DISCOVERY AND THERAPEUTIC PROMISE OF SELECTIVE ANDROGEN RECEPTOR MODULATORS FOR HORMONAL MALE CONTRACEPTION

DISSertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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*****

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ABSTRACT

There is an urgent need to develop new forms of contraception that men are willing to use. Currently, testosterone-based hormonal contraceptives represent the most promising approach. However, testosterone demonstrates little activity after oral administration due to rapid hepatic elimination, precluding its use in oral contraceptives for men. The long-term safety of testosterone, as it relates to psychological function, cardiovascular disease, and prostate disease, is also of concern. Non-steroidal selective androgen receptor modulators (SARMs) may provide an alternative to the use of testosterone, with the advantages of oral bioavailability, tissue selectivity, lack of influence on lipoproteins, and androgen receptor (AR) specificity. We hypothesized that SARMs would mimic the pharmacologic activity of testosterone in vivo and can safely replace testosterone as a component of hormonal male contraceptives.

SARMs discovered previously demonstrated potential applications in the treatment of muscle wasting, osteoporosis, and benign prostate hyperplasia in animal models. However, low or no CNS effects were observed, which precluded their use for hormonal male contraception as a single regimen. Novel AR ligands were designed and synthesized using integrated knowledge of molecular modeling, structure-activity-relationships, and pharmacokinetics and metabolism of known SARMs. In the current
studies, the abilities of these compounds to bind AR and to stimulate (agonist) or inhibit (antagonist) AR-mediated transcriptional activation (Chapter 2) were determined. Compounds with high AR binding affinity and potent stimulatory activity in transcriptional assays were further investigated in castrated rats (Chapter 3) for their pharmacologic effects. Key *in vitro* and *in vivo* structure-activity-relationships of these ligands were identified. Differences in the pharmacokinetics and metabolism between SARMs resulted in a contradiction between the *in vitro* and *in vivo* pharmacologic activity, and also provided rationale for further structural modifications (Chapter 4). Through our iterative, step-wise experimental paradigm, more potent SARMs were discovered using structural modifications of our lead compound. The effects of the potent SARMs on spermatogenesis, reproductive and peripheral organs, body composition, hormonal biomarkers, sexual behavior, and fertility were further investigated (Chapters 5 through 7). As a whole, these studies provide valuable information regarding the physiologic and pharmacokinetic factors governing the pharmacology and feasibility of SARMs for hormonal male contraception.
DEDICATION

To Jim, Our Parents, and Justin
ACKNOWLEDGMENTS

I would like to thank Dr. James T. Dalton, my mentor, teacher, and friend. I have been very fortunate to have had a great teacher in the past four years who stimulated my interest in drug discovery and androgen receptor ligands, guided my efforts to learn and explore in pharmaceutical areas, and gave me constant encouragement with his enthusiasm.

I am indebted to my collaborators, Drs Duane D. Miller, Dong-Jin Hwang, and Kiwon Chang for synthesizing compounds and scientific discussion. Dr. Randy J. Nelson gave generously and amusingly of his time and expertise on behavior studies. Without his help, I could not have written Chapter 6 and Chapter 7. I also benefited from stimulating discussion and encouragement from my other committee members, Drs Thomas D. Schmittgen and Duxin Sun. My sincere thanks also go to all my lab mates for their timely assistance and friendship.

Financially, my studies were supported by grants from NIH, GTx Inc, and a Presidential Fellowship at the Ohio State University. I would like to thank them.

Through all these years, I have been so blessed to learn and grow up with love and support from my husband, Jim J. Xiao, who is also a great scientific partner of me. I would like to thank our parents for their unconditional love, support, and consideration.
Without their tremendous help, I could not have finished my studies. I also thank my baby, Justin, for bringing so much joy and wonderful memory to my life.
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Major Field: Pharmacy

Studies in Pharmacokinetics, Pharmacodynamics, Drug Metabolism, and Pharmacology
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LIST OF ABBREVIATIONS

SARM Selective androgen receptor modulator
DHEA Dehydroepiandrosterone
DHT Dihydrotestosterone
AR Androgen receptor
DBD DNA binding domain
LBD Ligand binding domain
TAU Transcription activation unit
HSP Heat shock protein
NLS Nuclear localization signal
ARE Androgen response element
RNA-pol II RNA-polymerase II
GTFs General transcription factors
TBP TATA binding protein
TAFs TBP associating factors
TP Testosterone propionate
TE Testosterone enanthate
TB Testosterone buciclate
TU Testosterone undecanoate
TD Testosterone decanoate
MENT 7α-methyl-19-nortestosterone
BPH Benign prostate hyperplasia
CNS Central nervous system
GnRH Gonadotropin-releasing hormone
LH Luteinizing hormone
FSH Follicle stimulating hormone
WHO World Health Organization
CPA Cyproterone acetate
DMPA Depot medroxyprogesterone acetate
NETE Norethisterone enanthate
LNG Levonorgestrel
DSG Desogestrel
ENG Etonorgestrel
DNG Dienogest
[^3H]-MIB 17α-methyl[^3H] Mibolerone
<table>
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<tr>
<td>HAP</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>SARs</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>PEG 300</td>
<td>Polyethylene glycol-300</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>$T_{1/2}$</td>
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<tr>
<td>$AUC_{0-\infty}$</td>
<td>Area under the plasma concentration-time curve</td>
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<td>$V_{dss}$</td>
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<tr>
<td>$T_{max}$</td>
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<tr>
<td>$F_{p.o.}$</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
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<tr>
<td>MS$^n$</td>
<td>Multiple stage mass spectrometry</td>
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<tr>
<td>CID</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatograph</td>
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<tr>
<td>PEI</td>
<td>Post ejaculation interval</td>
</tr>
<tr>
<td>FM</td>
<td>Percent fat mass</td>
</tr>
<tr>
<td>LM</td>
<td>Percent lean mass</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
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<tr>
<td>DEXA</td>
<td>Dual energy x-ray absorptiometry</td>
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<tr>
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</tr>
<tr>
<td>SPG</td>
<td>Sperm Counts per gram of tissue</td>
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<td>NIH</td>
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CHAPTER 1

INTRODUCTION

1.1 ANDROGEN BIOSYNTHESIS AND THE ANDROGEN RECEPTOR (AR)

Androgens are necessary and essential for supporting male development and maintaining spermatogenesis and male secondary characteristics (1). More than 95% of circulating testosterone is synthesized and secreted by the Leydig cells in the testes (2, 3). Peripheral tissues, mainly the adrenal cortex, produce about 5% of testosterone. Circulating testosterone plays an important role in the regulation of androgen production via the hypothalamus-pituitary-testis axis. Figure 1.1 shows the steroidogenic pathways in the human testis and peripheral tissues. Steroidogenic reactions start from the translocation of cholesterol (C27) in the mitochondria and the production of pregnenolone (C21) by the cleavage of the side chain of cholesterol. Pregnenolone is translocated from the mitochondria to the smooth endoplasmic reticulum, where testosterone is synthesized via a stepwise reaction catalyzed by oxidative enzymes belonging to the cytochrome P450 family (4, 5). Adrenal glands mainly synthesize two testosterone precursors, dehydroepiandrosterone (DHEA) and androstenedione, which also function as weak androgens with 5 and 10% of the activity of testosterone, respectively. In peripheral tissues, two important and active metabolites of testosterone are formed, namely dihydrotestosterone (DHT) and estradiol mediated by the 5α-reductase and aromatase (CYP19) enzymes, respectively (Figure 1.1).

The pharmacologic activity of testosterone and DHT is predominantly mediated by the androgen receptor (AR), a ligand-dependent transcription factor. The full length AR is translated as a protein of approximately 918 amino acids (6, 7) and molecular
weight of 110 kDa (8). The AR belongs to the nuclear receptor superfamily and contains four functional domains. As illustrated in Figure 1.2, AR is composed with following domains: the N-terminal transactivation domain, the DNA binding domain (DBD), the hinge region, and the ligand binding domain (LBD). Transcriptional activation of the AR is primarily mediated by sequences in the N-terminal domain and a short region in the LBD, referred to as AF-1 and AF-2, respectively (9). The AF-1 domain is functionally independent from ligand and can be further divided into a transcription activation unit (TAU)-1 (amino acids 100-370) and TAU-5 (amino acids 360-528) (10). However, the precise mechanism of AF-1 domain is not well defined. In contrast, the AF-2 domain is ligand-dependent and indispensable for AR-mediated transcriptional activation as proved by deletion and mutation studies (11-14). The crystal structure of the AR LBD reveals that it forms twelve α helixes (H1 – H12) and one β-turn, resulting in a three-layer α helical sandwich (15, 16). The core of AF-2 is formed by helix H12. Upon binding with an agonist, the AR LBD is thought to form a compact conformation, in which H12 fold back as a lid to cover the ligand binding pocket and stabilize the binding surface for coactivators (17). On the other hand, binding with AR agonists is thought to prevent the AR LBD from forming such a compact and stable conformation as needed for transactivation.

1.2 MOLECULAR MECHANISM OF AR ACTIONS

Prior to ligand binding, the AR (apoAR) forms a heterocomplex with chaperone proteins like Heat Shock Proteins 90 (HSP90) and other co-chaperones (18, 19). The HSP90-AR complex protects the apoAR in an inactive state and also maintains its high binding affinity conformation. Mutation of HSP90 to HSP82 diminishes the high binding affinity of AR with its ligand compared to its wild-type complex (20). Figure 1.3 is a schematic that shows the molecular mechanism of AR actions. Binding with ligand triggers the dissociation of the AR from the chaperone protein, conformational changes in the LBD, formation of AR dimmers, and translocation of the AR-ligand (holoAR) complex into the nucleus via an intrinsic nuclear localization signal (NLS). The AR-
ligand complex then binds the enhancer region (androgen response element, ARE) located in the upstream promoter of target genes and interacts with several key components of the signaling cascade, including RNA-polymerase II (RNA-pol II), general transcription factors (GTFs), TATA binding protein (TBP), and TBP associating factors (TAFs) (21). The AR interacts with the transcriptional machinery by communication directly with GTFs or indirectly via coactivators (e.g., SRC-1) (22, 23), which in turn triggers production of the target mRNA and consequently protein synthesis (Figure 1.3).

In addition to the classical steroid receptor-mediated transcriptional activation, multiple lines of evidence support the existence of non-genomic effects of steroid receptors in the plasma membrane (24-27). Non-genomic effects of androgens were reported in osteoblasts (28), macrophage (29), adipose tissues (30), and Sertoli cells (31). However, the mechanisms of the non-genomic actions of steroid receptors are still poorly understood.

1.3 AR LIGANDS AND PHARMACOLOGY

Functionally, AR ligands can be divided into two categories-AR agonists (androgens) and AR antagonists (antiandrogens). Structurally, AR ligands can be further classified as steroidal and nonsteroidal compounds within each category. Testosterone and DHT are two naturally occurring androgens with a steroidal structure (Figure 1.4). Testosterone has been used for the treatment of hypogonadism for decades. However, testosterone undergoes rapid hepatic elimination (32) and is not oral bioavailable (33, 34) after oral administration. In order to prolong the action of testosterone, various testosterone derivatives were developed and clinically used, including 17α-methyltestosterone, testosterone propionate (TP), testosterone enanthate (TE), and testosterone buciclate (TB). Testosterone undecanoate (TU) was reported as an orally bioavailable androgen (35). However, its low efficacy in early clinical trials (36) of male contraception prevented its widespread use in this regard. To date, the main administration routes of steroidal androgens remain intramuscular injection, transdermal
patch, and pellet implantation. In addition to the treatment of hypogonadism, androgens have also been investigated intensively for hormonal male contraception (see Section 1.5) and androgen replacement for ageing men. Although testosterone demonstrated beneficial effects on the bone, cognitive function, body composition, muscle strength and function, mood, and sexual function in men, there are possible risks related to testosterone treatment including cardiovascular function, prostatic stimulation, changes in serum lipid and, erythropoiesis. The lack of large-scale trials of testosterone make it controversial and premature to make conclusions regarding the risk/benefit ratio of androgen replacement in aging men (37-40).

In men, the effects of testosterone on the growth of the reproductive tract (e.g., prostate) are amplified by conversion to DHT, which is a more potent androgen than testosterone. Co-administration of testosterone and a 5α-reductase inhibitor demonstrated that the androgenic activity of testosterone on the prostate and seminal vesicles is highly dependent on 5α-reductase activity, while the anabolic effect of testosterone in muscle occurs independently from conversion to DHT, indicating that the differential effect of testosterone action in androgenic and anabolic tissues is due to the high and low 5α-reductase activity in reproductive tissues and muscle, respectively (41-44). Recently, a tissue-selective steroidal androgen was discovered by the incorporation of a 7α-methyl group in a testosterone derivative. For instance, 7α-methyl-19-nortestosterone (MENT, Figure 1.4) demonstrates tissue selectivity with higher anabolic activity than androgenic activity both in rat (45) and in hypogonadal men (46). This tissue selectivity was mainly due to the inability of 5α-reductase to metabolize MENT.

Androgens are not only essential for the physiological development of the prostate, but also for the initiation and progression of prostate cancer. Antiandrogens are used as the first line treatment for prostate cancer by competitively binding with the AR in target tissues (47). Steroidal antiandrogens, such as cyproterone acetate and megestrol acetate (Figure 1.5), demonstrated mixed pharmacologic activity. Cyproterone acetate showed progestational activity and mixed AR agonist and antagonist activity (48). While megestrol acetate cross-reacts with the progesterone receptor, glucocorticoid receptor, and mineralocorticoid receptor (49-52). To date, these non-selective antiandrogens have
been largely replaced by nonsteroidal antiandrogens, including flutamide, nilutamide, and bicalutamide (Figure 1.5) due to their improved AR specificity, tissue selectivity, and pharmacokinetic properties. The use of nonsteroidal antiandrogens in prostate cancer treatment was recently reviewed (53-55).

1.4 NONSTEROIDAL SELECTIVE ANDROGEN RECEPTOR MODULATORS

In addition to the discovery of steroidal androgens using structural modifications of testosterone, another area in which progress has been made is the discovery of nonsteroidal androgens with multiple pharmacophores. Our laboratory was the first to report the discovery of nonsteroidal AR ligands structurally modified from known antiandrogens – flutamide and bicalutamide (56). The first generation of nonsteroidal androgens (e.g., acetothiolutamide) was the bicalutamide-derivatives, which displayed high binding affinity and potent activity in vitro (57) with poor pharmacologic activity in vivo. The contradictory in vitro and in vivo activity of acetothiolutamide was mainly due to its metabolic instability (58). Structural modification of the thio-ether linkage to an oxygen bridge (Figure 1.6) resulted in the identification, two potent and efficacious nonsteroidal AR ligands (S-1 and S-4) (59, 60) as selective androgen receptor modulators (SARMs). Unique characteristics of the second generation of nonsteroidal androgens include: AR specificity, tissue-selectivity, oral bioavailability, and amenability for further structural modifications. Key structure-activity relationships were analyzed to facilitate the identification and discovery novel AR ligands (61-63). Recently, we reported the potential application of SARMs in the treatment of benign prostate hyperplasia (BPH) (64) and osteoporosis (Kearbey, OSU dissertation, 2005). These SARMs work as partial agonists in androgenic tissues (e.g., prostate) and full agonists in anabolic tissues (e.g., levator ani muscle and bone) without affecting the hypothalamus-pituitary-testis axis. More recently, we investigated the ability of a novel SARM to regulate gonadotropins, testosterone, and spermatogenesis (65). Results suggested that novel SARMs (e.g. C-6, Figure 1.6) with potent central nervous system (CNS) effects can be designed by incorporating multiple substituents in the aromatic B-ring for potential use in hormonal
male contraception. Meanwhile, crystallography studies conducted in our laboratory suggest that a unique B-ring subpocket exists in the AR for SARMs that can be used to provide fundamental rationale for B-ring structural modifications (Bohl, OSU dissertation, 2005).

The concept of nonsteroidal androgens has significantly progressed since it was first defined by Dalton et al. in 1999 (66). Several other groups later reported similar transformation from nonsteroidal antagonists to agonists of compounds structurally related to 2-quinoline and coumarin (67, 68). Markedly increased bone mineral density (BMD) was observed after treatment of orchiectomized male rat with quinoline-related SARMs prostate-sparing property (69, 70).

1.5 HORMONAL MALE CONTRACEPTION

Unlike women, effective male contraception is restricted to physical methods, namely condoms and vasectomy. A variety of attempts have been made to produce pharmacologic, effective, reversible and side effect-free contraceptive methods for the male. Among them, only hormonal contraception has reached the stage of clinical development (71). Figure 1.7 illustrates the hypothalamus-pituitary-testis axis of androgen regulation. In the brain, gonadotropin-releasing hormone (GnRH) is released from the hypothalamus and stimulates the secretion of pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in a pulsatile fashion. In the testis, LH indirectly stimulates spermatogenesis through testosterone synthesized by Leydig cells, while FSH directly interacts with FSH receptors expressed in Sertoli cells and stimulates spermatogenesis (Figure 1.7). Both LH and FSH are required for quantitative and qualitative spermatogenesis (72). Testosterone and its aromatized metabolite (estradiol) negatively regulate circulating levels of testosterone and GnRH and LH production in the hypothalamus and pituitary, respectively. Activin and inhibin produced by Sertoli cells stimulate or inhibit, respectively, the secretion of FSH from the pituitary. (73). Theoretically, inhibition of one or more hormones involved in the hypothalamus-pituitary-testis axis will suppress spermatogenesis. GnRH secretion can be inhibited
either by exogenous androgens or by GnRH agonists. At the pituitary level, the stimulatory effect of GnRH can be blocked either by competitive binding of a GnRH antagonist with GnRH receptors or by binding of GnRH by a GnRH antibody before it interacts with its receptor. Progestins and exogenous androgens inhibit secretion of LH and FSH, while inhibin selectively down regulates FSH levels. Additionally, the effects of FSH on Sertoli cells can be blocked using either FSH immunization or FSH antagonists. However, the only clinically proven effective methods are androgen alone or androgen combined with either progestins or GnRH antagonists. The use of FSH immunization, FSH antagonists, and inhibin for hormonal male contraception is still under early investigation.

1.5.1 Testosterone Single Regimen

Testosterone was one of the first choices for male contraception due to its natural role in spermatogenesis. However, testosterone demonstrates little pharmacologic activity after oral administration due to from its rapid hepatic elimination. To prolong effect, testosterone implants and longer acting esters, including testosterone enanthate (TE), testosterone propionate (TP), testosterone buciclate (TB), testosterone undecanoate (TU), and testosterone decanoate (TD), were developed (Figure 1.4). Except for TU, the administration methods of most testosterone esters are limited to intramuscular injection (i.m.) (74-80) or surgical implantation of implants and pellets (81-83). In the 1990’s, two large international studies (n = 670) sponsored by the World Health Organization (WHO) (76, 77) tested TE (200 mg/week, i.m.) as a hormonal male contraceptive. In the first study (n = 271), azoospermia was achieved in 64% of men (76). In the second study (n = 357), 98% of men achieved as either azoospermia or severe oligozoospermia (< 3 million sperm/mL of ejaculation). The following efficacy studies demonstrated that the overall failure rate in preventing pregnancy by azoospermia and severe oligozoospermia was 1.4 per 100 person years (77), which was as the efficient as female pill and better than which can be afforded by condoms (12 to 15%). However, weekly injection and long-term side effects related to supraphysiologic levels of testosterone are not well tolerated. A single
intramuscular injection of TU provided normal serum testosterone levels for at least 6 weeks in hypogonadal men (35). Recently, the efficacy of male contraception using this longer-acting androgen was studied in China. Monthly injection of TU at a dose of 500 mg for 12 months demonstrated 97% success rate in achieving azoospermia and oligozoospermia and an overall failure rate in preventing pregnancy of 5.2 per 100 person years (78).

To prevent potential adverse effects of androgen treatment on the prostate, tissue selective steroidal androgens, such as 7α-methyl-19-nortestosterone (MENT), were also investigated for hormonal male contraception. MENT is a nonaromatizable steroidal androgen, which maintained libido in hypogonadal men (84) and demonstrated potent tissue-selective pharmacologic activity in animals (85) and men (46). The use of MENT for hormonal male contraception as a single regimen is being investigated in early-phase clinical trials (Table 1.1) (83).

1.5.2 Testosterone-Based Combination Regimens

In contrast to the high rate (91%) of azoospermia achieved in East Asian populations by testosterone alone, azoospermia was only observed in 50 to 70% of Caucasian men. To explain this heterogeneity in the spermatogenic response, multiple differences between responders and non-responders were studied and proposed, including the sensitivity of hypothalamus-pituitary-testis axis to the negative feedback signal from testosterone (86), intratesticular 5α-reductase activity (87), pretreatment hormonal status (88, 89), and the polymorphism of the common polyglutamine stretch (CAG repeats) length in exon 1 of the AR gene (90).

To increase the response rate of hormonal male contraception in Caucasians and to avoid those disadvantages caused by supraphysiologic doses of testosterone, other gonadotropin-suppressing substances (e.g., progestins and GnRH antagonists) were included and used as combination regimens. In principle, GnRH antagonists competitively bind to the GnRH receptor in the pituitary and consequently inhibit the secretion of gonadotropins. GnRH antagonist-testosterone regimens revealed more rapid
onset and complete suppression of spermatogenesis than testosterone alone (91). However, the widespread use of GnRH antagonists for hormonal male contraception is limited since these proteins are expensive to synthesize and difficult to deliver. On the contrary, progestins have been used as a key component of female contraception for decades. The administration of progestins alone in men resulted in faster and more potent gonadotropin suppression (92). However, nearly complete depletion of endogenous testosterone leads to loss of libido (93). Therefore, a physiological dose of testosterone was included during most clinical trials. Additionally, testosterone and progestins work synergistically in suppression of gonadotropins. The currently available progestins (Figure 1.8) include cyproterone acetate (CPA), depot medroxyprogesterone acetate (DMPA), norethisterone enanthate (NETE), levonorgestrel (LNG), desogestrel (DSG), etonorgestrel (ENG), and dienogest (DNG). Except for DMPA, all of the other progestins are orally active, making them good candidates for inclusion in a male contraceptive ‘pill’. Clinical trials of hormonal male contraception are aimed at finding the best androgen-progestin combination and the minimum effective dose of these compounds.

1.5.3 Testosterone Plus DMPA or CPA

It has been three decades since the first attempts to use DMPA or CPA for hormonal male contraception. As shown in Table 1.2, DMPA in combination with TE, 19-nortestosterone, or testosterone pellets achieved either azoospermia (50 to 100%) or oligozoospermia in all subjects (81, 94-98). In one major clinical study of 55 couples, no pregnancies occurred in a 426 person-months efficacy phase (98), suggesting testosterone-DMPA implantation is a promising approach for male contraception. However, disadvantages related to this method are a relatively high rate of extrusion and the requirement for a surgical procedure.

CPA is not only an oral bioavailable progestin, but also an antiandrogen, which provides dual effects in the suppression of spermatogenesis. A series of studies were performed to investigate oral CPA at doses ranging from 5 to 100 mg combined with a weekly injection (i.m.) of TE at 100 mg/week (99-101). Although small numbers of
subjects were enrolled in these studies, the combination regimen demonstrated high
efficacy, which was superior to that observed with TE alone (Table 1.3 vs Table 1.1). Unfortunately, the antiandrogenic effect of CPA led to a dose-dependent fall in
hemoglobin, hematocrit, and body weight. More recently, Meriggiola et al. observed that
TU alone or combined with lower doses of CPA maintains sperm suppression for 32
weeks without significant side effects (102). However, the efficacy and safety of this
prototype regimen need to be further investigated in larger populations.

1.5.4 Testosterone Plus LNG, DSG, ENG, NETE or DNG

LNG, DSG, ENG, NETE, and DNG are progestins that were structurally derived
from 19-nortestosterone. Testosterone-LNG and testosterone-DSG combinations for male
contraception have been extensively investigated. As shown in Table 1.4, weekly
injection of TE combined with LNG tablets or implants suppressed spermatogenesis to
azoospermia (61 to 93%) or severe oligozoospermia (89 to 100%) in men (74, 103, 104).
Although it offers more efficient activity than TE alone in Caucasian men (Table 1.4 vs
Table 1.1), again weekly injection is not applicable to routine or chronic clinical use.
Monthly injection of 250 mg of TU combined with LNG (250 µg/day, p.o.) achieved
either azoospermia (57%) or oligozoospermia (43%) in all subjects. However, no
significant difference was observed between TU-LNG and TU alone groups in the
suppression of spermatogenesis (79). Another study showed that preloading LNG for 4
weeks and then followed by 1000 mg of TU injection produces sustained suppression of
spermatogenesis to azoospermia (90%) in Chinese men (80). Similarly, LNG did not
offer significantly beneficial effects when TU-LNG was compared with TU alone
regimen. When the same dose of LNG was combined with a testosterone transdermal
patch, results were disappointing, indicating that the dose and administration route of
testosterone is critical to success for androgen-progestin combination regimens (104).

Table 1.5 shows the suppression of spermatogenesis following treatment with
testosterone esters combined with DSG (105-109). Among them, uniform azoospermia
was achieved in multiple studies using this testosterone-DSG combination regimen (105-
108), which was more effective than the testosterone-LNG regimen in suppression of spermatogenesis in Caucasians with similar side effects (e.g., weight gain and decrease in HDL-cholesterol). Moreover, a self-applied regimen was also investigated using testosterone transdermal patch plus DSG at doses of 75, 150, and 300 µg/day (110). However, the azoospermia rate was significantly lower than that of TE or T-pellet combined with the same dose of DSG. Therefore, the combination of long acting testosterone pellets (3 months) plus DSG appears to be a promising regimen for male contraception based on the results of small studies. Larger clinical trials using this regimen are necessary for further development of this regimen for hormonal male contraception.

More recently, male contraception studies have focused on newer androgen-progestin combination regimens, such as TU + NETE (111-113) and TD + ENG (114) (Table 1.6). NETE is a progestin containing partial AR agonist activity, approximately 10% of the activity of testosterone. Since both TU and NETE are long-acting hormones, TU (1000 mg) and NETE (200 or 400 mg) were dissolved in castor oil and injected at 6, 8, or 12-week intervals (111-113). More than 90% of subjects in each treatment achieved azoospermia with injection interval of 6 and 8 weeks. In contrast, only 38% subjects developed azoospermia with an injection interval of 12 weeks. The contraceptive effect of TU (1000 mg) plus NETE (200 mg) in men is promising and needs to be further evaluated in larger clinical trials. ENG is a potent progestin and the active metabolite of DSG following by oral administration. For the first clinical trial with T plus ENG (115), depot-testosterone (400 mg) was implanted at a 12-week interval along with one-time ENG (68, 136, and 204 mg) implant(s) into subjects. Although spermatogenesis was suppressed to lower than 3 million sperm per mL of ejaculate in almost all subjects, T-ENG (204 mg) was the only regimen that induced uniform azoospermia in men (116). Most recently, a multicenter phase II study (n = 112) of TD + ENG for male hormonal contraception was reported (114). TD at a dose of 400 mg was injected every 4 or 6 weeks combined with ENG at an oral dose of 300 µg for 48 weeks. As shown in Table 1.6, more frequent injections (4 vs 6-week) induced a higher rate of azoospermia (96 vs
83%) and more rapid onset. Therefore, TD (400 mg/4 weeks) plus ENG (300 µg) represents a promising approach for male contraception.

