UNIVERSITY OF TECHNOLOGY SYDNEY

The Use of Bioassays to Detect Designer Androgens in Sports Supplements

Elliot Russell Cooper

Bachelor of Biotechnology (UTS)

Master of Science in Medical Biotechnology (UTS)

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

at the University of Technology, Sydney

Supervisor: Dr Kristine C. Y. McGrath

Co-supervisors: Professor Alison K. Heather and Dr David van Reyk

Submitted June 2016

Certificate of Original Authorship

This thesis is the result of a research candidature undertaken at the University of Technology, Sydney as part of a doctoral degree. I certify that the work in this thesis has not previously been submitted for a degree.

I also certify that the work in this thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Elliot R. Cooper

30th June 2016

Acknowledgements

I would like to dedicate this thesis to my late brother, Harrison John Cooper. I wish you could be here to see me finish this PhD. I know you would be proud of me. We all miss you.

First and foremost, I must thank the endless love and support that my parents have given me throughout this PhD. Mum and Dad, thank you for always being there for me, through thick and thin.

To Alison Heather, thank you for all that you have taught me, and the opportunities you have given to me. You gave me a Masters project, this PhD project and my first job in science. I would definitely not be starting a career in science if it wasn't for you. Thank you for all the help and guidance you have given me over the years, and of course, the patience of dealing with me during times of stress! You are an amazing supervisor and mentor and your work ethic is remarkable.

To Kristine McGrath, you too have also been an amazing supervisor. Thank you for everything you have taught me in the lab, and for helping me all the times I needed help. You have had an extraordinary amount of patience with me, and for that I thank you. Your constant cheerfulness made it a pleasure to work with you in the lab.

There are also several other people I would like to thank for helping me throughout this PhD. I would like to thank Dr Rymantas Kazlauskas of the National Measurement Institute for providing the steroids and relevant information about them. The majority of work in this thesis would not be possible without your expertise. I would like to thank Dr David van Reyk for becoming my co-supervisor during my PhD. Your input during group meetings was always valued and I thank you for reading over my thesis.

I also want to thank some of the friends I have made throughout this PhD. Rob, Sam, Pat, Peter, Martin, Rosaline, Pamela, you have all been great friends. I have definitely enjoyed the last few years through the ups and downs of this PhD with you guys. Thanks for all your advice throughout the PhD and helping me switch off from work and relax.

I also want to thank other members of UTS. Dr George Herok, your attitude towards pro-doping (instead of anti) is an amusing one and it definitely helped make presenting my work more enjoyable, and your input was always appreciated. Dr Matt Padula and other members of the proteomics group for advice on the solid-phase extraction technique, which was crucial for a large part of my work. Dr Michael Johnson for your help on the IN-Cell Analyzer, which helped me get a lot of data. Dr Lani Li for your help and advice in the lab. You were the first person to teach me the yeast androgen bioassay five years ago, and I have been doing this assay ever since! I would also like to thank the members of the HUB group, for their advice during my presentations.

Lastly, I would like to thank UTS for allowing me to undertake a PhD and for providing me with financial support throughout the course of this PhD.

Table of Contents

List of Figures

List of Figures Continued

List of Tables

Abstract

Androgens are the most widely abused prohibited substances in sports. Detection of androgen abuse in sports relies on using sensitive gas chromatography tandem mass spectrometry-based techniques. These techniques require knowing the structure of the test compound in order to detect it. The last 15 years has seen the emergence of steroids with novel structures, termed designer steroids, which can bypass detection. In recent years, many of these designer steroids have appeared in sports supplements.

There is limited data on the safety and efficacy of designer steroids. Numerous studies report that consumption of sports supplements containing designer androgens are associated with a number of adverse health effects, including cholestatic jaundice. Furthermore, it is often not known if these designer androgens have beneficial anabolic activity.

The overall hypothesis of this thesis was that designer steroids contained within sports supplements are potent androgens. The main aim of this thesis was to assess the androgenic and anabolic activity of sports supplement-derived designer steroids using reporter gene androgen bioassays and a C2C12 myoblast cell model. Additionally, the Australian sports supplement market was screened for undeclared androgenic substances.

Chapters 3 and 4 investigated the androgenic bioactivity of 22 designer steroids utilising *in vitro* androgen bioassays. Chapter 3 aimed to assess the intrinsic androgenic bioactivity of the designer steroids using the *Saccharomyces cerevisiae*-based yeast androgen bioassay. It was determined that 45% of the sports supplements had strong androgenic activity. Chapter 4 tested these designer steroids in the HuH7 cell line to mimic hepatic metabolism. This chapter showed that several of these strong androgens remained potent or were activated into more

potent androgens after metabolism. Further, several intrinsically strong androgens were deactivated.

Chapter 5 assessed the anabolic potential of several potent designer androgens in a C2C12 myoblast cell line. This study demonstrated that five androgens which had strong AR bioactivity, also demonstrated a high anabolic potential, with significant increases in myotube hypertrophy, nuclei accretion and MHC expression.

Finally, Chapter 6 investigated the presence of undeclared androgenic substances in sports supplements available to the Australian market. Using the yeast and HuH7 androgen bioassays, it was shown that 5.3% (6/112) of the supplements had androgenic activity.

In conclusion, this thesis demonstrates that sports supplements contain potent androgens, and should be of concern to the general Australian population and athletes, due to the potential health risks associated with androgen abuse, and the potential for testing positive in a doping test.

Publications and Presentations

Publications

The use of tandem yeast and mammalian cell *in vitro* androgen bioassays to detect androgens in internet-sourced sports supplements

Elliot R. Cooper, Kristine C. Y. McGrath, Xiaohong Li, Omar Akram, Robert Kasz, Rymantas Kazlauskas, Malcolm D. McLeod, David J. Handelsman, Alison K. Heather

Drug Testing and Analysis, *2016, doi: 10.1002/dta.2000.*

Steroid extracts from nutritional sports supplements test positive for androgenic activity

Elliot R. Cooper, Xiaohong Li, Kristine C. Y. McGrath, Alison K. Heather

In preparation

***In vitro* androgen bioassays as a detection method for designer androgens**

Elliot R. Cooper, Kristine C. Y. McGrath, Alison K. Heather

Sensors, *2013, vol. 13: 2148-2163*

Presentations

Steroidal extracts from nutritional sports supplements sold in Australia test positive for androgenic activity

Elliot R. Cooper, Xiaohong Li, Kristine C. Y. McGrath, Alison K Heather

International Congress of Endocrinology/Endocrinology Society, 2014, Chicago, USA.

Poster Presentation

Nutritional sports supplements sold in Australia contain undeclared hormonal adulterants

Elliot R. Cooper, Xiaohong Li, Kristine C. Y. McGrath, Alison K Heather

New Horizons Conference, 2013, Royal North Shore Hospital, NSW, Australia.

Poster Presentation

List of Abbreviations

AAF	Adverse Analytical Findings
AAS	Anabolic Androgenic Steroid
ACTH	Adrenocorticotrophic Hormone
AF	Activation Function
AI	Aromatase Inhibitor
AR	Androgen Receptor
ARE	Androgen Response Element
BALCO	Bay Area Laboratory Co-Operative
CHO	Chinese Hamster Ovary
DBD	DNA Binding Domain
DHCMT	Dehydrochloromethyltestosterone
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DMT	Desoxymethyltestosterone
DNA	Deoxyribonucleic Acid
DSHEA	Dietary Supplement and Health Education Act
E	Epitestosterone
EC_{50}	Effective Concentration, 50%
EDC	Endocrine Disrupting Chemical
eIF3-f	Eukaryotic Translation Initiaion Factor 3 Subunit F
EPO	Erythropoietin
ER	Oestrogen Receptor
ESA	Erythropoiesis-Stimulating Agent
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FITC	Fluoroscein-Isothiocyanate
FoxO	Forkhead Box O

List of Abbreviations Continued

FSANZ	Food Standards Australia New Zealand
FSH	Follicle-Stimulating Hormone
GC-MS	Gas Chromatography-Mass Spectrometry
GDF-8	Growth and Differentiation Factor-8
GDR	German Democratic Republic
GMP	Good Manufacturing Practice
GnRH	Gonadotropin Releasing Hormone
GR	Glucocorticoid Receptor
HBOC	Haemoglobin-Based Oxygen Carrier
hCG	Human Chorionic Gonadotropin
hGH	Human Growth Hormone
HPG	Hypothalamic Pituitary Gonadal Axis
HSD	Hydroxysteroid Dehydrogenase
HSP	Heat Shock Protein
HuH7	Human Hepatocarcinoma
IGF-I	Insulin-Like Growth Factor-I
IOC	International Olympic Committee
IOC-MC	International Olympic Committee – Medical Commission
IRMS	Isotope Radio Mass Spectrometry
LBD	Ligand Binding Domain
LH	Luteinizing Hormone
LOH	Late Onset Hypogonadism
MAPK	Mitogen-Activated Protein Kinase
MHC	Myosin Heavy Chain
MR	Mineralocorticoid Receptor
mTOR	Mammalian (or Mechanistic) Target of Rapamycin
MuRF1	Muscle RING Finger 1
NTD	N-Terminal Domain

List of Abbreviations Continued

OD	Optical Density
ONPG	o-Nitrophenol-β-galactosidase
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PDGF	Platelet-Derived Growth Factor
PFC	Perfluorocarbon
PI3K	Phosphatidylinositol 3-Kinase
PIC	Pre-Initiation Complex
PKA	Protein Kinase A
PKC	Protein Kinase C
PR	Progestogen Receptor
PSA	Prostate Specific Antigen
RBC	Red Blood Cell
RIA	Radio-Immunoassay
RNA	Ribonucleic Acid
RP	Relative Potency
SARM	Selective Androgen Receptor Modulator
SEAP	Secreted Embryonic Alkaline Phosphatase
SERM	Selective Oestrogen Receptor Modulator
SHBG	Sex Hormone Binding Globulin
SPE	Solid Phase Extraction
T	Testosterone
T/E	Testosterone/Epitestosterone ratio
TGA	Therapeutic Goods Administration
TGF-β	Transforming Growth Factor β
THG	Tetrahydrogestrinone
TUE	Therapeutic Use Exemption
yEGFP	Yeast Enhanced Green Fluorescent Protein

List of Abbreviations Continued

YPD	Yeast Peptone Dextrose
WADA	World Anti-Doping Agency
WADC	World Anti-Doping Code

Chapter 1

Literature Review

Table of Contents

Table of Contents Continued

1.1 Introduction

Athletes have long sought the ability to enhance their performance in sports ever since the ancient Olympic Games and continue to do so in the modern era of elite competition. The modern day athlete is under an enormous amount of pressure, as their careers rely solely on their success. For the majority of athletes, their personalities feed off this pressure and is what drives them to compete at such a high level. Unfortunately, however, there are some that succumb to the pressure and look for alternate, prohibited means to ensure their success. Even before the days of modern science, athletes have known that certain foods and diets have the ability to increase performance in sport (Barroso *et al*, 2008; Sjöqvist *et al,* 2008). In recent times, athletes often turn to modern day sports supplementation to help increase their recovery and performance (Lun *et al*, 2012; Maughan *et al*, 2007). More cunningly, the use of exogenous hormones to enhance physical performance has been known since the early 1950s after the discovery of the role of the natural androgen, testosterone (T) in masculinisation (Handelsman, 2011a). While most sports supplements are not banned, the use of hormones, most stimulants, or substances that would otherwise artificially enhance physical performance is considered illegal in competition. The 'doping' with such substances is prohibited because they can unnaturally (and unfairly) enhance the performance of an athlete, take away the spirit of fair competition, or may be a potential health risk to the athlete (World Anti-Doping Agency, 2016).

While doping encompasses a range of prohibited substances (and methods), androgens remain the most widely abused substances in sport. Androgens, like T, are a group of lipophilic steroid hormones derived from cholesterol (Papadopoulos and Miller, 2012). They exert a number of physiological functions within the body which can be broadly classified into either

androgenic or anabolic. Their androgenic functions refer to their ability to augment the development of male sexual organs, facial and body hair, thickening of the vocal cord, and increase libido and aggression (Kicman, 2008). The anabolic effects of androgens refer to their ability to enhance skeletal muscle growth, increase lean muscle mass and reduce muscle protein catabolism (Kicman, 2008). These anabolic properties are highly desirable for many athletes as increases in muscle size and strength may lead to improved sporting performance and are the reason why androgens are the most widely abused performance enhancing substance in sport (Hartgens and Kuipers, 2004a).

In anti-doping tests, androgens are screened for using mass spectrometric-based techniques including gas chromatography tandem mass spectrometry (GC-MS), which rely on knowing the chemical structure of the compound. In recent years, there has been an influx of novel designer steroids used for doping in order to bypass screening tests. The majority of these designer steroids are being sold as 'sports supplements', freely available to consumers. There is little scientific knowledge regarding the safety and efficacy of these compounds, and therefore, may be a potential health risk to consumers.

While not currently accepted for routine screening tests, *in vitro* androgen bioassays are capable of detecting designer androgens in sports supplements (Akram *et al*, 2011). Bioassays rely on the biological activity and the activation of the androgen receptor (AR) in order to detect an androgenic molecule, rather than the requirement of knowing the chemical structure. Therefore, AR bioassays can determine the bioactivity a compound has with the AR through expression of a reporter protein. This can give an indication of the potency of the designer androgen, unlike MS-based detection methods.

The discovery that the first reported designer steroids, tetrahydrogestrinone (THG) and norbolethone, were potent androgens, has led to the overall hypothesis of this study that these newly emerging designer steroids found within sports supplements are potent androgens. This study aims to evaluate the sports supplement market for androgenic substances using *in vitro* androgen bioassays and to identify the prevalence of androgenic substances found in the Australian sports supplement market.

1.2 Doping in Sports

1.2.1 Early Accounts of Doping with Androgens in Sport

In the early 1950s, the US Olympic weightlifting team's physician, Doctor John Ziegler, learned of allegations that the Russian weightlifters were being administered exogenous T to increase their performance. As such, he began his own experiments with the synthetic androgen, Dianabol (Todd, 1987). These early experiments with androgens appeared to be effective, according to the athletes; despite the medical community intent on refuting that androgens can be performance enhancing (Wade, 1972). With the success of the Russian and US weightlifting teams, clandestine research and testing of androgens in athletes became more popular, and began spreading into other sports (Yesalis and Bahrke, 2002). After the unification of the East and West German states in 1989, it was found that the German Democratic Republic (GDR) undertook extensive research and use of doping programs with androgens in complete secrecy, beginning in the 1960s (Franke and Berendonk, 1997). The success of the GDR athletes (particularly the female athletes), came as a result of their intensive and secretive doping program, utilising a range of androgens including oral-Turinabol (dehydrochloromethyltestosterone; DHCMT), nandrolone, and testosterone

preparations (Franke and Berendonk, 1997). Although many of the athletes started developing harmful side effects as a result of androgen abuse (Froehner *et al*, 1999), the GDR continued pursuing their doping programs.

1.2.2 Development of Doping Regulations

With the aim of preventing the misuse and abuse of drugs by athletes, the International Olympic Committee (IOC) established a medical body in 1967 responsible for monitoring prohibited substances in the Olympic Games known as the Medical Commission (IOC-MC). The IOC-MC worked together with international sporting federations to compile a list of prohibited substances and methods that may enhance performance in athletes. Athletes would then been screened for these substances and methods. The first major screening tests were introduced in the 1972 Munich Olympic Games. However, only stimulants and narcotics were screened for during these tests (Fitch, 2008). The use of androgens was not prohibited until 1974, with the 1976 Montreal Games being the first Olympic Games implementing the use of radio-immunoassay (RIA) for androgen detection, developed two years prior (Beckett and Cowan, 1978; Brooks, 1975). The IOC also decided to implement the use of GC-MS. However, this was an expensive method for testing samples, since there were not many laboratories in the world at that time that had this equipment (Todd, 1987).

The implementation of the new RIA technique during the 1976 Montreal Games found only eight out of 275 tested athletes were positive for androgens (Todd, 1987). The relatively low percentage of positive findings gave a false perception of the reality of the drug problem in the athletic community (Wagner and Pedersen, 2014). This was further exacerbated during the 1980 Moscow Olympics where no samples tested positive for androgens (Fitch, 2008). As

it happens, many of the athletes had stopped taking the synthetic androgen analogues and instead switched to taking preparations of T itself right before competition to avoid detection, as exogenous T was not yet prohibited and screened for (Franke and Berendonk, 1997). This led to the development of testing the testosterone/epitestosterone (T/E) ratio by Manfred Donike, which, when tested, revealed that 20% of all athletes from the 1980 Moscow Games would have tested positive for T use. This was able to convince the IOC to add T to the list of prohibited substances in 1982 and implemented in the following year during the 1983 Pan American Games (Todd, 1987).

In 1999, the World Anti-Doping Agency (WADA) was formed to monitor and oversee anti-doping policies. In 2004, WADA assumed the roles of the IOC-MC and became the primary body dedicated to enforcing policies as well as accreditation of sports anti-doping laboratories. WADA currently oversees over 630 sports organisations (Barnes and Rainbow, 2013) and maintains 34 accredited anti-doping laboratories which carry out routine screening tests (World Anti-Doping Agency, 2016).

1.2.3 The Prohibited List

Since the IOC-MC (and now WADA) began to take a serious approach towards combating doping, the Prohibited List was created to compile a list of banned substances and methods that cannot be used by athletes prior to, and during, competition. Every year, this list is updated based on current scientific findings. The list is divided up into ten classes of prohibited substances and three classes of prohibited methods. There are also two classes of substances banned in particular sports only (Table 1.1). In order for a substance (or method) to qualify as prohibited, it must meet at least two of the following three criteria from the

World Anti-Doping Code (WADC): 1) it has the potential to enhance or enhances sports performance; 2) it represents a health risk for the athlete; or, 3) it is contrary to the spirit of the sport (Barroso *et al*, 2008). In 1992, a policy was made for an exception to this rule, known as the Therapeutic Use Exemption (TUE) policy. This allowed for the administration of prohibited substances for genuine medical conditions as long as they followed three criteria: 1) the athlete would experience significant impairment of health if the prohibited substances (medication) were withheld; 2) no enhancement of performance would result from the administration of the prohibited substance as medically prescribed; and 3) no permitted and practical alternative medication could be substituted for the prohibited substance (Fitch, 2008). The following section will describe the various classes of compounds on the WADA Prohibited List, based on the most recent version of this list (2016) (World Anti-Doping Agency, 2016).

1.2.3.1 Prohibited Substances

Over the last decade, the most commonly reported androgens abused are stanozolol, nandrolone, methandienone, and T (World Anti-Doping Agency, 2016). These belong to the class 'S1 Anabolic Agents'. This class comprises exogenous androgens, such as THG, stanozolol and boldenone as well as endogenous androgens such as T and dehydroepiandrosterone (DHEA). This class also includes other anabolic agents other than androgens, including selective androgen receptor modulators (SARMS), and the β2-agonist clenbuterol, which has been shown to have anabolic activity (Pleadin *et al*, 2010).

The next most frequently abused category is the 'S6 Stimulants'. This includes substances such as ephedrine, adrenaline, cocaine and amphetamines. Caffeine used to be part of this

list but was removed in 2004 due to the difficulty in determining a threshold between social uses and doping attempts (Del Coso *et al*, 2011; Thuyne *et al*, 2006). These substances are banned due to their performance enhancing effects in decreasing reaction times and increasing mental alertness. However, they are banned only during competition. Often, stimulants are stacked with other substances to further enhance their effect. Stacking with androgens, for example, can allow for a combination of alertness, aggression and explosive power and can be very beneficial in sports such as sprinting, wrestling, and boxing. Androgens can also be coupled with several other classes of drugs, such as those in the S4 and S2 categories. The S4 class includes hormone antagonists and modulators and involves compounds such as aromatase inhibitors (AI) and anti-oestrogens. The S2 class substances include peptide hormones, growth factors and related substances. Most notably from this class is the use of human growth hormone (hGH). This hormone is responsible for the growth of muscle and bones, as well as organs. It may therefore serve to be an anabolic hormone, helpful to the athlete in recovery and muscle growth and has been marketed as such since 1988 (Ehrnborg *et al*, 2000). This class includes other growth factors such as insulin-like growth factor-I (IGF-I) and platelet-derived growth factor (PDGF), and essentially have roles in muscle, tendon, ligament protein synthesis/degradation, vascularisation, energy utilisation, regenerative capacity and fibre type switching. Compounds in this class are often used synergistically with androgens for their anabolic effects, particularly hGH (Perls, 2009). It also involves insulins, corticotrophins, and gonadotropins. Gonadotropins like chorionic gonadotropin (hCH) and luteinizing hormone (LH) are only prohibited in males.

Out of all the S2 class substances, the subclass comprising erythropoiesis-stimulating agents (ESAs) is probably the most infamous, particularly in recent years with several 'blood doping'

scandals in cycling. Erythropoietin (EPO) is responsible for maturing red blood cell (RBC) precursors into mature RBCs and therefore, has the potential to enhance the bloods capability of transporting oxygen (Elliott, 2008; Diamanti-Kandarakis *et al* 2005). Despite their infamy, reported cases of their abuse are low, with 57 cases reported, making up 1.85% of all doping cases in 2014 (World Anti-Doping Agency, 2016); however, EPO is difficult to detect and may account for the low detection rate (Durussel *et al*, 2016).

The use of diuretics and masking agents in attempts to hinder the ability of analytical tests to detect prohibited substances is also banned. Diuretics increase the rate of production of urine. In sports doping, this is aimed at increasing the rate of which a drug is excreted from the system as well as diluting it in urine samples to avoid detection (Cadwallader *et al*, 2010). Other masking agents attempt to interfere with chemical analyses and hinder the accuracy of tests. For example, the use of glycerol had recently been detected in urine samples (Okano *et al*, 2014). Glycerol is introduced via catheters primarily to increase plasma volume and reduce haemoglobin concentration (Okano *et al*, 2014). Glycerol also has high polarization and is hydrophilic, which can be lead to poor retention on hydrophobic stationary phases during reverse-phase liquid chromatography, making it difficult to detect (Görgens *et al*, 2015). Certain medications are also prohibited due to the potential performance enhancing properties. Glucocorticoids used for anti-inflammatory purposes are permitted by inhalation (such as asthma inhalers), but are banned through other routes of administration to prevent systemic anti-inflammatory effects. Narcotics (S7) and cannabinoids (S8) are banned only during competition, despite being illegal. In 2014, the most reported substance in the S7 class was morphine, and the most reported substance in the S8 class was carboxy-THC (World Anti-Doping Agency, 2016).

There are several drugs that WADA does not ban for all sports. The classes P1 (alcohol) and P2 (β-blockers) are only banned in the following sports: archery, automobile, billiards, darts, golf, shooting (also banned outside of competition), skiing/snowboarding and ski jumping. Similarly, alcohol is banned only during competition, and only for the following sports: aeronautics, archery, automobile, karate, motorcycling, and powerboating.

1.2.3.2 Prohibited Methods

Certain methods, including enhancing oxygen transfer in the blood, gene doping, and chemical and or physical manipulation of samples are banned. Any manipulation with blood in an attempt to enhance its ability to transfer oxygen is strictly prohibited. This includes autologous, homologous and heterologous blood transfusions. It also includes artificially enhancing the uptake, transport or delivery of oxygen, including haemoglobin-based oxygen carriers (HBOC) and perfluorocarbons (PFC).

Probably one of the most devious ideas for doping has been the issue of gene doping. Genetic modification of any kind is banned. This includes the transfer of nucleic acids or nucleic acid sequences, using normal or genetically modified cells, or using agents that modify gene expression (Friedmann *et al*, 2010; Wells, 2008). However, findings of gene-doping cases have never before been reported by WADA.

Table 1.1 The Prohibited List. Overview of prohibited substances and methods of doping according to the WADA prohibited list of 2016. Adapted from Thevis *et al*, 2016 (Thevis *et al*, 2016).

Class	Sub-Group		Example
S0 Non-Approved Substances			Rycals (ARM036)
S1 Anabolic Agents	1)	Androgens	
		a) Exogenous	THG; Stanozolol; Boldenone
		b) Endogenous	T; DHEA
	2)	Other Anabolic Agents	Clenbuterol; SARMs
S2 Hormones and Related Substances	1)	Erythropoiesis-Stimulating Agents	EPO
	2)	Hypoxia-inducible factor stabilizers	Cobalt, xenon
	3)	hCG and LH	
	4)	Corticotrophins	LipPro (Humalog); Adrenocorticotrophic Hormone (ACTH)
	5)	GH, IGF-I and Related Compounds	Genotropin
S3 β2-Agonists			Fenoterol; Reproterol
S4 Hormone Antagonists and Modulators	1)	Aromatase Inhibitors	Formestane
	2)	SERMs	Tamoxifen
	3)	Other Anti-Estrogenic Substances	Clomiphene
	4)	Agents Modifying Myostatin Function(s)	Myostatin Inhibitors
	5)	Metabolic modulators	
S5 Diuretics and Other Masking Agents	1)	Masking Agents	probenecid
	2)	Diuretics	Acetazolamide
S6 Stimulants	1)	Non-Specified Stimulants	Cocaine; Amphetamine
	2)	Specified Stimulants	Ephedrine
S7 Narcotics			Morphine
S8 Cannabinoids			Marijuana
S9 Glucocorticosteroids			Dexamethasone
M1 Enhancement of Oxygen Transfer	1)	Administration or reintroduction of any quantity of blood	Autologous, homologous and heterologous blood; red blood cell products
	2)	Artificial Enhancement of Uptake, Transport or Delivery of Oxygen	PFCs; HBOCs
	3)	Intravascular manipulation of the blood	
M2 Chemical and Physical Manipulation	1)	Tampering	Catheterisation; Urine Substitution
	2)	Intravenous Infusion	
M3 Gene Doping	1)	Transfer of Nuclei Acids or Nucleic Acid Sequences	DNA; RNA
	2)	Use of normal or genetically modified cells	
P1 Alcohol			
P2 β-Blockers			Acebutolol; Atenolol

1.2.4 Statistics of Androgen Abuse in Different Sports

Since 2003, WADA has annually released the results of accredited doping laboratory test statistics of prohibited substances, as well as updating the list of prohibited substances (Thevis *et al*, 2016). It is clear that, although the number of adverse analytical findings (AAF) has increased, the percentage of all positive cases (of all prohibited substances and methods) remains approximately 2% of all samples tested (World Anti-Doping Agency, 2016). Also of note is that, not only have androgens been the most abused substance, the percentage of their abuse compared with other doping substances has increased over the last decade, peaking in 2009 with 63.5% of all reported findings due to androgens (World Anti-Doping Agency, 2016) (Figure 1.1). A study from the Doping Control Laboratory of Athens between 2005 and 2011 showed that of the 30,000 samples tested in that time period, 427 (approximately 1.6%) AAF occurred, with 54.8% of these AAFs resulting from androgens (Kioukia-Fougia *et al*, 2014). This study also showed that bodybuilding, weightlifting, football, and athletics were the most common sports that these AAFs were detected in. Another study between 2000 and 2009 showed that of 550 samples that were tested for anabolic steroids, 5.4% AAF were detected. Of these positive samples, 70% were from athletes involved in weightlifting sports. This was followed by 13.5% in both track and field sports and basketball and 3% in wrestling (Acevedo *et al*, 2011).

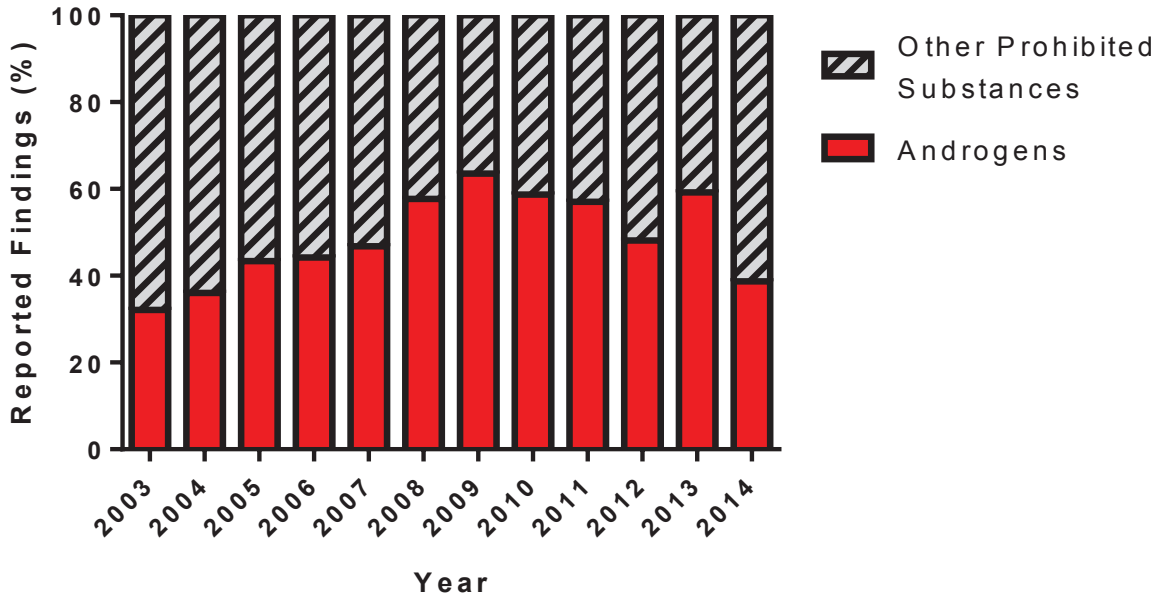


Figure 1.1 Percentage of adverse analytical findings in doping laboratories reported by the World Anti-Doping Agency that were classed as androgens between 2003 and 2014.

What is more difficult to determine, however, is the frequency of use of androgens by recreational and amateur athletes outside the realm of elite sport. Several studies exist reporting statistics of androgen use in gyms, fitness centres and schools. These studies utilise questionnaires and surveys which rely on the honesty of the respondents. This may therefore underrepresent the true number of people who abuse androgens. Such surveys have reported numbers as high as 5-6% of gym users use androgens (Leifman *et al*, 2011; Ozdemir *et al*, 2005; Kanayama *et al*, 2001). The number of unreported cases of doping has been

estimated to be as high as 12.5% (Simon *et al*, 2006) or even 33.3% (Santos *et al*, 2011), based on statistical modelling of the probability of unreported use. A recent meta-analysis suggests that the global prevalence (of both athletes and non-athletes) of androgen abuse is 3.3% (Sagoe *et al*, 2014). Androgen abuse is also prevalent amongst high school students, with reports stating levels between 2.4% and 6.3% (Dunn and White, 2011; van den Berg *et al*, 2007; Pallesen *et al*, 2006; Stilger and Yesalis, 1999; Faigenbaum *et al*, 1998; Kindlundh *et al* 1998) of students reported at least one instance of androgen use. These estimates have dropped considerably from a previous estimate of 11% in the late 1980s (Johnson *et al*, 1989). The prevalence of androgen abuse may, in part, be attributable to the ease of which androgens, and other anabolic agents can be purchased. Androgens and other anabolic agents can be sold over the internet or via telephone on the black market, allowing anyone to purchase them without question, including adolescents, and non-athletes (Cordaro *et al*, 2011).

1.3 Methods of Androgen Doping

1.3.1 Commonly Detected Androgens and How They Are Used

While androgens such as stanozolol and nandrolone are the most frequently detected, it is common practice for regular androgen users to use a cocktail of different androgens in order to take advantage of their different pharmacokinetic profiles (Choong *et al*, 2008; Hall and Hall, 2005). This 'stacking' of several androgens can result in dosages as high as 100 times the physiological dose of normal androgen concentrations in the blood (Pope Jr. *et al*, 2012; Ip *et al*, 2011; Evans, 1997). For example, a survey of 518 androgen users states that the average weekly dose of androgens were 1188.2 ± 1077.1 mg per week (Ip *et al*, 2011). Further, another

survey of 500 androgen users states that 12.6% of respondents were using greater than 2000 mg of T (or equivalent) per week with the highest reported use of 6000 mg (Parkinson and Evans, 2006). To put these dosages into perspective, a report by Bhasin *et al* showed that weekly dosages of T of ≥125 mg up to the highest concentration tested (600 mg) significantly increase muscled size. Moreover, muscular performance was increased at dosages ≥ 300 mg (up to 600 mg) in healthy young males over a 20 week treatment period (Bhasin *et al*, 2001a). Similarly, another study showed that 200 mg/week of nandrolone was able to significantly increase total body weight and fat-free mass compared to the placebo control in bodybuilders over an 8 week treatment period (Lichtenbelt *et al*, 2004).

Androgen users often self-report the use of androgens for a set period of time, followed by periods of time androgen free (Ip *et al*, 2011; Parkinson and Evans, 2006) This is known as 'cycling', and typically lasts for about 6-12 weeks, although this may differ depending on the steroid (Graham *et al*, 2008). A cycle generally involves taking increasing dosages of androgens over the course of the cycle, then taper off the androgen (Graham *et al*, 2008). The self-administration of androgens is often not medically supervised, and thus dosages and treatment periods can differ vastly. This has led to several inconsistencies among scientific studies, whereby dosages and treatment times differ.

Table 1.2 Commonly abused androgens and their routes of administration. Adapted from Barceloux and Palmer, 2013 (Barceloux and Palmer, 2013).

Administered Orally	Administered Intramuscularly
Ethylestrenol	Boldenone Undecylenate
Fluoxymesterone	Clostebol
Mesterolone	Drostanolone Proprionate
Metandienone	Methenolone Enanthate
Metenolone	Nandrolone Decanoate
Methandrostenolone	Nandrolone Phenprioniate
Methyltestosterone	Testosterone Cypionate
Methenolone acetate	Testosterone Enanthate
Mibolerone	Testosterone Priopionate
Norethandrolone	Testosterone Undecanoate
Oxandrolone	Trenbolone
Oxymetholone	
Stanozolol	

1.3.2 Direct Androgen Doping

Direct methods of androgen doping involve the administration of an androgen directly into the body. This includes both oral and parenteral administration. Depending on the chemical structure of the steroid, it may be necessary to inject intramuscularly, or it may be deliverable in an oral form (Kicman, 2008). Injectable forms of androgens generally stay in the blood for a longer period of time compared to their oral counterparts. This is because intramuscular injection allows the androgen to avoid the first pass metabolism of the liver and avoid the Phase I and II reactions for a longer period of time (Kicman, 2008). On the other hand, oral-forms are extensively metabolised by the liver before entering blood circulation and reaching the target sites (skeletal muscle). All androgens share a common 19-carbon

cyclopentanophenanthrene 4-ring structure with differences between androgens lying in the arrangement of side groups and double bonds within this 4-ring structure (Figure 1.2). The majority of interest in the androgen structure lies in the A-ring and the D-ring. In particular, the keto group at the C3 position, the C4-5 double bond, and the C17 hydroxyl group. The A-ring and D-ring are of interest because they represent not only the main sites of binding to the androgen receptor (AR), but are the main sites of metabolism and therefore lead to either activation or deactivation of the steroid (including conversion to oestrogens) (Shahidi, 2001; Schänzer, 1996).

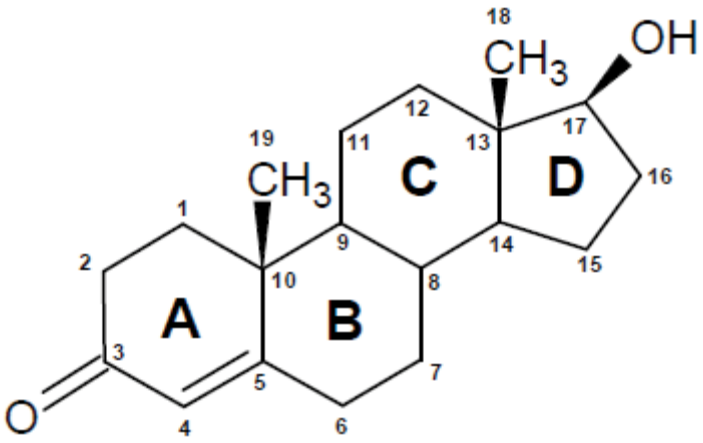


Figure 1.2 Steroid 19-carbon cyclopentanophenathrene 4-ring structure. Each ring is designated a letter and each carbon is designated a number.

The first stages in the metabolism of T involve Phase I reactions in the liver. These reactions generally make the androgen more polar to begin deactivating the compound. The first step is the irreversible reduction of the C4-5 double bond by the enzyme 5α/β reductase. This produces DHT from T. 5α-DHT is androgenic whilst the 5β-isomers are not. The reduction of the C3 keto group by 3α/β-hydroxysteroid dehydrogenase (HSD) rapidly follows to produce androstanediol isomers, although this reaction is reversible (Shahidi, 2001; Schänzer, 1996) (Figure 1.3).

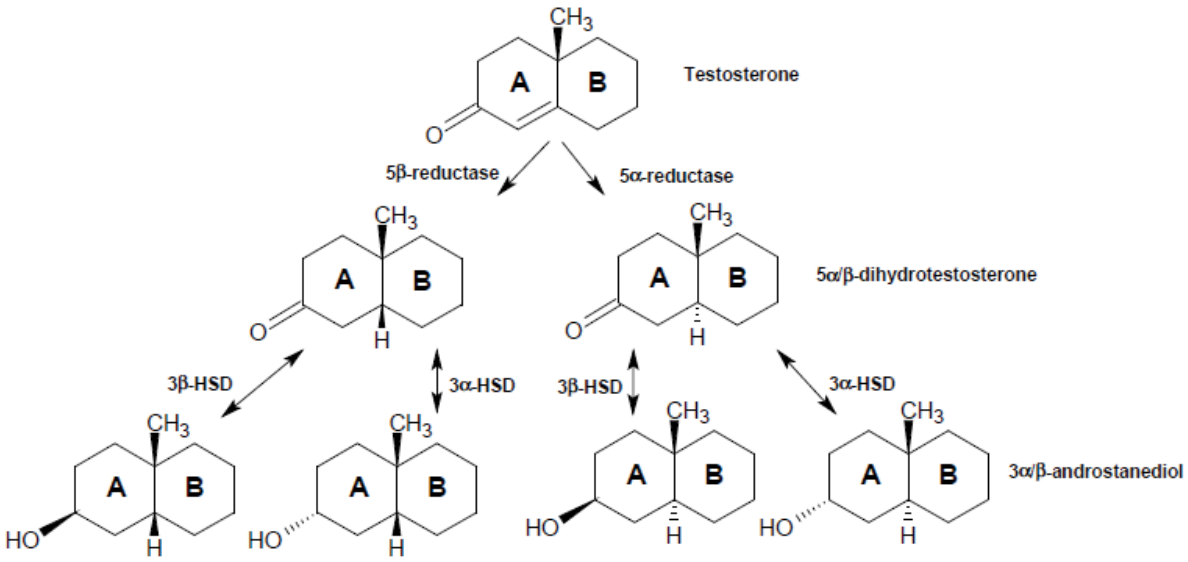


Figure 1.3 A-ring phase I metabolism. The C4-5 double bond is reduced to dihydrotestosterone by 5α/β-reductase. The C3 keto group is then reduced by 3α/β-HSD to form androstanediol.

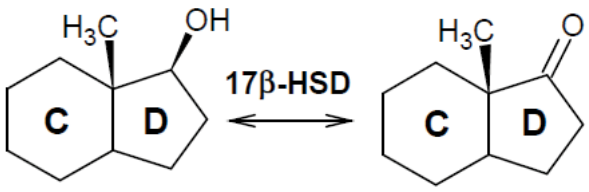


Figure 1.4 D-ring phase I metabolism. The C17 hydroxyl group is oxidised to form a 17-keto steroid by the enzyme 17β-HSD.

The next main Phase I reaction involves the 17β-hydroxyl group in the D-ring. In general, the 17β-hydroxyl group is oxidized to form a 17-keto steroid. This is reversibly catalysed by the enzyme 17β-HSD (Shahidi, 2001; Schänzer, 1996) (Figure 1.4). The Phase II metabolic reactions, also known as conjugation reactions, add glucuronic acid or sulphate to an androgen and its metabolites to assist in elimination from the body. However, not all androgens are conjugated before excretion. Conjugation mainly takes place in the A-ring or D-ring (Figure 1.5). In the A-ring, conjugation takes place on the C3-hydroxyl group. The 3α-isomer becomes conjugated with glucuronic acid while the 3β-isomer undergoes sulfatation. Conjugation in the D-ring takes place at the C17 position (Shahidi, 2001; Schänzer, 1996).

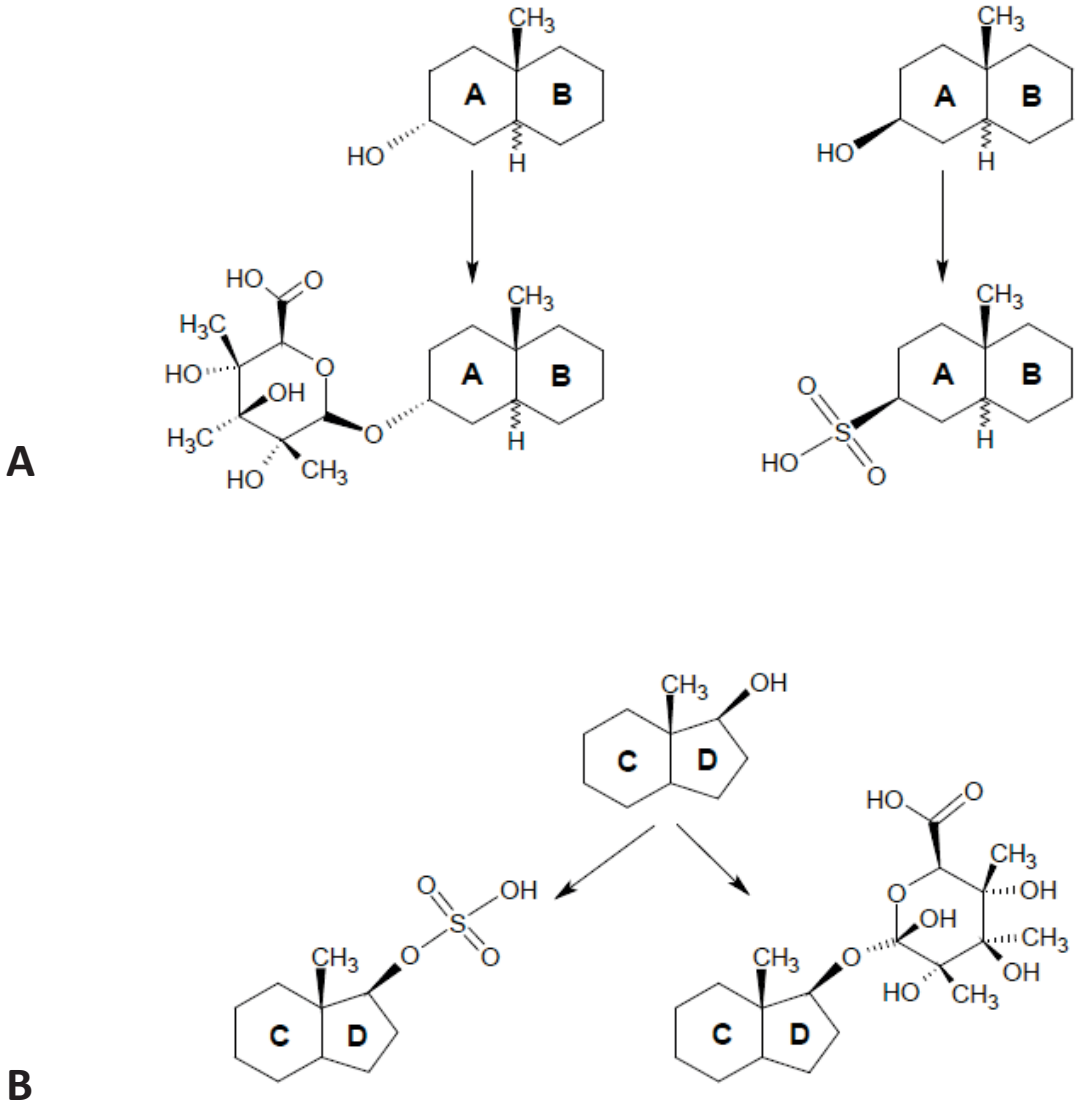


Figure 1.5 Phase II conjugation of androgens. A) Glucuronic acid and sulphate conjugation in the A-ring; B) Glucuronic acid and sulphate conjugation in the D-ring.

It is these general metabolic reactions which render oral administration of exogenous T ineffective. Therefore, androgens like stanozolol and methandienone have structural modifications to avoid, or delay, these kinds of reactions in an attempt to increase their half-life. By modifying the androgen backbone, the metabolism is altered and hence may serve to resist deactivation (Cadwallader *et al*, 2011). Therefore, androgens (or more specifically, T derivatives), can be categorised into three classes: A, B, or C. Class A androgens have an ester attached to the 17β-hydroxyl group in the D-ring and require intramuscular injection. Intramuscular injection avoids first pass metabolism and the androgen will be metabolised at a slower rate than their oral counterparts. Structural modifications to these androgens for increasing their half-life include the addition of esters (Ferriz and Vinsova, 2010; Liederer and Borchardt, 2005). Referring back to the example of T administration, it is known that adding an ester to T prolongs its half-life, hence making it more effective (Ferriz and Vinsova, 2010; Liederer and Borchardt, 2005). Therefore, T itself is not administered intramuscularly, but rather, an esterified form of T (or other androgen) is injected. Some examples of esters include proprionate, enanthate, decanoate, undecanoate, cypionate and acetate. Generally, the longer the ester chain is, the longer the half-life of the androgen. The esterification does not prevent metabolism once in the blood, but instead slows down the release of the androgen from the site of injection due to the increased fat solubility, ensuring a continual timed-release of the androgen into the blood. Therefore, it is common for commercial preparations of T (for illicit use) to involve a variety of T esters to take advantage of their pharmacokinetic profiles and their differing half-lives. This is noted on several websites that sell these preparations of T esters (Steroids Worldwide, 2008).

Class B androgens involve the alkylation of the 17α-hydroxyl position. These so-called 'α-alkylated androgens' can be administered orally, because the α-alkylation resists conjugation in the liver. In a similar fashion, Class C androgens are also alkylated, but in the A-, B-, or C-ring as opposed to the D-ring alkylation in Class B androgens. Class C androgens can also be administered orally, and the alkylation in rings other than D, serve to resist enzymatic conversion to DHT or oestradiol (Cadwallader *et al*, 2011).

Each method of direct doping, be it injecting or taking oral tablets, have their own benefits and drawbacks. The injectable forms are able to avoid first pass metabolism and enter the bloodstream quicker. An esterified form of this androgen is also able to prolong the half-life of the steroid. This means frequent injections are not necessary, but will depend on the individual androgen and ester as their half-lives will differ. The drawbacks of injecting are that there is a chance of infection occurring at the site of injection, due to using a non-sterile needle, for example. Also, the prolonged half-life can mean the androgen will stay in the blood system for a longer period of time. This inherently increases the chances of testing positive in a doping test, although this is only a concern if the person is subject to drug testing. The oral form, on the other hand, will not stay in the blood for as long and is completely metabolised, excreted and no longer able to be detected within 48-72 hours. Also, they do not require injection so they are more convenient to take and there is no risk of infection. However, oral forms are generally less effective due to their initial interaction with the enzymes in the gut and liver leading to deactivation and therefore dosing is more frequent to achieve physiological effects (Cadwallader *et al*, 2011; Shahidi, 2001; Schänzer, 1996; Schänzer et al, 1992).

1.3.3 Indirect Androgen Doping

Rather than the direct addition of exogenous hormones, indirect methods use other means to alter the hormonal balance within the body. A popular example of this is the blockade of oestrogens (Handelsman, 2008). This can occur through the use of aromatase inhibitors or oestrogen receptor (ER) blockers. Oestrogens are synthesised in the body by aromatisation of the androgens, androstenedione and T, by the enzyme aromatase. Certain compounds have been developed which inhibit the function of aromatase and are known as aromatase inhibitors (AI). AIs may be steroidal or non-steroid in structure. They may also bind reversibly or irreversibly (also known as a suicide substrate) to aromatase (Brodie and Njar, 1998). The principle behind AI abuse is that oestrogens are not formed, and as a result, leads to increased physiological concentrations of T (Mauras *et al,* 2003 and 2000). This is indirect androgen doping. Although there is a lack of evidence supporting an ergogenic effect (Handelsman, 2006), AIs have been incorporated into sports supplements and sold to athletes (Willoughby *et al*, 2007). Receptor blockers include selective oestrogen receptor modulators (SERMs), which bind to and antagonise the ER. SERMs are also prohibited by WADA (Hoffman *et al*, 2009).

1.4 Androgen Doping

1.4.1 Ergogenic and Anabolic Effects

Androgens enhance skeletal muscle growth and lean mass and increase muscle strength (Woodhouse *et al*, 2004). Normal levels of androgen production begin to decline naturally with age by about 1% per year in otherwise healthy older adults from around 40 years of age (Dean *et al*, 2004; Feldman *et al*, 2002). This can lead to late onset hypogonadism (LOH) and, along with other clinical symptoms, is associated with declining skeletal muscle size and strength. Several studies have shown that treatment with T can restore muscle size and strength in older men (Behre *et al*, 2012; Sheffield-Moore *et al*, 2011; Ferrando *et al*, 2002; Urban *et al*, 1995).

T treatment can also restore skeletal muscle size and strength in younger males, who have reduced muscle mass as a result of a number of disease states and conditions. These include, hypogonadism (Bhasin *et al*, 1997), HIV related muscle wasting (Gold *et al*, 2006), severe burns (Sundfeld *et al*, 2014) and other clinical settings (Woerdeman and de Ronde, 2011).

The anabolic effect of T was demonstrated more than 60 years ago, with experiments in castrated animals and androgen deficient human males, and thus paved the way for their abuse in sports, beginning in the early 1940s. Surprisingly, however, there was little evidence supporting the notion that dosing with androgens in healthy, eugonadal men would further increase the development of skeletal muscle tissue and strength. This led to speculation that androgens were ineffective as anabolic doping agents in healthy men (Fowler *et al*, 1965). Therefore, the medical and scientific community often refuted claims that androgens can be ergogenic in sport (Choong *et al*, 2011). Studies prior to 1990 generally incorrectly dismissed the ergogenic and anabolic properties of androgens due to inconsistencies in study design

that included not being randomized or blinded, not standardising energy and protein intake, or physical activity, as reviewed extensively by Hoffman *et al*, 2009 (Hoffman *et al*, 2009). Also, they did not accurately represent an athletes' typical androgen usage, with high dosages (Evans, 1997), and stacking of different androgens (Choong *et al*, 2008; Hall and Hall, 2005).

While the belief that "stacking" regimens will help maximize muscle growth, there has been little evidence supporting this, likely from the lack of clinical studies of long-term androgen stacking at supraphysiological doses coupled with intense exercise. However, in the last 25 years, there have been many studies showing that androgens in healthy eugonadal men can cause an increase in muscle size and lean body mass (Lichtenbelt *et al*, 2004; Storer *et al*, 2003; Hartgens *et al*, 2001a and 2001b; Bhasin et al, 1996). Whether or not these effects serve to be ergogenic in sport is difficult to determine, due to a limited base of controlled studies (Choong *et al*, 2008; Bhasin *et al*, 2003). Indeed, many of these studies have shown improvements in strength with exercises such as bench-press, leg-press and squat (Rogerson *et al*, 2007; Storer *et al* 2003; Giorgi *et al*, 1999; Bhasin *et al*, 1996), which would ultimately be ergogenic in strength dominating sports such as weightlifting. This, however, can be difficult to extrapolate to other sports.

Another facet of the ergogenic effects of androgens might not be the direct increase in muscle size and strength, but improvements in muscle recovery allowing for more frequent and intense training sessions (Bhasin *et al*, 2001b). Androgens are also known to stimulate erythropoiesis and increase haemoglobin and thus, enhance the oxygen-carrying capability of blood (Maggio *et al*, 2013). This could have a positive impact on training performance and recovery, but this has not been studied.

1.4.2 Androgenic Effects

The masculinising (androgenic) effects of androgens include the development of male sexual organs, facial and body hair, laryngeal enlargement and thickening of the vocal cord, and increased libido (Kicman, 2008). Early research with androgens sought to develop an androgen that would be suitable for clinical use in women and children, without presenting these unwanted androgenic effects (Gao and Dalton, 2007). Hence, a differentiation between the "anabolic" and "androgenic" effects was made, coining the term anabolic-androgenic steroid (AAS). Scientists therefore have worked to develop androgens which display a high anabolic/androgenic ratio. This is a loose term describing an androgen's ability to produce high levels of anabolic activity while presenting mild to low amounts of androgenic activity. Ultimately, these studies failed because the androgenic or anabolic activities were not able to be separated primarily because the physiological mechanisms remain the same throughout interaction with the AR (Handelsman, 2011b).

The most recent advancement in this endeavour has been the use of selective androgen receptor modulators (SARMs), which aim to treat patients without affecting prostate tissue in men or virilisation in women (Jones *et al*, 2010). This is a field of ongoing research, and there have been many SARMs developed (Crawford *et al*, 2016; Handlon *et al*, 2016; Saeed *et al*, 2016; Basaria *et al*, 2013; Gao and Dalton, 2007).

1.4.3 Side Effects

The use of androgens for non-medical purposes, such as doping, is considered abuse, and has been associated with a multitude of health-related side effects, both short- and long term. Abuse of androgens can lead to the enhancement of androgenic effects, including the enlarging of the prostate gland in males, baldness in men or hirsutism in women, acne, and enlarging of the vocal box and deepening of voice in women (Amsterdam *et al*, 2010; Kicman and Gower, 2003). It can also involve severe psychiatric disorders including "roid-rage" associated with increased aggression, depression and mood disorders (Oberlander and Henderson, 2012; Pagonis *et al*, 2006) as well as motivation for committing violent crimes and other risk-taking behaviour (Lundholm *et al*, 2010).

There are also a number of other health issues that have been reported with androgen abuse. Adverse cardiovascular effects have been suggested to be linked with chronic androgen abuse, as reviewed by Golestani *et al,* 2011 (Golestani *et al*, 2011), and Kaushik *et al*, 2010 (Kaushik *et al*, 2010). Androgen abuse has been suggested to be involved in premature atherosclerosis, myocardial infarction, cardiac hypertrophy and sudden cardiac death in young athletes (Pirompol *et al*, 2016; Far *et al*, 2012; Montisci *et al*, 2012; Ahlgrim and Guglin, 2009; Stergiopoulos *et al*, 2008; Fineschi *et al*, 2007). The majority of studies linking androgen abuse to these cardiovascular events are often anecdotal and single case reports so there is limited data. It remains unethical to complete studies of supraphysiological doses of androgens with the end point being cardiovascular disease in humans. However, other studies demonstrate androgens can unfavourably alter lipoprotein levels (Hartgens *et al*, 2004b), increase cholesterol uptake and foam cell formation (McCrohon *et al*, 2000), increase apoptosis and inflammation in endothelial cells (Death *et al*, 2004a; Ling *et al*, 2002), arterial

calcification (McRobb *et al*, 2009) and cardiac hypertrophy (Bissoli *et al*, 2009; Rocha *et al*, 2007).

Hepatotoxicity and related liver disorders are also associated with androgen abuse (Neri *et al*, 2011; Amsterdam *et al*, 2010). Androgens can cause stress on the liver by altering liver enzyme levels such as alkaline phosphatase, aspartate aminotransferase and alanine transaminase (Granados *et al*, 2014; Samaha *et al*, 2008). Liver dysfunctions lead to androgen-induced jaundice and cholestasis (Nasr and Ahmad, 2009), hepatic adenomas and haemorrhage (Patil *et al*, 2007; Bagia *et al*, 2000; Nakao *et al*, 2000), hepatocellular necrosis and hepatitis (Girgis *et al*, 2014; Wingert *et al*, 2010) and hepatocellular carcinoma (Kesler *et al*, 2014; Gorayski *et al*, 2008). As described for cardiovascular events, androgen-induced hepatotoxicity often presents as single-case and anecdotal reports. So far, there have been no reports on the hepatotoxic effects of parenteral use of T, however, orally-available preparations are toxic (Neri *et al*, 2011).

Another health concern with androgen abuse is hormonal balance and infertility. Normally, androgens (and steroid hormones, in general) are regulated by both negative and positive feedback in a system of endocrine glands together known as the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.6). This endocrine pathway is responsible for the secretion of gonadotropins, that is, hormones which act on the gonads to produce steroids. Specifically, these include: follicle-stimulating hormone (FSH), LH, and hCG (Saner-Amigh and Halvorson, 2010; Wu, 1992). Androgen abuse can disrupt this pathway, leading to suppression of the HPG axis. This can inhibit endogenous T production and spermatogenesis leading to infertility. Withdrawal of use of exogenous androgens can therefore lead to a period of androgen deficiency, while the HPG axis has not yet recovered (Handelsman, 2011a). Another side

effect of suppression of the HPG axis is testicular atrophy and erectile dysfunction. However, this effect is often reversible upon androgen withdrawal (Boregowda *et al*, 2011).

Another side effect of androgen use is the aromatisation of androgens leading to high levels of oestrogens in men. In men, this can cause an increase in fat, and the enlargement of mammary glands, leading to gynecomastia (Choong *et al*, 2011). Many androgen users will take anti-oestrogenic compounds such as AIs to prevent this.

