determine the accuracy of the standard curve (i.e. the standard graph was only used if $R_2 > 0.99$).

4.3.3 MCF-7 Proliferation Assay

The MCF-7 cells were provided by Dr John Lewis. Samantha Dudley is thanked for the maintenance cultures.

The MCF-7 cells were seeded, exposed and counted exactly as described in section 3.3.3.

4.4 Results

4.4.1 *in silico* Modelling Studies

The *in silico* model shows that M4B-4HP has significant structural analogy with E2 in the ER active site (Fig. 4.12). M4B-4HP orientates in the active site in such a way that the key binding groups of the ER (Glu 353, Arg 394, and His 524) are in a position that would likely allow for binding of M4B-4HP. The key active site residues of the ER active site: Glu 353, Arg 394, and His 524 have of 2.9, 3.3 and 2.8 Å respectively to M4B-4HP. Therefore, these residues would be likely to form hydrogen-bonds with M4B-4HP.

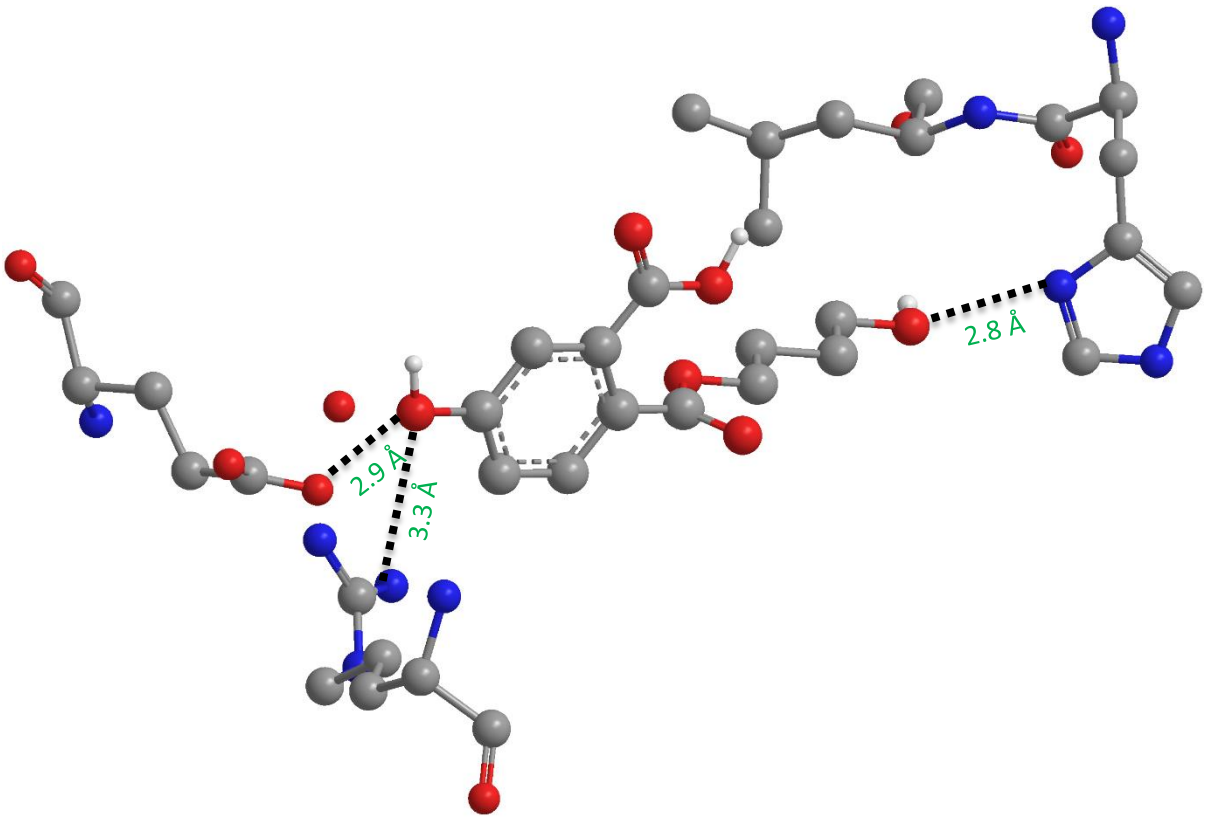


Figure 4.12: The in silico model of M4B-4HP overlayed in the active site with E2. There is structural analogy with E2 in the ER binding site.

4.4.2 Method Validation

4.4.2.1 Mobile phase

The two analytical standards available; DBP and MBP (the most likely metabolite) were effectively separated in the multi-step gradient by approximately 20 min (Fig. 4.13). The addition of FA into the mobile phase lowers the pH of the mobile phase (approximately pH 2.5). This protonates the molecules in each sample, ensuring consistent, sharp peaks.

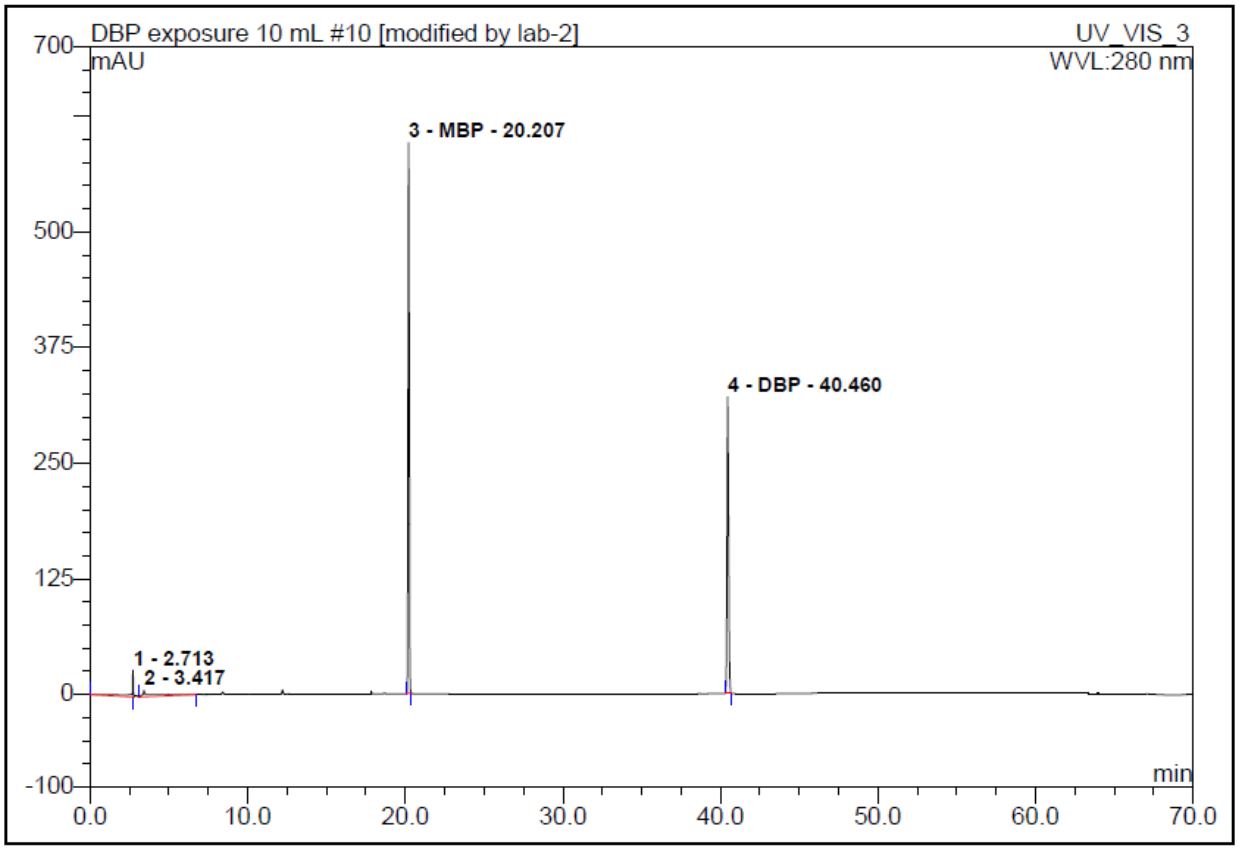


Figure 4.13: The MBP and DBP standards. The molecules are separated by approximately 20 mins.

4.4.2.2 Glucuronidase and Extraction Internal Controls

The success of the glucuronidase reaction is identified by presence of 4NP in the chromatogram. The cleavage of the glucuronide from 4NPDG yields 4NP. The 4NP standard ran at 14.0 min. This peak was found in all the glucuronidated extracts. The extraction internal standard was EE2. The EE2 standard ran at 22.65 min. This peak was found in all chromatograms.

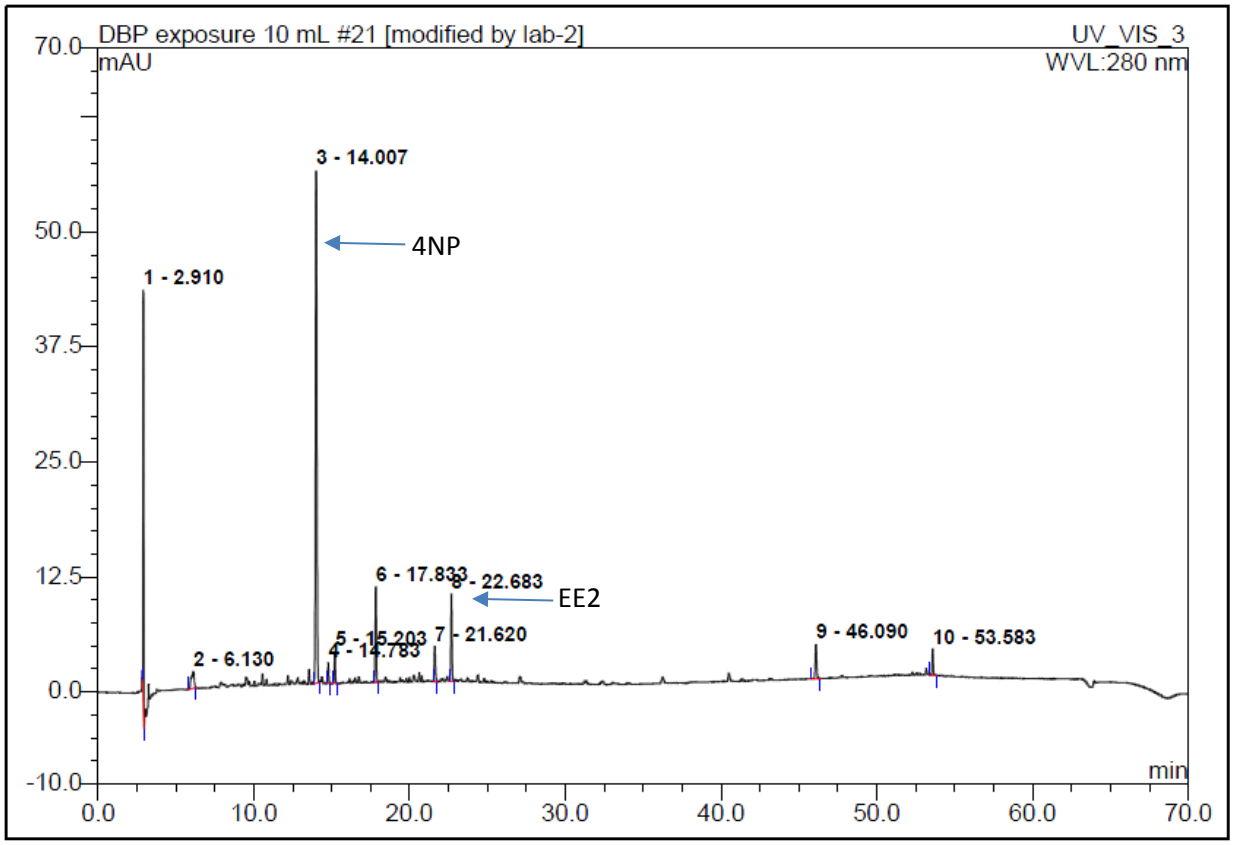


Figure 4.14: The chromatogram of a glucuronidated control extract. The 4NP and EE2 peaks are present indicating the glucuronidation and extraction was succesful

4.4.2.3 Standard Curves

The standard curves of DBP, MBP and EE2 all have good linear regression with R^2 values >0.999 (Fig. 4.15). The linear nature of the calibration graph allows for the reliable calculation of DBP and MBP concentrations

4.4.3 Xenobiotic Metabolism in LC-540 Cells

4.3.3.1 LC-540 Glucuronidation

LC-540 cells have high glucuronyl transferase activity and form a range of glucuronide excretion products (Fig. 4.16). The majority of excretion products exist as glucuronides.