1.5.5 Limitation of Testosterone-Based Hormonal Male Contraception

No major toxicological effects were reported in most clinical trials involving young, eugonadal subjects. The major limitations of using testosterone-based hormonal male contraception are inconvenient administration routes and steroidal related side effects, including decrease of HDL cholesterol, increase of hematologic parameters such as hemoglobin and hematocrit, increase of body weight, and acne. Although lower doses of testosterone were effectively used in testosterone-progestin combination regimens, similar but minor side effects were reported (see references in table 1.2 ~ 1.6). Additional concern of long-term treatment with testosterone is the potential risk of testosterone in the prostate and cardiovascular system (117), which needs to be evaluated in large prospective studies in the future.

1.6 SCOPE AND OBJECTIVES OF DISSERTATION

SARMs discovered in our laboratory offer potential use for androgen replacement with advantages such as high oral bioavailability, tissue selectivity (prostate-sparing effect), AR specificity, and absence of steroid-related side effects. We hypothesized that SARMs which mimic testosterone in regulating gonadotropin and testosterone levels could be used to achieve a male contraceptive ‘pill’, using SARM alone or in combination with progestins. This hypothesis was tested by an iterative and step-wise discovery paradigm.

The specific aims of this project were to: determine AR binding affinity and AR-mediated transcriptional activation in vitro of novel AR ligands with multiple substituents in the aromatic B-ring and analyze the in vitro structure-activity relationships (Chapter 2); examine the in vivo pharmacologic activity of AR ligands, analyze in vivo structure-
activity relationships, and select potent SARMs for male contraception studies (Chapter 3); illustrate the pharmacokinetics and metabolism of a SARM (C-3) in male rats (Chapter 4); characterize the pharmacologic activity of a SARM (C-6) in castrated and intact male rats and its effects on hormonal regulation and spermatogenesis (Chapter 5); examine the effects of C-6 on masculine sexual behavior in castrated male rats (Chapter 6); determine the effects of a SARM (C-31) on spermatogenesis, hormonal regulation, endocrine physiology, and male fertility (Chapter 7). In Chapter 8, conclusions and major findings of the current studies are summarized.
Figure 1. Steroidogenic Pathways in the Human Testis and Peripheral Tissues.
Figure 1. 2 Main Functional Domains of Androgen Receptor

AF: activation function; DBD: DNA binding domain; LBD: Ligand binding domain.
Figure 1. 3 Schematic of the Molecular Mechanism of AR Action.

After dissociation of heat shock proteins (hsp), the AR is translocated to the nucleus via an intrinsic nuclear localization signal, forms dimmers, and binds to specific DNA elements present as enhancers in upstream promoter sequences of androgen target genes. The AR complex directly or indirectly interacts with the transcription machinery, which triggers mRNA synthesis and consequently protein synthesis. A non-genomic pathway involving the classical AR via cross-talk with the Src/Raf-1/Erk-2 pathway is known.

Figure 1. 4 The Chemical Structures of Steroidal Androgens.
Figure 1. 5 The Chemical Structures of Steroidal and Nonsteroidal Antiandrogens.
Figure 1. The Chemical Structures of Recently Reported Nonsteroidal Androgens
Figure 1. 7 Hypothalamus-Pituitary-Testis Axis of Androgen Regulation.

The production of testosterone is regulated by hormones produced in the hypothalamus and pituitary. (+) represents positive feedback and (-) represents negative feedback.
<table>
<thead>
<tr>
<th>Androgen</th>
<th>Ethnicity</th>
<th>Androgen dose</th>
<th>Study size (n)</th>
<th>Azoospermia % (n)</th>
<th>Severe Oligozoospermia % (n)</th>
<th>Oligozoospermia % (n)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TE</strong></td>
<td>Caucasian</td>
<td>100 mg/week i.m.</td>
<td>18</td>
<td>30 (6)</td>
<td>22 (4)</td>
<td>6 (1)</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>200 mg/week i.m.</td>
<td>225</td>
<td>65 (157)</td>
<td>13 (29)</td>
<td>3.5 (8)</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>200 mg/week i.m.</td>
<td>357</td>
<td>75 (267)</td>
<td>n/a</td>
<td>23 (83)</td>
<td>76</td>
</tr>
<tr>
<td><strong>TU</strong></td>
<td>Chinese</td>
<td>500 mg/4 week i.m.</td>
<td>12</td>
<td>92 (11)</td>
<td>8 (1)</td>
<td>0 (0)</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Chinese</td>
<td>1000 mg/4 week i.m.</td>
<td>12</td>
<td>100 (12)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Chinese</td>
<td>1000 mg loading + 500 mg/4 week i.m.</td>
<td>305</td>
<td>93 (284)</td>
<td>2 (6)</td>
<td>2 (6)</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>1000 mg/4 week i.m.</td>
<td>14</td>
<td>50 (7)</td>
<td>29 (4)</td>
<td>7 (1)</td>
<td>78</td>
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<tr>
<td></td>
<td>Chinese</td>
<td>1000 mg/8 week i.m.</td>
<td>21</td>
<td>67 (14)</td>
<td>n/a</td>
<td>n/a</td>
<td>79</td>
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<td><strong>T Pellets</strong></td>
<td>n/a</td>
<td>400 mg</td>
<td>10</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>800 mg</td>
<td>10</td>
<td>40 (4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>80</td>
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<tr>
<td></td>
<td>n/a</td>
<td>1200 mg</td>
<td>9</td>
<td>56 (5)</td>
<td>44 (4)</td>
<td>0 (0)</td>
<td>81</td>
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<tr>
<td><strong>MENT Ac Implant</strong></td>
<td>n/a</td>
<td>1</td>
<td>12</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>82</td>
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<tr>
<td></td>
<td>n/a</td>
<td>2</td>
<td>11</td>
<td>18 (2)</td>
<td>n/a</td>
<td>18 (2)</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>4</td>
<td>12</td>
<td>58 (7)</td>
<td>n/a</td>
<td>8 (1)</td>
<td>82</td>
</tr>
</tbody>
</table>

Table 1. Recent Studies Using Androgen Single Regimen for Hormonal Male Contraception (n > 9).

Severe oligozoospermia: <1 million sperm/mL; Oligozoospermia: 1 to 3 million sperm/mL.
<table>
<thead>
<tr>
<th>Androgen + Progestin</th>
<th>Ethnicity</th>
<th>Androgen dose</th>
<th>Progestin dose</th>
<th>Study size</th>
<th>Azoospermia % (n)</th>
<th>Severe Oligozoospermia % (n)</th>
<th>Oligozoospermia % (n)</th>
<th>Reference</th>
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</thead>
<tbody>
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<td><strong>TE + DMPA</strong></td>
<td>Indonesian</td>
<td>100 mg/week i.m.</td>
<td>100 mg/4 week i.m.</td>
<td>10</td>
<td>100 (10)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Indonesian</td>
<td>200 mg/week i.m.×7, then every 3 weeks</td>
<td>25 mg/6 week i.m.</td>
<td>45</td>
<td>96 (43)</td>
<td>4 (2)</td>
<td>0 (0)</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Indonesian</td>
<td>250 mg/week i.m.</td>
<td>200 mg/4 week i.m.</td>
<td>10</td>
<td>100 (10)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>250 mg/week i.m.</td>
<td>200 mg/4 week i.m.</td>
<td>10</td>
<td>60 (6)</td>
<td>0 (0)</td>
<td>40 (4)</td>
<td>96</td>
</tr>
<tr>
<td><strong>19-Nortestosterone + DMPA</strong></td>
<td>Indonesian</td>
<td>200 mg/week i.m.×7, then every 3 weeks</td>
<td>25 mg/6 week i.m.</td>
<td>45</td>
<td>98 (44)</td>
<td>2 (1)</td>
<td>0 (0)</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>200 mg/3 week i.m.</td>
<td>250 mg/6 week i.m.</td>
<td>12</td>
<td>50 (6)</td>
<td>33 (4)</td>
<td>17 (2)</td>
<td>97</td>
</tr>
<tr>
<td><strong>T + DMPA</strong></td>
<td>n/a</td>
<td>800 mg Pellets</td>
<td>300 mg single dose i.m.</td>
<td>10</td>
<td>90 (9)</td>
<td>0 (0)</td>
<td>10 (1)</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>800 mg/4 or 6 month Pellets</td>
<td>300 mg/3 month i.m.</td>
<td>55</td>
<td>94 (53)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>98</td>
</tr>
</tbody>
</table>

Table 1. 2 Recent Studies Using Testosterone-DMPA for Hormonal Male Contraception (n >10).

Severe oligozoospermia: <1 million sperm/mL; Oligozoospermia: 1 to 3 million sperm/mL.
Table 1. 3 Recent Studies Using Androgen Plus CPA for Hormonal Male Contraception (n > 5).

Severe oligoospermia: <1 million sperm/mL; Oligoospermia: 1 to 3 million sperm/mL.
<table>
<thead>
<tr>
<th>Androgen + Progestin</th>
<th>Ethnicity</th>
<th>Androgen dose</th>
<th>Progestin dose</th>
<th>Study size (n)</th>
<th>Azoospermia % (n)</th>
<th>Severe Oligozoospermia % (n)</th>
<th>Oligozoospermia % (n)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TE + LNG</strong></td>
<td>Caucasian</td>
<td>100 mg/week i.m.</td>
<td>125 µg/day p.o.</td>
<td>18</td>
<td>61 (11)</td>
<td>28 (5)</td>
<td>6 (1)</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>100 mg/week i.m.</td>
<td>250 µg/day p.o.</td>
<td>18</td>
<td>78 (14)</td>
<td>11 (2)</td>
<td>0 (0)</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>100 mg/week i.m.</td>
<td>500 µg/day p.o.</td>
<td>18</td>
<td>67 (12)</td>
<td>11 (2)</td>
<td>17 (3)</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>100 mg/week i.m.</td>
<td>4 rods Norplant II</td>
<td>14</td>
<td>93 (13)</td>
<td>7 (1)</td>
<td>0 (0)</td>
<td>104</td>
</tr>
<tr>
<td><strong>TU + LNG</strong></td>
<td>Caucasian</td>
<td>1000 mg/6 week i.m.</td>
<td>250 µg/day p.o.</td>
<td>14</td>
<td>57 (8)</td>
<td>29 (4)</td>
<td>14 (2)</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Chinese</td>
<td>250 mg/4 week i.m.</td>
<td>2 rods Sino-Implant</td>
<td>16</td>
<td>38 (6)</td>
<td>0 (0)</td>
<td>6 (1)</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Chinese</td>
<td>500 mg/8 week i.m. (4 weeks after LNG implantation)</td>
<td>4 rods, 75mg/each</td>
<td>21</td>
<td>62 (?)</td>
<td>n/a</td>
<td>24 (?)</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Chinese</td>
<td>1000 mg/8 week i.m. (4 weeks after LNG implantation)</td>
<td>4 rods, 75mg/each</td>
<td>20</td>
<td>90 (19)</td>
<td>n/a</td>
<td>5 % (1)</td>
<td>79</td>
</tr>
<tr>
<td><strong>T+ LNG</strong></td>
<td>Mixed</td>
<td>2 patches/day Testoderm TTS</td>
<td>4 rods Norplant II</td>
<td>20</td>
<td>35 (7)</td>
<td>25 (5)</td>
<td>10 (2)</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>2 patches/day Testoderm TTS</td>
<td>125 µg/day p.o.</td>
<td>15</td>
<td>30 (5)</td>
<td>7 (1)</td>
<td>7 (1)</td>
<td>104</td>
</tr>
</tbody>
</table>

Table 1. 4 Recent Studies Using Androgen Plus LNG for Hormonal Male Contraception (n > 13).

Severe oligozoospermia: <1 million sperm/mL; Oligozoospermia: 1 to 3 million sperm/mL.
Table 1. 5 Recent Studies Using Androgen Plus DSG for Hormonal Male Contraception.

Severe oligozoospermia: < 1 million sperm/mL; Oligozoospermia: 1 to 3 million sperm/mL.
Table 1. 6 Recent Studies Using Androgen Plus NETE or ENG for Hormonal Male Contraception.

Severe oligozoospermia: < 1 million sperm/mL; Oligozoospermia: 1 to 3 million sperm/mL.
CHAPTER 2

IN VITRO STRUCTURAL ACTIVITY RELATIONSHIPS (SARS) OF NONSTEROIDAL ANDROGEN RECEPTOR LIGANDS WITH MULTIPLE-SUBSTITUENTS IN THE AROMATIC B-RING

2.1 INTRODUCTION

The androgen receptor (AR) belongs to a superfamily of nuclear receptor proteins that are ligand-dependent transcriptional factors. Binding of androgens triggers the conformational change of the AR to its active DNA-binding state, which further interacts with target genes and regulates gene transcription in the presence of appropriate cofactors (118). Androgens act on a number of different target organs by interacting with the AR expressed in those tissues. As such, the AR is a key modulator of processes involved in differentiation, homeostasis, and development of male secondary character (119). Androgen-related diseases include hypogonadism, male infertility, prostate cancer, delay of puberty, hirsutism, and male pattern baldness, etc.

According to their functional activity or chemical structures, androgen receptor ligands generally are classified as androgens or antiandrogens and steroidal or nonsteroidal ligands, respectively. Androgens are widely used for androgen deficient diseases, androgen replacement in aging men, and the development of hormonal male contraception. Two naturally occurring steroidal androgens are testosterone and its metabolite, 5α-dihydrotestosterone (DHT). However, the unfavorable physiochemical properties and steroidal related side effects of these androgens prevent their widespread use in the clinic. Antiandrogens are clinically used to treat androgen-sensitive diseases, including prostate cancer, hirsutism, and androgenic alopecia in women. One class of presently available antiandrogens are steroidal derivatives, such as cyproterone acetate
(120) and megestrol acetate (121), which possess mixed agonist and antagonist androgenic activity and cross-react with progesterone receptor. The other class of antiandrogens is nonsteroidal derivatives, such as flutamide, bicalutamide, and nilutamide, which block the AR specifically by competitive binding with the receptor and are widely used for prostate cancer treatment. Considerable efforts are still being made to develop antiandrogens more potent than the first pure antiandrogen (i.e., flutamide), since it is superior to steroidal compounds in terms of AR specificity, selectivity, and pharmacokinetic properties (122).

As compared to nonsteroidal antiandrogens, the discovery and development of nonsteroidal androgens was delayed for decades. During attempts to affinity label the AR, our laboratory identified the first class of nonsteroidal androgens, which are structural derivatives of flutamide and bicalutamide (56, 63). More recently, new nonsteroidal selective androgen receptor modulators (SARMs) were developed in an attempt to obtain derivatives with higher in vitro activity (57, 62, 123), in vivo selective pharmacologic activity (59), and better pharmacokinetic properties (58, 60, 124). In previous studies, we outlined the key structure-activity relationships (SARs) of bicalutamide-related androgen agonists to improve AR binding affinity and AR-mediated transcriptional activation. These include the importance of an electron-withdrawing group at the para-position of the A-ring, a methyl group linked to the chiral carbon (S-isomer for ether linked compounds and R-isomer for thio-ether linked compounds), an ether or thio-ether linkage, and an electronegative or acetamido group at the para-position of the B-ring. Although the size of the substituent on the B-ring was proved to be limited by both molecular modeling (61) and experiments (57), a novel SARM with a para-chloro group and a meta-fluoro group in the B-ring demonstrated more efficacious and potent pharmacologic activity than S-1, one of our known SARMs (65). Furthermore, the addition of electronegative and/or hydrophobic substituents in the B-ring may provide a feasible approach to modulate the hepatic metabolism or endocrine effects of SARMs, and identify new drug candidates for other therapeutic applications, including oral, male hormonal contraception. We therefore hypothesized that changing the type, position, and number of substituents in the B-ring would affect the binding affinity, functional activity, and tissue-selectivity of SARMs. The studies reported herein are the first to examine the
in vitro and in vivo pharmacologic effects of AR ligands with multiple substituents in the B-ring.

We investigated the effects and SARs of such structural modifications on AR binding affinity and AR-mediated transcriptional activation in vitro. This information reveals how important the B-ring structure is for AR binding and functional activity, information that is valuable for our understanding of the interaction between nonsteroidal androgen ligands and the subpocket within the AR binding site and that information provides candidates for in vivo pharmacologic studies.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and Animals

The S-isomer of synthetic AR ligands (Table 1) was synthesized in our laboratory with a purity greater than 99% using previously described methods (57, 60). [17α-methyl-3H] Mibolerone ([3H]-MIB, 84 Ci/mmol) and unlabeled MIB were purchased from PerkinElmer Life Sciences (Boston, MA). Hydroxyapatite (HAP) was purchased from Bio-Rad Laboratories (Hercules, CA). EcoLite (+) scintillation cocktail was purchased from ICN Research Products Division (Costa Mesa, CA). LipofectAMINE and Passive Lysis Buffer were purchased from Promega (Madison, WI). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Male Sprague-Dawley rats were purchased from Harlan Bioproducts for Science (Indianapolis, IN). All animals were maintained on a 12h light/dark cycle with food and water available ad libitum. The animal protocol was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

2.2.2 Buffers

Homogenization buffer contained 50 mM Tris, 1 mM potassium phosphate, and
0.1 mM phenylmethylsulfonyl fluoride (PMSF). Incubation buffer contained 10 mM Tris, 1.5 mM EDTA, 0.25 M sucrose, 10 mM sodium molybdate, and 1 mM PMSF. At 4 °C, the incubation buffer was adjusted to pH 7.4 using 1 M HCl. Washing buffer contained 50 mM Tris at pH 7.2. Beta-galactosidase buffer consisted of 200 mM sodium phosphate, 2 mM magnesium chloride, 100 mM B-mercaptopethanol, and 1.33 mg/ml ONGP. Luciferase assay buffer contained 25 mM glycylglycine, 15 mM magnesium chloride, and 5 mM ATP.

2.2.3 Preparation of Cytosolic ARs

Rat prostate cytosolic AR was prepared from ventral prostates of castrated male Sprague-Dawley rats using established methods (63). One day prior to the tissue preparation days, animals were castrated under anesthesia. The ventral prostate of each animal was dissected, removed from excess fat, and was minced with surgical blades. One ml of homogenization buffer was mixed with every 500 mg of prostate tissue. Prostate tissue was then homogenized in a mechanical homogenizer (Model PRO 200, Pro Scientific, Monroe, CT). The homogenate was centrifuged at 114000 g for 1 hour. The cytosolic supernatant, containing the AR, was decanted, aliquoted, and stored at -80°C. All process was carried out at 4°C.

2.2.4 In Vitro AR Binding Affinity

The AR binding affinity of synthetic AR ligands was determined using an in vitro radioligand competitive binding assay as previously described (62). An aliquot of AR cytosol was incubated with 1 nM of [3H]MIB and 1 mM of triamcinolone acetonide at 4 °C for 18 h in the absence or presence of ten increasing concentrations of the compound of interest (10^{-1} nM to 10^{4} nM). Triamcinolone was added into the incubation to block possible cross reaction between [3H]MIB and the progesterone receptor (PR). Nonspecific binding of [3H]MIB was determined by adding excess unlabeled MIB (1000 nM) to the incubate. After incubation, the AR-bound radioactivity was isolated using the HAP method (125). HAP pellets were centrifuged at 1000 g for 10 minutes and then
rinsed with washing buffer for three times. The bound radioactivity was extracted by an aliquot (1 mL) of ethanol at room temperature for 1 hour. The suspensions were centrifuged at 1000 g for 10 minutes and an aliquot (1 mL) of the supernatant was mixed well with 5 mL of scintillation cocktail. Radioactivity was counted in a Beckman LS6500 liquid scintillation counter (Beckman Instruments Inc., Irvine, CA).

2.2.5 In Vitro AR-Mediated Transcriptional Activation

The in vitro functional activities of nonsteroidal AR ligands were determined by the ability of each ligand to induce or suppress AR-mediated transcriptional activation, using a modification of the method of (62). On day one, CV-1 cells at 90% confluence were transiently transfected in T-175 flasks. The transfection was carried out in serum-free DMEM medium using LipofectAMINE according to the manufacturer’s instruction. Cells in each flask were transfected with 0.8 µg of a human AR expression construct (pCMVhAR; generously provided by Dr. Donald Tindall, Mayo Clinic), 4 µg of an androgen-dependent luciferase reporter construct (pMMTV-Luc, generously provided by Dr. Ron Evans, Salk Institute), and 4 µg of a β-galactosidase expression construct (pSV-b-galactosidase; Promega Corporation) for 10 h. Cells were allowed to recover for 12 h and were then seeded into 24-well plates at a density of 8×10⁴ cells/well and allowed to recover for an additional 10 h before drug treatment.

Cells were treated with the compound of interest (final concentrations ranging from 1 nM to 1,000 nM) in the presence or absence of 1 nM of DHT for 12 h. After drug treatment, cells were washed with ice-cold PBS twice and lysed with 110 µl/well of passive lysis buffer for 30 min at room temperature. An aliquot (50 µl) of cell lysate from each well was used for β-galactosidase assays and the other 50 µL of cell lysate was used for luciferase assays using the method previously described (62). An aliquot of (50 µL) of 1 mM beetle luciferin (Promega, Madison, WI) was added to 50 µL of cell lysate from each well and the luminescence was read by an AutoLumate LB953 luminometer (Wallace Inc., Gaithersberg, MD). Beta-galactosidase activity was determined by adding 50 µL of lysate to 50 µL of beta-galactosidase assay buffer. The optical density was
measured on a UV spectrophotometer (Cary Model 1E, Varian Associates, Sunnyvale CA) at a wavelength of 420 nm.

2.2.6 Data Analysis

2.2.6.1 AR Binding Affinity

The specific binding of \[^{3}H\]MIB at each concentration of the compound of interest was calculated by subtracting the nonspecific binding of \[^{3}H\]MIB, and expressed as the percentage of the specific binding in the absence of the compound of interest (B\(_0\)). The concentration of compound that reduced B\(_0\) by 50% (i.e., IC\(_{50}\)) was determined using WinNonlin (Pharsight Corporation, Mountain View, CA). The equilibrium binding constant (K\(_i\)) of the compound of interest was calculated by K\(_i\) = K\(_d\) \times IC\(_{50}\) / (K\(_d\) + L), where K\(_d\) was the dissociation constant of \[^{3}H\]MIB (0.19 ± 0.01 nM), and L was the concentration of \[^{3}H\]MIB used in the experiment (1 nM). The K\(_i\) value of each compound of interested was further compared.

2.2.6.2 AR-Mediated Transcriptional Activation

The transcriptional activity in each well was calculated as the ratio of luciferase activity to \(\beta\)-galactosidase activity to avoid variations caused by cell number and transfection efficiency. Transcriptional activity induced by compound of interest was expressed as the percentage of transcriptional activity induced by 1 nM of DHT.

2.3 RESULTS

2.3.1 In Vitro AR Binding Affinity

The structure-activity relationships (SARs) that define the interaction of nonsteroidal compounds with the AR have been studied intensively in our laboratories (56, 57, 60, 62, 63, 123) and by several other investigators (126-131). In our search for novel SARMs, a \(\textit{para}\)-fluoro substituted analog known as S-1 (59) was used as our
starting point. **S-1** was synthesized as a bicalutamide derivative and identified as a potent SARM with high *in vitro* and *in vivo* activities (64), and potential therapeutic application to benign prostatic hyperplasia. As shown in Figure 1, the key structural elements of **S-1** include an electron-deficient aromatic A-ring, a CH$_3$ linked to the chiral carbon (**S**-isomer), an ether linkage, and electronegative substituents on the aromatic B-ring. Recent crystallographic evidence suggests that the main difference between the AR binding pockets of steroidal androgens (e.g., DHT) and bicalutamide-related SARMs may be the unique interactions of the B-ring with additional amino acids near tryptophan 741 (132).

A series of nonsteroidal compounds with multiple substitutions on the B-ring were designed and synthesized to study the effects of structural modification on AR-binding affinity and functional activity. The AR binding affinity of synthetic compounds was determined using an *in vitro* radioligand competitive binding assay and were reported here as the inhibition constant (Ki) in Tables 1 through 3. Synthetic compounds showed a wide range of binding affinity with Ki values ranging from 1.0 to 51 nM, which provide an index of the relative binding affinities of compounds of interest.

Table 1 shows the Ki values of compounds with a *para*-nitro group in the A-ring and two substituents in the B-ring. Previous studies from our laboratories confirmed that with a single substituent in the B-ring, *para*-substitution was superior to *meta*-substitution in terms of AR binding (62, 123). Compared with the lead compound **S-1**, the incorporation of an additional fluoro, chloro, or methyl group at the *ortho*-position (i.e., R1 of the general structure shown in Table 1) of the B-ring increased or maintained AR-binding affinity, suggesting that a electron-deficient B-ring is more favorable. We also examined the influence of an additional *meta*-fluoro substituent (i.e., R2) on AR binding affinity of a series of compounds with varying *para*-substituent. The order of AR binding affinity was NO$_2$ > F > Cl > NHCOCH$_3$ (**C-11** > **C-3** > **C-6** > **C-7**), corroborating crystallographic evidence with bicalutamide that hydrogen bonding with *para*-substituent of SARMs is likely important for high affinity binding. AR binding affinity was optimum when there were two electron-withdrawing groups present in the *meta* and *para* positions of the aromatic B-ring. This is exemplified by the low Ki values (~ 1.0 nM) of **C-10** and **C-11**. For these two compounds, moving the chloro group or fluoro group from the *meta*-position (**C-10** and **C-11**) to the *ortho*-position (**C-9** and **C-12**) resulted in a ten-
fold decrease in AR binding affinity. However, this regioselectivity was not true for other pairs of compounds C-1/C-3, C-4/C-5, and C-6/C-8, indicating that this selectivity may be substituent-dependent.

Having demonstrated that AR binding affinity improved by incorporation of additional electron-withdrawing groups in the aromatic B-ring, we applied more halogen groups to the B-ring to further explore the effects of substitution positions and types on AR binding affinity. Compounds with three or five substituents in the B-ring were designed and synthesized. As shown in Table 2, when three fluoro groups were incorporated in the different positions of the B-ring, high AR binding affinity was maintained in each case. In particular, C-18, which bears fluoro groups at the 2, 4 and 5-positions of the B-ring, had significantly improved AR binding affinity with a Ki value as low as 1.0 nM. These three positions of the B-ring were also the best combination for chloro-substituted compounds (C-19 to C-22) in terms of binding affinity. However, in general, changing a fluoro group to a chloro group (C-13/C-19, C-14/C-20, C-17/C-21, and C-18/C-22) significantly decreased AR binding affinity, indicating that the size of substituents is critical when the B-ring has more than two substituents. To protect the aromatic B-ring from possible oxidation in vivo, all positions of the B-ring were occupied by introducing five halogen groups (C-23 and C-24). The excellent AR binding affinity of C-23 suggested that as many as five fluoro groups are well tolerated in the B-ring. Again, replacing the fluoro group of C-23 with a chloro group increased the Ki value but still maintained a reasonable AR binding affinity in vitro.

In previous studies, AR binding affinity was significantly improved by replacing the para-cyano group with a nitro group in the aromatic A-ring of hydroxyflutamide analogs (56), but it was not true in bicalutamide derivatives (57). To investigate the effects of such a change on the AR binding affinity of compounds with multiple substituents in the B-ring, some compounds listed in Table 1 and Table 2 were structurally modified as shown in Table 3. For compounds with two fluoro groups in the B-ring (C-25 and C-16), binding affinities were similar between analogs bearing a nitro group or a cyano group at the para-position of the A ring (compare C-25 vs C-1 and C-26 vs C-3). In all other cases, the cyano-substituted compounds exhibited at least a 2-
fold lower AR binding affinity than their corresponding nitro-substituted counterparts (pair C-27/C-9, C-28/C-10, and C-29/C-23).