Figure 1A

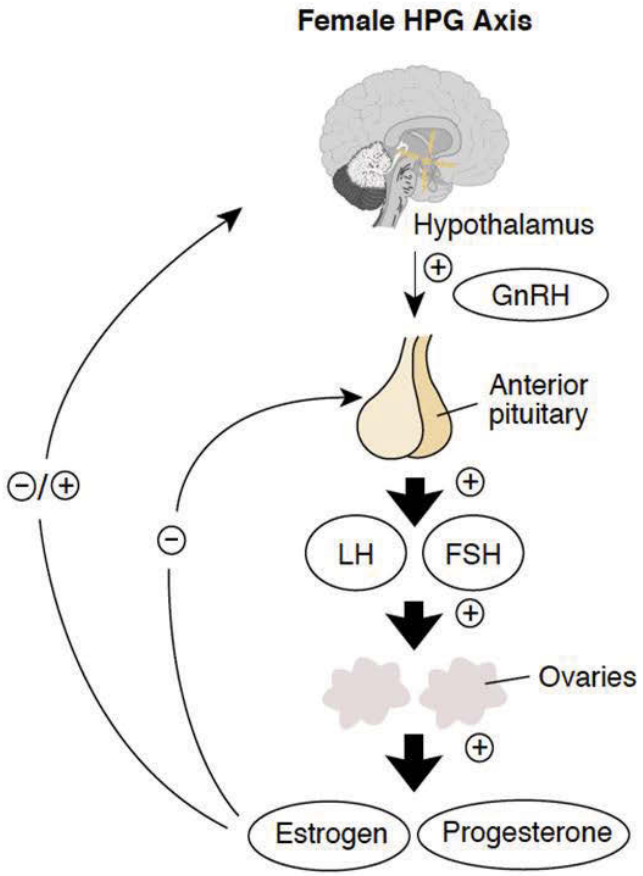


Figure 1B

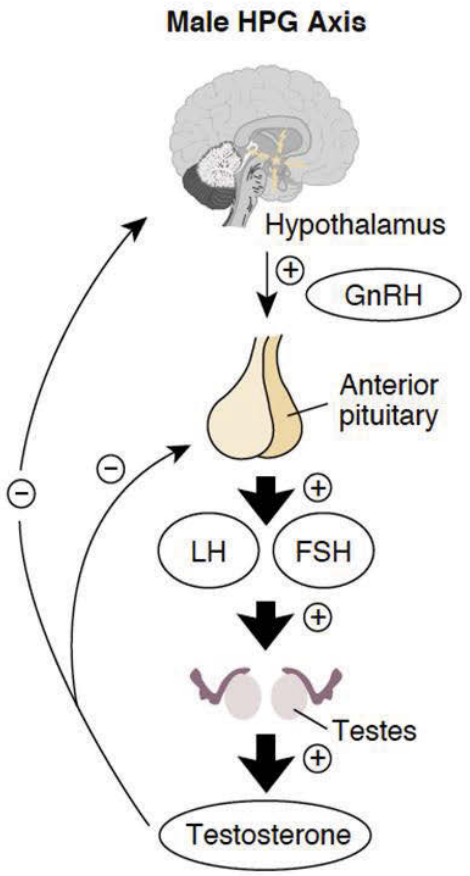


Figure 1.6 The Hypothalamic-Pituitary-Gonadal Axis of men and women. Adapted from Hiller-Sturmhöfel and Bartke, 1998

(Hiller-Sturmhöfel and Bartke, 1998)

1.5 Molecular Mechanisms of Androgens

1.5.1 The Androgen Receptor and the Canonical Pathway of Androgen Action

Androgens exert the majority of their effects by binding to the AR, which is in expressed in both males and females (McCrohon *et al*, 2000). AR is not limited to expression in androgenic tissues such as prostate and testis, but also includes peripheral tissues such as skeletal muscle (Ahtiainen *et al*, 2011), vascular cells (Death *et al*, 2004a), bone cells (Wiren *et al*, 1997), neuronal tissue (Yu *et al*, 2013) and liver tissue (Jiang *et al*, 2014). The AR is part of a nuclear receptor superfamily that comprises several other related, but differently structured receptors such as the ER-α/β; progestogen (PR); glucocorticoid (GR); and mineralocorticoid (MR) receptors (Wilson and McPhaul, 1996). Androgens exert their effects in the body mostly by binding to the AR and initiating a genomic pathway of transcriptional activation. The full length human AR is a ubiquitously expressed 110 kDa protein consisting of several functional domains. There also exists a shorter AR isoform (AR-A; 87 kDa) which has a truncated N-terminal Domain (NTD) (Liegibel *et al*, 2003) (Figure 1.7).

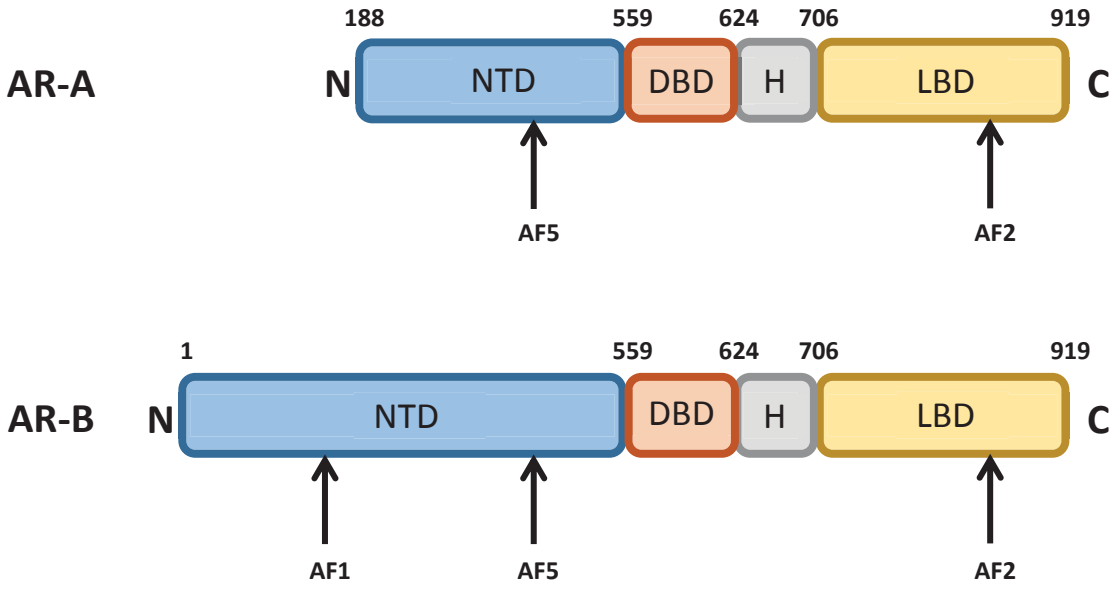


Figure 1.7 The androgen receptor. Structural domains of the two isoforms (AR-A and AR-B) of human AR. Numbers above the bars refer to the amino acid residues which separate the domains starting from the N-terminus (left) to C-terminus (right). NTD, N-terminal domain; DBD, DNA-binding domain; H, hinge region; LBD, ligand-binding domain; AF, Activation Function. Adapted from Li and Al-Azzawi, 2009 (Li and Al-Azzawi, 2009).

The native AR is located in the cytoplasm bound to heat shock proteins (HSP) and other chaperone proteins. Androgens will diffuse across the cell membrane and bind to the inactive AR on a region of the protein known as the ligand binding domain (LBD), located on the carboxy-terminal end of the protein. The LBD is conserved amongst species. Ligand (androgen) binding initiates a conformational change of AR and dissociates the HSP bound to the AR. The AR-ligand complex will then form a homodimer and, likely assisted by regulatory factors such as importin-α and importin-β, translocates to the nucleus (Wijngaart *et al*, 2012; Cutress *et al*, 2008). After entering the nucleus, the AR-ligand homodimer will bind to regulatory regions of DNA known as androgen response elements (AREs) via a centrally located domain on the AR known as the DNA-binding domain (DBD) (Figure 1.8). Like the LBD, the DBD is also conserved amongst species. The DBD is flanked by regions that assist in binding to the ARE, called nuclear localization signals. The AREs are located upstream of androgen dependent genes such as prostate specific antigen (PSA) and probasin (Bennett *et al*, 2010).

The DBD is linked to another domain known as the N-terminal Domain (NTD). The NTD contains two Activation Functions (AF; AF-1 and AF-5) and along with AF-2 located on the LBD, help stimulate transcription with the assistance of a number of cofactors and general transcriptional machinery such as RNA polymerase II (Cadwallader *et al*, 2011; Matsumoto *et al*, 2008). Cofactors such as histone modifying complexes and chromatin remodelling complexes help expose the chromatin structure and the formation of the preinitiation complex (PIC) assisting in efficient gene transcription (Li and Al-Azzawi, 2009). Cofactors that help stimulate gene transcription are referred to as co-activators. There also exist a number of co-repressors, cofactors that inhibit gene transcription. For a more in depth review on AR cofactors, see Heemers and Tindall (Heemers and Tindall, 2007).

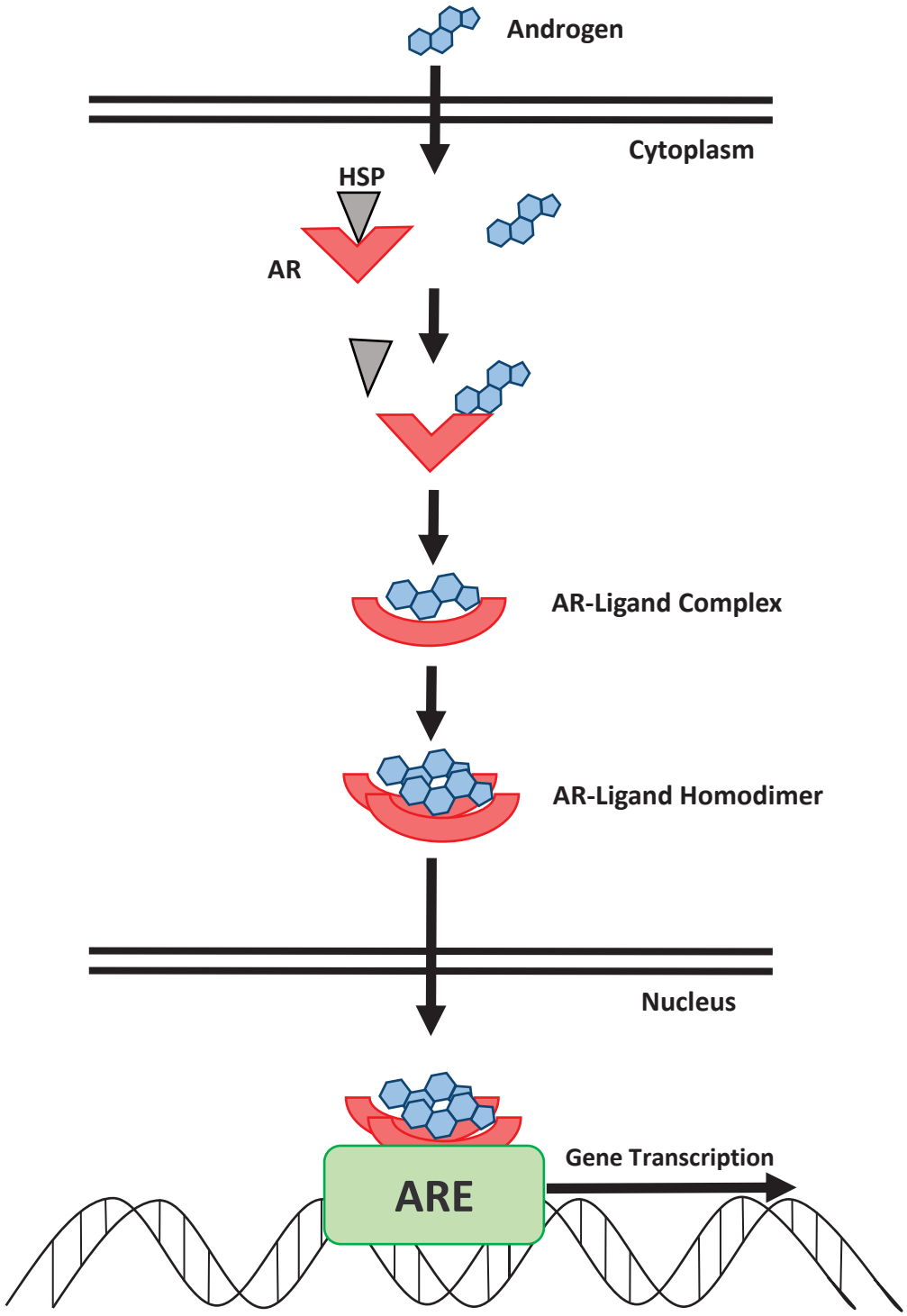


Figure 1.8 Canonical pathway of genomic androgen receptor action. Androgens diffuse through the cell membrane and bind to AR. Bound HSPs then dislocated from AR, and the AR-ligand complex forms a dimer. The AR-ligand homodimer then translocates to the nucleus and binds to the AREs present on DNA, which leads to downstream transcription of target genes.

1.5.2 Myotrophic Action of Androgens

Skeletal muscle exists as a bundle of muscle fibres highly organised in structure to facilitate a coordinated contraction in order to generate force output. Each individual muscle fibre is composed of many mononucleated precursor myoblast cells that have fused together into a multinucleated myotube (Figure 1.9). Skeletal muscle as a whole is composed of several different muscle fibre types (Type I, IIa, and IIx), or a combination of types which display different properties including the speed of contraction, oxidative or glycolytic capacity and fatigue resistance. For an extensive review on skeletal muscle fibre types and function, see Schiaffino and Reggiani, 2011 (Schiaffino and Reggiani, 2011). The nuclei in muscle fibres are post-mitotic and thus cannot divide. Therefore, growth in skeletal muscle tissue arises mainly from hypertrophy (growth in pre-existing muscle fibres) and hyperplasia (generation of new muscle fibres from a pool of satellite cells). Although androgens have been shown to increase muscle mass and strength (force output), the mechanisms underlying these myotrophic actions are not fully understood. There are a number of factors and mechanisms involved at both the cellular level and the intracellular levels and androgens have been shown to be involved in several of these factors and at several stages of skeletal muscle development. Skeletal muscle endocrinology regarding growth is complicated and is not fully understood. Therefore, this section aims to highlight some of the main mechanisms involved in androgen-mediated skeletal muscle growth and is not a complete discussion on skeletal muscle physiology.

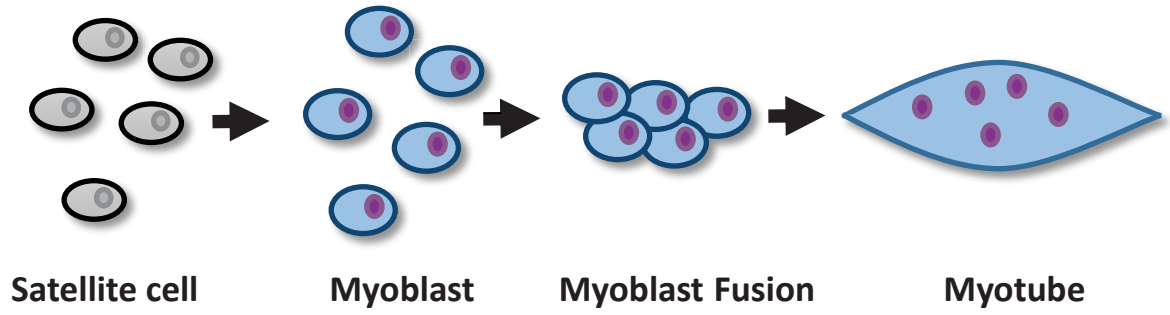


Figure 1.9 Skeletal muscle myotube formation. Quiescent satellite cells in the basal lamina of skeletal muscle are activated and differentiate into mononucleated myoblasts. Myoblasts then align and fuse to form a multinucleated myotube.

The increase in skeletal muscle hypertrophy is ultimately a result of a net increase in protein synthesis in the skeletal muscle fibre (Egerman and Glass, 2014). In order for this to occur, a number of anabolic stimuli must be present. This includes anabolic hormones such as androgens and IGF-I (Jacquemin et al, 2004; Crown *et al*, 2000), adequate nutrients, especially amino acids (specifically leucine) (Pasiakos and McClung, 2011; Norton *et al*, 2009) and mechanical overload (resistance exercise) (Gonzalez *et al*, 2015). The necessary increase in protein synthesis required to significantly increase muscle mass has a high demand for cellular energy and skeletal muscle, indeed, plays a large role in glucose and protein metabolism and

is insulin sensitive in healthy individuals (Akerstrom *et al*, 2014; Nisr and Affourtit, 2014). Therefore, increased muscle mass is difficult for the body to maintain unless these appropriate anabolic stimuli are present. Indeed, increases in catabolic hormones (Qin *et al*, 2010; Wu *et al*, 2010a), loss of anabolic hormones (Matheny and Nindl, 2011; Mauras *et al*, 1998) and muscle disuse (Camerino *et al*, 2015; You *et al*, 2015), (as well as various disease states) often lead to a reduction of muscle mass.

With respect to intracellular pathways that lead to increased protein synthesis, the phosphatidylinositol 3-kinase (PI3K)/Akt-mTOR pathway is the main pathway involved in stimulating skeletal muscle protein synthesis (Frost and Lang, 2007). This pathway involves phosphorylation of PI3K and Akt leading to downstream activation of mTOR and the protein kinase $p70^{S6k}$, increasing protein synthesis of various types of proteins including contractile proteins and cytoskeletal proteins (Wullschleger *et al*, 2006). The growth factor IGF-I has been shown to be a key positive regulator of Akt phosphorylation and several studies have shown that exogenous IGF-I treatment can lead to increased protein synthesis (Jacquemin *et al*, 2004) (Figure 1.10). Similarly, androgens have also been shown to stimulate the phosphorylation of Akt directly (Basualto-Alarcon *et al*, 2013), or indirectly by increasing IGF-I production leading to increased protein synthesis via this mechanism (Sculthorpe *et al*, 2012; Urban *et al*, 1995). Therefore, although IGF-I is considered an important anabolic stimuli, its presence is not essential in stimulating this pathway and the anabolic effect can be maintained by androgens. This effect was highlighted in a study by Serra *et al* whereby T was capable of stimulating skeletal muscle hypertrophy in the absence of IGF-I and GH (the key regulator of IGF-I production in the liver) *in vitro* in human skeletal muscle cells and *in vivo* in mice (Serra *et al*, 2011).

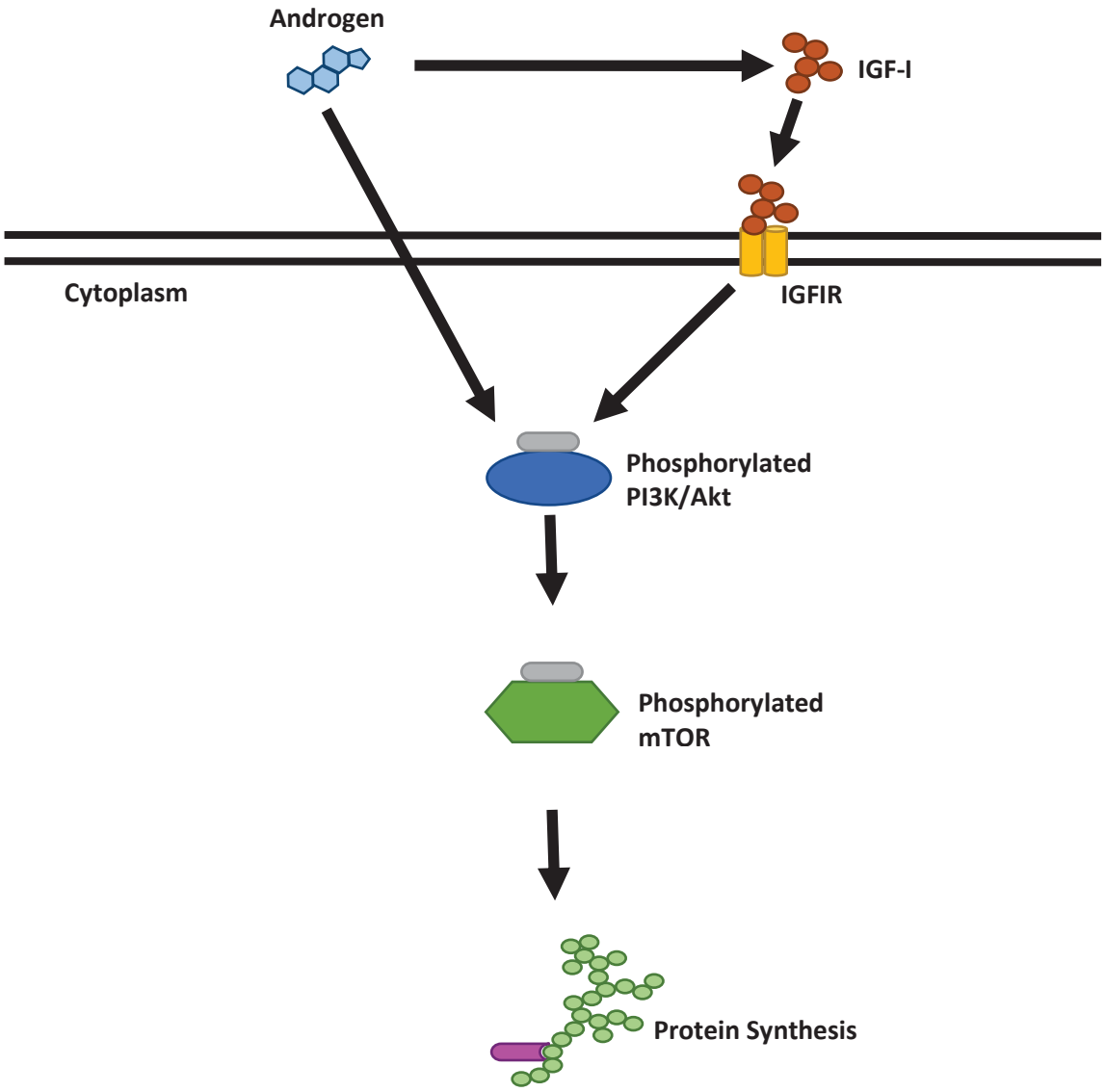


Figure 1.10 The IGF-Akt-mTOR pathway. Androgens activate PI3K/Akt via direct activation or through the IGFIR, which leads to downstream activation of mTOR. Activated mTOR drives protein synthesis in cells.

Androgens also exhibit crosstalk between various pathways involved in skeletal muscle protein catabolism and inhibitors of muscle growth (Figure 1.11). The negative regulator of muscle protein synthesis myostatin (growth differentiation factor-8; GDF-8) is a member of the transforming growth factor-β (TGF-β) superfamily and is a potent inhibitor of muscle protein synthesis. The potency of myostatin in inhibiting protein synthesis has been highlighted in studies with myostatin knockout models or genetic mutations in the myostatin gene which display massive growth in muscle tissue (Mendias *et al*, 2015; McPherron *et al*, 1997; McPherron and Lee, 1997). Myostatin has been shown to act via several mechanisms, including the inactivation of Akt (Morissette *et al*, 2009; Trendelenburg *et al*, 2009) as well as inhibiting the proliferation and differentiation of skeletal muscle myoblast cells (Trendelenburg *et al*, 2009). Androgens have been shown to be capable of inhibiting the action of myostatin through increased expression of follistatin (Mosler *et al*, 2012; Singh *et al* 2009), a glycoprotein capable of inhibiting the actions of myostatin (Amthor *et al*, 2004; Hill *et al*, 2002).

Androgens can also decrease the expression of atrophy related proteins, atrogin-1 (MAFbx) and MuRF1. Atrogin-1 and MuRF1 are muscle-specific E3 ubiquitin ligases that are part of the ubiquitin-proteasome pathway involved in intracellular degradation of key structural and contractile proteins such as myosin heavy and light chains, desmin, actin, and troponin I (Lokireddy *et al*, 2012; Polge *et al*, 2011; Fielitz *et al*, 2007; Cohen *et al*, 2009; Clarke *et al*, 2007; Kedar *et al*, 2004) as well as myogenic transcription factors such as MyoD and eukaryotic translation initiation factor 3 subunit F (eIF3-f) (Csibi *et al*, 2010; Tintignac *et al*, 2005). Through the activation of Akt, androgens can inhibit the activation of forkhead box O (FoxO) transcription factors, the upstream mediator of atrogin-1 and MuRF1 expression

(White *et al*, 2013; Stitt *et al*, 2004), thereby leading to decreased rates of protein degradation.

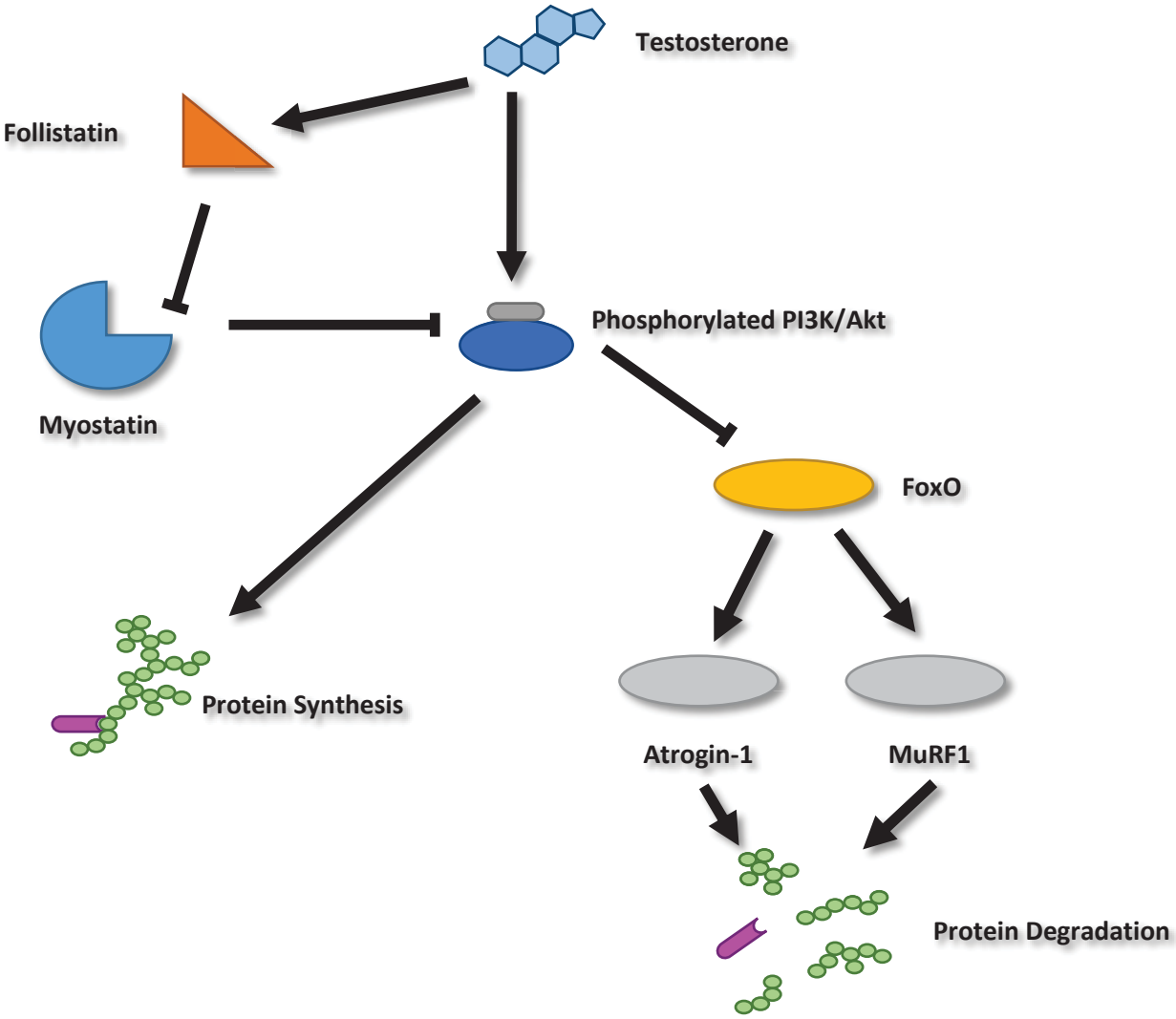


Figure 1.11 Pathways of skeletal muscle protein degradation. Proteins are degraded through E3 ubiquitin ligase enzymes Atrogin-1 and MuRF1, which are activated through the forkhead box O (FoxO) transcription factor. Testosterone can inactivate Atrogin-1 and MuRF1 through activation of PI3K/Akt, a negative regulator of the FoxO transcription factor. Testosterone also inhibits the actions of myostatin, which is a negative regulator of PI3K/Akt, through an increased production of follistatin.

The increased rates of protein synthesis and decreased rates of protein degradation mediated by androgens therefore leads to an increase in muscle mass. However, increases in muscle cell volume (hypertrophy) still has a restrictive limit, thought to be determined by the myonuclei content of the muscle cells (Qaisar *et al*, 2012). The theory that each myonuclei in the muscle fibre is accountable for a fixed amount of cell volume (and protein synthesis) is known as the myonuclear domain (Shenkman *et al*, 2010). Therefore, to further increase protein synthesis and ultimately increase cell volume (hypertrophy), skeletal muscle fibres must acquire new nuclei from myoblast fusion with pre-existing fibres, given that myotubes are post-mitotic. These myoblasts are derived from a population of muscle stem cells, termed satellite cells. These cells exist in a quiescent dormant state underneath the basal lamina and the sarcolemma of the muscle fibre (Tatsumi *et al*, 2001). Upon activation, these satellite cells re-enter the cell cycle and undergo asymmetric cell division resulting in two fates for the daughter cells: firstly, one population of the daughter cells become myoblasts which allow for regeneration and growth of the muscle fibres through fusion with pre-existing fibres (hypertrophy) or generate new fibres (hyperplasia); secondly, daughter cells will also form new satellite cells, exit the cell cycle, and resume quiescence so the reservoir of satellite cells is not depleted upon muscle regeneration (Sinha-Hikim *et al*, 2006 and 2003) (Figure 1.12).

Androgens play an important role in the regenerative capacity of satellite cells via increased differentiation and proliferation (fate 1 and 2, respectively) of satellite cells. While the exact mechanisms are not fully understood, androgens have been shown to increase the proliferation of satellite cells in order to replenish and even expand the satellite cell pool (Sinha-Hikim *et al*, 2006 and 2003; Joubert and Tobin, 1995). Similarly, androgen stimuli have been shown to enhance the differentiation of satellite cells into myoblasts and fusion with

muscle fibres resulting in hypertrophy (Sinha-Hikim *et al*, 2006 and 2003; Joubert and Tobin, 1995). It has been suggested that androgens increase the expression of myogenic genes that are involved in the proliferation (e.g. proliferating cell nuclear antigen or PCNA and Pax7) (Brown *et al*, 2009; Diel *et al*, 2008; Sinha-Hikim *et al*, 2006) and differentiation (e.g. Notch and myogenin) (Serra *et al*, 2011; Brown *et al*, 2009; Sinha-Hikim *et al*, 2006) of satellite cells.

Androgens are also capable of influencing the fate of other stem cells. Pluripotent mesenchymal stem cells are able to differentiate into multiple lineages such as skeletal muscle, bone, cartilage, and adipose cells, dependent upon the stimulus. Not only have androgens been shown to stimulate the myogenic skeletal muscle lineage, but they also inhibit the adipose lineage (Gupta *et al*, 2008; Singh *et al*, 2003) (Figure 1.13). However, it is estimated that myonuclei in skeletal muscle are not largely derived from activation of pluripotent mesenchymal stem cells. Satellite cells are the main stem cell that contribute myonuclei for hypertrophy. The inhibitory effect of fat cell formation has therefore been a proposed explanation, at least in part, for a decreased body fat composition commonly seen as a result of androgen treatment (Kadi, 2008; Singh *et al*, 2003).

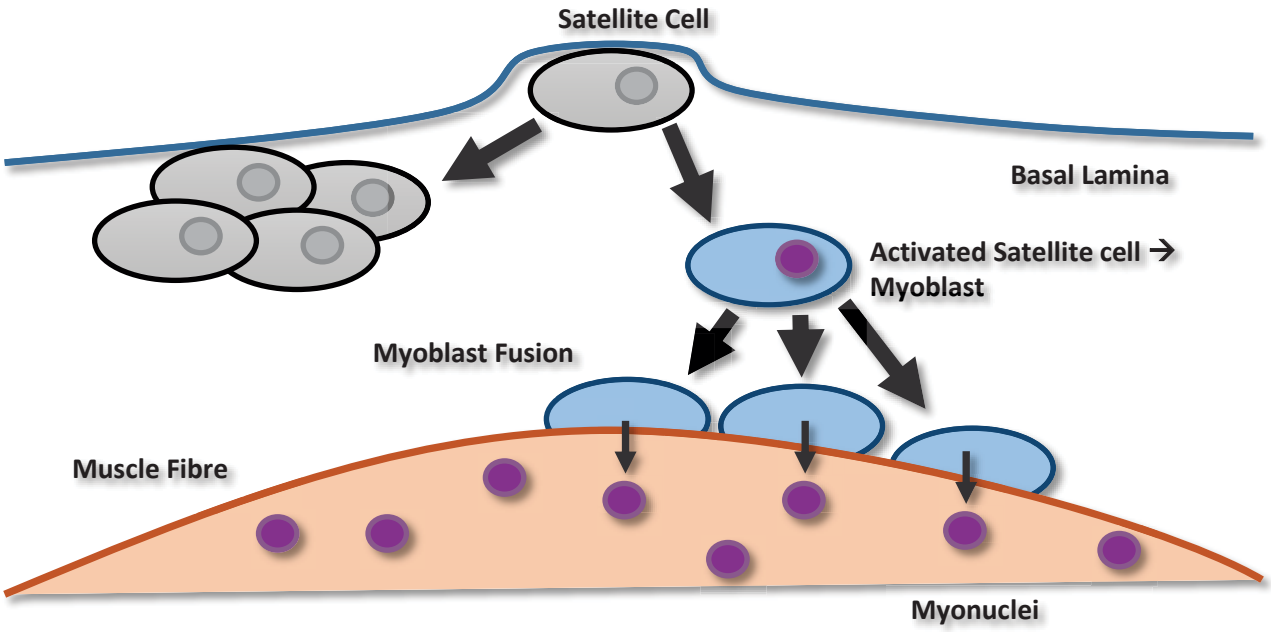


Figure 1.12 The fate of skeletal muscle satellite daughter cells. Muscle satellite cells enter the cell cycle and differentiate into myoblasts to allow nuclei donation for muscle hypertrophy or proliferate to expand the satellite cell pool.

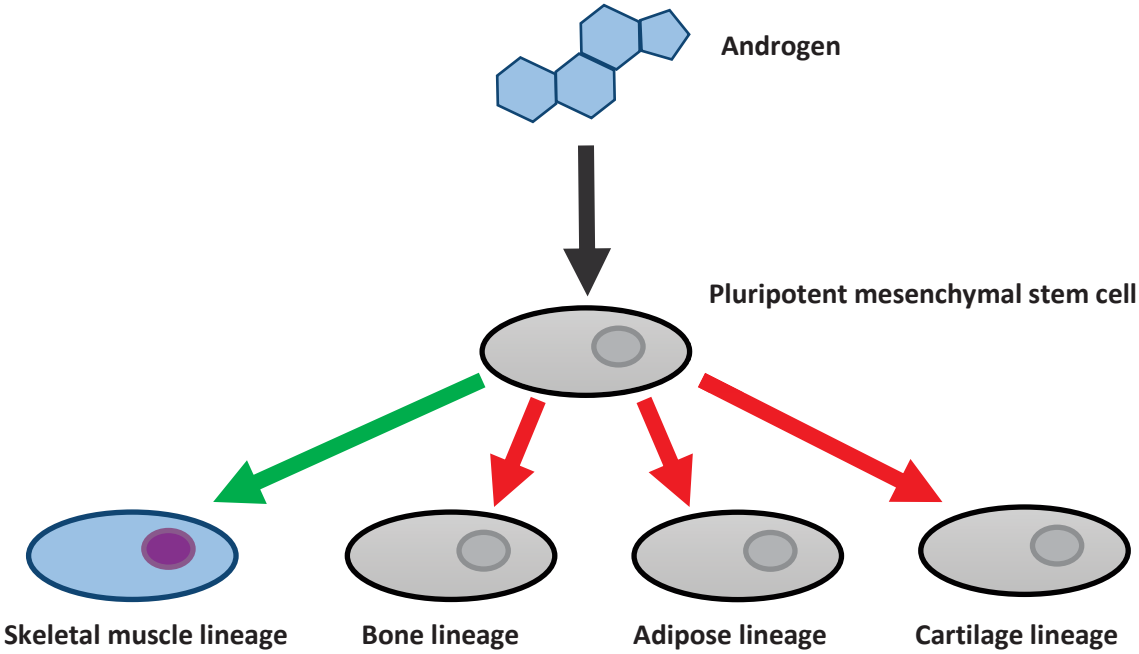


Figure 1.13 Mulitiple lineages of pluripotent mesenchymal stem cells. Androgens influence the activation of the muscle cell lineage over bone, adipose, and cartilage.

1.5.3 Non-Canonical Actions of Androgens

In addition to increasing muscle mass, androgens have also been noted to decrease fat mass, although the mechanisms are not fully elucidated. In addition to hindering the differentiation of mesenchymal stem cells into the adipose lineage, androgens have also been suggest to be directly lypolytic themselves. It is suggested that this action occurs via interaction with β_2-adrenoceptors and catecholamine-induced lipolysis (Xu *et al*, 1991 and 1990). For reviews, see Arner, 2005 (Arner, 2005) and Pergola, 2000 (Pergola, 2000).

Androgens may also be involved in a number of rapid, non-genomic mechanisms. Such mechanisms include increasing protein kinase A (PKA) and C (PKC) activity through interaction with sex hormone binding globulin (SHBG) receptor, and MAPK signalling cascades. Together, these cascade may influence AR transcriptional activity (Kim *et al*, 2005; Gioeli *et al*, 2002; Sadar, 1999). Androgens have also been shown to induce rapid increases in intracellular Ca^{2+} transport (Estrada *et al*, 2000), important for contractile activity of skeletal muscle. For more in depth reviews on the non-genomic actions of androgens, see Dubois *et al*, 2012 (Dubois *et al*, 2012), Foradori *et al*, 2008 (Foradori *et al*, 2008) and Michels and Hoppe, 2008 (Michels and Hoppe, 2008).

1.6 Methods of Detection

1.6.1 IOC and WADA Testing Methods

Biological samples that are to be screened, such as urine or blood, are obtained from an athlete and tested in an accredited anti-doping laboratory. Samples may be collected at any time, even outside of competition. This allows anti-doping authorities to prevent athletes from doping during their training periods prior to competition. Athletes may also be targeted, or randomly selected, at any time to provide a sample for testing (World Anti-Doping Agency, 2016). Doping authorities will incorporate a number of routine screening tests to detect any prohibited substance present. When screening for androgens, it is general practice to use GC-MS. GC-MS has been at the forefront of doping tests due to its high sensitivity and high specificity. Known prohibited substances are catalogued and placed into a database and their structures are compared to what is detected in a biological sample. That way it can be identified specifically what substance has been detected in an athlete's blood or urine test. GC-MS is so sensitive, it is capable of detecting trace amounts of a synthetic androgen months after the last administration and can detect concentrations in the pg/mL range (World Anti-Doping Agency, 2016). However, not all androgen abusers use synthetic androgens. WADA laboratory statistics show that T is one of the most commonly abused substances, and continues to be so (World Anti-Doping Agency, 2016). Distinguishing between high endogenous T levels and the exogenous administration of T initially involves the measurement of a naturally co-secreted hormone, epitestosterone (E). Natural T production is accompanied by a proportionate secretion of E. Therefore, a ratio should exist between E and T, and administration of T disrupts this ratio. This T/E ratio should only naturally fluctuate slightly due to variations in normal hormone homeostasis. Recognising this, WADA set out a

number of guidelines when considering the T/E ratio: (1) T/E value greater than or equal to 4; (2) concentrations of T or E greater than 200 ng/mL; (3) concentrations of androsterone or etiocholanolone greater than 10,000 ng/mL; and (4) concentrations of DHEA greater than 100 ng/mL. If any one of these criteria are met, samples are sent for further analysis using isotope radio mass spectrometry (IRMS). Commercially produced T has higher ^{13}C levels than endogenous androgens, and therefore can be distinguished. The principle of the T/E ratio can be applied to a number of other endogenous hormones, such as DHT, DHEA, or any other endogenous androgen (World Anti-Doping Agency, 2016).

1.7 Methods of Evasion

1.7.1 Designer Androgens

Detection of androgen use relies on detecting a known chemical structure at a measureable concentration in a biological sample. Therefore, any novel unknown chemical structure may bypass the whole GC-MS screening process. The designer androgen is one such bypass approach, and their emergence has allowed athletes to circumvent screening tests. Unlike other chemical synthetic androgens, which were initially designed for medical and/or veterinary use, designer androgens are purposefully created in a clandestine manner for the sole purpose of evading detection. Designer androgens first appeared on the scene in the early 2000s, with the discovery of a never before marketed steroid, norbolethone, detected in athlete's urine (Catlin *et al*, 2002). At around the same time, the same laboratory received syringes containing an oily substance, labelled "The Clear". Subsequent investigations into the

Bay Area Laboratory Co-Operative (BALCO) revealed that this oily syringe contained a novel designer steroid, tetrahydrogestrinone (THG) (Catlin *et al*, 2004). A later study revealed that this steroid to be a potent androgen and progestin (Death *et al*, 2004b). As a result of these investigations, several elite athletes, including Barry Bonds, Kelli White, Marion Jones and Tim Montgomery, linked to BALCO were found guilty of using a prohibited substance. This example demonstrates the lengths that athletes will go to in order to succeed in sport. Not only were novel substances being used by athletes, but they were synthesised to avoid detection (Kazlauskas, 2010).

Due to their clandestine nature, designer androgens including THG have minimal or no clinical studies regarding their ergogenic properties, and more importantly, toxicity and side effects. While THG is a true designer androgen, there are a number of androgens that have resurfaced since their initial conception for legitimate medical purposes, but were never marketed as such. Such androgens, including norbolethone, methyldrostanalone (superdrol), and prostanozol, were not initially included on the prohibited list and therefore, their resurfacing indicates attempts at doping with the aim of being missed by screening tests, much like a designer androgen (DEA Federal Register 44456).

1.7.2 Androgens and the Nutritional Supplement Market

In 1994, the Dietary Supplement and Health Education Act (DSHEA) in the US was passed and allowed for the sale of prohormones in sports dietary supplements (Delbeke *et al*, 2003). By definition, a prohormone itself has no or very little biological activity and requires enzymatic activation before eliciting its biological effects. The orally consumed prohormone would be activated when exposed to the liver before entering the general blood circulation (Ziegenfuss

et al, 2002). These prohormones include the natural androgen precursors, DHEA, 4-androstenedione, 4-androstenediol, 5-androstenediol, 19-norandrostenedione and 19-norandrostenediol. Prohormones were sold legally on the US and international markets, despite their continued ban by WADA. The use of prohormone supplementation was made particularly popular by the use of androstenedione by the baseball player, Mark McGwire (Brown *et al*, 2003). Since then, many supplement companies pushed the sale of these prohormones as anabolic agents suitable for building muscle mass (Broeder, 2003), although the efficacy of these supplements is debatable (Brown *et al*, 2006; Rasmussen *et al,* 2000). The legal loop-hole in marketing prohormones as sports supplements has been closed since 2004, and they are now illegal for sale in the U.S and require a prescription (Geyer *et al*, 2011). Regardless, many sports supplement companies still blatantly advertise the presence of androgens and prohormones (Cavalcanti *et al*, 2013; Parr *et al*, 2011a and 2007; Wingert *et al*, 2010; Okano *et al*, 2009).

1.8 *In Vitro* Androgen Bioassays as a Detection Method For Designer Androgens

Androgen bioassays are capable of measuring the hormonal activity of a substance based on its ability to bind to the AR, either *in vivo* or *in vitro*. Therefore, bioassays can detect androgenic activity of a substance via its biological activity and is not reliant on identifying the chemical structure, as is the case with standard MS-based screening tests. This means that any novel, unknown androgenic compounds that can theoretically bypass current screening tests, can be detected in a bioassay due to activation of the AR. Bioassays were initially used

to detect endocrine disrupting chemicals (EDCs) in the environment such as water streams, usually for oestrogenic activity but can be applied to measuring androgenic activity (Liscio *et al*, 2009). Testing the androgenic activity of androgens usually involves the use of an *in vivo* rodent model that has been orchidectomised, and the administration of an androgen should therefore result in the growth of androgen-dependent tissue which can be measured and weighed. This is known as the Hershberger assay, and has been a standard way of measuring the androgenic activity of compounds for over 70 years (Kennel *et al*, 2004). The test can be also modified to test for the anti-androgenic activity of chemicals. The Hershberger assay is not suitable for rapid screening due to its *in vivo* nature, and is also not suitable for all matrices such as testing an athlete's urine or blood. However, it has the benefit of analysing the metabolism of the compound as any metabolites produced can be analysed from the blood or urine. The move to *in vitro*-based androgen bioassays to evaluate the (anti)androgenic activity of samples is becoming more common, particularly with detecting androgens for growth promoting uses in livestock and their food. Akin to this, AR bioassays are also capable of screening human sports supplements for the addition of designer androgens and prohormones. *In vitro* AR bioassays can be categorised into two main types: cell proliferation assays and reporter gene assays.

1.8.1 Cell Proliferation Assays

Cell proliferation assays are comparable to the Hershberger assay in that they measure the proliferation of cells as opposed to muscle growth. Cell proliferation assays involve the measurement of radiolabelled nucleotides which are incorporated into the DNA during cell division, and therefore the amount of radiolabelled nucleotide that is incorporated is a direct measurement of cell proliferation. This is usually applied to test for (anti)oestrogenic activity

in breast cancer cell lines (Soto *et al*, 1995). This type of bioassay is not really feasible for sports doping tests due to the time involved in measuring cell growth (Connolly *et al*, 2011).

1.8.2 Reporter Gene Androgen Bioassays

Reporter gene AR bioassays involve genetically expressing human AR inside a host cell which would typically not express endogenous AR. The host is also transformed with a reporter vector and a minimal promoter regulated by AREs. This enables production of a reporter enzyme or protein which can be quantifiably measured upon stimulation of the AR by an AR agonist.

Host cells that are typically used are the yeast strain *Saccharomyces cerevisiae*, or a range of mammalian-based cell lines such as liver, kidney, bone, and breast cancer. These hosts are also capable of being transformed with a range of different constructs, each having differences in sensitivity and specificity. Typical cell line constructs used in AR reporter gene bioassays can be seen in Table 1.3 along with their varying sensitivities.

Table 1.3 Sensitivities of various androgen bioassays

Androgen	Construct	Cell Line/Species	EC_{50} (nM)	Author
Testosterone	MMTV/Luciferase AR	HEK293	0.3	Roy *et al*, 2006
	ARE/Luciferase	U2-OS	0.86	Houtman *et al*, 2009
	ARE/Luciferase	CHO	1.06	Araki *et al*, 2005
	ARE/β-galactosidase	*S. cerevisiae*	5	Death *et al*, 2005
	ARh-LBD-ASC1/ β-galactosidase	*S. cerevisiae*	15	Lee *et al*, 2003
	ARE/GFP	*S. cerevisiae*	23	Beck *et al*, 2008
5α-Dihydrotestosterone (DHT)	ARE/Luciferase	CHO	0.22	Araki *et al*, 2005
	ARh-LBD-ASC1/ β-galactosidase	*S. cerevisiae*	4.8	Lee *et al*, 2003
	ARE/GFP	*S. cerevisiae*	16	Beck *et al*, 2008
Methyltestosterone	ARE/Luciferase	CHO	0.7	Araki *et al*, 2005
	ARE/GFP	*S. cerevisiae*	1.2	Beck *et al*, 2008
4-Androstenedione	ARE/Luciferase	CHO	1.02	Araki *et al*, 2005
	AR-CALUX	U2-OS	4.5	Sonneveld *et al*, 2005
	ARE/Luciferase	*S. cerevisiae*	500	Michelini *et al*, 2005

With the growing concern of the clandestine development of designer androgens and prohormones, particularly with spiking and contamination concerns of sports supplements, *in vitro* androgen bioassays are a feasible approach for screening sports supplements. Several studies have demonstrated the effectiveness of androgen bioassays for screening supplements for anabolic activity (Akram *et al*, 2011; Plotan *et al*, 2011; Peters *et al*, 2010). One study even showed how a yeast-based androgen bioassay was able to show positive screens in two sports supplements initially missed by an MS-based screen (Rijk *et al*, 2009). While most *in vitro* androgen bioassays used for this approach have been yeast-based, mammalian cell-based androgens bioassays have their own set of advantages. Therefore, both types are necessary to better evaluate the androgenic activity of a sample, and will be further explained below.

1.8.2.1 Yeast Cell Androgen Bioassays

Androgen bioassays using yeast such as *S. cerevisiae* are typically less sensitive than their mammalian counterparts. This is due to yeast not endogenously expressing steroid receptors or metabolising enzymes. However, yeast-based androgen bioassays offer several advantages (as well as disadvantages) compared to mammalian cell-based androgen bioassays. Due to the absence of metabolising enzymes, the test compound is not metabolised by the yeast and as such, the intrinsic androgenic bioactivity of the compound can be evaluated. This, however, also means that any prohormones or compounds requiring metabolic activation cannot be detected using a yeast-based bioassay. Yeast-based AR bioassays are also cheap and easy to grow, making them suitable for high throughput applications. Most yeast-based AR bioassays are designed using an AR/ARE/β-galactosidase construct (Death *et al*, 2005). This is the most

sensitive of the varying types of yeast bioassays, and has an EC_{50} of about 5 nM for T. They are, however, generally more tedious and longer than other reporter proteins, with long incubation times, pre-assay preparation and cell lysis steps involved. To address this, other constructs have been produced, typically using a fluorescent protein (yeast enhanced green fluorescent protein [yEGFP]) or luciferase as a reporter instead of β-galactosidase. These constructs are less sensitive than β-galactosidase with EC_{50} values for T reported to be about 50 nM (Bovee *et al*, 2009a and 2007) and 10 nM (Leskinen *et al*, 2005), respectively.

However, they do not require any cell lysis steps and can, therefore, be easier to complete. To further this, the yEGFP construct is also cheaper than either the luciferase or the β-galactosidase reporter proteins as no enzyme substrate is required to produce the reporter readout, and requires only fluorescence. It also does not have the issue of enzyme inhibiting compounds which may also hinder enzyme function in luciferase-based assays (Sotoca *et al*, 2010). Certain compounds may bind to the firefly luciferase enzyme, inhibiting its activity (Auld *et al*, 2008).

1.8.2.2 Mammalian Cell Androgen Bioassays

Mammalian cell-based androgen bioassays are generally more sensitive with reported EC_{50} values often 10-fold lower (Houtman *et al*, 2009; Roy *et al*, 2006), but show less specificity than their yeast-counterparts. The lowering in specificity comes from two major differences between the two: the presence of metabolising enzymes, and receptor cross-talk (Bovee and Pikkemaat, 2009). Metabolising enzymes such as aromatase, 5α-reductase, and the HSD enzymes mean that the intrinsic androgenic activity of a compound cannot be established as it can be converted to a more or less potent form. Therefore, mammalian cell androgen

bioassays are capable of detecting prohormones. Cell lines can therefore give insight into how an androgen might behave *in vivo* in terms of metabolism, determining if the steroid remains active, or is deactivated. The degree to which this happens is dependent on the chosen cell line because the extent of the expression of these enzymes can vary between cell lines (Akram *et al*, 2011). The majority of cell types in the body express endogenous steroid receptors including the PR, ER and GR. Androgens are capable of exhibiting cross-talk with these receptors, particularly the PR and GR, and to a smaller extent, the ER. There have also been attempts using *ex vivo* liver preparations in order to better mimic *in vivo* metabolism (Rijk *et al*, 2012 and 2008). Such assays first involve incubating steroids with the liver fraction, then extracting the metabolites produced and exposing them to a yeast-based bioassay. This approach can also be used to detect prohormones and metabolites contained within sports supplement extracts.

1.9 Summary, Aims and Hypothesis

Androgens remain the most commonly abused performance enhancing drug used in sports. The high prevalence of use not only includes elite athletes, but also extends to amateur athletes and adolescents. Androgen abuse can be detected using GC-MS-based techniques if the structure of the compound of interest is known. The emergence of designer steroids, which have novel and initially unknown structures, are difficult to detect and present a major problem for doping authorities. Many of these compounds are being sold legally as sports supplements and have had no safety testing and therefore, may present a health hazard to consumers. Further, it is also unknown if these compounds are biologically active and effective as anabolic agents. Therefore, the overall aim of this project was to assess the

biological activity of designer steroids found within sports supplements. More specifically, the overarching hypothesis of this thesis is:

"**Designer steroids contained within sports supplements are potent androgens**"

The intrinsic androgenic bioactivity of 22 sports supplement-derived designer steroids was assessed in **Chapter 3** using the yeast androgen bioassay. We report that 45% of these designer steroids are potent androgens. **Chapter 4** extended on these findings by assessing the androgenic bioactivity of these 22 designer steroids after metabolism in the mammalian HuH7 androgen bioassay. This study demonstrated that several androgens are converted into more potent androgens while others are deactivated after metabolism. To determine if the potent androgens had anabolic activity, we next investigated the anabolic potential of several designer steroids in a mouse C2C12 myoblast cell model in **Chapter 5**.

Chapter 6 aimed to screen the Australian sports supplement market for undeclared androgenic substances using the yeast and HuH7 androgen bioassays. We report that 5.3% of the sports supplements screened contained undeclared androgenic substances.

Finally, **Chapter 7** contains a general conclusion of the results obtained throughout this thesis, and several limitations of the studies, highlighting the potential for future work.

Chapter 2

General Methods

Table of Contents

2.1 Yeast Culture

2.1.1 Materials and Solutions

Yeast Strain:

Saccharomyces cerevisiae, YPH500 (MATα, *ura*3-52, *lys*2-801, *ade*2-101, *trp*1-Δ63, *his*3-Δ200, *leu*2-Δ1)

Transformed with the following plasmids:

AR bioassay: YRpG2 (ARE-lacZ fusion) and YEpAR (AR cDNA)

PR bioassay: YRpG2 (PRE-lacZ fusion) and YEphPR-B (hPR-B cDNA)

YPD Medium (for reviving strains from frozen stocks)

Yeast extract (Cat # Y1625, Sigma Aldrich)	10 g
Peptone (Cat # J849, Amresco)	20 g

Medium was made up to 1 L with deionised water, autoclaved for 15 min and stored at 4 °C until required. Agar was added at a concentration of 1.5% (w/v) prior to autoclaving if plates were required. Glucose from a 20% (w/v) filter sterilised stock was added to a final concentration of 2% in YPD medium just before use.

Double drop-out leu-ura medium (DOB-leu-ura) (for AR bioassays)

DOB base powder (Cat # 4025-032, MP Biomedicals)	6.75 g
CSM-leu-ura (Cat # 4520-212, MP Biomedicals)	0.1675 g

The selective double drop-out medium was used for maintaining plasmids and for receptor assays. Medium was made up to 250 mL with deionised water and 1.5% (w/v) agar added prior to autoclaving if plates were required. Medium was autoclaved for 15 min, and stored at 4 °C.

Double drop-out trp-ura medium (DOB-trp-ura) (for PR bioassays)

DOB base powder (Cat # 4025-032, MP Biomedicals)	6.75 g
CSM-trp-ura (Cat # 4520-512, MP Biomedicals)	0.18 g

Medium was made up to 250 mL with deionised water and 1.5% (w/v) agar added prior to autoclaving if plates were required. Medium was autoclaved for 15 min, and stored at 4 °C.

ONPG (o-nitrophenol-β-galactoside) solution (4 mg/mL)

ONPG (Cat # N1127, Sigma Aldrich)	40 mg
60 mM $Na_2HPO_4.7H_2O$	16.1 g/L
40 mM $NaH_2PO_4.H_2O$	5.5 g/L

Forty mg of ONPG was dissolved in 10 mL of 100 mM phosphate buffer (60 mM $Na_2HPO_4.7H_2O$; 40 mM $NaH_2PO_4.H_2O$), aliquoted and stored at -20 °C. ONPG solution was warmed to 30 °C before use.

Z-buffer

$Na_2HPO_4.7H_2O$ (60 mM)	16.1 g
$NaH_2PO_4.H_2O$ (40 mM)	5.5 g
KCl (10 mM)	0.75 g
$MgSO_4.7H_2O$ (1 mM)	0.246 g
β-mercaptoethanol (50 mM)	2.7 mL

Solution was made up to 1 L with deionised water and the pH adjusted to 7.0. The solution was not autoclaved, and was stored at 4 °C.

Glycerol stocks

Yeast culture	1 mL
Glycerol (80% v/v)	0.5 mL

Glycerol stocks of yeast were made by mixing 1 mL of yeast culture (grown in selective DOB medium) with 0.5 mL glycerol (80% v/v) in 2 mL cryogenic tubes and stored at -80 °C.

2.1.2 β-galactosidase Assays

2.1.2.1 Androgen Receptor (AR) Bioassay

Yeast cells transformed with both the ARE-lacZ and AR plasmid were revived from frozen stocks on non-selective YPD agar before a single colony was streaked onto selective CSM-leu-ura agar plates and grown for 48-72 hours. CSM-leu-ura broth (50 mL) was inoculated with one colony from selective CSM-leu-ura agar plates and incubated overnight with orbital shaking (300 rpm) at 30 °C. The optical density (OD) of the culture was measured at a wavelength of 600 nm on an Infinite m200 Pro Tecan microplate reader. The culture was then diluted with fresh medium to obtain an OD of 0.5 to 0.7. Aliquots (500 µL) of the diluted yeast culture were then pipetted into a 24-well plate before the addition of 5 µL of $CuSO_4$ (10 mM stock) to induce AR expression via the CUP1 promoter. The respective steroid treatment (5 µL) was then added to the wells in triplicate. A testosterone dose-response curve with final concentrations ranging from $7x10^{-5}$ M to $1.31x10^{-10}$ M was included in duplicate in every assay for AR as well as duplicate ethanol vehicle controls. The determination of the concentrations used for each steroid are described in Chapter 3. The plates were incubated overnight at 30 °C with orbital shaking (300 rpm).

The following day the 24-well plates were placed on ice for 20 min to inhibit further yeast growth. The yeast were then resuspended before the density of yeast from each well was determined at 600 nm. The yeast cultures (400 µL) from each well were transferred to Eppendorf tubes and pelleted by centrifugation at 10,000 rpm for 5 min. The supernatant was carefully removed without disturbing the pellet and discarded. The pellet was resuspended in 200 µL of Z-buffer. Chloroform (15 µL) and SDS (0.1% w/v, 7.5 µL) were then added to permeabilise the yeast cells and the suspension was vortexed for 10 s. Tubes were then

warmed to 30 °C for 5 min before the assay was commenced. At time zero, 50 µL of ONPG substrate (4 mg/mL) was added and yellow colour developed for 30 min. The reaction was stopped by the addition of 125 µL of Na_2CO_3 (1M). Tubes were mixed briefly before allowing the chloroform to settle at the bottom of the tube for 5 min. Triplicate aliquots (100 µL) were removed from each tube being careful not to withdraw any chloroform from the bottom and transferred to a 96-well plate. Absorbance was measured at 550 and 420 nm and the β-galactosidase activity was determined according to the following equation:

$$\text{β-galactosidase activity (Miller units)} = \frac{1000\ x\ (OD420 - 1.75\ x\ OD550)}{Vol.(mL) x\ time\ (\text{minutes}) x\ OD600}$$

β-galactosidase activity was converted at each point to a percentage of activity of maximal testosterone.