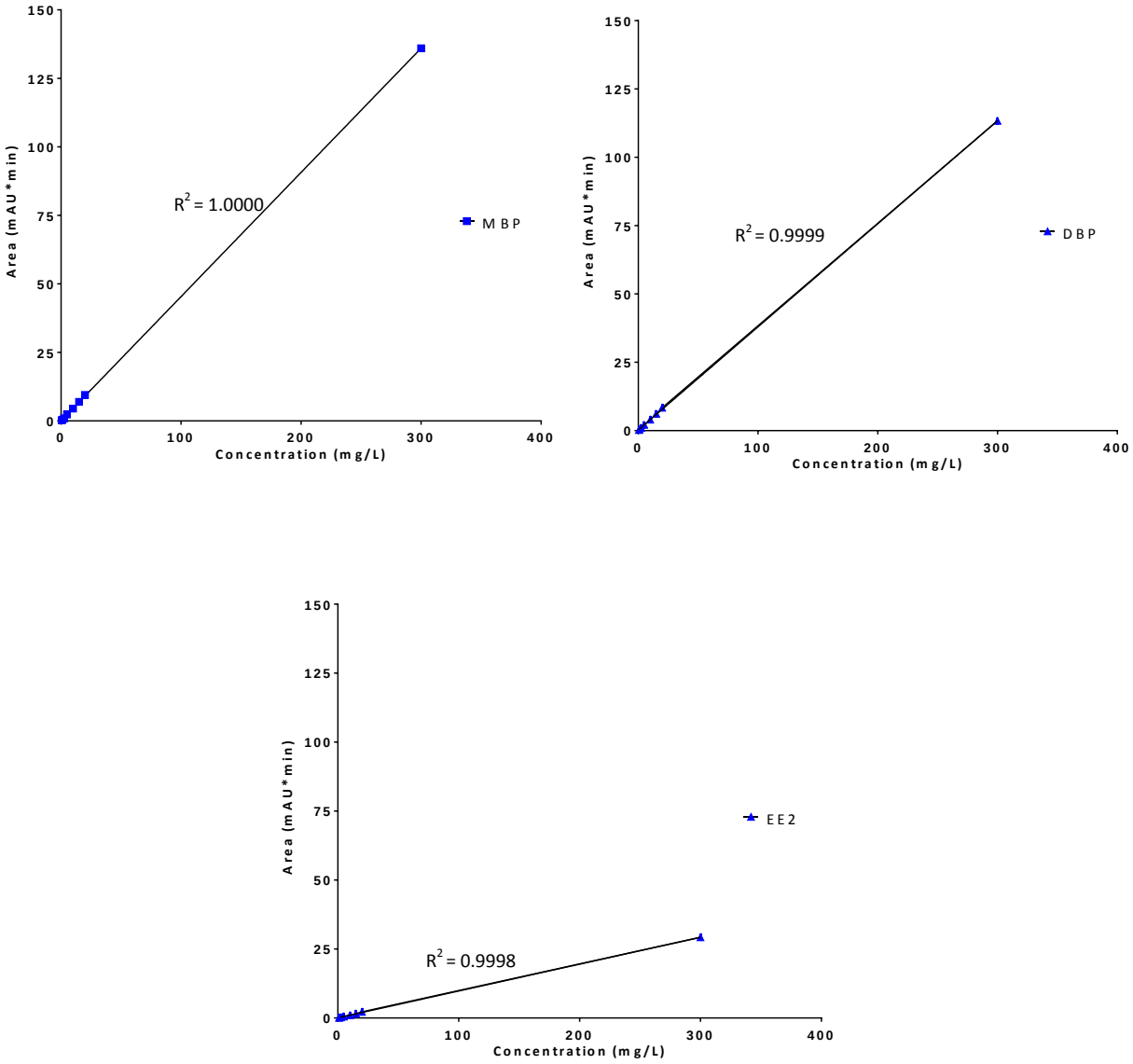
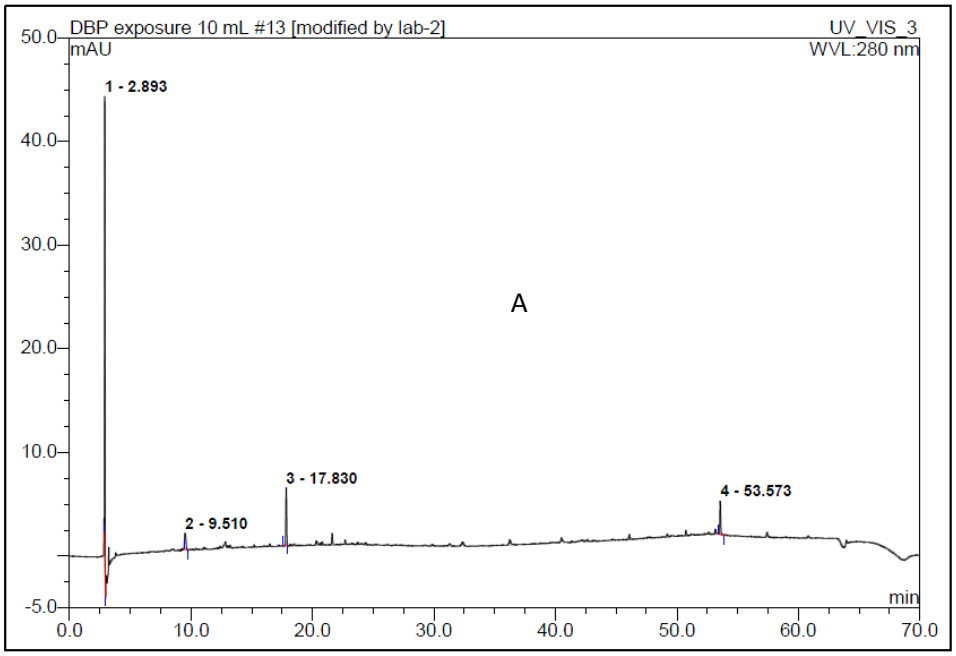


Figure 4.15: The calibration graphs of DBP, MBP and EE2. The R^2 values are all >0.999.



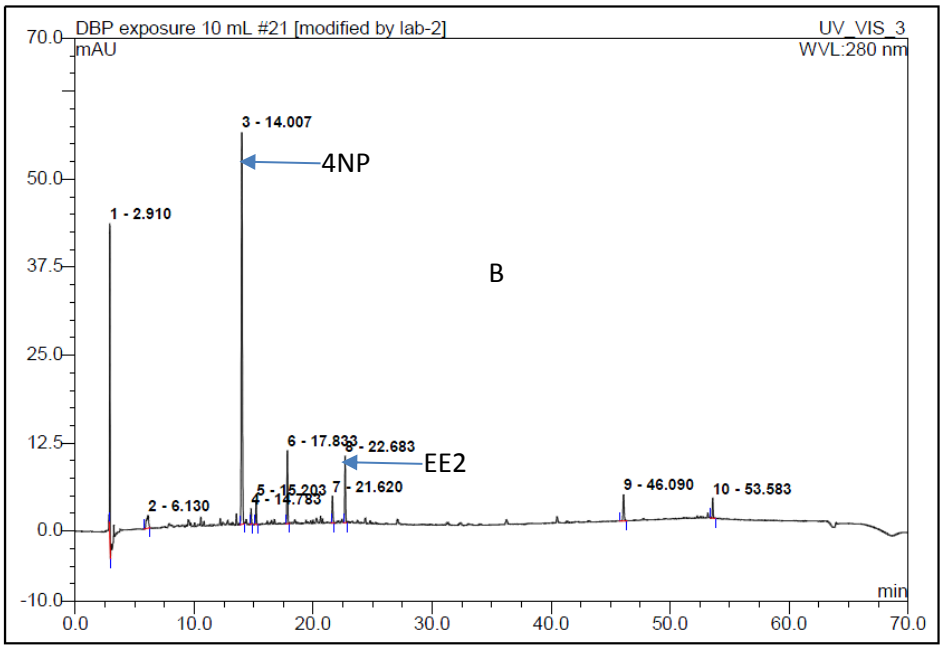


Figure 4.16: The comparison of extracts from untreated (A) and glucuronidase-treated (B) spent LC-540 MEM after 1 h incubation. The untreated media has few peaks. The glucuronidase-treated media demonstrates that LC-540 cells produce and excrete a range of glucuronides.

B

4.3.3.2 DBP metabolism

The metabolism of DBP in Leydig cells occurs primarily over 8 h. The exposed Leydig cells convert most of the DBP into MBP. The majority of MBP is subsequntly glucuronidated. Over the next 16 hours the levels of MBP decrease. At 24 h, some DBP remains unmeatbolised. There were no other detectable metabolites in either the untreated or glucuronidase treated groups.

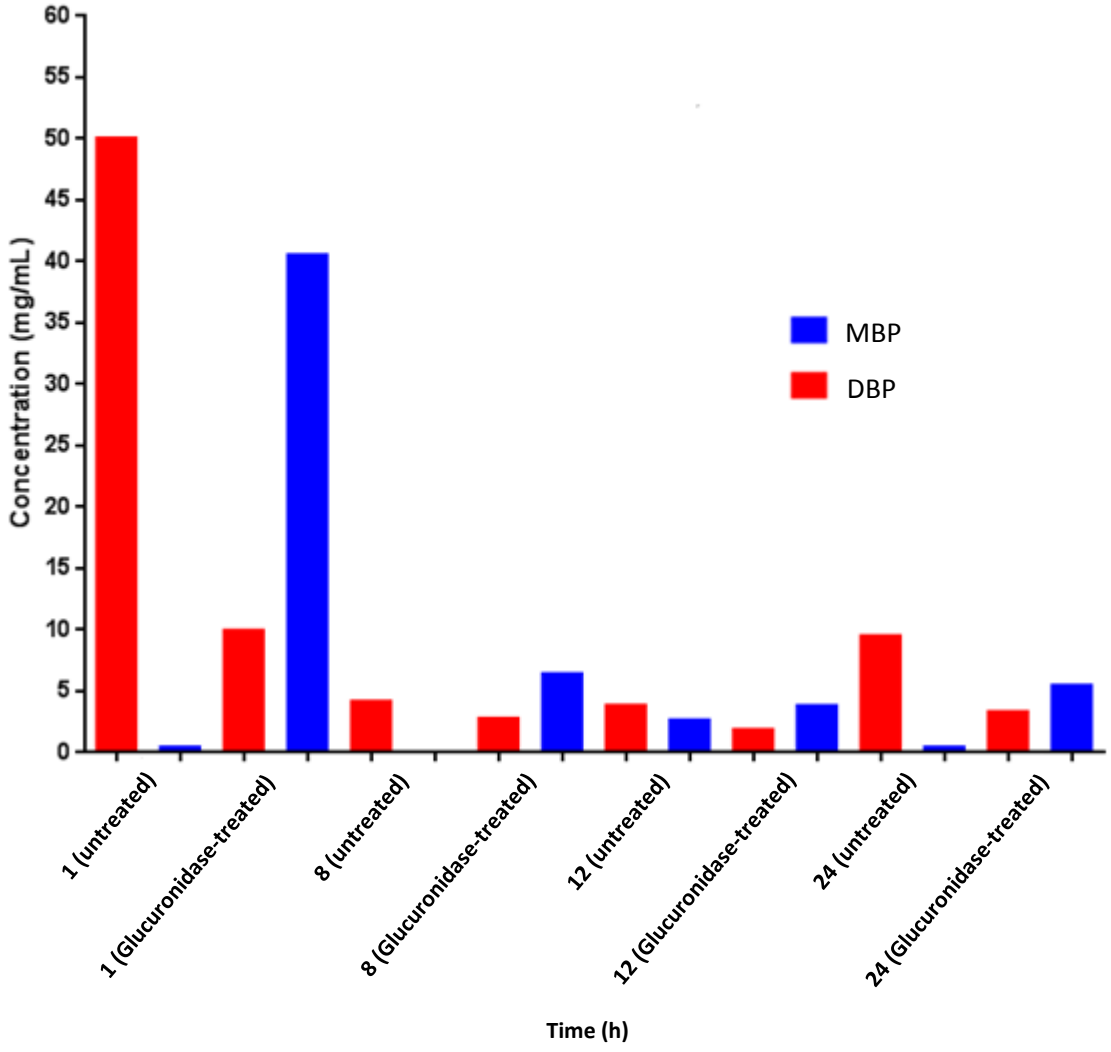


Figure 4.17: Levels of DBP and MBP in spent LC-540 MEM over 24 h. DBP is mostly metabolised over 8 h. The major metabolite detected was MBP. The levels of MBP also drop after 8 h.