2.3.2 *In Vitro* AR-Mediated Transcriptional Activation

Transcriptional activation induced by DHT plateaued at a concentration of 1 nM, as reported previously (56) and was used as a positive control (i.e., 100%) in each experiment to facilitate comparison to previous studies (62). Agonist activity and antagonist activity were determined in the absence or presence of 1 nM DHT, respectively. Ligands were defined as full AR agonists if compounds induced a similar level of transcriptional activation as that of 1 nM DHT in the agonist assay; ligands were defined as partial agonists if compounds induced a certain level ($\geq 10\%$) of transcriptional activation but still significantly lower than that of 1 nM DHT in the agonist assay; ligands were defined as antagonists if compounds induced a low level (< 10%) of transcriptional activation in the agonist assay and a significantly decreased level of transcriptional activation induced by 1 nM DHT in the antagonist assay (62).

Figure 2 shows the AR agonist activity of ligands with a para-nitro group in the A-ring and two substituents in the B-ring. All compounds examined in this series induced transcriptional activation in a concentration-dependent manner. In the presence of 1 nM DHT, no significant antagonist activity (data not shown) was observed for this series of compounds, suggesting that the introduction of two substituents does not interfere with the formation of the transcriptional active ligand binding domain (LBD) conformation. Except for C-12, the incorporation of two substituents in the aromatic B-ring maintained or improved the AR-mediated transcriptional activation as compared to previous studies (62). Compounds C-1 through C-11 therefore were identified as full agonists, while C-12 was identified as a partial agonist. Among this series of compounds, C-11 was one of the ligands with the highest AR binding affinity ($K_i = 1.0 \pm 0.02$ nM) and exhibited the most potent agonist activity. It is noteworthy that at a concentration of 10 nM, compounds with high binding affinity (e.g., $K_i < 5$) are as efficient as 1 nM DHT. Additional consistency between AR binding affinity and transcriptional activation is that the *meta-* and para-
positions of the B-ring are the optimal substituent positions for both binding and functional activities as exemplified by C-3, C-6, and C-11. However, inconsistency was also observed. For instance, C-10 (Ki ~ 1 nM) displayed less potent agonist activity than C-3 and C-6, which demonstrated 4 to 5-fold lower binding affinity than C-10. Also interestingly, moving the fluoro-group of C-11 from its meta-position to the ortho-position (C-12) of the B-ring caused a 10-fold increase of the Ki value but a more than 1000-fold decrease of the AR-mediated transcriptional activation. Although the presence of a fluoro group at the ortho-position of the B-ring was well tolerated in terms of binding with the AR, it appears to prevent the formation of transcriptionally active AR.

C-13 through C-24 are AR ligands with a para-nitro group in the A-ring and three or five substituents in the B-ring. AR-mediated transcriptional activity induced by these compounds is shown in Figure 3. Five compounds were identified as full agonists (C-13, C-14, C-18, C-22, and C-23), while the other seven compounds were identified as partial agonists. In antagonist assays, full agonists exhibited no significant effect on transcriptional activation induced by 1 nM DHT, while partial agonists demonstrated a concentration-dependent inhibition with about a 60% decrease for the most potent ligand (i.e., C-17) (data not shown). Obviously, for these compounds with more than two substituents in the B-ring, the substituent type, number, and/or positions are critical in terms of both binding affinity and functional activity. C-18 and C-22, two ligands that showed the optimal binding affinity, induced the most potent and efficacious transcriptional activation. It is important to note that the transcriptional activation induced by 1 nM C-18 was as potent and efficacious as that of DHT, although C-18 (Ki ~ 1 nM) demonstrated a 2~3-fold lower binding affinity than DHT (Ki ~ 0.4 nM). As the B-ring became bulkier due to incorporation of more substituents, we observed significant regioselectivity. Pairs of compounds (C-14/C-15, C-18/C-17, and C-22/C-21) were designed so that only a single substituent position was different in the B-ring between these two ligands. Moving the fluoro/chloro group from position 5 to position 6 (i.e., from R4 to R5 in the structure of Table 2) of the B-ring significantly decreased the transcriptional activation, indicating that the presence of two electron-withdrawing groups at both ortho-positions of the B-ring deteriorated the functional activity. For compounds C-19 to C-21, the low transcriptional activation was most likely due to their
low binding affinity. However, despite the higher binding affinity of compounds C-15 to C-17, functional activity induced by these compounds was lower than that of C-13 and C-14. Again, both binding affinity and the potential to form a transcriptionally active AR of a ligand are important to determine its functional activity. The incorporation of five fluoro groups in the B-ring was well tolerated for both binding affinity and functional activity. Changing the para-fluoro group of C-23 to a chloro group (C-24) significantly decreased its agonist activity.

In binding studies, all five ligands with a para-CN group in the A-ring demonstrated similar or lower binding affinity than their counterparts with a para-NO$_2$ group. In agonist assays, all five cyano-substituted compounds exhibited reasonably high agonist activity and were identified as full agonists, since no significant inhibition of activity induced by DHT was observed (data not shown). Comparing compounds with nitro-and cyano-substituents in the A-ring, it was obvious that the presence of the A-ring cyano group led to a slightly decreased functional activity in all cases, which correlated well with the binding affinity.

2.4 DISCUSSION

In the present study, we demonstrated that novel AR agonists with a wide range of in vitro and in vivo activities can be designed by structural modifications of the SARM pharmacophore. Early SAR work on hydroxyflutamide analogs (133) confirmed the importance of an electron-deficient aromatic A-ring and the substituents attached to the carbon atom bearing a tertiary hydroxyl group. Two years later, Tucker et al. (126) reported that the AR binding and antiandrogen activity of hydroxyflutamide and bicalutamide derivatives were optimum when the 4-substituent in the A-ring was either a cyano or nitro group and the 3-substituent was a chloro or trifluoromethyl group. Interestingly, partial androgen agonist activity was observed in some trifluoromethyl substituted compounds, indicating that AR agonists can be designed and developed by subtle structural modification (s) of known AR antagonists. When this concept was applied to discover novel bicalutamide-related AR agonists in our laboratories, important
in vitro SARs for the AR-binding affinity and agonist activity were further gained (62), including a para-nitro group in the A-ring, a trifluoromethyl group linked to the chiral carbon (R-isomer), a sulfide linkage, and a para-N-alkylamido group in the B-ring. However, when these compounds were tested in vivo, no pharmacologic activity was observed due to their unfavorable pharmacokinetic properties (58). Further structural modification was made to overcome this problem by changing the sulfide linkage to an ether bridge, which resulted in the successful discovery of SARMs (59). As shown in Figure 1, the lead compound of current studies (S-1) contains a para-nitro group and a meta-trifluoromethyl group in the A-ring, a methyl group linked to the chiral carbon (S-isomer), an ether linkage, and a para-fluoro substituent in the B-ring. Due to the difficult chemical synthesis and the small advantage of a trifluoromethyl group over a methyl group in terms of the AR binding affinity, a methyl group was linked to the chiral carbon. Results from our binding studies showed that the incorporation of multiple substituents in the B-ring was well tolerated. Four compounds with multiple electron-withdrawing substituents in the B-ring (C-10, C-11, C-18, and C-23) exhibited high AR binding affinity (Ki ~ 1.0 nM), which was even slightly higher than that of the endogenous androgen – testosterone. The significantly improved AR binding affinity was most likely either due to either increased hydrophobic interactions (C-10, C-18, and C-23) between halogen groups with the B-ring subpocket or the formation of hydrogen bonds formed between the para-nitro group (C-11) and the AR binding site. Crystallographic studies are underway in our laboratory to define the precise mechanism. Several structural modifications increased AR binding affinity, indicating that there is a free space surrounding the B-ring within the AR binding pocket, which is flexible to accommodate ligands with different B-ring. The interaction between the AR and bicalutamide-related compounds with multiple substituents in the B-ring is favorable if: 1) two electron-withdrawing groups are at the para- and meta-positions of the B-ring; 3) a H-bond acceptor is present at the para-position of the B-ring; 4) three substituents are at the 2-, 4-, and 5-positions of the B-ring; and 5) fluoro groups instead of chloro groups are incorporated in the B-ring for compounds with more than two substituents in that ring.

Structural studies on the ligand binding domain (LBD) of the AR revealed an α-helical sandwich fold consisting of 12 α-helices (H1-H12) and a short β-sheet (15, 16),
which was consistent with the canonical structure as those of the other nuclear hormone receptor LBDs (15, 16, 134-136). Comparison of the LBD structures of nuclear receptor in complex with an agonist and an antagonist suggested that receptor ligands trigger different conformation changes, especially the position of helix H12 and the unwinding of helix H11(134, 137). Upon binding with an agonist, helix H12 folds back, seals the ligand-binding pocket as a lid, and forms a surface to interact with coactivators. On the other hand, binding with an antagonist is thought to induce unwinding of helix H11 and allow helix H12 to disrupt the overall surface topography of AF-2 and/or the recruitment of coactivators (138). By incorporation of multiple substituents in the B-ring of our known SARM, novel AR agonists with higher binding affinity and pharmacologic activity were discovered, suggesting that the LBP around the B-ring area is flexible and that substituents with smaller atomic sizes can be well tolerated to stabilize a transcriptionally active LBP conformation via creation of new hydrophobic interactions or hydrogen bond. (56). It is noteworthy that significant regioselectivity was observed for compounds with three substituents in the B-ring. Functional activity changed between agonist and partial agonist by moving the fluoro/chloro groups along the aromatic ring and was optimized at the 2, 4, and 5-positions of the B-ring (C-18 and C-22). Since high AR binding affinity was maintained by incorporation of three fluoro groups in the B-ring (C-13 to C-18), functional results suggested that this regioselectivity may be related to the precise position of helix H12, strongly stabilized by pure agonist (C-18) or destabilized by partial agonist (C-15 to C-17). It is possible that an intermediate position of helix H12 exists for these partial agonists. Additional site-directed mutagenesis data with the AR suggests that the interaction between Thr877 of the AR and nonsteroidal ligands is important to determine the functional activity of this series of AR ligands. For example, the partial agonist, C-17, behaved as a potent and pure agonist in CV-1 cells co-transfected with mutant AR (T877A) and reporter plasmids (forthcoming report). Up to date, crystal structures are only available for the wild type AR-LBD complex with steroidal AR ligands (15, 16) but not with any nonsteroidal AR ligands. Chemical structures reported herein make it possible to study the detailed interactions between the aromatic B-ring and AR-LBP, especially within the unique subpocket of nonsteroidal compounds. Ongoing studies focused on this subject include molecular modeling, site-
directed mutagenesis, and X-ray crystallography to define the precise molecular mechanisms underlying SARM interactions with the AR and functional activity.

In summary, current studies revealed the in vitro SARs of AR ligands; proved that structural modification in the B-ring of our known SARMs is a feasible method to discover and develop novel SARMs with higher AR binding affinity and in vitro functional activity.
Figure 2. 1 Chemical Structures of Bicalutamide and S-1.
Table 2. AR Binding Affinity of Nonsteroidal AR Ligands with a *para*-NO₂ in the A-ring and Two Substituents in the B-ring.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>Ki a (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>* S-1</td>
<td>H</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>C-1</td>
<td>F</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>C-2</td>
<td>CH₃</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td>C-3</td>
<td>H</td>
<td>F</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>C-4</td>
<td>H</td>
<td>Cl</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>10.3 ± 2</td>
</tr>
<tr>
<td>C-5</td>
<td>Cl</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>5.2 ± 0.9</td>
</tr>
<tr>
<td>C-6</td>
<td>H</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>C-7</td>
<td>H</td>
<td>F</td>
<td>NHC(O)CH₃</td>
<td>H</td>
<td>H</td>
<td>6.7 ± 0.9</td>
</tr>
<tr>
<td>C-8</td>
<td>F</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>C-9</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>9.7 ± 2</td>
</tr>
<tr>
<td>C-10</td>
<td>H</td>
<td>Cl</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>1.0 ± 0.09</td>
</tr>
<tr>
<td>C-11</td>
<td>H</td>
<td>F</td>
<td>NO₂</td>
<td>H</td>
<td>H</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>C-12</td>
<td>F</td>
<td>H</td>
<td>NO₂</td>
<td>H</td>
<td>H</td>
<td>11 ± 3</td>
</tr>
</tbody>
</table>

* The Ki value of **S-1** was previously reported in (59)

a The AR binding affinity (Ki) of each compound was determined using a competitive binding assay as described in the *Materials and Methods* section. Data represent the mean ± S.D. of three experiments.
Table 2. 2 AR Binding Affinity of Nonsteroidal AR Ligands with a \textit{para}-\text{NO}_2 in the A-ring and Three or Five Substituents in the B-ring.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>Ki$^a$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>* S-1</td>
<td>H</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>C-13</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>C-14</td>
<td>F</td>
<td>F</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>C-15</td>
<td>F</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>F</td>
<td>7.5 ± 1</td>
</tr>
<tr>
<td>C-16</td>
<td>H</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>H</td>
<td>4.0 ± 0.9</td>
</tr>
<tr>
<td>C-17</td>
<td>F</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>F</td>
<td>5.9 ± 0.6</td>
</tr>
<tr>
<td>C-18</td>
<td>F</td>
<td>H</td>
<td>F</td>
<td>F</td>
<td>H</td>
<td>0.97 ± 0.1</td>
</tr>
<tr>
<td>C-19</td>
<td>Cl</td>
<td>Cl</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>C-20</td>
<td>Cl</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>C-21</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>C-22</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>Cl</td>
<td>H</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>C-23</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>C-24</td>
<td>F</td>
<td>F</td>
<td>Cl</td>
<td>F</td>
<td>F</td>
<td>11 ± 0.8</td>
</tr>
</tbody>
</table>

* The Ki value of \textit{S-1} was previously reported in (59)

$^a$ The AR binding affinity (Ki) of each compound was determined using a competitive binding assay as described in the \textit{Materials and Methods} section. Data represent the mean ± S.D. of three experiments.
<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>Ki (^a) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>* S-1</td>
<td>H</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>C-25</td>
<td>F</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>C-26</td>
<td>H</td>
<td>F</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>C-27</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>20 ± 0.2</td>
</tr>
<tr>
<td>C-28</td>
<td>H</td>
<td>Cl</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>C-29</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>6.8 ± 0.4</td>
</tr>
</tbody>
</table>

Table 2. 3 AR Binding Affinity of Nonsteroidal AR Ligands with a \(\text{para}\)-CN in the A-ring and Multiple-Substituents in the B-ring.

* The Ki value of \(\text{S-1}\) was previously reported in (59)

\(^a\) The AR binding affinity (Ki) of each compound of interested was determined using a competitive binding assay as described in the \textit{Materials and Methods} section. Data represent the mean ± S.D. of three experiments.
CV-1 cells were transfected with a human AR plasmid, an androgen-responsive luciferase reporter plasmid, and a constitutively expressed β-galactosidase plasmid in a T-175 flask using LipofectAMINE. After transfection, cells were plated onto 24-well plates and allowed to recover for 12 h before drug treatment. Cells were then treated with vehicle or increasing concentrations of AR ligands alone or together with 1 nM of DHT for 24 h. Luciferase activity in each well was normalized with the β-galactosidase activity and then expressed as the percentage of that induced by 1 nM DHT. Each bar represents mean ± S.D. (n=3).

Figure 2. AR-mediated Transcriptional Activation of Nonsteroidal AR Ligands with a \textit{para}^-\text{NO}_2\text{in the A-ring and Two Substituents in the B-ring.}
Figure 2. 3 AR-mediated Transcriptional Activation of Nonsteroidal AR Ligands with a para-NO₂ in the A-ring and Three or Five Substituents in the B-ring.

CV-1 cells were transfected with a human AR plasmid, an androgen-responsive luciferase reporter plasmid, and a constitutively expressed β-galactosidase plasmid in a T-175 flask using LipofectAMINE. After transfection, cells were plated onto 24-well plates and allowed to recover for 12 h before drug treatment. Cells were then treated with vehicle or increasing concentrations of AR ligands alone or together with 1 nM of DHT for 24 h. Luciferase activity in each well was normalized with the β-galactosidase activity and then expressed as the percentage of that induced by 1 nM DHT. Each bar represents mean ± S.D. (n=3).
Figure 2. 4 AR-mediated Transcriptional Activation of Nonsteroidal AR Ligands with a \textit{para}-CN in the A-ring and Multiple Substituents in the B-ring.

CV-1 cells were transfected with a human AR plasmid, an androgen-responsive luciferase reporter plasmid, and a constitutively expressed $\beta$-galactosidase plasmid in a T-175 flask using LipofectAMINE. After transfection, cells were plated onto 24-well plates and allowed to recover for 12 h before drug treatment. Cells were then treated with vehicle or increasing concentrations of AR ligands alone or together with 1 nM of DHT for 24 h. Luciferase activity in each well was normalized with the $\beta$-galactosidase activity and then expressed as the percentage of that induced by 1 nM DHT. Each bar represents mean $\pm$ S.D. (n=3).
CHAPTER 3

IN VIVO STRUCTURAL ACTIVITY RELATIONSHIPS (SARS) OF NONSTEROIDAL ANDROGEN RECEPTOR LIGANDS WITH MULTIPLE-SUBSTITUENTS IN THE AROMATIC B-RING

3.1 INTRODUCTION

Endogenous androgens, mainly testosterone and dihydrotestosterone (DHT), play important roles for male development and maintenance of male secondary characteristics. Pharmacologic effects of androgens include androgenic (masculinizing) effects and anabolic (tissue-building) effects. In men, androgenic effects are those that relate to the growth of male reproductive tract or to the development of secondary sexual characteristics, including increases in the length and diameter of the penis, development of the prostate and scrotum, and the appearance of the pubic, auxiliary, and facial hair (139). Anabolic effects of testosterone are the changes that occur in the nonreproductive tract tissue, such as bone, larynx, libido and sexual potential, muscle, and fat distribution. Immediately after the discovery of testosterone, its anabolic activity was recognized as a potential therapeutic application for patients with protein-deficient conditions (140). However, the androgenic activity of testosterone prevented its usage in women and children. The potential therapeutic value of the anabolic activity of testosterone and its commercial availability stimulated many investigators to discover anabolic reagents, using structural modifications of steroidal androgens.

Testosterone (Figure 3.1, a) undergoes multiple metabolism pathways in vivo and demonstrates differential effects on target tissues. Two active metabolites, 17β-estradiol (Figure 3.1, b) and 5α-dihydrotestosterone (DHT, Figure 3.1, c), are from aromatization or reduction of the Δ4 bond of testosterone, respectively. In many tissues, including
certain areas of the brain, liver, adipose tissue, and testis, the aromatase converts testosterone to estradiol (141, 142), which in turn exerts its effects via estrogen receptors. In male reproductive tracts and skin, testosterone undergoes 5α-reduction to DHT, which amplifies the apparent potency of its precursor(44, 143). Studies using specific 5α-reductase inhibitors revealed that the androgenic activity of testosterone on ventral prostate and seminal vesicles significantly decreased but no changes were observed on muscle, indicating that this differential effect of testosterone action is due to the high and low 5α-reductase activity in reproductive tissues and muscle, respectively (41-44). Recently, tissue-selective steroidal androgen was discovered by incorporation of 7α-methyl substituent in testosterone derivatives. For instance, 7α-methyl-19-nortestosterone (MENT) demonstrate tissue selectivity with higher anabolic activity than androgenic activity both in rat (45) and in hypogonadal men (46). This tissue selectivity was mainly due to the inability of 5α-reductase to metabolize MENT.

Although the discover of anabolic-androgenic steroids with tissue-selectivity provide potential alternative to testosterone for androgen replacement and male contraception (85), these compounds can only be administered via pellet implantation or transdermal patches due to their poor pharmacokinetic and physiochemical properties. On the other hand, potent and efficacious selective androgen receptor modulators (SARMs) reported by Yin et. al. are superior in terms of physicochemical, pharmacokinetic properties, and flexibility of structural modifications. Potential therapeutic applications include treatment for hypogonadism, delayed puberty, protein-deficiency induced by HIV/AIDS, male contraception, and aging males.

Our previous in vitro structure-activity relationship (SAR) studies of bicalutamide-related AR ligands revealed that compounds with higher AR binding affinity and in vitro functional activity can be successfully achieved by introduction of multiple electron-withdrawing substituents in the aromatic B-ring. We hypothesized that some AR ligands with improved AR binding affinity and/or in vitro functional activity will exhibit more potent and efficacious pharmacologic activity then the lead compound in vivo.
3.2 MATERIALS AND METHODS

3.2.1 Animals

Male Sprague-Dawley rats were purchased from Harlan Bioproducts for Science (Indianapolis, IN). All animals were maintained on a 12h light/dark cycle with food and water available ad libitum. The animal protocol was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

3.2.2 In Vivo Pharmacologic Activity of Novel AR Ligands in Castrated Male Rats

The in vivo pharmacologic activity of each synthetic androgen receptor ligand was examined in five male Sprague-Dawley rats weighing approximately 200 g using Hershberger assay (144). Animals were castrated via a scrotal incision under anesthesia 24 h prior to drug treatments and received daily subcutaneous injections of compounds of interest at a dose rate of 1 mg/day for 14 days. All compounds of interest were freshly dissolved in vehicle containing DMSO (5%, v/v) in PEG 300 before dose administration. Additional two groups of animals with or without castration received vehicle only and served as castrated or intact control groups, respectively. Animals were sacrificed at the end of the treatment. Plasma samples were collected and stored at –80 °C for future use. The ventral prostate, seminal vesicles, and levator ani muscle were removed, cleared of extraneous tissue, and weighed. All organ weights were normalized to body weight and compared. The weights of prostate and seminal vesicles were used to evaluate androgenic activity, while the levator ani muscle weight was used as a measurement of anabolic activity. Compounds with higher anabolic activity than androgenic activity were identified as SARMs.

3.2.3 In Vivo Pharmacodynamics of SARMs in Castrated Male Rats

Some SARMs identified by method described in 3.2.2 were selected and further examined for pharmacodynamics of these compounds in castrated male rats. For each
compound of interest, 20 Sprague-Dawley rats weighing approximately 200 g were purchased and randomly divided into four groups with five rats per group. Group 1 through 4 received daily subcutaneous injection of SARM of interest at a dose rate of 0.1, 0.3, 0.5, and 0.75 mg/day, respectively. Combined the pharmacologic activity obtained at a dose rate of 1 mg/day (3.2.2), dose-response relationship of each SARM on androgenic tissues and anabolic tissue were simulated using a pharmacodynamic model - Sigmoidal Emax model with a baseline effect and WinNonlin software (Pharsight Corporation, Mountain View, CA). The maximal response (Emax) induced by each SARM and the dose rate of individual SARM that induced 50% of its maximal response (ED50) were obtained and compared. The Emax value indicated the efficacy of each compound, whereas the ED50 indicated its potency. The relative efficacy of each compound to testosterone propionate (TP) was defined as the ratio of (Emax of the compound) to (Emax of TP). The relative potency was defined as the ratio of (ED50 of TP) to (ED50 of the compound). The Emax and ED50 values of TP in each organ were previously reported by Yin et al. (59).

3.2.4 Statistical Analyses

All statistical analyses were performed using single-factor ANOVA with the α value set a priori at p < 0.05.

3.3 RESULTS

3.3.1 In Vivo Pharmacologic Activity of Novel AR Ligands in Castrated Male Rats

As we have shown that certain structural modifications in the B-ring of nonsteroidal AR ligands improved the binding affinity and functional activity in vitro. However, high binding affinity and agonist activity not always predict high pharmacologic activity in vivo as showed by Yin et al. (58). Therefore, the pharmacologic effects of selected ligands on prostate, seminal vesicles, and levator ani muscle were further evaluated in castrated male rats at a dose rate of 1 mg/day. Since replacing a para-
nitro with a cyano group in the A-ring decreased the binding affinity and transcriptional activity of these compounds in most cases, compounds selected for in vivo studies all bear a para-nitro group in the A-ring. Figure 3.2 and figure 3.3 showed the effects of novel AR ligands in prostate and seminal vesicles, respectively, while figure 3.4 showed the anabolic activity (levator ani muscle) of AR ligands of interest. Due to the depletion of endogenous testosterone, castration lead to a rapid reduction of the weight in prostate, seminal vesicles, and levator ani muscle to 6.2, 8.1, and 41% of intact levels, respectively. According to the potency of AR agonists, the weights of androgen-dependent organs would remain low, partially, or fully maintained after drug treatment.

Figure 3.2 shows B-ring chemical structures of novel AR ligands selected for in vivo pharmacologic activity studies. For compounds with two substituents in the B-ring, the position and size of substituents clearly played an important role in the pharmacologic activity in vivo. Introduction of a fluoro group at the para-position (C-1) resulted an equipotent pharmacologic activity as that of S-1, suggesting that a small electron-withdrawing group at this position is well tolerated. However, changing this ortho-fluoro group to a methyl group (C-2) significantly decreased its anabolic activity, consisting with the SAR from the AR binding studies. Interestingly, when the ortho-fluoro group was moved to the meta-position of the B-ring (C-3), it demonstrated much higher pharmacologic activity than C-1, largely due to the improved transcriptional activation instead of the AR binding affinity. The ortho/meta-regioselectivity was further illustrated by the potencies of C-6, C-10, and C-11 compared with those of C-8, C-9, and C-12. When the meta-fluoro group of C-3 was replaced by a chloro group (C-4), significantly decreased pharmacologic activity was observed, indicating that the size of meta-substituent is critical. Besides S-1, Yin et al. (59) identified another more potent SARM (S-4), which bears a relatively larger substituent (acetamido group) at the para-position of the B-ring. Therefore, larger substituents were also incorporated into that position to discover new SARs in current studies. Replacing the para-fluoro group with a chloro group resulted in a 3-fold increase in androgenic activity (pairs C-1/C-8 and C-3/C-6) and 30% increase in anabolic activity (pair C-1/C-8). As we previously reported (59, 65), the maximum effect of our SARMs in levator ani muscle plateaued about 120 – 140% of the intact control level, therefore, the great improved pharmacologic effects of C-6 over
**C-3** were only shown in the prostate and seminal vesicles but not in the muscle. Although at a dose rate of 1mg/day, **C-6** demonstrated both high androgenic and anabolic activity, a complete dose-response study has shown that **C-6** remain tissue-selectivity over the physiologic dose range (Chapter 5). Surprisingly, replacing the *para*-fluoro group of **C-3** with a nitro group (**C-11**) decreased the pharmacologic effect, in spite of the fact that the AR binding affinity of **C-11** was three-fold higher than that of **C-3 in vitro**. This relatively low activity of **C-11** may be due to its rapid metabolic property *in vivo* since both nitro groups on the A-ring and B-ring are two mobile groups *in vivo* metabolism (Chen et al, in preparation).