2.1.2.2 Progestogen Receptor (PR) Bioassay

A single colony of yeast transformed with both the PRE-lacZ and PR-B plasmid was picked from the non-selective YPD agar plate and streaked onto selective CSM-trp-ura agar plates and grown for 48-72 hours. CSM-trp-ura broth (50 mL) was inoculated with one colony from selective CSM-trp-ura agar plates and incubated overnight with orbital shaking (300 rpm) at 30 °C. The OD of the culture was measured at a wavelength of 600 nm on an Infinite m200 Pro Tecan microplate reader. The culture was then diluted with fresh medium to obtain an OD of 1.0. Aliquots (500 µL) of the diluted yeast culture were then pipetted into a 24-well plate before the addition of 5 µL of $CuSO_4$ (10 mM stock) to induce PR expression. The respective steroid treatment (5 µL) was then added to the wells in triplicate. A progesterone dose-response curve with final concentrations ranging from 3.18×10^{-4} M to 6×10^{-10} M was

included in duplicate in every assay for PR as well as duplicate ethanol vehicle controls. The plates were incubated overnight at 30 °C with orbital shaking (300 rpm).

The following day the 24-well plates were placed on ice for 20 min to inhibit further yeast growth. The yeast were then resuspended before the density of yeast from each well was determined at 600 nm. The yeast cultures (400 µL) from each well were transferred to Eppendorf tubes and pelleted by centrifugation at 10,000 rpm for 5 min. The supernatant was carefully removed without disturbing the pellet and discarded. The pellet was resuspended in 400 µL of Z-buffer. Chloroform (24 µL) and SDS (0.1% w/v, 12 µL) were then added to permeabilise the yeast cells and the suspension was vortexed for 10 s. Tubes were then warmed to 30 °C for 5 min before the assay was commenced. At time zero, 80 µL of ONPG substrate (4 mg/mL) was added and yellow colour developed for 30 min. The reaction was stopped by the addition of 200 µL of Na_2CO_3 (1M). Tubes were mixed briefly before allowing the chloroform to settle at the bottom of the tube for 5 min. Duplicate aliquots (100 µL) were removed from each tube being careful not to withdraw any chloroform from the bottom and transferred to a 96-well plate. Absorbance was measured at 550 and 420 nm and the β-galactosidase activity was determined according to the equation described above. β-galactosidase activity was converted at each point to a percentage of activity of maximal progesterone.

2.2 Solid Phase Extraction

The amount of each supplement used for the extraction was dependent upon the manufacturer's recommended serving size (Chapter 6, Table 6.2). Steroid extracts were

prepared from sports supplements using a standard solid phase extraction (SPE) method, with minor alterations (Rijk *et al*, 2009). Briefly, capsules were emptied and solid tablets were pulverised using a mortar and pestle. The powders were suspended in water/methanol 1:1 v/v and dissolved by sonication for 5 min. The suspension was then centrifuged at 3000 g for 10 min and the pellet was discarded. The pH of the supernatant was adjusted to 4.8 using 4N acetic acid and sodium acetate. The supernatant then underwent SPE using a C18 column (0.5 g Bond Elut, Agilent Technologies) that was previously conditioned with 4 mL methanol/sodium acetate (pH 4.8). After the sample was loaded, the columns were washed sequentially with 2 mL water, 1.5 mL sodium carbonate (10% w/v), 2 mL water, and 2 mL water/methanol (1:1, v/v). The columns were then air-dried and the sample was eluted with 4 mL acetonitrile. The eluate was concentrated by evaporation then resuspended in 50 µL 100% ethanol.

2.3 Tissue Culture

2.3.1 Materials and Solutions

Cell lines

Human hepatocarcinoma cells (HuH7) and mouse myoblast cells (C2C12) were purchased from ATCC (Manassas, VA).

Phosphate-buffered saline (PBS)

PBS (Cat# 09-2051-100, Astral Scientific) was made up to 1 L in deionised water according to the manufacturer's instructions (1 tablet/100 mL) and autoclaved for 20 min.

Dulbecco's modified eagle medium (DMEM) (For HuH7 cell line)

HuH7 cells were cultured in DMEM (Cat# 11995065, Life Technologies) containing high glucose (4500 mg/L) L-glutamine (584 mg/L), sodium pyruvate (110 mg/L) and phenol red indicator supplemented with:

Fetal calf serum (Cat # 10099-141, Gibco Life Technologies)	10% (v/v)
Penicillin/Streptomycin (Cat # 15140-122, Gibco Life Technologies)	100 U/mL

DMEM (HuH7 cell line) (AR SEAP bioassay)

HuH7 cells stably transfected with the human AR expression plasmid and the enhancer/ARE/SEAP reporter plasmid were cultured in DMEM (Cat# 11995065, Life Technologies) containing high glucose (4500 mg/L), L-glutamine (584 mg/L), sodium pyruvate (110 mg/L) without phenol red indicator supplemented with:

Charcoal-stripped fetal calf serum	10% (v/v)
Penicillin/Streptomycin (Cat # 15140-122, Gibco Life Technologies)	100 U/mL
Puromycin dihydrochloride (Cat # A11138-02, Gibco Life Technologies)	5.5 µg/mL (HuH7)

DMEM growth media (For C2C12 cells)

C2C12 cells were grown in DMEM (Cat# 11965092, Life Technologies) without sodium pyruvate containing high glucose (4500 mg/L), L-glutamine (584 mg/L) and phenol red indicator supplemented with:

Fetal calf serum (Cat # 10099-141, Gibco Life Technologies)	20% (v/v)
Penicillin/Streptomycin (Cat # 15140-122, Gibco Life Technologies)	100 U/mL

DMEM differentiation media (For C2C12 cells)

C2C12 cells were differentiated in DMEM (Cat# 11965092, Life Technologies) without sodium pyruvate containing high glucose (4500 mg/L), L-glutamine (584 mg/L), and phenol red indicator supplemented with:

Charcoal-stripped horse serum (Cat # 16050-122, Gibco Life Technologies)	2% (v/v)
Penicillin/Streptomycin (Cat # 15140-122, Gibco Life Technologies)	100 U/mL

2.3.2 Charcoal Stripping

To strip fetal calf serum of endogenous steroids, 625 mg of activated charcoal (Cat # C9175, Sigma Aldrich) was first washed by adding 250 mL PBS. The 250 mL was separated into five falcon tubes, and then centrifuged for 20 min at 2000 rpm. The PBS was discarded and the charcoal resuspended in a total volume of 50 mL FCS (10 mL/tube). The 10 mL of resuspended activated charcoal in each tube were then combined into one tube and stripped by heating at 55 °C for 30 min. The tube was then centrifuged at 2000 rpm for 20 min and the FCS decanted into a clean falcon and filter sterilised before adding to medium.

2.3.3 Cell Culturing

To revive cells from frozen stocks, cells were quickly thawed in a 37 °C water bath before transferring to a T75 cm^2 flask containing at least 15 mL of appropriate media. The next morning, the media was aspirated and cells were washed once with 5 mL PBS to remove DMSO and non-viable cells before replacing with fresh media.

Cells were cultured in T175 cm^2 flasks in appropriate media. Every second day, cells were washed once with 10 mL PBS and replenished with fresh media. At 80-90% confluence HuH7 cells were passaged 1 in 5 into new flasks to propagate cells. For the C2C12 cell line, cells were passaged at 50-60% confluence to minimise cell-cell contact and cell fusion causing partial differentiation. For passaging, cells were washed with 10 mL PBS followed by incubation with 5 mL trypsin EDTA (Cat # 15400054, Life Technologies) at 37 °C for 2 to 3 min to detach cells. Once cells had lifted, the trypsin was deactivated with an equal volume of serum-containing media and centrifuged at 3000 rpm for 5 min. The supernatant was aspirated and the cell pellet was resuspended in appropriate media and split into new flasks or counted and seeded for experimental procedures.

Frozen stocks for each cell line were prepared by trypsinising and centrifugation (3000 rpm for 5 min) as described above. The supernatant was discarded, and the cell pellet was resuspended in cold media containing 40% FCS. An equal volume of cold media containing 20% DMSO (Cat # D2650, Sigma Aldrich) was added and gently mixed. The cell suspension was then transferred to cryopreservation tubes (Cat # 72.379, Starstedt) (1 mL per tube) and stored in a CoolCell (Cat # BCS-136, Biocision) at -80 °C for at least 24 hours before being transferred to a liquid N_2 tank.

2.3.4 Trypan Blue Staining For Cell Viability and Quantification With Haemocytometer

To assess cell viability, 10 µL of trypan blue was added to 10 µL of cell suspension and counted using a haemocytometer. Non-viable cells were differentiated by incorporation of trypan blue indicating a compromised cell wall integrity and were not included in the cell count. A cell count of $1x10^5$ cells/mL was used for experiments unless otherwise stated.

2.3.5 Mammalian AR Bioassays

2.3.5.1 Secreted Embryonic Alkaline Phosphatase (SEAP) AR Bioassay

HuH7 cells stably transfected with the human AR expression vector and the enhancer/ARE/SEAP reporter gene construct (Akram *et al*, 2011) were cultured in appropriate DMEM media supplemented with the selective antibiotic puromycin (5.5 µg/mL). Cells were seeded into a 96-well plate ($1x10^5$/mL) with a volume of 200 µL/well in phenol-red free DMEM media for the AR bioassay (described in section 2.3.1). After 24 hours, cells were treated with steroids (diluted in 100% ethanol, with a final concentration of 1%) at various concentrations by adding 2 µL in triplicate and incubated for 24 hours. After incubation with steroid treatments, 25 µL of culture supernatant from each well was aliquoted into white opaque 96-well plates in duplicate. The plate was then heated to 65 °C for 35 min to deactivate endogenous alkaline phosphatase before placing on ice for 3 to 5 min and then allowed to equilibrate to room temperature. The assay was initiated by the addition of 50 µL SEAP substrate reagent (Cat # 631737, Clontech) to each well. Wells were incubated for 35 min at room temperature and protected from light before measuring luminescence on the Infinite m200 Pro Tecan microplate reader. A testosterone dose-response curve with final

concentrations ranging from $7x10^{-5}$ M to $1.31x10^{-10}$ M was included in duplicate in every assay as well as duplicate ethanol vehicle controls. The determination of the concentrations used for each steroid are described in Chapter 4.

2.4 Immunocytochemistry

2.4.1 Materials and Solutions

TBST

Tris Base	13.9 g
Tris HCL	60.6 g
NaCl	60.6 g
Tween 20	5 mL

A 10x stock solution was made up to 1 L with deionised H_2O.

3% skim milk blocking solution

Skim milk powder	3 g
1x TBST	100 mL

2.4.2 Immunocytochemistry

C2C12 cells were washed 3x with PBS before fixing with ice-cold methanol for 10 min at room temperature. Following fixation, cells were washed 3x with PBS and non-specific binding was blocked with 3% skim milk blocking solution for 3x 5 min incubations. After blocking, cells were again washed 3x with PBS before incubating with the primary antibody as described in Table 2.1 for 1.5 hours at room temperature. After incubation with the primary antibody, cells were washed 3x with PBS and secondary antibody (Table 2.1) was incubated for 1.5 hours at room temperature, protected from light exposure. Nuclei were counterstained with DAPI for 30 minutes before image analysis was performed on the IN Cell Analyzer 2200, GE Healthcare.

Table 2.1 Antibodies and optimised conditions used in immunocytochemistry

Primary Antibody	Optimised Concentration
Anti-sarcomeric myosin MF-20 (Developmental Studies Hybridoma Bank)	0.75 µg/mL
Secondary Antibody	**Dilution**
Fluoroscein-isothiocyanate-conjugated anti-mouse IgG H&L secondary antibody, Abcam (Cat# ab6785)	1/1000

Chapter 3

Evaluation of the Intrinsic Androgenic Bioactivity of Sports Supplement-Derived Designer Steroids Using the Yeast Androgen Bioassay

Table of Contents

3.1 Introduction

Despite the banning of prohormone-based sports supplements in the United States, the last 10 years has seen the continual sale of androgens on the sports supplement market (Abbate *et al*, 2014). The last decade has also seen the emergence of new designer steroids being sold in sports supplements (Cavalcanti *et al*, 2013). These designer steroids include steroids produced in the 1950s and 1960s for pharmaceutical purposes but were never commercialised, and have since re-emerged in the last decade (Diel *et al*, 2007). There is also an increase in newly developed steroids that have been introduced solely for the sports supplement market (Parr *et al*, 2011a). These steroid molecules have likely emerged on the sports supplement market in the last few years as attempts to bypass the illegality of selling androgens as sports supplements, as well as avoiding detection in anti-doping tests (Joseph and Parr, 2015).

There exists very little information on these designer steroids in the literature, particularly in terms of safety and their efficacy in eliciting anabolic effects (Diel *et al*, 2007). There is a lot of evidence that modifications to the steroid structure can alter activity and binding affinity to the AR (McRobb *et al*, 2008), as reviewed by Fragkaki *et al* (Fragkaki *et al*, 2009) and Kicman (Kicman, 2008). Therefore, without scientific investigation, there is no evidence that a new structure will be physiologically effective. These designer steroids are therefore being marketed without proper knowledge of their effects *in vitro* or *in vivo*.

Given that these designer steroids are sold as pharmacological agents with no scientific testing, it is not clear that they are androgenic. To test for intrinsic androgenic potency, androgen bioassays can be utilised. One of the most feasible androgen bioassays is that based on yeast cells. Yeast cells are ideal because they do not endogenously express AR or steroid

metabolising enzymes. An androgen receptor expression vector and an ARE/reporter vector can then be introduced into the yeast cells to exploit the basic genomic androgen pathway in order measure AR bioactivity, in an environment where there is no cross-reactivity with other steroid receptors or metabolism of the test steroid (Gaido *et al*, 1997).

The yeast-based androgen bioassay has been successfully used in determining the relative androgenic potency of steroids (McRobb *et al*, 2008). Our laboratory group utilises a yeast-based androgen bioassay using an ARE/β-galactosidase reporter vector which has been used to determine the androgenic potency of a number of synthetic steroid compounds (McRobb *et al*, 2008; Death *et al*, 2005). Further, yeast-based androgen bioassays have been used in determining the intrinsic androgenic bioactivity of a number of steroids derived from nutritional supplements, including human sports supplements (Akram *et al*, 2011) as well as livestock-based feed supplements (Rijk *et al*, 2011).

Given the emergence of designer steroids in internet-sourced sports supplements, we collected 22 of these. It was hypothesized that these designer steroids would be potent androgens, designed for the positive anabolic effects but with different structures to avoid GC-MS or other chemical-based assay detection. The aim of this study was to test the intrinsic androgenic bioactivity of these designer steroids in the yeast-based androgen bioassay.

3.2 Methods

3.2.1 Steroids Used in This Study

A total of 22 steroid molecules were extracted from sports supplements and tested for their androgenic bioactivity. T and DHT were used as reference controls. The steroid molecules are listed in Table 3.1. Nineteen of the sports supplements that declared the presence of an androgen were purchased online from www.bodybuilding.com. The steroids 2α,3α-epithio-17α-methylandrostane-17β-ol and 2β,3β-epithio-17α-methylandrostane-17β-ol were synthesised by BDG Synthesis (B Dent Global, Wellington, New Zealand) while 17α-methylandrostane-17β-ol-3-one hydrazone was obtained from the National Measurement Institute (NMI), North Ryde, Australia. Concentrations of all steroids used was calculated from molecular weights and the mass of the steroid dissolved in ethanol.

3.2.2 Steroid Extraction from Sports Supplements

Steroid extraction from the sports supplements was performed by Dr. Rymantas Kazlauskas at the NMI. Briefly, steroids were extracted from the powder contents with 3 volumes of a dichloromethane/methanol mixture (1:1), evaporating the solvent then chromatographing the residue on silica gel. The compounds were eluted with dichloromethane/*t*-butylmethylether mixtures, and separation was followed by thin-layer chromatography or by GC-MS on the fractions. Fractions with fairly pure materials were recrystallized from mixtures of solvents such as toluene, *t*-butylmethylether and hexane. Structures were determined from GC-MS and ^{1}H NMR and ^{13}C NMR data. For all supplements, the data was consistent with the steroid indicated on supplement labels. All steroids were dissolved in 100% ethanol for subsequent assay.

3.2.3 Plasmids and Reporter Gene Constructs

The full-length hAR-cDNA expression plasmid that encodes the hAR receptor fused to the CUP1 metallothionen promoter and the ARE-β-galactosidase reporter plasmid that has androgen response elements upstream of *LacZ*, the β-galactosidase reporter gene, were kindly provided by Professor D. P. McDonnell (Duke University Medical Centre, Durham, NC). Yeast strain YPH500 (MATα, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1) was co-transformed with both plasmids by standard alkali transformation. Co-transformed yeast cell were maintained in CSM-leu-ura selective medium (Methods section 2.1.1).

3.2.4 Yeast Culture

Yeast transformants were grown overnight at 30 °C with orbital shaking (300 rpm) in CSM-leu-ura (MP Biomedicals) selective media. The yeast culture was then sub-cultured in fresh medium and grown to early mid-log phase (OD_{600} = 0.5-0.7). (Methods section 2.1.2.1).

3.2.5 Yeast Androgen Bioassay

For the yeast androgen bioassay, cells in mid-log phase growth were diluted in selective medium to OD_{600} = 0.5-0.7 (CSM-leu-ura) plus 100 µM $CuSO_4$ to induce receptor expression via the CUP1 promoter. Yeast cells were then treated with T and DHT concentrations ranging from $7x10^{-5}$ M to $1.3x10^{-10}$ M or supplement-derived steroids with concentrations ranging from $2.3x10^{-3}$ M to $1.3x10^{-10}$ M. All steroids were diluted in ethanol with a final concentration of 1%. Yeast cells were incubated overnight at 30 °C with vigorous orbital shaking (300 rpm)

before being lysed and assayed for β-galactosidase activity across 6 independent experiments (Methods section 2.1.2.1).

3.2.6 Determination of Steroid EC_{50} Values and Relative Potencies

A dose-response curve for the reference androgen, DHT, was performed each time the androgen bioassay was performed as a positive control. The potency of each steroid was determined by measuring the half maximal effective concentration (EC_{50}), the concentration at which the steroid elicits 50% of its maximal response in the bioassay. The EC_{50} values were determined from a sigmoidal curve fit using GraphPad Prism 6 software. The EC_{50} values for each test steroid were then compared to DHT to determine relative potency to DHT. The relative potency was determined using the equation:

$$\frac{EC50\ [Dihydrotestosterone]}{EC50\ [Test\ Steroid]}\ \text{X}\ 100\%$$

Table 3.1 List of steroids used in this study

Product/Trivial Name	Chemical Name
Dihydrotestosterone and Derivatives	
Dihydrotestosterone (DHT)	17β-hydroxy-5α-androstan-3-one
Ultradrol (Methylstenbolone)	2, 17α-dimethyl-17β-hydroxy-5α-androst-1-ene-3-one
The One (Hydrazone)	17α-methylandrostane-17β-ol-3-one hydrazone
The One (Oxime)	17α-methylandrostane-17β-ol-3-one oxime
Furazadrol (C isomer)	17β-hydroxyandrostano-[3,2-c] isoxazole
Furazadrol (D isomer)	17β-hydroxyandrostano-[3,2-d] isoxazole
P-Plex (Madol)	17α-methylandrost-2-ene-17β-ol
Superdrol (Methasterone)	2α,17α-dimethylandrostane-3-one-17β-ol
Prostanozol	[3,2-c] pyrazole-5α-androstan-17β-ol
Epistane (2α, 3α-epithio)	2α,3α-epithio-17α-methylandrostane-17β-ol
Epistane (2β, 3β-epithio)	2β,3β-epithio-17α-methylandrostane-17β-ol
Testosterone and Derivatives	
Testosterone (T)	17β-hydroxy-androst-4-en-3-one
Mechabol (Methylclostebol)	4-chloro-17α-methyl-17β-hydroxyandrost-4-ene-3-one
Jungle Warfare	17α-methylandro-4,6-diene-17β-ol-3-one
H-Drol (DHCMT, Oral-Turinabol)	4-chloro-17α-methyl-androst-1,4-diene-3,17β-diol
Testabol	4-hydroxytestosterone-17-acetate
Androstenedione Derivatives/Prohormones	
LG Formadrol Extreme	6α-methylandrost-4-ene-3,17-dione
1, 4 AD Bold 200 (Boldione)	Androst-1,4-diene-3,17-dione
DS Rebound XT	Androst-1,4,6-triene-3,17-dione
Tren 250	Estra-4,9-diene-3,17-dione
Novadex XT	Androst-4-ene-6,17-dione-3-ol
Other	
Finaflex 1-Andro	3β-hydroxy-5α-androst-1-ene-17-one
1-Androsterone	1-androsten-3β-hydroxy-17-one
Finaflex 550-XD	3β-hydroxy-19norandrost-4-ene-17-one

3.3 Results

A total of 22 steroids that were derived from sports supplements were evaluated for their intrinsic AR bioactivity in the yeast androgen bioassay. Sigmoidal dose-response curves were generated for each steroid to determine the EC_{50} values. The EC_{50} values were then used to calculate the relative potency (RP) to DHT.

3.3.1 Androgenic Bioactivity of DHT-Derived Steroids

The endogenous androgens T and DHT were used as references for potent AR activators. The calculated EC_{50} values were 4.68 nM and 2.38 nM, respectively, which is in keeping with previous findings for this assay (McRobb *et al*, 2008). Out of the 22 steroids tested in the yeast androgen bioassay, ten were DHT-derivatives and the EC_{50} and RP values were calculated for each steroid from three different assays (Figure 3.1). There were six steroids with strong RPs ranging from 19.93% to 106.7% (Figure 3.2 A). Three steroids were moderately strong with RP values less than 12% (Figure 3.2 B). One steroid was a weak androgen with an RP more than 1000-fold lower than DHT (Figure 3.2 C). The molecular structures of these ten steroids are shown in Figure 3.3.

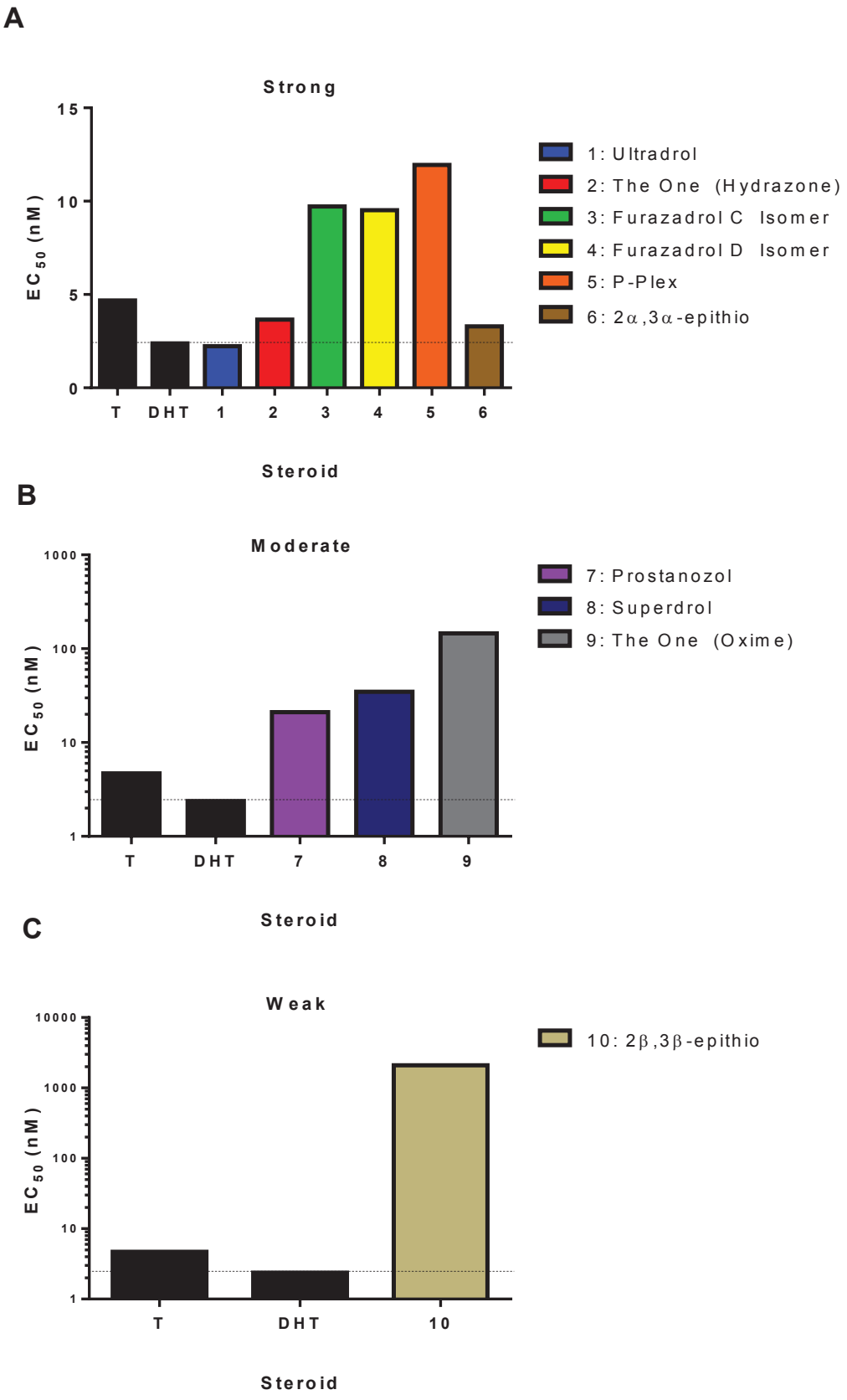

A
Strong
15
10
5
0
EC50 (nM)
T
DHT
1
2
3
4
5
6
Steroid
1: Ultradrol
2: The One (Hydrazone)
3: Furazadrol C Isomer
4: Furazadrol D Isomer
5: P-Plex
6: 2α,3α-epithio
B
Moderate
1000
100
10
1
EC50 (nM)
T
DHT
7
8
9
Steroid
7: Prostanozol
8: Superdrol
9: The One (Oxime)
C
Weak
10000
1000
100
10
1
EC50 (nM)
T
DHT
10
Steroid
10: 2β,3β-epithio

Figure 3.1

Figure 3.1 EC_{50} values of DHT-derived steroids. DHT-derived steroids were tested in the yeast androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. Steroids with EC_{50} above the dotted line were less potent than DHT, while steroids with EC_{50} below the dotted line were more potent than DHT. A) Steroids that had strong intrinsic androgenic bioactivity; B) steroids that had moderate intrinsic androgenic bioactivity; and C) steroids that had weak intrinsic androgenic bioactivity.

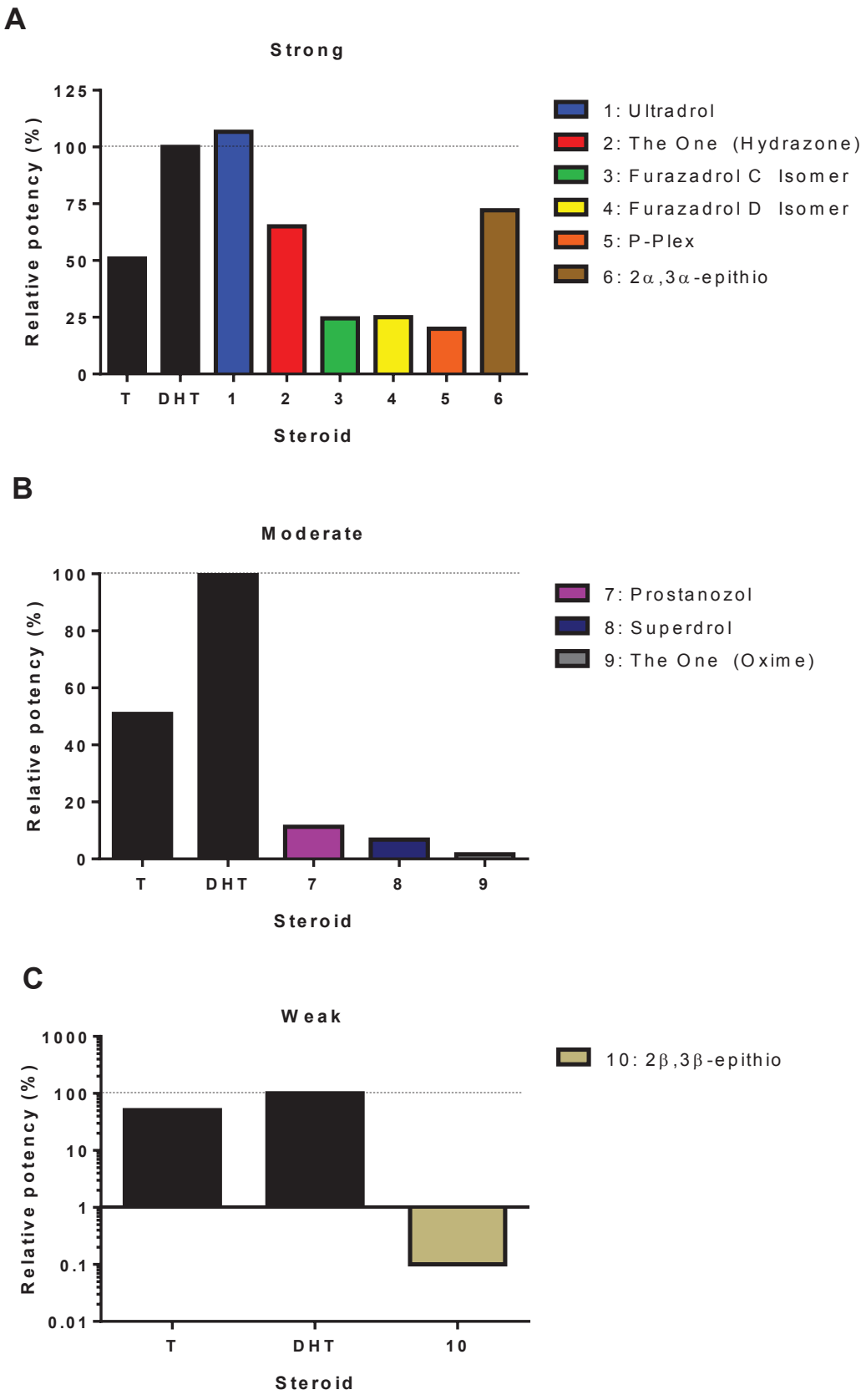

A
Strong
Relative potency (%)
125
100
75
50
25
0
T
DHT
1
2
3
4
5
6
Steroid
1: Ultradrol
2: The One (Hydrazone)
3: Furazadrol C Isomer
4: Furazadrol D Isomer
5: P-Plex
6: 2α,3α-epithio
B
Moderate
Relative potency (%)
100
80
60
40
20
0
T
DHT
7
8
9
Steroid
7: Prostanozol
8: Superdrol
9: The One (Oxime)
C
Weak
Relative potency (%)
1000
100
10
1
0.1
0.01
T
DHT
10
Steroid
10: 2β,3β-epithio

Figure 3.2

Figure 3.2 Relative potency of DHT-derived steroids. DHT-derived steroids were tested in the yeast androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. The relative potency (RP) of each steroid to DHT was calculated. Steroids with RP above the dotted line were more potent than DHT, while steroids with RP below the dotted line were less potent than DHT. A) Steroids that had strong androgenic bioactivity; B) steroids that had moderate androgenic bioactivity; and C) steroids that had weak androgenic bioactivity.

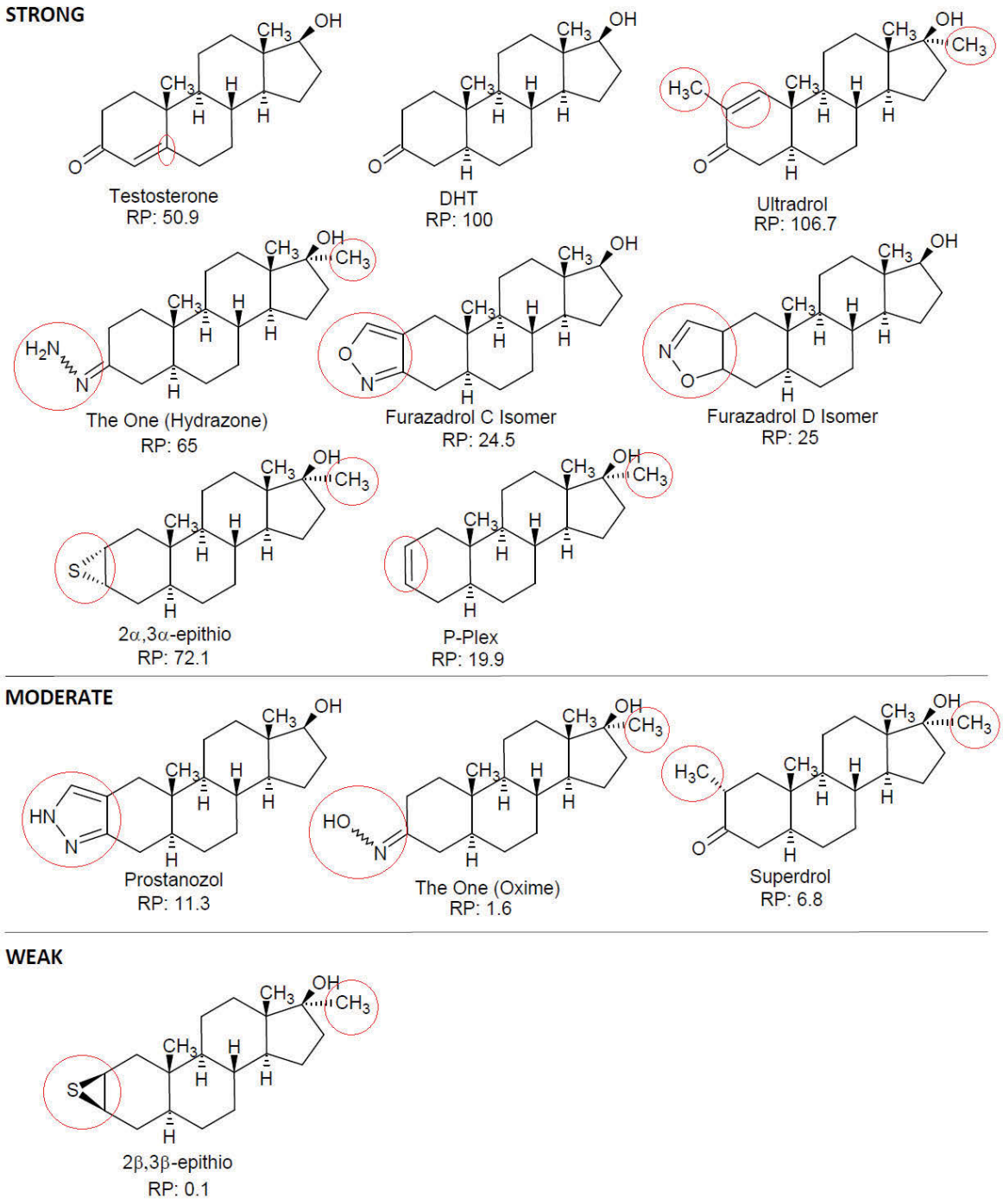


Figure 3.3 Molecular structures of DHT-derived steroids. Red circles indicate structural differences to DHT. RP indicates relative potency of steroids compared to DHT.

3.3.2 Androgenic Bioactivity of T-Derived Steroids

Four of the tested steroids were derivatives of T (Figure 3.4). Two of these steroids were strong activators of AR with RP values of 24.2 and 26.5% (Figure 3.5 A). One steroid was a moderate activator of AR with an RP of less than 12% while one steroid was a weak activator with an RP of less than 0.1% (Figures 3.5 B and C). The molecular structures of these four steroids are shown in Figure 3.6.

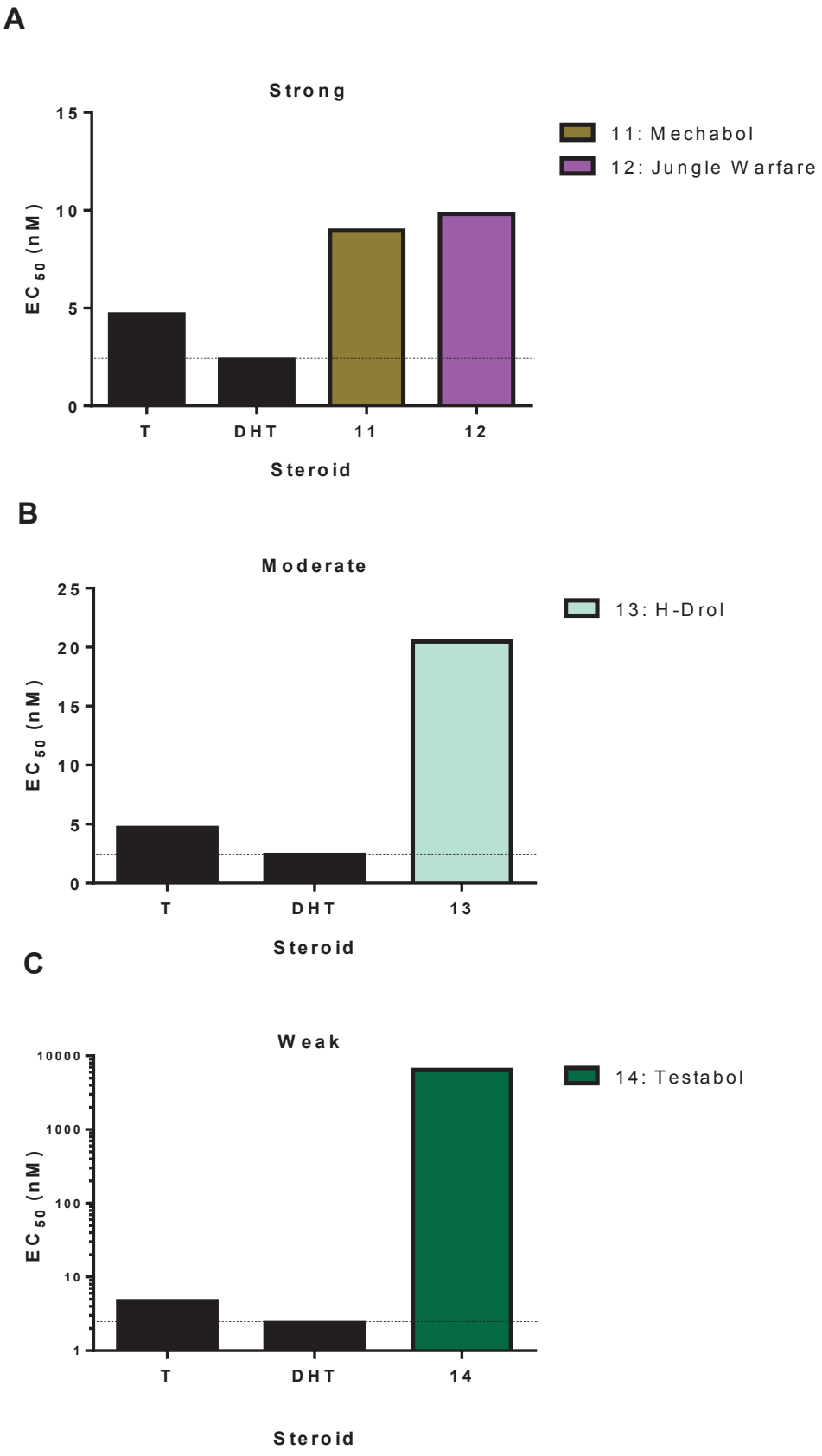

A
Strong
15
10
5
0
EC50 (nM)
T
DHT
11
12
Steroid
11: Mechabol
12: Jungle Warfare
B
Moderate
25
20
15
10
5
0
EC50 (nM)
T
DHT
13
Steroid
13: H-Drol
C
Weak
10000
1000
100
10
1
EC50 (nM)
T
DHT
14
Steroid
14: Testabol

Figure 3.4

Figure 3.4 EC_{50} values of T-derived steroids. T-derived steroids were tested in the yeast androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. Steroids with EC_{50} above the dotted line were less potent than DHT, while steroids with EC_{50} below the dotted line were more potent than DHT. A) Steroids that had strong androgenic bioactivity; B) steroids that had moderate androgenic bioactivity; and C) steroids that had weak androgenic bioactivity.

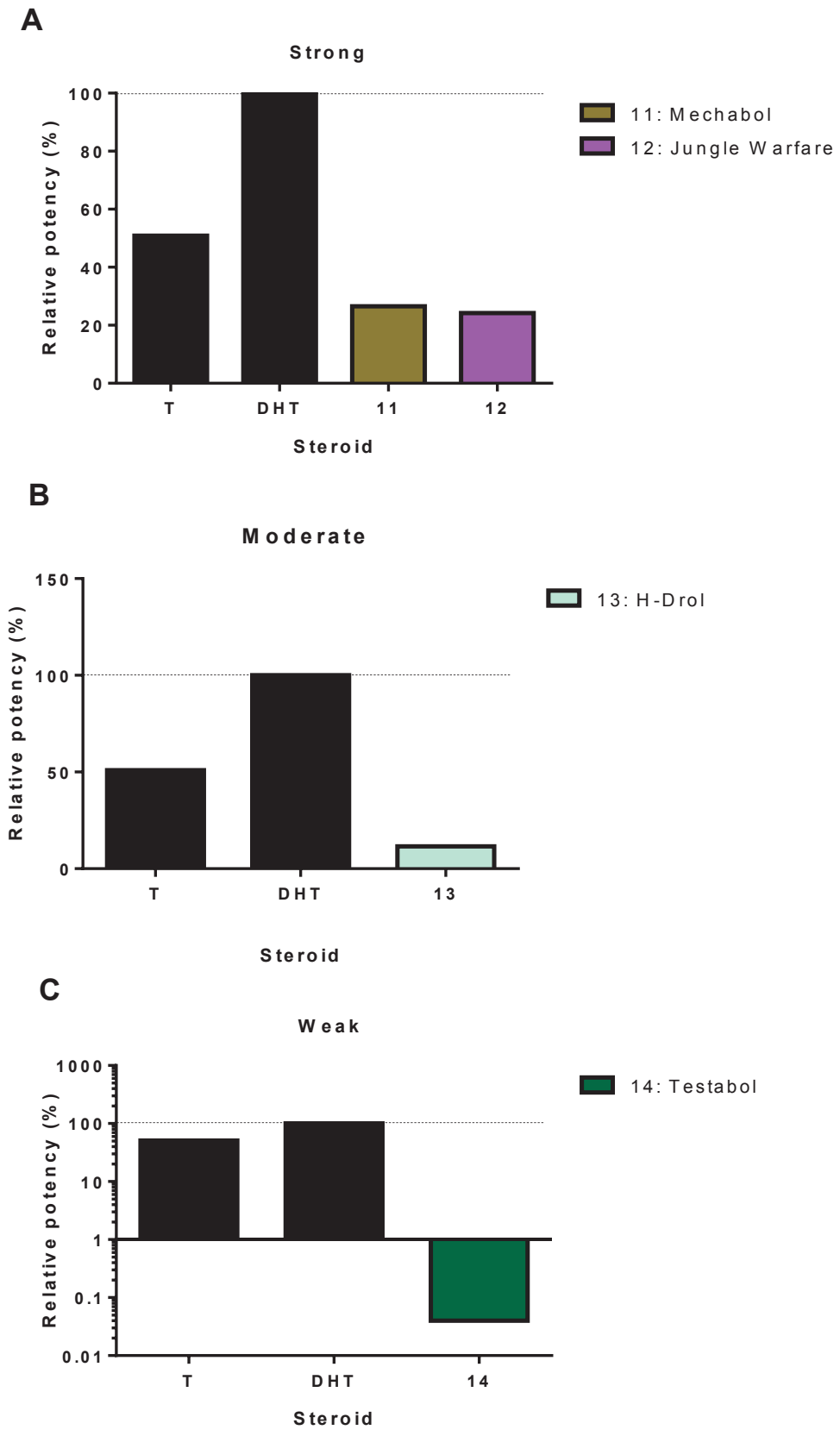

A
Strong
Relative potency (%)
100
80
60
40
20
0
T
DHT
11
12
Steroid
11: Mechabol
12: Jungle Warfare
B
Moderate
Relative potency (%)
150
100
50
0
T
DHT
13
Steroid
13: H-Drol
C
Weak
Relative potency (%)
1000
100
10
1
0.1
0.01
T
DHT
14
Steroid
14: Testabol

Figure 3.5

Figure 3.5 Relative potency of T-derived steroids. T-derived steroids were tested in the yeast androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. The relative potency (RP) of each steroid to DHT was calculated. Steroids with RP above the dotted line were more potent than DHT, while steroids with RP below the dotted line were less potent than DHT. A) Steroids that had potent androgenic bioactivity; B) steroids that had moderate androgenic bioactivity; and C) steroids that had weak androgenic bioactivity.

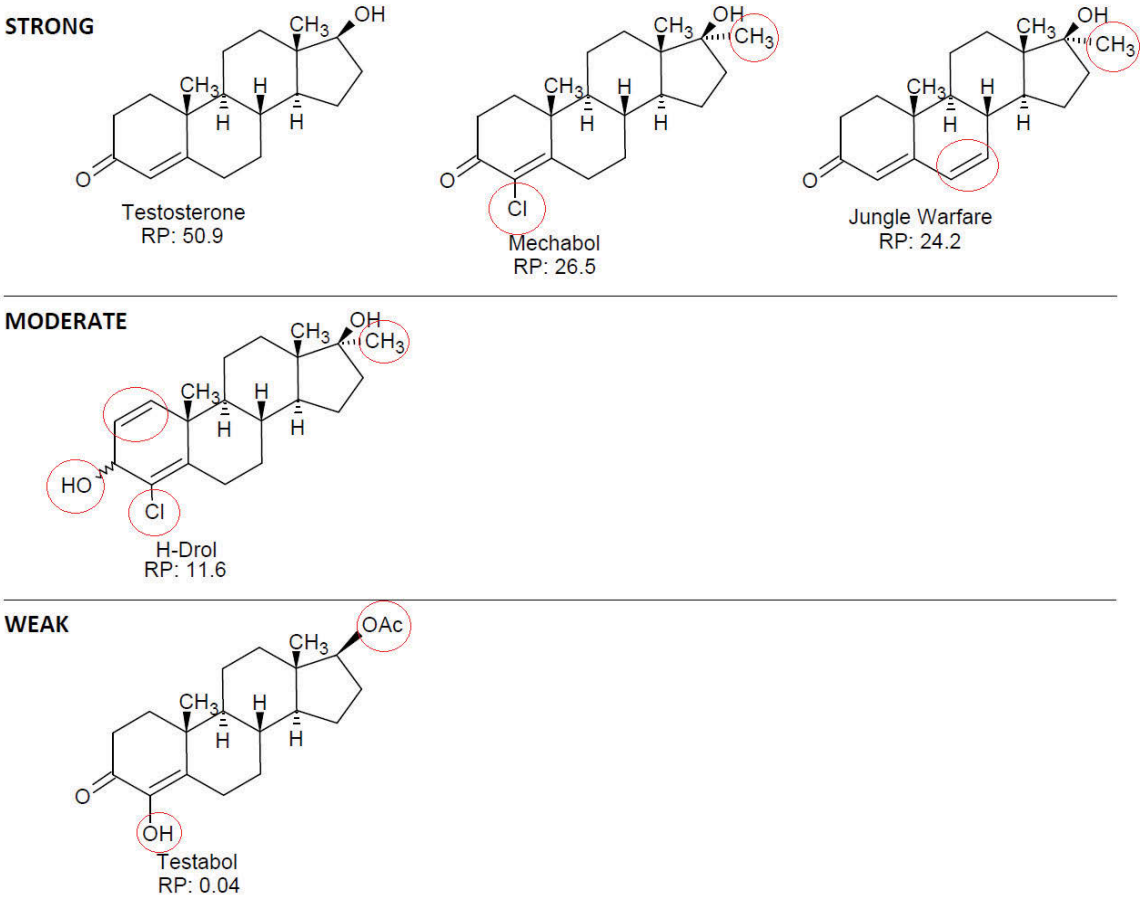


Figure 3.6 Molecular structures of T-derived steroids. Red circles indicate structural differences to T. RP indicates relative potency of steroids compared to DHT.

3.3.3 Androgenic bioactivity of androstenedione-derived steroids

Five of the steroids were derivatives of the proandrogen, androstenedione (Figure 3.7). One steroid was a strong activator with an RP of roughly 50% (Figure 3.8 A). Two steroids were moderate activators of AR with RP values ranging from 1.3% to 2.6% while the other two steroids were weak activators of AR with RP values ranging from 0.1% to 0.001% (Figure 3.8 B and C). The molecular structures of these five steroids are shown in Figure 3.9.

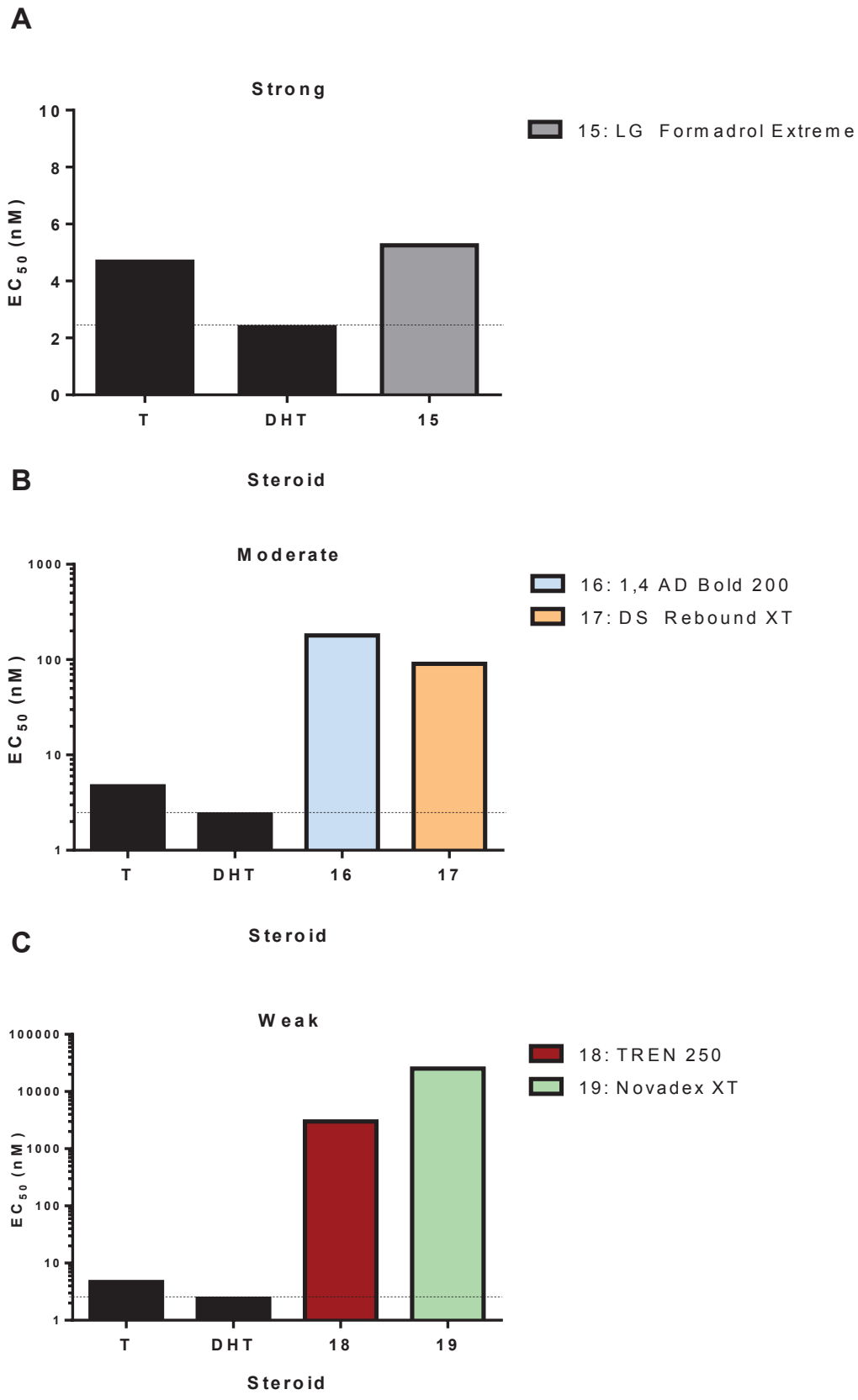

A
Strong
10
8
6
4
2
0
EC50 (nM)
T
DHT
15
15: LG Formadrol Extreme
B
Steroid
Moderate
1000
100
10
1
EC50 (nM)
T
DHT
16
17
16: 1,4 AD Bold 200
17: DS Rebound XT
C
Steroid
Weak
100000
10000
1000
100
10
1
EC50 (nM)
T
DHT
18
19
18: TREN 250
19: Novadex XT
Steroid

Figure 3.7

Figure 3.7 EC_{50} values of androstenedione-derived steroids. Androstenedione-derived steroids were tested in the yeast androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. Steroids with EC_{50} above the dotted line were less potent than DHT, while steroids with EC_{50} below the dotted line were more potent than DHT. A) Steroids that had strong androgenic bioactivity; B) steroids that had moderate androgenic bioactivity; and C) steroids that had weak androgenic bioactivity.

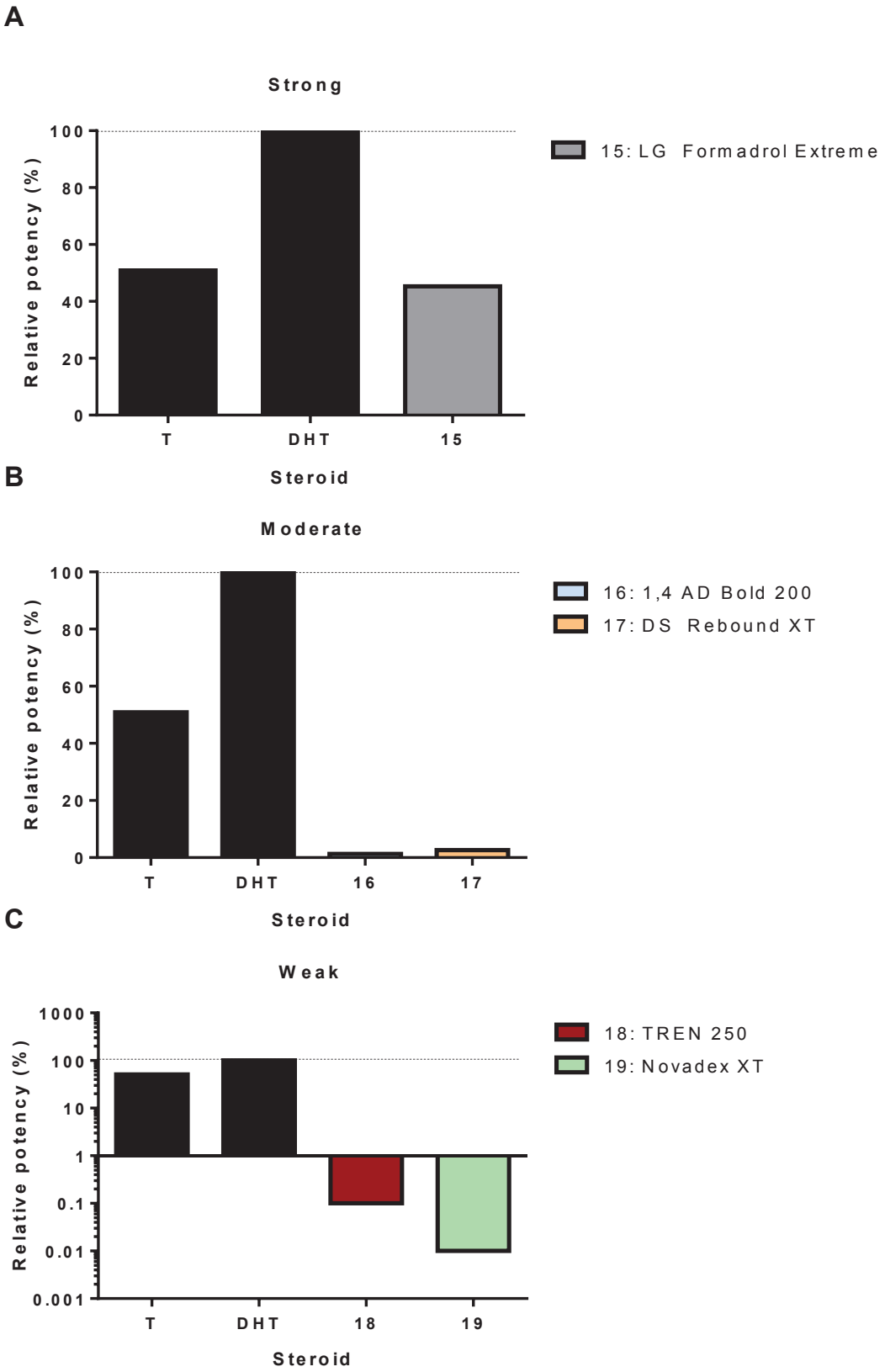

A
Strong
Relative potency (%)
100
80
60
40
20
0
T
DHT
15
Steroid
15: LG Formadrol Extreme
B
Moderate
Relative potency (%)
100
80
60
40
20
0
T
DHT
16
17
Steroid
16: 1,4 AD Bold 200
17: DS Rebound XT
C
Weak
Relative potency (%)
1000
100
10
1
0.1
0.01
0.001
T
DHT
18
19
Steroid
18: TREN 250
19: Novadex XT

Figure 3.8

Figure 3.8 Relative potency of androstenedione-derived steroids. Androstenedione-derived steroids were tested in the yeast androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. The relative potency (RP) of each steroid to DHT was calculated. Steroids with RP above the dotted line were more potent than DHT, while steroids below the dotted line were less potent than DHT. A) Steroids that had potent androgenic bioactivity; B) steroids that had moderate androgenic bioactivity; and C) steroids that had weak androgenic bioactivity.