4.4.4 MCF-7 Proliferation Assay

The MCF-7 cells are able to grow in culture over 8 days and have a typical sigmoidal growth curve (Fig. 4.18). DBP stimulates significant increases in cell division at the general population and occupational daily exposure levels. Interestingly, the general population exposure level (i.e. the lower dose) had a greater effect than the occupational exposure level. The general population exposure level had an approximately 70% increase in final cell number compared to controls. The occupational exposure level increased the final cell number approximately 25% over controls

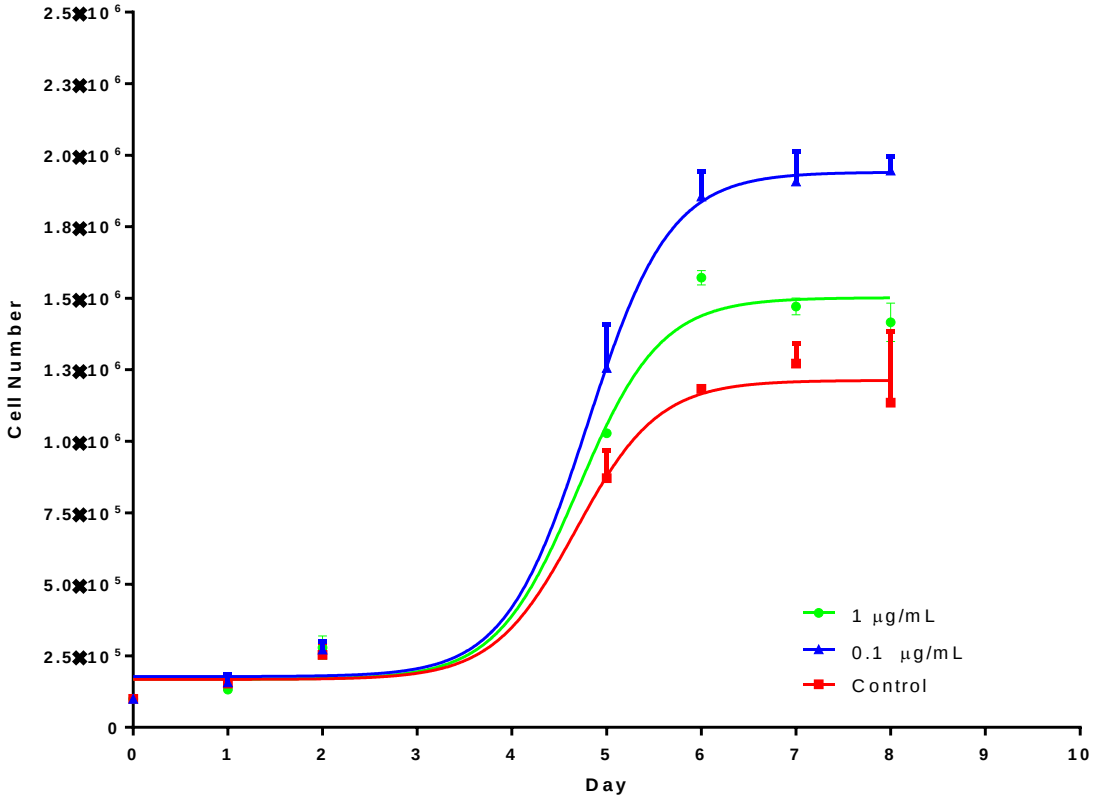


Figure 4.18: The MCF-7 proliferation in response to daily and occupational exposure levels of DBP. The cell increases maximum growth by 70% and 25% for the 0.1 and 1 µg/mL exposure levels respectively

4.5 Discussion

There remains little conjecture in the literature concerning the anti-androgenic like effects of DBP. However, there is no consensus on the potential of DBP being estrogenic. The present study produces conflicting evidence: estrogenic metabolite/s were not detected in any extract of the culture medium of LC-540-exposed; however, a MCF-7 proliferation assays demonstrated a slight an estrogenic effect. There are many questions to consider; namely, how reliable is the *in silico* modelling? Was the analytical methodology sensitive enough to detect M4B-4HP? Are there other factors that influenced the MCF-7 proliferation assay?.

4.5.1 *in silico* Modelling

4.5.1.1 The Use of M4B-4HP

The in silico model focused on structural homology between M4B-4HP and E2 in the active site of the ER. M4B-4HP was chosen as it is a logical product of Phase I metabolism of DBP *in vivo;* i.e. the oxidation of the phenyl and alkyl groups of MBP, the major metabolite of DBP. These oxidations occur commonly in the metabolism of a range of xenobiotics. Therefore, it is entirely logical that this product would be produced in some capacity. The key question is: how much of this metabolite is produced and is it excreted by the cell?

4.5.1.2 in silico Software

The modelling software used was ChemBio 3D, a relatively cheap and user friendly modelling program that has been used previously to investigate the molecular mimicry of molecules in the active site of receptors.[220] The software runs PDB coordinates and allows molecules to be overlayed on other molecules bound in the active site (i.e. agonists). This allows a molecule of interest to be viewed in an active site in a probable conformation *in vivo*. This information can allow for many postulations regarding the potential for that molecule to an agonist. Unlike more sophisticated software (e.g. Schrödinger),[221] the interactions with the protein itself are not considered. The sole focus is on the best

fit model of the two overlayed compounds. The lack of protein interactions impacts the overall reliability of the model.

There are key structural features of E2 that xenoestrogens mimic that allow them to bind to the hydrophobic binding pocket of the ER: the phenolic hydroxyl, a hydrophobic backbone and an aliphatic carbon. M4B-4HP does possess these key features and therefore has estrogenic potential (Fig. 4.12). It is these features that allow the molecule to have a structural analogy with E2 in the *in silico* model. Therefore, it can be assumed that M4B-4HP would have very similar interactions with the hydrophobic binding pocket of E2.

The model system has some key limitations but does give some insights into the potential estrogenicity of M4B-4HP. As is the case with all models systems, the model must be backed up with experimental evidence. In terms of estrogenicity, this is the MCF-7 proliferation assay. However, its estrogenicity is irrelevant if it is not produced *in vivo.* The use of Schrödinger along with ChemBio 3D would improve the validity of the model.

4.5.2 DBP Metabolism in LC-540 Cells

The investigation of metabolism of DBP in the present study had three main goals: to evaluate the major metabolites of DBP in LC-540 cells, to ascertain if LC-540 cells have the potential for Phase II metabolism (i.e. glucuronidation and sulfonation) and the detection of M4B-4HP.

4.5.2.1 Phase II metabolism in LC-540 Cells

A major Phase II metabolism processes in mammals including rats is the glucuronidation of xenobiotics.[222] There are other minor conjugates such as amino acid conjugates and sulfonates. These modifications are designed to facilitate the excretion of the xenobiotics from the cell; usually involving ABC transporters. The functional metabolism of xenobiotics has implications in the viability of the LC-540 models system. If the cells cannot modify and excrete DBP, the unmodified DBP is free to exert a

greater toxic effect; i.e. unmetabolised DBP would affect the Leydig cells more than would normally occur *in vivo*. Leydig cells *in vivo* metabolise and excrete xenobiotics quickly (usually between 6-36 h). The LC-540 cells are tumour cells and therefore, susceptible to reductions in expression of multiple metabolism genes (e.g. CYPs and transferases).[221] However, it is evident that LC-540 cells retain the expression of glucuronyl transferases as the concentrations of a variety of excretion products are much higher in the glucuronidase treated extracts (Fig. 4.16). The present study clearly demonstrates that there is minimal MBP in non-treated extracts compared with the extracts treated with glucuronidase (>90%). This suggests that the majority of MBP is glucuronidated. This is in contrast with DBP metabolism studies involving the oral administration to rats.[216] The urinary levels of MBP in rats had over 90% free MBP. The present study only investigated the metabolism of DBP in Leydig cells; however, Foster et al.[216] studied the urinary metabolites. The urinary metabolites are comprised of mutiple metabolism processes in other organs (e.g. via the liver, gut etc.). It is highly likely that these other systems favour excretion of free MBP of MBP-glucuronides. It is clear that MBP is harmful to Leydig cells; therefore, LC-540 cells may glucuronidate the MBP in response to a toxic effect exerted on the cells. As MBP is not harmful to the liver and most other organs, there is little need to detoxify to the extent needed by Leydig cells.

The present study utilised a β-glucuronidase enzymatic digest of the spent culture medium. The β-glucuronidase used in the experiments was isolated from helix pomatia and has minor sulfatase activity. To ensure all glucuronides present in the spent culture medium were deconjugated, the concentration was set at the standard of 1 unit/µL culture medium. The standard buffer was 0.1 M sodium acetate pH 5.0. The enzymatic reaction requires a pH range of 4.0-5.0 and so the culture medium was added 1:1 v/v which yields a pH of 5.0. The reaction limited to 2 h and clenched by adjusting the pH to approximately 7.4 using NaOH. This prevented the hydrolysis of DBP and MBP (and potentially other metabolites) which can occur in aqueous solution under acidic conditions. There is some apparent hydrolysis in the 1

h Glucuronidase-treated extract. The same media was divided evenly into the un-treated and glucuronidase treated groups; however, the levels of DBP are much lower in the glucuronidase treated groups, a 5-fold decrease. It is clear that the conditions for deconjugation of glucuronides are unfavourable for esters under acidic conditions.

4.5.2.2 DBP Metabolism

The present study used HPLC to investigate the metabolites of DBP following exposure of LC-540 cells. DBP is metabolism begins rapidly and after 1 h, approximately 40% of the DBP is converted to MBP-glucuronides. Over the next 8-24 h, most of the DBP is metabolised, with only trace amounts remaining. Interestingly, the levels of MBP decrease rapidly during the same time period. The reduction in MBP concentration is likely due to the MBP-glucuronides re-entering the cell and being further metabolised. Therefore, this observation is an artefact of the experiment as the MBP *in vivo* would be readily transported away from the Leydig cells and excreted in the urine.[216] There was no detection of M4B-4HP in any of the samples using HPLC. However, M4B-4HP would almost certainly be excreted as a glucuronide. There is the possibility that the acidic conditions of the glucuronidase digest could have degraded M4B-4HP. This molecule is likely to be produced in small amounts and therefore, following acid degradation, M4B-4HP could possibly be below the limit of detection.

Other studies investigating DBP metabolism found trace amounts of oxidised MBP metabolites (e.g. 4-hydroxy-MBP). [204] The present study did not find any of these compounds in trace amounts. This may be due to the possible hydrolysis of these metabolites during the Glucuronidase treatment.

There were some limitations of the present study. The metabolism experiment was not done in replicate. This was due to time constraints. The metabolism of DBP in LC-540 cells occurs primarily between 1-8 h. There was no analysis of between this time period. As the majority of DBP metabolism occurs during this period, subsequent metabolism studies should evaluate the levels of DBP and MBP

over this timeframe. HPLC may not be sensitive enough to detect the minor metabolites of DBP. A more sensitive analytical methodology such as liquid chromatography-mass spectrometry (LC-MS) could increase the limit of detection. It might be possible identify M4B-4HP and other metabolites using LC-MS.