Partial agonists (**C-12**, **C-14**, **C-15**, and **C-18**) identified *in vitro* failed to show any activity *in vivo*. Besides the low functional activity identified *in vitro*, fast metabolism rate of these compounds could also cause its inactivity *in vivo*. Consistent with results of *in vitro* studies, 2, 4, and 5-position in the B-ring are the optimal locations to incorporate three electron-withdrawing substituents. Among compounds with three substituents in the B-ring, only **C-19** and **C-23** exhibited high activity *in vivo*. **C-19** demonstrated significantly higher androgenic and anabolic activity than **S-1**, which **C-23** selectively increased the androgenic activity and maintained the anabolic activity as compared with **S-1**. Obviously, changing the type and position of substituents in the B-ring altered the androgenic activity/anabolic activity ratio in rats. Although the incorporation of five fluoro groups in the B-ring significantly improved the AR binding affinity and transcriptional activation *in vitro*, the *in vivo* pharmacologic activity was similar as that of **S-1**.

### 3.3.2 *In Vivo* Pharmacodynamics of SARMs in Castrated Male Rats

Pharmacologic results from a single high dose (1 mg/day) treatment revealed that structural modifications in the B-ring not only changed the efficacy but also the tissue selectivity of SARMs. **C-6** demonstrated equal efficacy in reproductive tissues and muscle. Due to its unique ‘non-tissue selectivity’ at a dose rate of 1 mg/day, **C-6** was studied separately and discussed in Chapter 5. Dose-response relationships of **C-1, C-3, C-10, and C-23** were further studied for additional *in vivo* SARs.
The pharmacologic activity of C-1, C-3, C-10, and C-23 was in a dose-dependent manner in three organs examined. Figure 3.6 shows the androgenic activity and anabolic activity of C-1 in castrated male rats, which is in a dose-dependent manner. The weights of prostate, seminal vesicles, and levator ani muscle were maximally maintained by C-1 to 21.9, 10.4, and 80.2% of those in intact control animals, respectively (Figure 3.6 B). Accordingly, the ED50 values of C-1 in these three organs were 0.33 ± 0.1, 0.66 ± 0.03, and 0.42 ± 0.09 mg/day, respectively (Figure 3.6 B). The significantly higher activity of C-1 in the muscle than that in the prostate and seminal vesicles indicate that C-1 is an efficacious SARM in rats. The only structural difference between C-1 and our lead compound – S-1 was the presence of a fluoro group at the ortho position of the B-ring of C-1. As reported previously, the Emax values of S-1 in the prostate, seminal vesicles, and levator ani muscle were 14.9, 13.4, and 74.3% of those in the intact animals, respectively; the ED50 values of S-1 in the prostate, seminal vesicles, and levator ani muscle were 0.42 ± 0.04, 0.38 ± 0.26, and 0.44 ± 0.01 mg/day, respectively (59). Such structural modification slightly increased activity in prostate, maintained high anabolic activity in muscle, and tissue-selectivity in rats.

Figure 3.7 shows the pharmacologic activity of C-3 in castrated male rats at dose rates ranging from 0.1 to 1 mg/day. The maximal effects of this compound in the prostate, seminal vesicles, and levator ani muscle were 59%, 27%, and 132% of those observed in intact animals, respectively. ED50 values of C-3, using non-linear regression analysis, in the prostate, seminal vesicles, and levator ani muscle were 0.65 ± 0.4, 0.58 ± 0.04, and 0.33 ± 0.09 mg/day, respectively. Table 3.1 lists Emax values, ED50 values, and relative efficacy and potency of compound of interest. Structurally compare with S-1, C-3 bears an additional fluoro group at the meta position of the B-ring. Such structural modification resulted in significantly improved relative efficacy (Emax) as indicated by a 4-fold and a 1.8-fold increase in the prostate and levator ani muscle, respectively (Table 3.1). As to the relative potency, changes in each organ were not that obvious. The significant increase of the relative efficacy in the prostate was also observed in animals treated with C-10 (Figure 3.8 A), suggesting that di-halogen substituents in the meta and para position of the B-ring induced more profound increase in androgenic activity than anabolic activity. Although AR-binding affinity of C-10 (Ki = 1.0 nM) is greater than
that of C-3 \((K_i = 3.4 \, \text{nM})\), C-10 revealed relatively lower pharmacologic activity than C-3 \textit{in vivo}. This discrepancy may due to the significantly lower intrinsic activity of C-10 as illustrated in the transcriptional activation studies.

As shown in Figure 3.9 B and Table 3.1, the Emax values of this compound in the prostate, seminal vesicles, and levator ani muscle were 20.4, 12.7, and 70.8\% of those observed in intact animals, respectively. While the ED_{50} values of C-24 in the prostate, seminal vesicles, and levator ani muscle were 0.63 ± 0.04, 0.62 ± 0.07, and 0.70 ± 0.003 mg/day, respectively. Although the pharmacologic activity of C-24 (Figure 3.9 A) was comparable with that of S-1, it was surprisingly lower than what we expected. C-24 bears five fluoro groups in the B-ring, which was originally designed to prevent possible oxidation metabolism on the B-ring \textit{in vivo}. In comparison with S-1, both AR-binding affinity and \textit{in vitro} AR-mediated transcriptional activation were significantly improved. Especially, the Ki value of C-5 was as low as 1 nM. This unpredicted low pharmacologic activity indicated that the pharmacokinetic properties of this compound may be significantly different from those of S-1 in rats.

### 3.4 DISCUSSION

We have successfully discovered a series of novel AR ligands (Chapter 2) with high AR-binding affinity and AR-mediated transcriptional activation using structural modifications of our lead compound, S-1, which is a potent and efficacious SARM previously identified in our laboratory (59). However, the correlation between \textit{in vitro} function and \textit{in vivo} pharmacologic activity was poor as reported by Yin el. al. (58). The purposes of the structural modifications of our study was to improve \textit{in vivo} activity of AR ligands by increasing AR binding affinity, intrinsic functional activity, and/or plasma exposure.

Compounds selected for \textit{in vivo} pharmacologic activity studies all demonstrated high AR binding affinity \textit{in vitro} with Ki values smaller than 15 nM. Since the AR-mediated transcriptional activation \textit{in vitro} does not always a correct predictor of \textit{in vivo} activity (58), compounds identified as partial agonists from \textit{in vitro} studies were also included in the current study. Despite of the similarity in chemical structures and high
AR binding affinity *in vitro*, this series of compounds revealed a wide range of pharmacologic activity in castrated rats. After castration, organ weights of prostate and levator ani muscle decreased to 6.2 and 40.9% of those in the intact animals, respectively. Partial agonists, C-12, C-14, C-15, and C-18, failed to demonstrate any pharmacologic activity *in vivo*, indicating that the AR-mediated transcriptional activation assay is an important screening step to select potential candidates for *in vivo* studies in the future.

Among active compounds, C-2 maintained the weight of ventral prostate and levator ani muscle at 11.4% and 51.5% of those in intact animals, respectively and exhibited both the lowest androgenic activity and anabolic activity in male rats. The highest androgenic activity (83.4%) and anabolic activity (123%) were observed in animals treated with C-6 and C-18, respectively. This observation indicates that the introduction of different size and type of electron-withdrawing substituents in the B-ring not only change the potency but also alter the tissue selectivity of compounds of interest. For decades, differential effects in tissues have been clearly illustrated between testosterone and dihydrotestosterone (DHT). DHT shows more profound androgenic activity in the prostate and seminal vesicles than testosterone. The underlying mechanisms of differential effects between these two androgens are still mysteries. To investigate possible mechanisms, several studies have been done both *in vivo* and *in vitro*. Using 5α-reductase inhibitor to block the conversion between testosterone to DHT significantly diminished the potent effects of DHT in ventral prostate (41, 145-149), suggesting that DHT bears higher androgenic activity than testosterone. *In vitro* assays reveals that DHT demonstrated higher AR-binding affinity (150) and AR-mediated transcriptional activation (151). Moreover, two kinds of androgen response elements (AREs) were identified to response differentially to testosterone and DHT (152), which provides a possible molecular mechanism for the difference between these two androgens. Mechanisms of the tissue-selectivity among nonsteroidal AR modulators are as interesting as those between nonsteroidal AR modulators and endogenous steroidal androgens. Since compounds of interest demonstrated high AR binding affinity and similar physiochemical properties, the high androgenic activity of C-6 and C-19 may due to their higher intrinsic activity, unique binding mode with AR, and recruitment of co-factors.
In conclusion, *in vivo* pharmacologic results demonstrated that pharmacologic activity of a SARM is determined by several ligand characteristics, including AR binding affinity, transcriptional activation, and metabolic stability. The Androgenic activity/anabolic activity ratio of this series of compounds can be altered by incorporation of halogen at the *para*-position of the B-ring. The effects of these compounds on hormonal regulation, including luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone may also be changed by structural modifications.
Figure 3.1 Chemical Structures of Testosterone, Estradiol-17β, and 5α-dihydrotestosterone (DHT).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure of the B-ring</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-1</td>
<td><img src="image" alt="Structure S-1" /></td>
</tr>
<tr>
<td>C-1</td>
<td><img src="image" alt="Structure C-1" /></td>
</tr>
<tr>
<td>C-2</td>
<td><img src="image" alt="Structure C-2" /></td>
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<tr>
<td>C-3</td>
<td><img src="image" alt="Structure C-3" /></td>
</tr>
<tr>
<td>C-4</td>
<td><img src="image" alt="Structure C-4" /></td>
</tr>
<tr>
<td>C-6</td>
<td><img src="image" alt="Structure C-6" /></td>
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<tr>
<td>C-8</td>
<td><img src="image" alt="Structure C-8" /></td>
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</tr>
<tr>
<td>C-24</td>
<td><img src="image" alt="Structure C-24" /></td>
</tr>
</tbody>
</table>

Figure 3.2 The B-ring Chemical Structure of Novel AR Ligands Examined for *In vivo* Pharmacologic Activity.
Figure 3. The Effect of Novel AR Ligands on the Prostate in Castrated Male Rats.
Figure 3. 4 The Effect of Novel AR Ligands on Seminal Vesicles in Castrated Male Rats.
Figure 3. 5 The Effect of Novel AR Ligands on the Muscle in Castrated Male Rats.
Figure 3. 6 Pharmacologic Effects of C-1 in Castrated Male Rats (A) and Dose-Response Relationships (B).
Figure 3. 7 Pharmacologic Effects of C-3 in Castrated Male Rats (A) and Dose-Response Relationships (B).
Figure 3.8 Pharmacologic Effects of C-10 in Castrated Male Rats (A) and Dose-Response Relationships (B).
Figure 3.9 Pharmacologic Effects of C-24 in Castrated Male Rats (A) and Dose-Response Relationships (B).
Table 3.1 Comparison of Androgenic and Anabolic Activities of Novel SARMs to TP.

<table>
<thead>
<tr>
<th>Organs</th>
<th>Treatment</th>
<th>Emax (% of Intact Control)</th>
<th>Relative Efficacy</th>
<th>ED&lt;sub&gt;50&lt;/sub&gt; (mg/day)</th>
<th>Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androgenic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>TP*</td>
<td>120.6 ± 13</td>
<td>1.0</td>
<td>0.13 ± 0.03</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>C-1</td>
<td>21.9 ± 3.5</td>
<td>0.18</td>
<td>0.33 ± 0.1</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>C-3</td>
<td>59 ± 22</td>
<td>0.49</td>
<td>0.65 ± 0.4</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>C-10</td>
<td>48.5 ± 4</td>
<td>0.40</td>
<td>0.59 ± 0.07</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>C-24</td>
<td>20.4 ± 1.1</td>
<td>0.17</td>
<td>0.63 ± 0.04</td>
<td>0.21</td>
</tr>
<tr>
<td>Seminal Vesicles</td>
<td>TP*</td>
<td>70.0 ± 19</td>
<td>1.0</td>
<td>0.12 ± 0.02</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>C-1</td>
<td>10.4 ± 0.4</td>
<td>0.15</td>
<td>0.66 ± 0.03</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>C-3</td>
<td>27 ± 1.6</td>
<td>0.39</td>
<td>0.58 ± 0.04</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>C-10</td>
<td>27.2 ± 0.2</td>
<td>0.39</td>
<td>0.82 ± 0.006</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>C-24</td>
<td>12.7 ± 1.4</td>
<td>0.18</td>
<td>0.62 ± 0.07</td>
<td>0.19</td>
</tr>
<tr>
<td>Anabolic</td>
<td>TP*</td>
<td>104.2 ± 10.1</td>
<td>1.0</td>
<td>0.15 ± 0.03</td>
<td>1.0</td>
</tr>
<tr>
<td>Levator Muscle</td>
<td>C-1</td>
<td>80.2 ± 7.5</td>
<td>0.77</td>
<td>0.42 ± 0.09</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>C-3</td>
<td>132 ± 12</td>
<td>1.27</td>
<td>0.33 ± 0.09</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>C-10</td>
<td>93.9 ± 8</td>
<td>0.90</td>
<td>0.43 ± 0.07</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>C-24</td>
<td>70.8 ± 0.1</td>
<td>0.68</td>
<td>0.70 ± 0.003</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* The Emax and ED<sub>50</sub> values of TP were previously reported by Yin et. al. (59)
CHAPTER 4

PRECLINICAL PHARMACOKINETICS AND METABOLISM OF C-3 IN THE RAT

4.1 INTRODUCTION

Clinically, the first choice for androgen replacement therapy is the endogenous androgen (testosterone). However, testosterone demonstrates negligible oral bioavailability and a very short half-life (153) due to rapid hepatic metabolism (32-34). To increase in vivo drug exposure, testosterone was structurally modified to testosterone esters, such as testosterone propionate, testosterone enanthate, and testosterone buciclat, etc. However, these esters also require intramuscular injection on a weekly basis (76, 77). Orally bioavailable testosterone derivatives (e.g., testosterone undecanoate, TU) were developed and used for androgen replacement in hypogonadal men. However, TU requires multiple doses per day and produces widely variable serum testosterone levels (35, 154). As such, testosterone undecanoate is still administered intramuscularly for clinical use.

Besides the development of androgens using structural modifications of testosterone, nonsteroidal androgens were discovered based on several pharmacophores (60, 62, 67, 155-157). In our laboratories, the first series of androgen receptor ligands (e.g., acetothiolutamide) exhibited similar androgen receptor (AR) binding affinity as that of testosterone and high in vitro functional activity. Surprisingly, these compounds were inactive in vivo. The metabolism of acetothiolutamide was further studied and shown to be responsible for the contradiction between in vitro and in vivo activity (58). Three major metabolism pathways of acetothiolutamide were identified, including oxidation of
the thio-ether linkage, hydrolysis of the amide bond, and sulfate conjugation. A second generation of androgen receptor ligands were designed and synthesized with improved pharmacokinetic properties by structural optimization of acetothiolutamide. Replacing the thio-ether linkage of acetothiolutamide significantly decreased its hepatic metabolism. S-1 and S-4 (Figure 4.1.) were identified as potent and efficacious selective androgen receptor modulators (SARMs) (59). Therefore, the integration of knowledge related to in vitro and in vivo structure activity relationships, pharmacokinetics, and metabolism of known compounds is a useful tool to discover novel SARMs.

Previous pharmacologic studies illustrated that novel SARMs can be designed by introduction of multiple substituents in the aromatic B-ring of our lead compound, S-1. Comparing C-3 with S-1, the only structural difference between these compounds is an additional fluoro group presented at the meta position of the B-ring of C-3. No significant difference was observed between these two compounds in terms of AR binding affinity. However, S-1 and C-3 exhibited significant different in vitro agonist activity, with a concentration of 10 nM inducing 99% and 43% of the activation induced by 1 nM of DHT, respectively. Furthermore, C-3 demonstrated much higher in vivo pharmacologic activity than S-1. For instance, the Emax values of C-3 in the prostate, seminal vesicles, and levator ani muscle were 59%, 27%, and 132% of those observed in the control group, respectively, while the Emax values of S-1 in the prostate, seminal vesicles, and levator ani muscle were 15%, 13%, and 75% of those in control animals, respectively. The significantly increased in vivo pharmacologic activity of C-3 may due to multiple mechanisms, such as increased intrinsic functional activity and/or improved pharmacokinetic properties. In the present studies, we examined the pharmacokinetics and metabolism of C-3 in male rats.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Male Sprague-Dawley rats, weighing approximately 250 g, were purchased from Harlan Bioproducts for Science (Indianapolis, IN). All animals were maintained on a 12h light/dark cycle with food and water available ad libitum. The animal protocol was
reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

4.2.2 Chemicals

\( \text{C-3} \) was synthesized using the methods described by Marhefka et al. (60). Chemical purity was confirmed using elemental analysis, mass spectrometry, and proton nuclear magnetic resonance. Dimethyl sulfoxide (DMSO), polyethylene glycol-300 (PEG 300), ketamine, xylazine, and acetonitrile were purchased from Sigma Chemical Co (St Louis, MO) and ethanol was purchased from Pharmco Products (Brookfield, CT).

4.2.3 Preclinical Pharmacokinetics of \( \text{C-3} \)

The pharmacokinetics of \( \text{C-3} \) were examined in five male SD rats weighing approximately 250 g after a dose of 10 mg/kg by intravenous injection. A catheter was implanted in the right external jugular vein of each animal 24 h prior to drug administration under anesthesia. \( \text{C-3} \) was dissolved in a vehicle containing DMSO (5%, v/v) in PEG 300 and administered to animals through the jugular vein catheter. Immediately after the drug administration, the catheter was flushed three times with saline (3× the volume of the dosing solution) before blood sampling. Blood samples (~200 µL) were then withdrawn through the jugular vein catheter at 5, 10, 30, 60, 90, 120, 240, 480, and 720 min after the i.v. dose. Blood samples were centrifuged at 1,000 g, 4°C for 10 min. Plasma samples were prepared and stored at -20°C until HPLC analysis.

4.2.4 HPLC Methods

An aliquot (100 µL) of each plasma sample from the pharmacokinetic studies was spiked with 10 µL of an internal standard (a structural analog of \( \text{C-3} \)) and mixed well with 1 mL of acetonitrile. After centrifugation at 16,000 g, 4°C for 10 min, the supernatant was collected and evaporated. The residues were reconstituted in 120 µL of
mobile phase. An aliquot of each sample was injected into a Nova-pak C\textsubscript{18} column (3.9 × 150 mm, 4 µm particle size) purchased from Waters Corporation (Milford, MA). The HPLC system consisted of a model 515 HPLC pump (Waters), a model 717 plus autosampler (Waters), and a model 486 absorbance detector (Waters). HPLC separation was performed using an isocratic mobile phase (H\textsubscript{2}O/Acetonitrile: 46/54, v/v) at a flow rate of 1 mL/min. The UV absorbance of eluents was monitored at 215 nm. Calibration standards were prepared in blank rat plasma with C-3 concentrations ranging from 0.1 to 50 µg/mL. The recoveries of this compound over the calibration range were from 96.8% to 98.1%. The intra- and inter-day coefficients of variation of the assay were lower than 4.6% and 9.9%, respectively. The limit of quantitation of the HPLC assay was 0.1 µg/mL.

4.2.5 Pharmacokinetic Data Analysis

The plasma concentration-time data were analyzed using noncompartmental methods and WinNonlin software (Pharsight, Mountain View, CA). The terminal half-life (T\textsubscript{1/2}) was calculated as T\textsubscript{1/2} = 0.693/\(\lambda\), where \(\lambda\) was the first-order rate constant describing the terminal decline in plasma drug concentration. The area under the plasma concentration-time curve (AUC\textsubscript{0-\text{\(\infty\)}}) was calculated using the trapezoidal method with extrapolation to time infinity. The plasma clearance (CL) was calculated as CL = Dose\textsubscript{i,v.}/AUC\textsubscript{0-\text{\(\infty\)}}\textsubscript{i,v.}, where the Dose\textsubscript{i,v.} and AUC\textsubscript{0-\text{\(\infty\)}}\textsubscript{i,v.} were the intravascular dose and the corresponding area under the plasma concentration-time curve from time 0 to infinity, respectively. The apparent volume of distribution at equilibrium (Vd\textsubscript{ss}) was calculated as Vd\textsubscript{ss} = CL · MRT, where the MRT was the mean residence time following the intravenous bolus dose.

4.2.6 Metabolism of C-3 in the Male Rat

A catheter was implanted in the right external jugular vein of adult male Sprague-Dawley rats 24 h prior to drug administration. C-3 was freshly dissolved in vehicle
containing DMSO (20%, v/v) in PEG 300 and administered to animals through the jugular vein catheter at a dose of 50 mg/kg. Each animal was individually housed in a Nalgene® metabolism cage. Urine and fecal samples were collected 24 h prior to the dose and for up to 24 h post after the dose. Samples were stored at –20°C until HPLC/MS<sup>n</sup> analysis.

Urine samples were allowed to completely thaw at room temperature and centrifuged at 3,000 g, 4°C for 10 min before using the sample preparation method described by Yin et al (58). In brief, an aliquot (10 ml) of supernatant from each collection interval was extracted four times with the same volume of ethyl acetate. The combined organic and aqueous phase from these extractions were evaporated separately to dryness under reduced pressure. Before sample analysis, the residues were reconstituted with mobile phase consisting of H<sub>2</sub>O/Acetonitrile (46/54, v/v). Each reconstituted sample was filtered through a membrane filter (0.45 µm, Millipore Corporation). An aliquot (20 µl) of the filtrate was injected in the HPLC/MS system and analyzed for metabolites of C-3 using the method described below.

Similarly, fecal samples, approximately 8 g, were homogenized with a mechanical homogenizer after adding 10 ml of distilled water (58). The homogenates were further extracted three times with a mixture of methanol/ethyl acetate (2:1, v/v). After each extraction, samples were centrifuged at 3,000 g, 4°C for 10 min. The aqueous phase was collected, combined, and evaporated under reduced pressure. The resulting semi-solid residues were then mixed well with methanol, and an aliquot (200 µl) of each methanol extract was filtered through the same membrane mentioned above and used for LC/MS<sup>n</sup> analysis.

4.2.7 LC-MS<sup>n</sup> Analysis

The LC/MS system used consisted of a Surveyor Pump, Surveyor Autosampler, and a LCQ Deca ion-trap mass spectrometer (ThermoFinnigan Corp, San Jose, CA) coupled with an electrospray ionization (ESI) source. The negative ion mode was used, and the general system parameters were tuned with the parent compound C-3 except for those used in collision-induced dissociation (CID), in which case the collision energy and
collision time for each ion were optimized. The temperature of the heated capillary was set at 300 °C, the spray voltage was 4.0 kV, and the sheath gas flow rate and auxiliary gas flow rate were 74 and 50 ml/min. Full MS scans in the range m/z 50-1000, selected ion monitoring (SIM) scans and MS^n scans of ions of interest were conducted as needed. Collision energy levels (He) and activation times for the MS^n analysis were adjusted for the target compounds individually.

For LC/MS, a reversed-phase column (5µm, 2mm × 250 mm, Ultrasphere ODS C18 column, (Deerfield, IL) was used with a gradient mobile phase consisting of acetonitrile and water at a flow rate of 100 µl/min. The gradient was initiated with 5% acetonitrile for 5 min, followed by a linear increase to 70% acetonitrile over 10 min, which was kept constant for 15 min. Then the mobile phase was linearly returned to the original condition over 3 min, and was maintained for 7 min. The cycle time for each analysis was 40 min.

Accurate mass determinations were performed using a Micromass Q-TOPTM II (Micromass, Wythenshawe, UK) equipped with an orthogonal electrospray source (Z-spray) operated in negative ion mode. Sodium iodide was used for mass calibration for the range m/z 50-1000. Data acquisition was controlled by Xcaliber software. Mass spectra data was analyzed by Metabolite ID software, version 1.0 and Mass Frontier software, version 3.0 (Thermo Electron Corp. San Jose, CA).

4.2.8 Statistical Analyses

All statistical analyses were performed using single-factor ANOVA with the α value set a priori at p < 0.05.

4.3 RESULTS
4.3.1 Preclinical Pharmacokinetics of C-3

The only structural difference between C-3 and the lead compound S-1 is an additional fluoro group at the meta position of the B-ring presented in C-3. However, the in vivo pharmacologic activity of C-3 was much greater than that of S-1. The different
pharmacokinetic properties of these two compounds may contribute to the observed difference in their pharmacologic activity. As shown in Figure 4.2, the mean plasma concentration-time profiles of C-3 and S-1 declined biexponentially in rats after a single i.v. bolus dose of 10 mg/kg. Pharmacokinetic parameters of these two compounds were analyzed using noncompartmental methods and WinNolin software (Version 4.0, Pharsight Corporation).

Table 4.1 lists the pharmacokinetic parameters of C-3 and S-1 in male rats. The terminal half-life (T$_{1/2}$) and total body clearance of C-3 was 197 min and 5.37 ± 1.2 mL/min/kg. The average steady state volume of distribution for C-3 (1.34 L/kg) was about twice that of the total body water (0.67 L/kg). The AUC of C-3 (1.81 ± 0.28 mg · min/L) was significantly less than that of S-1 (2.55 ± 0.27 mg · min/mL), suggesting that the increased pharmacologic activity of C-3 was not due to increased in vivo drug exposure.

4.3.2 Profiling and Identification of Urinary Metabolites of C-3 in the Rat

The purpose of this study was to qualitatively identify the metabolites of C-3 in male rats. The metabolic profiles of C-3 in urine and feces after high dose IV administration of a high (50 mg/kg) intravenous dose to rats were determined by ESI mass spectrometry after separation by reversed phase HPLC. The total ion spectra of post-dose urine samples were compared with blank urine samples taken prior to dosing to identify drug-related peaks. Molecular ions of interest were then subjected to LC/MS$^n$ analysis for structural identification. Figure 4.3 shows the HPLC/ESI-MS chromatogram of the organic extracts of the blank and 24 h post-dose urine samples. Five major peaks observed in the 24 h post-dose urine samples were absent in the blank urine samples, indicating that these peaks were drug-related components (Figure 4.4).

Molecular ion at m/z 419 ([M-H]$^-$) of the parent compound. (Retention time 21.7 min) Initial scan of these peaks for molecular ions revealed a peak with [M-H]$^-$ of m/z 419. This peak was identified as the parent compound, which was based on its ESI/MS$^2$-MS$^3$ scans of the appropriate fragment ions. The mass spectra of this peak were identical in every respect to those of the synthetic standard. Collision-induced dissociation (CID)
of C-3 generated three fragmentation ions at m/z 129, 261, and 289 as shown in Figure 4.5. The base peak ion at m/z 261 resulted from the cleavage of the chiral carbon-methylene carbon bond, with a loss of the methyl group. The cleavage at the bond between the ether linkage and the methylene carbon generated two fragmentation ions at m/z 129 and 289. The fragmentation ion at m/z 289 was generated by the cleavage mentioned above, followed by a loss of a hydrogen atom, and formed the epoxide product. Further CID of the fragment ion at m/z 289 (LC/MS$^3$) yielded an abundant granddaughter ion at m/z 205 and a less abundant ion at m/z 261. Since no peak ion at m/z 205 was detected during LC/MS scan, the contribution from the ion at m/z 289 to the formation of the ion at m/z 261 was minor. Figure 4.6 shows the proposed fragmentation pattern of C-3.

Molecular ion at m/z 403 ([M-H]$^-$) of M1. (Retention time 22.3 min) The [M-H]$^-$ ion at m/z 403 was 16 Da less than that of the parent compound, suggesting it to be a reduced product of C-3 and was identified as the reduction product of C-3 in which the nitro group was converted to a nitroso group (Table 4.2). The retention time of M1 was slightly longer than that of the parent compound, indicating that M1 is more hydrophobic than C-3. The [M-H]$^-$ at m/z 403 was 16 Da less than that of the parent compound, suggesting it to be a reduced product of C-3. Its MS$^2$ spectrum showed fragment peaks at m/z 129, 189, 245, and 273 with the fragment ion at m/z 245 as the base peak, consistent with the cleavage pattern of the parent compound. The simultaneous detection of peaks at m/z 129 for M1 and the parent compound suggested no metabolic changes in the B-ring. The existence of a fragment ion of 189 m/z in the MS$^2$ spectrum of M1 and MS$^3$ spectrum of the 273 m/z fragment ion further supports the assignment of M1 as the nitroso reduction product. Thus the only possible reduction, which would yield two fragment peaks containing an aromatic A-ring at m/z 245 and 273, was to reduce the nitro group to a nitroso group at the para position of the A-ring.