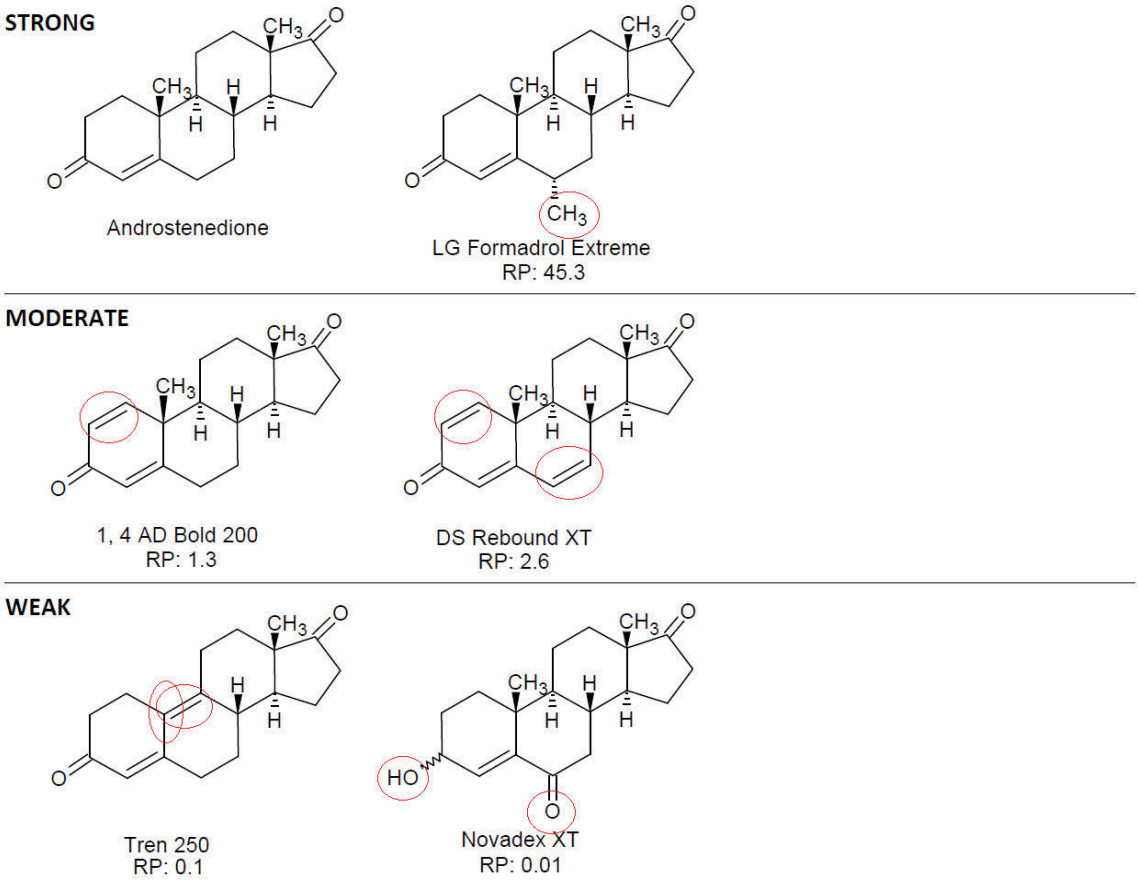


Figure 3.9 Molecular structures of androstenedione-derived steroids. Red circles indicate structural differences to androstenedione. RP indicates relative potency of steroids compared to DHT.

3.3.4 Androgenic bioactivity of remaining steroids

Three steroids that were not classed as DHT-, T-, or androstenedione-derivatives were tested for their androgenic bioactivity in the yeast androgen bioassay (Figure 3.10). Two steroids, which were very similar in structure to each other, displayed moderate and strong AR activation with RP values between 10 and 60%, respectively (Figure 3.11 A and B). The third steroid demonstrated moderate AR activation with an RP value of less than 0.01% (Figure 3.11 B). The molecular structures of these three steroids are shown in Figure 3.12.

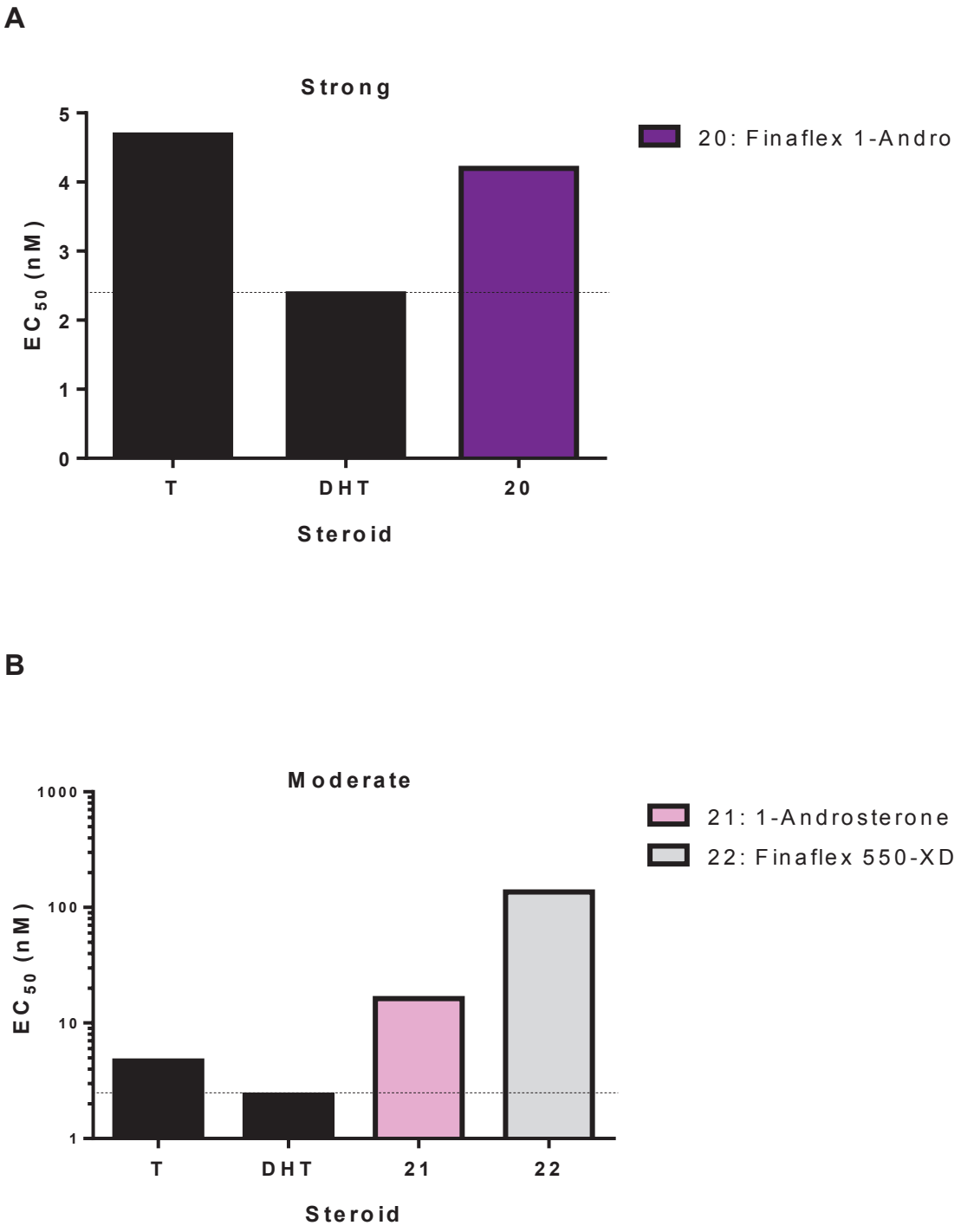

A
Strong
EC50 (nM)
5
4
3
2
1
0
T
DHT
20
Steroid
20: Finaflex 1-Andro
B
Moderate
EC50 (nM)
1000
100
10
1
T
DHT
21
22
Steroid
21: 1-Androsterone
22: Finaflex 550-XD

Figure 3.10

Figure 3.10 EC_{50} values of remaining steroids. Three steroids not classed as DHT-, T-, or androstenedione-derivatives were tested in the yeast androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. Steroids with EC_{50} above the dotted line were less potent than DHT, while steroids with EC_{50} below the dotted line were more potent than DHT. A) Steroids that had potent androgenic bioactivity; and B) steroids that had moderate androgenic bioactivity.

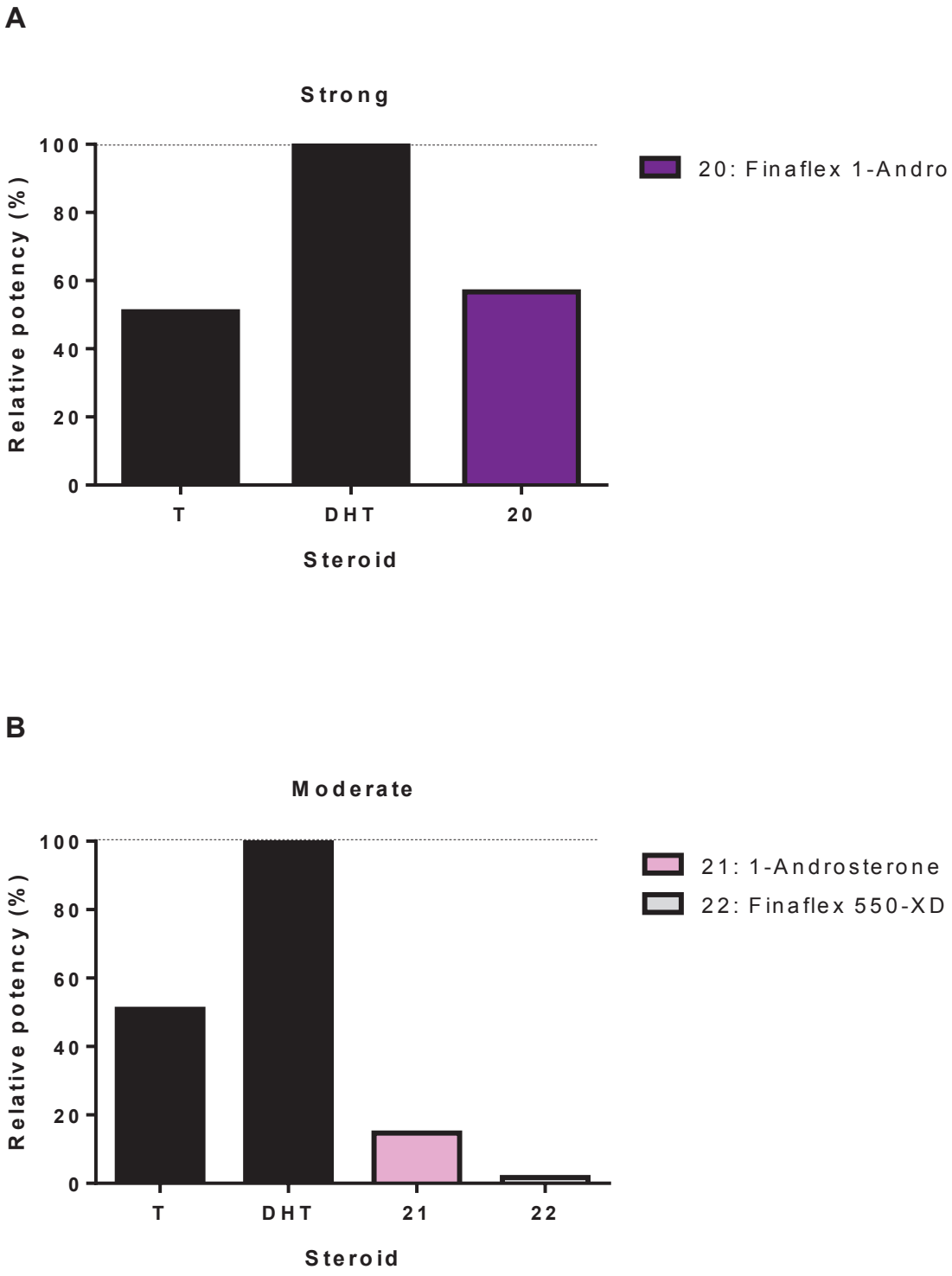

A
Strong
Relative potency (%)
100
80
60
40
20
0
T
DHT
20
Steroid
20: Finaflex 1-Andro
B
Moderate
Relative potency (%)
100
80
60
40
20
0
T
DHT
21
22
Steroid
21: 1-Androsterone
22: Finaflex 550-XD

Figure 3.11

Figure 3.11 Relative potency of remaining steroids. Three steroids not classed as DHT-, T-, or androstenedione-derivatives were tested in the yeast androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. The relative potency (RP) of each steroid to DHT was calculated. Steroids with RP above the dotted line were more potent than DHT, while steroids with RP below the dotted line were less potent than DHT. A) Steroids that had strong androgenic bioactivity; and B) steroids that had moderate androgenic bioactivity.

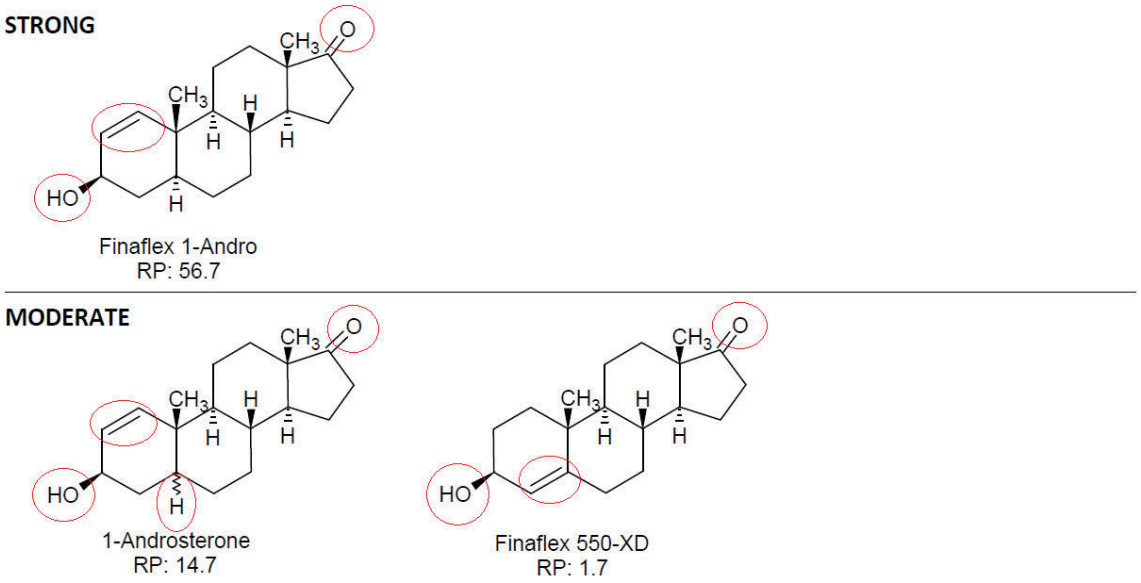


Figure 3.12 Molecular structures of the remaining steroids. Red circles indicate structural differences to DHT. RP indicates relative potency of steroids compared to DHT.

3.4 Discussion

Novel designer steroids are often marketed in sports supplements. Such steroids are sold despite a lack of safety and efficacy data. Indeed, it is often not even known if these designer steroids activate AR and elicit an androgenic response. In this study, 22 designer steroids extracted from sports supplements that were purchased over the internet were tested for androgenic bioactivity. Androgenic bioactivity was determined using an *in vitro* yeast-based androgen bioassay. Of the 22 designer steroids tested, 10 were strong activators of AR, 8 were moderately potent androgens, while 4 were weak androgens.

The most important finding of this study was that approximately 45% (10/22) of the designer steroids were determined to be strong activators of AR. These steroids with strong intrinsic androgenic bioactivity are being sold in sports supplements without clinical safety data. These supplements are available to the general public without proper medical supervision and are real concern due to the potential health risks associated with steroid use (Girgis *et al*, 2014; Wingert *et al*, 2010).

Although there is minimal scientific data showing a beneficial anabolic effect with these compounds, consumers still use androgen-containing sports supplements. This is despite a lack of information regarding the potential health risks associated with their use. It has been well established that certain structural modifications to the steroid can be hepatotoxic. In particular, 17α-methylation has been known to be hepatotoxic (Neri *et al*, 2011). This structural modification is added to improve oral bioavailability, and is present on the majority of the steroids tested in this study. The hepatotoxic effects of these steroids has been highlighted in several case reports involving the moderately potent androgen, Superdrol (methasterone) (Nasr and Ahmad, 2009; Jasiurkowski *et al*, 2006). Two separate clinical

reports have suggested that consumption of the "recommended" dose of a Superdrol product resulted in the development of cholestatic jaundice and IgA nephropathy (Jasiurkowski *et al*, 2006) and severe cholestasis and renal failure (Nasr and Ahmad, 2009). In both cases the males involved had no prior medical history. A more recent report has been linked to the steroid dymethazine (a Superdrol dimer linked by an azine bond) and Ultradrol whereby an otherwise healthy male developed cholestatic jaundice after consuming the "recommended" dosage of the sports supplement containing these two androgens (Agbenyefia *et al*, 2014). In all cases, the patients ingested the contents of one bottle at the recommended dosage and duration, highlighting the danger of regularly taking androgen-containing sports supplements.

Considering the potent androgens that were identified, Ultradrol and The One (Hydrazone) are of particular interest because there has been no prior indication of bioactivity. For Ultradrol, metabolism and excretion studies are reported. These involve a single oral dose ingestion of an Ultradrol capsule in human males. Over a 7 day period, two long term metabolites were measured from collected urine using GC-MS, while the parent compound was rapidly eliminated within 45 hours (Cavalcanti *et al*, 2013). Another study using chimeric mice with humanized livers also involved oral ingestion of a single dose and urine was collected after 48 hours and analysed (Geldof *et al*, 2014). In this study, the previous two long-term metabolites detected by Cavalcanti *et al* could not be detected, but an additional two were detected. The parent compound Ultradrol could not be detected. This may suggest some differences in metabolism between humans and the chimeric humanized liver mouse model. In both cases, the parent compound is rapidly metabolised. The present findings now add to the metabolism data showing that Ultradrol possess potent androgenic activity. For The One

(Hydrazone), there have been no other studies. This steroid is a DHT molecule with an azine substitution at the C3 position and 17α-methylation and is a potent androgen. It was not known how this azine group would affect AR activity, although substitutions at this position on the steroid have been associated with high AR activity (Cawley *et al*, 2015). Interestingly, when the azine group is replaced by an oxime group (for The One (Oxime)), there is a dramatic loss in androgenic activity and is about 100-fold less potent than DHT.

A number of the other DHT-derived steroids were developed originally for clinical use but were never approved. These have emerged as designer steroids in the sports supplement market, and include P-Plex, prostanozol, furazadrol, and 2α,3α-epithio. P-Plex and prostanozol are listed on the prohibited substances list (WADA, The 2016 prohibited list). This is not the first indication that these steroids have intrinsic androgenic potential. Miyake *et al* describe epitiostanol (the non 17-methylated parent compound of epithio) to have modest androgenic-myotrophic activities when administered subcutaneously in rodents (Miyake *et al*, 1974). Unfortunately, these early reports could not be accessed and so the details of the androgenic and myotrophic activities cannot be described here. A later study with Epithio demonstrates a potent anti-oestrogenic effect, by causing a reduction of rat mammary cancer cells in a hormone-dependent model (Watanabe *et al*, 1995). It is possible that the "modest androgenic-myotrophic" activity of epithio is due to aromatase inhibition causing a subsequent increase in serum T levels, but this is yet to be fully elucidated. The results of this study may, at least in part, suggest that the potent AR activation of 2α,3α-epithio may play a role in potentiating an anabolic effect. In stark contrast, however, the β-isomer of epithio was a weak androgen, indicating little AR activation in the β-configuration.

More recently, direct anabolic effects have been demonstrated for prostanozol and P-Plex. Subcutaneous injections of P-Plex were shown to restore muscle mass of levator ani muscle to normal levels in orchidectomised rats in a Hershberger assay. The authors also demonstrated strong AR binding and activity using a receptor binding assay and yeast androgen bioassay, respectively (Diel *et al*, 2007). Similar findings were reported for prostanozol by the US Drug Enforcement Administration, whereby prostanozol demonstrated high binding affinity and AR receptor activity in a receptor binding assay and a mammalian cell-based androgen bioassay, respectively (DEA Federal Register 44456). The US Drug Enforcement Administration then describe that prostanozol "exhibited considerably less androgenic/anabolic activity than testosterone" in levator ani muscle in a castrated rat assay, suggesting the intrinsically potent prostanozol may produce weak androgenic metabolites *in vivo* when injected subcutaneously. In the next chapter, metabolism of prostanozol is explored further.

Several of the potent androgens in this study were derived from testosterone. This includes Jungle Warfare and Mechabol. Jungle Warfare is a 17α-methylated testosterone analogue with a 6-7 carbon double bond that was detected in a dietary supplement in 2011 (Parr *et al*, 2011a). These authors describe the metabolism of Jungle Warfare in a male human urine excretion study where one capsule was ingested and urine was collected for up to 11 days. There was approximately 6% recovery of the parent compound in the urine samples by the end of the 11 days, indicating that Jungle Warfare is heavily metabolised *in vivo*. Here, it is shown for the first time that this steroid is an intrinsically potent androgen. The metabolism of Jungle Warfare will be explored further in chapter 4. Mechabol (methylclostebol) is a designer steroid that is a 17α-alkylated version of clostebol. Several studies from the 1950s

report the anabolic action of clostebol in humans via a measurement of nitrogen retention, as reviewed by Camerino and Sciaky (Camerino and Sciaky, 1975), but there are no studies of Mechabol that report an anabolic effect. Methylclostebol is not a new steroid, as it was reported to be used by East German athletes during the late 60s and early 70s (Franke and Berendonk, 1997). Typically, 4-chlorinated androgens like methylclostebol have been associated with high anabolic activity (Fragkaki *et al*, 2009; Camerino and Sciaky, 1975). Given that clostebol has proven anabolic activity, it is likely that Mechabol would also demonstrate strong AR bioactivity. The present finding for the yeast AR bioassay represents the first report of Mechabol as a potent intrinsic androgen.

While the majority of testosterone-derivatives tested in this study demonstrated potent AR activity, Testabol did not. Testabol is an esterified testosterone analogue with an acetate group attached to the C17 position, and a hydroxyl group on the C4 position. It is known that the C17 position is crucial for AR-ligand binding (Mindnich *et al*, 2004; Vihko *et al*, 2001), and it may be that the bulky acetate group is sterically hindering binding of the steroid is to the AR. It is therefore in keeping that Testabol was found to be a weak intrinsic androgen in the yeast AR bioassay as yeast cells do not express the necessary enzymes to cleave off the acetate group.

Five of the androgens tested in this study were derived from androstenedione, a proandrogen. Our laboratory group has previously shown that androstenedione has an RP of approximately 4.2% (relative to DHT) (Akram *et al*, 2011). The 5 androstenedione derivatives tested in this study were therefore not expected to be highly androgenic because yeast cells do not express the enzymes required to convert the proandrogens into potent androgenic metabolites. Surprisingly, LG Formadrol Extreme did demonstrate strong AR bioactivity. The

structure of the steroid in the LG Formadrol Extreme supplement differs from androstenedione by only an α-methyl group at the C6 position, indicating that this change structurally favours AR activation. LG Formadrol Extreme was originally reported in 1940. Although for the purposes of this discussion the original article could not be obtained, Campbell *et al* references the original paper stating that the androgenic activity was "about the same as that of the parent compound [androstenedione]" (Campbell *et al*, 1958). The interpretation of this statement is unclear but the new results with the yeast AR bioassay suggest that in contrast to this early result, androgenic activity of LG Formadrol Extreme is greater than that of the parent compound.

Three steroids that were not classed as derivatives of the endogenous androgens, DHT, T, or androstenedione were also tested in this study. Two of these androgens, 1-Androsterone and Finaflex 1-Andro are moderate and potent activators of AR, respectively. They are of similar structure and differ by the stereochemistry of the hydrogen at the C5 position. Finaflex 1-Andro has a defined 5α-hydrogen, while 1-Androsterone does not (Table 3.1). Therefore, 1-Androsterone may contain a mixture of both α- and β-isomers, and may explain the difference in AR bioactivity between these two steroids. Interestingly, a study by Parr *et al* showed that the supplement 1-Androsterone contained only the 5α-isomer using NMR and GC-electrospray ionisation-mass spectrometry data (Parr *et al*, 2011b). This suggests that perhaps the 1-Androsterone supplement tested in this study is from a different batch and may contain β-isomer impurities. Further work needs to be done in order to determine stereochemistry of the C5 hydrogen of the steroid in 1-Androsterone tested in this study.

A recent study by Granados *et al* has shown that the 5α-isomer has anabolic and ergogenic properties in trained males by increasing lean body mass and increasing back squat one

repetition maximum exercise performance in young males, supporting the finding of high androgenic bioactivity in this study (Granados *et al*, 2014). Further blood analysis also revealed markers of cardiovascular, liver, and kidney dysfunction, such as: increased LDL and lowered HDL levels; increased serum creatinine and aspartate transaminase and decreased serum albumin and alkaline phosphatase, indicating stress on the liver; as well as decreased glomerular filtration rate of the kidneys (Granados *et al*, 2014). This study was conducted over a 1 month period and the prohormone supplement was taken as directed by the manufacturer, further highlighting the health risks of androgen-containing supplements. The third steroid, Finaflex 550-XD, is not reduced at the C5 position and has a C4-C5 carbon double bond, as well as not having a C1-C2 double bond like 1-Androsterone and Finaflex 1-Andro. Finaflex 550-XD is roughly 100-fold less potent than DHT.

Several steroids in this study had very weak androgenic activity and therefore represent steroids that do not have potent intrinsic androgenic bioactivity. However, if steroid metabolism will increase the androgenic bioactivity by producing androgenic metabolites remains unknown. Metabolism is a key issue when considering all of these results. Metabolism effects could alter *in vivo* androgenic potency by increase or decreasing androgenic effects subsequent to metabolite formation. While the yeast androgen bioassay is suitable for screening steroids for intrinsic androgenic potential, it alone may not be sufficient to detect all designer steroids as it is possible that proandrogens will not be detected by the yeast androgen bioassay. Therefore a more thorough screening process needs to incorporate a mammalian cell-based counter-part in parallel so androgenic bioactivity can be assessed both with and without steroid metabolism.

3.5 Summary and Conclusions

This study shows for the first time that several steroids sold on the sports supplement market are potent androgens. The yeast androgen bioassay provided a suitable first-pass screen for determining the androgenic potency of these steroids. The lack of endogenous steroid receptors and steroid metabolising enzymes in yeast allowed for the intrinsic bioactivity of each steroid to be determined. Of the 22 tested, 10 were potent androgens and potentially represent a safety risk. Moreover, they represent a sports doping threat.

Chapter 4

Evaluation of the Androgenic Bioactivity of Sports Supplement-Derived Designer Steroids Using the HuH7 Androgen Bioassay

Table of Contents

4.1 Introduction

The yeast androgen bioassay has identified steroids that were derived from sports supplements which have intrinsic bioactivity. However, the yeast androgen bioassay provided no insight into possible *in vivo* biological activity of the steroids as yeast cells have no steroid metabolism capacity. It is possible that subjected to metabolism the steroids may be converted to more potent androgens. Alternatively, they may be deactivated. This chapter explores metabolic activation and deactivation of the sports supplement steroids.

Hepatic metabolism is very important for final biological activity of androgens and proandrogens (Ferriz and Vinsova, 2010). Proandrogens require enzymatic conversion before becoming biologically active, while androgens may be further activated or deactivated (Liederer and Borchardt, 2005). Dehydroepiandrosterone, androstenedione and androstenediol are naturally occurring endogenous proandrogens that are commonly sold as sports supplements (Brown *et al*, 2006). These 'supplements' are sold on the premise that they will increase endogenous testosterone levels via enzymatic conversion. Many new designer steroids are marketed in sports supplements as proandrogens. However, the biological activities of these steroids are often not known and thus, it is not known if these designer steroids are proandrogens.

In vitro reporter gene bioassays based in some mammalian cells provide a basic model of metabolism (Houtman *et al*, 2009; Roy *et al*, 2006). While androgen metabolising enzymes are expressed in most cells, hepatic cells are primarily responsible for steroid metabolism (Lootens *et al*, 2009). Our laboratory group has demonstrated that an *in vitro* androgen bioassay using a human liver cell line (human hepatocarcinoma, HuH7) was suitable for determining the potential metabolic activation or deactivation of a range of sports

supplement-derived steroids (Akram *et al*, 2011). We showed that HuH7 cells highly express known androgen metabolising enzymes, including hydroxysteroid dehydrogenases, reductases, and aromatase enzymes (Table 4.1) (Akram *et al*, 2011).

Unfortunately, the metabolic capacity of the HuH7-based androgen bioassay is a double-edged sword. Steroidogenic enzymes may deactivate androgens that are intrinsically potent, making their detection difficult if only this assay was performed. Therefore, the use of the HuH7 androgen bioassay serves to compliment the yeast androgen bioassay. The yeast bioassay measures the intrinsic androgenic bioactivity whilst the HuH7 bioassay measures potential potency after metabolism. The aim of this study was to test the 22 sports supplement-derived steroids for metabolic activation or inactivation using the HuH7 androgen bioassay, thus, determining the potential *in vivo* potency for each steroid. It was hypothesized that a proportion of the sports supplement-derived steroids would be proandrogens.

Table 4.1. Steroid metabolising enzymes expressed by HuH7 cells.

Enzyme	Function	References
3α-HSD 1	DHT to 3α-androstanediol	Penning *et al*, 1996; Penning *et al*, 2004
	Androstenedione to T	Penning *et al*, 2003
	Oestrone to 17β-oestradiol	Penning *et al*, 2003
3α-HSD 2	Androstenedione to T	Penning *et al*, 2003
	DHT to 3α-Androstanediol	Penning *et al*, 1996; Penning *et al*, 2004
	Oestrone to 17β-oestradiol	Penning *et al*, 2004
3α-HSD 3	DHT to 3α-androstanediol	Penning *et al*, 2004
	3α-androstanediol to DHT	Penning *et al*, 2004
	Androstenedione to T	Penning *et al*, 2004
	Oestrone to 17β-oestradiol	Penning *et al*, 2004
3β-HSD 1	DHEA to Androstenedione	Thomas *et al*, 2004
	DHT to 3β-Androstanediol	Mason *et al*, 1997
	5-Androstenediol to T	Mason *et al*, 1997
3β-HSD 2	DHEA to Androstenedione	Mizrachi and Auchus, 2009
	DHT to 3β-Androstanediol	Mason *et al*, 1997
	5-Androstenediol to T	Mason *et al*, 1997
17β-HSD 1	Oestrone to 17β-oestradiol	Mizrachi and Auchus, 2009
17β-HSD 2	T to Androstenedione	Mizrachi and Auchus, 2009
	17β-oestradiol to E1	Mizrachi and Auchus, 2009
	5-androstenediol to DHEA	Mindnich *et al*, 2004
	DHT to 5α-androstanedione	Mizrachi and Auchus, 2009
17β-HSD 3	Androstenedione to T	Labrie *et al*, 2000
17β-HSD 4	17β-oestradiol to oestrone	Mindnich *et al*, 2004
	5-Androstenediol to DHEA	Mindnich *et al*, 2004
17β-HSD 6	3α-Androstanediol to androsterone	Labrie *et al*, 2000
	DHT to Androstanedione	Labrie *et al*, 2000
	T to Androstenedione	Labrie *et al*, 2000
17β-HSD 7	Oestrone to 17β-oestradiol	Mindnich *et al*, 2004
17β-HSD 8	17β-oestradiol to oestrone	Mindnich *et al*, 2004
	T to Androstenedione	Mindnich *et al*, 2004
Aromatase	T to 17β-oestradiol	Stocco, 2012
	Androstenedione to oestrone	Stocco, 2012
5α-Reductase 1	T to DHT	Kazmierczak *et al*, 2006
5α-Reductase 2	T to DHT	Kazmierczak *et al*, 2006

4.2 Methods

4.2.1 Mammalian Cell Culture

Human hepatocarcinoma (HuH7) cells stably co-transfected with both the human AR expression plasmid and the enhancer/ARE/SEAP reporter plasmid (Akram *et al*, 2011) were cultured in DMEM (Gibco, Life Technologies) containing high glucose (4500 mg/L), L-glutamine (584 mg/L), sodium pyruvate (110 mg/L), 10% FCS and puromycin dihydrochloride (5.5 µg/mL). Once confluent, cells were seeded at a concentration of $1x10^5$ cells/mL in 96-well plates with phenol-red free DMEM supplemented with 10% charcoal-stripped FCS, as described (Methods section 2.3.3).

4.2.2 HuH7 Androgen Bioassay

After overnight culture of cells in phenol-red free DMEM, cells were treated with steroids in triplicate over a concentration range of 2.3^{-3} M to 1.3^{-10} M (in a final volume of 2 µL steroid/well) for 24 hours. After 24 hours, cell culture supernatant was assayed for SEAP activity using SEAP substrate reagent (Clontech), as described (Methods section 2.3.5.1) across 6 independent experiments.

4.2.3 Determination of Steroid EC_{50} Values, Relative Potencies, and Activation Factors

A DHT standard dose-response curve was included in triplicate with every AR bioassay. The EC_{50} values were determined from a sigmoidal curve fit using GraphPad Prism 6 software. The EC_{50} values for each test steroid were used to determine the relative potency (RP) of the test

steroid with respect to DHT. AR bioactivity of steroid relative to DHT was determined using the equation:

$$\frac{EC50\ [Dihydrotesterone]}{EC50\ [Test\ Steroid]}\ x\ 100\%$$

To determine the activation of the test steroids, the following equation was used:

$$Activation\ Factor = \frac{EC50\ steroid\ [Yeast]}{EC50\ steroid\ [HuH7]}$$

If the activation factor was <1, then the inverse of this equation was used to calculate the deactivation factor.

4.3 Results

A total of 22 steroids that were derived from sports supplements were evaluated for their AR bioactivity in the HuH7 androgen bioassay. Sigmoidal dose-response curves were generated for each steroid to determine the EC_{50} values. The EC_{50} values were then used to calculate the relative potency (RP) to DHT. Activation (or deactivation) factors were calculated to determine if HuH7-mediated metabolism increased or decreased the RP of the steroid.

4.3.1 Androgenic Bioactivity of DHT-Derived Steroids

The endogenous androgen DHT was used as a reference for potent AR activation. The calculated EC_{50} value was 7.51 nM. Out of the 22 steroids tested in the HuH7 androgen

bioassay, ten were DHT-derivatives and the EC_{50} and RP values were calculated for each steroid (Figure 4.1). There were four steroids with strong RPs ranging from 62 to 393.1% (Figure 4.2 A). Five steroids were moderately strong with RP values ranging from 1.6 to 22.3% (Figure 4.2 B). One steroid was a weak androgen with an RP more than 1000-fold lower than DHT (Figure 4.2 C).

Of the ten DHT-derivatives, six were intrinsically strong androgens. Three of these androgens were activated further in the HuH7 androgen bioassay with activation factors ranging from 1.2 to 3.4 (Figure 4.3). One intrinsically strong androgen was deactivated but still remained a strong androgen. The other two intrinsically strong androgens were deactivated into moderately strong androgens (Figure 4.3).

There were three androgens that were intrinsically moderately strong. Two of these androgens were deactivated but remained moderately strong, while the third was deactivated into a weak androgen. The remaining steroid was intrinsically a weak androgen that was activated into a moderately strong androgen in the HuH7 androgen bioassay (Figure 4.3).

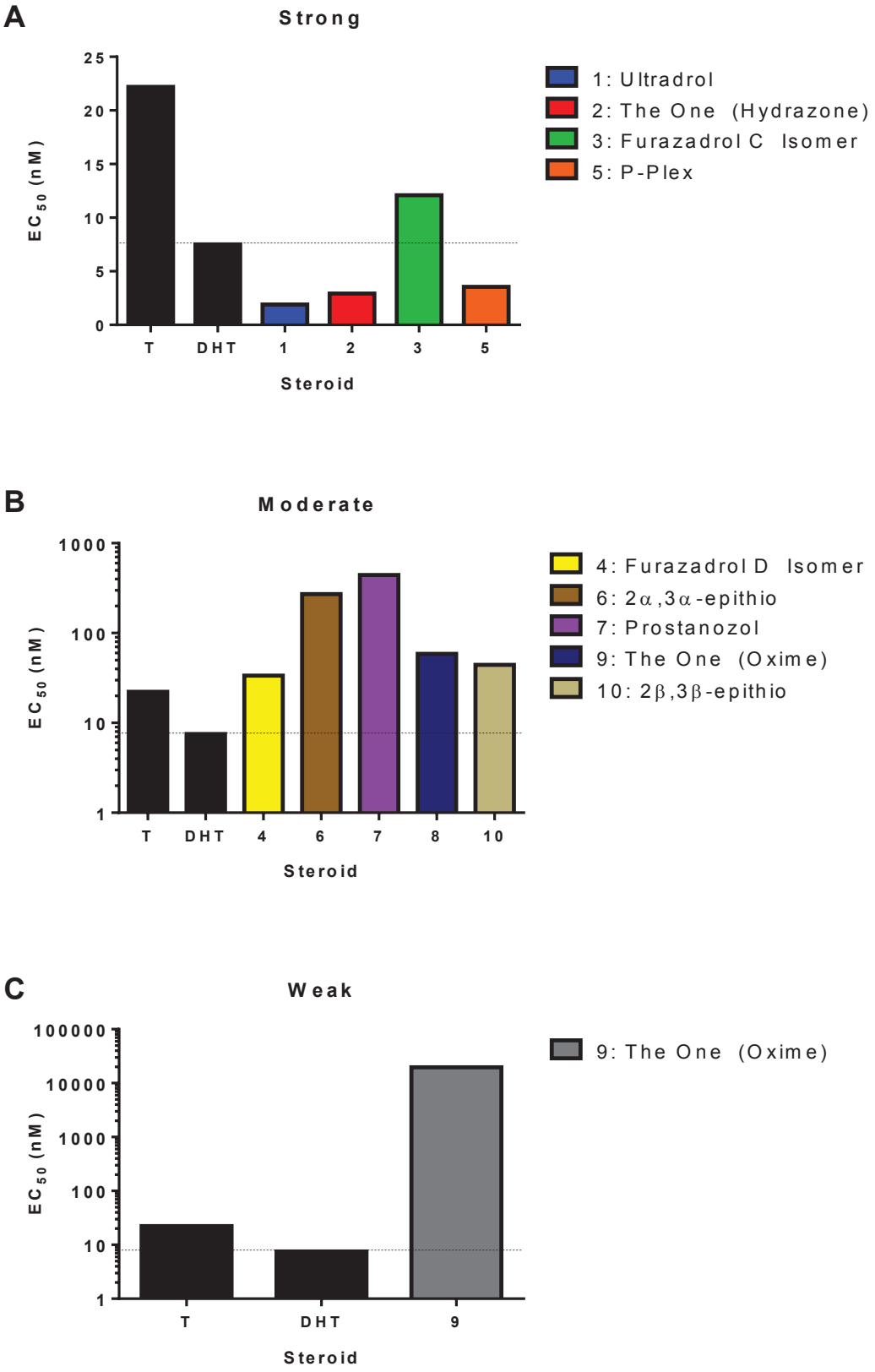

A
Strong
25
20
15
10
5
0
EC50 (nM)
T
DHT
1
2
3
5
Steroid
1: Ultradrol
2: The One (Hydrazone)
3: Furazadrol C Isomer
5: P-Plex
B
Moderate
1000
100
10
1
EC50 (nM)
T
DHT
4
6
7
8
10
Steroid
4: Furazadrol D Isomer
6: 2α,3α-epithio
7: Prostanozol
9: The One (Oxime)
10: 2β,3β-epithio
C
Weak
100000
10000
1000
100
10
1
EC50 (nM)
T
DHT
9
Steroid
9: The One (Oxime)

Figure 4.1

Figure 4.1 EC_{50} values of DHT-derived steroids. DHT-derivative steroids were tested in the HuH7 androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. Steroids with EC_{50} values above the dotted line were less potent than DHT, while steroids with EC_{50} values below the dotted line were more potent than DHT. A) Steroids that had strong AR bioactivity; B) steroids that had moderate AR bioactivity; and C) steroids that had weak AR bioactivity.

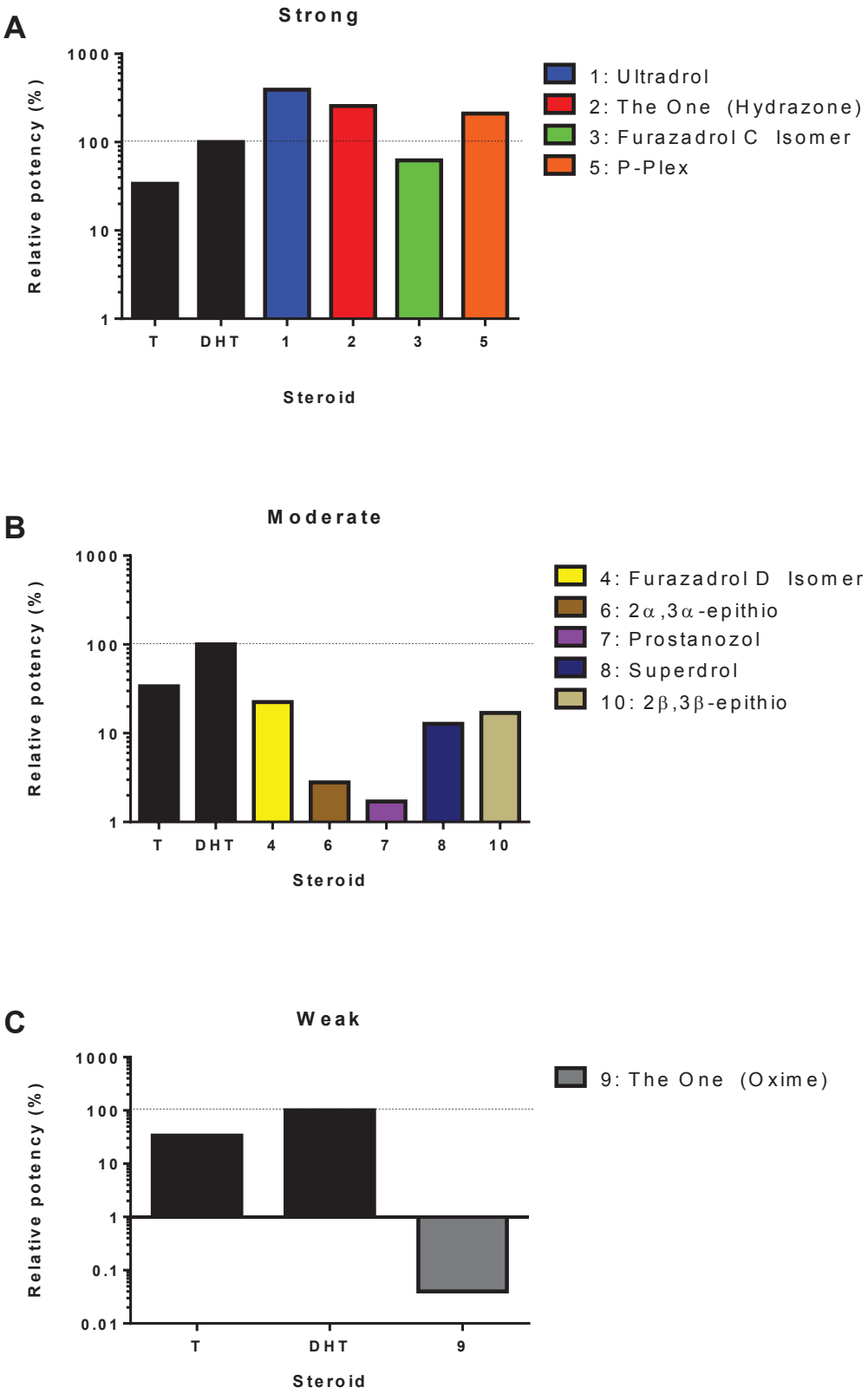

A
Strong
Relative potency (%)
1000
100
10
1
T
DHT
1
2
3
5
Steroid
1: Ultradrol
2: The One (Hydrazone)
3: Furazadrol C Isomer
5: P-Plex
B
Moderate
Relative potency (%)
1000
100
10
1
T
DHT
4
6
7
8
10
Steroid
4: Furazadrol D Isomer
6: 2α,3α-epithio
7: Prostanozol
8: Superdrol
10: 2β,3β-epithio
C
Weak
Relative potency (%)
1000
100
10
1
0.1
0.01
T
DHT
9
Steroid
9: The One (Oxime)

Figure 4.2

Figure 4.2 Relative potency of DHT-derived steroids. DHT-derived steroids were tested in the HuH7 androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. The relative potency (RP) of each steroid to DHT was calculated. Steroids with RP values above the dotted line were more potent than DHT, while steroids with RP below the dotted line were less potent than DHT. A) Steroids that had strong AR bioactivity; B) steroids that had moderate AR bioactivity; and C) steroids that had weak AR bioactivity.

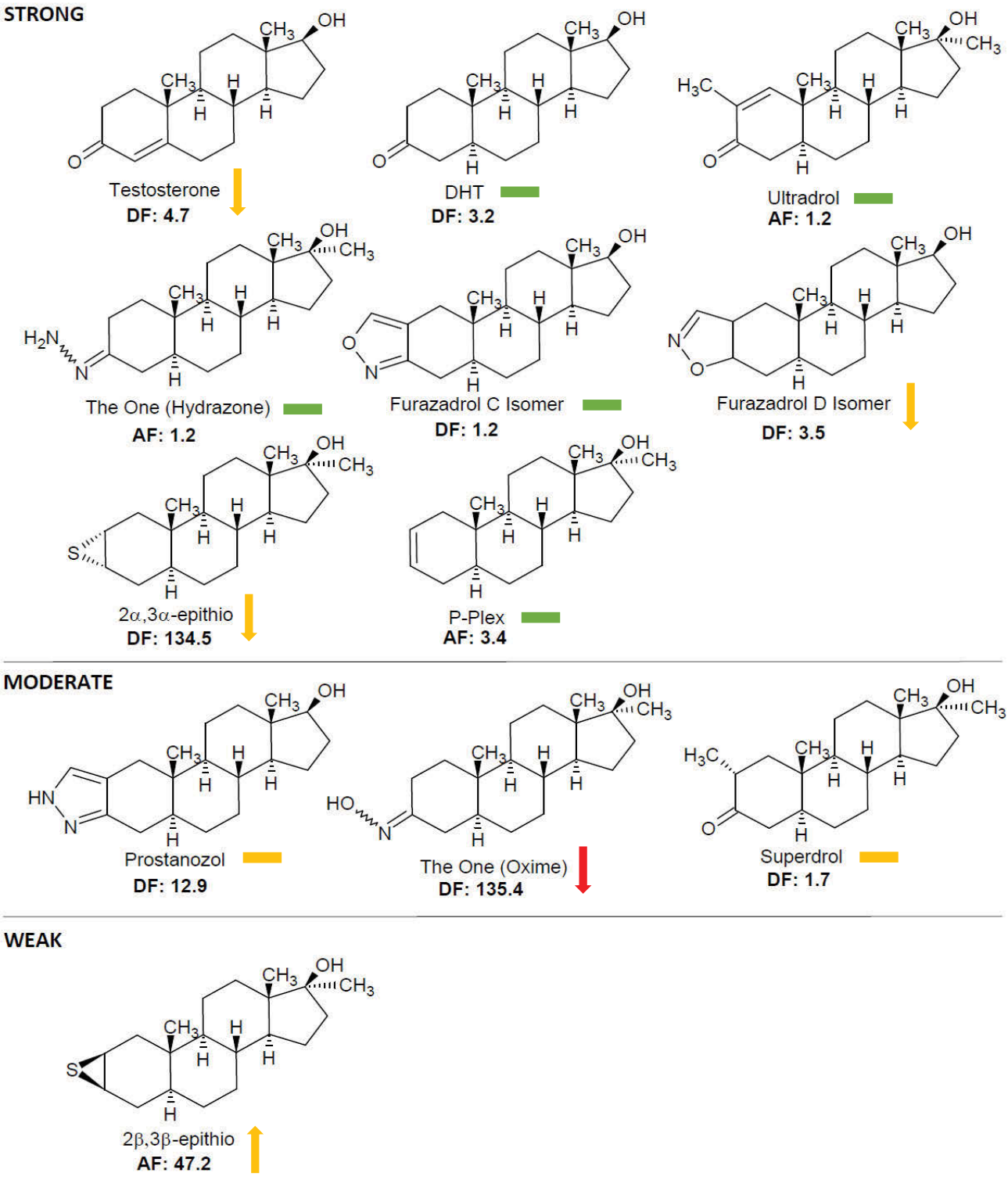

STRONG
Testosterone
DF: 4.7
DHT
DF: 3.2
Ultradrol
AF: 1.2
The One (Hydrazone)
AF: 1.2
Furazadrol C Isomer
DF: 1.2
Furazadrol D Isomer
DF: 3.5
2α,3α-epithio
DF: 134.5
P-Plex
AF: 3.4
MODERATE
Prostanozol
DF: 12.9
The One (Oxime)
DF: 135.4
Superdrol
DF: 1.7
WEAK
2β,3β-epithio
AF: 47.2

Figure 4.3

Figure 4.3 Metabolism in the HuH7 androgen bioassay altered the bioactivity of DHT-derived steroids. This figure shows the activation factor (AF) or deactivation factor (DF) of each steroid that was classed intrinsically as either strong, moderate, or weak. A downward arrow indicates that a steroid was deactivated into a weaker androgen, and an upward arrow indicates that a steroid was activated into a stronger androgen. A dash indicates the steroid remained in the same group. The colour of the symbol indicates which group the steroid belongs to as a result of being activated or weakened: Green = strong; Yellow = moderate; and red = weak.

4.3.2 Androgenic Bioactivity of T-Derived Steroids

Three of the T-derived steroids were strong activators of AR (Figure 4.4). These three steroids had RP values ranging from 253.7 to 1028.8% (Figure 4.5 A). One steroid was intrinsically moderately strong that was activated into a strong androgen, while the other two were intrinsically strong androgens that were activated further (Figure 4.6). A fourth steroid was a weak activator of AR with an RP of 0.01% (Figure 4.5 B). This steroid was intrinsically weak and was deactivated further in the HuH7 androgen bioassay (Figure 4.6).

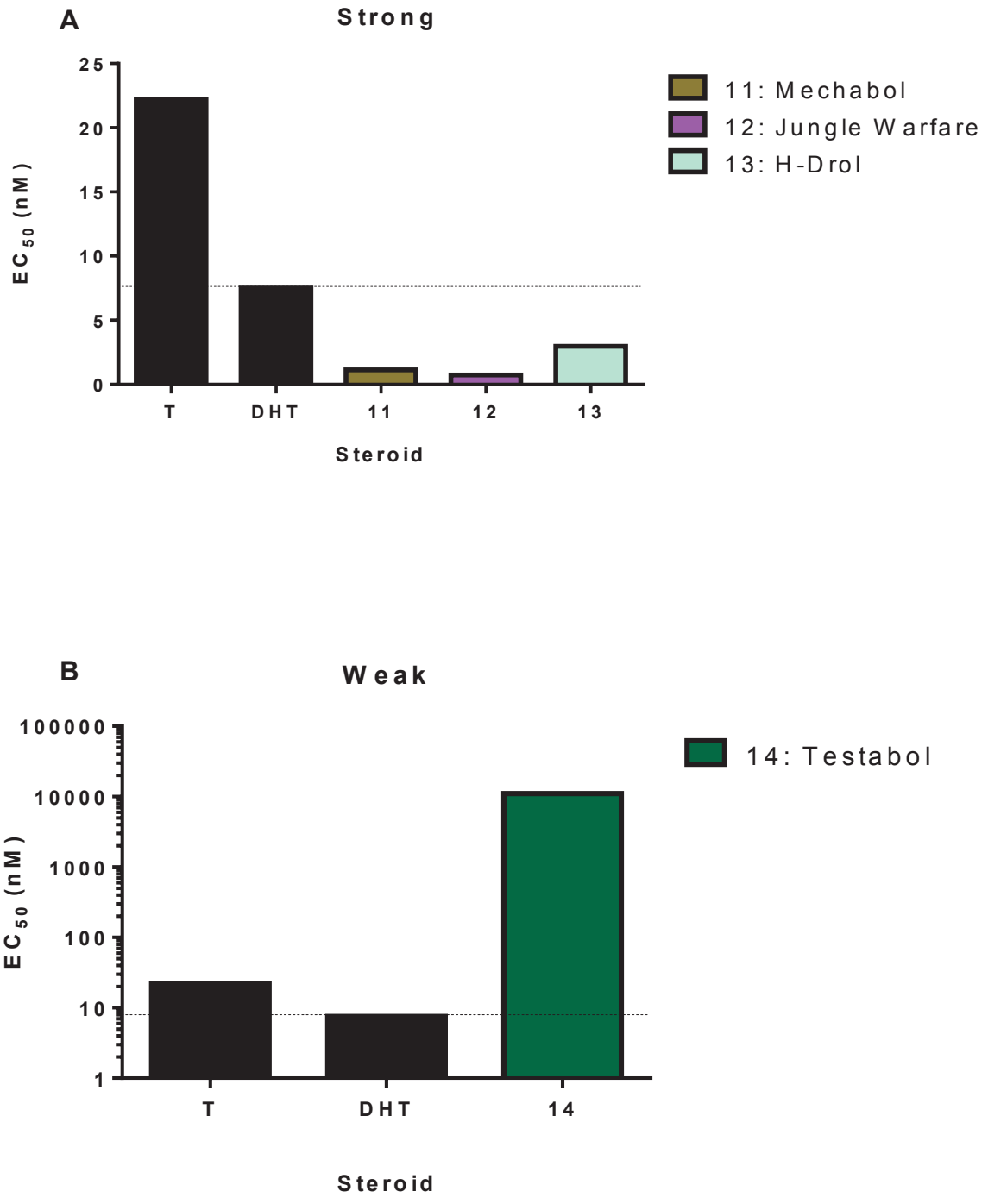

A
Strong
25
20
15
10
5
0
EC50 (nM)
T
DHT
11
12
13
Steroid
11: Mechabol
12: Jungle Warfare
13: H-Drol
B
Weak
100000
10000
1000
100
10
1
EC50 (nM)
T
DHT
14
Steroid
14: Testabol

Figure 4.4

Figure 4.4 EC_{50} values of T-derived steroids. T-derived steroids were tested in the HuH7 androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. Steroids with EC_{50} values above the dotted line were less potent than DHT, while steroids with EC_{50} values below the dotted line were more potent than DHT. A) Steroids that had strong AR bioactivity; and B) steroids that had weak AR bioactivity.

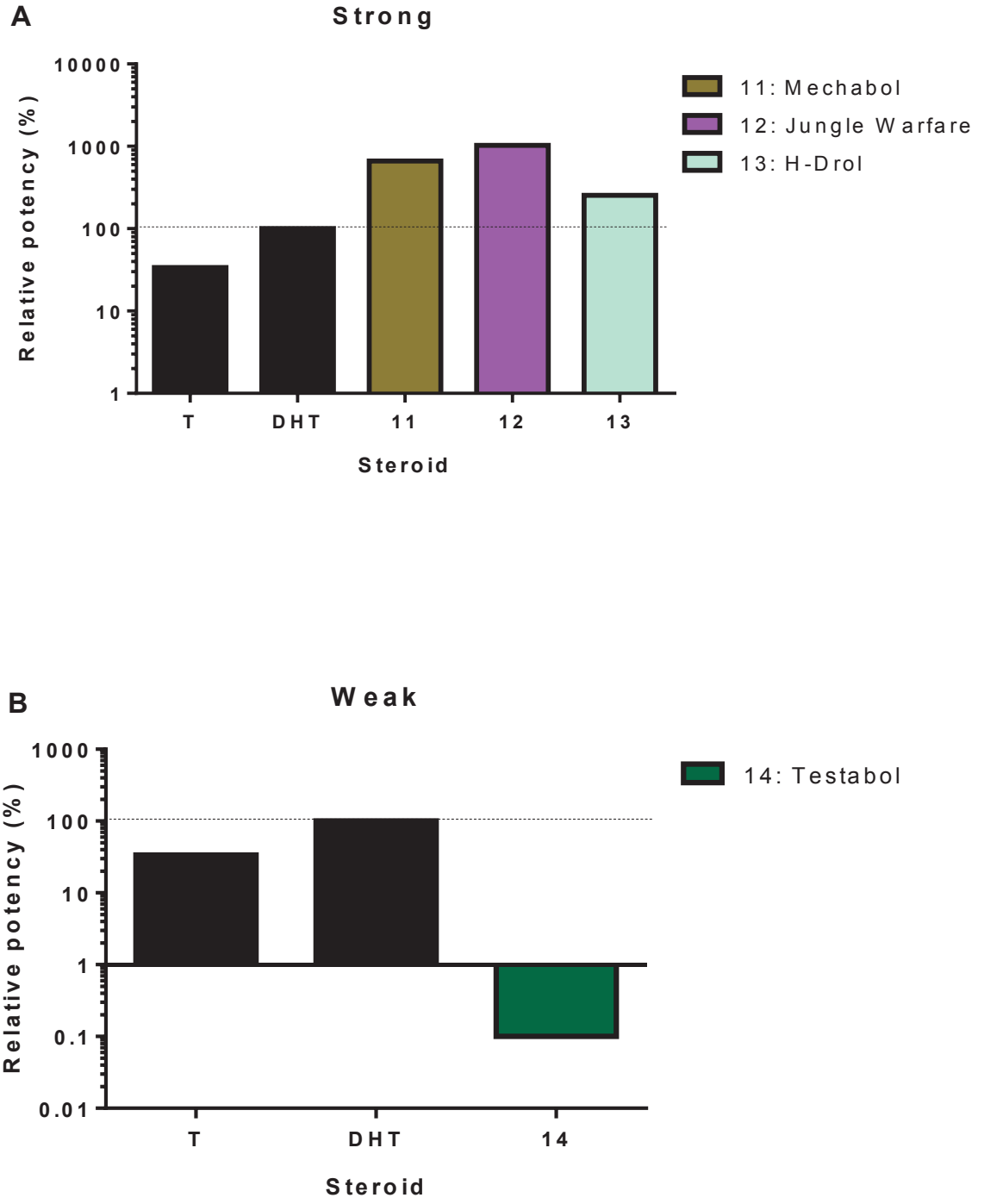

A
Strong
Relative potency (%)
10000
1000
100
10
1
T
DHT
11
12
13
Steroid
11: Mechabol
12: Jungle Warfare
13: H-Drol
B
Weak
Relative potency (%)
1000
100
10
1
0.1
0.01
T
DHT
14
Steroid
14: Testabol

Figure 4.5

Figure 4.5 Relative potency of T-derived steroids. T-derived steroids were tested in the HuH7 androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. The relative potency (RP) of each steroid to DHT was calculated. Steroids with RP above the dotted line were more potent than DHT, while steroids with RP below the dotted line were less potent. A) Steroids that had strong AR bioactivity; and B) steroids that had weak AR bioactivity.