While no M4B-4HP was detected, the estrogenic effects of it, or some other molecule is implied in the MCF-7 proliferation assay.

4.5.3 MCF-7 Proliferation Assay

MCF-7 cells are a breast cancer cell line with expression of ERs α and β.[223] Upon binding to E2 (or other xenoestrogens) the ERs dimerize and enter the nucleus to upregulate the expression of cell cycle genes to increase cell proliferation. MCF-7 cells have been used to determine the estrogenicity of compounds in culture.[224] The MCF-7 proliferation assay has a distinct advantage over the YES assay; the ability to metabolise the exposure compound. This is vital when the compound is a pro-estrogenic (i.e. is converted to an estrogenic molecule via metabolism), as is likely the case with DBP. Indeed, many studies have overlooked the estrogenic potential of DBP as assays like the YES assay do not produce conclusive results (i.e. the YES assay demonstrates little DBP estreogenicity).[61] However, when you evaluate the possible metabolic routes *in vivo*, it is clear that the biosynthesis of an estrogenic metabolite is possible. No study to date has published a MCF-7 proliferation assay involving DBP. The present study does not find such any metabolite in extracted culture medium of DBP-exposed LC-540 cells. However, the effects of an estrogenic molecule can be seen in the MCF-7 proliferation assay.

The results of the MCF-7 proliferation assay demonstrate that DBP has a significant effect on the growth rate of MCF-7 cells in culture. The cells were exposed to the normal population (0.1 µg DBP/mL) and occupational (1 µg DBP/mL) daily exposure levels. The greater effect was observed in the normal

population exposure level which had the greatest effect on MCF-7 proliferation compared to the controls and occupational exposure level. The occupational exposure level did increase the growth of the MCF-7 cells but to a much smaller extent than the normal population exposure level. This could be due to the MCF-7 cells sensitivity to DBP; i.e. there is an initial 'die off'. This is followed by an estrogenic stimulation of DBP to increase proliferation after the cells recover. There is also the possibility of clonal evolution; i.e. after the initial die off, the remaining cells are more resistant to DBP compared to the daily exposure level. This effect is observable in effects of some drugs which have a maximum effect dose. Any dose above this level has significantly lower effect.[225]

An interesting observation in the MCF-7 proliferation assay is the effect of DBP continues throughout the 8 day course of the experiment despite DBP being mostly metabolised at 24 h. This suggests the possibility of epigenetic modification. As described in section 2.1.5.2, MCF-7 cells are prone to epigenetic modifications in culture. There is a strong possibility DBP is effecting the epigenome as exposure to DBP is known to cause a wide range of epigenetic modifications *in vivo.*[81]

The MCF-7 proliferation assay has limitations. Firstly, cells *in vitro* are highly variable and have irregular growth patterns. Therefore, without more evidence, (e.g. detection of estrogenic metabolites in spent MEM) the estrogenic effect of DBP is not conclusive. The errors associated with cell counting (see Section 2.5.1). Other methods of cell quantification should be used in future work to validate the cell counts.

The present study has demonstrated that LC-540 cells have a robust Phase I and Phase II metabolism that converts DBP to MBP. The presence of M4B-4HP was not detected; however, its effects are implied in an MCF-7 proliferation assay. More work is needed to fully evaluate the metabolism of DBP in LC-540 cells; including a more sensitive analytical methodology to determine metabolites below the limit of detection of HPLC.

Chapter 5– Effects of DBP on Steroid Biosynthesis

5.1 Introduction

The biosynthesis of steroid hormones is essential in many higher organisms to regulate metabolism, healthy sexual development, cellular stress, immune functionality and maintain reproductive health.[65] Steroids are endocrine signalling lipid hormones with a conserved sterol structure of three fused six-membered rings and one five-membered ring.[226,227] Steroids can be found in most higher organisms such as animals,[228] plants[229] and fungi.[230] The many hundreds of steroids discovered go date are all biosynthesized from squalene. Squalene is synthesized via the cyclization of lanosterol and cycloartenol in animals and plants respectively.[230,231] The production of steroids in animals is regulated by the steroidogenesis biosynthetic pathway. Active steroid hormones are synthesized in the gonads, adrenal glands and placenta;[65] beginning at early gestation in humans with production of progesterone and continuing throughout life.[232]

Four main classes of steroids in humans are known; namely, the androgens (e.g. testosterone), estrogens (e.g. E2), the progestogens (e.g. progesterone) and the glucocorticoids (e.g. cortisol).[233] These steroids are produced in different glands and bound to the steroid binding globulins (e.g. testosterone binding globulin) which transport the steroids through plasma.[234] Steroids circulate the body, acting on specific tissues. Steroids may have profound impacts on the tissues they interact with. Therefore, their biosynthesis is highly regulated.[66] The unregulated production of steroids has been linked to the feminization of men via disruption of the androgen:estrogen ratio.[235] The regulation of steroid biosynthesis is controlled in part by signalling hormones excreted from the pituitary gland and gene regulatory effects.[66] However, exposure to some environmental EDCs can disrupt this regulation and perturb some steroid specific tissues.

To understand how the effects of environmental EDCs on steroidogenesis and steroid dependant tissues, it is essential to understand the regulation, synthesis and function of steroids hormones.

5.1.1 Cholesterol biosynthesis

In all organisms, all steroid hormones are synthesized from the lipid cholesterol[230]. This essential lipid is of course found in the membranes of all cells and is a precursor to many other bioactive molecules (e.g. vitamin D).[236] The synthesis of cholesterol (Fig. 5.1) occurs primarily in the liver via a multi-step pathway beginning with the cyclization of squalene.[230] Firstly, squalene is oxidized by a monooxygenase to form Squalene-2,3-epoxide. The straight chain terpeneoid squalene folds into a sterol-like conformation and cyclizes via the enzyme 2,3-oxidosqualene:lanosterol cyclase. This yields the typical sterol fused ring structure (i.e. three six membered rings and a single five membered ring) of lanesterol.[230] Several more enzymatic modifications occur before cholesterol in synthesized. The final step (inside the liver) is the acetylation step to yield a cholesterol ester.[237,238] Lipoproteins carry cholesterol and cholesterol esters in plasma, delivering them to all tissues[15] including the adrenal glands and gonads to undergo steroidogenesis. However, cholesterol cannot freely enter the inner mitochondrial membrane to undergo the first step of steroidogenesis. A series of signalling cascades must first occur first to ensure transport of cholesterol to the site of steroidogenesis.

5.1.2 Gonadotropin Regulation of Steroidogenesis

The regulation of steroidogenesis in the testis is highly dependent upon the actions of gonadotropin hormones secreted by the pituitary gland. The periodic secretion of LH induces the steroidogenic function upon binding to the LHR on the cell surface of steroidogenic cells.[66] The binding of to the LHR initiates a cascade of cAMP-mediated events.[239] The products of steroidogenesis also regulate the expression of hormone secretion (e.g. testosterone down regulates LH production).[240] Overall there is tightly regulated complex feedback machinery ensuring the healthy production of steroids.

5.1.2.1 Hormonal Stimulation

The hypothalamus initiates the first step of steroidogenesis by secreting the gonadotropin-releasing hormone (GnRH), a decapeptide which binds to receptors on the surface of the pituitary gland.[241] The binding of GnRH induces intracellular events that synthesize and release several gonadotropins; namely,

LH and follicle-stimulating hormone (FSH), the latter of which primarily aids spermatogenesis.[241] The released LH binds to the LHR, a specific high-affinity transmembrane GPCR.[239,242] Like many GPCRs, LHR is a heterotrimer consisting of an α, β, and γ subunits.[243,244] The α subunit binds guanine triphosphate (GTP) or guanine diphosphate (GDP). Binding of LH to the LHR causes rapid exchange of GTP for GDP which, in turn facilitates the dissociation of α-subunit. The α subunit binds to adenylate cyclase, inducing rapid cAMP synthesis. However, the low GTPase activity eventually hydrolyses the GTP on the α-subunit, leading to dissociation from adenylate cyclase and reformation of the GPCR trimer. The increased cellular cAMP levels activate several kinases.[244] These kinases phosphorylate several promotors to stimulate and initiate steroidogenesis.[16]

There are two distinct cellular responses to increased cAMP levels; namely acute and chronic effects. The acute response is the rapid transport of cholesterol into mitochondrial membranes by the StAR. [245,246]

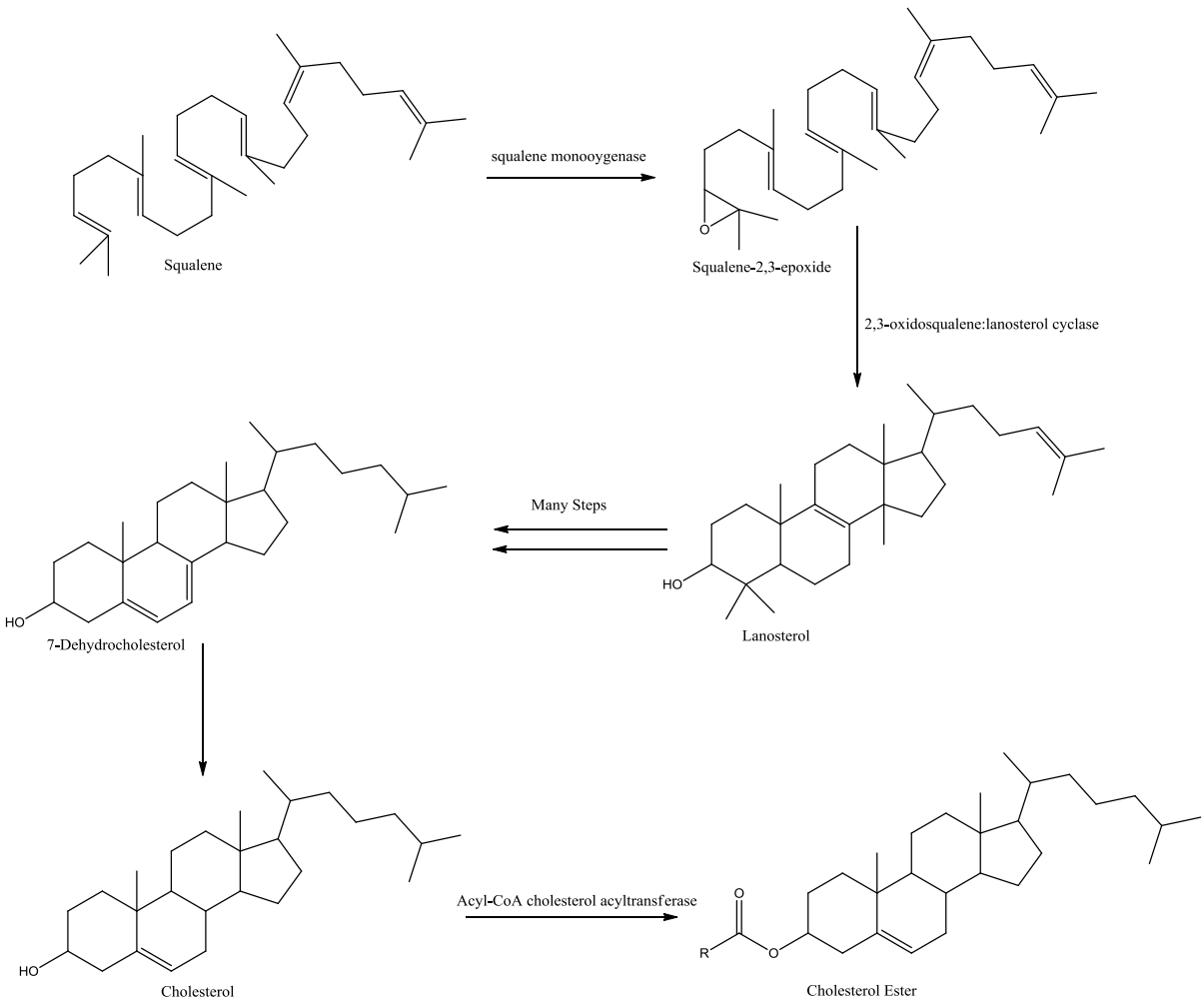


Figure 5.1: The biosynthesis of cholesterol. The terpene squalene is first oxidised before cyclising into a sterol conformation. Lanosterol undergoes many other biotransformations to produce cholesterol esters which are transported around the body.