Molecular ion at m/z 485 ([M-H]$^-$) of M2. (Retention time 11.42 min) The [M-H]$^-$ ion at 485 m/z corresponded to a metabolite (M2) with two metabolic alterations; namely (1) hydroxylated B-ring and (2) an A-ring in which the nitro group was reduced and then conjugated with sulfate. Fragmentation of this molecular ion produced a base peak at m/z 405 and less abundant ions at m/z 145, 259, and 339 (Table 4.2). The proposed sulfate
conjugate was first confirmed by the loss of 80 Da., a mass that corresponds to SO$_3$, from the molecular ion at m/z 485 to an ion at m/z 405 and the fragment ion at m/z 339 to ion at m/z 259. The fragment ion at m/z 145, suggesting that oxidation occurred within the aromatic B-ring, was supported by the 16 m/z. increase in the corresponding fragment ion at m/z 129 produced by the parent compound. Further CID of the 405 m/z fragment ion yielded two ions of m/z 145 and 259 with the former being the base peak in the spectrum, which further supported the conclusion that the sulfate conjugate did not occur in the B-ring. The fragment ion at m/z 259 was 30 Da. less than the corresponding fragment ion at m/z 289 produced by the parent compound and suggested the reduction of the nitro group to an amine group in the A-ring. The overall fragmentation pattern of M3 proved that it was a B-ring hydroxylated, A-ring reduced sulfate conjugate. However, the site of oxidation in the B-ring could not be determined.

Molecular ion at m/z 485 ([M-H]$^-$) of M3. (Retention time 11.91 min) The [M-H]$^-$ ion showed the same m/z as that M2 but contained two metabolic alterations in the A-ring; namely (1) oxidation of the A-ring and (2) reduction and subsequent sulfate conjugation of the nitro group in the A-ring of C-3. Similar to M2, fragmentation of the molecular ion yielded a base peak at m/z 405 and less abundant peaks at m/z 275, 385, and 129 (Table 4.2). The loss of a SO$_3$ (80 Da.) from the molecular ion to the peak at m/z 405 confirmed that M3 was a sulfate conjugate. The presence of a fragment ion at m/z 129 suggested that the B-ring remained the same as that in the parent compound. Further CID of the ion at m/z 405 produced a base peak at m/z 275 and other fragment ions at m/z 129, 255, and 385. Consistent with parent compound, cleavage at the bond between the ether linkage and the methylene carbon generated ions at m/z 129 and 275. The latter ion was 14 Da. less than the fragment ion at m/z 289 observed upon fragmentation of the parent compound, which was proposed herein as the combination of the following changes: 1) B-ring nitro reduction to an amine group and 2) oxidation of the amine group or hydroxylation of the aromatic A-ring. The oxidation site in the A-ring was not certain. Interestingly, ions at m/z 385 and 255 derived from the fragment ion at m/z 405 were absent in the parent fragmentation pathways, indicating that the structural changes in the A-ring of M3 induced different dissociation pattern upon CID as proposed in Figure 4.6. The loss of a fluoro group from the trifluoromethyl carbon and a hydrogen from the
amine group of the ion at m/z 405 yielded the product ions at m/z 385 and 129. This proposed fragmentation pattern was further supported by the ion at m/z 255 (Figure 4.7).

**Molecular ion at m/z 499 ([M-H]⁻) of M4.** (Retention time 13.22 min) The [M-H]⁻ ion at m/z 499 was assigned as the sulfate conjugate of B-ring oxidized M1. The loss of 80 Da. from the molecular ion at m/z 499 to produce the fragment ion at m/z 419, which was identical in mass to the molecular ion of C-3, indicated that this molecular ion is a sulfate conjugate. However, the presence of a fragment ion at m/z 145 suggested that the B-ring has been oxidized. Further CID of the fragment ion at m/z 145 suggested that the B-ring has been oxidized. Further CID of the fragment ion at m/z 419 yielded product ions at m/z 145, 189, 245, and 273, consisting with the fragmentation pattern of M1. The sulfate conjugation occurred in the oxidized B-ring, as suggested by the presence of the 145 m/z ion upon fragmentation of the 499 and 419 m/z ions.

**Molecular ion at m/z 515 ([M-H]⁻) of M5.** (Retention time 13.12 min) The [M-H]⁻ ion at m/z 515 was assigned as the B-ring oxidated metabolite which had also undergone sulfate conjugation at the chiral hydroxyl group (C3). The loss of 80 Da. from the molecular ion at m/z 515 to produce the fragment ion at m/z 435 indicated that this molecular ion was a sulfate conjugate. In addition to this base peak ion, product ions at m/z 145, 289, and 370 were also observed. The fragment ions at m/z 145 and 370 were from the cleavage between the ether linkage and the methylene carbon, indicating that the molecular ion bears an oxidized B-ring and that the sulfate conjugation occurred at the tertiary hydroxyl group linked to the chiral carbon. Upon CID, the fragment ion at m/z 435 further yielded product ions at m/z 145, 205, 261, and 289, which matched well with the fragmentation pattern of C-3 except for the oxidized B-ring product ion.

4.3.3 Profiling and Identification of 0- to 24-h Fecal Metabolites in Rat

The mass spectra of the organic extract of the blank and 24 hr rat fecal samples are shown in Figure 4.8. No molecular ions corresponding to the parent compound or other major metabolites observed in the 24 h urine sample were detected. Two molecular ions associated with the parent compound at m/z 389 (Figure 4.8; c) and 405 (Figure 4.8; d) were extracted and identified by LC/MS/MS.
**4.4 DISCUSSION**

Several generations of SARMs were developed by structural optimization of the first generation compounds. The first generation of AR ligands (e.g. acetothiolutamide, Figure 4.1) demonstrated high _in vitro_ activity, but minor _in vivo_ pharmacologic activity in animals (58). Metabolism studies identified three major metabolism pathways of acetothiolutamide in rats, including oxidation, hydrolysis, and sulfation (Figure 4.9.A). Oxidation of the thio-ether linkage happened rapidly and diminished its AR agonist activity _in vivo_. Hydrolysis of the amide bond of the para-acetamido group in the B-ring of acetothiolutamide produced an amine metabolite, which acted as an AR antagonist activity _in vitro_. Furthermore, multiple positions of the aromatic B-ring were susceptible to oxidation reactions _in vivo_. Previous _in vitro_ structure-activity relationship of AR ligands showed that AR binding affinity and transcriptional activity were relatively lower for compounds with a para-cyano group than their nitro group containing counterparts. To improve the _in vivo_ metabolic stability and pharmacologic activity, structural modification of acetothiolutamide included: 1) replacing the thio-ether linkage with an oxygen bridge; 2) changing the para-acetamido group to other electron-withdrawing groups according the structure-activity relationships of SARMs; 3) replacing the para-
The second generation of SARMs (e.g., S-1 and S-2, Figure 4.2) demonstrated potent pharmacologic activity in vivo and much greater drug exposure than that of acetothiolutamide. Additional structural modification in the B-ring of S-1 (e.g., C-3) further improved the pharmacologic activity of SARMs in vivo (Chapter 3), which was likely due to several factors, including AR binding affinity, intrinsic functional activity, and pharmacokinetic properties. The pharmacokinetics of C-3 were then investigated. In contrast to what we expected, the plasma concentration – time curves of C-3 and S-1 were similar after an i.v. dose at 10 mg/day. The clearance of C-3 was about 30% higher than that of S-1 (5.37 vs 4.00 ml/min/kg) and consequently the total AUC of C-3 was 70% of AUC of S-1. Besides the difference in pharmacokinetics, the binding affinity of S-1 and C-3 were 6.1 ± 0.2 nM and 3.4 ± 0.6, respectively. Moreover, the in vitro functional activity of C-3 (99%) was two-fold higher than that of S-1 (43%). Therefore, the higher in vivo pharmacologic activity of C-3 than S-1 was likely due to improved binding affinity and intrinsic functional activity rather than the pharmacokinetic properties of this compound.

Previous pharmacokinetic studies with radioactive-labeled SARMs showed that this series of compounds undergoes extensive hepatic elimination in rats. Although the terminal half-life (3.3 h) of C-3 in rats is moderate, it is likely too short for once per day treatment (e.g., hormonal male contraception). The metabolism of C-3 was studied in rats for further structural optimization. Seven phase I or phase II metabolites were identified in urine and fecal samples 24 h after i.v. administration of C-3 at a dose of 50 mg/kg. C-3 was metabolized in rats through three major metabolism pathways: nitro-reduction in the A-ring, oxidation in the A or B-ring, and sulfate conjugation. A rational metabolic schematic of C-3 in rats was proposed in Figure 4.10. The para-nitro group was first reduced to a nitroso group and produced a reduced metabolite, M1, of C-3. Moreover, the nitroso group was completely reduced to an amine group, resulting another reduced metabolite, M6. Oxidation reaction occurred in the aromatic rings of the parents and its reduced metabolites. Sulfate conjugate happened rapidly to those oxidation products and formed final metabolites, M2, M3, M4, M5, and M7. Similar to the metabolism pattern of acetothiolutamide (58), sulfate conjugates were the only phase II metabolites of C-3.
detected by current study. In addition to the hydroxyl group introduced into the aromatic rings during oxidation, the hydroxyl group linked to the chiral carbon of **C-3** was also a possible site for sulfate conjugation. If that was the case, a metabolite ion at m/z 499 would be detected and its mass spectra of LC/MS<sup>n</sup> should match those of **C-3** after dissociating with the sulfate group. However, the only metabolite ion at m/z 499 showed a typical B-ring with a hydroxyl group and a reduced A-ring. Therefore, the sulfate conjugate site was uniformly assigned to the hydroxyl group in the A or B-ring.

In conclusion, the higher *in vivo* pharmacologic activity of **C-3** than **S-1** was likely due to its increased intrinsic functional activity, but not increased drug exposure than **S-1**. Nitro reduction and oxidation were the major phase I metabolism pathways of **C-3**, followed with rapid sulfate conjugation. Replacing the nitro group with a cyano group may increase the metabolic stability of **C-3** and maintain or increase its *in vivo* pharmacologic activity.
Figure 4.1 Chemical Structures of Bicalutamide, Acetothiolutamide, **S-1**, **S-4**, and **C-3**

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>X</th>
<th>Y</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-Bicalutamide</td>
<td>CN</td>
<td>SO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>H</td>
<td>F</td>
</tr>
<tr>
<td>Acetothiolutamide</td>
<td>CN</td>
<td>S</td>
<td>H</td>
<td><strong>NHC(O)CH&lt;sub&gt;3&lt;/sub&gt;</strong></td>
</tr>
<tr>
<td>S-1</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>O</td>
<td>H</td>
<td>F</td>
</tr>
<tr>
<td>S-4</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>O</td>
<td>H</td>
<td><strong>NHC(O)CH&lt;sub&gt;3&lt;/sub&gt;</strong></td>
</tr>
<tr>
<td>C-3</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>O</td>
<td>F</td>
<td>F</td>
</tr>
</tbody>
</table>
Figure 4. 2 Mean Plasma Concentration-Time Profiles of C-3 and S-1 in Male Rats.

Each data point represents the mean ± SD of five rats. (N = 5)

* Data was provided by Wu et al.
Table 4.1 Mean Pharmacokinetic Parameters of C-3 and S-1 in Male Rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>S-1</th>
<th>C-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{AUC}_{0 \rightarrow \infty} ) (min * mg/mL)</td>
<td>2.55 ± 0.27</td>
<td>1.81 ± 0.28</td>
</tr>
<tr>
<td>( T_{1/2} ) (min)</td>
<td>248</td>
<td>197</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>363 ± 51</td>
<td>250 ± 37</td>
</tr>
<tr>
<td>CL (mL/min/kg)</td>
<td>4.00 ± 0.50</td>
<td>5.37 ± 1.2</td>
</tr>
<tr>
<td>( V_{ss} ) (L/kg)</td>
<td>1.46 ± 0.35</td>
<td>1.34 ± 0.82</td>
</tr>
</tbody>
</table>

Dose: 10 mg/kg, i.v.
Data represent the mean ± SD of five rats. (\( N = 5 \))
* Data was provided by Di et al.
Figure 4. 3 HPLC/ESI-MS Chromatograms of Extracts from Rat Urine Samples

A: Blank urine samples; B: Urine samples taken 24 h after an i.v. dose of C-3; C: 24 h samples subtracted from the background of the blank urine samples.
Figure 4. 4 HPLC/ESI-MS Chromatograms of Extracts from 24 h Rat Urine Samples.

(a) Total ion chromatogram (TIC); (b) Extracted ion chromatogram (XIC) of the parent drug C-3 at m/z 419; (c) XIC of one reduction product M1 at m/z 403; (d) XIC of two reduction and sulfate conjugate M2 and M3 at m/z 485; (e) XIC of one sulfate conjugate M4 at m/z 499; (f) XIC of one oxidation and sulfate conjugate M5 at m/z 515.
Figure 4.5 Mass spectrum of **C-3** obtained from LC/MS/MS.
Figure 4. 6 Proposed Fragmentation Pattern of C-3

Structural information was determined by LC/MS\(^2\) and LC/MS\(^3\) fragmentation of the parent compound, C-3.
<table>
<thead>
<tr>
<th>Molecular Ions [M-H]</th>
<th>Product Ions Observed (m/z Values)</th>
<th>Assigned Structure and Proposed Fragmentation Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>419 (C-3)</td>
<td>129, 261*, 289</td>
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</tr>
<tr>
<td>289</td>
<td>205*, 261</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>261</td>
<td>188, 218, 205*</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>403 (M1)</td>
<td>129, 189, 245, 273*</td>
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</tr>
<tr>
<td>273</td>
<td>189*, 245</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>485 (M2)</td>
<td>145, 339, 259, 405*</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>405</td>
<td>145*, 259</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>485 (M3)</td>
<td>129, 385, 405*</td>
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<tr>
<td>405</td>
<td>129, 255, 275*, 385</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>499 (M4)</td>
<td>145, 189, 245, 419*</td>
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<td>419</td>
<td>145*, 189, 245, 273</td>
<td><img src="image" alt="Structure" /></td>
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<tr>
<td>515 (M5)</td>
<td>145, 289, 370, 435*</td>
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<tr>
<td>435</td>
<td>145*, 205, 261, 289</td>
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</table>

Table 4. 2 Product Ions of C-3 and Its Metabolites from the 24 h Rat Urine Sample

*a Denotes a base peak in the product ion mass spectra.
Figure 4. 7 Proposed Fragmentation Pattern of M3

Structural information was determined by LC/MS$^2$ and LC/MS$^3$ fragmentation of M3.
Figure 4. 8 HPLC/ESI-MS Chromatograms of Extracts from Rat Fecal Samples.

(a) Total ion chromatogram (TIC) of blank fecal sample; (b) TIC of 24 h fecal sample; (c) Extracted ion chromatogram (XIC) of one A-ring reduction product M6 at \( m/z \) 389; (d) XIC of one A-ring reduction B-ring hydroxylation product M7 at \( m/z \) 405.
<table>
<thead>
<tr>
<th>Molecular Ions [M-H]</th>
<th>Product Ions Observed (m/z Values)</th>
<th>Assigned Structure and Proposed Fragmentation Pattern</th>
</tr>
</thead>
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<tr>
<td>419 (C-3)</td>
<td>129, 261a, 289</td>
<td>![Diagram of C-3]</td>
</tr>
<tr>
<td>289</td>
<td>205a, 261</td>
<td>![Diagram of 289]</td>
</tr>
<tr>
<td>261</td>
<td>188, 218, 205a</td>
<td>![Diagram of 261]</td>
</tr>
<tr>
<td>389 (M6)</td>
<td>129a, 175, 259</td>
<td>![Diagram of 389]</td>
</tr>
<tr>
<td>405 (M7)</td>
<td>145a, 255, 275, 385</td>
<td>![Diagram of 405]</td>
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Table 4. 3 Product Ions of C-3 and Its Metabolites from the 24 h Rat Fecal Sample.

*a Denotes a base peak in the product ion mass spectra.
Figure 4. 9 Metabolism Sites of Acetothiolutamide and C-3 in the Rat.
Figure 4. 10 Proposed Metabolism Pathways of C-3 in Male Rats.

Structures in parenthesis are proposed intermediates.
CHAPTER 5

A SELECTIVE ANDROGEN RECEPTOR FOR HORMONAL MALE CONTRACEPTION

5.1 INTRODUCTION

Worldwide population growth and social awareness of reproductive health have stimulated a great deal of research in contraception. Although contraceptive pills have been available for women for decades, male contraception remains restricted to physical methods, such as condoms and vasectomy. However, many polls show that men would be more willing to be involved in family planning if appropriate contraceptive methods (e.g., a male contraceptive ‘pill’) were available (158, 159). Although the mechanism of spermatogenesis is not completely understood, inhibition of LH and FSH production is known to interrupt spermatogenesis. To date, the most practical method of male contraception is the hormonal approach. Potential regimens include androgen alone, androgen combined with progestins, and androgen combined with gonadotropin hormone releasing hormone (GnRH) analogs (160, 161). Among them, androgen-progestin combinations remain the most promising approach. Because androgens are essential for male development and the maintenance of male secondary characteristics, such as bone mass, muscle mass, fat tissue distribution, and spermatogenesis (119), an androgen supplement is a required component of hormonal male contraception. However, the lack of orally bioavailable and safe androgens has been a major limitation in the search for hormonal male contraceptives. The main disadvantages of steroidal androgens (e.g., testosterone and its esters) are their undesirable physicochemical and pharmacokinetic properties. Testosterone and the majority of its esters must be administered by intramuscular injection, implant, and/or transdermal patch. Besides their inconvenient
means of administration, steroidal androgens are associated with a variety of undesirable side effects. In fact, the major dose-limiting side effects observed in recent clinical trials of male contraception were androgen-related (161).

Several approaches to overcome the limitations of using testosterone preparations have been explored. One major approach focused on structural modifications of steroidal androgens to develop long-acting androgens. Another approach is nonsteroidal selective androgen receptor modulators (SARMs). In 1998, during our search for androgen receptor (AR) affinity labels, we discovered and reported a group of nonsteroidal androgens, which are derivatives of two known antiandrogens – bicalutamide (Fig. 1) and flutamide (56). In recent years, we determined important structure-activity relationships for AR binding affinity and transcriptional activation (57, 62), pharmacokinetics and key metabolism pathways of SARMs (58), and identified dozens of SARMs that demonstrate tissue-selective anabolic and androgenic in vivo pharmacologic effects, but are devoid of the side effects commonly associated with testosterone therapy (59, 60). The discovery of SARMs therefore provides a unique alternative for androgen replacement therapy with advantages including oral bioavailability, flexibility of structural modification, AR specificity, lower activity in the prostate, and the lack of steroid-related side effects.

Earlier studies demonstrated that minor structural modification of SARMs could result in dramatic changes in their in vitro and in vivo pharmacologic activity. To date, most of the SARMs reported in our laboratories are able to mimic various pharmacologic activities of testosterone in peripheral tissues, but are unable to do so in the central nervous system (Yin et al., 2003a). We hypothesized that novel SARMs can also be designed and synthesized to mimic the effects of testosterone on the hypothalamus-pituitary-testis axis. Such compounds would represent an important step toward the discovery and development of SARMs for hormonal male contraception. The present studies were designed to characterize the preclinical pharmacology of one such novel SARM.
5.2 MATERIALS AND METHODS

5.2.1 Chemicals and Animals

The S-isomer of C-6 (Fig. 1) was synthesized in our laboratory with a purity greater than 99% using previously described methods (57, 60). [17α-methyl-3H] Mibolerone ([3H]-MIB, 84 Ci/mmol) and unlabeled MIB were purchased from PerkinElmer Life Sciences (Boston, MA). Phosphate-buffered saline (PBS, catalog# P5368, consisting of 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Male Sprague-Dawley rats were purchased from Harlan Bioproducts for Science (Indianapolis, IN). All animals were maintained on a 12 h light/dark cycle with food and water available ad libitum. The animal protocol was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

5.2.2 In Vitro Pharmacologic Activity

Rat prostate cytosolic AR was prepared from ventral prostates of castrated male SD rats using established methods (63). The AR binding affinity of C-6 was determined using an in vitro radioligand competitive binding assay as previously described (62). In brief, an aliquot of AR cytosol was incubated with 1 nM [3H]MIB and 1 mM triamcinolone acetonide at 4 °C for 18 h in the absence or presence of increasing concentrations of DHT or C-6 (10⁻¹ nM to 10⁴ nM). Nonspecific binding of [3H]MIB was determined by adding excess unlabeled MIB (1000 nM) to the incubate. The separation of bound and free radioactivity at the end of incubation was achieved by the hydroxyapatite (HAP) method as previously described (Yin et al., 2003b), then the radioactivity was counted in a liquid scintillation counter (Model LS6500, Beckman Instruments Inc, Palo Alto, CA). The specific binding of [3H]MIB at each concentration of DHT or C-6 (B) was further calculated by subtracting the nonspecific binding of [3H]MIB, and expressed as the percentage of the specific binding in the absence of DHT or C-6 (B₀). Competitive displacement curves of DHT and C-6 were constructed with B
on the vertical axis and ligand concentration (C) on the horizontal axis. The concentration of compound that reduced the B₀ by 50% (IC₅₀) was determined by computer-fitting the data to the following equation using WinNonlin (Pharsight Corporation, Mountain View, CA): \( B = B₀ × [1 - \frac{C}{IC₅₀ + C}] \). The equilibrium binding constant (Ki) of DHT or C-6 was calculated by \( K_i = K_d × \frac{IC₅₀}{(K_d + L)} \), where Kd was the dissociation constant of [³H]MIB (0.19 ± 0.01 nM, previously determined by Mukherjee et al., 1999), and L was the concentration of [³H]MIB used in the experiment (1 nM).

The in vitro AR-mediated transcriptional activity of C-6 was measured using a modification of the method of (62). Monkey kidney cells, CV-1, were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air in DMEM containing 10% fetal bovine serum (FBS). At ~90% confluence, cells were transfected in serum-free medium using Lipofect AMINE (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells in each T-175 flask were transfected with 0.8 μg of a human AR expression construct (pCMVhAR; generously provided by Dr. Donald Tindall, Mayo Clinic), 8 μg of an androgen-dependent luciferase reporter construct (pMMTV-Luc, generously provided by Dr. Ron Evans, Salk Institute), and 8 μg of a β-galactosidase expression construct (pSV-β-galactosidase; Promega Corporation). After 3~5 h of transfection, cells were washed with serum-free medium and allowed to recover for 10 h in DMEM medium containing 0.2% FBS. Transfected cells were trypsinized, centrifuged, and counted. Cells were then plated into 24-well plates at a density of 8×10⁴ cells/well. Before drug treatment, cells were allowed to attach to plates for 12 h. The agonist activity and antagonist activity of C-6 were determined by incubating cells with C-6 (1, 10, 100, 1000 nM) in the absence and presence of DHT (1 nM), respectively. In each experiment, vehicle control and positive control (activity induced by 1 nM of DHT) were included. For each treatment, compounds were dissolved in ethanol, and then diluted with medium to the desired concentrations. The final concentration of ethanol in each well was ≤ 0.05%.

After 24 h drug treatment, medium was removed by aspiration. Cells were washed twice with ice-cold PBS and lysed with 110 μL/well of Reporter Lysis Buffer (Promega Corporation) at room temperature for 30 min. An aliquot (50 μL) of cell lysate from each
well was used for β-galactosidase assays and the other 50 μL of cell lysate was used for luciferase assays using the method previously described (62). All results were expressed as the ratio of (luciferase activity/β galactosidase activity) to avoid variations caused by cell number and transfection efficiency.

5.2.3 In vivo Pharmacologic Activity

5.2.3.1 Androgenic and Anabolic Activity of C-6 in Castrated and Intact Male Rats

Forty-five male SD rats weighing approximately 200 g were purchased and randomly divided into nine groups with five rats per group. One day before the start of treatment, groups 1 through 8 were castrated via a scrotal incision under anesthesia. Group 9 served as an intact vehicle control. C-6 was dissolved in vehicle containing DMSO (5%, v/v) in PEG 300. Groups 1 through 7 received daily subcutaneous injections of C-6 at a dose rate of 0.05, 0.1, 0.3, 0.5, 0.75, 1, and 3 mg/day, respectively. Groups 8 and 9 received vehicle. In another study, three groups of intact male rats were treated with vehicle, testosterone propionate (TP), or C-6 at a dose rate of 0.5 mg/day. This dose rate was chosen based on the results from the aforementioned study in castrated rats. Rats were sacrificed after 14 days of treatment. Serum samples were collected and stored at –80 °C. The ventral prostate, seminal vesicles, and levator ani muscle were removed, cleared of extraneous tissue, and weighed. All organ weights were normalized to body weight and compared. The weights of prostate and seminal vesicles were used to evaluate androgenic activity, while the levator ani muscle weight was used as a measurement of anabolic activity. Non-linear regression analysis was performed to obtain the maximal response (Emax) induced by C-6 and the dose rate of C-6 that induced 50% of the maximal response (ED50) using the sigmoid E\text{max} model with a baseline effect parameter and WinNonlin software (Pharsight Corporation, Mountain View, CA). The value of Emax was constrained to an upper limit of 130%, slightly above the highest change in tissue weight observed in our study. Serum levels of LH and FSH were measured by the National Hormone & Peptide Program (Dr. A. Parlow, Torrance, CA) using RIA kits with rLH-NIDDK-RP-3 and r-FSH-NIDDK-RP-2 as reference standards, respectively.
Serum testosterone concentrations were measured using commercially available EIA kits (DSL, Webster, TX).

5.2.3.2 *In Vivo* Antireproductive Activity of **C-6** in Intact Male Rats

Fourteen mature, male Sprague Dawley rats (90 days old) were randomly assigned to two groups and received vehicle (5% DMSO + 95% PEG 300, n=6) or **C-6** dissolved in vehicle (n=8) at a dose rate of 1 mg/day. Compounds were freshly prepared immediately before dosing and delivered to animals via daily s.c. injections (100 µL/day) for 70 days. At the end of treatment, animals were sacrificed under anesthesia. Body weight was recorded at autopsy. Prostate, seminal vesicles, right testis, and right epididymis (divided in the middle of corpus epididymis to caput and cauda epididymis) were collected and weighed. All organ weights were normalized with body weights and compared. Blood was also collected. Serum samples were prepared and stored at -80°C until used. One testis from each rat was decapsulated and homogenized in PBS (pH 7.4). To obtain spermatozoa, the cauda epididymis was macerated in PBS (pH 7.4). Advanced spermatids (step 17-19) in the testis and the spermatozoa in cauda epididymis were counted with a hemacytometer. Results were expressed as number of spermatids per testis and spermatozoa per cauda epididymis, respectively. Testosterone concentrations in serum were measured with a commercially available EIA kit (DSL, Webster, TX).