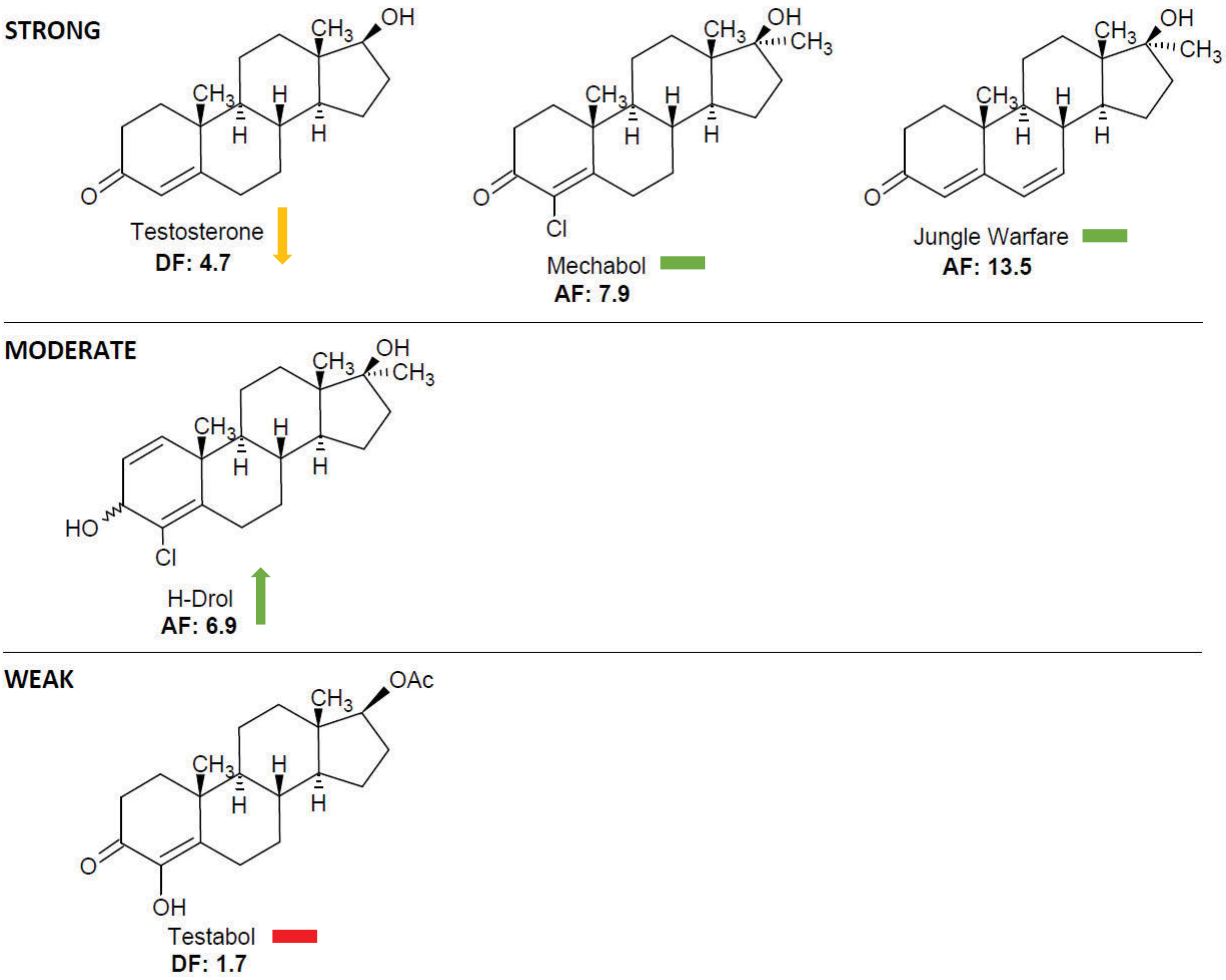


Figure 4.6 Metabolism in the HuH7 androgen bioassay altered the bioactivity of T-derived steroids. This figure shows the activation factor (AF) or deactivation factor (DF) of each steroid that was classed intrinsically as either strong, moderate, or weak. A downward arrow indicates that a steroid was deactivated into a weaker androgen, and an upward arrow indicates that a steroid was activated into a stronger androgen. A dash indicates the steroid remained in the same group. The colour of the symbol indicates which group the steroid belongs to as a result of being activated or deactivated: Green = strong; Yellow = moderate; and red = weak.

4.3.3 Androgenic Bioactivity of Androstenedione-Derived Steroids

Five of the steroids were derivatives of the proandrogen, androstenedione (Figure 4.7). Of the five androstenedione derivatives, two were moderately strong AR activators, with RP values ranging from 8.4 to 22.2% (Figure 4.8 A). The other three were weak activators of AR (Figure 4.8 B). One steroid was inactive in the HuH7 androgen bioassay and so an RP value could not be calculated, while the other two had RP values ranging from 0.1 to 0.5%.

One of the moderately strong steroids was intrinsically moderately potent that was activated further, while the other was intrinsically a weak androgen that was activated by over 33-fold (Figure 4.9). Of the three weak androgens in the HuH7 bioassay, one was intrinsically a strong androgen that was deactivated by over 1371-fold, while the other was intrinsically moderately strong that was deactivated by approximately 16-fold (Figure 4.9). The steroid which did not have any activity in the HuH7 bioassay was intrinsically a weak androgen.

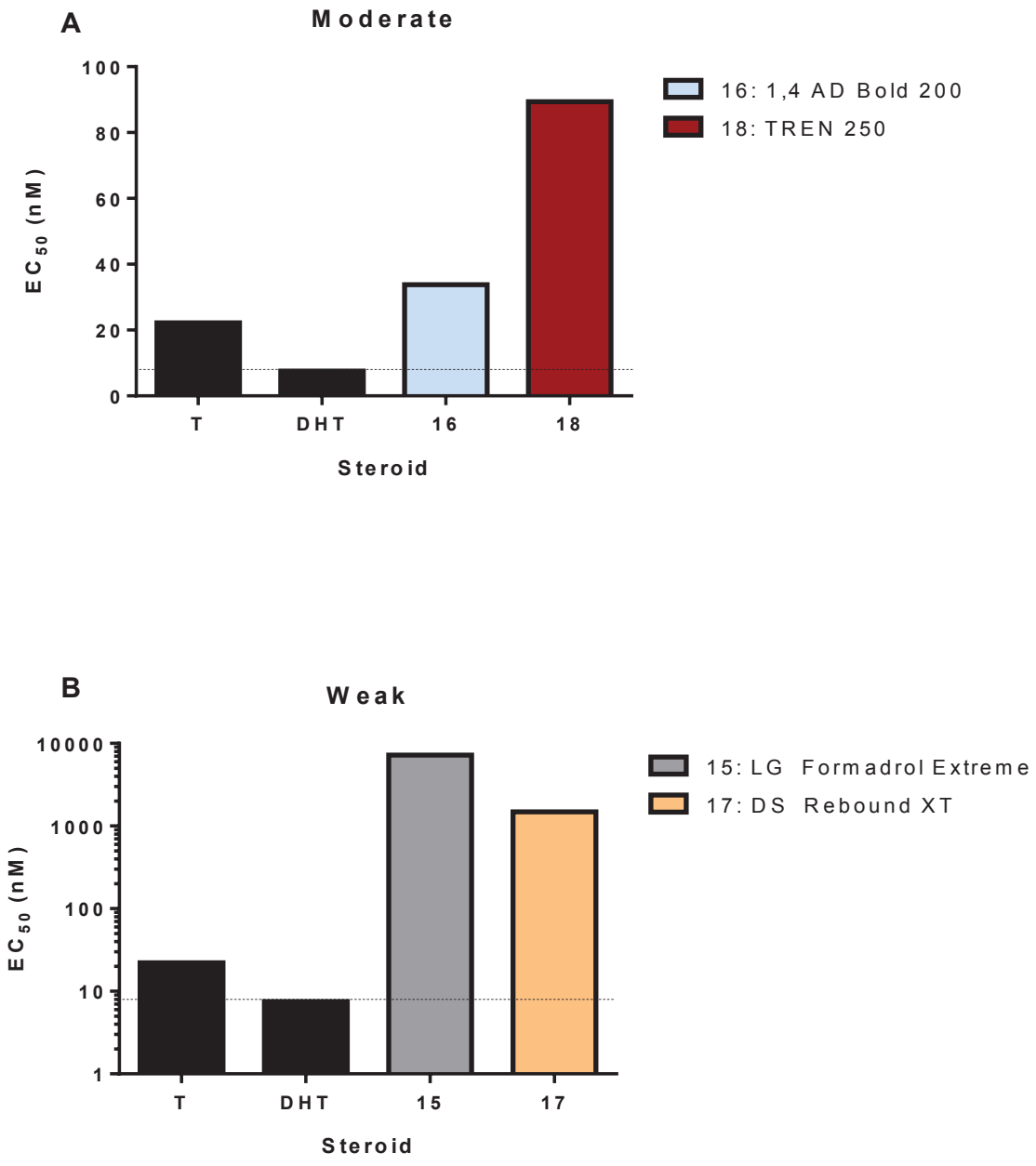
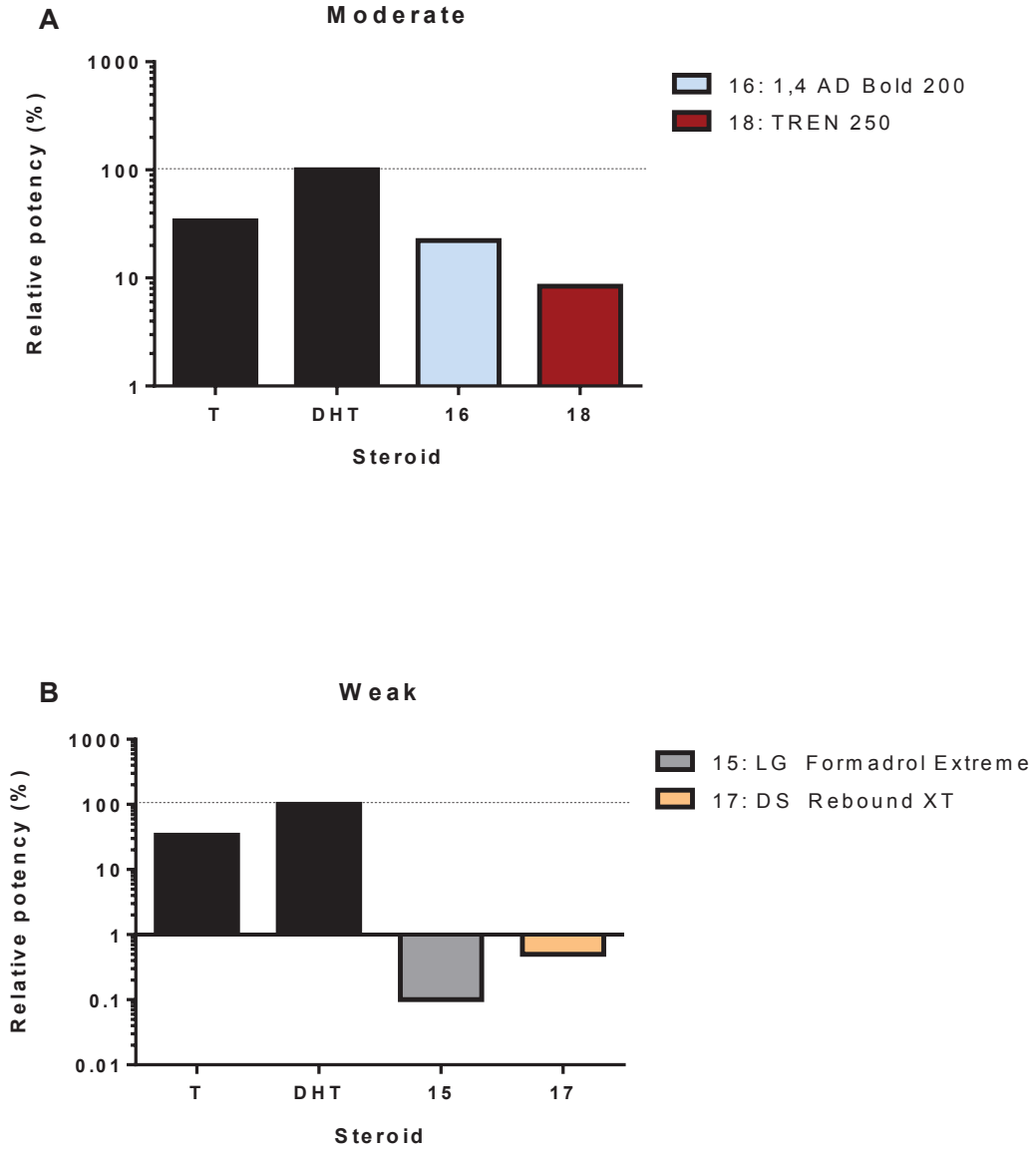


Figure 4.7 EC_{50} values of androstenedione-derived steroids. Androstenedione-derived steroids were tested in the HuH7 androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. Steroids above the dotted line were less potent than DHT while steroids below the dotted line were more potent than DHT. A) Steroids that had moderate AR bioactivity; and B) steroids that had weak AR bioactivity.



Figure

4.8 Relative potency of androstenedione-derived steroids. Androstenedione-derived steroids were tested in the HuH7 androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. The relative potency (RP) of each steroid to DHT was calculated. Steroids with RP above the dotted line were more potent than DHT, while steroids below the dotted line were less potent than DHT. A) Steroids that had moderate androgenic bioactivity; and B) steroids that had weak androgenic bioactivity.

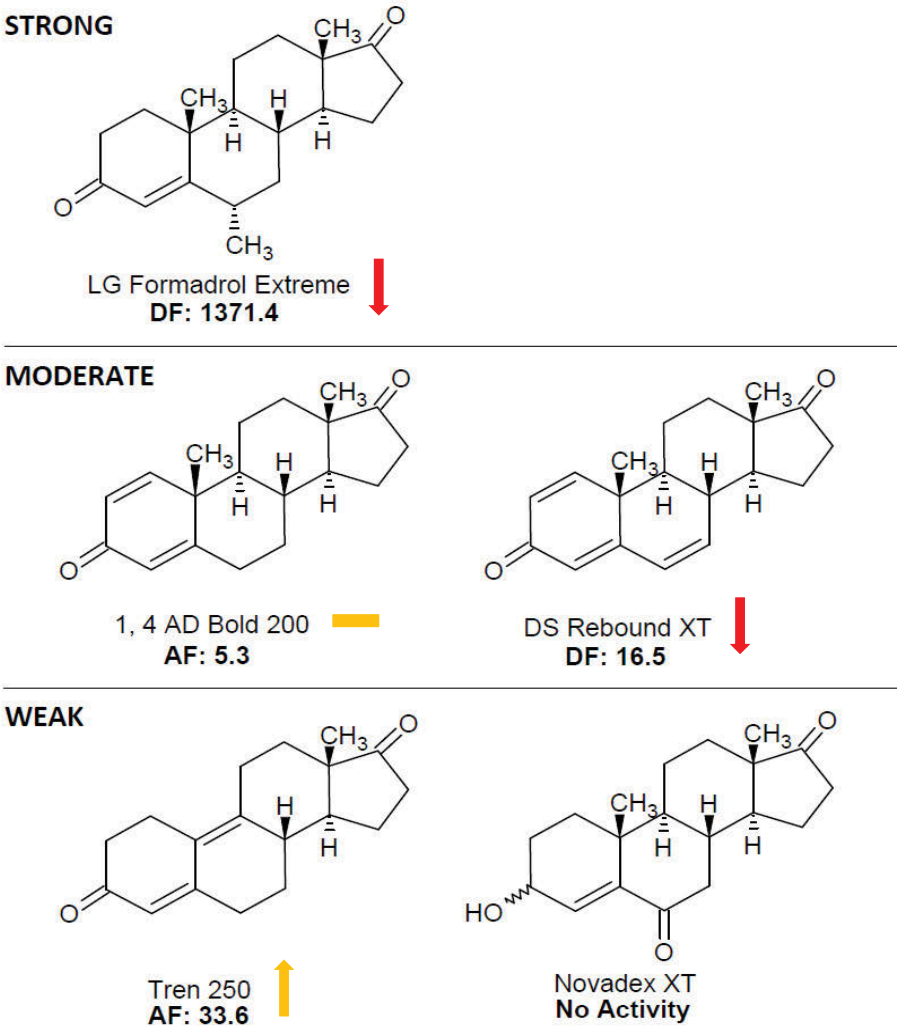


Figure 4.9 Metabolism in the HuH7 androgen bioassay altered the bioactivity of androstenedione-derived steroids. This figure shows the activation factor (AF) or deactivation factor (DF) of each steroid that was classed intrinsically as either strong, moderate, or weak. A downward arrow indicates that a steroid was deactivated into a weaker androgen, and an upward arrow indicates that a steroid was activated into a stronger androgen. A dash indicates the steroid remained in the same group. The colour of the symbol indicates which group the steroid belongs to as a result of being activated or deactivated: Green = strong; Yellow = moderate; and red = weak.

4.3.4 Androgenic Bioactivity of Remaining Steroids

Three steroids that were not classed as DHT-, T-, or androstenedione-derivatives were tested for their androgenic bioactivity in the HuH7 androgen bioassay (Figure 4.10). The three steroids were all moderate activators of AR, with RP values ranging from 6.9 to 42.5% (Figure 4.11). One of these androgens was intrinsically strong, that was deactivated over 4-fold to a moderate AR activator. The other two were intrinsically moderately strong activators (Figure 4.12).

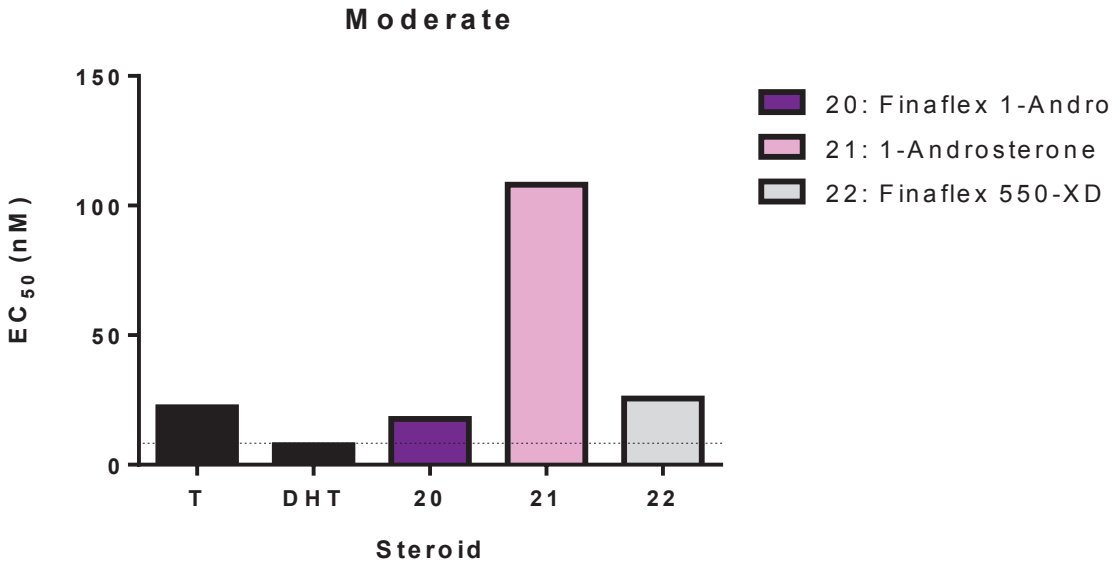


Figure 4.10 EC_{50} values of remaining steroids. Three steroids that were not classed as DHT-, T- or androstenedione-derivatives were tested in the HuH7 androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. Steroids with EC_{50} values above the dotted line were less potent than DHT, while steroids with EC_{50} values below the dotted line were more potent than DHT.

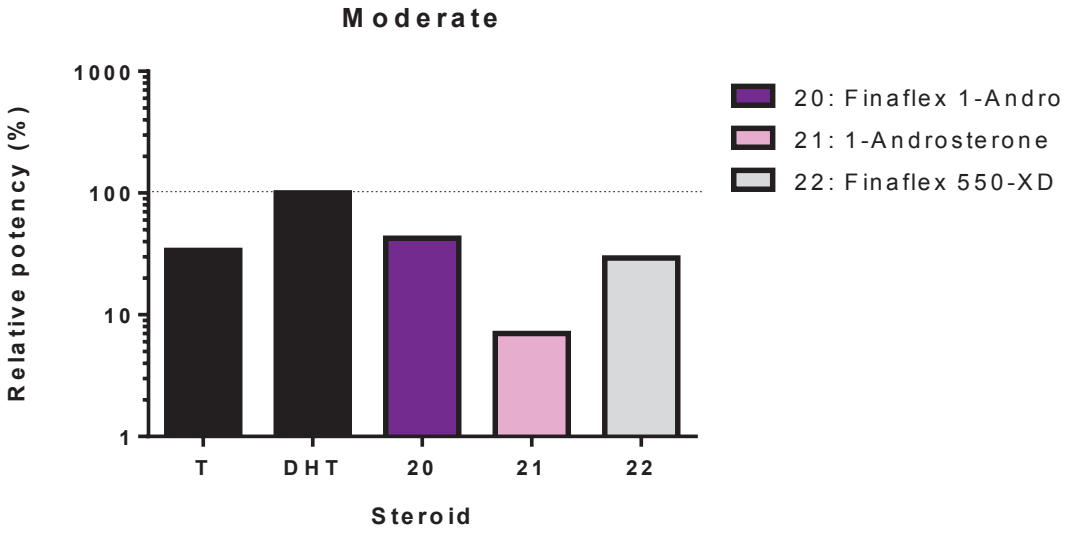


Figure 4.11 Relative potency of remaining steroids. Three steroids not classed as DHT-, T-, or androstenedione-derivatives were tested in the yeast androgen bioassay and EC_{50} values were calculated from sigmoidal dose-response curves. T and DHT were included as reference androgens. The relative potency (RP) of each steroid to DHT was calculated. Steroids with RP above the dotted line were more potent than DHT, while steroids with RP below the dotted line were less potent than DHT.

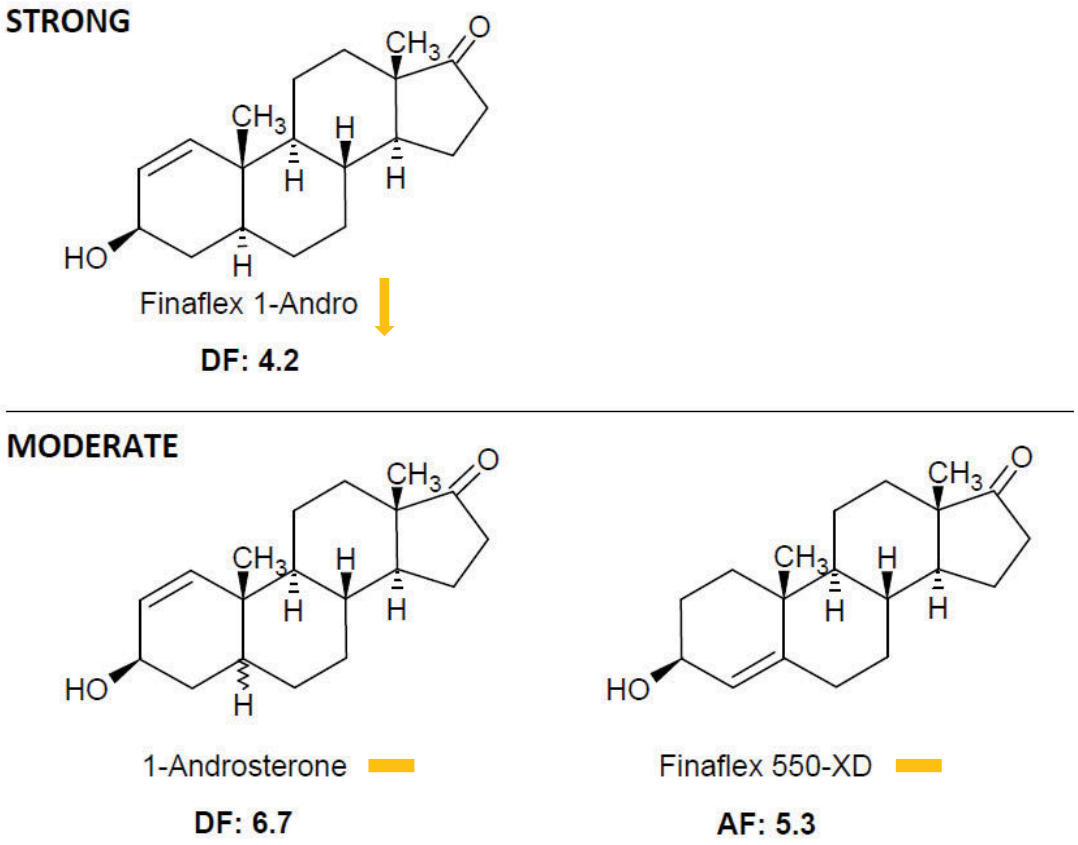


Figure 4.12 Metabolism in the HuH7 androgen bioassay altered the bioactivity of remaining steroids. This figure shows the activation factor (AF) or deactivation factor (DF) of each steroid that was classed intrinsically as either strong, moderate, or weak. A downward arrow indicates that a steroid was deactivated into a weaker androgen, and an upward arrow indicates that a steroid was activated into a stronger androgen. A dash indicates the steroid remained in the same group. The colour of the symbol indicates which group the steroid belongs to as a result of being activated or deactivated: Green = strong; Yellow = moderate; and red = weak.

4.4 Discussion

In this study, 22 sports supplement-derived steroids were assessed for their potential androgenic bioactivity in the HuH7 bioassay. This is because metabolism may activate or inactivate the steroid. Importantly, this screen allows for the detection of inactive proandrogens which may not be detected in the yeast androgen bioassay. This provides additional information to the intrinsic androgenic bioactivity of the steroids. Of the 10 intrinsically strong androgens tested, 6 remained strong activators of AR, while 4 were deactivated. Additionally, there was 1 moderately strong androgen that was activated into a potent androgen, while 2 were deactivated into weak androgens. Further, 2 androgens that were intrinsically weak were activated into moderately potent androgens.

The most important finding of this study was that H-Drol was activated into a potent androgen by metabolism. Therefore, liver cell metabolism is able to produce active metabolites more androgenic than the parent compound. This could potentially classify this androgen as a prohormone, which is being sold as a sports supplement, despite being banned for sale as such, as well as being banned by WADA for use in sports.

H-Drol is a known anabolic steroid that was first synthesised in 1960 and was the most abused androgen by East Germany (Schänzer *et al*, 1996). Several reports exist describing the metabolism of H-Drol in humans. Dürbeck *et al* report the detection of 3 main hydroxylated metabolites and no parent compound after a single ingestion in man using GC-MS and capillary columns (Dürbeck *et al*, 1983). Additional metabolites were later reported by Schänzer *et al* (Schänzer *et al*, 1996), and Sobolevsky and Rodchenkov (Sobolevsky and Rodchenkov, 2012) in human urine excretion studies. This study shows that H-Drol is more potent after exposure to HuH7 cells that metabolise it into potent androgenic metabolites.

High doses of this androgen have been associated with adverse side effects such as the development of intratesticular leiomyosarcoma (Froehner *et al*, 1999).

Along similar lines to H-Drol, Tren 250 and 2β, 3β-epithio were converted from very weak androgens into moderately potent androgens. This indicates that these androgens are also prohormones, although the active metabolites are not strongly androgenic. This was also seen with 1, 4 AD Bold 200 and Finaflex 550-XD, which increased in androgenic activity but remained moderately strong androgens. Therefore, measuring the androgenic activity of the active metabolites after metabolism is key to recognising the full bioactivity potential of androgens. This high bioactivity potential of androgens may be missed if using only the yeast androgen bioassay.

The findings of this study are in keeping with *in vivo* studies in which metabolism may activate androgens into more potent androgens. Early studies show several androgens had between 59 and 800% RP of the control androgen, methyltestosterone, in growth of levator ani muscle, when administered by gavage to orchidectomised rats (Kincl and Dorfman, 1963). In humans, oral consumption of oxymethelone has been associated with increases in fat-free mass and strength in older men (Schroeder *et al*, 2003) and haemodialysis patients (Supasyndh *et al*, 2013). In another example, old, but otherwise healthy men and women, were given oxandrolone orally for 14 days, resulting in an increase in skeletal muscle protein synthesis (Sheffield-Moore *et al*, 2006). In young, healthy men, oral administration of 1-Androsterone increased lean body mass and strength over a 4 week duration (Granados *et al*, 2014). Together, these reports indicate that androgens can be effective when subjected to first pass metabolism after being administered orally. Therefore, it is possible that the designer

androgens tested in this study which were activated in the HuH7 androgen bioassay may be physiologically effective.

While it is more interesting to determine that metabolism converts some steroids into more potent active metabolites, it was also found that some were inactivated into weaker androgens. LG Formadrol Extreme was intrinsically a potent androgen that was inactivated into a very weak androgen. Similarly, The One (Oxime) and DS Rebound XT were moderately strong androgens that were inactivated into weak androgens. Therefore, detection of these androgens could potentially be missed if using the HuH7 androgen bioassay alone. Thus, it is necessary to screen androgens with both the yeast androgen bioassay and the HuH7 androgen bioassay.

These findings are similar to *in vivo* studies of several androgens showing that androgens may be inactivated by metabolism after oral consumption and first pass metabolism. In a study by Rasmussen *et al*, the endogenous proandrogen androstenedione was given orally as a supplement to six healthy males for 5 days. Measured plasma samples did not show elevated levels of testosterone, but instead found elevated levels of oestradiol, indicating that the androstenedione was not activated into the more potent testosterone, but was inactivated by metabolism (via aromatisation) (Rasmussen *et al*, 2000). Parr *et al* demonstrated that the prohormone 19-norandrostenedione was unable to significantly stimulate growth of the androgen responsive skeletal muscle levator ani in rats when given orally, but did after subcutaneous injection, indicating that this steroid was inactivated after metabolism from the liver (Parr *et al*, 2009a). Interestingly, the authors did not detect major differences in metabolites in serum and plasma samples for both subcutaneous and oral routes of administration. This was also shown with the potent androgen methyl-1-testosterone (Parr

et al, 2011c). Parr *et al* report that methyl-1-testosterone was intrinsically a potent androgen in a yeast androgen bioassay and was able to stimulate growth of levator ani muscle after subcutaneous injection in rats. However, no effect was observed after oral administration, indicating its inactivation. Oral ingestion still did however, exhibit signs of toxicity via measurement of tyrosine aminotransferase expression (a marker of liver toxicity). With regards to androgens tested in this study, Superdrol has been shown to have decreased anabolic activity after oral administration compared to when administered subcutaneously in rats, indicating poor absorption and rapid metabolism (DEA Federal Register 44456). This steroid has been linked to various adverse side effects, including cholestatic jaundice and IgA nephropathy (Jasiurkowski *et al*, 2006) and severe cholestasis and renal failure (Nasr and Ahmad, 2009). This highlights that even though a steroid may be deactivated, it can still produce unwanted side effects, and should be of concern for consumers.

Of major interest is that some of the intrinsically strong designer androgens remained strong androgens and are activated into very potent androgens after metabolism. The designer androgens, Ultradrol and The One (hydrazone), did not greatly increase in potency and their bioactivities only increased by approximately 20% from their intrinsic bioactivity. As there is no data reported on The One (hydrazone), it is unknown if this slight increase in bioactivity is due to the steroid resisting metabolism, or if the metabolites produced have similar androgenic activity to the parent compound. However, both the 5α-reduction and the 17α-methyl group are known to resist aromatisation and metabolism on the A-ring and D-ring, respectively (Kicman, 2008; Schänzer, 1996). This may suggest that The One (hydrazone) is not heavily metabolised, although this needs to be determined. On the other hand, it is known that Ultradrol is rapidly metabolised. Both Cavalcanti *et al* and Geldof *et al* identified two

long-term metabolites (different metabolites in each study) in excreted urine samples from humans and chimeric mice with humanized livers, respectively, with no parent compound being detected by 48 hours in both studies (Cavalcanti *et al*, 2013; Geldof *et al*, 2014). The results from this study therefore suggest that these metabolites are highly androgenic, even though the parent compound is rapidly metabolised.

In contrast, the strong designer androgens Mechabol and Jungle Warfare are activated by metabolism by a much larger extent, with increases in bioactivity by approximately 800 and 1350%, respectively. This suggests that these two androgens are heavily metabolised and produce highly androgenic metabolites. Parr *et al* report that only 6% of the parent compound of Jungle Warfare is detected in human male urine samples after 11 days (Parr *et al*, 2011a). Similarly, Mechabol is also heavily metabolised, as Lootens *et al* report with an excretion study of Mechabol in chimeric mice with humanized livers. In the excretion study, urine was collected 24 hours after administration of Mechabol, in which the parent compound and several hydroxylated metabolites were detected, which included the known androgen, promagnon (Lootens *et al*, 2011).

Determining bioactivity after metabolism is an important factor when considering the potential physiological activity of androgens. This is because metabolism can drastically alter the bioactivity, as was shown in this study. Those androgens that were deactivated from metabolism would likely not be physiologically very effective, at least through oral route of administration due to metabolism in the liver. However, those androgens that remained potent androgens or became potent androgens after metabolism would possibly have a strong physiological effect at the target site (i.e. skeletal muscle). The potential for the potent

androgens identified in this study to have a strong anabolic effect in skeletal muscle is explored further in the next chapter.

4.5 Summary and Conclusions

This study shows for the first time that several designer androgens are activated into potent androgenic metabolites after metabolism. The HuH7 androgen bioassay provided a suitable screen into determining the androgenic bioactivity of the designer steroids after metabolism. In contrast to the yeast androgen bioassay, the presence of steroid metabolising enzymes in the HuH7 cells allowed for the determination of bioactivity after metabolism. Of the 22 steroids tested, 7 were potent androgens. These designer androgens could potentially have potent physiological effects *in vivo*, but this is not known for many of these compounds, despite being sold as performance enhancing substances.

Chapter 5

Determination of the Anabolic Potential of Designer Androgens in an *In Vitro* Mouse Skeletal Muscle Myoblast Cell Model

Table of Contents

5.1 Introduction

Androgens play a crucial role in regulating skeletal muscle mass and have been abused by athletes for over 60 years. It has been shown that treating healthy eugonadal men with androgens such as testosterone and nandrolone can enhance skeletal muscle mass beyond normal levels (Lichtenbelt *et al*, 2004; Bhasin *et al*, 2001a). Skeletal muscle fibre hypertrophy is caused by increases in net muscle protein synthesis, which is facilitated by enhanced myonuclear accretion in the syncytial muscle fibre by precursor satellite cells (Qaisar *et al*, 2012). Skeletal muscle strength is also improved in response to androgens, typically as a result from the increased expression of contractile proteins such as myosin heavy chain isoforms (Miller *et al*, 2015; Frese *et al*, 2011).

Although mechanisms detailing how androgens exert their anabolic effects in skeletal muscle are not fully understood, it has been suggested that the AR plays a central role (Hughes *et al*, 2015; Basualto-Alarcon *et al*, 2013). *In vitro* studies of skeletal muscle myoblast cell models have shown hypertrophy in response to testosterone, the effect of which is negated by specific AR antagonists (Hughes *et al*, 2015; Basualto-Alarcon *et al*, 2013). These findings have been supported by *in vivo* studies involving AR-knockout mice showing impaired muscle strength and size in comparison to normal mice (Dubois *et al*, 2014; MacLean *et al*, 2008). Together, these studies show an important role of AR activation in skeletal muscle anabolism.

While androgens such as testosterone and nandrolone have been extensively studied for their anabolic effects, very little is known about the anabolic effects of designer steroids. In Chapters 3 and 4, 18 designer steroids derived from sports supplements were determined to be moderate or strong AR activators using the HuH7 androgen bioassay. This suggests that these designer androgens may retain their bioactivity when consumed orally in a sports

supplement and stimulate AR present in skeletal muscle. In this chapter, the hypothesis that these potent designer steroids may potentiate an anabolic effect in skeletal muscle is tested.

Traditionally, androgenic and anabolic effects of steroids are assessed using an *in vivo* animal model such as the Hershberger assay. In this assay, the animal is orchidectomised to minimise endogenous androgen production, and the weight of androgen-dependent tissue is weighed after a treatment period with the exogenous steroid (Kennel *et al*, 2004). This allows the measurement of androgenic tissue such as prostate and seminal vesicles, as well assessment of anabolic activity via the measurement of skeletal muscle, typically levator ani muscle (Dalbo *et al*, 2016; Kennel *et al*, 2004). However, due to the use of animals and ethical considerations using *in vivo* models, these screens are not high throughput.

There are also several *in vitro* models used for studying the anabolic effects of steroids. Such models often involve primary human skeletal muscle as well as primary and established skeletal muscle cell lines of rodents (Palsgaard *et al*, 2009; Clarke *et al*, 2007; Jacquemin *et al*, 2004). In this study, such a model was used to assess the anabolic potential of 5 designer androgens. The steroids chosen were those that tested positive for both the yeast and HuH7 AR bioassay. The assay used was based on mouse myoblast C2C12 cells. Specifically, myotube formation including the size and number of myotubes and myonuclear accretion as well as myosin heavy chain (MHC) expression was measured.

5.2 Methods

5.2.1 C2C12 Cell Culture

Mouse myoblast C2C12 cells were cultured in growth media (DMEM, Gibco Life Technologies) containing high glucose (4500 mg/L), L-glutamine (584 mg/L) without sodium pyruvate, and supplemented with 20% FCS. Cells were seeded at low confluence (~15,000 cells/ cm^2) in a 24-well plate and were grown to 100% confluence in growth media (3-4 days). After reaching 100% confluence, cells were washed with PBS and cultured in differentiation media (DMEM, Gibco Life Technologies) containing high glucose (4500 mg/L), L-glutamine (584 mg/L) without sodium pyruvate, and supplemented with 2% charcoal-stripped adult horse serum, as described (Methods section 2.3.3). Cells were treated daily with steroids diluted in ethanol (final concentration <1%) with fresh media, for 5 days to allow myotube formation.

5.2.2 Immunocytochemistry

After myotube formation, cells were washed 3x with PBS before fixing with ice-cold methanol for 10 min at room temperature. Following fixation, cells were washed 3x with PBS and non-specific binding was blocked with 3% skim milk blocking solution for 3x 5 min incubations. After blocking, cells were again washed 3x with PBS before incubating with the primary anti-sarcomeric myosin heavy chain MF-20 antibody (Developmental Studies Hybridoma Bank, University of Iowa) for 1.5 hours at room temperature. An optimising experiment was performed to determine the optimal primary antibody concentration and also to check specificity of the primary and secondary antibodies, as described below (Figure 5.1). After incubation with the primary antibody, cells were washed 3x with PBS and the fluorescein-isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (Abcam, ab6785) (1/1000) was incubated for 1.5 hours at room temperature, protected from light exposure.

Nuclei were counterstained with DAPI for 30 minutes before image analysis was performed on the IN Cell Analyzer 2200, GE Healthcare, as described (Methods section 2.4.2). For each treatment, 15 images were taken from random fields of view generated by the IN Cell Analyzer 2200 software and analysed using ImageJ software.

5.2.3 Assessment of Myotube Area

Myotubes were defined as cells that stained positive for sarcomeric MHC expression and contained a minimum of 3 nuclei. The MF-20 antibody recognises all MHC isoforms. Measurement of myotube area was conducted using the polygon selection tool in ImageJ to trace the edges of myotubes and measuring the total area of pixels. An average was taken over 15 random fields of view per treatment, across 10 independent experiments.

5.2.4 Threshold of MHC Expression

Measurement of total MHC expression was conducted using a colour threshold in ImageJ. Total MHC expression was measured in both defined myotubes as well as undifferentiated myoblasts expressing MHC. An average was taken over 15 random fields of view per treatment, across 10 independent experiments.

5.2.5 Statistical Analysis

Data are presented as mean ± SEM. Treatments were compared using a One-way ANOVA and Dunnet's posthoc test. Non-parametric data was compared using Kruskal-Wallis test with Dunn's posthoc test, where appropriate. A value of $p < 0.05$ was considered to be statistically significant.

5.3 Results

Five designer steroids were tested for their anabolic potential in a mouse myoblast C2C12 cell line. Ultradrol, The One (Hydrazone), P-Plex, and Mechabol were shown to be potent activators in the yeast androgen bioassay and H-Drol was moderately potent. All five steroids were potent activators of AR in the HuH7 androgen bioassay. The endogenous androgen, DHT, was used as a positive control. All treatments were compared to an ethanol-vehicle control.

5.3.1 Optimising Antibody Concentration and Specificity

To determine the optimal concentration of primary antibody, differentiated myotubes were fixed and stained as described above, using a range of primary antibody concentrations. It was determined that 0.75 µg/ mL was optimal for staining of MHC positive C2C12 cells. To determine the specificity of the primary antibody binding to the fluorescent secondary antibody and ensure no background fluorescence was detected, differentiated C2C12 cells were stained with or without the primary and secondary antibody. It was determined that fluorescence was only detected in the presence of primary and secondary antibody together, indicating no background fluorescence or unspecific binding of the secondary antibody. Additionally, there was negligible fluorescence detected in undifferentiated C2C12 myoblasts. A minimal amount of MHC expression is expected in undifferentiated myoblasts in these conditions due to the high cell confluence causing partial differentiation to occur upon cell to cell contact (Figure 5.1).

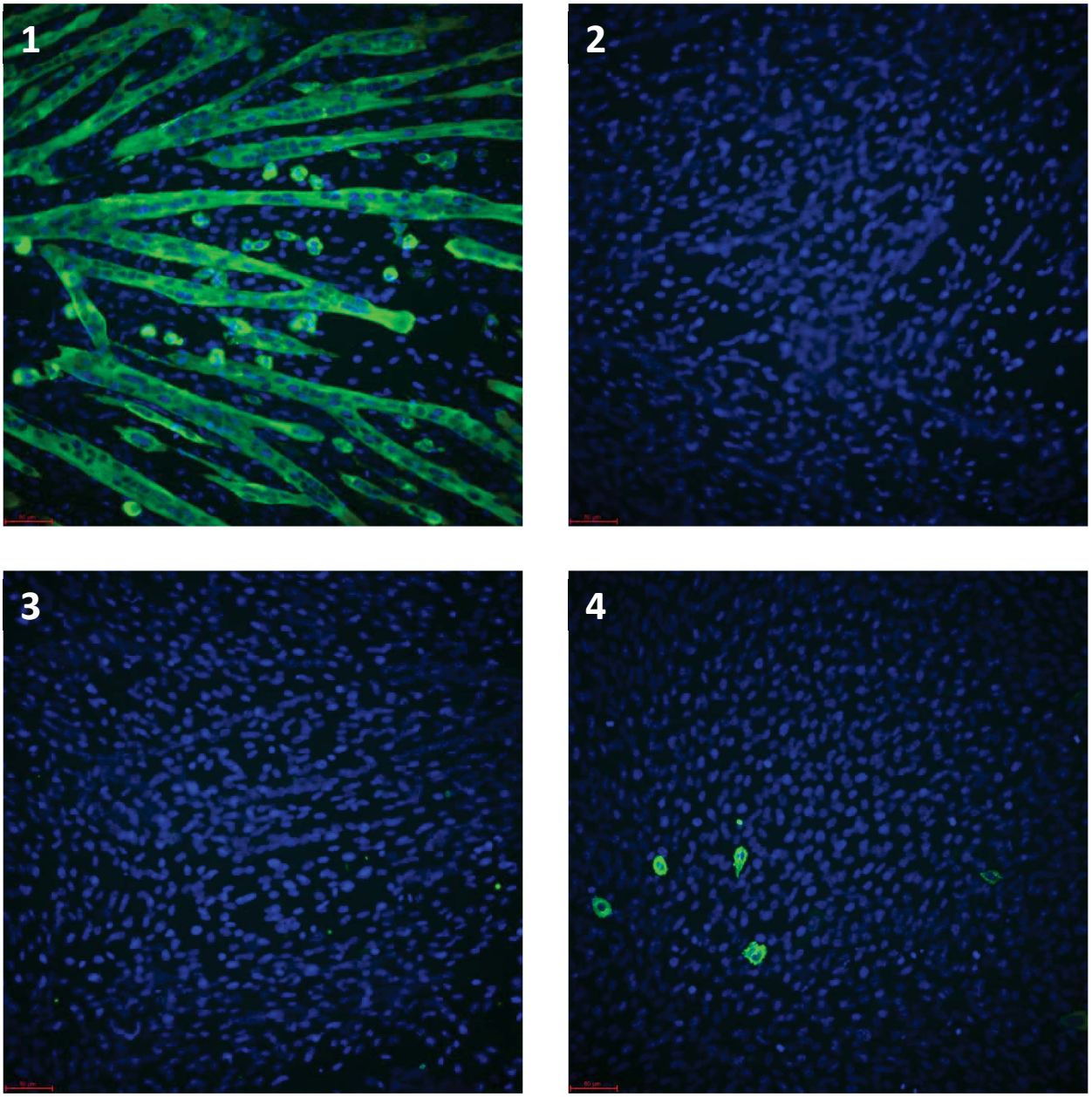


Figure 5.1 Immunocytochemistry control reactions to verify efficacy and specificity of primary and secondary antibodies. Representative image of control immunocytochemistry reactions. Primary antibody 0.75 µg/ mL; secondary antibody (1/1000). 1) Positive control primary antibody plus secondary antibody with DAPI and differentiated myotubes; 2) negative control (no secondary antibody); 3) negative control (no primary antibody); and 4) negative control (undifferentiated myoblasts). Images are at 20x magnification. Scale bar: 60 µM.

5.3.2 Assessment of Myotube Area in Response to Designer Androgens

To assess the ability of the designer androgens to promote myotube hypertrophy by increasing the morphological size of the myotubes, C2C12 cells were treated with steroids at concentrations of 4, 40, and 400 nM. The area of the myotubes was then measured compared to the control. The results are expressed as a percentage compared to the vehicle control, which was normalised to 100%. The positive control, DHT, produced significant increases in average myotube area with all concentrations tested (Figure 5.2). There were no significant differences observed between DHT treatments. Treatments with the five designer androgens also significantly increased relative myotube area at 40 and 400 nM (4 nM was not tested for these steroids) (Figure 5.2). Representative images of myotube formation for ethanol and DHT treatments, and one of the designer androgens can be seen in Figure 5.3.

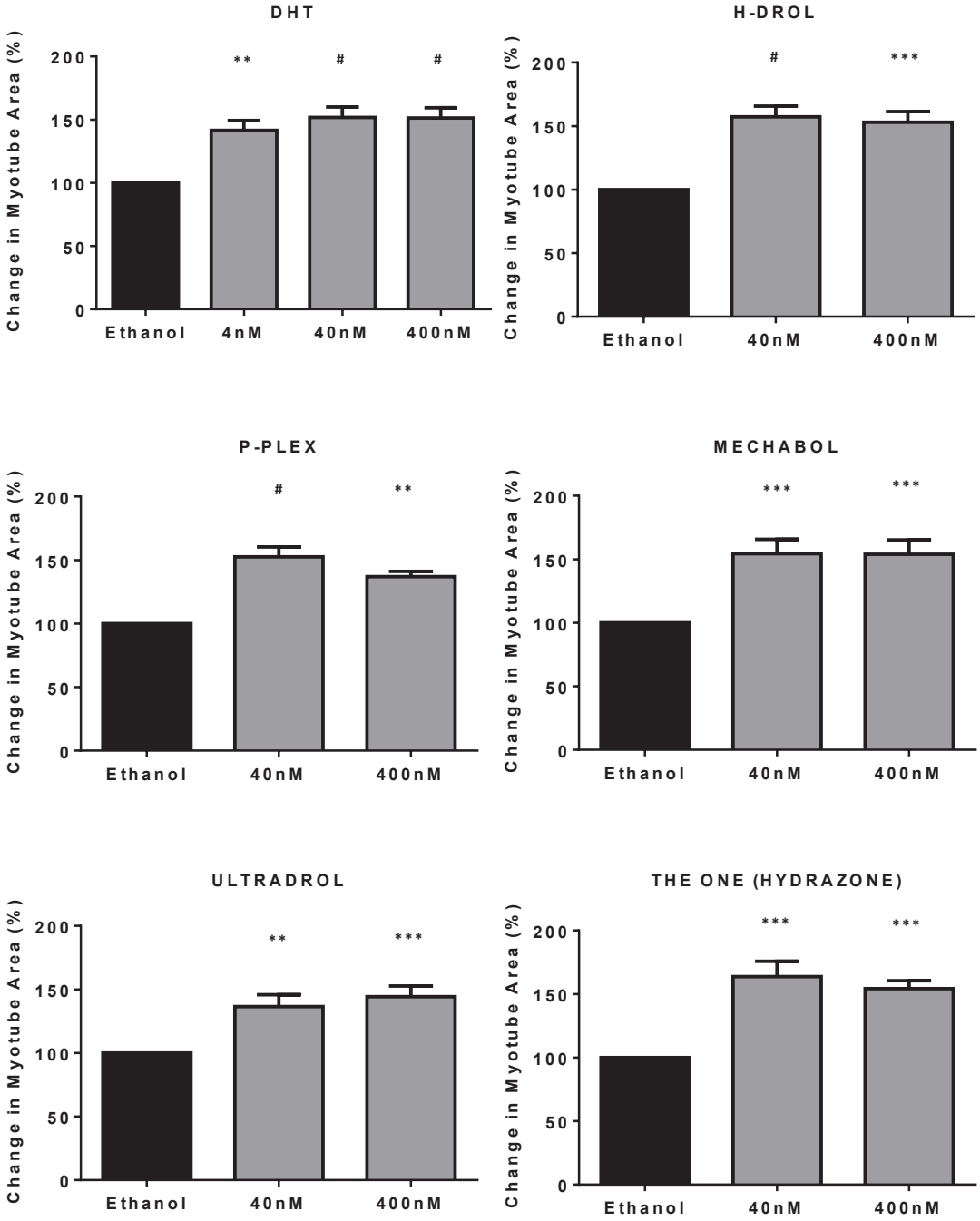


Figure 5.2 Designer androgens increase myotube area. C2C12 cells were differentiated for 5 days and treated with designer androgens at 40 and 400 nM each day, and cross-sectional area of the myotubes was measured. Treatment with designer androgens (40 or 400 nM) increased mean ± SEM myotube area, compared to control. ** $p<0.005$; *** $p<0.001$; # $p<0.0001$.

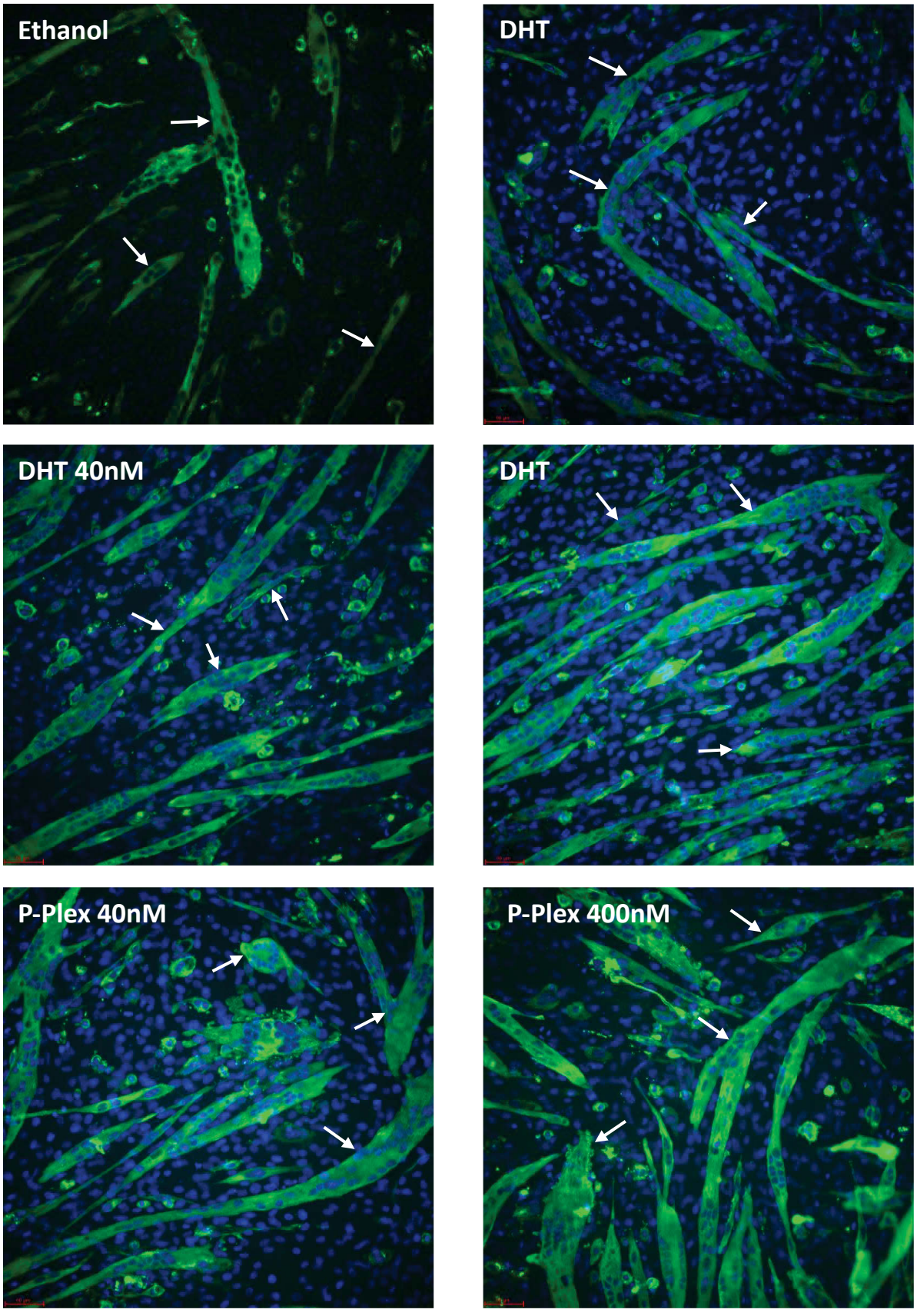


Figure 5.3

Figure 5.3 Representative images of myotube formation in C2C12 cells. C2C12 cells were differentiated for 5 days and treated with designer androgens at 40 and 400 nM or DHT at 4, 40, and 400 nM. Ethanol was used as a vehicle control. Myotubes were stained with sarcomeric MHC (green) and myonuclei were counterstained with DAPI (blue). Myotubes were defined as cells that were positive for MHC expression and contained a minimum of three nuclei. An average of myotube size was taken over 15 random fields of view per treatment, across 10 independent experiments. This figure shows a representative image of the vehicle control and the positive control, DHT, and one of the designer androgens (P-Plex). Examples of myotubes measured in each treatment group are indicated by a white arrow.

5.3.3 Quantification of Myotube Formation in Response to Designer Androgens

It was next sought to determine if the designer androgens influenced the number of myotubes formed during 5 days of differentiation. The mean number of myotubes per field of view per treatment were counted and compared to the vehicle control. DHT was used as an endogenous androgenic control and was shown to not increase myotube number. Similarly, no effect was measured for any of the designer androgens tested (Figure 5.4).

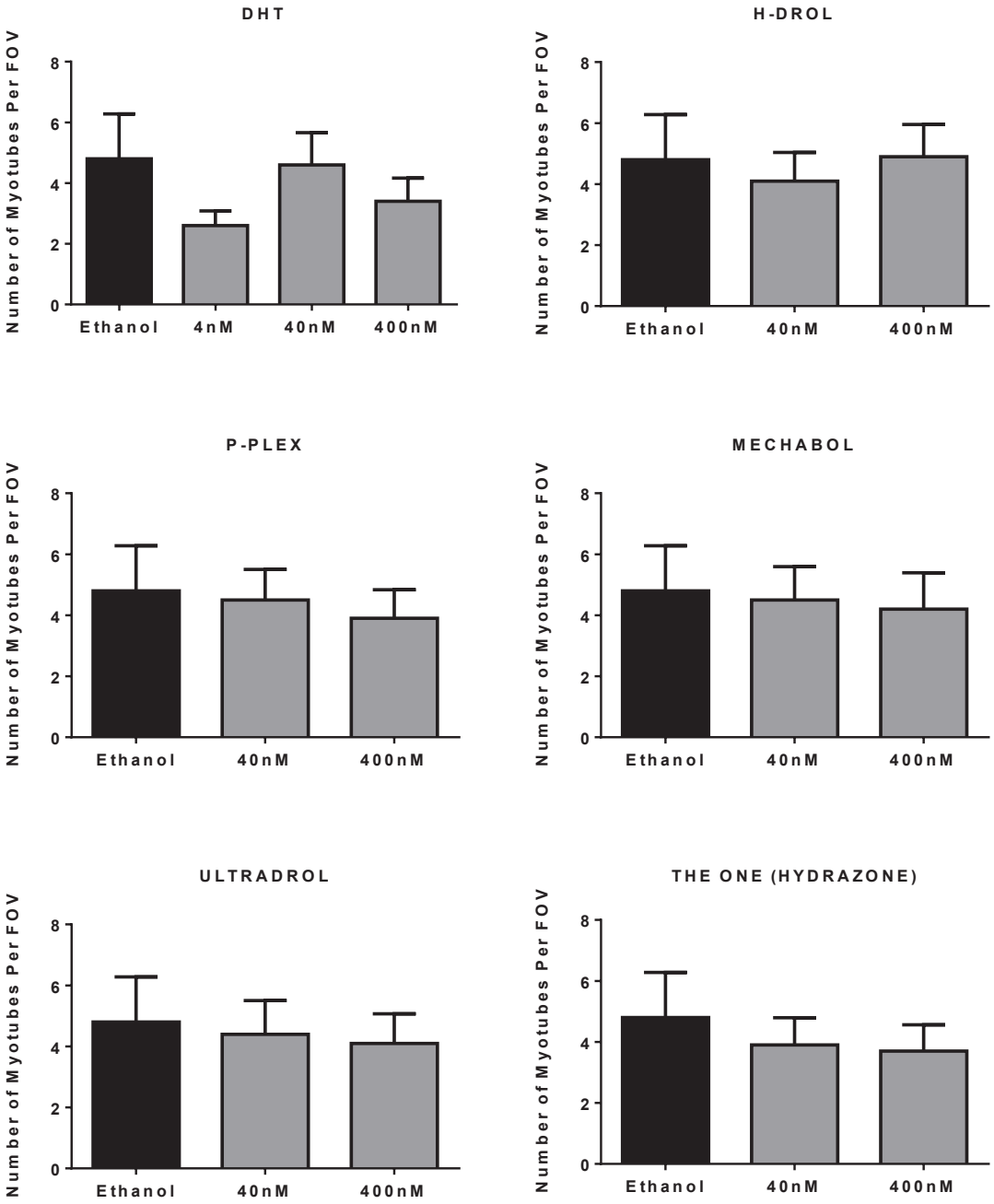


Figure 5.4 Designer androgens had no effect on myotube number. Counts of defined myotubes per field of view were averaged for each steroid treatment and compared to the ethanol control. No significant difference was determined by One-way ANOVA and Dunnet's posthoc test.