5.1.2.2 StAR

StAR is an essential protein in the regulation of steroidogenesis. The activity of StAR directly induces a 6-fold increase in steroidogenic activity.[248] StAR is a 37 kDA protein belonging to the StAR-related lipid transfer (START) domain protein family.[249,250] START proteins have a conserved α/β structured lipid binding domain (i.e. the START domain) comprises of ~210 residues. This domain represents the binding pocket specific for cholesterol.[251] StAR has been a difficult protein to study as the purification procedure is difficult and costly.[249,252] This in some way has contributed to the mechanism of StAR action being far from fully solved. It is known that StAR is up-regulated by the cAMP-dependant kinase action which phosphorylates promoters, increasing its transcription of steroidogenic enzymes.[253] The proposed mechanism of action goes via pH-dependant binding and release of cholesterol. At neutral pH to slightly alkaline pH (i.e. in the cytosol), StAR binds to cholesterol and acts upon the outer mitochondrial membrane, facilitating its transport into the inner mitochondrial membrane.[254-256] The pH is more acidic in the inner mitochondrial membrane (pH 5). This leads to protonation of the C-terminal α-helix, essentially opening the gate and releasing cholesterol.

5.1.2.3 Chronic stimulation of Steroidogenic Enzymes

The actions of LH and cAMP are essential for the chronic stimulation of steroidogenic enzymes.[239] It is clear that the actions of cAMP dependant kinases[257] phosphorylate steroidogenic promoters such as steroidogenic factor 1 (SF1).[258] The role of SF1 and other promoters has been demonstrated by the increased *de novo* synthesis of several steroidogenic enzymes; $P450_{SCC}$ and CYP17.[180,259] In the absence of cAMP, CYP17 protein synthesis has been shown to cease in cultured Leydig cells after 48 h.[260] Interestingly, in the same study, $P450_{SCC}$ was able to maintain some *de novo* synthesis.[260] However, a reduction in CYP17 severely limits the production of androgen or estrogen hormones as CYP17 catalyses a key reaction in steroidogenesis (see section 5.1.3.2). The stimulation of cAMP appears to be non-essential for the expression of 3β-HSD in cultured Leydig cells.[261] This enzyme has been shown to be expressed at normal levels following the removal of cAMP stimulus in cultured Leydig cells. These

cultured cells were able to produce progesterone (a product of 3β-HSD), but had little to no androgenic capacity.[261] The role of cAMP in the stimulation of androgen synthesis is almost certainly regulatory mechanism to prevent the unregulated biosynthesis of androgen hormones.

5.1.2.4 Downregulation of Steroidogenesis

Gonadal steroids have been show to act as a feedback regulators of the episodic release of GnHR from the hypothalamus. E2 is known to be an important regulator in GnHR[262,263] with surges of secretion observed following administration of E2 in pre-ovulatory ewes.[264] E2 plays a dual role in GnHR surges; firstly, during the first four hours, E2 increases synthesis and insertion of GnHR to its receptor[265,266]. Secondly, E2 stimulates a sustained GnHR surge 12-15 afters after administration.[267] The role of E2 in up-regulating GnHR stimulation and activation is linked to the estrous cycle in women. During this cycle, large quantities of E2 are needed for the gametes to become estrous (i.e. fertile). Therefore, up-regulation of GnHR secretion stimulates increased production of E2 in the ovum.

Progesterone has been shown to decrease the frequency of GnHR pulses *in vivo.*[268] It has been suggested that progesterone acts to reduce the number of GnHRs on the pituitary gland.[241] This has been shown in cultured ovine pituitary cells which had decreases in GnHR protein and mRNA.[269] The role of progesterone is essential during the luteal phase of the estrous cycle to ensure the gametes are developed and fertile. As E2 stimulates ovulation, it would be essential to reduce the levels of E2 significantly during this phase.

In men, there is a complex system of regulation involving the GnHR down-regulation from both testosterone and E2.[270] This has been demonstrated in humans via the suppression of E2 and testosterone synthesis (via chemical castration in healthy men). The reduction in the sex hormones increased LH and FSH levels 3-fold.[271] The mechanism of down-regulation of LH expression in thought to be via the reduction of GnHR pulses accompanied with decreases in the sensitivity of the pituitary gland

to respond to GnHR.[271] A down-regulation is expected in males as the overproduction of androgen hormones results in hypogonadism in males and possibly causes precocious puberty. The down-regulation by E2 may be a response to prevent feminization by preventing the production of E2 via the shutdown of steroidogenesis.

The regulation of steroidogenesis is complex and involves a feedback mechanism involving a variety of endocrine glands. The process of steroidogenesis inside the cell is complex with many branching chains in the pathway.

5.1.3 Steroidogenesis

The steroidogenesis biosynthetic pathway (Fig. 5.2) produces a variety of tissue specific products with key roles in the endocrine system.[233] This section will discuss in the enzymes involved in steroidogenesis.

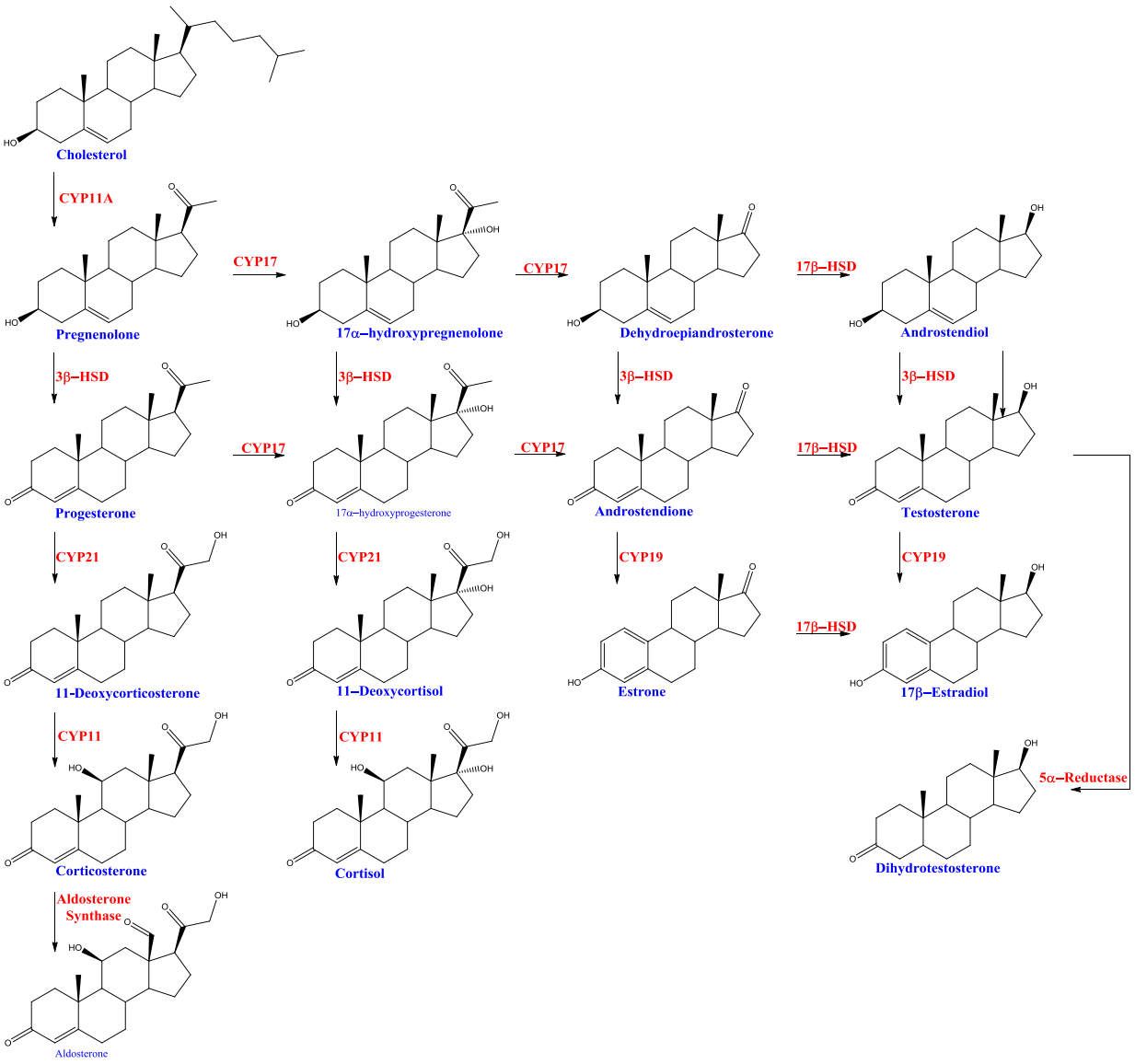


Figure 5.2: The steroidogenesis biosynthetic pathway. The synthesis of steroids uses a variety of enzymes in a tissue dependant manner. Sex hormones, androgens and estrogens are synthesised in the testis and ovum respectively. The glucocorticoids are synthesized in the adrenal cortex.

5.1.3.1 P450scc

The first catalytic reaction of steroidogenesis is the conversion of cholesterol to pregnenolone (Fig. 5.3) via the enzyme $P450_{scc}$.[233] The ~140 kDa protein is found in the inner mitochondrial matrix. The reaction occurs via two sequential oxidations at C22 followed by C20. This is followed by cleavage between C20

and C21 resulting in pregnenolone and isocaprotic aldehyde.[272,273] All three steps in the catalytic reaction occur in the same active site and require a molecule of O_2 and NADPH[274,275]. The reaction takes place in the inner mitochondrial membrane where these cofactors are produced and are in abundance.

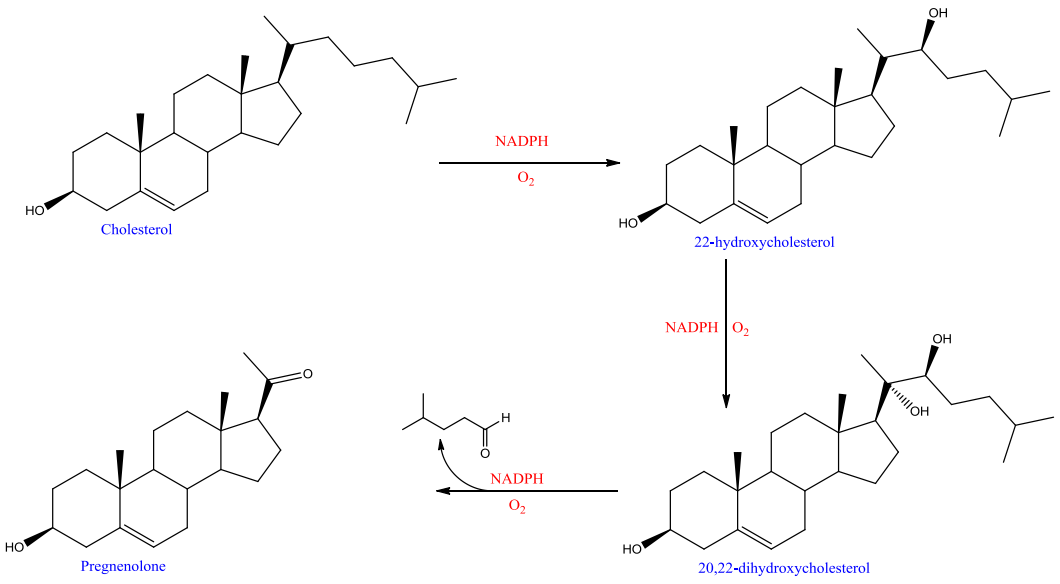


Figure 5.3: The reaction catalysed by CYP11A. The carbon chain of cholesterol is oxidised twice before being cleaved to form pregnenolone.