5.2.4 Pharmacokinetics of **C-6** in the Rat

The pharmacokinetics of **C-6** was examined in male SD rats weighing approximately 250 g after a dose of 10 mg/kg by intravenous injection or oral gavage. A catheter was implanted in the right external jugular vein of each animal 24 h prior to drug administration. **C-6** was dissolved in a vehicle containing DMSO (10%, v/v) in PEG 300 and administered to animals through the jugular vein catheter (i.v. group) or oral gavage (p.o. group). For animals in the i.v. group, the catheter was flushed three times with saline (3× the volume of the dosing solution) before blood sampling. Blood samples (~200 µL) were then withdrawn through the jugular vein catheter at 5, 10, 30, 60, 90, 120,
180, 360, 720, 1440, and 1800 min after the i.v. dose and at 20, 30, 60, 90, 120, 240, 360, 
480, 720, 1080, 1440, and 1800 min after the p.o. dose. Blood samples were immediately 
centrifuged at 1,000 g, 4°C for 10 min. Plasma samples were prepared and stored at -
20°C until HPLC analysis.

5.2.5 HPLC Method

An aliquot (100 µL) of each plasma sample from the pharmacokinetic studies was 
spiked with 10 µL of an internal standard (a structural analog of C-6) and mixed well 
with 1 mL of acetonitrile. After centrifugation at 16,000 g, 4°C for 10 min, the 
supernatant was collected and evaporated. The residues were reconstituted in 120 µL of 
mobile phase. An aliquot of each sample was injected into a Nova-pak C18 column (3.9 × 
150 mm, 4 µm particle size) purchased from Waters Corporation (Milford, MA). The 
HPLC system consisted of a model 515 HPLC pump (Waters), a model 717 plus 
autosampler (Waters), and a model 486 absorbance detector (Waters). HPLC separation 
was performed using an isocratic mobile phase (H2O/Acetonitrile: 46/54, v/v) at a flow 
rate of 1 mL/min. The UV absorbance of eluents was monitored at 298nm. Calibration 
standards were prepared in blank rat plasma with C-6 concentrations ranging from 0.1 ~
50 µg/mL. The recoveries of this compound over the calibration range were from 93.4% 
to 100.4%. The intra- and inter-day coefficients of variation of the assay were lower than 
2.9% and 8.9%, respectively. The limit of quantitation of the HPLC assay was 0.1 
µg/mL.

5.2.6 Pharmacokinetic Data Analyses

The plasma concentration-time data were analyzed using noncompartmental 
methods and WinNonlin software (Pharsight, Mountain View, CA). The terminal half-
life (T1/2) was calculated as T1/2 = 0.693/λ, where λ was the terminal elimination constant. 
The area under the plasma concentration-time curve (AUC0-∞) was calculated using the
trapezoidal method with extrapolation to time infinity. The plasma clearance (CL) was calculated as \( CL = \frac{\text{Dose}_{i.v.}}{\text{AUC}_{0-\infty, i.v.}} \), where the Dose_{i.v.} and AUC_{0-\infty, i.v.} were the intravascular dose and the corresponding area under the plasma concentration-time curve from time 0 to infinity, respectively. The apparent volume of distribution at equilibrium (Vdss) was calculated as \( Vdss = CL \cdot MRT \), where the MRT was the mean residence time following the intravenous bolus dose. The peak plasma concentration (C_{max}) and time to reach the peak concentration (T_{max}) after a p.o. dose were obtained directly from the plasma concentration-time curves. Oral bioavailability (F_{p.o.}) was defined as \( F_{p.o.} = \frac{\text{AUC}_{0-\infty, p.o.} \cdot \text{Dose}_{i.v.}}{\text{AUC}_{0-\infty, i.v.} \cdot \text{Dose}_{p.o.}} \), where the Dose_{p.o.} and AUC_{0-\infty, p.o.} were the oral dose and the corresponding area under the plasma concentration-time curve from time 0 to infinity after p.o. administration, respectively.

5.2.7 Statistical Analyses

All statistical analyses were performed using single-factor ANOVA with the \( \alpha \) value set a priori at \( p < 0.05 \).

5.3 RESULTS
5.3.1 In vitro Pharmacologic Activity

Previous studies demonstrated that structural modification(s) of the A-ring, linkage, and/or the B-ring of nonsteroidal SARMs affect their in vitro and in vivo pharmacologic activity. Compared to bicalutamide, C-6 has a NO2 at the para-position of the A-ring, an ether linkage, and a chloro and a fluoro substituent at the para-position and meta-position of the B-ring, respectively (Fig. 1). The AR binding affinity of C-6 was determined using a competitive binding assay. Dihydrotestosterone (DHT) and C-6 displaced \(^3\)H-MIB from AR binding sites (Fig. 2). The Ki values of DHT and C-6 were 0.45 \( \pm \) 0.2 nM and 4.9 \( \pm \) 0.3 nM, respectively. Although the binding affinity of C-6 was less than one-tenth that of DHT, it bound the AR with 2-fold higher affinity than R-bicalutamide (i.e., Ki, Bicalutamide = 11.0 nM) (162) and 4~8-fold higher affinity than
hydroxyflutamide (128, 163), indicating that C-6 interacted with the AR with high binding affinity.

Upon binding to the AR, C-6 induced a concentration-dependent increase in AR-mediated transcriptional activation (Fig. 3, panel A). The transcriptional activity induced by C-6 plateaued at concentrations equal to or greater than 10 nM (~100% of activity that was induced by 1 nM of DHT). The activity in the vehicle-treated wells was less than 3% of the transcriptional activation induced by 1 nM of DHT. C-6 did not significantly inhibit transcriptional activity induced by DHT (Fig. 3, panel B) even at a concentration as high as 1,000 nM. These results clearly demonstrated that C-6 binds to the AR with high affinity and potently stimulates AR-mediated transcriptional activity in vitro.

5.3.2 In vivo Pharmacologic Activity
5.3.2.1 Androgenic and Anabolic Activity of C-6

We then examined the in vivo androgenic and anabolic activities of C-6. In castrated male rats, the androgenic activity was evaluated by the ability of C-6 to maintain the weights of ventral prostate and seminal vesicle, while levator ani muscle weight was used to assess anabolic activity. Castration resulted in a significant reduction in the weights of ventral prostate, seminal vesicle, and levator ani muscle to 8.5%, 6.5%, and 34% of that observed in intact animals, respectively. The administration of C-6 increased the weights of these tissues in a dose-dependent manner (Fig. 4A). At the highest dose rate (3 mg/day), the weights of all three organs were increased to about 130% of those observed in intact controls. Nonlinear regression analysis of the dose-response relationships showed that the ED$_{50}$ values of C-6 were $0.77 \pm 0.06$, $0.88 \pm 0.1$, and $0.17 \pm 0.04$ mg/day in the ventral prostate, seminal vesicle, and levator ani muscle, respectively (Fig. 4B). These results clearly revealed the potent and efficacious androgenic and anabolic activity of C-6 in male rats. The ED$_{50}$ value of C-6 in the levator ani muscle ($0.17 \pm 0.04$ mg/day) was about 4 to 5-fold less than its ED$_{50}$ values in prostate and seminal vesicle ($0.77 \pm 0.06$ mg/day and $0.88 \pm 0.14$ mg/day, respectively). At a dose rate of 0.3 mg/day, C-6 maintained the weight of levator ani muscle at a weight not different from that observed in intact controls. The effect of C-6 in the ventral
prostate and seminal vesicle was about 30% of that observed in intact controls at this
dose rate. Results suggested that C-6 acts as a tissue-selective androgen with more potent
anabolic activity than androgenic activity. The efficacy (maximum effect) of C-6 in
anabolic and androgenic tissues was about the same.

We also determined the serum levels of LH and FSH in animals treated with C-6,
and compared them to the levels of those hormones observed in intact and castrated
control animals. As shown in Table 1, castration led to a significant elevation in LH (8.4
± 2.5 ng/mL) and FSH (63.4 ± 5.0 ng/mL) levels due to the lack of negative feedback
inhibition by testosterone. C-6 caused a dose-dependent decrease in LH and FSH levels
and restored the levels of these hormones back to physiological levels at dose rates as low
as 0.3 mg/day and 0.5 mg/day, respectively.

These data led us to immediately examine the pharmacologic activity of C-6 in
intact male rats to answer an important question: Would C-6 mimic the ability of
exogenous testosterone to inhibit the synthesis of endogenous testosterone? We chose a
dose rate of 0.5 mg/day for this study. This dose was the minimum dose required to fully
suppress LH and FSH, and yet maintain normal muscle mass in castrated rats. In a 14-day
study (Fig. 5), testosterone propionate (TP) stimulated growth in all three organs, while
C-6 demonstrated tissue-selective pharmacologic effects in intact rats. The weights of the
ventral prostate and seminal vesicles were significantly decreased to about 50% of those
observed in intact controls, while the weights oflevator ani muscle remained the same as
those observed in intact controls. Surprisingly, the pharmacologic effects of C-6 in intact
rats were nearly identical to those found in castrated rats at this dose rate (0.5 mg/day)
(Fig. 5). Based on this finding, we predicted that C-6 reduced serum testosterone
concentration to levels similar to those observed in castrated rats. Results showed that the
average testosterone concentration in the intact control group was 4.47 ± 1.66 ng/mL.
Testosterone concentrations in three animals of the C-6-treated group were below the
detection limit (0.04 ng/mL) of the assay, while testosterone concentrations in the two
remaining animals were 0.11 ng/mL and 0.46 ng/mL. Clearly, C-6 potently and rapidly
inhibited endogenous testosterone synthesis in male rats.

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5.3.2.2 Antireproductive Activity of \textbf{C-6}

In this pilot study, we evaluated the effects of \textbf{C-6} on the reproductive system after long-term (1 mg/day, 10 weeks) treatment in adult male rats. Body and organ weights in control and \textbf{C-6}-treated rats are summarized in Table 2. No significant difference in the mean body weight was observed after \textbf{C-6} treatment. \textbf{C-6} caused a significant reduction in the size of the prostate, seminal vesicles, testis and epididymis to 68, 83, 60, and 64% of control values, respectively. Similar to our observations in castrated animals, \textbf{C-6} significantly increased the weight of levator ani muscle to 120% of that observed in controls. As shown in Figure 6, the mean sperm counts in both testis and cauda epididymis were significantly reduced to about 25% of those observed in the intact control group. Because of the marked decline in testicular and epididymal weight, the changes in serum testosterone level were further evaluated. The average testosterone concentration in intact control animals was 1.06 ± 0.78 ng/mL. These concentrations are within the normal range of testosterone concentration reported from male rats with a similar age (164, 165). Animals in \textbf{C-6}-treated group showed significantly suppressed testosterone levels that were lower than the quantitation limit (0.04 ng/mL) of the EIA kits. Clearly, the reduction of endogenous testosterone production was the main reason for the suppressed reproductive organ weights and sperm number in intact animals. Although a high dose rate (1 mg/day) of \textbf{C-6} was used in this study, low activity was observed in the prostate and seminal vesicles due to the tissue selectivity of \textbf{C-6}. In conclusion, \textbf{C-6} was able to mimic the effects of exogenous testosterone on spermatogenesis in male rat, while maintaining its tissue selectivity after chronic treatment. Additional studies over a wider dose range are warranted to more fully describe the effects of \textbf{C-6} on spermatogenesis and male reproductive activity.

5.3.3 Pharmacokinetics of \textbf{C-6} in the Rat

To develop a possible male contraceptive ‘pill’, high oral bioavailability and a suitable half-life of the compound are required. Therefore, the pharmacokinetics of \textbf{C-6}
were examined after i.v. and p.o. administration to male rats after a single dose of 10 mg/kg.

The time-courses of the changes in plasma concentrations of C-6 following i.v. and p.o. administration are shown in Figure 7, and the pharmacokinetic parameters are listed in Table 3. Following intravenous administration, C-6 concentrations declined and remained detectable until 30 h after the dose. The terminal $T_{1/2}$ of C-6 in male rats was 6.3 h. The systemic clearance (CL) and steady state volume distribution (Vdss) were 0.72 mL/min · kg and 473 mL/kg, respectively. C-6 appeared rapidly (within 5 minutes) in the systemic circulation after p.o. administration, suggesting gastric absorption of the drug. Oral absorption was prolonged with maximum plasma concentrations forming a plateau over 6 to 12 h post-dose. Plasma concentrations of C-6 diminished with a mean terminal $T_{1/2}$ of 5.6 h. This terminal $T_{1/2}$ was smaller than that observed after i.v. administration, an observation which was likely due to individual variation of plasma drug concentration after p.o. administration. However, statistical analysis revealed no significant difference between these two values. The bioavailability of C-6 following oral administration was 76%.

5.4 DISCUSSION

Earlier studies revealed that known nonsteroidal AR ligands (mainly bicalutamide derivatives) could be structurally modified to obtain a series of compounds with diverse in vitro (62) and in vivo pharmacologic activities (59). Subsequent research in our laboratories has focused on the discovery of novel SARMs as alternatives for testosterone replacement therapy and the treatment of muscle wasting diseases, osteoporosis, prostate hyperplasia (64), and hormonal male contraception. However, unlike testosterone, the SARMs that we have reported to date showed little to no effects on the hypothalamic-pituitary-testis axis, precluding their ability to regulate gonadotropins, serum testosterone levels, or serve as components of a male contraceptive regimen. Our previous studies showed that the aromatic B-ring is much more amenable to structural modification than other positions in the structure (60, 62). We used this and other structure-activity
relationships uncovered in our research to design and synthesize additional series of SARMs in the hopes of identifying compounds with enhanced central activity. Typically, these compounds were S-isomers with a structure that bears a NO$_2$ and a CF$_3$ at the para-position and meta-position of the A-ring, respectively, a CH$_3$ linked to the chiral carbon, an ether linkage, and an electronegative substituent(s) in the aromatic B-ring. C-6 was one of these AR ligands.

When we compared the *in vitro* activity of C-6 with one of our known SARMs (i.e., (S)-3-(3-fluorophenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethylphenyl) propionamide (S-1)) (59), we found that the di-halogen (para-Cl and meta-F) substituents in the aromatic B-ring retained high AR binding affinity (i.e., $K_i$, C-6 = 4.9 nM vs $K_i$, S-1 = 6.1 nM). Notably, at a concentration of 10 nM, C-6 showed 2 to 3-fold higher transcriptional activation than S-1 (i.e., 118% vs 43% of the activity induced by 1 nM of DHT). These findings were consistent with our prediction regarding the flexibility of structural modification in the B-ring. When C-6 was tested in the castrated male rat model, it produced more potent and efficacious *in vivo* pharmacologic activity than S-1. Although C-6 exhibited profound effects on all three androgen-dependent organs, it remained tissue selective. The potency (i.e., ED$_{50}$) and efficacy (i.e., $E_{max}$) of C-6 and testosterone propionate (TP) (59) in levator ani muscle were comparable, while the potency of C-6 in prostate and seminal vesicles was 6 to 7-fold less than that of TP. Further, the ED$_{50}$ value for C-6 in the levator ani muscle was 0.17 mg/day, or about 5-fold lower than that in the prostate and seminal vesicle. Thus, in castrated rats, a dose of C-6 (i.e., 0.3 mg/day) that will fully maintain the weight of levator ani muscle will only partially maintain the weight of the prostate and seminal vesicles. The enhanced pharmacologic activity of C-6 compared to our previously reported SARM (i.e., S-1) might have resulted from several factors, including: binding affinity, transcriptional activity, drug disposition, metabolism rate, and/or nuclear retention time. During *in vitro* studies, C-6 showed higher transcriptional activity but similar binding affinity to S-1. The *in vivo* total body clearance of C-6 was 0.72 mL/min/kg, which was significantly smaller than that of S-1 (Data not published). Therefore, the increased efficacy and potency of C-6 in rats may have been due to its higher intrinsic activity and greater *in vivo* exposure.
Due to the detection limit of the LH RIA assay and the limited sample volumes, LH concentrations in the high dose drug-treated group and intact group were undetectable. However, it was clear that the antigonadotropic activity of C-6 was dose-dependent and more potent than TP (59). Others showed that testosterone and DHT inhibit LH secretion in response to LHRH stimulation in rats (166) in cultures of rat anterior pituitary fragments (167) and anterior pituitary cells (168). Studies using 5α-reductase inhibitors revealed that the conversion of testosterone to DHT is not critical to the antigonadotropic activity of testosterone. This conclusion is further supported by our current studies since C-6 does not undergo 5α-reduction.

It is well known that exogenous testosterone interferes with spermatogenesis indirectly by decreasing pituitary gonadotropin secretion via negative feedback on the hypothalamus-pituitary-testis axis in different species (169-172). However, the effects of testosterone on spermatogenesis were biphasic depending on the dosage of androgen administered (173, 174). At low doses, androgen suppresses gonadotropin secretion, decreases testicular steroidogenesis, and then subsequently inhibits spermatogenesis. At higher androgen doses, endogenous testosterone levels remain suppressed but the exogenous androgen is sufficient to directly supports spermatogenesis. In our pilot antireproductive study, for the first time, we demonstrated that the administration of a nonsteroidal androgen receptor modulator (C-6) to adult male rats resulted in marked suppression of spermatogenesis. C-6-treatment reduced peripheral testosterone concentrations to undetectable levels, significantly suppressed spermatogenesis, reduced the size of testis and epididymis, and increased the weight of levator ani muscle. The magnitude of antireproductive effects of C-6 could, like testosterone, be dose-dependent. Only one dose rate was examined in this pilot study. Although the androgenic activity of C-6 was much lower than testosterone and its esters, this dose of C-6 may still be sufficient to partially maintain spermatogenesis in rats. To date, our knowledge about the role of SARMs on the reproductive system is limited. Further dose-response relationships of SARMs on spermatogenesis, fertility, and libido are needed to understand and fully develop their potential for use in hormonal male contraception.

In conclusion, C-6 is a novel SARM that was identified during studies to define structure-activity relationships for AR binding. C-6 bound the AR with high affinity and
stimulated AR-mediated transcriptional activation to a similar extent as DHT. \textit{In vivo} studies showed that \textbf{C-6} demonstrated tissue-selective pharmacologic activity, with more potent anabolic than androgenic activity. The high oral bioavailability and potent ability of \textbf{C-6} to suppress gonadotropins, testosterone, and spermatogenesis suggest that \textbf{C-6} is a promising candidate for hormonal male contraception either alone or in combination with a progestin.
Figure 5.1 Chemical Structures of Bicalutamide, S-1, and C-6.
Figure 5. 2 Competitive Displacement Curves for DHT and C-6.

Various concentrations of DHT or C-6 were incubated with $^3$H-MIB (1 nM) for 18 hr as described in the Material and Methods section. Each data point represents the mean ± S.D. of triplicate measurements, expressed as a percentage of specific binding. The IC$_{50}$ was estimated using nonlinear regression and the inhibitory effect sigmoid $E_{max}$ model and defined as the ligand concentration that caused 50% reduction of the binding of $^3$H-MIB.
Figure 5. 3 AR-Mediated Transcriptional Activity Induced by DHT and C-6.

CV-1 cells were transfected with a human AR plasmid, an androgen-responsive luciferase reporter plasmid, and a constitutively expressed β-galactosidase plasmid in a T-125 flask using LipofectAMINE. After transfection, cells were plated onto 24-well plates and allowed to recover for 12 h before drug treatment. Cells were then treated with vehicle or increasing concentrations of C-6 alone or together with 1 nM of DHT for 24 h. Luciferase activity in each well was normalized with the β-galactosidase activity and then expressed as the percentage of that induced by 1 nM DHT. Each bar represents mean ± S.D. (n=3).
Figure 5. 4 Androgenic and Anabolic Activities of C-6 in Castrated Male Rats.

Castrated male rats received C-6 at a dose rate ranging from 0.05 to 3 mg/day by daily subcutaneous injection for 14 days. Organ weights were normalized with body weight and were expressed as the percentage of the weights in the intact control group. Each bar represents the mean ± S.D. (n=5/group). The letters “I” and “C” above each error bar represent a significant difference between the group and intact control group or castrated control group, respectively, as analyzed by single-factor ANOVA with \( p < 0.05 \).
Figure 5. 5 Dose Response Relationship of C-6 in Castrated Male Rats.

Dose-response curves, $E_{\text{max}}$, and ED$_{50}$ in this figure were obtained by nonlinear least-square regression analysis.
Figure 5. 6 Androgenic and Anabolic Activities of TP and C-6 in Male Rats.

Intact male rats received vehicle, testosterone propionate (TP), or C-6 at a dose rate of 0.5 mg/day by daily subcutaneous injection for 14 days. Additional five male rats were castrated 24 h prior to the treatment and received C-6 at the same dose rate by daily subcutaneous injection for 14 days. Organ weights were normalized with body weight, expressed as the percentage of the weights in the intact control group (100%, the reference line), and compared. Each bar represents the mean ± S.D. (n=5/group). The letter “I” above each error bar represents a significant difference between the group and intact control group as analyzed by single-factor ANOVA with $p < 0.05$. 

---

% of Control Organ Weight

0  20  40  60  80  100  120  140  160  180

TP, Intact  C-6, Intact  C-6, Castrated

Prostate

TP, Intact  C-6, Intact  C-6, Castrated

Seminal Vesicle

TP, Intact  C-6, Intact  C-6, Castrated

Levator Ani Muscle
Figure 5. 7 Effects of C-6 on Spermatogenesis in Intact Male Rats.

Adult male rats received C-6 at a dose rate of 1 mg/day by daily subcutaneous injection for 10 weeks. One testis and one epididymis from each animal were collected and weighed. Sperm contents in the testis and cauda epididymis were examined as described in the Material and Methods section.

* Significant difference between groups, as analyzed by single-factor ANOVA with $p < 0.05$. Each bar represents the mean ± S.D. (n = 6 to 8/group).
Figure 5. Mean Plasma Concentration-Time Profile of C-6 in Male Rats.

Plasma concentrations of C-6 were measured using HPLC. Data were expressed as microgram per milliliter, and each point represents the mean ± S.D. (n = 5/group).
Table 5.1 Effects of C-6 on LH and FSH in Castrated Male Rats.

Letter I and C indicate a significant difference between the group and intact control group or castrated control group, respectively, as analyzed by single-factor ANOVA with $p < 0.05$. Values are mean ± SE (n = 5/group).
Table 5.2 Effects of C-6 on Body Weight and Androgen-Dependent Organ Weights.

* Significant difference between groups, analyzed by single-factor ANOVA with $p < 0.05$. Values are mean ± SE. Figures in parentheses indicate number of animals.

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<th>Group</th>
<th>Body Weight (g)</th>
<th>Normalized Organ Weight (% of Control)</th>
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<td>Epididymis</td>
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<td>$\text{AUC}_{0\rightarrow\infty}$ (min*mg/ml)</td>
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Table 5. 3 Average Pharmacokinetic Parameters of C-6 in Male Rats.

Values are mean ± SE (n = 4 or 5/group).
CHAPTER 6

EFFECTS OF A SELECTIVE ANDROGEN RECEPTOR MODULATOR (C-6) ON MUSCULAR SEXUAL BEHAVIOR IN MALE RATS

6.1 INTRODUCTION

Masculine sexual behavior in male rats is highly dependent on hormones. Testosterone is the primary steroid hormone secreted by the testes. Administration of synthetic testosterone to castrated male rats fully restored masculine sexual behavior (175). In the brain and peripheral tissues, testosterone is extensively metabolized to estradiol and 5α-dihydrotestosterone (DHT) by aromatase and 5α-reductase, respectively (176). The role of estrogen versus DHT in mediating the activation of mating behavior in male rats is controversial. In the hypothalamic and limbic structures of adult male rats, estrogen (177, 178), DHT (179), and their specific receptors (180-182) are present. Using castrated male rats, multiple lines of evidence indicated that estrogen stimulates masculine sexual behavior in a less quantitative and qualitative manner than testosterone (183-187). Furthermore, the aromatase inhibitor, androst-1,4,6,-triene-3,17-dione (ATD), inhibits the stimulation of ejaculation by testosterone in castrated male rats by blocking the conversion of testosterone to estradiol (188). On the other hand, administration of DHT, a nonaromatizable androgen, only activates minimal levels of masculine sexual behavior in castrated male rats (189, 190). While neither estradiol benzoate (EB) nor DHT fully restore mating behavior in castrated male rats when used alone, combined administration of estrogen and DHT are as effective as testosterone in stimulating sexual behavior in rats (185, 191, 192). It has been suggested that the failure of DHT to
stimulate masculine sexual behavior in male rats is due to its unfavorable pharmacokinetic properties. To test this hypothesis, other synthetic androgens [e.g., methyltrienolone (R1881) and 7α-methyl-19-nortestosterone (MENT)] with better in vivo stability were also investigated for their effects on sexual behavior in male rats. Androgens with no estrogenic metabolites, such as R1881, fail to activate mating in castrated male rats as readily as testosterone or androgen plus estrogen (193). However, androgens with estrogenic metabolites, such as MENT, fully restore mating in castrated male rats (194). Taken together, these data suggest mating behavior in the castrated male rat requires the central effects of estrogen and peripheral effects of androgen.

Selective androgen receptor modulators (SARMs) discovered in our laboratories demonstrate no estrogenic activity with high androgen receptor specificity. An ideal SARM for androgen replacement therapy would mimic the pharmacologic activity of testosterone and be devoid of testosterone-related side effects. For the purpose of hormonal male contraception, SARMs should mimic the negative feedback actions of testosterone on the hypothalamic-pituitary-gonadal axis to suppress gonadotropins yet support peripheral organs like muscle and bone that require androgen. However, SARMs that can completely inhibit endogenous testosterone production and spermatogenesis must also support or maintain male sexual behavior. C-6 demonstrated potent and rapid inhibition of gonadotropins and testosterone in castrated and intact male rats, respectively (65). To date, rats remain the best small animal model for hormonal male contraception. We examined the effects of C-6 on masculine sexual behavior in rats as a first step toward designing studies to understand the therapeutic potential of SARMs in hormonal male contraception and the true role of androgens in masculine sexual behavior in rats. The results of these studies unequivocally establish the critical role of androgens and estrogens in sexual behavior in rats and are reported herein.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Mature, male Sprague-Dawley rats (BW, ~300 g) and female SD rats (BW, ~200 g) were purchased from Harlan Bioproducts for Science (Indianapolis, IN). All animals were maintained on a 12h light/dark cycle with food and water available ad libitum. The
animal protocol was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

6.2.2 Chemicals

C-6 was synthesized using the methods described by Marhefka et al. (60). Chemical purity was confirmed using elemental analysis, mass spectrometry, and proton nuclear magnetic resonance. Dimethyl sulfoxide (DMSO), polyethylene glycol-300 (PEG 300), ketamine, xylazine, estrogen benzoate (EB), progesterone and acetonitrile were purchased from Sigma Chemical Co (St. Louis, MO) and ethanol was purchased from Pharmco Products (Brookfield, CT).