5.3.4 Quantification of Myosin Heavy Chain Expression in Response to Designer Steroids

To determine if the designer androgens increased the expression of sarcomeric MHC, a marker of differentiation, total MHC expression was quantified. Measurements of MHC positive cells included differentiated myotubes as well as undifferentiated myoblasts (i.e. did not contain a minimum of three nuclei). All treatments were compared to the vehicle control, which was normalized to 100% MHC expression. The endogenous control, DHT, increased total MHC expression at 400 and 40 nM, but not 4 nM. H-Drol, Mechabol and The One (Hydrazone) increased total MHC expression compared to the vehicle control at both 40 and 400 nM (Figure 5.5). P-Plex and Ultradrol increased total MHC expression at 40 nM, but not at 400 nM.

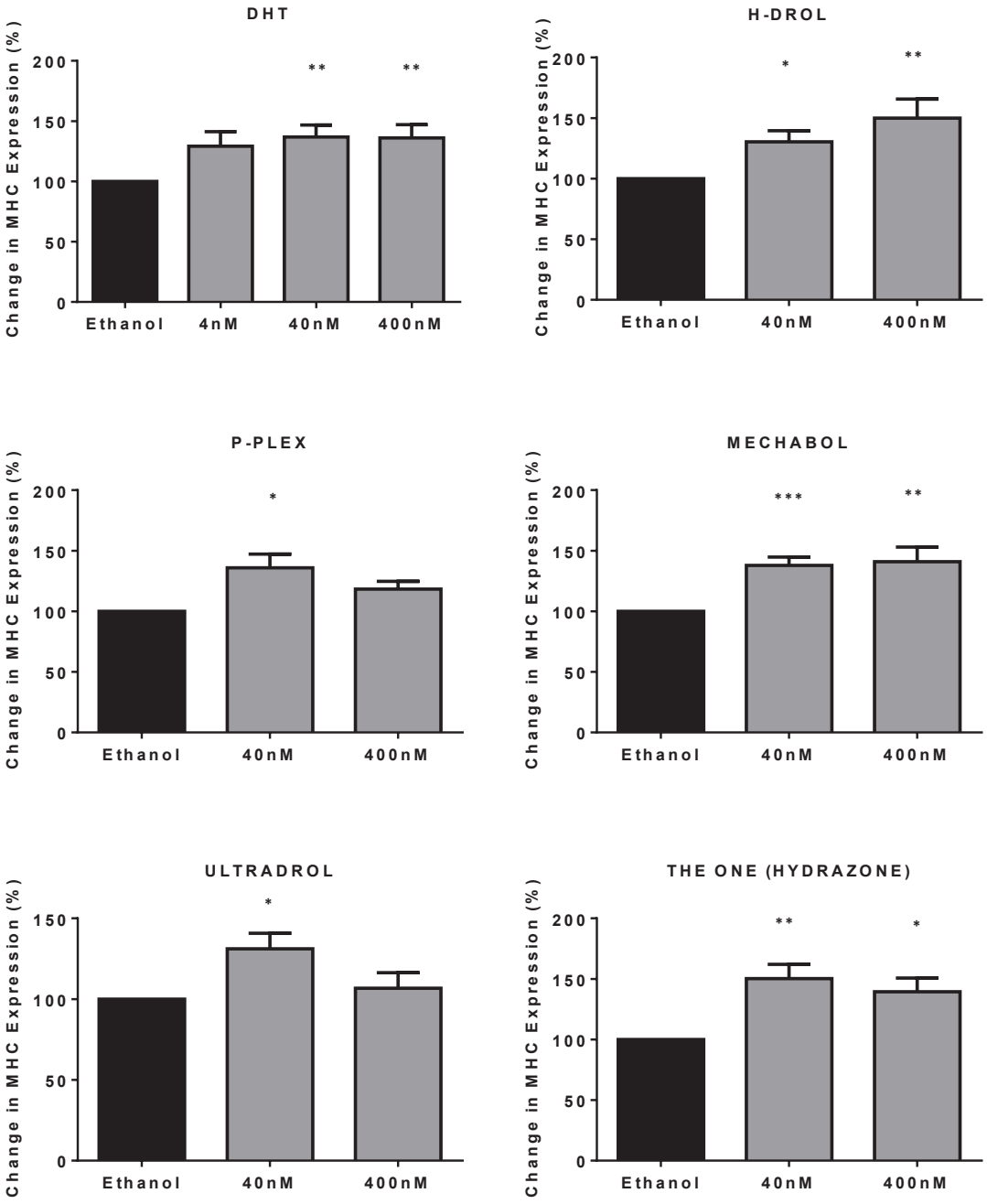


Figure 5.5 Designer androgens increased total MHC expression. Treatment with DHT, H-Drol, Mechabol and The One (Hydrazone) increased mean ± SEM MHC expression at 40 and 400 nM compared to the vehicle control. Treatment with Ultradrol and P-Plex increased mean ±

SEM MHC expression at 40 nM, but not 400 nM, compared to the vehicle control. * P <0.05; ** P <0.005; *** P <0.001.

5.3.5 Assessment of Nuclei Accretion in Response to Designer Androgens

To assess myonuclear accretion in the formed myotubes in response to the designer androgens, nuclei were counted in each myotube and then myotubes were grouped according to the number of nuclei. Between 3 and 5 nuclei per myotube was considered 'low'; between 6 and 9 nuclei per myotube was considered 'medium'; and greater than 9 nuclei per myotube was considered 'high' (Figure 5.6). The number of myotubes in each group was then expressed as a percentage of total number of myotubes. This classification is based on that used by Deane *et al*, 2013, but modified slightly (Deane *et al*, 2013). DHT treatment of 400 nM showed a significant difference in the number of myotubes in the 'medium' group compared to the control, but not at 4 or 40 nM. DHT did not change the number of myotubes in the 'low' or 'high' group (Figure 5.7). This was also seen with P-Plex (Figure 5.8). Treatment with H-Drol showed significantly less myotubes in the 'low' group compared to the control at 40 and 400 nM (Figure 5.8). It also showed significantly more myotubes in the 'medium' and 'high' group compared to the control. Both The One (Hydrazone) and Mechabol showed significantly less myotubes in the 'low' group compared to the control and significantly more myotubes in the 'medium' group compared to the control (Figure 5.8). No significant changes were observed in the 'high' group. Ultradrol did not alter nuclei accretion compared to the control (Figure 5.9).

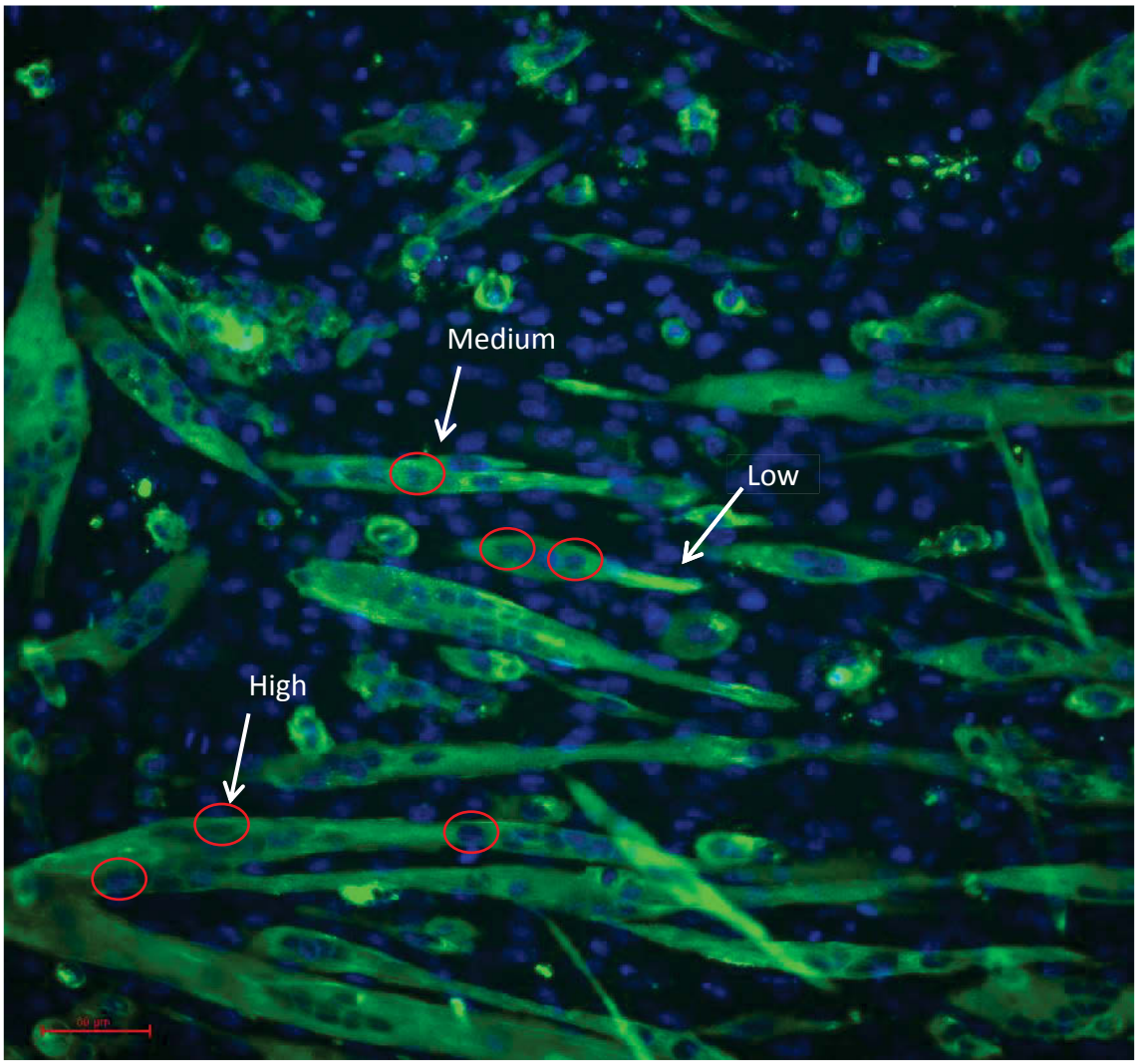


Figure 5.6 Myotubes grouped into number of myonuclei. Myonuclei were counted in each myotube and grouped based on the number of nuclei. 'Low' = 3-5 myonuclei, 'medium' = 6-9 myonuclei, and 'high' = >9 myonuclei. Red arrows indicate examples of nuclei that were counted.

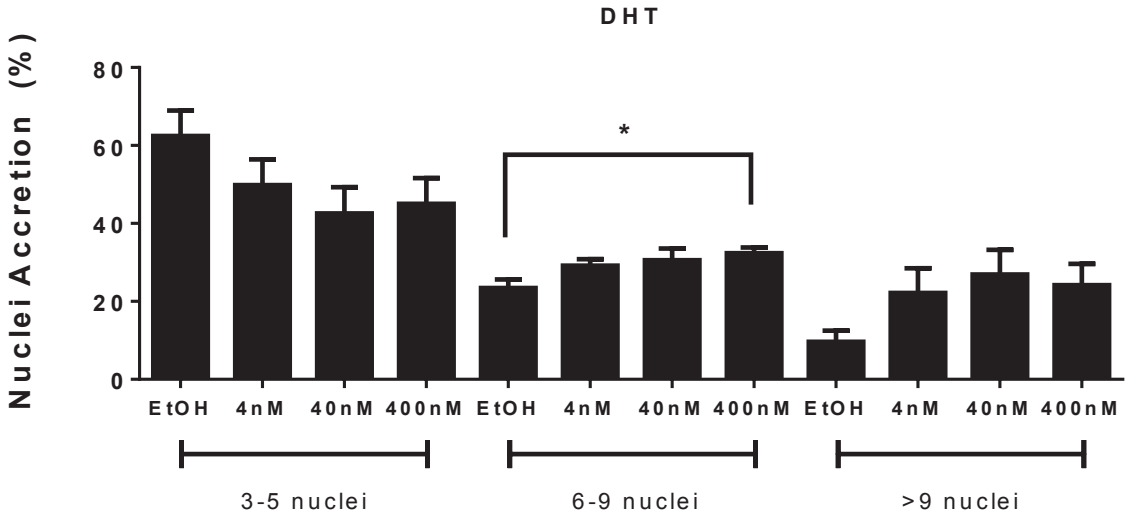


Figure 5.7 Nuclear accretion in C2C12 myotubes was increased by DHT. The number of nuclei were counted in each myotube and grouped into low (3-5 nuclei), medium (6-9 nuclei), or high (>9) nuclear accretion, and expressed as a percentage of total number of myotubes. DHT at 400 nM had significantly higher nuclear accretion in the medium group compared to the control. * $p < 0.05$.

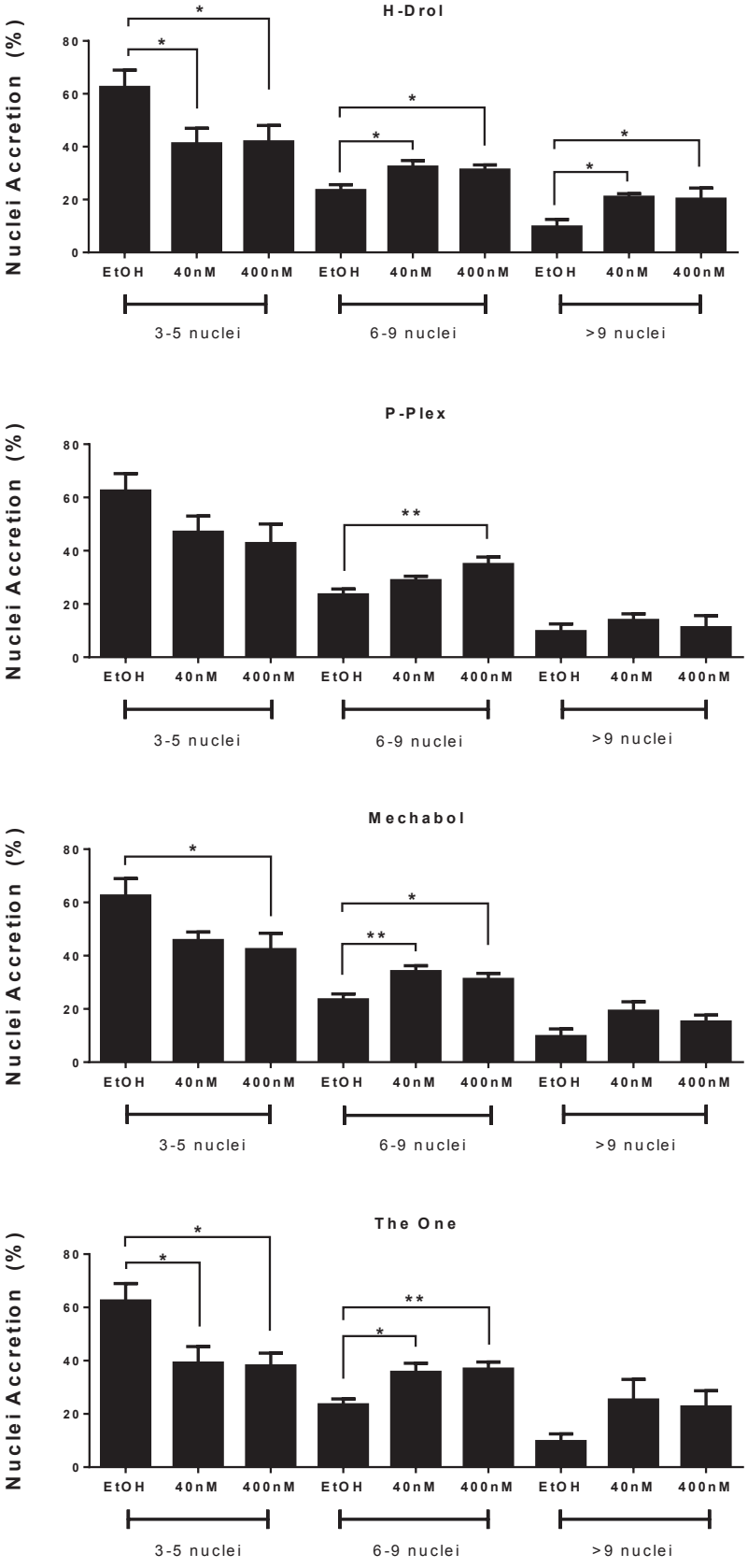

H-Drol
P-Plex
Mechabol
The One
Nuclei Accretion (%)
0
20
40
60
80
EtOH
40nM
400nM
3-5 nuclei
6-9 nuclei
>9 nuclei
*
**

Figure 5.8

Figure 5.8 Nuclear accretion in C2C12 myotubes was increased by designer androgens. The number of nuclei were counted in each myotube and grouped into low (3-5 nuclei), medium (6-9 nuclei), or high (>9) nuclear accretion, and expressed as a percentage of total number of myotubes. The H-Drol, The One (Hydrazone), and Mechabol) had significantly less nuclear accretion in the low group compared to the control. P-Plex, H-Drol, The One (Hydrazone), and Mechabol) had significantly more nuclear accretion in the medium group compared to the control. H-Drol had significantly more nuclear accretion in the high group compared to the control using a One-way ANOVA and Dunnet's posthoc test. * $p<0.05$; ** $p<0.005$.

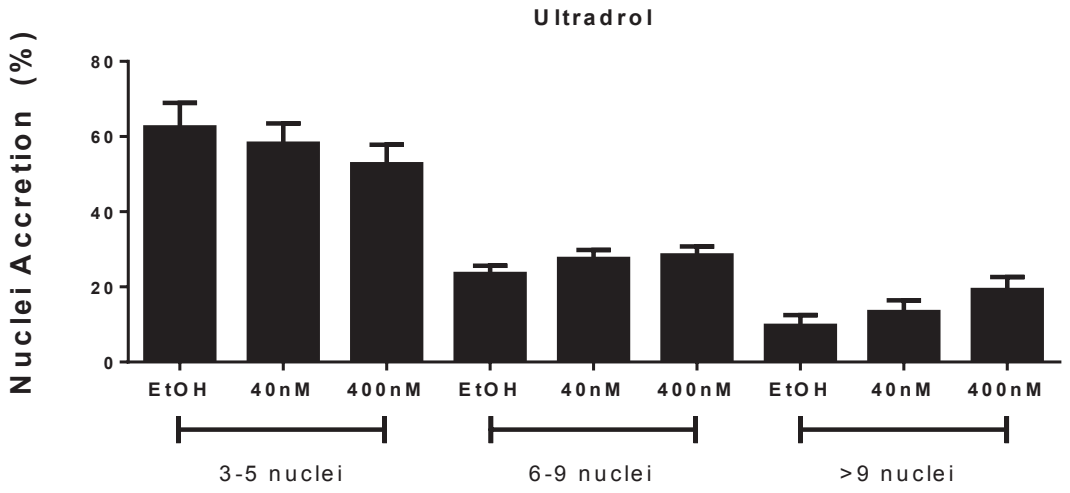


Figure 5.9 Nuclear accretion in C2C12 myotubes was not increased by Ultradrol. The number of nuclei were counted in each myotube and grouped into low (3-5 nuclei), medium (6-9 nuclei), or high (>9) nuclear accretion, and expressed as a percentage of total number of myotubes. Ultradrol did not significantly alter the number of nuclei accretion in the low, medium or high group at either 40 or 400 nM compared to the control using a One-way ANOVA and Dunnet's posthoc test.

5.4 Discussion

The isolation and synthesis of testosterone in the 1930s led to the aim of synthesising androgens capable of achieving an optimal balance between beneficial anabolic effects and negative androgenic effects (Gao and Dalton, 2007). This led to the development of many synthetic testosterone and dihydrotestosterone analogues. Many of these steroids were unsuccessful and were not used clinically. However, regardless of the lack of safety and efficacy data, athletes began, and continue to, abuse some of them in sport. Further, there have been recent attempts in the clandestine synthesis of novel testosterone and dihydrotestosterone analogues that were not created for clinical use, but solely for abuse in sports (Parr *et al*, 2011b). The first reported designer steroids were tetrahydrogestrinone and norbolethone, in the early 2000s (Catlin *et al*, 2002 and 2004), and newer steroids continue to emerge in sports supplements (Rahnema *et al*, 2015; Parr *et al*, 2011a, 2011b, 2011c and 2009b). These steroids, and those created in the 1950s and 1960s, are being abused by athletes, despite having minimal scientific reporting on efficacy and safety.

In this study, five designer androgens were assessed for their anabolic potential using a C2C12 mouse myoblast cell model. The five androgens, H-Drol, P-Plex, Ultradrol, Mechabol and The One (Hydrazone) were studied because of their potent AR activation in the yeast and HuH7 androgen bioassays. This led to the hypothesis that these androgens would potentiate an anabolic effect in skeletal muscle given that they are potent AR activators after HuH7 liver cell metabolism.

Androgens are well characterised for their anabolic properties. Androgens have been reported to significantly increase the cross-sectional area of skeletal muscle myotubes, (Basualto-Alarcon *et al*, 2013; Wu *et al*, 2010b), muscle fibres in animals (Serra *et al*, 2013;

Yarrow *et al*, 2011; Brown *et al*, 2009), and in human skeletal muscle (Eriksson *et al*, 2005; Lichtenbelt *et al*, 2004; Sinha-Hikim *et al*, 2002). However, the exact mechanisms are not fully understood. Thus, assessment of anabolic activity (*in vitro* and *in vivo*) often involves measurements of several markers. These include expression of contractile proteins such as MHC (Frese *et al* 2011), increased myonuclei (Eriksson *et al*, 2005; Sinha-Hikim *et al*, 2003 and 2006), and hypertrophy of muscle fibres or myotubes (Eriksson *et al*, 2005; Sinha-Hikim *et al*, 2002, 2003, and 2006). Therefore, to assess the anabolic potential of the designer androgens, these markers were measured in the C2C12 mouse myoblast model.

This study shows for the first time that H-Drol, P-Plex, Mechabol, Ultradrol and The One (Hydrazone) have potent anabolic activity in a C2C12 cell model. The effects of the designer steroids are comparable to DHT. These findings suggest that designer steroids in sports supplements are potent anabolic agents, and have important implications for anti-doping authorities, due to the widespread use of sports supplements by athletes (Maughan *et al*, 2007).

The findings of this study are in agreement with other cell culture models showing anabolic activity of androgens. Several reports have indicated testosterone increases myotube size by up to approximately 50% *in vitro*, supporting the findings of this study (Hughes *et al*, 2015; Deane *et al*, 2013; Basualto-Alarcon *et al*, 2013; Wu *et al*, 2010b). Similarly, reports have shown increases in myonuclei content in myotubes (Hughes *et al*, 2015; Deane *et al*, 2013), and increases in MHC expression (Singh *et al*, 2003), as measured in this study. The increased myonuclei content is important to sustain increased protein synthesis during hypertrophy (Egner *et al*, 2013). The increased protein content and mass of muscle fibres during hypertrophy is largely due to increases of contractile proteins, like MHC (Balagopal *et al*,

1997). Therefore, the C2C12 model used in this study is a valid model for screening androgens and assessing anabolic activity as increases in these anabolic markers were measured.

Although the findings of this study agree with other *in vitro* reports, the C2C12 model has several limitations in determining anabolic activity compared to *in vivo* models. Firstly, myotube hypertrophy is limited due to a fixed growth surface area. This may help explain why myotube hypertrophy is restricted to an approximately 50% increase in size, both in this study, as well as other *in vitro* studies (Deane *et al*, 2013; Basualto-Alarcon *et al*, 2013). In *in vivo* studies, testosterone has been shown to have a greater increase in hypertrophy. Increases in cross-sectional area of muscle fibres have been reported to be as high as 69 (Brown *et al*, 2009) and 77% (Egner *et al*, 2013) in mice gastrocnemius muscle and rat soleus muscle compared to controls, respectively, although other studies have reported relative changes comparable to that found in this study (Eriksson *et al*, 2005; Sinha-Hikim *et al*, 2002, 2003 and 2006). This indicates that androgens have the potential to induce hypertrophy to a greater extent *in vivo* compared to *in vitro* models. It has also been noted that androgens such as testosterone display dose-response relationships between androgen concentrations and skeletal muscle hypertrophy *in vivo* (Sinha-Hikim *et al*, 2002; Storer *et al*, 2008 and 2003; Bhasin *et al*, 2005), but a dose-response relationship was not observed in this study. Thus, the 40 nM doses of androgens already reached the "saturable" amount of myotube hypertrophy in this culture model. Forty nM represents 4 to 10 times the physiological concentration so this finding is not unexpected. Secondly, continual myotube hypertrophy is sustained by a replenishment of myoblast cells which donate extra nuclei. This will only occur for a limited time *in vitro* as the myoblasts will partially fuse on cell to cell contact and no longer proliferate. Thus, the myotubes only have a finite source of myonuclei for growth, whereas *in*

vivo, satellite cells are capable of replenishing the myoblast cell pool (Zhang *et al*, 2015; Bellamy *et al*, 2014). Therefore, the *in vitro* C2C12 model is not suitable for determining the full anabolic potential of a compound, but rather that it has anabolic activity *per se* and thus, warrants further investigation *in vivo*. This can limit the need for using animal models as the *in vitro* model can be used as a high throughput screen of anabolic activity for novel compounds.

The results for P-Plex are in agreement with previous *in vivo* studies showing anabolic activity. In 1964, Kincl and Dorfman showed that P-Plex had 437% RP compared to methyltestosterone in stimulating levator ani muscle growth in rats after oral administration (Kincl and Dorfman, 1964). In a more recent study, Diel *et al* demonstrated that P-Plex restored absolute weight of levator ani muscle in orchidectomised rats to normal baseline levels, comparable to the androgen control, testosterone proprionate when injected subcutaneously (Diel *et al*, 2007). In a similar assay, Frese *et al* demonstrated that P-Plex restored levator ani muscle in orchidectomised rats, but to a significantly less extent than testosterone proprionate (Frese *et al*, 2011). These reports validate the findings in this study suggesting that the C2C12 model is appropriate in determining the anabolic potential of designer androgens.

5.5 Summary and Conclusions

The emergence of designer androgens as anabolic agents in sports has highlighted the importance of anti-doping screening to adopt proactive measures as well as reactive counter-measures to combat illicit drug use in sports. Unfortunately, the detection of a novel designer androgen typically indicates that the compound is already being abused by athletes in sports, such as tetrahydrogestrinone and norbolethone (Catlin *et al*, 2004 and 2002). This is also true

with companies manufacturing sports supplements containing designer steroids on a large scale ready for purchase.

However, the identification of a novel designer steroid does not result in an immediate banning of the compound in sport. Banning of a substance by WADA in sport requires that the substance is performance enhancing, detrimental to user health, or both (Barroso *et al*, 2008). Unfortunately, this information is often very limited and with many novel designer steroids there exists no data. This is of particular concern with regards to sports supplements containing designer steroids for both sports doping purposes as well as user health.

With the recent insurgence of designer steroids in sports supplements, it was sought to determine the anabolic potential of a number of potent AR activating designer steroids as determined in the previous chapters. The designer steroids H-Drol, P-Plex, Mechabol, Ultradrol, and The One (Hydrazone) were rapidly screened for their anabolic potential in a C2C12 myoblast cell model in this study. It was shown for the first time that these steroids displayed anabolic activity in a C2C12 cell model, and the results for P-Plex are in agreement with previous studies demonstrating significant levator ani hypertrophy in rodent models (Frese *et al*, 2011; Diel *et al*, 2007; Kincl and Dorfman, 1964). In conclusion, screening designer steroids with the yeast and HuH7 androgen bioassays together with this C2C12 cell model can be used to identify steroids that have anabolic activity. This can then help identify steroids which should require further testing *in vivo*, thus limiting the use for animal models. Moreover, additional testing in the C2C12 model with androgens that have had *in vivo* testing may reveal a correlation between anabolic activity *in vitro* and *in vivo*. This may help predict if an androgen has anabolic activity *in vitro* it will also be anabolic *in vivo* and further limit the use of animals. This may be useful in higher throughput screening assays of new compounds.

Chapter 6

Evaluation of the Australian Sports Supplement Market for the Presence of Undeclared Androgenic Substances Using Yeast and Mammalian Cell Androgen Bioassays

Table of Contents

6.1 Introduction

The sports supplement market is an exponentially growing industry due to its popularity amongst athletes and the general population (Maughan *et al,* 2007). The sports supplement market has been estimated to be worth $142.1B USD and expected to reach $204.8B USD by 2017 (Transparency market research, accessed 2014). Most regulatory bodies, such as the Food and Drug Administration (FDA) in the US, class sports supplements as food products, and not medicinal products. They are therefore not subject to strict manufacturing guidelines such as those enforced on medicinal products (Brownie, 2005). Because of the poor manufacturing guidelines, unregulated practices can lead to the contamination of sports supplements with molecules not specified on the product label, including androgens (Thuyne *et al*, 2006). Most regulation of sports supplements occurs after the product is on the market, making it difficult to monitor sports supplements, and in some instances even allow previously banned products to re-enter the market (Cohen *et al*, 2014).

Several reports have demonstrated that the contamination of sports supplements with androgens can occur as a result of cross contamination from manufacturing equipment and transport vessels, as well as contaminated raw ingredients (Judkins *et al*, 2010). Contaminated sports supplements may contain varying concentrations of androgens ranging from trace amounts (ng range/serving) to relatively high, supratherapeutic concentrations (mg range/serving) (Geyer *et al*, 2004 and 2003). There are also reported instances of the intentional addition of androgens to sports supplements. Many marketers of these sports supplements bypass regulatory bodies using unscrupulous strategies including the declaration of the androgen under the guise of a fake or unofficial name (Parr *et al*, 2011a and 2007), or the addition of the androgen without any declaration on the product label (Geyer *et al*, 2008).

The presence of non-declared androgens in sports supplements is a major concern for consumers. Primarily, there are the potential health risks associated with androgen abuse. Several clinical reports have suggested that sports supplements containing androgens can cause serious acute hepatic complications (Khurana and Dasanu, 2014; Nasr and Ahmad, 2009; Shah *et al,* 2008; Jasiurkowski *et al,* 2006). For example, Jasiurkowski *et al* reported the development of severe cholestatic jaundice and IgA nephropathy after an athlete consumed the "recommended" dose of a sports supplement containing the androgen, methasterone (Jasiurkowski *et al*, 2006). Other reports have indicated similar findings with methasterone (Khurana and Dasanu, 2014; Nasr and Ahmad, 2009; Shah *et al,* 2008) as well as other oral-based synthetic androgens such as those found in sports supplements (Agbenyefia *et al,* 2014; Girgis *et al*, 2014; Vilella *et al*, 2013; Wingert *et al,* 2010). Many of these open label androgen-containing supplements "recommend" use of the supplement for short periods of time, usually around 4 to 6 weeks to minimise the risk of adverse side effects (Agbenyefia *et al*, 2014; Vilella *et al*, 2013; Jasiurkowski *et al*, 2006). By contrast, regular sports supplements that do not declare androgens can often be taken indefinitely (Maughan *et al*, 2011). Therefore, non-declared androgens may be unknowingly taken for long periods of time by consumers, increasing the risk of adverse health effects.

Sports supplements with undeclared androgens may also be a problem for athletes that undergo routine or random drug testing. A positive doping test may occur as a result of consuming a sports supplement containing an androgen (Maughan, 2005). Although it is the athletes responsibility to know the contents of all supplements they use, if a supplement contains an undeclared androgen the athlete faces a lengthy legal battle to prove this (Striegel *et al*, 2005). This is a real concern as it has been corroborated by a number of reports. Watson

et al reported that ingestion of 5 µg of 19-norandrostenedione from a "contaminated" creatine supplement resulted in the detection of the metabolite 19-norandrosterone in the urine of 75% (15/20) of volunteers in excess of the WADA 2 ng/ mL threshold. Reducing this to 2.5 µg of 19-norandrostenedione resulted in 25% urine samples testing positive 6 hours after ingestion (Watson *et al*, 2009). Similar findings were also reported by van der Merwe and Grobbelaar and Catlin *et al*, highlighting that even trace contaminations present a high risk for athletes for testing positive in a doping test (van der Merwe and Grobbelaar, 2005; Catlin *et al*, 2000).

During the last decade, several studies have reported that there is a 10-25% prevalence of sports supplements containing undeclared androgens (Plotan *et al*, 2011; Judkins *et al*, 2007; Martello *et al*, 2007; Geyer *et al*, 2004). Many of these studies were based in Europe and North America. Given that the number of products available on the global sports supplement market has increased exponentially over the last few years (Gilard *et al*, 2015), the current prevalence of undeclared androgens in sports supplements is not known. Furthermore, the US recently introduced the Designer Anabolic Steroid Control Act (2014), which targeted a number of newer designer steroids that were being sold on the supplement market, classing them as anabolic steroids which therefore made their sale illegal. Manufacturer's that were selling these products must now cease, which consequently may lead to these products being sold without declaration of the androgen.

To date, no screening of the Australian sports supplement market for the presence of undeclared androgens has been completed. In Australia, sports supplements are regulated by the Therapeutic Goods Administration (TGA) and Food Standards Australia New Zealand (FSANZ) (Brownie, 2005). These two regulatory bodies maintain similar guidelines for sports

supplements to that of the FDA in the US in that they are considered food products and not medicinal. They are still subject to Good Manufacturing Practices (GMP), however, and this may help reduce the risk of contamination (Baylis *et al*, 2001). However, 6.4% of doping violations between 2006 and 2013 in Australia have been associated with supplement use (Outram and Stewart, 2015). Given the 10-25% prevalence of undeclared androgens in the European and US markets coupled with the association of positive doping tests in Australia with sports supplements, it was hypothesized that the Australian sports supplement market may also contain undeclared androgens in products marketed to the public. The aim of this study was to screen sports supplements available on the Australian market for undeclared intrinsic androgenic substances using the *in vitro* yeast cell-based androgen bioassay and the HuH7 mammalian cell-based androgen bioassay to detect proandrogens. The androgenic activity of any positive samples were then tested in the yeast progestogen bioassay to determine cross reactivity with the progesterone receptor.

6.2 Methods

6.2.1 Details of Sports Supplements Tested in This Study

Sports supplements (n=112) were randomly purchased from 7 different stores. Four of these stores were based in Sydney; 2 were based in Queensland; and 1 online US store that sold sports supplements in Australia. The sports supplements included protein powders, amino acids, creatine powders, fat metabolisers, 'testosterone- and growth-hormone boosters', carbohydrates, stimulant/nitric oxide 'pre-workout'-based supplements and vitamin and herbal extracts (Table 6.1). The only inclusion factor for the supplements was that the labelled ingredients did not declare an androgen. The sports supplements were in the form of powders, capsules or tablets. One supplement was a liquid. The sports supplements represented 77 different companies. 11 companies were based in Australia. One company was Canadian and another company was from New Zealand. The remaining companies were based in the US. Five of the US-based companies did market androgens declared in other products, so there is a chance of cross-contamination from manufacturing processes. A supplement which declared the presence of the androgen, DHEA, was purchased in Sydney and was used as an androgen-positive control supplement in this study.

Table 6.1 The type of sports supplements screened in this study. *No. of products tested* denotes the number of products screened belonging to the respective product category. *No. of different companies* shows how many companies these products encompass in that respective product category.

Product category	No. of products tested	No. of different companies
Protein Powders	25	19
Stimulants/Nitric Oxide (Pre-workouts)	24	21
Amino Acids	12	12
Testosterone and Growth Hormone Boosters	18	11
Fat Metabolisers	9	9
Carbohydrates	2	2
Creatine Formulations	6	6
Vitamin and Herbals Extracts	16	16

6.2.2 Steroid Extraction Procedure

The amount of each supplement used for the extraction was dependent upon the manufacturer's recommended serving size (Table 6.2). Steroid extracts were prepared from sports supplements using a standard solid-phase extraction (SPE) method, with minor alterations (Rijk *et al*, 2009). Briefly, capsules were emptied and solid tablets were pulverised using a mortar and pestle. The powders were suspended in water/methanol 1:1 v/v and dissolved by sonication for 5 min. The suspension was then centrifuged at 3000 g for 10 min and the pellet was discarded. The pH of the supernatant was adjusted to 4.8 using 4N acetic acid and sodium acetate. The supernatant then underwent SPE using a C18 column (0.5 g Bond Elut, Agilent Technologies) that was previously conditioned with 4 ml methanol/sodium acetate (pH 4.8). After the sample was loaded, the columns were washed sequentially with 2 mL water, 1.5 mL sodium carbonate (10% w/v), 2 mL water, and 2 mL water/methanol (1:1, v/v). The columns were then air-dried and the sample was eluted with 4 mL acetonitrile. The eluate was concentrated by evaporation then resuspended in 50 µL 100% ethanol.

Table 6.2 Details of sports supplements screened in this study. This table designates a number to each of the supplements tested in this study along with product information including the country of origin, product type, and serving size.

Suppl. No.	Company	Country of Origin	Type	Serving Size	Suppl. No.	Company	Country of Origin	Type	Serving Size
1	1	US	Amino Acids	23 g	**31**		US	Pre-Workout	6.4 g
2	2	US	Creatine	2 capsules	**32**	18	US	Protein Powder	27 g
3	3	US	Testosterone Booster	1 Capsule	**33**	19	US	Pre-Workout	10 g
4		US	Creatine	4 Capsules	**34**	20	New Zealand	Pre-Workout	2 capsules
5		US	Testosterone Booster	3 Capsules	**35**	21	US	Protein Powder	30 g
6		US	Testosterone Booster	1 Capsule	**36**		US	Amino Acids	1.9 g
7	4	Australia	Pre-Workout	15.5 g	**37**	22	US	Pre-Workout	5 g
8	5	US	Creatine	5 g	**38**	23	Australia	Pre-Workout	2 capsules
9	6	Australia	Pre-Workout	12 g	**39**	24	US	Protein Powder	50 g
10	7	US	Testosterone Booster	6 capsules	**40**	25	US	Testosterone Booster	1 capsule
11		US	Testosterone Booster	1 capsule	**41**		US	Testosterone Booster	1 capsule
12	8	Australia	Amino Acids	1 g	**42**	26	US	Pre-Workout	30 g
13		US	Fat Metaboliser	1 capsule	**43**		US	Pre-Workout	2 g
14		US	Protein Powder	35 g	**44**	27	US	Testosterone Booster	10 g
15	9	US	Protein Powder	30 g	**45**	28	US	Amino Acids	1 g
16		US	Protein Powder	23 g	**46**	29	US	Amino Acids	4 tablets
17	10	US	Testosterone Booster	1 capsule	**47**	30	US	Growth Hormone Booster	3 capsules
18		US	Testosterone Booster	1 capsule	**48**	31	US	Protein Powder	45 g
19		US	Testosterone Booster	1 capsule	**49**	32	US	Testosterone Booster	3 capsules
20		US	Pre-Workout	8 g	**50**	33	US	Amino Acids	12 g
21	11	US	Protein Powder	23 g	**51**	34	US	Pre-Workout	13 g
22		US	Protein Powder	23 g	**52**		US	Fat Metaboliser	3 capsules
23	12	Australia	Protein Powder	25 g	**53**	35	US	Fat Metaboliser	4.5 g
24		US	Protein Powder	25 g	**54**	36	Canada	Creatine	5 g
25	13	US	Pre-Workout	5 g	**55**		US	Protein Powder	118 g
26		US	Fat Metaboliser	4 g	**56**		US	Protein Powder	32 g
27	14	US	Pre-Workout	5.7 g	**57**		US	Carbohydrate	40 g
28	15	US	Testosterone Booster	2 capsules	**58**	37	Australia	Creatine	5 g
29	16	US	Amino Acids	14 g	**59**		US	Protein Powder	24 g
30	17	US	Pre-Workout	6.4 g	**60**	38	US	Amino Acids	

Table 6.2 Continued

Suppl. No.	Company	Country of Origin	Type	Serving Size	Suppl. No.	Company	Country of Origin	Type	Serving Size
61		US	Protein Powder	36 g	**87**		US	Creatine	5 g
62		US	Pre-Workout	6 g	**88**	54	US	Protein Powder	30 g
63	39	US	Fat Metaboliser	3 capsules	**89**	55	US	Pre-Workout	3.65 g
64	40	US	Pre-Workout	3 capsules	**90**	56	Australia	Protein Powder	24 g
65	41	US	Amino Acids	9 g	**91**	57	US	Fat Metaboliser	2 capsules
66		US	Protein Powder	36 g	**92**	58	US	Carbohydrate	41 g
67		US	Protein Powder	30.4 g	**93**		US	Pre-Workout	19 g
68		US	Protein Powder	39 g	**94**	59	US	Fat Metaboliser	1 capsule
69	42	US	Testosterone Booster	1 capsule	**95**	60	US	Fat Metaboliser	1.95 g
70	43	US	Pre-Workout	6 g	**96**	61	Australia	Protein Powder	22 g
71		US	Pre-Workout	29 g	**97**	62	US	Vitamins and Herbal Extracts	2 g
72	44	US	Pre-Workout	1 tablet	**98**	63	US	Vitamins and Herbal Extracts	4 g
73		US	Amino Acids	1 tablet	**99**	64	US	Vitamins and Herbal Extracts	7.5 g
74		US	Pre-Workout	1 tablet	**100**	65	US	Vitamins and Herbal Extracts	3 g
75	45	US	Fat Metaboliser	2 capsules	**101**	66	US	Vitamins and Herbal Extracts	3 g
76	46	US	Pre-Workout	14 g	**102**	67	US	Vitamins and Herbal Extracts	12 g
77		US	Protein Powder	27 g	**103**	68	US	Vitamins and Herbal Extracts	10 g
78	47	US	Testosterone Booster	4.4 g	**104**	69	US	Vitamins and Herbal Extracts	11 g
79	48	US	Pre-Workout	8 g	**105**	70	US	Vitamins and Herbal Extracts	4 g
80		US	Growth Hormone Booster	8 g	**106**	71	US	Vitamins and Herbal Extracts	4.5 g
81		US	Testosterone Booster	5 g	**107**	72	US	Vitamins and Herbal Extracts	9 g
82	49	US	Protein Powder	30 g	**108**	73	US	Vitamins and Herbal Extracts	2 capsules
83	50	US	Amino Acids	13 g	**109**	74	US	Vitamins and Herbal Extracts	11 g
84	51	Australia	Protein Powder	29 g	**110**	75	US	Vitamins and Herbal Extracts	12.5 g
85	52	Australia	Protein Powder	75 g	**111**	76	US	Vitamins and Herbal Extracts	5 g
86	53	US	Amino Acids	4 tablets	**112**	77	Australia	Vitamins and Herbal Extracts	50 mL

6.2.3 Yeast Cell Culture

Yeast transformants were grown overnight at 30 °C with orbital shaking (300 rpm) in CSM-leu-ura (androgen bioassay, BIO 101, Inc) or CSM-trp-ura (progestogen bioassay, BIO 101) selective media. The yeast culture was then subcultured in fresh medium and grown until early mid-log phase (OD_{600} = 0.5-0.7 for androgen bioassay; 1.0 for progestogen bioassay) (Methods 2.1.2).

6.2.4 Yeast Cell-Based Androgen and Progestogen Bioassays

For the androgen and progestogen bioassays, yeast from mid-log phase growth was diluted in selective medium to OD_{600} = 0.5-0.7 (CSM-leu-ura; AR) or 1.0 (CSM-trp-ura; PR) plus 100 µM $CuSO_4$ to induce receptor expression via the CUP1 promoter. Yeast cells were then treated with testosterone (T) or progesterone (P) (5 µL per well) concentrations ranging from $7x10^{-5}$ M to $1.3x10^{-10}$ M or $1x10^{-6}$ M to $2x10^{-10}$ M respectively, and yeast cells were incubated overnight at 30 °C with vigorous orbital shaking (300 rpm) before yeast cells were lysed and assayed for β-galactosidase activity, as previously described (Death *et al*, 2004b) (Methods 2.1.2). For sports supplement extracts, yeast cells were treated in duplicate with 5 µL per well in a 24-well plate. Following overnight incubation, yeast cells were lysed and assayed for β-galactosidase activity. The androgenic activation of the sports supplement was determined according to the equation:

$$\text{β-galactosidase activity (Miller units)} = \frac{1000\ x\ OD_{420} - 1.75\ x\ OD_{550}}{Vol.(mL) x\ time\ (min) x\ OD_{600}}$$

6.2.5 HuH7 Cell Culture

Human hepatocarcinoma (HuH7) cells stably co-transfected with the human AR-puromycin and enhancer/ARE/SEAP expression plasmids (Akram *et al*, 2011) were cultured in DMEM (Gibco, Life Technologies) containing high glucose (4500 mg/L), L-glutamine (584 mg/L) and sodium pyruvate (110 mg/L) and supplemented with 10% FCS and puromycin dihydrochloride (5.5 µg/mL). Once confluent, cells were seeded at a concentration of $1x10^5$ cells/mL in 96-well plates with phenol-red free DMEM supplemented with 10% charcoal-stripped FCS, as described (Methods section 2.3.3).

6.2.6 HuH7 Androgen Bioassay

The HuH7 androgen bioassay cells were seeded in 96-well plates ($1x10^5$ cells/mL) and grown in a volume of 200 µL/well phenol red-free DMEM media supplemented with 10% charcoal-stripped FCS and 5.5 µg/mL puromycin dihydrochloride. After 24 hours, cells were treated with T concentrations ranging from $7x10^{-5}$ M to $1.3x10^{-10}$ M and incubated for a further 24 hours. For sports supplement extracts, cells were treated in triplicate with 2 µL/well and also incubated for 24 hours. After incubation, 25 µL of culture supernatant was aliquoted into white opaque 96-well plates before being heated to 65 °C for 35 min to deactivate endogenous alkaline phosphatase. The plate was then cooled on ice for 3 to 5 min before equilibrating to room temperature. SEAP activity was measured by adding 50 µL SEAP reagent (Clontech) and incubating for 35 min at room temperature before measuring luminescence using the Infinite m200 Pro microplate reader (Tecan) (Methods 2.5.3.1).

6.2.7 Decision Limit (CCα) and Detection Capability (CCβ)

To determine the CCα and CCβ of the yeast androgen bioassay, a known androgen-free sports supplement (Supplement 54, a creatine product) spiked with dihydrotestosterone (DHT) was used. Twenty unspiked sports supplement samples and two sets of 20 DHT spiked sports supplement samples were prepared. The DHT concentration in the spiked samples was $2x10^{-9}$ M or $2.8x10^{-7}$ M. The spiked samples underwent SPE and were assayed with either the yeast androgen bioassay or HuH7 androgen bioassay as described above. The CCα was calculated as the mean β-galactosidase (or luminescence) activity of the 20 blank sports supplement samples plus 2.33 times the corresponding standard deviation. The CCβ was calculated as the CCα plus 1.64 times the standard deviation of the mean β-galactosidase (or luminescence) activity of the 20 spiked sports supplement samples (Plotan *et al*, 2012).

6.2.8 Viability Assays

To determine if the sports supplement extracts affected yeast growth, a growth curve was performed. To do this, yeast from mid-log phase growth was diluted in selective medium to OD_{600} = 0.1 and 200 µL was added per well in a 96-well plate. Yeast were then treated in triplicate with 2 µL of T (5 nM) or sports supplement extract, and incubated at 30 °C with orbital shaking (300 rpm). The optical density (OD_{600}) of the yeast was measured every hour for 20 hours to generate a growth curve. The slope (R^2) of the growth curve of the supplement extract was then compared to T using GraphPad Prism 6.0 nonlinear regression analysis.

6.2.9 Statistical Analysis

Unless otherwise indicated, results are presented as mean ± SEM and comparative statistical tests were performed using a Student's t-test.

6.3 Results

6.3.1 Steroid Recovery Evaluation

To assess the recovery of steroids from the extraction procedure, two 1 g portions of a sports supplement (Supplement 54) negative for androgens were spiked with $2.5x10^{-6}$ M or $3.5x10^{-9}$ M DHT at the beginning of the sample preparation, prior to SPE (pre-spike). Two other 1 g portions underwent SPE extraction and after elution with acetonitrile were spiked with either $2.5x10^{-6}$ M or $3.5x10^{-9}$ M DHT (post-spike). The activities of the pre- and post-spike samples were assessed in the yeast androgen bioassay to determine the percent recovery of DHT from the extraction procedure. Figure 6.1 demonstrates that for both $2.5x10^{-6}$ M (high) and $3.5x10^{-9}$ M (low) DHT concentrations there was 100 ± 0.96% and 102 ± 0.97% steroid recovery, respectively. This indicates that the pre-spike androgen bioactivity was equal to that of the post-spike androgen bioactivity for both concentrations demonstrating that there was no androgen loss during the extraction procedure.

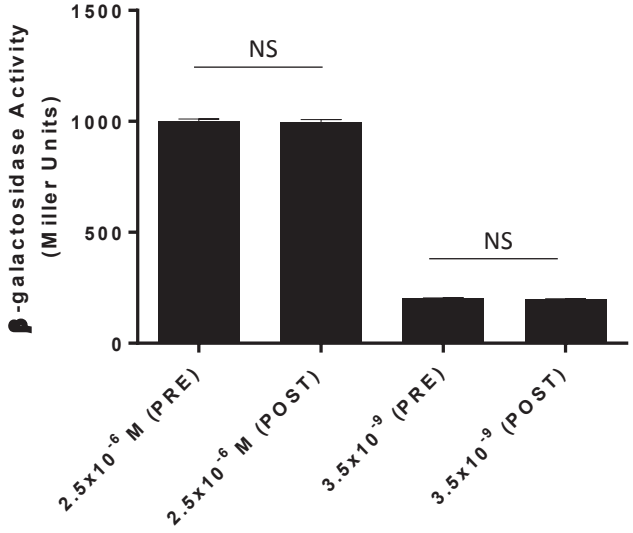


Figure 6.1 Determining the efficacy of the SPE extraction procedure used in this study. 1 g of a sports supplement was spiked with $2.5x10^{-6}$ (high) or $3.5x10^{-9}$ (low) M DHT, prior to extraction (PRE) and after (POST) extraction. The extracts were then assayed in the yeast androgen bioassay. Results are presented as mean ± SEM of 3 independent experiments. The POST extractions were then compared to the PRE extractions using a Student's t-test, where NS = not significant.

6.3.2 Decision Limit (CCα) and Detection Capability (CCβ)

The CCα and CCβ for DHT were determined in the yeast and HuH7 androgen bioassay to determine the minimum DHT concentration needed to elicit a detectable positive response in the androgen bioassays (Table 6.3). Based on the calculated CCβ, DHT can be detected at a minimum concentration of $3.9x10^{-10}$ M or $8x10^{-11}$ M for the yeast or HuH7 androgen bioassay, respectively.

Table 6.3 Yeast and HuH7 androgen bioassay decision and detection limits. The decision limit and detection capability for DHT in the yeast and HuH7 androgen bioassays.

	Bioassay	
	Yeast	**HuH7**
Decision limit (CCα)	4.1 (β-galactosidase Miller units)	19.7 (RLU)
Detection capability (CCβ)	6.7 (β-galactosidase Miller units)	32.3 (RLU)

6.3.3 Androgen Bioactivity Measurements

6.3.3.1 Yeast Androgen Bioassay

The androgen bioactivity of sports supplement extracts was determined using the yeast androgen bioassay. T was used as a reference androgen to show androgenic bioactivity in the bioassay. The EC_{50} of T was previously calculated to be 5 nM (Chapter 3) and was used in this study. A known DHEA supplement was used as a positive control for the extraction procedure of the sports supplement extracts. The DHEA supplement demonstrated approximately 50% bioactivity of T (5 nM) and is in keeping with what was expected as DHEA is known to be a weaker androgen than T (Figure 6.2). This data demonstrated that both the extraction procedure and bioassay were functioning effectively and were capable of detecting androgenic molecules in supplement extracts.

The 112 sports supplement extracts were next tested. Of the 112 extracts, 24 belonged to the 'pre-workout' category and it was found that 3 (Supplement 30, 34 and 94) of these extracts demonstrated significant androgenic bioactivity (Figure 6.2) ($p<0.0001$). Supplements 30, 34,

and 94 will be referred to as supplement B, E and F, respectively for the remainder of this chapter. These three products were from different companies. Eighteen supplements belonging to the 'testosterone and growth hormone-booster' category were also tested, and it was found that 2 (Supplement 5 and 3) of these extracts demonstrated significant androgenic bioactivity (Figure 6.2) ($p<0.0001$). These supplements will be referred to as supplement A and D, respectively for the remainder of this chapter. These two supplements were from the same company. A third supplement (supplement 4) from this company, a creatine product, also demonstrated significant androgenic bioactivity ($p<0.0001$) and represented 1/6 of the creatine products tested in this study (Figure 6.2). Supplement 4 will be referred to as supplement C for the remainder of this chapter. There were 0 products that demonstrated androgenic bioactivity from the protein (0/25), amino acids (0/12), carbohydrates (0/2), fat metaboliser (0/9) or vitamin and herbal extracts (0/16) categories.

Together, the 6 positive supplement extracts represent 4 different companies. Five of the supplements (A-D, F) were manufactured by companies based in the US, while 1 supplement (supplement E) was manufactured by a company based in New Zealand. There were 0 products (out of 11) manufactured in Australia that tested positive.

It should be noted that 3/112 supplement extracts led to yeast cytotoxic effects that meant the androgenic bioactivity of these extracts could not be reliably assayed, as the R^2 values from the growth curve assay were significantly different ($p<0.0001$) from the T control (Figure 6.3). These 3 extracts were supplement 91 (fat metaboliser), 49 (testosterone-booster) and 62 (pre-workout).

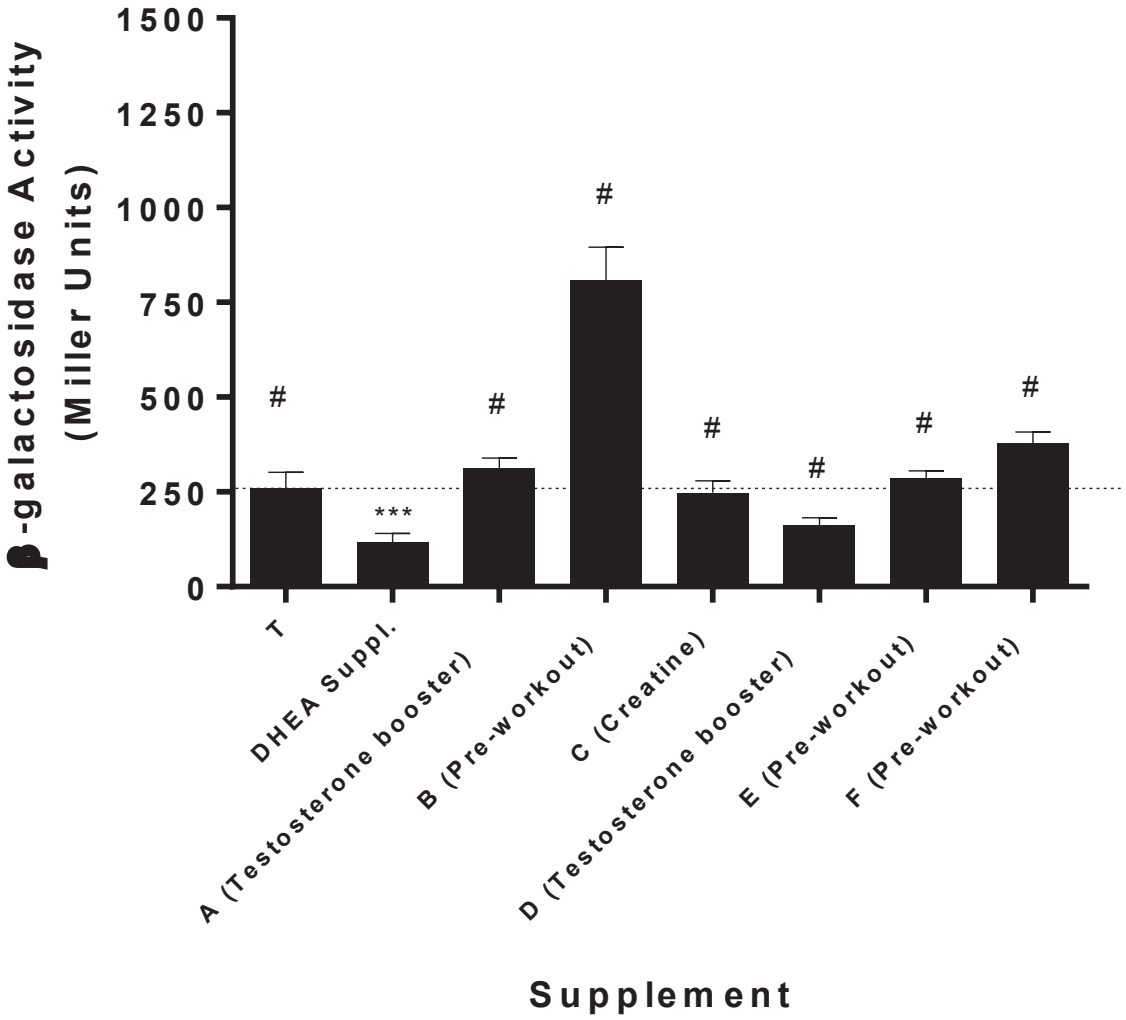


Figure 6.2 Bioactivity of sports supplement extracts as measured in the yeast androgen bioassay. Sports supplement extracts were tested for bioactivity using the yeast androgen bioassay. Results are presented as mean ± SEM of 3 independent experiments. T (5 nM, EC_{50} concentration) was included as a reference value while the DHEA-containing supplement (DHEA suppl.) was included as an androgen positive control supplement. *** = $p<0.001$; # = $p<0.0001$.