There is a single gene that codes for the approximately 60 kDa protein; namely *cyp11a1.*[276] *Cyp11a* is expressed in all steroidogenic tissues; e.g. the gonads, adrenal cortex and placenta.[233] Interestingly, pregnenolone is synthesized in the brain suggesting expression of *cyp11a1* in the brain.[277] Pregnenolene is a neurosteroid that has been demonstrated to act as an allosteric inhibitor of several neuroreceptors (e.g. Gamma-aminobutyric acid, GABA) which protects the brain from the toxic effects of external agonists of these receptors.[278,279] However, in the steroidogenic tissues it is predominantly metabolized to one of two other steroids; namely progesterone and 17α-hydroxyprogesterone by the enzymes 3β-HSD and CYP17 respectively.

5.1.3.2 CYP17

CYP17 has dual functionality in the steroidogenic pathway, with two different substrates (Fig. 5.4).[233] Both reactions oxidise their respective substrates at C17 followed by further oxidation at the same position, resulting in cleavage of the C17-C20 bond. This yields the C19 steroids, androstenedione and dehydroepiandronstenedione (DEHA).[280] Each reaction is a two-step process whereby the C17 of progesterone or pregnenolone is hydroxylated to form the intermediate 17α-hydroxyprogesterone or 17α-hydroxypregnenolone.[280] The C17-C20 bond is cleaved via the oxidative formation of the ketone producing the C19 steroid with concomitant loss of formaldehyde.[281] As with all CYP enzymatic reactions, CYP17 utilizes an O_2 and NADPH molecule. A single gene codes for CYP17 and the approximately 57 kDa protein is found in the endoplasmic reticulum; where, the necessary co-factors for catalysis are present.[282]

Androstenedione has no major biological function (except as a metabolic intermediate to testosterone and other sex hormones). DEHA like androstenedione is also commonly a metabolic intermediate; however, it also has a wide range of biological applications and is the most abundant steroid found in human plasma.[283] DEHA, like pregnenolone is a neurosteroid having similar allosteric inhibitory functions on the GABA receptor (among others).[283]

As androstenedione and DEHA are intermediates to the sex hormones, CYP17 is expressed in the gonads. Interestingly, DHEA has been shown to up-regulate glucocorticoid synthesis as such is expressed in the adrenal glands despite not being an intermediate in glucocorticoid metabolism.

The multi-substrate functionality of CYP17 is not uncommon of the steroidogenic enzymes. In fact, 3β-HSD catalyses four different substrates into several products; including the biological essential progesterone and minor amounts of testosterone.[233]

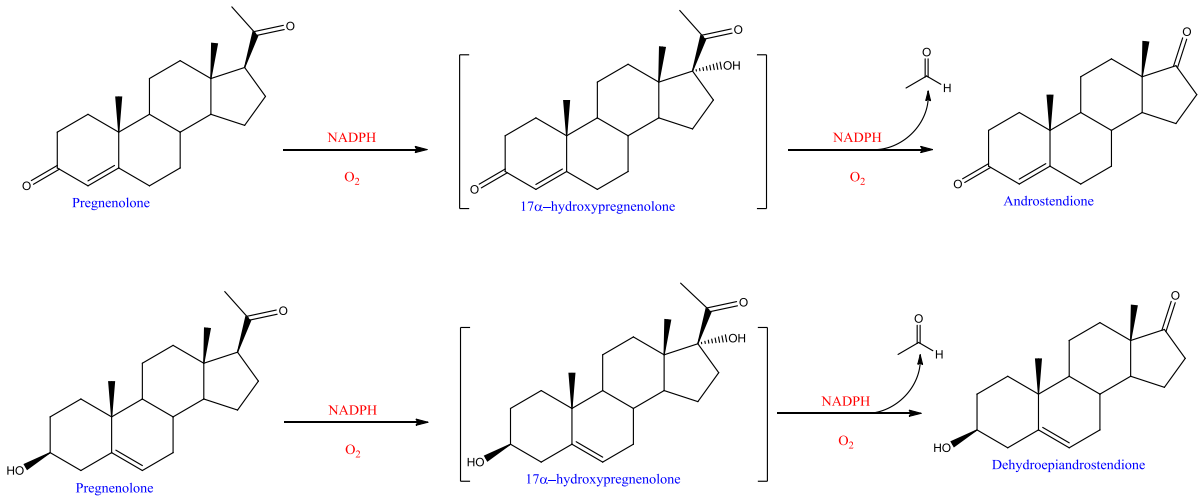


Figure 5.4: The catalytic reactions of CYP17. There is an initial hydroxylation at C17 followed by a second oxidation step to cleave formaldehyde and form a keto group at C17

5.1.3.3 3β-HSD

The ketone at C3 is essential for the biochemical functionality of many steroids (e.g. testosterone, progesterone and cortisol). 3β-HSD catalyses the conversion of the C3 alcohol to a keto group is. The enzymatic reaction is a two-step process to convert the C3 alcohol to a ketone and transfer the alkene from the C5 to the C4 position via an isomerase (Fig. 5.5).[284] Unlike the steroidogenic CYPs, 3β-HSD utilizes the cofactor NAD^+ which is reduced to NADH during catalysis.[284] 3β-HSD is coded for by multiple genes that synthesize a protein of approximately 44 kDa.[285,286] There are multiple isoforms of 3β-HSD (all found in the SEM). All the isoforms perform the same catalytic reactions; however, they are tissue specific (e.g. the human isoform I is found in the placenta, breast and skin, isoform II is located in the adrenal gland, ovary and testis).[287,288]

There are four products catalysed by 3β-HSD: progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone.[233] The role of testosterone (see Chapter 2) and androstenedione (section 5.1.3.2) have been discussed. Progesterone is an essential steroid in the female reproductive

cycle whereby it ensures the development of fertile gametes before ovulation. Both progesterone and 17α-progesterone are important intermediates in the metabolism of the glucocorticoids (see below).

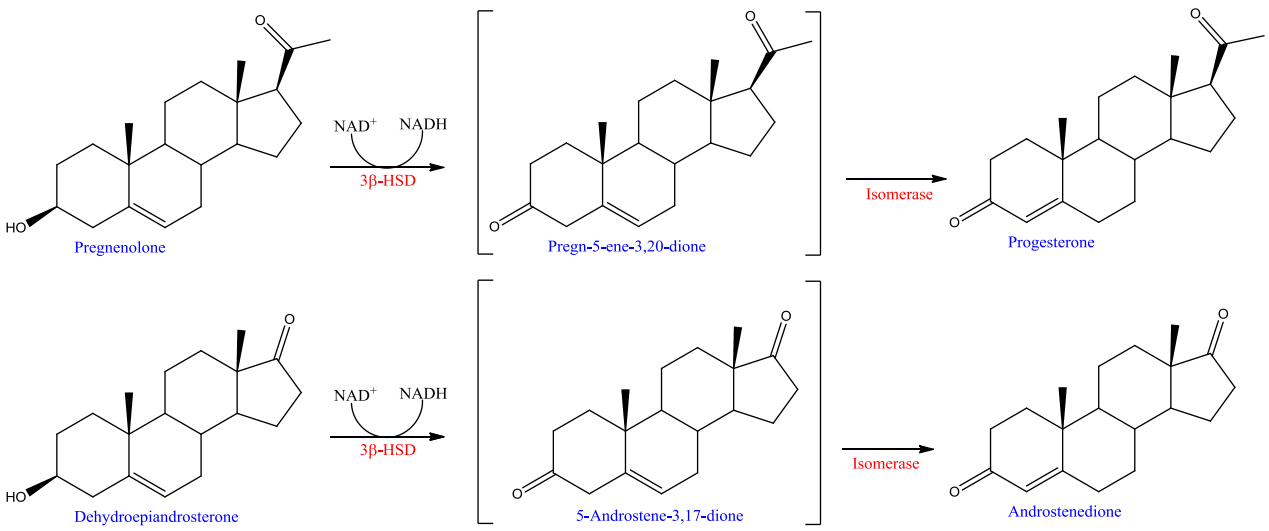


Figure 5.5: The oxidation of the C3 alcohol of pregnenolone and androstenedione by 3β-HSD. This reaction requires the cofactor NAD^+ which is reduced in the reaction. The next step is the isomeration of the alkene which migrates from C5 to C4

5.1.3.4 CYP21 and CYP11

The multifunctional glucocorticoids are synthesised by the adrenal glands. They are essential in the regulation of the immune system, metabolism and maintaining bodily homeostasis.[65] They are metabolised form two progestogen intermediates; namely progesterone and 17α-hydroxyprogesterone into 11-deoxycorticosterone and 11-deoxycortisol respectively (Fig. 5.6).[233] The initial reaction is catalysed by CYP21. CYP21 hydroxylates the progestogens at C21 utilizing a NADPH and 0_2 molecule.[233] The approximately 56 kDa protein is coded by two genes, with only one gene (*cyp21a*) producing the active enzyme.[289] CYP21 (like most steroidogenic CYPs) is located in the endoplasmic reticulum.

Following catalysis, 11-deoxycorticosterone and 11-deoxycortisol are transported to inside the mitochondria to be further metabolised into active glucocorticoids.

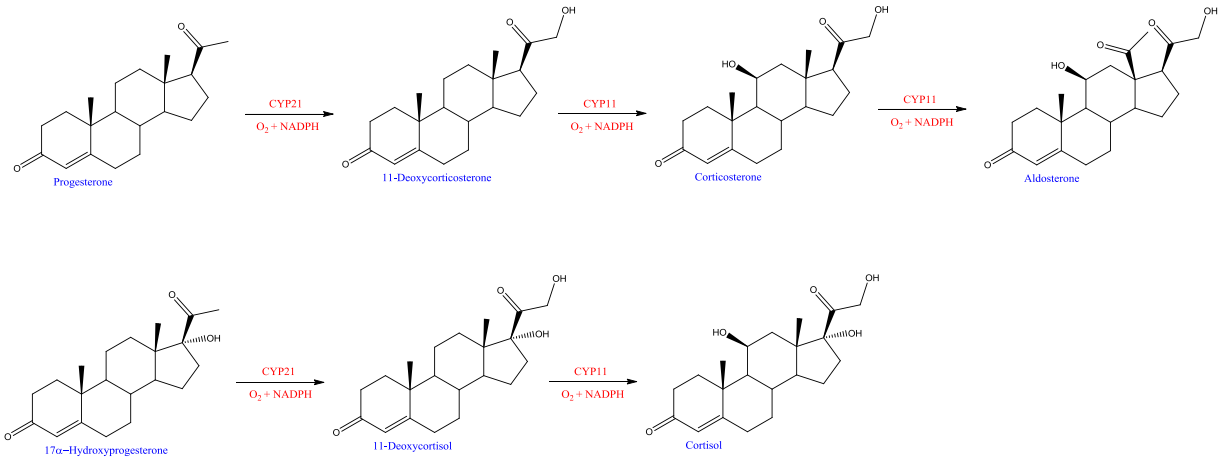


Figure 5.6: The metabolism of glucocorticoids. The synthesis of active glucocorticoids has two hydroxylation steps; firstly at C21 then at C22.

The second step in glucocorticoid synthesis is the β-hydroxylation at C11 by CYP11. The reaction occurs in the inner mitochondrial membrane to yield the active hormones aldosterone and cortisol (Fig. 5.6).[290] There are two genes that code for two isoforms of CYP11 with approximately 97% sequence homology; namely, *cyp11b1* and *cyp11b2.*[291]

5.1.3.5 17β-HSD

17β-HSD plays a major role in the synthesis of both androgens and estrogens. The functionality of 17β-HSDs is limited to sex hormone synthesis (it has no role in glucocorticoid synthesis).[233] The enzymatic reaction occurs via the reduction of the C17 ketone to yield a β-alcohol.[292] The steroid dehydrogenase utilizes NADH. Perhaps the most important reaction catalysed by 17β-HSD is the conversion of androstenedione to testosterone. A reaction essential for the healthy sexual growth and development in males (Fig. 5.7).[292]

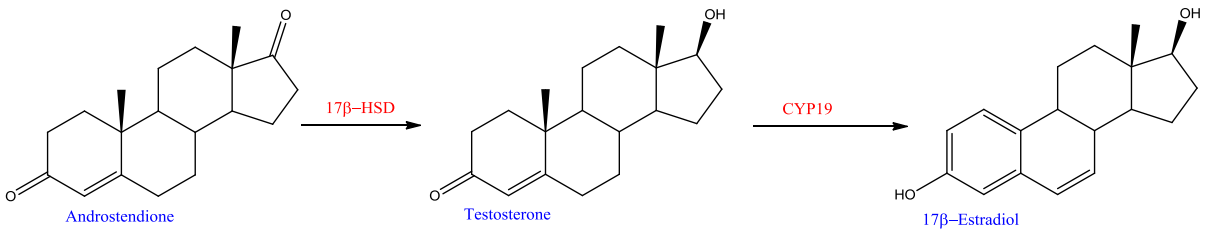


Figure 5.7: The primary synthesis of the sex hormones. The first step involves the reduction of the 7-keto group to form testosterone. In males, this is typically the end point. In females, testosterone is further metabolised by CYP19 to yield E2.