6.2.3 Effects of C-6 on Male Sexual Behavior in Castrated Rats

All sexual behavior tests were conducted in the early part of the dark portion of the light/dark cycle under dim red light for illumination. Each test began by placing a male into a glass observation box (25 × 47 × 29 cm). Five minutes later, a receptive female was introduced to the observation cage. During a 30 min observation period, the latency and frequency of all mounts (with palpation and pelvic thrusts), intromissions, and ejaculations were recorded. All female rats were ovariectomized under anesthesia with ketamine/xylazine (83/17 mg/kg, w/w). Sexual receptivity was induced by subcutaneous injections of EB (100 µg) at 24 h and 48 h prior to the test and progesterone 4 h prior to the test at a dose of 500 µg. The following behavioral indices were defined as: Mount Frequency (MF), number of mounts per 30-min test; Intromission Frequency (IF), number of intromissions per 30-min test; Ejaculation Frequency (EF), number of ejaculations per 30-min test; Mount Latency (ML), time from female’s introduction to first mount or 1800 sec for nonresponders; Intromission Latency (IL), time from female’s introduction to the first intromission or 1800 s for nonresponders; Ejaculation Latency (EL), time from the first intromission to the first ejaculation or 1800 s for nonresponders; Post-Ejaculation Interval (PEI), time from the first ejaculation to the first intromission of the next series or 1800 s for nonresponders.
On the day after the last behavioral test, male rats were anesthetized and sacrificed. Animals were weighted, and then osmotic pumps were removed and checked for proper delivery. Prostate, seminal vesicles, and levator ani muscle were collected, clear of extraneous fat tissues, weighed, and compared. All organ weights were expressed as a percentage of body weight for comparison between groups.

6.2.3.1 Behavior Studies in Sexually Naïve Male Rats

Sexually naïve male rats were castrated via a single scrotal incision while anesthetized with ketamine/xylazine (83/17 mg/kg, w/w). An additional five animals received a sham operation and served as intact controls. Four weeks after surgery, an osmotic pump containing vehicle (10% DMSO in PEG 300) was implanted subcutaneously at the lower back area of each animal in the intact control group. Osmotic pumps containing either C-6 or C-6 plus EB were implanted subcutaneously into animals in drug treatment groups (N = 5). The target delivery doses of C-6 and EB were 1 mg/day and 5µg/day, respectively. This dose of EB was reported to be insufficient to fully restore masculine sexual behavior in male rats (191, 192, 195). However, it was an effective dose when combined with DHT at a dose of 1 mg/day. The sexual behavior of each animal was tested one day prior to and at 7-day intervals after implantation of the osmotic pumps. Results from five tests were reported.

6.2.3.2 Behavior Studies in Sexually Experienced Male Rats

The sexual behavior of sexually experienced male rats treated with C-6 was also examined. Male animals included in these studies had ejaculated in three preliminary screening tests (1, 5, and 9 days prior to castration). An osmotic pump containing C-6 at a target dose rate of 1 mg/day was implanted into five animals immediately after castration. An additional five animals served as an intact control group and received sham operation and osmotic pumps containing only vehicle (10% DMSO in PEG 300). The sexual behavior of each animal was tested seven days after implantation of the osmotic pump and at 4-day intervals thereafter for the next 27 days.
6.2.4 Statistical Analysis

All statistical analyses were performed using single-factor ANOVA with the α value set a priori at $p < 0.05$.

6.3 RESULTS

6.3.1 Effects of C-6 in Sexually Naïve Rats

None of the animals showed any sexual activity during the pretreatment test (Figures 6.1 to 6.3). As expected on the basis of numerous previous studies (191), mating behavior of males was greatly reduced in castrated male rats four weeks castration. In the current study, sexually naive males from the intact control group also showed low mounts, intromissions, and ejaculations on the first two tests. Animals in the intact control group and the C-6 + EB group demonstrated progressively increased measures of mating behavior over a five-week period: mounts Figure 6.1-A) increased first, then intromissions (Figure 6.2-A), and then ejaculations (Figure 6.3-A). Treatment with C-6 alone had no effect on mounting, intromissions, or ejaculations as compared to castrated control group. By contrast, mounting and intromission rates of animals treated with C-6 + EB were significantly higher than the castrated control group at all time points, beginning with the second week of treatment. Compared with the intact control group, C-6 + EB-treated animals displayed lower mounting and intromission rates and longer ML (Figure 6.1-B) and IL (Figure 6.2-B) during the tests conducted before treatment and after one week of treatment. However, by the second week of treatment, animals in the C-6 + EB group demonstrated significantly higher mounting and intromission rates and significantly shorter post-ejaculation interval (Figure 6.3-C) than those of intact control animals. There were no significant differences in sexual behavior between the intact control group and C-6 + EB group during the last two weeks of the study (i.e., weeks 3 and 4). It is well known that mount and intromission rates and latencies are common measures of sexual motivation, whereas the ability to ejaculate is a common measure of
sexual performance in the male rats (196). These studies suggest that treatment with \textbf{C-6} + EB stimulates mating behavior in castrated male rats to a similar but slower extent as compared to intact controls. Since \textbf{C-6} alone failed to stimulate any mating behavior, the increased sexual motivation observed in animals treated with \textbf{C-6} + EB by the second week post-treatment was due to EB or the synergistic effects of androgen and estrogen.

At the conclusion of the behavioral studies with sexually naïve rats, we sacrificed the animals and determined their body weight and the weight of androgen-responsive tissues. As shown in Figure 6.4, animals treated with \textbf{C-6} + EB demonstrated significantly lower body weights than animals in the other treatment groups, suggesting that estrogen affected the growth of these animals. After long-term castration (60 days), the weights of the prostate, seminal vesicles, and levator ani muscle in castrated animals treated with vehicle alone decreased to 3.4\%, 7.2\%, and 42.7\% of those observed in intact animals. Weights of the prostate, seminal vesicles, and levator ani muscle were increased to 24.6\%, 57.1\%, and 111\% of the intact values after \textbf{C-6} treatment. Combination treatment with \textbf{C-6} + EB increased the weights of the prostate, seminal vesicles, and levator ani muscle to 39.5\%, 69.8\%, and 127\% of those observed in intact animals. Although the increase of organ weights was slightly greater in \textbf{C-6} + EB-treated groups than \textbf{C-6} alone group, it was not statistically different. This difference was mainly due to the significant lower body weight of animals that received \textbf{C-6} + EB since all organ weights were normalized by the body weight. These results were consistent with the androgenic and anabolic activity of \textbf{C-6} that we observed during pharmacologic studies. The small (i.e., 5 \textmu g/day) dose of EB significantly decreased the body weight without interfering with the pharmacologic activity and tissue selectivity of \textbf{C-6}.

6.3.2 Effects of \textbf{C-6} in Sexually Experienced Rats

The studies described above show that sexually naïve intact rats require several experiences with receptive females before demonstrating full mating behavior. Therefore, we later examined the effects of \textbf{C-6} on mating behavior in sexually experienced male rats after castration. All animals used in this study ejaculated in at least one of the mating tests conducted 1, 5, and 9 days prior to castration. After castration, all parameters of
mating behavior were progressively decreased in the vehicle control and \textit{C-6}-treated groups, presumably due to the lack of endogenous testosterone. Figure 6.5 shows the percentage of castrated animals that displayed mounting, intromission, and ejaculation after treatment with vehicle or \textit{C-6}. In both groups, ejaculations declined first, followed by intromissions, and then mounts. On the last two tests, animals in both groups demonstrated a high rate of mounts. Only one animal in the vehicle control group exhibited intromissions, while 3 to 4 animals in the \textit{C-6}-treated group showed intromissions. Animals in the vehicle control group failed to ejaculate on the last two tests. Although the number of ejaculations also declined in animals that received \textit{C-6}, at least two animals in the \textit{C-6}-treated group (\(N = 5\)) ejaculated. These results clearly demonstrated that \textit{C-6} can partially maintain mating behavior in sexually experienced animals when treatment was initiated immediately after castration.

No difference in body weight was observed between the two groups at the end of treatment. The pharmacologic activity of \textit{C-6} in androgen-dependent organs was similar to that observed in previous studies (data not shown).

\section*{6.4 DISCUSSION}

There has been a long debate over the ability of estrogen, either alone or in combination with DHT, to restore mating behavior in castrated male rats (183, 191, 192, 195, 197-199). The dose of EB used in these studies (5 \(\mu\)g/day) is insufficient to restore mating behavior alone but is known to be effective when given in combination with DHT (183, 191, 192). In sexually naïve rats, the only treatment to produce an equivalent mating behavior to that observed in the intact controls was \textbf{C-6} (1 mg/day) + EB (5 \(\mu\)g/day). Neither castrated controls nor \textbf{C-6}-treated animals displayed measurable mating behavior during one pretreatment test and four tests conducted during drug treatment. Thus, \textbf{C-6} failed to stimulate mating behavior in long-term castrated male rats, which is in agreement with reports using non-aromatizable androgens, such as DHT (189, 190) and R1881 (193). Due to its androgen receptor specificity, high dose rate (1 mg/day) used, and relatively low total body clearance of \textbf{C-6} in rats (65), it seems unlikely that the
inability of **C-6** to stimulate mating behavior was the result of insufficient levels of androgen acting on target androgen receptors. When **C-6** was administrated with 5 µg/day of EB, animals exhibited significantly greater sexual motivation by the second week of treatment and then reached a similar pattern of mating behavior as that observed in the intact control by the third week of treatment. Previous pharmacologic studies showed that it takes about two weeks for androgen-dependent organs to reach their minimal sizes after castration. In turn, it appears to take a similar amount of time to fully recover androgen activity in target organs, which may explain the observed transiently increase in sexual motivation (mounting and intromission) but delays in sexual performance (ejaculation) in sexually naïve animals treated with **C-6** + EB. We recorded the weights of the prostate, seminal vesicles, and levator ani muscle at the end of behavior studies. Both **C-6** alone and **C-6** + EB treatments significantly increased muscle weights to sizes greater than those of intact control, indicating that **C-6** exerted potent effects in the stimulation of the genitals. Clearly, combination treatment with estrogen and androgen restored sexual motivation and organ weights in peripheral organs.

To date, the mechanisms by which EB alone or EB in the combination with DHT stimulate mating behavior are not well understood. One possibility is that estrogen maintains the normal basal level of extracellular dopamine, an important neurotransmitter for sexual behavior, in the medial preoptic area (MPOA) of the brain at a level that sufficient for suboptimal copulation (200-202). In the presence of androgen and estrogen, castrated animals have normal basal dopamine levels and exhibit female-induced increases in dopamine to the same extent as testosterone-treated animals (200). Several characteristics of **C-6** make it more advantageous for behavioral studies, including its lack of cross-reactivity with other nuclear receptors, unique tissue selectivity, and high androgen receptor binding affinity. Studies using intracranial implantation of **C-6** might provide important insight to the mechanisms by which androgen and estrogen regulate sexual behavior, and should be seriously considered as a component of future studies.

Butera and Czaja reported that DHT failed to maintain copulatory behavior in castrated male rats, in spite of the fact that it fully maintained peripheral structures (203). Consistently, our studies show that **C-6**, a potent SARM, failed to induce mating behavior in sexually naïve rats. However, it partially maintained mating behavior in
sexually experienced male rats. For example, C-6 completely maintained mounting and partially maintained intromission and ejaculation in sexually experienced male rats. It remains unknown whether this partial maintenance was due to the delayed disappearance of mating behavior or a true suboptimal status. However, the inability of castrated males to ejaculate and suggests that the study was conducted for a sufficient duration. Studies of greater duration and that include intact and EB-treated groups will be necessary in the future to unequivocally answer this question.

It is well known that the relative contributions of androgen and estrogen to the activation of masculine sexual behavior differ among mammalian species. For instance, estrogen shows potent activation of mating behavior in multiple castrated animal species, including rats (184, 188, 197), hamsters (204), pigs (205), deer (206), sheep (207), and cows (208), but not in castrated guinea pigs (197) or rhesus monkeys (209). By contrast, DHT failed to stimulate any mating behavior in castrated male rats. However, it was quite effective in guinea-pigs (210), mice (211), rabbits (212), and rhesus monkeys (213, 214), but not in cynomolgus monkey (215) or stumptailed monkeys (216). Therefore, the effects of androgen and estrogen on sexual behavior in human can not be predicted by animal studies. Furthermore, sexual libido in humans is highly regulated by multiple social factors and much less dependent on hormones than other animal species.

The purpose of these studies was to define the appropriate treatment regimens prior to the design and conduct of a study to examine the feasibility of using a SARM for hormonal male contraception. Due to the fact that no male antifertility compounds have yet been developed, there is a lack of knowledge about which species or test systems best extrapolate to humans. Since all the compounds that have been reported to have male antifertility activity are active in the rat, it remains the primary small animal model for male contraception studies. Lack of sexual libido or appropriate mating behavior would confound the results of contraceptive efficacy studies (i.e., mating is a prerequisite for pregnancy). These studies with C-6 alone and in combination with EB demonstrate: a) both androgen and estrogen are required to fully maintain masculine sexual behavior in castrated male rats; b) immediate SARM treatment can partly maintain or support sexual behavior in castrated male rats; c) contraception studies of SARMs in rats should include EB to maintain sexual libido.
Figure 6. Changes in Mounting Behavior as a Function of Time and Drug Treatment.

The number of mounts (Panel A) and mount latency (Panel B) were determined prior to treatment (P1) and at weekly intervals for up to four weeks after implantation of an osmotic pump containing vehicle (circles), C-6 (closed triangles), or C-6 + EB (open triangles). ML of animals in castrated and C-6 alone groups was not analyzed because too few animals performed the behavior. Each value represents the mean ± s.d. of four or five rats. * indicates a significant difference from intact control group as tested by single-factor ANOVA (p < 0.05).
Figure 6. 2 Changes in Intromission Behavior as a Function of Time and Drug Treatment.

The number of intromissions (Panel A) and intromission latency (Panel B) were determined prior to treatment (P1) and at weekly intervals for up to four weeks after implantation of an osmotic pump containing vehicle (circles), C-6 (closed triangles), or C-6 + EB (open triangles). IL of animals in castrated and C-6 alone groups was not analyzed because too few animals performed the behavior. Each value represents the mean ± s.d. of four or five rats. * indicates a significant difference from intact control group as tested by single-factor ANOVA (p < 0.05).
Figure 6. 3 Changes in Ejaculation Behavior as a Function of Time and Drug Treatment

The number of ejaculations (Panel A), ejaculation latency (Panel B), and post-ejaculation latency (Panel C) were determined prior to treatment (P1) and at weekly intervals for up to four weeks after implantation of an osmotic pump containing vehicle (circles), C-6 (closed triangles), or C-6 + EB (open triangles). EL of animals in castrated and C-6 alone groups was not analyzed because too few animals performed the behavior. Each value represents the mean ± s.d. of five rats. * indicates a significant difference from intact control group as tested by single-factor ANOVA (p < 0.05).
Figure 6. 4 Body Weights and Normalized Organs Weights of Animals in Sexually Naïve Male Rats.

The organ weights were normalized with body weight and were expressed as the percentage of the weights in the intact control group. Each bar represents the mean ± S.D. (n=5/group). The letters “I” and “C” above each error bar represent a significant difference between the group and intact control group or castrated control group, respectively, as analyzed by single-factor ANOVA with $p < 0.05$. 
Figure 6.5 The Percentage of Castrated Male Rats Displayed Mounting (Panel A), Intromission (Panel B), and Ejaculation (Panel C).

Three mating tests were performed at 9, 5, and 1 day prior to castration. The dotted vertical line indicates the day of castration. Six mating tests were performed at 7, 11, 15, 19, 23, and 27 days after osmotic pump implantation.
CHAPTER 7

IN VITRO AND IN VIVO CHARACTERIZATION OF A SELECTIVE ANDROGEN RECEPTOR MODULATOR (SARM), C-31, FOR HORMONAL MALE CONTRACEPTION

7.1 INTRODUCTION

The negative feedback loop between testosterone synthesized in the testis and gonadotropins released from the pituitary was first discovered in 1932 (217). In the following years, numerous studies proved the ability of androgens and anabolic steroids to inhibit endogenous testosterone synthesis (218-220). Although the detailed mechanism regarding hormonal regulation of spermatogenesis is still not fully understood, it is widely employed as the main principle of hormonal male contraception. Much higher testosterone concentrations are required to maintain normal spermatogenesis in the testis than are needed in the systemic circulation to maintain muscle mass, bone structure, and male secondary characteristics. The goal of hormonal male contraception is to suppress luteinizing hormone (LH) and follicle stimulating hormone (FSH), resulting in a depletion of intratesticular testosterone and suppression of spermatogenesis. However, depletion of endogenous androgen production results in a physiologic need for androgen replacement (221). The most clinically advanced approaches for male hormonal contraception use testosterone alone (76, 77) or in the combination of progesterone or gonadotropin-releasing hormone (GnRH) analogs.

In previous studies, we identified two potent selective androgen receptor modulators (SARMs), S-1 and S-4 (59), with potential use for androgen-related diseases, including muscle wasting, osteoporosis, and benign prostate hyperplasia (BPH) in animal models (64). However, these SARMs showed low or no effects in the central nervous
system (CNS), which precluded their usage as a single regimen for hormonal male contraception. More recently, we solved the x-ray crystal structure of the androgen receptor (AR) ligand-binding domain bound to bicalutamide and our nonsteroidal SARMs and proved that these nonsteroidal ligands bind in a unique subpocket within the AR that is not shared by steroidal AR ligands. The aromatic B-ring of S-1 in the wide type AR is accommodated in close proximity to the indole ring of Trp-741 of the AR. Multiple electron-withdrawing groups were introduced to the B-ring of our lead compound-S-1. Such structural modifications not only maintained high AR binding affinity and \textit{in vitro} functional activity, but also improved the \textit{in vivo} pharmacologic activity of these compounds in castrated male rats. For example, when an additional fluoro group was introduced to the meta position in the B-ring of S-1, this structural modification led to a novel SARM (C-3), which was more efficacious (Emax) than S-1 in maintaining the weights of prostate (59% vs 15%) and levator ani muscle (132% vs 75%) in castrated animals. This increased efficacy was not due to improved AR binding affinity or pharmacokinetics of C-3, but was through increased functional activity. \textit{In vivo} metabolism studies revealed that the major metabolites of C-3 arose from the step-wise reaction of the nitro group in the A-ring of C-3. Although structure-activity relationship studies showed that changing the nitro group to a cyano group slightly decreases AR binding affinity and \textit{in vitro} functional activity, changing the nitro group of C-3 to a cyano group should make the pharmacokinetic properties of this compound more desirable without compromising significant functional activity.

Another novel SARM (C-6), which bears a \textit{para}-nitro group in the A-ring, a \textit{para}-chloro group and a \textit{meta}-fluoro group in the B-ring, was identified as the first SARM with the ability to regulate gonadotropin levels and testosterone concentrations in castrated and intact male rats, respectively (65). Pharmacokinetic studies showed that C-6 is orally bioavailable (76%) with a fairly long terminal half-life (6.3 h) at a dose of 10 mg/kg in male rats. Additional pharmacologic studies of C-6 demonstrated its profound suppressive effects on spermatogenesis in intact male rats after 70-days of treatment at 1 mg/day by s.c. daily injections. We hypothesized that a new molecule with the B-ring of C-6 and a cyano-group in the A-ring (C-31) might be a more promising candidate for
hormonal male contraception, due to its structural properties that favor high intrinsic pharmacologic activity and improved pharmacokinetics.

In the current studies, the \textit{in vitro} and \textit{in vivo} characteristics of \textbf{C-31} were examined, including \textit{in vitro} AR binding affinity, AR-mediated transcriptional activation, and pharmacologic activity in castrated male rats. The pharmacologic activity of \textbf{C-31} for hormonal male contraception was further investigated in male rats. To maintain libido, estrogen benzoate (EB) at a dose rate of 5\(\mu\)g/day was combined with \textbf{C-31} at doses ranging from 0.05 to 0.75 mg/day by s.c. daily injections for 70 days. The contraceptive properties of \textbf{C-31} were evaluated by examining the ability of this SARM to suppress spermatogenesis and reduce pregnancy rates during mating trials. The effects of \textbf{C-31} on body weight, androgen-dependent organ weights, body composition, and hormonal regulations were also reported. These studies are the first to fully demonstrate the ability of SARMs to completely suppress spermatogenesis, maintain peripheral androgenic tissues, and serve as a major step forward development of effective oral male contraception.

\section*{7.2 MATERIALS AND METHODS}
\subsection*{7.2.1 Animals}
Mature male Sprague-Dawley rats (BW, \(~300\) g) and female Sprague-Dawley rats (BW, \(~200\) g) were purchased from Harlan Bioproducts for Science (Indianapolis, IN). All animals were maintained on a 12h light/dark cycle with food and water available \textit{ad libitum}. The animal protocol was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

\subsection*{7.2.2 Chemicals}
\textbf{C-31} was synthesized using the methods described by Marhefka \textit{et al.} (60). Chemical purity was confirmed using elemental analysis, mass spectrometry, and proton nuclear magnetic resonance. Unlabeled mibolerone (MIB) and 17\(\alpha\)-methyl-\textsuperscript{3}H Mibolerone (\textsuperscript{3}H-MIB, 84 Ci/mmol) were purchased from PerkinElmer Life Sciences
(Boston, MA). Ethanol was purchased from Pharmco Products (Brookfield, CT). All other chemicals used in current studies were purchased from Sigma Chemical Co (St. Louis, MO).

7.2.3 \textit{In Vitro} AR Binding Affinity and Transcriptional Activation of \textbf{C-31}

Cytosolic AR was prepared from the ventral prostates of male Sprague-Dawley rats 24 h after castration. The AR binding affinity of \textbf{C-31} was determined using the same radio-labeled competitive binding assay as described in the Method Section of Chapter 2. The \textit{in vitro} functional activity of \textbf{C-31} was determined and characterized using a co-transfection assay previously described in the Method Section of Chapter 2.

7.2.4 \textit{In Vivo} Pharmacologic Activity of \textbf{C-31} in Castrated Male Rats

The \textit{in vivo} pharmacologic activity of \textbf{C-31} was examined in five male Sprague-Dawley rats weighing approximately 200 g. Animals were castrated via a scrotal incision under anesthesia 24 h prior to drug treatments and received daily subcutaneous injections of \textbf{C-31} at a dose rate of 0.01, 0.05, 0.1, 0.5, 1, and 3 mg/day for 14 days. \textbf{C-31} was freshly dissolved in vehicle containing DMSO (5%, v/v) in PEG 300 immediately before dose administration. An additional two groups of animals with or without castration received vehicle only and served as castrated or intact control groups, respectively. Animals were sacrificed at the end of the treatment. Plasma samples were collected and stored at –80 °C until use. The ventral prostate, seminal vesicles, and levator ani muscle were removed, cleared of extraneous tissue, and weighed. All organ weights were normalized to body weight and compared. The weights of prostate and seminal vesicles were used to evaluate androgenic activity, while the levator ani muscle weight was used as a measurement of anabolic activity. Non-linear regression analysis was performed to obtain the maximal response (Emax) induced by \textbf{C-31} and the dose rate of \textbf{C-31} that induced 50% of that maximal response (ED$_{50}$) using the sigmoid $E_{\text{max}}$ model with a baseline effect parameter and WinNonlin software (Pharsight Corporation, Mountain View, CA). No upper or lower boundary was used for this modeling.
7.2.5  *In Vivo* Pharmacologic Activity of C-31 in Castrated Male Rats

7.2.5.1  Animals and Treatment

Forty-two male Sprague Dawley rats (90 days old) were randomly assigned to seven groups \((N = 6/\text{group})\). Group 1 was the intact control group and received vehicle \((5\% \text{ DMSO} + 95\% \text{ PEG 300})\). Group 2 was the estradiol benzoate (EB) alone group and received EB at a dose of 5 \(\mu\text{g/day}\). Animals in groups 3 to 7 were treated with C-31 at dose rates of 0.05, 0.1, 0.3, 0.5, and 0.75 mg/day, respectively, in combination with EB (5 \(\mu\text{g/day}\)). Compounds were freshly prepared immediately before dosing and delivered to animals via daily s.c. injections \((100 \mu\text{L/day})\) for 70 days. Mating trials were conducted during the last week of treatment for animals in groups 1, 2, 4, and 5. On day 69, the total body composition of each animal was determined using dual energy x-ray absorptiometry \((\text{DEXA})\) \((\text{GE, Lunar Prodigy™})\).

7.2.5.2  Tissue and Blood Sample Collection and Preparation

At the end of treatment, animals were sacrificed under anesthesia. Body weight was recorded at autopsy. Prostate, seminal vesicles, right testis, and right epididymis (divided in the middle of corpus epididymis to caput and cauda epididymis) were collected and weighed. All organ weights were normalized with body weights and compared. Blood samples were collected in plastic tubes, stored at room temperature for 40 min, and centrifuged at 3,000 g for 10 min to prepare serum samples. Serum samples were aliquoted and stored at -80°C until hormonal assays. One testis from each rat was removed and weighed. After decapsulation, testicular parenchyma was homogenized in a volume of PBS \((\text{pH 7.4})\) that was equivalent to its weight. An aliquot of each testicular homogenate was used to count advanced spermatids \((\text{step 17-19})\) in the testis with a hemacytometer. Results were expressed as the number of spermatids per mL. The remaining testicular homogenates was centrifuged at 3,000 g for 10 min. The supernatant of each sample was collected and used to determine the intratesticular testosterone concentrations. The other testes were carefully dissected and fixed in Bouin’s Solution for 24 h. Tissue samples were washed repeatedly with lithium-saturated 70% ethanol.
until completely decolored. The testes were cut into small (~ 0.2-cm) transverse slices and preserved in 70% ethanol. One slice from the middle region of the testis was processed by routine paraffin embedding for histological analysis of spermatogenesis and morphology.

7.2.5.3 Hormone Assays

Serum levels of LH and FSH and intratesticular testosterone concentrations were measured by the University of Virginia, Center for Research in Reproduction (Charlottesville, VA) using RIA kits. The sensitivity of LH, FSH, and intratesticular testosterone RIA kits were 0.040, 1.0, and 0.025 ng/mL, respectively. Testosterone concentrations in serum were measured with a commercially available EIA kit (DSL, Webster, TX). The detection limit of this EIA kit is 7.5 pg/mL.

7.2.5.4 Whole Body DEXA Analyses

One day prior to the end of treatment, body weight (BW), total body bone mineral density (BMD), percent fat mass (FM), bone mineral content (BMC), bone mineral area (BMA), and lean mass (LM) were determined by dual energy x-ray absorptiometry (DEXA) (GE, Lunar Prodigy™) using the small animal software (Lunar enCORE, version 6.60.041). For scanning, the animals were anesthetized with ketamine: xylazine (87:13 mg/kg) and positioned in a prone position as demonstrated in Figure 7.1. The total body data was obtained by selecting an area encompassing the entire animal as the region of interest during data processing.

7.2.5.5 Mating Trials

During the last week of treatment, each male rat from groups 1, 2, 4, and 5 was housed with two sexually active female rats. Female rats were individually housed after mating trials with free access to water and standard food chow. The female rats were allowed to bring the pregnancy to term. Upon delivery, numbers of the mother rats and litters were counted. A male rat was defined as fertile if it impregnated either one or two female partners.
7.2.6 Pharmacokinetics of **C-31** in Rats

7.2.6.1 Animals and Blood Sample Collection and Preparation

The pharmacokinetics of **C-31** were examined in male Sprague-Dawley rats weighing approximately 250 g after a dose of 10 mg/kg by intravenous injection or oral gavage. A catheter was implanted in the right external jugular vein of each animal 24 h prior to drug administration. **C-31** was dissolved in a vehicle containing DMSO (10%, v/v) in PEG 300 and administered to animals through the jugular vein catheter (i.v. group) or oral gavage (p.o. group). For animals in the i.v. group, the catheter was flushed three times with saline (3× the volume of the dosing solution) before blood sampling. Blood samples (~ 200 µL) were then withdrawn through the jugular vein catheter at 5, 10, 30, 60, 90, 120, 180, 360, 720, 1440, and 1800 min after the i.v. dose and at 20, 30, 60, 90, 120, 240, 360, 480, 720, 1080, 1440, and 1800 min after the p.o. dose. Blood samples were immediately centrifuged at 1,000 g, 4°C for 10 min. Plasma samples were prepared and stored at -20°C until HPLC analysis.