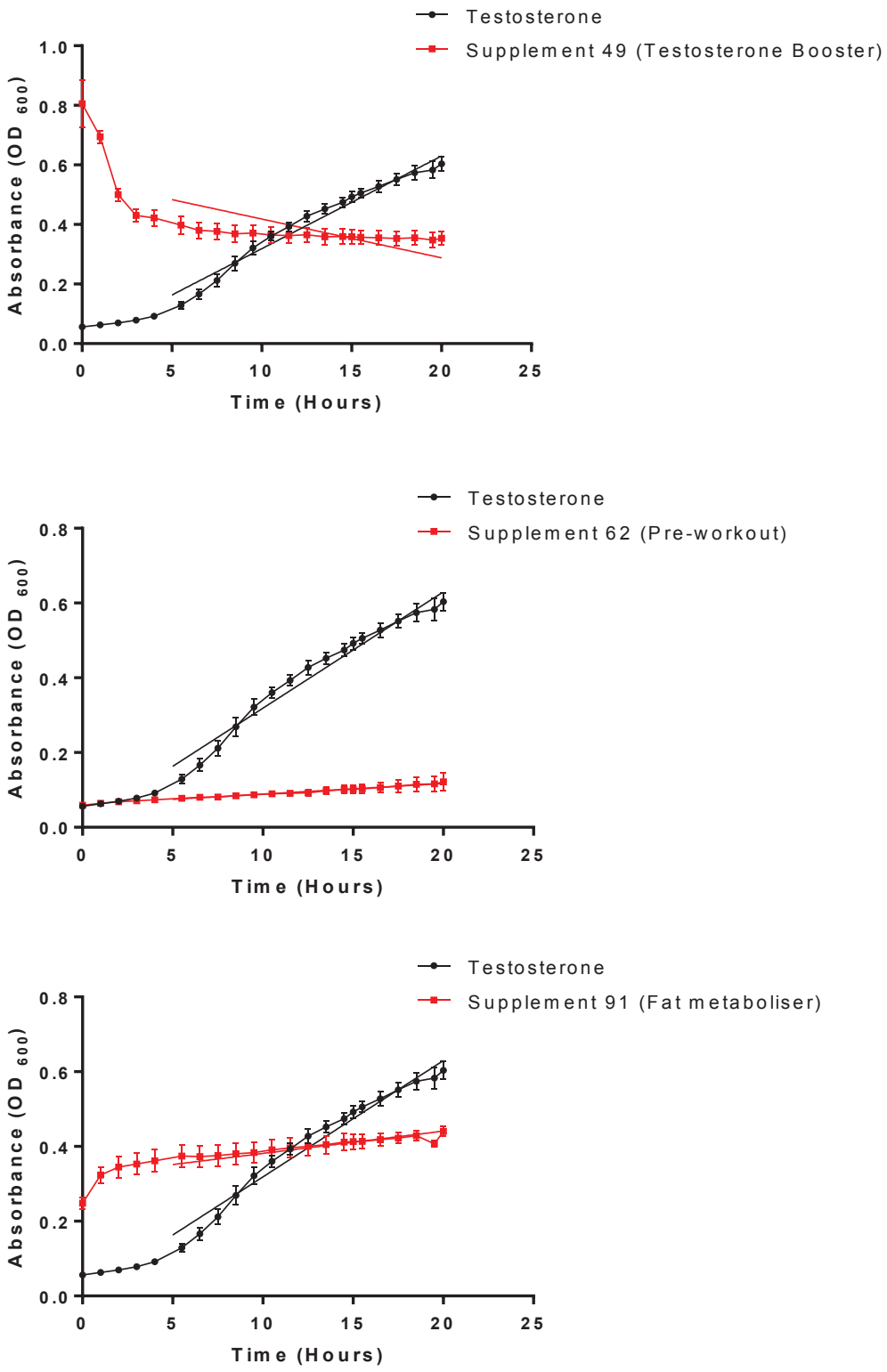

Testosterone
Supplement 49 (Testosterone Booster)
Absorbance (OD 600)
Time (Hours)
Testosterone
Supplement 62 (Pre-workout)
Absorbance (OD 600)
Time (Hours)
Testosterone
Supplement 91 (Fat metaboliser)
Absorbance (OD 600)
Time (Hours)

Figure 6.3

Figure 6.3 Viability growth curves of supplement extracts 49, 62 and 91. Sports supplement extracts were tested for toxicity in the yeast androgen bioassay. Results are presented as mean ± SEM of 3 independent experiments. Due to the lag period of growth, the gradients were calculated from 5 hours onwards. The R^2 of the supplement extract was compared to T.

6.3.3.2 HuH7 Androgen Bioassay

Sports supplements may be contaminated with proandrogens that are not intrinsically potent because they require metabolic activation (Rijk *et al*, 2008). Such proandrogens would be difficult to detect in the yeast androgen bioassay. By contrast, the HuH7 liver cells express steroid metabolising enzymes and therefore increase the chance for the detection of proandrogens (Akram *et al*, 2011). Furthermore, the HuH7 androgen bioassay can be used to test if the positive supplement extracts from the yeast androgen bioassay are metabolised into stronger androgens or inactivated to weak androgens by liver cell metabolism.

Figure 6.4 shows that the DHEA-containing supplement was positive for androgenic bioactivity in the HuH7 androgen bioassay, as expected. Of the 112 extracts, 5 were positive for androgenic bioactivity, while the remaining supplement extracts did not display any androgenic bioactivity (Figure 6.4). The 5 extracts that tested positive in the HuH7 androgen bioassay were 5/6 positive extracts from the yeast androgen bioassay screen. Strikingly, 4/5 of these supplements were markedly androgenic, with androgenic bioactivity stronger than T. Interestingly, supplement F showed no androgenic bioactivity in the HuH7 androgen bioassay, despite being positive in the yeast androgen bioassay. This suggests the androgen contained in supplement F was deactivated by liver cell metabolism.

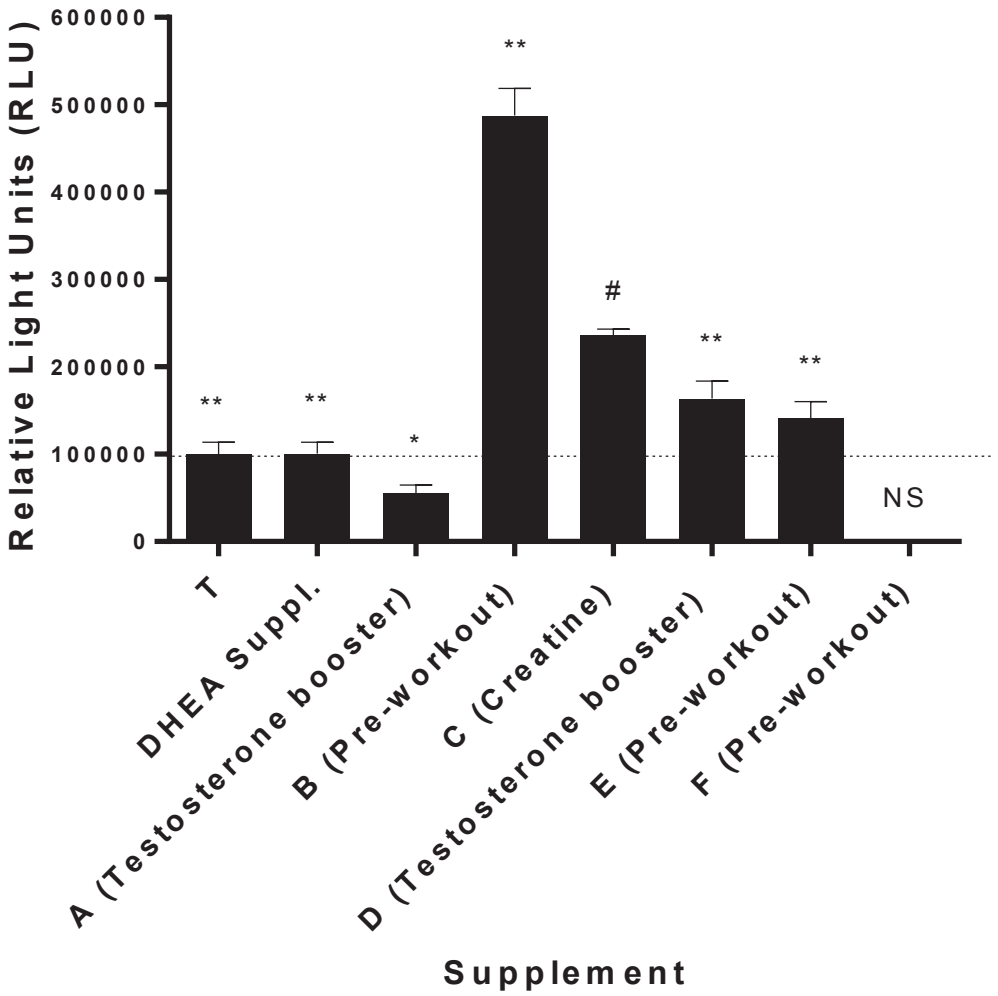


Figure 6.4 Bioactivity of sports supplement extracts as measured in the HuH7 androgen bioassay. One hundred and twelve sports supplement extracts were tested for AR bioactivity in the HuH7 androgen bioassay. T (5 nM, EC_{50} concentration) was included as a reference value while the DHEA-containing supplement (DHEA Suppl.) was included as an androgen-positive control supplement. Results are presented as mean ± SEM from triplicates of one experiment. * = $p < 0.05$, ** = $p < 0.01$, # = $P < 0.0001$, and NS = not significant.

6.3.4 Progestogen Bioactivity Screen

6.3.4.1 PR Dose-Response Curve

To establish a reference value of progestogenic activity for comparison to sports supplement extracts in the yeast progestogen bioassay, the EC_{50} of progesterone was required. To determine the EC_{50} value of progesterone, a dose-response curve was performed with progesterone concentrations ranging from $1x10^{-6}$ M to $2x10^{-10}$ M. The EC_{50} was determined to be 3.8 nM (Figure 6.5).

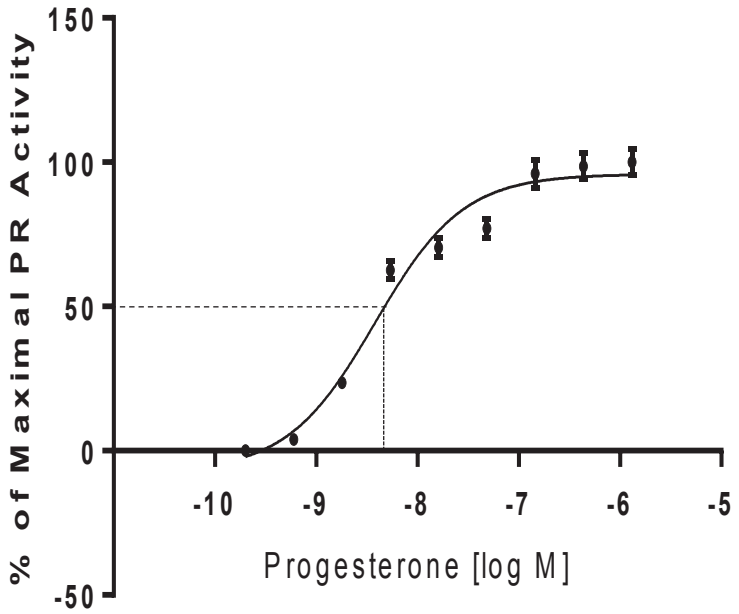


Figure 6.5 Progesterone dose-response curve. A sigmoidal-dose response curve was used to determine the EC_{50} of progesterone in the yeast progestogen bioassay over a concentration range of $1x10^{-6}$ M to $2x10^{-10}$ M. Results are presented as mean ± SEM from 6 independent experiments.

6.3.4.2 Yeast Progestogen Bioassay

The AR and PR show high homology at the protein level, especially in the ligand binding domain (Ojasoo *et al*, 1987). This results in progesterone and progestins able to bind and activate AR. Therefore, to ensure AR activation as detected in the androgen bioassays was by an androgen rather than a progestin (or progesterone), the positive supplement extracts were tested using a yeast progestogen bioassay. Of the 6 supplement extracts tested, supplements B to E did not demonstrate any PR bioactivity (Figure 6.6). Supplement A showed very weak progestogenic activity (compared to the EC_{50} of progesterone), while supplement F showed relatively strong progestogenic activity, albeit still only about 70% of the bioactivity of elicited by the EC_{50} concentration of progesterone.

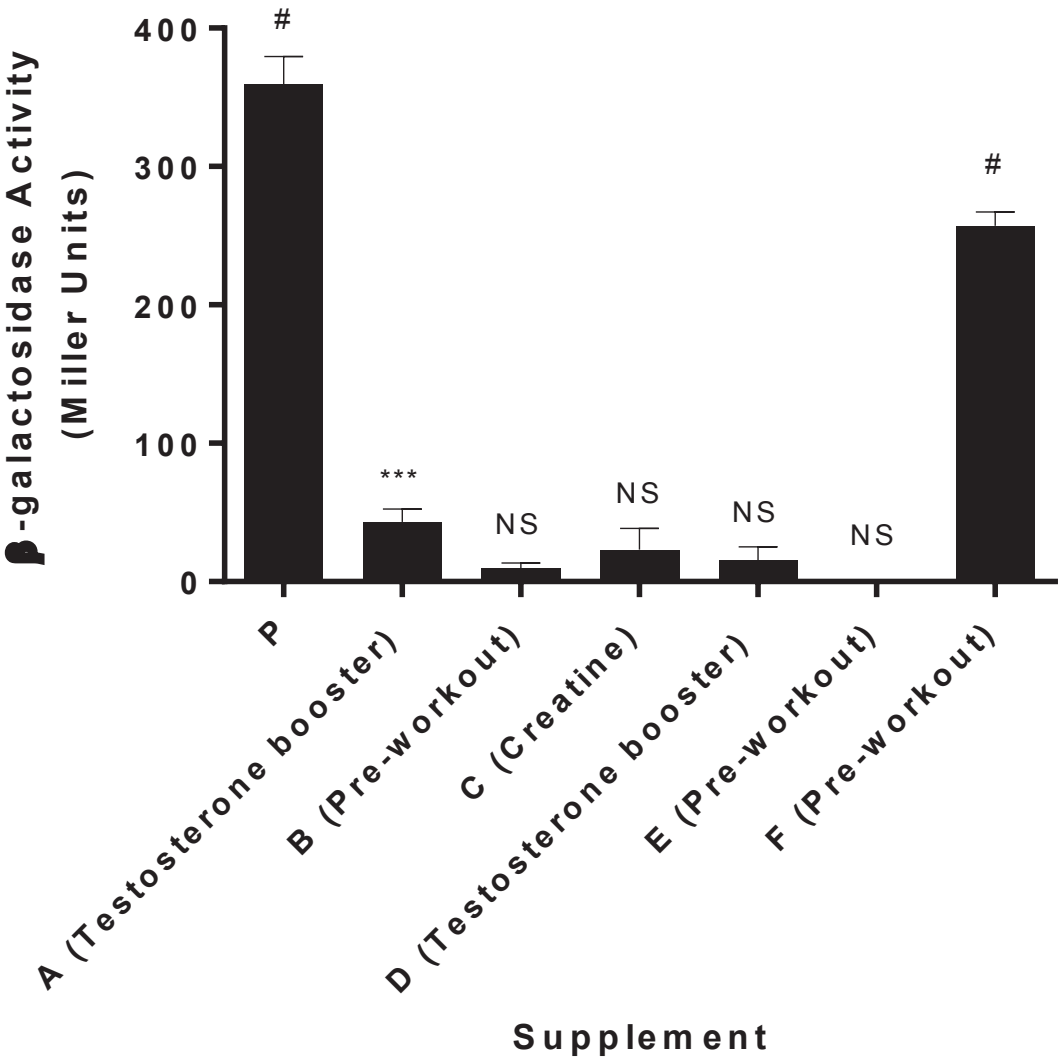


Figure 6.6 Bioactivity of sports supplement extracts as measured in the yeast progestogen bioassay. Sports supplement extracts that were positive in the yeast androgen bioassay were tested for PR activation in the yeast progestogen bioassay. Results are presented as mean ± SEM from 3 independent experiments. P (3.8 nM, EC_{50} concentration) was included as a positive control. *** = p <0.001, # = P <0.0001, and NS = not significant.

6.4 Discussion

This study shows for the first time that 5.4% (6/112) sports supplements marketed to the Australian population contain androgenic molecules, despite no declaration of an androgen on the product label. Most supplements (74/112) tested were purchased over the counter in Sydney-based stores while others (38/112) were purchased from online stores including two from Queensland and one US-based online store. A tandem screening approach was used based on the yeast- and HuH7 androgen bioassays. The yeast androgen bioassay detected 6 supplement extracts that were capable of intrinsically activating AR. Of these 6 supplement extracts, 5 remained potent activators of AR after steroid metabolism in the HuH7 androgen bioassay. One supplement extract was deactivated after steroid metabolism in the HuH7 androgen bioassay. There were no additional intrinsically weak androgenic supplement extracts that were detected in the HuH7 androgen bioassay after metabolism. The finding of 6 intrinsically potent androgenic supplement extracts has important implications for users of sports supplements because of potential health issues and the risk of a positive doping test.

The most important findings of this study is that extracts from 6/112 sports supplements had potent androgenic activity. High concentrations of androgens in sports supplements have potential health risks for consumers. Oral androgens are reported to cause liver complications such as jaundice and cholestasis, hepatic adenomas and haemorrhages, hepatocellular necrosis and hepatitis (Khurana and Dasanu, 2014; Nasr and Ahmad, 2009; Shah *et al,* 2008; Jasiurkowski *et al,* 2006). There have been several reports of acute hepatic complications due to the consumption of the "recommended" dose of androgen-containing sports supplements (Agbenyefia *et al*, 2014; Nasr and Ahmad, 2009; Jasiurkowski *et al,* 2006). Although the concentrations (or identity) of the androgenic molecules found within the 6 positive sports

supplements in this study are unknown, the fact that they would be detected by the androgen bioassay suggest they are at a concentration greater than nM range and the potential health risks for consumers should be of concern.

Androgens in sports supplements also raise the risk for a urine sample to test positive for a banned substance during routine drug testing. There have been several reports of contaminated sports supplements leading to a positive drug test. Merwe and Grobbelaar showed that consumption of 1 capsule of a sports supplement containing 8.4 – 31.8 µg/capsule of 19-nor-androstenedione and 0.1 – 0.4 µg/ capsule of 4-androstenedione would lead to a positive test for the detection of the metabolite of 19-nor-4-androstenedione, 19-norandrosterone, for up to 36 hours (Merwe and Grobbelaar, 2005). Similarly, Catlin *et al* showed that ingestion of a sports supplement contaminated with 10 µg of 19-norandrostenedione would cause a positive urine sample under IOC guidelines (2 ng/ mL for males) for 20 out of 24 athletes for up to 8 hours post consumption (Catlin *et al*, 2000).

The 112 sports supplements tested show a lower rate of detection of undeclared androgens than what has been previously reported for the USA- and European markets (Judkins *et al*, 2007; Martello *et al*, 2007; Geyer *et al*, 2004). In 2004, Geyer *et al* reported that the countries with the highest prevalence of undeclared androgenic molecules in 'non-hormonal' products are the Netherlands, Austria, the UK, the US and Italy, with percentages of 25.8%, 22.7%, 18.9%, 18.8% and 14.3%, respectively (Geyer *et al*, 2004). However, not all countries had high rates, and were in keeping with the finding of this study of 5.4%. Belgium, France, and Norway all reported lower than 7% prevalence rates, albeit only 30 products were tested from each country. Similarly, van der Merwe and Grobbelaar found 6.7% (2/30) supplements contained undeclared androgens in the South African market (Merwe and Grobbelaar, 2004). Other

countries such as Switzerland, Sweden and Hungary showed no undeclared androgens, although only 13, 6, and 2 products each, respectively, were tested (Geyer *et al*, 2004) and this may under represent the real situation. This underrepresentation is evidenced by a later study in Switzerland showing that 4.6% (3/65) of supplements tested contained undeclared androgens (Baume *et al*, 2006).

The data above represents a screen of US and several European markets from over a decade ago, before the ban of sale of prohormones in the US. Given that the sale of prohormones in sports supplements has been banned since 2004, the frequency of contamination in sports supplements may be lower. However, there have been few large scale supplement screens in recent years. Determining the prevalence of contaminated or undeclared androgens in sports supplements is therefore difficult. In 2007, Martello *et al* reported that 10% (6/60) supplements purchased in Italy were positive for an androgenic molecule, showing a similar, but lower rate of detection than in the earlier Geyer *et al* study for the Italian market (Martello *et al*, 2007). Alarmingly, Judkins *et al* reported that 25% (13/52) supplements purchased in the US contained an undeclared androgen using GC-MS methods; which is higher than the previous estimate by Geyer *et al* for the US (Judkins *et al*, 2007). More recently, Plotan *et al* reported that 19% (12/63) supplements purchased on the Irish market contained undeclared androgenic molecules detected using an AR bioassay (Plotan *et al*, 2011). Therefore, the 5.4% of positive supplements detected in this study appears to be low compared to that reported for other countries.

The yeast androgen bioassay is appropriate for detecting intrinsically potent androgens such as T or DHT due to the lack of steroid metabolising enzymes. The yeast androgen bioassay has been previously used to detect potent androgens in a variety of matrices, including food

supplements (human and animal feed) (Akram *et al*, 2011; Rijk *et al*, 2009 and 2008), hair samples (Becue *et al*, 2011), and environmental samples (Chou *et al*, 2015; Pickford *et al*, 2015). It therefore served as a suitable primary screen for androgenic substances in sports supplements for this current study. The results suggest that six products from the Australian market contained intrinsically potent androgenic molecules. This was confirmed by the HuH7 androgen bioassay where 5/6 products still showed potent androgenic activity, even after being subjected to liver cell metabolism. The remaining steroid extract was deactivated indicating the non-declared steroid is readily metabolised to inactive metabolites. Of the 5 that remained potent, 4 of them increased in potency with metabolism suggesting the formation of strong androgenic metabolites.

The combined data from the dual androgen bioassay approach provides more information than either bioassay alone. For example, the liver cell metabolism allows insight into the potential activation or inactivation of the androgenic molecule as may happen *in vivo*. By contrast, the yeast cell provides intrinsic androgenic potency, something that can be missed with the liver cell assay due to the rapid formation of inactive metabolites. Although in the supplements tested no proandrogens were detected, the HuH7 androgen bioassay has previously been shown by our laboratory to successfully identify oxyguno (4-chloro-17α-methyl-etioallochol-4-ene-17β-ol-3,11-dione) as a proandrogen (Akram *et al*, 2011).

The limitations of the current study include the lower sensitivity of detection of the androgen bioassays. The detection limit for the bioassays is in the nM range, whereas for conventional GC-MS-based techniques, detection is in the order of pM. Therefore, GC-MS is much more likely to detect trace amounts of contaminating androgens. However, typically, deliberately added androgens are at higher concentrations than what would be expected from

contamination of a sports supplement (Geyer *et al*, 2003; Parr *et al*, 2007). Geyer *et al* reported that more than 28% (27/94) positive supplements contained between 0.01 and 0.2 µg /g supplement (Geyer *et al*, 2004). Similar concentrations were reported by van der Merwe and Grobbelaar (van der Merwe and Grobbelaar, 2004). These low concentrations, when diluted during extraction and application in the bioassay, will not be detected by the yeast and HuH7 androgen bioassays. This however, gives more importance to the positive findings in this study because to be detected they need to be at greater than nM concentrations. Deliberate addition of androgens to sports supplements have been reported to be as high as 16.8 mg/ g (Parr *et al*, 2007) and 28.93 mg/ g (Geyer *et al*, 2003) of supplement. Further testing is required to determine the concentrations of the androgenic molecules as well as several batches of each supplement in order to confirm if the androgenic molecules were deliberately added. A further limitation of the study is that the identity of the androgenic molecule is unknown as well as the exact concentration.

6.5 Summary and Conclusions

In conclusion, marketed sports supplements in Australia may contain undeclared androgenic molecules. This may be an issue for elite athletes, or other athletes that consume these inadvertently, due to the potential of testing positive for banned substances (Merwe and Grobbelaar, 2005; Thuyne and Delbeke, 2004). There are also a number of potential health risks associated with androgens (Agbenyefia *et al*, 2014; Girgis *et al*, 2014; Vilella *et al*, 2013; Wingert *et al*, 2010), highlighting the danger with their inadvertent consumption. This is an issue that authorities and consumers need to be aware of given the increase in popularity of

sports supplements over recent years. Future work needs to be done in order to identify the structure of the androgenic molecules that are responsible for the positive results.

Chapters 7

Conclusions

7.1 Summary, Conclusions and Limitations

Androgens are the most widely abused prohibited substances in sports. The extent of androgen abuse spans from elite Olympic athletes to amateur athletes, both adults and adolescents (Dunn and White, 2011; Leifman *et al*, 2011). Detection of androgens for anti-doping purposes involves gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (LC)-based methods (Abushareeda *et al*, 2014). These techniques require structural knowledge of the test compound in order to be detected. This criteria led to the development of 'designer' steroids with novel, unknown structures for abuse by athletes in sports to avoid detection.

Designer steroids first appeared in doping samples in the early 2000s, with the detection of norbolethone in 2002 (Catlin *et al*, 2002) and tetrahydrogestrinone in 2004 (Catlin *et al*, 2004). These designer steroids were synthesised and used in a clandestine fashion by select athletes. The use of designer steroids has since evolved to their incorporation in sports supplements that are mass produced and available to the general public. Many of these designer steroids were initially synthesised in the 1950s and 1960s for clinical purposes, but were never marketed. There are also several novel designer steroids that have only ever been detected in sports supplements.

There is limited data on the safety and efficacy of designer androgens. Numerous deleterious side effects, including cholestatic jaundice, (Agbenyefia *et al*, 2014; Nasr and Ahmad, 2009; Jasiurkowski *et al*, 2006) have been associated with the consumption of sports supplements containing designer androgens. Furthermore, it is often not known if consumption of these designer androgens results in a beneficial anabolic effect. These sports supplements are of

major concern for anti-doping authorities due to the ease of access of these products for athletes.

Also of concern is that some sports supplements may contain androgens without any indication on the product label. Several studies have reported sports supplements may contain unlabelled androgens (Thuyne *et al*, 2006). The origin of these androgens may be through accidental contamination of low concentrations (Geyer *et al*, 2004), or deliberate additions of high concentrations of androgens (Parr *et al*, 2007). The presence of unlabelled androgens is therefore of concern to the general public and also anti-doping authorities due to the potential health risks associated with androgen abuse and the potential for a positive doping test.

The main aim of this thesis was to assess the androgenic and anabolic activity of designer steroids derived from sports supplements using *in vitro* androgen bioassays.

Chapter 3 assessed the androgenic bioactivity of 22 designer steroids derived from sports supplements using the *in vitro* yeast androgen bioassay. The yeast androgen bioassay uses *Saccharomyces cerevisiae* as the host strain, which does not endogenously express steroid receptors or steroid metabolising enzymes. This allowed for the determination of intrinsic androgenic bioactivity of the test compounds. This study demonstrated that 45% of the designer steroids had potent intrinsic androgenic bioactivity.

Chapter 4 assessed the androgenic bioactivity of these 22 designer steroids using the *in vitro* HuH7 androgen bioassay. The HuH7 cell line is a human liver cell line and was used as a host cell line for this bioassay due to the presence of steroid metabolising enzymes. This therefore allowed the assessment of androgenic bioactivity after metabolism. Complimenting Chapter 3, this study demonstrated that H-Drol was activated into a potent androgen, while 6 other

steroids remained potent androgens. Together, Chapter 3 and Chapter 4 demonstrated that sports supplements may contain potent androgens, freely available to athletes and the general public.

The hypothesis that these potent androgens would have an anabolic effect on skeletal muscle was addressed in Chapter 5. Chapter 5 assessed the anabolic potential of 5 designer steroids which displayed strong AR bioactivity after metabolism in a mouse C2C12 myoblast cell model. This chapter demonstrated that H-Drol, Ultradrol, P-Plex, Mechabol and The One (Hydrazone) had anabolic activity in the C2C12 myoblast model, through increases in myotube hypertrophy, myonuclei accretion, and MHC expression.

Finally, Chapter 6 determined the prevalence of unlabelled androgenic substances in sports supplements available to the Australian public. Sports supplements underwent solid-phase extraction for steroidal compounds and the extracts were then tested in the yeast androgen bioassay. This study demonstrated that 5.3% (6/112) sports supplements were positive for androgenic activity. When the extracts were tested in the HuH7 androgen bioassay, 5 of these extracts were activated further, whilst one supplement extract was deactivated. The results of this chapter suggest that while the percentage of positive findings was low, it is still of concern for the general public and athletes due to the possible positive doping test and also the potential side effects associated with androgen abuse.

There are a number of limitations to the studies undertaken in this thesis which may be addressed in future work. Firstly, while the C2C12 myoblast model is a suitable screen for determining the anabolic potential of steroids, the full anabolic potential cannot be established. For this to be determined, *in vivo* models need to be used. Thus, future work may entail *in vivo* models with the designer androgens that demonstrated high AR bioactivity, and

high anabolic activity in the C2C12 model. This data may help validate the C2C12 model in identifying steroids with anabolic activity.

Secondly, more sports supplements can be screened for unlabelled androgenic substances to achieve greater power for the study in Chapter 6. Although the number of sports supplements screened was larger than many other similar studies conducted in other countries, the sports supplement market is an ever increasing market. Therefore, increasing the sample size of products tested may give a more accurate representation of the prevalence of androgenic substances present in sports supplements. Additionally, only one batch of each supplement was tested. It would be useful to test multiple batches of those supplements that tested positive. This would help determine if the positive findings were consistently identified across multiple batches, and help determine if the androgenic substances were deliberately added to the sports supplements.

Furthermore, the androgenic substances detected in the sports supplement extracts were not identified in this study. Further work can be done to determine the identity and concentrations of the compounds responsible for eliciting the androgenic response. This may identify a novel compound that is being added to sports supplements. Determining the concentration of the compounds may also help determine if the compounds were deliberately added or were likely an accidental contamination, as the concentrations would be checked across various batches (if present).

In summary, some sports supplements freely available to the general public may contain designer steroids. These designer steroids have very limited data concerning safety and efficacy. The data presented in this thesis suggests that several of these designer steroids are potent androgens, with high AR bioactivity and anabolic activity in a C2C12 myoblast cell

model. Furthermore, the Australian market may contain a small percentage of androgenic substances not identified on the product label. These findings should be of concern to anti-doping authorities, as well as the Australian public.

Although GC-MS-based techniques are highly sensitive, they are not suitable for identifying novel compounds, such as designer steroids, as part of routine doping control. Biological-based assays such as the androgen bioassay are more suited to detecting the presence of unknown androgenic compounds due to not being reliant on knowing chemical structure. The bioassay could therefore work in parallel to chemical-based detection methods to identify novel androgenic compounds. Unfortunately, however, the androgen bioassay in its current format is not suited to high throughput screening of samples. Furthermore, in light of these findings, it may be possible for supplement companies to produce new supplements containing micro-doses of novel designer androgens which would be undecetectable in the androgen bioassay. Although these doses would likely not result in a beneficial anabolic effect for the consumer, it may still cause a positive doping test (once the novel steroid had been identified) as well potential harmful side-effects. Therefore, there is a need to develop an assay that is more sensitive than the current bioassays in use. More importantly, androgen bioassays need to be more high throughput in order for them to be successfully incorporated into routine anti-doping screening.

Furthermore, this thesis demonstrates the need for further regulation of the sports supplement market. In most countries, sports supplements are classed as food products and not medicinal products, and therefore the manufacturing guidelines are not as strict (Brownie *et al*, 2005). This is also true with Australian manufacturing standards of sports supplements (Brownie *et al*, 2005). The overt addition of potent androgens in sports supplements

(Chapters 3, 4 and 5) demonstrates the ease of which steroids may be incorporated into sports supplements and sold to the general public. Although there have been recent legislative attempts at prohibiting this, such as the Designer Anabolic Steroid Control Act of 2014 in the United States, there are little preventative measures of these products being manufactured and sold. The results of Chapter 6 further augment the need for tighter regulation in the manufacturing of sports supplements.

Finally, it is important for athletes and the general public to be made more aware of the potential risks associated with sports supplements. Better educational programs should emphasise to both athletes and general public that sports supplements may contain androgens and that not only do athletes risk testing positive for a banned substances, but also of the serious potential health risks associated with androgen use.

It can thus be concluded that androgen bioassays are suitable for detecting designer androgens due to activation of the androgen receptor and are not reliant on knowing the chemical structure like GC-MS-based methods. Sports supplements available on the Australian market may contain potent androgens, and athletes and the general public should be aware of the potential risks of sports supplements.

Chapter 8

References

1. 44456 Federal Register, **Classification of two steroids, prostanozol and methasterone, as schedule III anabolic steroids under the Controlled Substances Act**, Vol. 77, No. 146. Monday July 30th, 2012. https://www.federalregister.gov/articles/2012/07/30/2012-18495/classification-of-two-steroids-prostanozol.

2. Abbate V, Kicman AT, Evans-Brown M, McVeigh J, Cowan DA, Wilson C, Coles SJ, Walker CJ, 2014, **Anabolic steroids detected in bodybuilding dietary supplements – a significant risk to public health,** *Drug Testing and Analysis*, 7: 609-618.

3. Abushareeda W, Fragkaki A, Vonaparti A, Angelis Y, Tsivou M, Saad K, Krajem S, Lyris E, 2014, **Advances in the detection of designer steroids in anti-doping***, Bioanalysis*, 6 (6): 881-896.

4. Acevedo P, Jorge JC, Cruz-Sanchez A, Amy E, Barreto-Estrada L, 2011, **A Ten-year assessment of anabolic steroid misuse among competitive athletes in Puerto Rico***, West Indian Medical Journal*, 60 (5): 531-535.

5. Agbenyefia P, Arnold CA, Kirkpatrick III R, 2014, **Cholestatic jaundice with the use of methylstenbolone and dymethazine, designer steroids found in Super DMZ Rx 2.0 "nutritional supplement": A case report***, Journal of Investigative Medicine High Impact Case Reports* 2 (2): 1-4.

6. Ahlgrim C, Guglin M, 2009, **Anabolic and cardiomyopathy in a bodybuilder: Case report and literature review***, Journal of Cardiac Failure*, 15 (6): 496-500.

7. Ahtiainen JP, Hulmi JJ, Kraemer WJ, Lehti M, Nyman K, Selänne H, Alen M, Pakarinen A, Komulainen J, Kovanen V, Mer AA, Häkkinen K, 2011, **Heavy resistance exercise training and skeletal muscle androgen receptor expression in younger and older men***, Steroids*, 76: 183-192.

8. Akerstrom T, Laub L, Vedel K, Brand CL, Pedersen BK, Lindqvist AK, Wojtaszewski JF, Hellsten Y, 2014, **Increased skeletal muscle capillarization enhances insulin sensitivity**, *American Journal of Physiology Endocrinology & Metabolism*, 307: E1105-E1116.

9. Akram ON, Bursill C, Desai R, Heather AK, Kazlauskas R, Handelsman DJ, Lambert G, 2011, **Evaluation of androgenic activity of nutraceutical-derived steroids using mammalian and yeast in vitro androgen bioassays**¸ *Analytical Chemistry*, 83: 2065-2074.

10. van Amsterdam J, Opperhuizen A, Hartgens F, 2010, **Adverse health effects of anabolic-androgenic steroids**, *Regulatory Toxicology and Pharmacology*, 57: 117-123.

11. Amthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R, Patel K, 2004, **Follistatin complexes myostatin and antagonises myostatin-mediated inhibition of myogenesis**, *Developmental Biology*, 270: 19-30.

12. Araki N, Ohno K, Takyoshi M, Lida M, 2005, **Evaluation of a rapid in vitro androgen receptor transcriptional activation assay using AR-EcoScreen cells**, *Toxicology in Vitro*, 19: 335-352.

13. Arner P, 2005, **Effects of testosterone on fat cell lipolysis. Species differences and possible role in polycystic ovarian syndrome**, *Biochimie*, 87: 39-43.

14. Auld DS, Southall NT, Jadhav A, Johson RL, Diller DJ, Simeonov A, Austin CP, Inglese J, 2008, **Characterization of chemical libraries for luciferase inhibitory activity**, *Journal of Medicinal Chemistry*, 51: 2372-2386.

15. Bagia A, Hewitt PM, Morris DL, 2000, **Anabolic steroid-induced hepatic adenomas with spontaneous haemorrhage in a bodybuilder**, *Australian and New Zealand Journal of Surgery*, 70: 686-687.

16. Balagopal P, Rooyackers OE, Adey DB, Ades PA, Nair KS, 1997, **Effects of aging on in vivo synthesis of skeletal muscle myosin heavy-chain and sarcoplasmic protein in humans**, *The American Journal of Physiology*, 273 (4): E790-800.

17. Barceloux DG, Palmer RB, 2013, **Anabolic-Androgenic Steroids,** *Disease-a-Month*, 59: 226-248.

18. Barroso O, Mazzoni I, Rabin O, 2008, **Hormone abuse in sports: the antidoping perspective,** *Asian Journal of Andrology*, 10 (3): 931-402.

19. Basaria S, Collins L, Dillon EL, Orwoll K, Storer TW, Miciek R, Ulloor J, Zhang A, Eder R, Zientek H, Gordon G, Kazmi S, Sheffield-Moore M, Bhasin S, 2013, **The safety, pharmacokinetics, and effects of LGD-4033, a novel nonsteroidal oral, selective androgen receptor modulator, in healthy young men**, *Journal of Gerontology: Medical Sciences*, 68 (1): 87-95.

20. Basualto-Alarcon C, Jorquera G, Altamirano F, Jaimovich E, Estrada M, 2013, **Testosterone signals through mTOR and androgen receptor to induce muscle hypertrophy**, *Medicine & Science in Sports & Exercise*, 45 (9): 1712-1720.

21. Baume N, Mahler N, Kamber M, Mangin P, Saugy M, 2006, **Research of stimulants and anabolic steroids in dietary supplements**, *Scandinavian Journal of Medicine & Science in Sports*, 16: 41-48.

22. Baylis A, Cameron-Smith D, Burke LM, 2001, **Inadvertent doping through supplement use by athletes: Assessment and management of the risk in Australia**, *International Journal of Sport Nutrition and Exercise Metabolism*, 11: 365-383.

23. Beck V, Reiter E, Jungbauer A, 2008, **Androgen receptor transactivation assay using green fluorescent protein as a reporter**, *Analytical Biochemistry*, 373: 263-271.

24. Beckett AH, Cowan DA, 1978, **Misuse of drugs in sport**, *British Journal of Sports Medicine*, 12 (4): 185-194.

25. Becue I, Bovee TFH, Van Poucke C, Groot MJ, Nielen MWF, Van Peteghem C, 2011, **Applicability of a yeast bioassay in the detection of steroid esters in hair**, *Analytical Bioanalytical Chemistry*, 399: 1031-1039.

26. Behre HM, Tammela TLJ, Arver S, Tolra JR, Bonifacio V, Lamche M, Kelly J, Hiemeyer F, Giltray EJ, Gooren LJ, 2012, **A randomized, double-blind, placebo controlled trial of testosterone gel on body composition and health-related quality-of-life in men with hypogonadal to low-normal levels of serum testosterone and symptoms of androgen deficiency over 6 months with 12 months open-label follow up**, *The Aging Male*, 15 (4): 198-207.

27. Bellamy LM, Joanisse S, Grub A, Mitchell CJ, McKay BR, Phillips SM, Baker S, Parise G, 2014, **The acute satellite cell response and skeletal muscle hypertrophy following resistance training**, *PLOS One*, 9 (10): e109739.

28. Bennett NC, Gardiner RA, Hooper JD, Johnson DW, Gobe GC, 2010, **Molecular cell biology of androgen receptor signalling**, *The International Journal of Biochemistry & Cell Biology*, 42: 813-827.

29. Van den Berg P, Neumark-Sztainer D, Cafri G, Wall M, 2007, **Steroid use among adolescents: Longitudinal findings from Project EAT**, *Pediatrics*, 119 (3): 476-486.

30. Bhasin S, Woodhouse L, Casaburi R, Singh AB, Mac RP, Lee M, Yarasheski KE, Sinha-Hikim I, Dzekov C, Dzekov J, Magliano L, Storer TW, 2005, **Older men are as responsive as young men to the anabolic effects of graded doses of testosterone on the skeletal muscle**, *The Journal of Clinical Endocrinology & Metabolism*, 90 (2): 678-688.

31. Bhasin S, Woodhouse L, Storer TW, 2003, **Androgen effects on body composition,** *Growth Hormone & IGF Research*, 13: S63-S71.

32. Bhasin S, Woodhouse L, Casaburi R, Singh AB, Bhasin D, Berman N, Chen X, Yarasheski KE, Magliano L, Dzekov C, Dzekov J, Bross R, Phillips J, Sinha-Hikim I, Shen R, Storer TW, 2001a, **Testosterone dose-response relationships in healthy young men***, American Journal of Physiology Endocrinology & Metabolism*, 281: E1172-E1181.

33. Bhasin S, Woodhouse L, Storer TW, 2001b, **Proof of the effect of testosterone on skeletal muscle**, *Journal of Endocrinology*, 170: 27-38.

34. Bhasin S, Storer TW, Berman N, Yarasheski KE, Clevenger B, Phillips J, Lee PW, Burnell TJ, Casaburi R, 1997, **Testosterone replacement increases fat-free mass and muscle size in hypogonadal men***, Journal of Clinical Endocrinology & Metabolism*, 82 (2): 407-413.

35. Bhasin S, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, Burnell TJ, Tricker R, Shirazi A, Casaburi R, 1996, **The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men***, The New England Journal of Medicine*, 335 (1): 1-7.

36. Bissoli NS, Medeiros ARS, Santos MCS, Busato VCW, Jarske RD, Abrue GR, Moyses MR, de Andrade TU, 2009, **Long-term treatment with supraphysiological doses of nandrolone decanoate reduces the sensititivity of Bezold-Jarish reflex control of heart rate and blood pressure***, Pharmacological Research*, 59: 379-384.

37. Boregowda K, Joels L, Stephens JW, Price DE, 2011, **Persistent primary hypogonadism associated with anabolic steroid abuse***, Fertility and Sterility*, 96 (1): e7-e8.

38. Bovee TFH, Bor G, Heskamp HH, Lasaroms JJP, Sanders MB, Nielen MWF, 2009, **Validation and application of a yeast bioassay for screening androgenic activity in calf urine and feed**, *Analytica Chimica Acta*, 637: 225-234.

39. Bovee TFH, Pikkemaat MG, 2009, **Bioactivity-based screening of antibiotics and hormones**, *Journal of Chromatography A*, 1216: 8035-8050.

40. Bovee TFH, Helsdingen RJR, Hamers ARM, van Duursen MBM, Nielen MWF, Hoogenboom RLAP, 2007, **A new highly specific and robust yeast androgen bioassay for the detection of agonists and antagonists**, *Analytical and Bioanalytical Chemistry*, 389: 1549-1558.

41. Broeder CE, 2003, **Oral andro-related prohormone supplementation: Do the potential risks outweigh the benefits?** *Canadian Journal of Applied Physiology*, 28 (1): 102-116.

42. Brodie AMH, Njar VCO, 1998, **Aromatase inhibitors in advanced breast cancer: Mechanism of action and clinical implications**, *Journal of Steroid Biochemistry & Molecular Biology*, 66: 1-10.

43. Brooks RV, Firth RG, Sumner NA, 1975, **Detection of anabolic steroids by radioimmunoassay**, *British Journal of Sports Medicine,* 9: 89-92.

44. Brown D, Hikim APS, Kovacheva EL, Sinha-Hikim I, 2009, **Mouse model of testosterone-induced muscle fiber hypertrophy: involvement of p38 mitogen-activated protein kinase-mediated Notch signalling**, *Journal of Endocrinology*, 201: 129-139.

45. Brown GA, Vukovich M, King DS, 2006, **Testosterone prohormone supplements**, *Medicine & Science in Sports & Exercise*, 38 (8): 1451-1461.

46. Brown WJ, Basil MD, Bocarnea MC, 2003, **The influence of famous athletes on health benefits and practices: Mark McGwire, child abuse prevention, and androstenedione**, *Journal of Health Communication: International Perspectives*, 8 (1): 41-57.

47. Brownie S, 2005, **The development of the US and Australian dietary supplement regulations. What are the implications for product quality?** *Complementary Therapies in Medicine*, 13: 191-198.

48. Cadwallader AB, Lim CS, Rollins DE, Botre F, Amy B. Cadwallader, Carol S. Lim, Douglas E. Rollins, Francesco Botre, 2011, **The androgen receptor and its use in biological assays: looking toward effect-based testing and its applications,** *Journal of Analytical Toxicology*, 35: 594-607.

49. Cadwallader A, de la Torre X, Tieri A, Botre F, 2010, **The abuse of diuretics as performance-enhancing drugs and masking agents in sport doping: pharmacology, toxicology and analysis**, *British Journal of Pharmacology*, 161: 1-16.

50. Camerino GM, Desaphy J-F, Bellis MD, Capogrosso RF, Cozzoli A, Dinardo MM, Caloiero R, Musaraj K, Fonzino A, Conte E, Jagerschmidt C, Namour F, Liantonio A, Luca AD, Camerino DC, Pierono S, 2015, **Effects of nandrolone in the counteraction of skeletal muscle atrophy in a mouse model of muscle disuse: Molecular biology and functional evaluation**, *PLOS One*, 10 (6): e0129686.

51. Camerino B, Sciaky R, 1975, **Structure and effects of anabolic steroids**, *Pharmacology & Therapeutics: Part B*, 1 (2): 233-275.

52. Campbell JA, Babcock JC, Hogg JA, 1958, **6-methyl steroids in the androstane series**, *Journal of the American Chemical Society*, 80 (17): 4717-4721.

53. Catlin DH, Sekera MH, Ahrens BD, Starcevic B, Chang Y-C, Hatton CK, 2004, **Tetrahydrogestrinone: discovery, synthesis, and detection in urine**, *Rapid Communications in Mass Spectrometry*, 18: 1245-1249.

54. Catlin DH, Ahrens BD, Kucherova Y, 2002, **Detection of norbolethone, an anabolic steroid never marketed, in athletes' urine**, *Rapid Communications in Mass Spectrometry*, 16: 1273-1275.

55. Catlin DH, Leder BZ, Ahrens B, Starcevic B, Hatton CK, Green GA, Finkelstein JS, 2000, **Trace contamination of over-the-counter androstenedione and positive urine test results for a nandrolone metabolite**, *Journal of the American Medical Association*, 284 (20): 2618-2621.

56. Cavalcanti GDA, Leal FD, Garrido BC, Padilha MC, Neto FRDA, 2013, **Detection of designer steroid methylstenbolone in "nutritional supplement" using gas chromatography and tandem mass spectrometry: Elucidation of its urinary metabolites**, *Steroids*, 78: 228-233.

57. Cawley AT, Blakely K, Waller CC, McLeod MD, Boyd S, Heather A, McGrath KC, Handelsman DJ, Willis AC, 2015, **Detection and metabolic investigations of a novel designer steroid: 3-chloro-17α-methyl-5α-androstan-17β-ol**, *Drug Testing and Analysis*, doi: 10.1002/dta.1832.

58. Choong K, Jasuja R, Basaria S, Storer TW, Bhasin S, 2011, **Androgen Abuse,** in Ghigo et al (eds) *Hormone Use and Abuse by Athletes pp* 63-87.

59. Choong K, Lakshman KM, Bhasin S, 2008, **The physiological and pharmacological basis for the ergogenic effects of androgens in elite sports**, *Asian Journal of Andrology*, 10 (3): 351-363.

60. Chou P-H, Lin Y-L, Liu T-C, Chen K-Y, 2015, **Exploring potential contributors to endocrine disrupting activities in Taiwan's surface waters using yeast assays and chemical analysis***, Chemosphere*, 138: 814-820.

61. Clarke BA, Drujan D, Willis MS, Murphy LO, Corpina RA, Burova E, Rakhilin SV, Stitt TN, Patterson C, Latres E, Glass DJ, 2007, **The E3 ligases MuRF1 degrade myosin heavy chain protein in dexamethasone-treated skeletal muscle***, Cell Metabolism*, 6: 376-385.

62. Cohen PA, Maller G, DeSouza R, Neal-Kababick J, 2014, **Presence of banned drugs in dietary supplements following FDA recalls***, Journal of the American Medical Association*, 312 (16): 1691-1693.

63. Cohen S, Brault JJ, Gygi SP, Glass DJ, Valenzuela DM, Gartner C, Latres E, Goldberg AL, 2009, **During muscle atrophy, thick, but not thin, filament components are degraded by MuRF1-dependent ubiquitylation***, The Journal of Cell Biology*, 185 (6): 1083-1095.

64. Connolly L, Ropstad E, Verhaegen S, 2011, ***In vitro*** **bioassays for the study of endocrine-disrupting food additives and contaminants***, Trends in Analytical Chemistry*, 30 (2): 227-238.

65. Cordaro FG, Lombardo S, Cosentino M, 2011, **Selling androgenic anabolic steroids by the pound: identification and analysis of popular websites on the internet,** *Scandinavian Journal of Medicine & Science in Sports*, 21: e247-e259.

66. Coso JD, Munoz G, Munoz-Guerra J, 2011, **Prevalence of caffeine use in elite athletes following its removal from the World Anti-Doping Agency list of banned substances***, Applied Physiology, Nutrition and Metabolism*, 36: 555-561.

67. Crawford J, Prado CMM, Johnston MA, Gralla RJ, Taylor RP, Hancock ML, Dalton JT, 2016, **Study design and rationale for the Phase 3 clinical development program of**

Enobosarm, a Selective Androgen Receptor Modulator, for the prevention and treatment of muscle wasting in cancer patients (POWER Trials), *Current Oncology Reports*, 18: 37.

68. Crown AL, He XL, Holly JMP, Lightman SL, Stewart CEH, 2000, **Characterisation of the IGF system in a primary adult human skeletal muscle cell model, and comparison of the effects of insulin and IGF-I on protein metabolism**, *Journal of Endocrinology*, 167: 403-415.
69. Csibi A, Cornille K, Leibovitch M-P, Poupon A, Tintignac LA, Sanchez AMJ, Leibovitch SA, 2010, **The translation regulator subunit eIF3f controls the kinase-dependent mTOR signaling required for muscle differentiation and hypertrophy in mouse**, *PLoS ONE*, 5 (2): e8994.
70. Cutress ML, Whitaker HC, Mills IG, Stewart M, Neal DE, 2008, **Structural basis for the nuclear import of the human androgen receptor**, *Journal of Cell Science*, 121 (7): 957-968.
71. Dalbo VJ, Roberts MD, Mobley CB, Ballmann C, Kephart WC, Fox CD, Santucci VA, Conover CF, Beggs LA, Balaez A, Hoerr FJ, Yarrow JF, Borst SE, Beck DT, 2016, **Testosterone and trenbolone enanthate increase mature myostatin protein expression despite increasing skeletal muscle hypertrophy and satellite cell number in rodent muscle**, *Andrologia*, doi: 10.1111/and.12622.
72. Dean JD, Carnegie C, Rodzvilla J, Smith T, 2004, **Long-term effects of Testim 1% testosterone gel in hypogonadal men**, *Reviews in Urology*, 6 (6): S22-S29.
73. Deane CS, Hughes DC, Sculthorpe N, Lewis MP, Stewart CE, Sharples AP, 2013, **Impaired hypertrophy in myoblasts is improved with testosterone administration**, *Journal of Steroid Biochemistry & Molecular Biology*, 138: 152-161.

74. Death AK, McGrath KCM, Handelsman DJ, 2005, **Valproate is an anti-androgen and anti-progestin,** *Steroids*, 70: 946-953.

75. Death AK, McGrath KCM, Sader MA, Nakhla S, Jessup W, Handelsman DJ, Celermajer DS, 2004a, **Dihydrotestosterone promotes vascular cell adhesion molecule-1 expression in male human endothelial cells via a nuclear factor-κB-dependent pathway***, Endocrinology*, 145 (4): 1889-1897.

76. Death AK, McGrath KCM, Kazlauskas R, Handelsman DJ, 2004b, **Tetrahydrogestrinone is a potent androgen and progestin***, The Journal of Clinical Endocrinology & Metabolism*, 89 (5): 2498-2500.

77. Delbeke FT, Eenoo PV, Thuyne WV, Desmet N, 2003, **Prohormones and sport***, Journal of Steroid Biochemistry & Molecular Biology*, 83: 245-251.

78. Diamanti-Kandarakis E, Konstantinopolous PA, Papailiou J, Kandarakis SA, Andreopolos A, Sykiotos GP, 2005, **Erythropoietin abuse and erythropoietin gene doping***, Sports Medicine*, 35 (10): 831-840.

79. Diel P, Baadners D, Schlüpmann K, Velders M, Schwarz JP, 2008, **C2C12 myoblastoma cell differentiation and proliferation is stimulated by androgens and associated with a modulation of myostatin and Pax7 expression***, Journal of Molecular Endocrinology*, 40: 231-241.

80. Diel P, Friedel A, Geyer H, Kamber M, Laudenbach-Leschowsky U, Schänzer W, Thevis M, Vollmer G, Zierau O, 2007, **Characterisation of the pharmacological profile of desoxymethyltestosterone (Madol), a steroid misused for doping***, Toxicology Letters*, 169: 64-71.

81. Dubois V, Laurent MR, Sinnesael M, Cielen N, Helsen C, Clinckemalie L, Spans L, Gayan-Ramirez G, Deldicque L, Hespel P, Carmeliet G, Vanderschueren D, Claessens F, 2014,

A satellite cell-specific knockout of the androgen receptor reveals myostatin as a direct androgen target in skeletal muscle, *The FASEB Journal*, 28: 2979-2994.

82. Dubois V, Laurent M, Boonen S, Vanderschueren D, Claessens F, 2012, **Androgens and skeletal muscle: Cellular and molecular mechanisms underlying the anabolic actions**, *Cellular and Molecular Life Sciences*, 69: 1651-1667.
83. Dunn M, White V, 2011, **The epidemiology of anabolic-androgenic steroid use among Australian secondary school students**, *Journal of Science and Medicine in Sport*, 14: 10-14.
84. Dürbeck HW, Buker I, Scheulen B, Telin B, 1983, **GC and capillary column GC/MS determination of synthetic anabolic steroids. II. 4-chloro-methandienone (oral turinabol) and its metabolites**, *Journal of Chromatographic Science*, 21 (9): 405-10.
85. Durussel J, Haile DW, Mooses K, Daskalaki E, Beattie W, Mooses M, Mekonen W, Ongaro N, Angila E, Patel RK, Padmanabhan N, McBride MW, McClure JD, Pitsiladis YP, 2016, **Blood transcriptional signature of recombinant human erythropoietin administration and implication for antidoping strategies**, *Physiological Genomics*, 48: 202-209.
86. Egerman MA, Glass DJ, 2014, **Signaling pathways controlling skeletal muscle mass**, *Critical Reviews in Biochemistry and Molecular Biology*, 49 (1): 59-68.
87. Egner IM, Bruusgaard JC, Eftestol E, Gundersen K, 2013, **A cellular memory mechanism aids overload hypertrophy in muscle long after an episodic exposure to anabolic steroids**, *Journal of Physiology*, 591 (24): 6221-6230.
88. Ehrnborg C, Bengtsson B-A, Rosen T, 2000, **Growth hormone abuse**, *Bailliere's Clinical Endocrinology and Metabolism*, 14 (1): 71-77.

89. Elliott S, 2008, **Erythropoiesis-stimulating agents and other methods to enhance oxygen transport**, *British Journal of Pharmacology*, 154: 529-541.

90. Estrada M, Liberona JL, Miranda M, Jaimovich E, 2000, **Aldosterone- and testosterone-mediated intracellular calcium response in skeletal muscle cell cultures**, *American Journal of Physiology Endocrinology and Metabolism*, 279: E132-E139.

91. Eriksson A, Kadi F, Malm C, Thornell L-E, 2005, **Skeletal muscle morphology in power-lifters with and without anabolic steroids**, *Histochemistry and Cell Biology*, 124: 167-175.

92. Evans NA, 1997, **Gym and tonic: a profile of 100 male steroid users**, *British Journal of Sports Medicine*, 31: 54-58.

93. Faigenbaum AD, Zaichkowsky LD, Gardner DE, Micheli LJ, 1998, **Anabolic steroid use by male and female middle school students**, *Pediatrics*, 101 (5): E6.

94. Far HRM, Agren G, Thiblin I, 2012, **Cardiac hypertrophy in deceased users of anabolic androgenic steroids: an investigation of autopsy findings**, *Cardiovascular Pathology*, 21: 312-316.

95. Feldman HA, Longcope C, Derby CA, Johannes CB, Araujo AB, Coviello AD, Bremner WJ, McKinlay JB, 2002, **Age trends in the level of serum testosterone and other hormones in middle-aged men: Longitudinal results from the Massachusetts Male Aging Study**, *The Journal of Clinical Endocrinology & Metabolism*, 87 (2): 589-598.

96. Ferrando AA, Sheffield-Moore M, Yeckel CW, Gilkison C, Jiang J, Achacosa A, Lieberman SA, Tipton K, Wolfe RR, Urban RJ, 2002, **Testosterone administration to older men improves muscle function: molecular and physiological mechanisms**, *American Journal of Physiology Endocrinology & Metabolism*, 282: E601-E607.

97. Ferriz JM, Vinsova J, 2010, **Prodrug design of phenolic drugs,** *Current Pharmaceutical Design*, 16: 2033-2052.

98. Fielitz J, Kim M-S, Shelton JM, Latif S, Spencer JA, Glass DJ, Richardson JA, Bassel-Duby R, Olson EN, 2007, **Myosin accumulation and striated muscle myopathy result from the loss of muscle RING finger 1 and 3***, The Journal of Clinical Investigation*, 117 (9): 2486-2495.