There are multiple tissue specific isoforms of 17β-HSD. Each isoform is synthesized by a specific gene to produce a protein of approximately 37 kDa.[293] The isoforms are primarily found in the gonads and placenta. Each isoform catalysed a specific reaction; e.g. 17β-HSD3, the enzyme responsible for testosterone synthesis is found exclusively in the testis.[294] The role of testosterone has been highlighted extensively in Chapter 2.

17β-HSD is a highly regulated as over expression of *hsd17b3* could yield too much testosterone, particularly in prepubescent boys. Therefore, the expression of the genes is directly regulated by LH action. However, any changes in the expression of this gene could result in significant reductions in testosterone production, with potentially negative effects on male growth and development.

5.1.3.6 CYP19

The activity of CYP19 in the ovaries is essential for the healthy sexual development of females. CYP19 catalyses the aromatization of testosterone (Fig. 5.7).[295] The reaction is a multi-step process; firstly, two oxidations of the C19 methyl group are followed by loss of the formic acid.[295,296] The final step is aromatization of the A ring to yield E2. Each step requires a single NADPH and O_2 molecule.

CYP19 is coded by a single gene (*cyp19a1*) which yields a 58 kDa protein.[295,297] The expression of CYP19 is limited to the ovary, placenta and Leydig cells.[298,299] Much like testosterone, the synthesis of E2 is tightly regulated by LH (as evidenced by the increased production via stimulation with gonadotropin

analogues). The regulation of *cyp19a1* is essential to prevent feminization in men as an increase in CYP19 expression would likely result in increased excess aromatization of testosterone. This could reduce testosterone levels and disrupt the androgen:estrogen ratio.

The regulation of steroidogenesis is essential in the healthy growth and sexual development of humans. From early gestation until late adulthood, steroidogenesis is regulated by a variety of compounds that stimulate the up-regulation of the steroidogenic genes. However, exposure to compounds (e.g. DBP) has the potential to disrupt the regulation of these genes. This may disrupt the healthy sexual growth and development of humans.

5.2 Research objectives

The aim of this described in this chapter is to investigate the effects of DBP on the gene expression and metabolism of the steroidogenesis pathway in cultured LC-540 cells.

This will be achieved through following research objectives:

1. Analyse and quantify the gene expression changes in key genes of the steroidogenesis pathway following exposure to DBP utilizing Nanostring® nCounter technology.
2. Determine and measure any effects of gene changes on the testosterone production in LC-540 cells following exposure to DBP utilizing a developed ELISA protocol.
3. Bring all the data together in the present study to determine the potential mechanisms of toxicity of DBP exposure in cultured cells.

5.3 Methods

5.3.1 Preparation of Exposure Standards and Media

This procedure was carried out as described in section 3.3.1

5.3.2 Preparation of LC-540 Cells

LC-540 cells (6 flasks) were removed from incubation and placed in the laminar flow cabinet. The spent MEM was aspirated and the cells washed with PBS (5 mL). The PBS was aspirated and 2.5% v/v aq Trypsin (4 mL) was added to each flask. The cells were left at 37°C for 10 min until the cells fully detached. The trypsin was inactivated with MEM (10 mL) and removed via centrifugation (4000 xg/5 min) and vacuum aspirated. The cell pellets were suspended in MEM (20 mL) and pipetted into a fresh 75 cm^2 culture flask. The cells were incubated for 24 h.

5.3.3 Exposure of LC-540 Cells

This procedure was carried out as described in section 3.3.2. The cells were incubated for 72 h following exposure.

5.3.4 Cell Counting

The 12 flasks were removed from incubation and placed in the laminar flow cabinet. The spent MEM was collected into 50 mL centrifuge tubes and frozen at -20°C for testosterone analysis (see 5.3.7). The cells were washed with PBS (5 mL) to inactivate any remaining the MEM. The PBS was aspirated and 2.5% aq v/v trypsin (4 mL) was added. The cells were left to incubate for 10 min until the cells fully detached (as observed via inverted microscope). The trypsin was inactivated with MEM (16 mL) and the cell suspension was transferred into a 50 mL centrifuge tube. The suspension was then vortex mixed to break up any cell clumps. A 1 mL aliquot was taken. A 10 µL aliquot was mixed 1:1 in trypan blue and counted using a hemocytometer (see section 2.3.4.1). The cells were stored at -80°C prior to gene expression analysis.

5.3.5 RNA Extraction

The RNA extraction procedure was carried out in conjunction with Dr Ellen Podivinsky. This section represents joint work.

Pelleted cells were removed from -80°C and gently thawed on ice. The cells were suspended at approximately 15,000 cells/µL in RTL lysis buffer supplemented with 1% v/v 2-mercaptoethanol. Cells were suspended and lysed by gentle vortex mixing followed by aspiration using a 1 mL micropipettor tip. Genomic DNA was removed from the samples by centrifugation through a gDNA removal column at 8000xg (max) for 30 s. Aliquots ere added into Eppendorf tubes and stored at -80°C prior to analysis.

5.3.6 Gene Expression Assay

The gene expression assay was carried out by NZ Genomics Ltd (Dunedin, NZ).

5.2.6.1 Code Sets

A custom nCounter CodeSet of reporter and capture probes for rat genes was obtained from Nanostring Technologies. The CodeSet also contained 6 proprietary positive Control probes and their RNA targets, plus 8 proprietary negative Control probes; provided by Nanostring Technologies (Seattle, Washington, USA).

5.2.6.2 Gene Expression Assay

Gene expression was assayed using the NanoStrings Technologies nCounter Gene Expression Assay system. Cell lysate from approximately 15,000 cells was processed in a 5 µL total reaction volume using the standard nCounter gene expression protocol for this sample type. Data acquisition was performed using a GEN2 Digital Analyzer with the "Max" Field of View setting (555 images per sample; 5 hour scan per cartridge).

5.2.6.3 Data and Statistical Analysis

Raw data (RCC files) exported from the Digital Analyzer were QC checked and normalised using nSolver Analysis software.

A background 'no-gene expression' (NE) threshold level was calculated from negative control NanoString nCounter® data, with the mean of negative control + 1 standard deviation taken as the baseline threshold for gene expression. Expression levels for target genes were normalized against *Actb* and *Hprt1* endogenous control gene expression levels. Gene expression fold-change was calculated for normalized NanoString nCounter® data for exposed cultures compared to control cultures and a Student's t-test was used to determine the probability that differences between control and exposed cultures were significant. The threshold for a fold change was a fold change >1.4 and a P value < 0.075.

5.3.7 MEM extraction

The spent MEM was thawed at 4°C overnight. MEM (5 mL) was filtered through a 0.22 µm cellulose acetate sterile syringe filter. The media was extracted as described in section 4.3.2.4. The extract was dissolved in testosterone buffer (500 µ) and stored at 4°C prior to testosterone analysis.

5.3.8 Testosterone Analysis

The testosterone ELISA was performed by Dr John Lewis at the CHL, Christchurch, New Zealand. The assay was performed using the standard procedure.[300]

5.4 Results

5.4.1 Changes in Expression of Key Testosterone Genes

The control exposure group (i.e. 0 µg DBP/mL) was used as the zero point in determining the fold changes. Data were normalised against two housekeeping genes; namely *Actb* and *Hprt1*. Three major changes in gene expression were found (Fig. 5.8). Firstly, *hsd17b3* had a 2.7-fold and 3.1-fold decrease at 0.1 µg DBP/mL and 1 µg DBP/mL respectively. Secondly, *cyp19a1* was found to have a 4.6-fold increase at 1 µg DBP/mL exposure level. The final gene expression change was of *AR*, which had modest fold increases of 1.1 and 1.4 at 0.1 µg DBP/mL and 1 µg DBP/mL respectively. The expression of *esr1, esr2, lhcgr, cyp21a1* and *cyp11b2* remained unchanged.

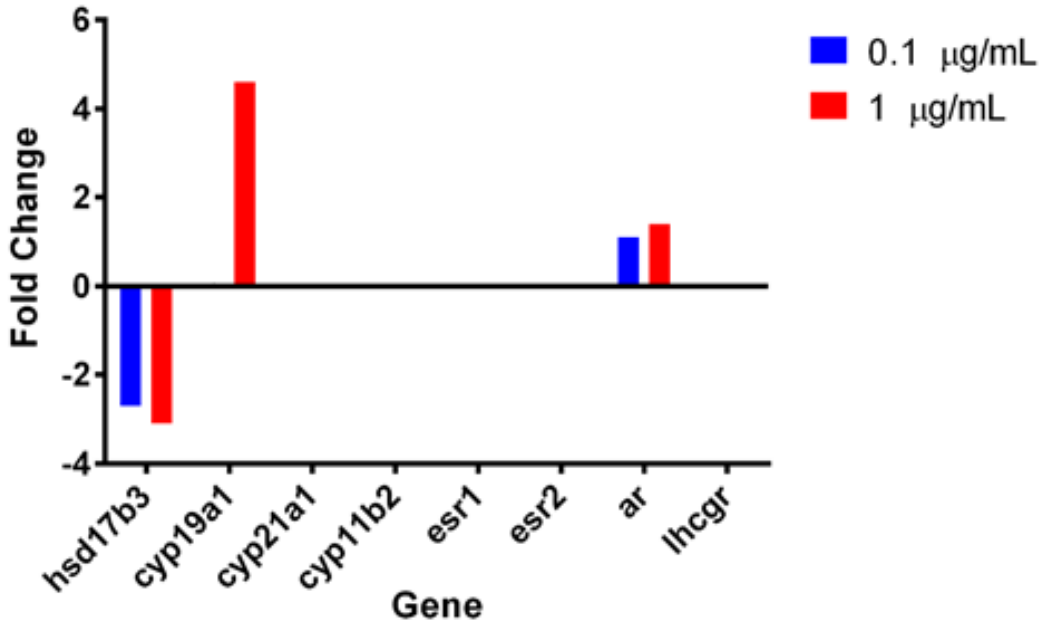


Figure 5.8: The gene expression changes of key genes in testosterone biosynthesis. The gene *hsd17b3* is approximately 3-fold lower in both exposure groups. The genes *cyp19a1* and *ar* were up-regulated by 4.6-fold and 1.4-fold respectively in the higher exposure level. Many genes remained unchanged including genes for glucocorticoid synthesis, ERs and LHRs.

5.4.2 Testosterone production in LC-540 Cells following DBP exposure

The testosterone production in LC-540 cells was measured via ELISA (Fig. 5.9). The levels of testosterone are significantly decreased in both exposure levels following the 72 h incubation with DBP. The testosterone levels are normalised against cell count. The control group has an average testosterone concentration of 79.9 pg/10^6 cells. Following DBP exposure the testosterone concentration decreases to 42.4 and 38.7 pg/10^6 cells for the 0.1 µg DBP/mL and 1 µg DBP/mL exposure levels respectively.