99. Fineschi V, Riezzo I, Centini F, Silingardi E, Licata M, Beduschi G, Karch SB, 2007, **Sudden cardiac death during anabolic steroid abuse: morphologic and toxicologic findings in two fatal cases of bodybuilders***, International Journal of Legal Medicine*, 121: 48-53.

100. Fitch KD, 2008, **Androgenic-anabolic steroids and the Olympic Games***, Asian Journal of Andrology*, 10 (3): 384-390.

101. Foradori CD, Weiser MJ, Handa RJ, 2008, **Non-genomic actions of androgens***, Frontiers in Neuroendocrinology*, 29: 169-181.

102. Fowler Jr. WM, Gardner GW, Egstrom GH, 1965, **Effect of an anabolic steroid on physical performance of young men***, Journal of Applied Physiology*, 20 (5): 1038-1040.

103. Fragkaki G, Angelis YS, Koupparis M, Tsantili-Kakoulidou A, Kokotos G, Georgakopoulos C, 2009, **Structural characteristics of anabolic androgenic steroids contributing to binding to the androgen receptor and to their anabolic and androgenic activities applied modifications in the steroidal structure**, *Steroids*, 74: 172-197.

104. Franke WW, Berendonke B, 1997, **Hormonal doping and androgenisation of athletes: a secret program of German Democratic Republic government**, *Clinical Chemistry*, 43 (7): 1262-1279.

105. Frese S, Velders M, Schleipen B, Schänzer W, Bloch W, Diel P, 2011, **Myosin heavy chain expression pattern as a marker for anabolic potency: desoxymethyltestosterone (madol), norandrostenedione and testosterone repress MHC-IIb expression and stimulate MHC-IId/x expression in orchiectomised rat gastrocnemius muscle**, *Arch Toxicol*, 85: 635-643.

106. Friedmann T, Rabin O, Frankel MS, 2010, **Gene doping and sport**, *Science*, 327: 647-648.

107. Froehner M, Fischer R, Leike S, Hakenberg OW, Noack B, Wirth MP, 1999, **Intratesticular leiomyosarcoma in a young man after high dose doping with oral-Turinabol***, Cancer*, 86 (8): 1571-5.

108. Frost RA, Lang CH, 2007, **Protein kinase B/Akt: a nexus of growth factor and cytokine signaling in determining muscle mass***, Journal of Applied Physiology* 103: 378-387.

109. Gaido KW, Leonard LS, Lovell S, Gould JC, Babai D, Portier CJ, McDonnell DP, 1997, **Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay***, Toxicology and Applied Pharmacology*, 143: 205-212.

110. Gao W, Dalton JT, 2007, **Expanding the therapeutic use of androgens via selective androgen receptor modulators (SARMs)***, Drug Discovery Today*, 12 (5/6): 241-248.

111. Geldof L, Lootens L, Polet M, Eicher D, Campbell T, Nair V, Botre F, Meuleman P, Lerous-Roels G, Deventer K, Eenoo PV, 2014, **Metabolism of methylstenebolone studied with human liver microsomes and the uPA $^{+/+}$-SCID chimeric mouse model***, Biomedical Chromatography*, 28: 974-985.

112. Geyer H, Braun H, Burke LM, Stear SJ, Castell LM, 2011, **A-Z of nutritional supplements: dietary supplements, sports nutrition foods and ergogenic aids for health and performance – Part 22***, British Journal of Sports Medicine*, 45: 752-754.

113. Geyer H, Parr MK, Koehler K, Mareck U, Schänzer W, Thevis M, 2008, **Nutritional supplements cross-contaminated and faked with doping substances***, Journal of Mass Spectrometry*, 43: 892-902.

114. Geyer H, Parr MK, Mareck U, Reinhart U, Schrader Y, Schänzer W, 2004, **Analysis of non-hormonal nutritional supplements for anabolic-androgenic steroids – Results of an international study***, International Journal of Sports Medicine*, 25: 124-129.

115. Geyer H, Bredehöft M, Mareck U, Parr MK, Schänzer W, 2003, **High doses of the anabolic steroid metandienone found in dietary supplements***, European Journal of Sport Science*, 3 (1): 1-5.

116. Gilard V, Balayssac S, Tinaugus A, Martins N, Martino R, Malet-Martino M, 2015, **Detection, identification and quantification by ^{1}H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance**, *Journal of Pharmaceutical and Biomedical Analysis*, 102: 476-493.

117. Gioeli D, F SB, Kwiek JJ, Aaronson D, Hancock M, Catling AD, White FM, Christian RE, Settlage RE, Shabanowitz J, Hunt DF, Weber MJ, 2002, **Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites***, The Journal of Biological Chemistry*, 277 (32): 29304-29314.

118. Giorgi A, Weatherby RP, Murphy PW, 1999, **Muscular strength, body composition and health responses to the use of testosterone enanthate: A double blind study**, *Journal of Science and Medicine in Sport*, 2 (4): 341-355.

119. Girgis CM, Kuo I, Handelsman DJ, 2014, **Acute hepatitis and fevers in an amateur body-builder: A new complication of synthetic androgen abuse?** *Endocrine Practice*, 20 (8): e130-e133.

120. Gold J, Batterham MJ, Rekers H, MK Harms, Guerts TBP, Helmyr PME, de Mendonca JS, Carvalho LHF, Panos G, Pinchera A, Aiuti F, Lee C, Horban A, Gatell J, Phanuphak P, Prasithsirikul W, Gazzard B, Bloch M, Damner SA, 2006, **Effects of nandrolone decanoate compared with placebo or testosterone on HIV-associated wasting**, *HIV Medicine*, 7: 146-155.

121. Golestani R, Slart RHJA, Dullaart RPF, Glaudemans AWJM, Zeebregts CJ, Boersma HH, Tio RA, Dierckx RAJO, 2012, **Adverse cardiovascular effects of anabolic steroids: pathophysiology imaging**, *European Journal of Clinical Investigations*, 42 (7): 795-803.

122. Gonzalez AM, Hoffman JR, Townsend JR, Jajtner AR, Wlls AJ, Beyer KS, Willoughby DS, Oliveira LP, Fukuda DH, Fragala MS, 2015, **Association between myosin heavy chain protein isoforms and intramuscular anabolic signaling following resistance exercise in trained men**, *Physiological Reports*, 3 (1): e12268.

123. Gorayski P, Thompson CH, Subhash HS, Thomas AC, 2008, **Hepatocellular carcinoma associated with recreational anabolic steroid use**, *British Journal of Sports Medicine*, 42: 74-75.

124. Görgens C, Guddat S, Orlovius A-K, Sigmund G, Thomas A, Thevis M, Schänzer W, 2015, **"Dilute-and-inject" multi-target screening assay for highly polar doping agents using hydrophilic interaction liquid chromatography high resolution/high accuracy mass spectrometry for sports drug testing**, *Analytical and Bioanalytical Chemistry*, 407: 5365-5379.

125. Graham MR, Davies B, Grace FM, Kicman A, Baker JS, 2008, **Anabolic steroid use. Patterns of use and detection of doping**, *Sports Medicine*, 38 (6): 505-525.

126. Granados J, Gillum TL, Christmas KM, Kuennen MR, 2014, **Prohormone supplement 3β-hydroxy-5α-androst-1-en-17-one enhances resistance training gains but impairs user health**, *Journal of Applied Physiology*, 116: 560-569.

127. Gupta V, Bhasin S, Guo W, Singh R, Miki R, Chahan P, Choong K, Tchonia T, Lebrasseur NK, Flanagan JN, Hamilton JA, Viereck JC, Nurala NS, Kirkland JL, Jasuja R, 2008, **Effects of dihydrotestosterone on differentiation and proliferation of human mesenchymal stem cells and preadipocytes**, *Molecular and Cellular Endocrinology*, 296: 32-40.

128. Hall RCW, Hall RCW, 2005, **Abuse of supraphysiological doses of anabolic steroids**, *Southern Medical Journal*, 98: 550-555.

129. Handelsman DJ, 2011a, **Androgen misuse and abuse**, *Best Practice & Research Clinical Endocrinology & Metabolism*, 25: 377-389.

130. Handelsman DJ, 2011b, **Commentary: Androgens and "anabolic steroids": The one-headed Janus,** *Endocrinology*, 152 (5): 1752-1754.

131. Handelsman DJ, 2008, **Indirect androgen doping by oestrogen blockade in sports**, *British Journal of Pharmacology*, 154: 598-605.

132. Handelsman DJ, 2006, **Clinical review: The rationale for banning human chorionic gonadotropin and estrogen blockers in sport**, *The Journal of Clinical Endocrinology & Metabolism*, 91 (5): 1646-1653.

133. Handlon AL, Schaller LT, Leesnitzer LM, Merrihew RV, Poole C, Ulrich JC, Wilson JW, Cadilla R, Turnbull P, 2016, **Optimizing ligand efficiency of selective androgen receptor modulators (SARMs)**, *Medicinal Chemistry Letters*, 7: 83-88.

134. Hartgens F, Kuipers H, 2004a, **Effects of androgenic-anabolic steroids in athletes***, Sports Medicine*, 34 (8): 513-554.

135. Hartgens F, Rietjens G, Keizer HA, Kuipers H, Wolffenbuttel BHR, 2004b, **Effects of androgenic-anabolic steroids on apolipoproteins and lipoprotein (a)***, British Journal of Sports Medicine*, 38: 253-259.

136. Hartgens F, Lichtenbelt WDVM, Ebbing S, Vollaard N, Rietjens G, Kuipers H, 2001a, **Androgenic-anabolic steroid-induced body changes in strength athletes,** *The Physician and Sportsmedicine*, 29 (1): 49-65.

137. Hartgens F, Lichtenbelt WDVM, Ebbing S, Vollaard N, Rietjens G, Kuipers H, 2001b, **Body composition and anthropometry in bodybuilders: regional changes due to Nandrolone decanoate administration,** *International Journal of Sports Medicine*, 22 (3): 235-241.

138. Heemers HV, Tindall DJ, 2007, **Androgen receptor (AR) coregulators: A diversity of functions converging on and regulating the AR transcriptional complex***, Endocrine Reviews*, 28 (7): 778-808.

139. Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y, 2002, **The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum***, The Journal of Biological Chemistry*, 277 (43): 40735-40741.

140. Hiller-Sturmhöfel S, Bartke A, 1998, **The endocrine system. An Overview***, Alcohol Health & Research World*, 22 (3): 153-164.

141. Hoffman JR, Kraemer WJ, Bhasin S, Storer TW, Ratamess NA, Haff GG, Willoughby DS, Rogol AD, 2009, **Position stand on androgen and human growth hormone use***, Journal of Strength and Conditioning Research*, 23 (5): S1-S59.

142. Hughes DC, Stewart CE, Sculthorpe N, Dugdale HF, yousefian F, Lewis MP, Sharples AP, 2015, **Testosterone enables growth and hypertrophy in fusion impaired myoblasts that display myotube atrophy: deciphering the role of androgen and IGF-I receptors***, Biogerontology*, 17 (3): 619-639.

143. Houtman CJ, Sterk SS, van de Heijning MPM, Brouwer A, Stephany RW, van der Burg B, Sonneveld E, 2009, **Detection of anabolic androgenic steroid abuse in doping control using mammalian reporter gene bioassays,** *Analytica Chimica Acta*, 637: 247-258.

144. Ip EJ, Barnett MJ, Terenowicz MJ, Perry PJ, 2011, **The Anabolic 500 survey: Characteristics of male users versus nonusers of anabolic-androgenic steroids for strength training***, Pharmacotherapy*, 31 (8): 757-766.

145. Jacquemin V, Furling D, Bigot A, Butler-Browne GS, Mouly V, 2004, **IGF-1 induces human myotube hypertrophy by increasing cell recruitment***, Experimental Cell Research*, 299: 148-158.

146. Jasiurkowski B, Raj J, Wisinger D, Carlson R, Zou L, Nadir A, 2006, **Cholestatic jaundice and IgA nephropathy induced by OTC muscle building agent Superdrol**, *American Journal of Gastroenterology*, 101: 2659-2662.

147. Jiang X, Kanda T, Nakamoto S, Miyamura T, Wu Shuang, Yokosuka O, 2014, **Involvement of androgen receptor and glucose-regulated protein 78 kDa in human hepatocarcinogenesis***, Experimental Cell Research*, 323: 326-336.

148. Jones A, Hwang DJ, Duke III CB, He Y, Siddam A, Miller DD, Dalton JT, 2010, **Nonsteroidal selective androgen receptor modulators enhance female sexual motivation***, The Journal of Pharmacology and Experimental Therapeutics*, 334 (2): 439-448.

149. Johnson MD, Jay S, Shoup B, Rickert VI, 1989, **Anabolic steroid use by male adolescents**, *Pediatrics*, 83 (6): 921-924.

150. Joseph JF, Parr MK, 2015, **Synthetic androgens as designer supplements**, *Current Neuropharmacology*, 13: 89-100.

151. Joubert Y, Tobin C, 1995, **Testosterone treatment results in quiescent satellite cells being activated and recruited into cell cycle in rat levator ani muscle**, *Developemental Biology*, 169: 286-294.

152. Judkins CMG, Teale P, Hall DJ, 2010, **The role of banned substance residue analysis in the control of dietary supplement contamination**, *Drug Testing and Analysis*, 2: 417-420.

153. Judkins C, Hall D, Hoffman K, 2007, **Investigation into supplement contamination levels in the US market,** Cambridgeshire, UK: HFL, Ltd. ([accessed Oct 21, 2015]), available at http://pop.supplementsafetynow.org/files/pdfs/HFL-supplement-research-report.pdf.

154. Kadi F, 2008, **Cellular and molecular mechanisms responsible for the action of testosterone on human skeletal muscle. A basis for illegal performance enhancement**, *British Journal of Pharmacology*, 154: 522-528.

155. Kanayama G, Gruber AJ, Pope Jr. HG, Borowiecki JJ, Hudson JI, 2001, **Over-the-counter drug use in gymnasiums: an underrecognized substance abuse problem?**, *Psychotherapy and Psychosomatics*, 70: 137-140.

156. Kaushik M, Sontineni SP, Hunter C, 2010, **Cardiovascular disease and androgens: A review**, *International Journal of Cardiology*, 142: 8-14.

157. Kazlauskas R, 2010, **Designer steroids**, In D. Thieme and P. Hemmersbach (eds), *Doping in Sports*, Handbook of Experimental Pharmacology 195. Pp 155-183.

158. Kazmierczak W, Skalba P, Dabkowska-Huc A, Samojedny A, 2006, **Expression of aromatase and 5-α-reductase genes in endometrial adenocarcinoma**, *Pathology – Research and Practice*, 202: 789-791.

159. Kedar V, McDonough H, Arya R, Li H-H, Rockman HA, Patterson C, 2004, **Muscle-specific RING finger 1 is a bona fide ubiquitin ligase that degrades cardiac troponin I**, *Proceedings of the National Academy of Sciences*, 101 (52): 18135-18140.

160. Kennel PF, Pallen CT, Bars RG, 2004, **Evaluation of the rodent Hershberger assay using three reference endocrine disruptors (androgen and antiandrogens)**, *Reproductive Toxicology*, 18: 63-73.

161. Kesler T, Sandhu RS, Krishnamoorthy S, 2014, **Hepatology: Hepatocellular carcinoma in a young man secondary to androgenic anabolic steroid use**, *Journal of Gastroenterology and Hepatology*, 29: 1852.

162. Khurana A, Dasanu CA, 2014, **Hepatitis associated aplastic anemia: Case report and discussion**, *Connecticut Medicine*, 78 (4): 493-495.

163. Kicman AT, Gower DB, 2003, **Anabolic steroids in sport: biochemical, clinical and analytical perspectives**, *Annals of Clinical Biochemistry*, 40: 321-356.

164. Kicman AT, 2008, **Pharmacology of anabolic steroids**, *British Journal of Pharmacology*, 154: 502-521.

165. Kim J, Jia L, Stallcup MR, Coetzee GA, 2005, **The role of protein kinase A pathway and cAMP responsive element-binding protein in androgen receptor-mediated transcription at the prostate-specific antigen locus**, *Journal of Molecular Endocrinology*, 34: 107-118.

166. Kincl FA, Dorfman RI, 1963, **Anabolic-androgenic potency of various steroids in a castrated rat assay**, *Steroids*, 3:1.

167. Kindlundh AMS, Isacson DGL, Berglund L, Nyberg F, 1998, **Doping among high school students in Uppsala, Sweden: A presentation of the attitudes, distribution, side effects, and extent of use**, *Scandinavian Journal of Social Medicine*, 1: (71-74).

168. Kioukia-Fougia N, Fragkaki A, Kiousi P, Leontiou I-P, Dimopoulou H, Tsivou M, Lyris E, Georgakopolous C, 2014, **A synopsis of the adverse analytical and atypical findings between 2005 and 2011 from the Doping Control Laboratory of Athens in Greece**, *Journal of Analytical Toxicology*, 38: 16-23.

169. Labrie F, Luu-The V, Lin S-X, Simard J, Labrie C, 2000, **Role of 17β-hydroxysteroid dehydrogenases in sex steroid formation in peripheral intracrine tissues**, *Trends in Endocrinology & Metabolism*, 11 (10): 421-427.

170. Lee HJ, Lee YS, Kwon HB, Lee K, 2003, **Novel yeast bioassay system for detection of androgenic and antiandrogenic compounds**, *Toxicology in Vitro*, 17: 237-244.

171. Leifman H, Rehnman C, Sjöblom E, Holgersson S, 2011, **Anabolic androgenic steroids – Use and correlates among gym users – an assessment study using questionnaires and observations at gyms in the Stockholm region**, *International Journal of Environmental Research and Public Health*, 8: 2656-2674.

172. Leskinen P, Michelini E, Picard D, Karp M, Virta M, 2005, **Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices**, *Chemosphere*, 61: 259-266.

173. Li J, Al-Azzawi F, 2009, **Mechanism of androgen receptor action**, *Maturitas*, 63: 142-148.

174. Lichtenbelt WDVM, Hartgens F, Vollaard NBJ, Ebbing S, Kuipers H, 2004, **Bodybuilder's body composition: Effect of nandrolone decanoate**, *Medicine & Science in Sports & Exercise*, 36 (3): 484-489.

175. Liederer BM, Borchardt RT, 2005, **Enzymes involved in the bioconversion of ester-based prodrugs,** *Journal of Pharmaceutical Sciences*, 95: 1177-1195.

176. Liegibel UM, Sommer U, Boercsoek I, Hilscher U, Bierhaus A, Schweikert HU, Nawroth P, Kasperk C, 2003, **Androgen receptor isoforms AR-A and AR-B display functional differences in cultured human bone cells and genital skin fibroblasts***, Steroids*, 68: 1179-1187.

177. Ling S, Dai A, Williams MRI, Myles K, Dilley RJ, Komesaroff PA, Sudhir K, 2002, **Testosterone (T) enhances apoptosis-related damage in human vascular endothelial cells**, *Endocrinology*, 143 (3): 1119-1125.

178. Liscio C, Magi E, Carro MD, Suter MJ-F, Vermeirssen ELM, 2009, **Combining passive samplers and biomonitors to evaluate endocrine disrupting compounds in a wastewater treatment plant by LC/MS/MS and bioassay analysis***, Environmental Pollution*, 157: 2716-2721.

179. Lokireddy S, Wijerupag I, Wijesoma W, Sze SK, McFarlane C, Kambadur R, Sharma M, 2012, **Identification of atrogin-1-targeted proteins during the myostatin-induced skeletal muscle wasting***, American Journal of Physiology Cell Physiology*, 303: C512-C529.

180. Lootens L, Meuleman P, Leroux-Roels G, Eenoo PV, 2011, **Metabolic studies with promagnon, methylclostebol and methasterone in the uPA$^{+/+}$-SCID chimeric mice***, Journal of Steroid Biochemistry & Molecular Biology*, 127: 374-381.

181. Lootens L, Eenoo PV, Meuleman P, Pozo OJ, Renterghem PV, Leroux-Roels F, Delbeke FT, 2009, **Steroid metabolism in chimeric mice with humanized liver***, Drug Testing and Analysis*, 1: 531-537.

182. Lun V, Erdman KA, Fung TS, Reimer RA, 2012, **Dietary supplementation practices in Canadian High-performance athletes**, *International Journal of Sport Nutrition and Exercise Metabolism*, 22: 31-37.

183. Lundholm L, Kall K, Wallin S, Thiblin I, 2010, **Use of anabolic androgenic steroids in substance abusers arrested for crime**, *Drug and Alcohol Dependence*, 111: 222-226.

184. MacLean HE, Chiu WSM, Notini AJ, Axell A-M, Davey RA, McManus JF, Ma C, Plant DR, Lynch GS, Zajac JD, 2008, **Impaired skeletal muscle development and function in male, but not female, genomic androgen receptor knockout mice**, *The FASEB Journal*, 22: 2676-2689.

185. Maggio M, Snyder PJ, Ceda GP, Milaneschi Y, Luci M, Cattabiani C, Masoni S, Vignali A, Volpi R, Laurentani F, Peachey H, Valenti G, Cappola AR, Longo D, Ferrucci L, 2013, **Is the haematopoietic effect of testosterone mediated by erythropoietin? The results of a clinical trial in older men**, *Andrology*, 1 (1): 24-8.

186. Martello S, Felli M, Chiarotti M, 2007, **Survey of nutritional supplements for selected illegal anabolic steroids and ephedrine using LC-MS/MS and GC-MS methods, respectively**, *Food Additives and Contaminants*, 24 (3): 258-265.

187. Mason JI, Keenay DS, BirdIM, Rainey WE, Morohashi K-I, Sucheta SL, Melner MH, 1997, **The regulation of 3β-hydroxysteroid dehydrogenase expression**, *Steroids*, 62: 164-168.

188. Matheny Jr. RW, Nindl BC, 2011, **Loss of IGF-IEa or IGF-IEb impairs myogenic differentiation**, *Endocrinology*, 152 (5): 1923-1934.

189. Matsumoto T, Shiina H, Kawano H, Sato T, Kato S, 2008, **Androgen receptor functions in male and female physiology,** *Journal of Steroid Biochemistry & Molecular Biology*, 109: 236-241.

190. Maughan RJ, Greenhaff PL, Hespel P, 2011, **Dietary supplements for athletes: Emerging trends and recurring themes**, *Journal of Sports Sciences*, 29 (S1): S57-S66.

191. Maughan RJ, Depiesse F, Geyer H, 2007, **The use of dietary supplements by athletes**, *Journal of Sports Sciences*, 25 (S1): S103-S113.

192. Maughan RJ, 2005, **Contamination of dietary supplements and positive drug tests in sport**, *Journal of Sports Sciences*, 23 (9): 883-889.

193. Mauras N, Lima J, Patel D, Rini A, Di Salle E, Kwok A, Lippe B, 2003, **Pharmacokinetics and dose finding of a potent aromatase inhibitor, Aromasin (Exemestane), in young males**, *The Journal of Clinical Endocrinology & Metabolism*, 88 (12): 5951-5956.

194. Mauras N, O'Brien KO, Klein KO, Hayes V, 2000, **Estrogen suppression in males: Metabolic effects**, *The Journal of Clinical Endocrinology & Metabolism*, 85 (7): 2370-2377.

195. Mauras N, Hayes V, Welch S, Rini A, Helgeson K, Dokler M, Veldhuis JD, Urban RJ, 1998, **Testosterone deficiency in young men: Marked alterations in whole body protein kinetics, strength, and adiposity**, *Journal of Clinical Endocrinology and Metabolism*, 83 (6): 1886-1892.

196. McCrohon JA, Death AK, Nakhla S, Jessup W, Handelsman DJ, Stanley KK, Celermajer DS, 2000, **Androgen receptor expression is greater in macrophages from male than from female donors. A sex difference with implications for atherogenesis**, *Circulation*, 101: 224-226.

197. McPherron AC, Lawler AM, Lee S-J, 1997, **Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member**, *Nature*, 387: 83-90.

198. McPherron AC, Lee S-J, 1997, **Double muscling in cattle due to mutations in the myostatin gene**, *Proceedings of the National Academy of Sciences USA*, 94: 12457-12461.

199. McRobb L, Handelsman DJ, Heather AK, 2009, **Androgen-induced progression of arterial calcification in apolipoprotein E-null mice is uncoupled from plaque growth and lipid levels**, *Endocrinology* 150 (2): 841-848.

200. McRobb L, Handelsman DJ, Kazlauskas R, Wilkinson S, McLeod MD, Heather AK, 2008, **Structure-activity relationships of synthetic progestins in a yeast-based *in vitro* androgen bioassay**, *Journal of Steroid Biochemistry & Molecular Biology*, 110: 39-47.

201. Mendias CL, Lynch EB, Gumucido JP, Flood MD, Rittman DS, Pelt DWV, Roche SM, Davis CS, 2015, **Changes in skeletal muscle and tendon structure and function following genetic inactivation of myostatin in rats**, *Journal of Physiology*, 593 (8): 2037-2052.

202. Van der Merwe PJ, Grobbelaar E, 2005, **Unintentional doping through the use of contaminated nutritional supplements**, *South African Medical Journal*, 95 (7): 510-511.

203. Van de Merwe PJ, Grobbelaar E, 2004, **Inadvertent doping through nutritional supplements is a reality**, *Sports Medicine*, 16 (2): 3-7.

204. Michelini E, Leskinen P, Virta M, Karp M, Roda A, 2005, **A new recombinant cell-based bioluminescent assay for sensitive androgen-like compound detection**, *Biosensors and Bioelectronics*, 20: 2261-2267.

205. Michels G, Hoppe UC, 2008, **Rapid actions of androgens**, *Frontiers in Neuroendocrinology*, 29: 182-198.

206. Miller MS, Bedrin NG, Ades PA, Palmers BM, Toth MJ, 2015, **Molecular determinants of force production in human skeletal muscle fibres: effects of myosin isoform expression and cross-sectional area**, *American Journal of Physiology Cell Physiology*, 308: C473-C484.

207. Mindnich R, Möller G, Adamski J, 2004, **The role of 17 beta-hydroxysteroid dehydrogenases**, *Molecular and Cellular Endocrinology*, 218: 7-20.

208. Miyake T, Hori T, Kato G, Ide M, Uchida N, Yamaguchi K, 1974, **2α,3α-epithio-5α-androstan-17β-YL 1-methoxycyclopentyl ether (1036-S), a new orally active anti-estrogenic steroid**, *Steroids*, 23 (6): 929-937.

209. Mizrachi D, Auchus RJ, 2009, **Androgens, estrogens, and hydroxysteroid dehydrogenases**, *Molecular and Cellular Endocrinology*, 301: 37-42.

210. Montisci M, Mazloum RE, Cecchetto G, Terranova C, Ferrara SD, Thiene G, Basso C, 2012, **Anabolic androgenic steroids abuse and cardiac death in athletes: morphological and toxicological findings four fatal cases**, *Forensic Science International*, 217: e13-e18.

211. Mosler S, Pankratz C, Seyfried A, Piechotta M, Diel P, 2012, **The anabolic steroid methandienone targets the hypothalamic-pituitary-testicular axis and myostatin signaling in a rat training model**, *Archives of Toxicology*, 86: 109-119.

212. Morissette MR, Cook SA, Buranasombati C, Rosenberg MA, Rosenzweig A, 2009, **Myostatin inhibits IGF-I-induced myotube hypertrophy through Akt**, *American Journal of Physiology Cell Physiology*, 297: C1124-C1132.

213. Nasr J, Ahmad J, 2009, **Severe cholestasis and renal failure associated with the use of the designer steroid Superdrol (methasterone): A case report and literature review,** *Digestive Diseases and Sciences*, 54: 1144-1146.

214. Nakao A, Sakagami K, Nakata Y, Komazawa K, Amimoto T, Nakashima K, Isozaki H, Takakura N, Tanaka N, 2000, **Multiple hepatic adenomas caused by long-term administration of androgenic steroids for aplastic anemia in association with familial adenomatous polyposis**, *Journal of Gastroenterology*, 35: 557-562.

215. Neri M, Bello S, Bonsignore A, Cantator S, Riezzo I, Terillazzi E, Fineschi V, 2011, **Anabolic androgenic steroids abuse and liver toxicity,** *Mini-Reviews in Medicinal Chemistry*, 11: 430-437.

216. Nisr RB, Affourtit C, 2014, **Insulin acutely improves mitochondrial function of rat and human skeletal muscle by increasing coupling efficiency of oxidative phosphorylation**, *Biochimica et Biophysica Acta*, 1837: 270-276.

217. Norton LE, Laymen DK, 2006, **Leucine regulates translation initiation of protein synthesis in skeletal muscle after exercise**, *Journal of Nutrition*, 136: 533S-S537.

218. Oberlander JG, Henderson LP, 2012, **The *Sturm und Drang* of anabolic steroid use: angst, anxiety, and aggression**, *Trends in Neurosciences*, 35 (6): 382-392.

219. Ojasoo T, Delettre J, Mornon JP, Turpin-vanDycke C, Raynaud JP, 1987, **Towards the mapping of the progesterone and androgen receptors**, *Journal of Steroid Biochemistry*, 27 (1-3): 255-269.

220. Okano M, Nishitani Y, Kageyama S, 2014, **Possibility of analytical finding of glycerol caused by self-catheterization in doping control**, *Drug Testing and Analysis*, 6: 1151-1154.

221. Okano M, Sato M, Ikekita A, Kageyama S, 2009, **Analysis of non-ketoic steroids 17α-methylepithiostanol and desoxymethyltestosterone in dietary supplements**, *Drug Testing and Analysis*, 1: 518-525.

222. Outram S, Stewart B, 2015, **Doping through supplement use: A review of the available empirical data**, *International Journal of Sport Nutrition and Exercise Metabolism*, 25: 54-59.

223. Ozdemir L, Nur N, Bagcivan I, Bulut O, Sumer H, Tezeren G, 2005, **Doping and performance enhancing drug use in athletes living in Sivas, mid-Anatolia: A brief report**, *Journal of Sports Science and Medicine*, 4: 248-252.

224. Pagonis TA, Angelopolous NV, Koukoulis GN, Hadjichristodoulou CS, 2006, **Psychiatric side effects induced by supraphysiological doses of combinations of anabolic steroids correlate to the severity of abuse**, *European Psychiatry*, 21: 551-562.

225. Pallesen S, Josendal O, Johnsen B-H, Larsen S, Molde H, 2006, **Anabolic steroid use in high school students**, *Substance Use & Misuse*, 41: 1705-1717.

226. Palsgaard J, Brown AE, Jensen M, Borup R, Walker M, Meyts PD, 2009, **Insulin-like growth factor I (IGF-I) is a more potent regulator of gene expression than insulin in primary human myoblasts and myotubes**, *Growth Hormone & IGF Research*, 19: 168-178.

227. Papadopoulos V, Miller WL, 2012, **Role of mitochondria in steroidogenesis**, *Best Practice & Research Clinical Endocrinology & Metabolism*, 26: 771-790.

228. Parkinson AB, Evans NA, 2006, **Anabolic androgenic steroids: A survey of 500 users**, *Medicine & Science in Sports & Exercise*, 38 (4): 644-651.

229. Parr MK, Fußhöller G, Schlörer N, Opfermann G, Geyer H, Rodchenkov G, Schänzer W, 2011a, **Detection of Δ6-methyltestosterone in a "dietary supplement" and GC-MS/MS investigations on its urinary metabolism**, *Toxicology Letters*, 201: 101-104.

230. Parr MK, Opfermann G, Geyer H, Westphal F, Sönnichsen FD, Zapp J, Kwitkowska D, Schänzer W, 2011b, **Seized designer supplement named "1-androsterone":**

Identification as 3β-hydroxy-5α-androst-1-en-17-one and its urinary elimination, *Steroids*, 76: 540-547.

231. Parr MK, Blatt C, Zierau O, Hess C, Gutschow M, Fussholler G, Opfermann G, Schänzer W, Diel P, 2011c, **Endocrine characterization of the designer steroid methyl-1-testosterone: investigations on tissue-specific anabolic-androgenic potency, side effects, and metabolism**, *Endocrinology*, 152 (12): 4718-4728.
232. Parr MK, Laudenbach-Leschowsky U, Hofer N, Schänzer W, Diel P, 2009a, **Anabolic and androgenic activity of 19-norandrostenedione after oral and subcutaneous administration – Analysis of side effects and metabolism**, *Toxicology Letters*, 188: 137-141.
233. Parr MK, Gütschow M, Daniels J, Opfermann G, Thevis M, Schänzer W, 2009b, **Identification of steroid isoxazole isomers marketed as designer supplement**, *Steroids*, 74: 322-328.
234. Parr MK, Geyer H, Hoffmann B, Köhler K, Mareck U, Schänzer W, 2007, **High amounts of 17-methylated anabolic-androgenic steroids in effervescent tablets on the dietary supplement market**, *Biomedical Chromatography*, 21: 164-168.
235. Pasiakos SM, McClung JP, 2011, **Supplemental dietary leucine and the skeletal muscle anabolic response to essential amino acids**, *Nutrition Reviews*, 69 (9): 550-557.
236. Patil JJ, O'Donahoe B, Loyden CF, Shanahan D, 2007, **Near-fatal spontaneous hepatic rupture associated with anabolic androgenic steroid use: a case report**, British Journal of Sports Medicine, 41: 462-463.

237. Penning TM, Jin Y, Steckelbroeck S, Rizner TL, Lewis M, 2004, **Structure-function of human 3α-hydroxysteroid dehydrogenases: genes and proteins**, *Molecular and Cellular Endocrinology*, 215: 63-72.

238. Penning TM, Jin Y, Heredia VV, Lewis M, 2003, **Structure-function relationships in 3α-hydroxysteroid dehydrogenases: a comparison of the rat and human isoforms**, *Journal of Steroid Biochemistry & Molecular Biology*, 85: 247-255.

239. Penning TM, Pawlowski JE, Schlegel BP, Jez JM, Lin H-K, Hoog SS, Bennett MJ, Lewis M, 1996, **Mammalian 3α-hydroxysteroid dehydrogenases**, *Steroids* 61: 508-523.

240. Pergola GD, 2000, **The adipose tissue metabolism: Role of testosterone and dehydroepiandrosterone**, *International Journal of Obesity*, 24 (2): S59-S63.

241. Perls TT, 2009, **Growth hormone and anabolic steroids: athletes are the tip of the iceberg,** *Drug Testing and Analysis*, 1: 419-425.

242. Peters RJB, Rijk JCW, Bovee TFH, Nijrolder AWJM, Lommen A, Nielen MWF, 2010, **Identification of anabolic steroids and derivatives using bioassay-guided fractionation, UHPLC/TOFMS analysis and accurate mass database searching**, *Analytica Chimica Acta*, 664: 77-88.

243. Pickford DB, Jones A, Valez-Pelez A, Iguchi T, Mitsui N, Tooi O, 2015, **Screening breeding sites of the common toad (*Bufu bufo*) in England and Wales for evidence of endocrine disrupting activity**, *Ecotoxicology and Environmental Safety*, 117: 7-19.

244. Pleadin J, Vulic A, Persi N, Vahcic N, 2010, **Clenbuterol residues in pig muscle after repeat administration in a growth-promoting dose**, *Meat Science*, 86: 733-737.

245. Plotan M, Elliott CT, Oplatowska M, Connolly L, 2012, **Validation and application of reporter gene assays for the determination of estrogenic and androgenic endocrine**

disruptor activity in sport supplements, *Analytical and Bioanalytical Chemistry*, 403: 3057-3067.

246. Plotan M, Elliott CT, Scippo ML, Muller M, Antignac J-P, Malone E, Bovee TFH, Mitchel S, Connolly S, 2011, **The application of reporter gene assays for the detection of endocrine disruptors in sports supplements**, *Analytica Chimica Acta*, 700: 34-40.
247. Polge C, Heng A-E, Jarzaguet M, Ventadour S, Claustre A, Combaret L, Bechet D, Matondo M, Uttenweiler-Joseph S, Monsarrat B, Attaix D, Taillandier D, 2011, **Muscle actin is polyubiquitinylated *in vitro* and *in vivo* and targeted for breakdown by the E3 ligase MuRF1**, *The FASEB Journal*, 25: 3790-3802.
248. Pope Jr. HG, Kanayama G, Hudson JI, 2012, **Risk factors for illicit anabolic-androgenic steroid use in male weightlifters: A cross-sectional cohort study**, *Biological Psychiatry*, 71: 254-261.
249. Pirompol P, Teekabut V, Weerachatyanukul W, Bupha-Intr T, Wattanapermool J, 2016, **Supra-physiological dose of testosterone induces pathological cardiac hypertrophy**, *Journal of Endocrinology*, 229 (1): 13-23.
250. Qaisar R, Renaud G, Morine K, Barton ER, Sweeney HL, Larsson L, 2012, **Is functional hypertrophy and specific force coupled with the addition of myonuclei at the single muscle fiber level?** *The FASEB Journal*, 26: 1077-1085.
251. Qin W, Pan J, Wu Y, Baumen WA, Cardozo C, 2010, **Protection against dexamethasone-induced muscle atrophy is related to modulation by testosterone of FOXO1 and PGC-1α**, *Biochemical and Biophysical Research Communications*, 403: 473-478.

252. Rahnema CD, Crosnoe LE, Kim ED, 2015, **Designer steroids – over-the-counter supplements and their androgenic component: review of an increasing problem**, *Andrology*, 3 (2): 150-155.

253. Rasmussen BB, Volpi E, Gore DC, Wolfe RR, 2000, **Androstenedione does not stimulate muscle protein anabolism in young healthy men**, *The Journal of Clinical Endocrinology & Metabolism*, 85 (1): 55-59.

254. Rijk JCW, Bovee TFH, Peijnenburg AACM, Groot MJ, Rietjens IMCM, Nielen MWF, 2012, **Bovine liver slices: A multifunctional *in vitro* model to study the prohormone dehydroepiandrosterone (DHEA)**, *Toxicology in Vitro*, 26: 1014-1021.

255. Rijk JCW, Ashwin H, van Kuijk SJA, Groot MJ, Heskamp HH, Bovee TFH, Nielen MWF, 2011, **Bioassay based screening of steroid derivatives in animal feed and supplements**, *Analytica Chimica Acta*, 700: 183-188.

256. Rijk JCW, Bovee TFH, Wang S, Van Poucke C, Peteghem CV, Nielen MWF, 2009, **Detection of anabolic steroids in dietary supplements: The added value of an androgen yeast bioassay in parallel with a liquid chromatography-tandem mass spectrometry screening method**, *Analytica Chimica Acta*, 637: 305-314.

257. Rijk JCW, Bovee TFH, Groot MJ, Peijnenburg AACM, Nielen MWF, 2008, **Evidence of the indirect hormonal activity of prohormones using liver S9 metabolic bioactivation and an androgen bioassay**, *Analytical and Bioanalytical Chemistry*, 392: 417-425.

258. Rocha FL, Carmo EC, Roque FR, Hashimoto NY, Rossoni LV, Frimm C, Aneas I, Negrao CE, Krieger JE, Oliveira EM, 2007, **Anabolic steroids induce cardiac renin-angiotensin system and impair the beneficial effects of aerobic training in rats**, *Journal of Physiology: Heart and Circulator Physiology*, 293: H3757-H3583.

259. Rogerson S, Weatherby RP, Deakin GB, Meir RA, Coutts RA, Zhou S, Marshal-Gradisnik SM, 2007, **The effect of short-term use of testosterone enanthate on muscular strength and power in healthy young men**, *Journal of Strength and Conditioning Research*, 21 (2): 354-361.

260. Roy P, Franks S, Read M, Huhtaniemi IT, 2006, **Determination of androgen bioactivity in human serum samples using a recombinant cell based *in vitro* bioassay,** *Journal of Steroid Biochemistry & Molecular Biology*, 101: 68-77.

261. Sader MD, 1999, **Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways**, *The Journal of Biological Chemistry*, 274 (12): 7777-7783.

262. Saeed A, Vaught GM, Gavardinas K, Matthews D, Green JE, Losada PG, Bullock HA, Calvert NA, Patel NJ, Sweetana SA, Krishnan V, Henck JW, Luz, JG, Wang Y, Jadhav P, 2016, **2-chloro-4-[[(1R,2R)-2-hydroxy-2-methyl-cyclopentyl]amino]-3-methyl-benzonitrile: A transdermal selective androgen receptor modulator (SARM) for muscle atrophy**, *Journal of Medicinal Chemistry*, 59: 750-755.

263. Sagoe D, Phil M, Molde H, Andreassen CS, Torsheim T, Pallesen S, 2014, **The global epidemiology of anabolic-androgenic steroid use: A meta-analysis and meta-regression analysis**, *Annals of Epidemiology*, 24 (5): 383-98.

264. Samaha AA, Nasser-Eddine W, Shatila E, Haddad JJ, Wazne J, Eid AH, 2008, **Multi-organ damage induced by anabolic steroid supplements: A case report and literature review**, *Journal of Medical Case Reports*, 2: 340-345.

265. Saner-Amigh KJ, Halvorson LM, 2011, **Andrology and fertility assessment**, *Lab Medicine*, 42: 41-50.

266. Santos AM, Sergio M, Rocha P, da Silva MF, 2011, **Illicit use and abuse of anabolic-androgenic steroids among Brazilian bodybuilders**, *Substance Use & Misuse*, 46: 742-748.

267. Shahidi NT, 2001, **A review of the chemistry, biological action, and clinical applications of anabolic-androgenic steroids,** *Clinical Therapeutics*, 23: 1355-1390.

268. Schänzer W, Opfermann G, Donike M, 1992, **17-epimerization of 17α-methyl anabolic steroids in humans: metabolism and synthesis of 17α-hydroxy-17β-methyl steroids**, *Steroids*, 57: 537-550.

269. Schänzer W, 1996, **Metabolism of anabolic androgenic steroids**, *Clinical Chemistry*, 42 (7): 1001-1020.

270. Schänzer W, Horning S, Opfermann G, Donike M, 1996, **Gas Chromatography/Mass Spectrometry identification of long-term excreted metabolites of the anabolic steroid 4-chloro-1,2-dehydro-17α-methyltestosterone in humans**, *Journal of Steroid Biochemistry & Molecular Biology*, 57 (5/6): 363-376.

271. Schiaffino A, Reggiani C, 2011, **Fiber types in mammalian skeletal muscles**, *Physiology Reviews*, 91: 1447-1531.

272. Schroeder ET, Singh A, Bhasin S, Storer TW, Azen C, Davidson T, Martinez C, Sinha-Hikim I, Jaque SV, Terk M, Sattler F, 2003, **Effects of an oral androgen on muscle and metabolism in older, community-dwelling men**, *American Journal of Physiology Endocrinology and Metabolism*, 284: E120-E128.

273. Sculthorpe N, Solomon AM, Sinanan ACM, Bouloux P-M G, Grace F, Lewis MP, 2012, **Androgens affect myogenesis *in vitro* and increase local IGF-1 expression**, *Medicine & Science in Sports & Exercise*, 44 (4): 610-615.

274. Serra C, Tangherlini F, Rude S, Lee Daniel, Toraldo G, Sandor NL, Zhang A, Jasuja R, Bhasin S, 2013, **Testosterone improves the regeneration of old and young mouse skeletal muscle**, *Journal of Gerontology A: Biological Sciences and Medical Science*, 68 (1): 17-26.

275. Serra C, Bhasin S, Tangherlini F, Barton ER, Ganno M, Zhang A, Shansky J, Vandenburgh HH, Travison TG, Jasuja R, Morris C, 2011, **The role of GH and IGF-I in mediating anabolic effects of testosterone on androgen-responsive muscle**, *Endocrinology*, 152: 193-206.

276. Shah NL, Zacharias I, Khettry U, Afdhal N, Gordon FD, 2008, **Methasteron-associated cholestatic liver injury: Clinicopathologic findings in 5 cases**, *Clinical Gastroenterology and Hepatology*, 6 (2): 255-258.

277. Sheffield-Moore M, Dillon EL, Casperson SL, Gilkison CR, Paddon-Jones D, Durham WJ, Grady JJ, Urban RJ, 2011, **A randomized pilot study of monthly cycled testosterone replacement or continuous testosterone replacement versus placebo in older men**, *Journal of Clinical Endocrinology & Metabolism*, 96 (11): E1831-E1837.

278. Sheffield-Moore M, Paddon-Jones D, Casperson SL, Gilkison C, Volpi E, Wolf SE, Jiang J, Rosenblatt JI, Urban RJ, 2006, **Androgen therapy induces muscle protein anabolism in older women**, *The Journal of Clinical Endocrinology & Metabolism*, 91 (10): 3844-3849.

279. Shenkman BS, Turtikova OV, Nemirovskaya TL, Grigoriev AI, 2010, **Skeletal muscle activity and the fate of myonuclei**, *Acta Naturae*, 2 (5): 59-65.

280. Simon P, Striegel H, Aust F, Dietz K, Ulrich R, 2006, **Doping in fitness sports: estimated number of unreported cases and individual probability of doping**, *Addiction*, 101: 1640-1644.

281. Singh R, Bhasin, Braga M, Artaza JN, Pervin S, Taylor WE, Krishnan V, Sinha SK, Rajavashisth TB, Jasuja R, 2009, **Regulation of myogenic differentiation by androgens: Cross talk between androgen receptor/β-catenin and follistatin/transforming growth factor-β signaling pathways**, *Endocrinology*, 150: 1259-1268.

282. Singh R, Artaza JN, Taylor WE, Gonzalex-Cadavid NF, Bhasin S, 2003, **Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent cells through an androgen receptor-mediated pathway**, *Endocrinology*, 144 (11): 5081-5088.

283. Sinha-Hikim I, Cornford M, Gaytan H, Lee ML, Bhasin S, 2006, **Effects of testosterone supplementation on skeletal muscle fiber hypertrophy and satellite cells in community-dwelling older men**, *The Journal of Clinical Endocrinology & Metabolism*, 91 (8): 3024-3033.

284. Sinha-Hikim I, Roth SM, Lee MI, Bhasin S, 2003, **Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men**, *American Journal of Physiology Endocrinology & Metabolism*, 285: E197-E205.

285. Sinha-Hikim I, Artaza J, Woodhouse L, Gonzalez-Cadavid N, Singh AB, Lee MI, Storer TW, Casaburi R, Shen R, Bhasin S, 2002, **Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy**, *American Journal of Physiology Endocrinology and Metabolism*, 283: E154-E164.

286. Sjöqvist F, Garle M, Rane A, 2008, **Use of doping agents, particularly anabolic steroids, in sports and society**, *The Lancet*, 371: 1872-1882.

287. Sobolevsky T, Rodchenkov G, 2012, **Detection and mass spectrometric characterization of novel long-term dehydrochloromethyltestosterone metabolites in human urine**, *Journal of Steroid Biochemistry & Molecular Biology*, 128: 121-127.

288. Sonneveld E, Jansen HJ, Riteco JAC, Brouwer A, van der Burg B, 2005, **Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays**, *Toxicological Sciences*, 83: 136-148.

289. Soto AM, Sonnenshein C, Chung KL, Fernandez MF, Olea N, Serrano FO, 1995, **The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants**, *Environmental Health Perspective*, 103 (7): 113-122.

290. Sotoca AM, Bovee TFH, Brand W, Velikova N, Boeren S, Murk AJ, Vervoort J, Rietjens IMCM, 2010, **Superinduction of estrogen receptor mediated gene expression in luciferase reporter gene assays is mediated by a post-transcriptional mechanism**, *Journal of Steroid Biochemistry & Molecular Biology*, 122: 204-211.

291. Steroids Worldwide, **Depo Test 250 10 mL**, available online: http://www.steroidsworldwide.com/depo-test-10ml-p-5.html (accessed March 2016).

292. Stergiopoulos K, Brennan JJ, Mathews R, Setaro JF, Kort S, 2008, **Anabolic steroids, acute myocardial infarction and polycythemia: A case report and review of the literature**, *Vascular Health and risk Management*, 4 (6): 1475-1480.

293. Stilger VG, Yesalis CE, 1999, **Anabolic-androgenic steroid use among high school football players**, *Journal of Community Health*, 24 (2): 131-145.

294. Stocco C, 2012, **Tissue physiology and pathology of aromatase**, *Steroids*, 77: 27-35.

295. Storer TW, Woodhouse L, Magliano L, Singh AB, Dzekov C, Dzekov J, Bhasin S, 2008, **Changes in muscle mass, muscle strength, and power but not physical function are related to testosterone dose in healthy older men**, *Journal of the American Geriatrics Society*, 56 (11): 1991-1999.

296. Storer TW, Magliano L, Woodhouse L, Lee ML, Dzekov J, Casaburi R, Bhasin S, 2003, **Testosterone dose-dependently increases maximal voluntary strength and leg power, but does not affect fatigability or specific tension**, *The Journal of Clinical Endocrinology & Metabolism*, 88 (4): 1478-1485.

297. Striegel H, Vollkommer G, Horstmann T, Niess AM, 2005, **Contaminated nutritional supplements – legal protection for elite athletes who tested positive: A case report from Germany**, *Journal of Sports Sciences*, 23 (7): 723-726.

298. Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, Glass DJ, 2004, **The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors**, *Molecular Cell*, 14: 395-403.

299. Sundfeld D, Real S, Reis RP, Piccolo MS, Okamato RH, Gragnan A, Ferreira LM, 2014, **Oxandrolone use in adult burn patients. Systematic review and meta-analysis,** *Acta Cirurgiga Brasileira*, 29 (3): 68-76.

300. Supasyndh O, Satirapoj B, Aramwit P, Viroonudomphol D, Chaipraser A, Thanachatwej V, Vanichakarn S, Kopple JD, 2013, **Effect of oral anabolic steroid on muscle strength and muscle growth in hemodialysis patients**, *Clinical Journal of the American Society of Nephrology* 8 (2): 271-279.

301. Tatsumi R, Sheehan SM, Iwasaki H, Hattori A, Allen RE, 2001, **Mechanical stretch induces activation of skeletal satellite cells *in vitro***, Experimental Cell Research, 267: 107-114.

302. Thevis M, Kuuranne T, Walpurgis K, Geyer H, Schänzer W, 2016, **Annual banned-substance review: analytical approaches in human sports drug testing**, *Drug Testing and Analysis*, 8: 7-29.

303. Thomas JL, Duaz WL, Addlagatta A, Kacsoh Balint, Brandt SE, Norris WB, 2004, **Structure/function aspects of human 3β-hydroxysteroid dehydrogenases**, *Molecular and Cellular Endocrinology*, 215: 73-82.

304. Thuyne WV, Eenoo PV, Delbeke FT, 2006, **Nutritional supplements: prevalence of use and contamination with doping agents**, *Nutrition Research Reviews*, 19: 147-158.

305. Thuyne WV, Delbeke FT, 2004, **Validation of a GC-MS screening method for anabolizing agents in solid nutritional supplements**, *Biomedical Chromatography*, 18: 155-159.

306. Tintignac LA, Lagirand J, Batonnet S, Sirri V, Leibovitch MP, Leibovitch SA, 2005, **Degradation of MyoD mediated by the SCF (MAFbx) ubiquitin ligases**, *The Journal of Biological Chemistry*, 280 (4): 2847-2856.

307. Todd T, 1987, **Anabolic steroids: The gremlins of sport**, *Journal of Sport History*, 14 (1): 87-107.

308. Transparency Market Research, Nutraceuticals product market: global market size, segment and country analysis and forecast (2007-2017), http://www.transparencymarketresearch.com/global-nutraceuticals-product-market.html (accessed 20 April 2014).

309. Trendelenburg AU, Meyer A, Rohner D, Boyle J, Hatakeyama S, Glass DJ, 2009, **Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size**, *American Journal of Physiology Cell Physiology*, 296: C1258-C1270.

310. Urban RJ, Bodenburg YH, Gilkison C, Foxworth J, Coggan AR, Wolfe RR, Ferrando A, 1995, **Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis**, *American Journal of Physiology Endocrinology & Metabolism*, 269: E820-E826.

311. Vihko P, Isomaa V, Ghosh D, 2001, **Structure and function of 17β-hydroxysteroid dehydrogenase type 1 and 2**, *Molecular and Cellular Endocrinology*, 171: 71-76.

312. Wade N, 1972, **Anabolic steroids: Doctors denounce them, but athletes aren't listening**, *Science*, 176: 1399-1403.

313. Vilella AL, Limsuwat C, Williams DR, Seifert SF, 2013, **Cholestatic jaundice as a result of combination designer supplement ingestion**, *The Annals of Pharmacotherapy*, 47: e33.

314. Wagner U, Pedersen KM, 2014, **The IOC and the doping issue – An institutional discursive approach to the organizational identity construction**, *Sport Management Review*, 17: 160-173.

315. Watanabe Y, Sawada N, Isomura H, Satoh H, Hirata K, Mori M, 1995, **Estrogen-depleted condition induces apoptosis of rat mammary cancer cells after entering the S-phase of the cell cycle**, *Cell Structure and Function*, 20: 125-132.

316. Watson P, Judkins C, Houghton E, Russell C, Maughan RJ, 2009, **Urinary nandrolone metabolite detection after ingestion of a nandrolone precursor**, *Medicine & Science in Sports & Exercise*, 41 (4): 766-772.

317. Wells DJ, 2008, **Gene doping: the hype and the reality,** *British Journal of Pharmacology*, 154: 623-631.

318. White JP, Gao S, Puppa MJ, Sato S, Welle SL, Carson JA, 2013, **Testosterone regulation of Akt/mTORC1/FoxO3a signaling in skeletal muscle**, *Molecular and Cellular Endocrinology*, 365: 174-186.

319. Willoughby DS, Wilborn C, Taylor L, Campbell W, 2007, **Eight weeks of aromatase inhibition using the nutritional supplement Novedex XT: Effects in young, eugonadal men,** *International Journal of Sport Nutrition and Exercise Metabolism*, 17: 92-108.

320. Wingert N, Tavakoli H, Yoder E, 2010, **Acute hepatitis and personality change in a 31-year-old man taking prohormone supplement SUS500**, *Psychosomatics*, 51: 340-344.

321. Woerdeman J, de Ronde W, 2011, **Therapeutic effects of anabolic androgenic steroids on chronic diseases associated with muscle wasting,** *Expert Opinion on Investigational Drugs*, 20 (1): 87-97.

322. World Anti-Doping Agency, **The 2016 prohibited list**, available online: https://wada-main-prod.s3.amazonaws.com/resources/files/wada-2016-prohibited-list-en.pdf (accessed April 2016).

323. World Anti-Doping Agency, available online: https://www.wada-ama.org/en (accessed March 2016).

324. Woodhouse LJ, Gupta N, Bhasin M, Singh AB, Ross R, Phillips J, Bhasin S, 2004, **Dose-dependent effects of testosterone on regional adipose tissue distribution in healthy young men**, *The Journal of Clinical Endocrinology & Metabolism*, 89 (2): 718-726.

325. Van de Wijngaart DJ, Dubbink HJ, van Royen ME, Trapman J, 2012, **Androgen receptor coregulators: Recruitment via the coactivator binding groove**, *Molecular and Cellular Endocrinology*, 352: 57-69.

326. Wilson CM, McPhaul MJ, 1996, **A and B forms of the androgen receptor are expressed in a variety of human tissues**, *Molecular and Cellular Endocrinology*, 120: 51-57.

327. Wiren KM, Zhang X, Chang C, Keenan E, Orwoll ES, 1997, **Transcriptional up-regulation of the human androgen receptor by androgen in bone cells**, *Endocrinology*, 138 (6): 2291-2300.

328. Wu Y, Zhao W, Zhao J, Zhang Y, Qin W, Pan J, Baumen WA, Blitzer RD, Cardozo C, 2010a, **REDD1 is a major target of testosterone action in preventing dexamethasone-induced muscle loss**, *Endocrinology*, 151 (3): 1050-1059.

329. Wu Y, Baumen WA, Blitzer RD, Cardozo C, 2010b, **Testosterone-induced hypertrophy of L6 myoblasts is dependent upon Erk and mTOR**, *Biochemical and Biophysical Research Communications*, 400: 679-683.

330. Wu FCW, 1992, **Testicular steroidogenesis and androgen use and abuse**, *Bailliere's Clinical Endocrinology and Metabolism*, 6 (2): 373-403.

331. Wullschleger S, Loewith R, Hall MN, 2006, **TOR signaling in growth and metabolism**, *Cell,* 124: 471-484.

332. Xu X, Pergola GD, Bjorntorp P, 1991, **Testosterone increases lipolysis and the number of β-andrenoceptors in male rat adipocytes**, *Endocrinology*, 128 (1): 379-382.

333. Xu X, Pergola GD, Bjorntorp P, 1990, **The effects of androgens on the regulation of lipolysis in adipose precursor cells**, *Endocrinology*, 126 (2): 1229-1234.

334. Yarrow JF, Conover CF, McCoy SC, Lipinska JA, Santillana CA, Hance JM, Cannady DF, Van Pelt TD, Sanchez J, Conrad BP, Pingel JE, Wronski TJ, Borst SE, 2011, **17β-hydroxyestra-4,9,11-trien-3-one (trenbolone) exhibits tissue selective anabolic activity: effects on muscle, bone, adiposity, haemoglobin, and prostate**, *American Journal of Physiology Endocrinology & Metabolism*, 300: E650-E660.

335. Yesalis CE, Bahrke MS, 2002, **History of doping in sport***, International Sports Studies*, 24 (1): 42-76.

336. You J-S, Anderson GB, Dooley MS, Hornberger TA, 2015, **The Role of mTOR signaling in the regulation of protein synthesis and muscle mass during immobilization***, Disease Models & Mechanisms*, 8 (9): 1059-69.

337. Yu I-C, Lin H-Y, Liu N-C, Sparks JD, Yeh S, Gang L-Y, Chen L, Chang C, 2013, **Neuronal androgen receptor regulates insulin sensitivity via suppression of hypothalamic NF-κB-mediated PTP1B expression***, Diabetes*, 62: 411-423.

338. Zhang P, Liang X, Shan T, Jiang Q, Deng C, Zheng R, Kuang S, 2015, **mTOR is necessary for proper satellite cell activity and skeletal muscle regeneration***, Biochemical and Biophysical Research Communications*, 463: 102-108.

339. Ziegenfuss TM, Berardi JM, Lowery LM, Antonio J, 2002, **Effects of prohormone supplementation in humans: A review***, Canadian Journal of Applied Physiology*, 27 (6): 628-645.