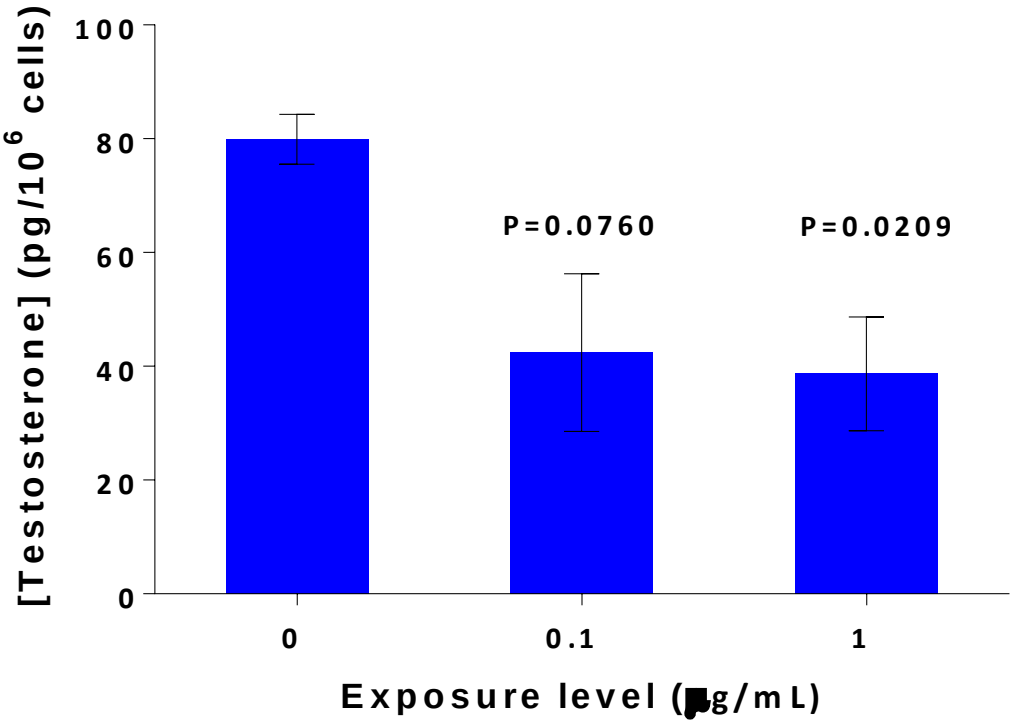


Figure 5.9: The production of testosterone per million LC-540 cells following exposure to DBP. The testosterone producing capacity decreases from approximately 80 pg/10^6 to approximately 42 and 38 pg/10^6 in the exposure groups.

5.5 Discussion

5.5.1 Gene Expression Changes

Nanostring nCounter technology is a relatively new methodology for analyses of gene expression. The assay itself bridges the two more conventional gene expression assays; namely microarray and real time polymerase chain reaction (RT-PCR). The assay itself has a key advantage; i.e. the sensitivity of RT-PCR without the need to convert RNA to cDNA. As such, Nanostring nCounter technology provides an excellent opportunity to analyse the effects of DBP on genes in cultured Leydig cells. The present study clearly demonstrates the gene expression changes in response to DBP exposure.

5.5.1.1 Downregulation of hsd17b3

The gene expression of hsd17b3 was downregulated 2.7 and 3.1 fold following exposure to 0.1 µg DBP/mL and 1 µg DBP/mL respectively (Fig. 5.8). The protein coded for by *hsd17b3*, 17β-HSD, is a critical enzyme in testosterone biosynthesis as it converts androstenedione into testosterone in Leydig cells. The downregulation of this gene may lead to a reduction in the transcription of 17β-HSD, leading to a reduced capacity of the Leydig cell to produce testosterone.

As discussed in chapter 2, testosterone is critical in the growth, development and maintenance of the male phenotype beginning at early gestation and continuing throughout life. During early gestation, testosterone and DHT act upon ARs to promote the transcription of growth factors. These growth factors ensure the development of the Wolffian ducts to form the testes and external genitalia. This process is highly sexually dimorphic as the same tissues become the female genitalia in female embryos when under control of E2. The descent of the testes into the scrotum is entirely dependent of the paracrine signalling induced regression of ECMs of gubernaculum cells (which prevent the teste form entering the scrotum). If there is inadequate testosterone produced by FLCs in the embryo, it is likely that the guburnaculum cells will not fully regress. The result of this is likely undescended testis (i.e. cryptorchidism). Cryptorchidism has been widely demonstrated to be a result of embryological

exposure to DBP. Therefore, the gene expression changes of *hsd17b3* could explain the pathogenesis of this disease in rats exposed to DBP.

Testosterone is also essential in the development of the penis, which is dependant of the activation of ARs in penile glans and the urethral epithelial cells. Therefore, if testosterone production is decreased, there is the possibility that there could be an inadequate development of the urethra. This is known as hypospadias and is commonly observed following embryological exposure to DBP. A key observation of the present study is that the down-regulation of *hsd17b3* occurs at both exposure levels; i.e. at the normal population and occupational daily exposure levels. Therefore, the present study demonstrates that the exposure to DBP at the TDI can affect Leydig cells via gene regulatory changes. These changes are likely to have effects on male embryos.

Reduced capacity to produce testosterone via the down-regulation of *hsd17b3* is a very probable cause of the feminizing effects of DBP exposure. However, the gene expression results also give rise to the possibility of increased E2 synthesis which could significantly affect male growth and development.

5.5.1.2 Upregulation of CYP19

The gene expression results demonstrate that exposure to DBP at the occupational daily exposure level (1 µg DBP/mL) upregulates the expression of *cyp19a1*, the gene that codes for the enzyme CYP19 (also known as aromatase). The primary functionality of this enzyme is the conversion of testosterone into E2. The production of E2 is directly responsible for feminization of cells and organisms. Therefore, upregulation of this gene could have a two-fold role in the disruption of male reproductive health. Firstly, along with the reductions in *hsd17b3* expression, an increase in the expression of *cyp19a1* would further decrease the capacity of a Leydig cell to produce adequate testosterone (such effects of this highlighted above). Secondly, an subsequent increases in E2 production would have the potential to disrupt the androgen:estrogen ratio. This is particularly damaging to the developing embryo. The initial

phase of teste descent requires the regression of the CSL (see chapter 2). While testosterone does not play a major role in this process (INSL3 is the key androgenic factor), the CSL can be reinforced by the activity of E2. In a developing male embryo, this could prevent total regression of the CSL and lead to cryptorchidism.

Overall the dual regulatory changes of *hsd17b3* and *cyp19a1* demonstrate a mechanism of reduced testosterone synthesis and an increased potential of feminization. It is important to note that there were no major changes in gene expression of esr1 or esr2 which would be a strong indicator of increased E2 synthesis. Both *esr1* and *esr2* were up-regulated by 1.4 and 1.9 fold respectively. However, the respective P values were 0.186 and 0.707 and so fell well below the threshold for a statistically significant fold change. Interestingly, the up-regulation of *AR* expression was statistically significant.

5.1.1.3 Increased AR Expression

The expression of *AR* was increased at the higher exposure level by 1.4 fold with a P value of 0.03. Such an increase *AR* expression seems counterintuitive as any reductions in testosterone biosynthesis would presumably reduce the expression of the AR. However, the observed increase in AR expression may in fact be an indicator of reduced testosterone synthesis or feminizing pressure. This has been shown in other studies which demonstrate increases in *AR* in prostate cells following exposure to the xenoestrogen, BPA.[70] If the production of testosterone reduces, the cell could possibly increase AR expression in a potential scavenger mechanism in an attempt to increase the possibility of testosterone binding to and activating the AR.

5.1.1.4 The Unchanged Expression of Several Key Genes

Several key genes remained unchanged following exposure to DBP including *cyp11b1, cyp21a1* and *lhcgr*. These negative results provide insight allow for the potential elimination of several mechanisms of toxicity. The unchanged expression of *cyp11b1 and cyp21a1* despite reductions in *hsd17b3* suggest that the steroidogenic pathway may not change the direction of flux in response to changes in the genes

responsible for testosterone synthesis. This contradicts previous reports of increased cortisol synthesis following DBP exposure.[67] This may be due to the cell type differences; the present study used LC-540 cells whereas the other study used R2C cells.

The unchanged expression of *lhcgr* also suggests that the reductions in testosterone likely do not occur via reductions in the hormonal stimulation via gonadotropins.

5.5.2 Reduction in Testosterone Production in DBP-exposed LC-540 Cells

Many hundreds of genes can change following exposure to potentially toxic compounds. However, such changes in gene expression do not always carry through to the translational level. Indeed, the proteomics and metabolomics can be considered stronger indicators of the effects of exposure to such compounds. The proteomics are perhaps more difficult to measure, especially in LC-540 cells as the enzymes are almost certainly at very low concentrations. This can prevent the analysis via Western Blotting. Therefore, it is essential to determine metabolism of testosterone in the culture medium following exposure to DBP. If there are changes in metabolism combined with changes in gene expression strongly implies that protein synthesis is also affected.

The ELISA analysis of used culture medium found that there was a significant drop in the production in testosterone when compared to the no exposure group (Fig. 5.9). The base level of testosterone production was found to be approximately 80 pg/10^6 cells. The cells exposed to DBP had approximately half the capacity to produce testosterone at 42 pg/10^6 cells and 38 pg/10^6 cells at 0.1 µg DBP/mL and 1 µg DBP/mL exposure levels respectively. The slight variability in testosterone production between the two exposure levels matches the gene expression results which had a mere 0.4 fold difference in *hsd17b3* expression.

The levels of E2 were not analysed during this thesis. As the levels of testosterone are much lower in LC-540 cells than primary cells, if the gene changes of *cyp19a1* translated into increased aromatase activity

there is the possibility of changes in the levels of E2. However, as the levels would be extremely low, ELISA or radioimmunoassay would not be sensitive enough. A potential method could be isotope dilution mass spectrometry. More work needs to be to develop this method for the detection of E2.

There are several limitations in the research described in this chapter. Firstly, the gene expression assay only focused on two exposure levels. This prevents any identification of a dose-response relationship. Further work needs to be done to analyse the gene expression changes following DBP exposure at a range of concentrations. The gene expression assay only analyses a few genes in steroidogenesis. This limited scope does not allow for the full elucidation of the gene expression changes following DBP exposure. More work needs to be done to evaluate the effects of DBP on StAR, 3β-HSD and other steroidogenic CYPs. The lack of proteomics prevents the absolute conclusion that the gene changes to *hsd17b3* or *cyp19a1* carry through to the protein level. The ELIZA data suggest the genes are likely changed but a reduction in testosterone could be due to number of effects on steroidogenesis. Western Blot analysis must be done in the future to evaluate the effects of the gene changes of 17β-HSD and CYP19.

6.0 Conclusion

The LC-540 cells are a robust model system. The present study used them to significant success as a model system for the toxicity of DBP *in vivo.* The biochemistry of LC-540 coupled with their ease in culture provided an excellent basis for the other experiments in the present; i.e. the ability to produce adequate testosterone and maintain their biochemistry in long term cultures.

DBP metabolism in LC-540 cells is in contrast to other metabolism studies in rats. This is due to the excess glucuronidation of MBP. The presence of estrogenic molecule, M4B-4HP was not detected. However, more work is needed to determine it is produced. The presence of M4B-4HP (or another estrogenic metabolite) was implied by the estrogenic activity in MCF-7 cells following exposure to DBP.

The present study presents a wide range of data that demonstrates the dual mechanism of toxicity of DBP in cultured cells. Firstly, DBP alters the expression of key genes in testosterone biosynthesis. The expression of *hsd17b3* is significantly reduced. ELIZA analysis suggests that this down-regulation diminishes the capacity of LC-540 cells to produce testosterone. The expression of *cyp19a1* is simultaneously up-regulated. This could metabolise the already reduced testosterone levels into E2. This could have two roles, further reduction of the testosterone levels with the concomitant feminizing pressure via increases in E2 levels. The second mechanism of toxicity is via the minor conversion of DBP into M4B-4HP. This likely acts as a xenoestrogen, feminizing tissues and cells.

It is therefore possible that exposure to DBP at normal population exposure levels results in male genital developmental changes consistent with the increased rates of hypospadias and cryptorchidism previously reported.[179] Prolonged indirect exposure from the ingestion, inhalation and dermal absorption might lead to developmental changes to the general population.

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