7.2.6.2 HPLC Method

An aliquot (100 µL) of each plasma sample from the pharmacokinetic studies was spiked with 10 µL of an internal standard (**C-3**) and mixed well with 1 mL of acetonitrile. After centrifugation at 16,000 g, 4°C for 10 min, the supernatant was collected and evaporated. The residues were reconstituted in 120 µL of mobile phase. An aliquot of each sample was injected into a Nova-pak C18 column (3.9 × 150 mm, 4 µm particle size) purchased from Waters Corporation (Milford, MA). The HPLC system consisted of a model 515 HPLC pump (Waters), a model 717 plus autosampler (Waters), and a model 486 absorbance detector (Waters). HPLC separation was performed using an isocratic mobile phase (H2O/Acetonitrile: 46/54, v/v) at a flow rate of 1 mL/min. The UV absorbance of eluents was monitored at 298nm. Calibration standards were prepared in blank rat plasma with **C-31** concentrations ranging from 0.1 ~ 50 µg/mL. The recoveries of this compound over the calibration range were from 90.1% to 105.5%. The intra- and
inter-day coefficients of variation of the assay were lower than 3.6% and 7.0%, respectively. The limit of quantitation of the HPLC assay was 0.1 µg/mL.

7.2.6.3 Pharmacokinetic Data Analyses

The plasma concentration-time data were analyzed using noncompartmental methods and WinNonlin software (Pharsight, Mountain View, CA). The terminal half-life ($T_{1/2}$) was calculated as $T_{1/2} = 0.693/\lambda$, where $\lambda$ was the rate constant describing the terminal phase decline in C-31 plasma concentrations. The area under the plasma concentration-time curve ($AUC_{0-\infty}$) was calculated using the trapezoidal method with extrapolation to time infinity. The plasma clearance (CL) was calculated as $CL = \text{Dose}_{i.v.}/AUC_{0-\infty,i.v.}$, where the Dose$_{i.v.}$ and AUC$_{0-\infty,i.v.}$ were the intravascular dose and the corresponding area under the plasma concentration-time curve from time 0 to infinity, respectively. The apparent volume of distribution at equilibrium ($V_{dss}$) was calculated as $V_{dss} = CL \cdot \text{MRT}$, where the MRT was the mean residence time following the intravenous bolus dose. The peak plasma concentration ($C_{max}$) and time to reach the peak concentration ($T_{max}$) after a p.o. dose were obtained directly from the plasma concentration-time curves. Oral bioavailability ($F_{p,o.}$) was defined as $F_{p,o.} = (AUC_{0-\infty,p.o.} \cdot \text{Dose}_{p,o.})/(AUC_{0-\infty,i.v.} \cdot \text{Dose}_{i.v.})$, where the Dose$_{p,o.}$ and AUC$_{0-\infty,p.o.}$ were the oral dose and the corresponding area under the plasma concentration-time curve from time 0 to infinity after p.o. administration, respectively.

7.2.7 Statistical Analyses

All statistical analyses were performed using single-factor ANOVA with the $\alpha$ value set a priori at $p < 0.05$. 
7.3 Results

7.3.1 In Vitro AR Binding Affinity and Transcriptional Activation of C-31

The AR binding affinity of AR ligands was determined using a competitive binding assay. DHT was used as the positive control in each assay and the Ki value of DHT was 0.45 ± 0.2 nM. Figure 7.2 shows the chemical structures and in vitro pharmacology of S-1, C-6, and C-31. The introduction of two halogen groups in the aromatic B-ring maintained or increased AR binding affinity. Both C-6 and C-31 showed higher binding affinity than S-1. C-31 was structurally modified from C-6 by changing the para-nitro group of C-6 to a cyano group, which resulted about 2-fold higher binding affinity than that of C-6. However, previously discussed structure-activity relationships (SARs) based on five pairs of compounds suggested that cyano-substituted compounds exhibited lower AR binding affinity than their corresponding nitro-substituted counterparts. This inconsistency indicated that this known SAR might be substituent dependent.

The ability of C-31 to induce AR-mediated transcriptional activation was determined using a co-transfection assay in CV-1 cells. Upon binding to the AR, C-31 induced a concentration-dependent increase in AR-mediated transcriptional activation. Data listed in Figure 7.2 was the transcriptional activity induced by each compound at a final concentration of 10 nM and is reported as the percentage of activity observed for 1 nM of DHT. Compared with our lead compound (S-1), compounds with di-halogen substituents in the B-ring (C-6 and C-31) exhibited significantly increased in vitro functional activity. Although the transcriptional activation induced by C-6 was higher than that of C-31, the difference was not statistically significant.

7.3.2 In Vivo Pharmacologic Activity of C-31 in Castrated Male Rats

The in vivo pharmacologic activity of C-31 was further evaluated in castrated male rats after 14-day treatment. Previous pharmacologic studies of C-6 revealed that the maximum effects of C-6 in prostate and seminal vesicles were about 130% of those observed in intact controls at a dose of 3 mg/day. The highest dose rate of C-31 used in
the current study was also 3 mg/day. The ED$_{50}$ values of C-6 in prostate, seminal vesicles, and levator ani muscle were 0.78 ± 0.06, 0.88 ± 0.1, and 0.17 ± 0.04 mg/day, respectively. According to the rationale of the structural modification, we expected to see higher potency in animals treated with C-31 than C-6. Therefore, the lowest dose rate of C-31 used in this study was 0.01 mg/day. Figure 7.3 shows the androgenic activity (prostate and seminal vesicles) and anabolic activity (levator ani muscle) of C-31 in castrated male rats at doses ranging from 0.01 mg/day to 3 mg/day. After castration, the weight of prostate, seminal vesicles, and levator ani muscle dramatically decreased to 5.7%, 6.5%, and 34% of control values. Administration of C-31 to castrated animals led to dose-dependent increases in androgen-dependent organ weights. The maximum effect (Emax) of C-31 in the prostate, seminal vesicles, and levator ani muscle was 138 ± 21%, 144 ± 1%, and 129 ± 4% of those observed in intact controls, respectively. Accordingly, the ED$_{50}$ of C-31, calculated with non-linear regression analysis of dose-response relationship, in the prostate, seminal vesicles, and levator ani muscle was 0.43 ± 0.18, 0.41 ± 0.0070, and 0.079 ± 0.010 mg/day, respectively (Figure 7.4). As compared to C-6, C-31 demonstrated 2-fold higher potency (ED$_{50}$) in all three organs without significantly increased efficacy (Emax) or change in androgenic/anabolic activity in vivo. Considering the in vitro characteristics of C-31, this increased in vivo potency was possibly due to its higher AR binding affinity than C-6. Another possibility was the improved in vivo drug exposure after administration, which would be verified by the results of pharmacokinetic studies.

7.3.3 In Vivo Antireproductive Activity of C-31 in Male Rats

After identification of C-31 as a more potent SARM than C-6, the in vivo antireproductive activity of this compound was immediately investigated in intact male rats. Previous studies of the effects of C-6 on masculine mating behavior showed that C-6 alone partially maintained mating behavior in sexually experienced rats, but failed to stimulate any mating behavior in sexually naïve male rats after long-term castration. A small amount of estrogen in combination with SARM is sufficient and effective to fully restore mating behavior in long-term castrated male rats. To examine the efficacy of male
contraception, mating trials were included at end of the treatment. Maintenance of libido plays a key role in mating and reproductive studies. Animals in group 1 served as vehicle controls. Animals in groups 2 through 7 were treated with EB at a dose of 5 µg/day in combination with C-31 at a dose of 0, 0.05, 0.1, 0.3, 0.5, or 0.75 mg/day, respectively (Table 7.1). The doses of C-31 were chosen based on the pharmacologic activity of this compound in castrated male rats, and covered the whole range of the observed therapeutic window. At the highest dose rate tested herein (0.75 mg/day), C-31 maintained the weight of the prostate and seminal vesicles to values similar to those observed in intact animals. Notably, at the dose rate of 0.1 mg/day, C-31 selectively maintained the weight of levator ani muscle at the intact control level, while its effects on the prostate and seminal vesicles were lower than 30% of those observed in intact controls.

7.3.3.1 Whole Body DEXA Analyses

Figure 7.5 shows the body weight of animals measured after 69 days of treatment. Body weight in the EB-treated group was significantly less as compared to the vehicle control, indicating the effects of estrogen in male body growth. In the presence of EB, C-31 significantly increased body weight at the lowest doses (i.e., 0.05 mg/day), but further decreased body weight at intermediate doses (i.e., 0.5 and 0.75 mg/day). The ‘bell-shaped’ change in body weight most likely reflected the mixed effect of hormones on body composition, including estrogen, endogenous testosterone, and SARM. However, the mean body weight of all treatment groups was significantly less than that of intact controls. Subsequent comparisons of body composition and organ weights were therefore normalized with body weight. Besides the body weight, body composition was also analyzed using DEXA. As shown in Figure 7.6, the whole body bone mineral density (BMD) was increased in all drug treatment groups as compared to the intact vehicle-treated control group, which is in agreement with the known effects of estrogen on bone. Additionally, a further increase in BMD was observed in animals treated with EB + C-31 (0.05 mg/day). The effects of treatment on the percentage of fat mass (FM) are shown in Figure 7.7. EB alone significantly increased FM compared to the intact control. Co-
administration of EB and C-31 decreased FM in a dose-dependent manner. At doses equal or higher than 0.5 mg/day, FM was significantly less than that of intact controls. FM was linearly correlated to the dose of C-31, with $R^2 = 0.9825$. Correspondingly, the lean mass (LM) also exhibited a dose-dependent increase after EB + C-31 treatment (Figure 7.8).

7.3.3.2 Effects of Treatment on Androgen-Dependent Organs

During screening studies, dozens of compounds demonstrated potent activity and tissue selectivity in castrated male rats after two weeks of treatment. However, the current studies were the first time to investigate the androgenic and anabolic activity of C-31 in intact animals with co-administration of estrogen. Significant inhibition of prostate and seminal vesicle growth was observed in the group treated with EB alone as shown in Figure 7.9, suggesting treatment with EB decreased systemic concentrations of testosterone. The effect of co-administered C-31 was biphasic, meaning that C-31 further decreased the weight of prostate and seminal vesicles at a dose of 0.05 and 0.1 mg/day, but stimulated re-growth of these organs at higher doses (e.g., ≥ 0.3 mg/day). At a dose of 0.75 mg/day, the prostate weight was fully maintained and the seminal vesicles weight was significantly higher as compared to the intact controls. In contrast to the effect of C-31 on the peripheral sexual organs, EB caused a non-significant decrease in the weight of testis and epididymis (Figure 7.10), indicating that a high level of endogenous testosterone exists locally in these organs. Again, a biphasic effect of C-31 on the testis and epididymis was observed. Since the levator ani muscle weight highly depends on the systemic concentrations of testosterone, EB alone reduced its weight to 62% of that observed in intact control (Figure 7.11). Co-administration of C-31 increased levator ani muscle weight in a dose-dependent manner and fully maintained the muscle weight at a dose as low as 0.1 mg/day.
7.3.3.3 Effects of Treatment on Spermatogenesis and Hormonal Regulations

The effect of combined treatment with C-31 and EB on spermatogenesis was evaluated by counting the number of homogenization-resistant, advanced (step 17-19) spermatids in the testis from control and drug-treated rats. As shown in Figure 7.12, mean sperm counts were expressed as number of spermatids per testis (SPT, upper panel) or as the number of spermatids per gram of tissue (SPG, lower panel) after normalization with testis weight. No differences in the mean sperm counts (SPT or SPG) were noticed between EB-treated and intact control animals. Co-administration of C-31 with EB caused a biphasic effect on sperm counts in testis, which was consistent with the androgenic activity of C-31 in the peripheral and sexual organs. The greatest inhibition of spermatogenesis was observed in animals treated with 5 µg/day of EB plus 0.1 mg/day of C-31 (Group 4). Four out of six animals were azoospermic and sperm counts in the remaining two animals were lower than 1.5 million sperm per mL of testicular homogenate. Higher doses of C-31 actually supported spermatogenesis in a dose-dependent manner, resulting a similar mean sperm count as observed in the group treated with EB alone at the highest dose of C-31 used (Group 7, Figure 7.12, A). Notably, the mean sperm counts of animals in Group 7 were greater than those observed in intact and EB alone groups after normalization with testis weights, suggesting that SARMs might also be useful to restore fertility in androgen-deficient males.

Intratesticular testosterone levels and serum concentrations of LH, FSH, and testosterone were determined using RIA assays and summarized in Table 7.2. The average serum concentration of LH in the intact control group was 0.21 ± 0.050 ng/mL. Drug treatments caused a marked suppression of serum LH to levels that were lower than the assay detection limit (0.04 ng/mL). The average serum concentration of FSH in the intact control group was 5.6 ± 1.2 ng/mL. In contrast to LH, significantly lower levels of FSH were only apparent in animals treated with the highest doses of C-31 (i.e., 0.5 and 0.75 mg/day). The average serum concentration of testosterone in the intact control group was 2.2 ± 1.1 ng/mL. EB alone caused a significant inhibition in the average concentration of serum testosterone to 0.35 ± 0.37 ng/mL. In the presence of C-31, serum testosterone level was further suppressed in a dose-dependent manner. Serum
testosterone concentrations were below the detection limit of this assay at **C-31** doses of 0.1 mg/day or higher, in combination with EB. Accordingly, **C-31** induced similar trends in the intratesticular testosterone levels (Table 7.2). The mean intratesticular testosterone concentration of animals in the intact group, EB group, and EB plus 0.05 mg of **C-31** group was 91.4 ± 50.3, 22.8 ± 29.9, and 11.7 ± 14.5 ng/mL, respectively. Although the minimum dose of **C-31** to completely suppress intratesticular testosterone production was 0.3 mg/day, the most remarkable decreases in spermatogenesis were observed in animals treated with EB plus 0.1 mg/day of **C-31**. Sixty-seven percent of the animals that received EB plus 0.1 mg/day of **C-31** achieved azoospermia (4/6) and the remaining animals achieved oligozoospermia (2/6) with an average intratesticular testosterone concentration at 4.36 ± 6.57 ng/mL. Although increased doses of **C-31** (0.3 to 0.75 mg/day) completely suppressed intratesticular testosterone production, spermatogenesis was either partially or completely maintained due to the androgenic activity of **C-31** in the testes

7.3.3.4 Mating Trials

Previous pharmacologic studies showed that the levator ani muscle of castrated rats was maintained at the intact control level in animals that received **C-31** at a dose of 0.1 or 0.3 mg/day for 14 days, identifying these doses as the “physiologic” doses of **C-31**. Mating trials were performed during the last week of treatment for animals in groups 1, 2, 4, and 5, receiving vehicle, EB alone, EB plus 0.1 mg of **C-31**, and EB plus 0.3 mg of **C-31**, respectively. The efficacy of hormonal male contraception was evaluated by the fertility rate of male rats in the mating trials. One hundred percent of the animals in the vehicle-treated intact control group were fertile. Four of five (80%) male rats were fertile after treatment with EB alone. The average numbers of litter in intact control and EB alone group were 14 ± 2 and 13 ± 2, respectively. All male rats, treated with EB plus 0.1 mg of **C-31**, were infertile, while only one out of six male rats treated with EB plus 0.3 mg of **C-31** was fertile and impregnated a female rat with five litters.
7.3.4 Pharmacokinetics of \textbf{C-31} in the Rat

The time-courses of the changes in plasma concentrations of \textbf{C-31} following i.v. and p.o. administration are shown in Figure 7.13, and the pharmacokinetic parameters are listed in Table 7.3. Following intravenous administration, \textbf{C-31} concentrations declined and remained detectable until 48 h after the dose. The terminal $T_{1/2}$ of \textbf{C-31} in male rats was 10.9 h. The systemic clearance (CL) and steady state volume distribution (Vdss) were 0.87 mL/min/kg and 655 mL/kg, respectively. \textbf{C-31} appeared rapidly in the systemic circulation after p.o. administration. Oral absorption was prolonged with maximum plasma concentrations forming a plateau from 6 to 10 h after dose administration. Plasma concentrations of \textbf{C-31} diminished with a mean terminal $T_{1/2}$ of 11.9 h. Statistical analysis revealed no significant difference between the values of $\lambda$ after i.v. and p.o. doses. The bioavailability of \textbf{C-31} following oral administration was 95.9%, indicating the near complete absorption after oral administration.

7.4 DISCUSSION

Among the experimental methods for male contraception, the most promising approach is testosterone-based hormone therapy. Spermatogenesis is highly regulated by hormones through the hypothalamus-pituitary-testis axis. The goal of hormonal male contraception is to suppress spermatogenesis by interrupting the action of one or more hormones involved in the hypothalamus-pituitary-testis axis, including GnRH, LH, FSH, and testosterone. Testosterone is commonly chosen for hormonal contraception because of its ability to replace peripheral androgens while inhibiting LH, FSH, and intratesticular testosterone levels via endogenous negative feedback mechanisms. Although testosterone alone showed an overall safety rate higher than that afforded by condoms in an Asian population, the azoospermia rate in Caucasian population was only two thirds of that observed in Asians (76, 77). Other anti-gonadotropin substances, such as progesterone and GnRH analogs, were used in combination with testosterone to achieve more rapid and complete suppression of spermatogenesis (reviewed in Chapter 1). For hormonal
male contraception, the disadvantages of using testosterone include inconvenient dosing routes (e.g., i.m. injection or implantation) and unwanted effects on the prostate, coagulation system, and lipid metabolism (reviewed in Chapter 1).

In the current studies, multiple in vitro and in vivo assays were used to characterize the feasibility of C-31 for hormonal male contraception. First, C-31 was identified as a potent AR agonist with high AR binding affinity and specificity in vitro. In the following in vivo study using castrated male rats, C-31 revealed the most potent and efficacious activity among SARMs with multiple substituents in the B-ring. The levator ani muscle weight was maintained at values similar to that observed in the intact control group using C-31 at doses of 0.1 to 0.3 mg/day in castrated male rats. Previous masculine behavior studies (Chapter 6) showed that SARM alone can not fully maintain or induce mating behavior in castrated rats. However, SARM induced mating behavior in long-term castrated rats when co-administrated with a low dose (5 µg/day) of EB. To maintain the libido of animals during or after treatment, SARM and EB combination regimens were used for studies of hormonal contraception and mating in intact male rats. Efficacy endpoint for these studies included androgen-dependent organ weights, body composition, total body bone mineral density, spermatogenesis, serum levels of LH, FSH, and testosterone, and intratesticular testosterone concentrations. Consistent with prior literature reports (222, 223), EB alone significantly decreased LH concentration and testosterone levels due to its negative feedback signals. Consequently, EB alone caused a significant decrease in the body weight, peripheral and reproductive organ weights, and lean muscle mass, which was mainly due to the suppressed circulating levels of testosterone. In animals that received EB + SARM combination regimens, their physiologic needs of androgen were replace by C-31, as evidenced by the fully maintained levator ani muscle and partially maintained prostate and seminal vesicles. Furthermore, animals treated with EB + C-31 were leaner than intact controls with lower average body weight and significantly lower percent fat mass. An inverse correlation existed between the dose rate of C-31 and the percent of fat mass in rats. Both in man (224) and animals (225, 226), androgen levels were negatively associated with leptin concentrations. Our previous studies with S-1 showed that administration of SARM significantly decreased leptin levels in castrated male rats (Chen et al. unpublished data).
Findings from the current studies further support our hypothesis that SARM may mimic the pharmacologic actions of testosterone on body weight and body composition.

In adult animals, testicular size is mainly determined by the intratesticular testosterone concentration and is the most common endpoint to assess quantitative maintenance of spermatogenesis with minor inter laboratory variations (223, 227). Testosterone concentrations in the testis are 40-fold and 250-fold higher than those in serum in the rat and men, respectively. The levels of intratesticular testosterone in normal, healthy male rats range from 60 to 400 ng/mL Considerable variations were observed between animals and studies. Nevertheless, studies have shown that much lower intratesticular levels of testosterone are sufficient to maintain various degrees of spermatogenesis (228). To date, it is still not clear why such high local concentrations of testosterone are physiologically needed or what is the minimum level of testosterone to maintain spermatogenesis. In the current studies, EB alone remarkably suppressed the intratesticular testosterone levels to 25% of those in intact animals. However, no significant inhibition in the weights of testis or epididymis was observed after EB treatment, presumably suggesting that the suppressed intratesticular concentration of testosterone was still sufficient to maintain normal spermatogenesis. Indeed, the sperm counts of animals treated with EB alone were similar to those of intact animals. Testosterone levels were suppressed to a further extent and in a dose-dependent manner when \textbf{C-31} was added to the treatment regimens. However, the overall effects of the combination regimens on spermatogenesis were biphasic. In the absence of testosterone, EB + \textbf{C-31} fully maintained spermatogenesis, indicating that \textbf{C-31} mimics the effects of testosterone on spermatogenesis. A similar biphasic phenomenon in spermatogenesis was reported by studies using testosterone-based hormonal male contraception (170). At the highest dose (0.75 mg/day) we studied herein, \textbf{C-31} maintained spermatogenesis with an average but subnormal testicular weight of 75 ± 7% of intact control values. This finding is consistent with a number of studies using testosterone to restore spermatogenesis in rats made azoospermic either by hypophysectomy or gonadotropin withdrawal (229-232). A possible explanation for this observation is that measurement of homogenization-resistant sperm number overestimates the true sperm counts caused by abnormal retention or failed release of step 19 spermatids beyond stage VIII, the most subtle abnormal
changes of spermatogenesis associated with various experimental conditions (233-235). Histological analysis of testis and epididymis from animals after drug treatment is ongoing in our laboratory. Furthermore, EB plus 0.75 mg of C-31 (group 7) not only inhibited LH levels, but also significantly suppressed FSH concentrations. It is well known that FSH directly affects Sertoli cells, which in turn directly determine the size of the testis. The lack of a physiologic level of FSH may have partially contributed to the subnormal size of the testis. Additionally, synergistic effects of testosterone and estradiol on suppression of spermatogenesis were reported both in the rat (223) and in man (236). Although the current studies clearly demonstrated the promise of SARMs for hormonal male contraception, it remains unknown whether SARM and estradiol have a similar synergistic inhibition in spermatogenesis or not.

In summary, C-31 was identified as a potent and efficacious SARM in castrated male rats with significantly higher anabolic activity than androgenic activity. In intact male rats, EB plus C-31 demonstrated potential use for hormonal male contraception. Either azoospermia or oligozoospermia was successfully achieved in the EB plus 0.1 mg/day C-31 combination regimen with a 100% infertile rate observed in the efficacy study. Unlike testosterone which showed biphasic effects in the testis and epididymis and nonselective increase in peripheral reproductive tracts (e.g., prostate and seminal vesicles), C-31 displayed biphasic effects in all organs mentioned above. Additionally, C-31 selectively increased levator ani muscle weight and improved body composition. In conclusion, this is the first study to show that azoospermia can be induced using EB plus SARM combination therapy, in which, EB was used to maintain libido without affecting quantitative spermatogenesis. This therapy selectively decreased weights of prostate, seminal vesicles, testis, and epididymis, retained muscle weight, decreased fat and increased lean mass, and increased BMD. Furthermore, C-31 demonstrated high oral bioavailability (97%). The identification of EB plus C-31 combination therapy represents an important step forward toward the “male pill”.
Figure 7.1 Animal Positioning for DEXA Scan.
Figure 7. 2 Chemical Structures, AR Binding Affinity, and In vitro Transcriptional Activation of S-1, C-3, C-6, and C-31.

AR binding affinity of compound of interest was determined using a radioligand competitive assay. The ability of compound of interest to induce the AR-mediated transcriptional activation was determined using a co-transfection assay in CV-1 cells. Transcriptional activity induced by each compound at 10 nM was reported as the percentage of activity observed for 1 nM of DHT. Each value represents the mean ± s.d. of three replicates. * Data were reported previously by Yin et al. (59).

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Figure 7.3 The Androgenic Activity and Anabolic Activity of \textbf{C-31} in Castrated Male Rats.

\textbf{C-31} was administrated to animals via daily s.c. injections for 14 days with doses ranging from 0.01 mg/day to 3 mg/day. All organ weights were normalized with body weight and presented as the percentage of intact control. Each Bar represents the mean ± s.d. of five rats. I and C indicate a significant difference from intact control and castrated control groups as tested by single-factor ANOVA ($p < 0.05$).
Figure 7. 4 Dose-Response Curves of C-31 in Castrated Male Rats.

$E_{\text{max}}$ and ED$_{50}$ in this figure were obtained by nonlinear least-square regression analysis. Each value represents the mean ± s.d. of five rats.

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Prostate, Seminal Vesicles, Levator Ani Muscle
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Table 7.1 Summary of the Dosing Groups
Figure 7. 5 Body Weights at Day 69 (DEXA).

Intact, male Sprague-Dawley rats received daily subcutaneous doses of the indicated drugs for 69 days. Each bar represents the mean ± S.D. (n=5 or 6/group). The letters “I” and “E” above each error bar represent a significant difference between the group and intact control group and EB group, respectively, as analyzed by single-factor ANOVA with p < 0.05.
Figure 7.6 Whole Body Bone Mineral Density (BMD) at Day 69 (DEXA).

Intact, male Sprague-Dawley rats received daily subcutaneous doses of the indicated drugs for 69 days. Each bar represents the mean ± S.D. (n=5 or 6/group). The letters “I” and “E” above each error bar represent a significant difference between the group and intact control group and estrogen-treated group, respectively, as analyzed by single-factor ANOVA with $p < 0.05$. 
Figure 7. 7 Percentage of Fat Mass at Day 69 (DEXA).

Intact, male Sprague-Dawley rats received daily subcutaneous doses of the indicated drugs for 69 days. Each bar represents the mean ± S.D. (n=5 or 6/group). The letters “I” and “E” above each error bar represent a significant difference between the group and intact control group and estrogen-treated group, respectively, as analyzed by single-factor ANOVA with $p < 0.05$. 
Figure 7.8 The Linear Correlation Between the Dose Rate of C-31 and Body Composition (% of Fat Mass and % of Fat Free Mass).
Figure 7.9 The Weight of Prostate (A) and Seminal Vesicles (B).

Intact, male Sprague-Dawley rats received daily subcutaneous doses of the indicated drugs for 69 days. Each bar represents the mean ± S.D. (n = 5 or 6/group). The letters “I” and “E” above each error bar represent a significant difference between the group and intact control group and estrogen-treated group, respectively, as analyzed by single-factor ANOVA with $p < 0.05$. 

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Intact, male Sprague-Dawley rats received daily subcutaneous doses of the indicated drugs for 69 days. Each bar represents the mean ± S.D. (n = 5 or 6/group). The letters “I” and “E” above each error bar represent a significant difference between the group and intact control group and estrogen-treated group, respectively, as analyzed by single-factor ANOVA with $p < 0.05$. 

Figure 7. 10 The Weight of Testis (A) and Epididymis (B).
Intact, male Sprague-Dawley rats received daily subcutaneous doses of the indicated drugs for 69 days. Each bar represents the mean ± S.D. (n = 5 or 6/group). The letters “I” and “E” above each error bar represent a significant difference between the group and intact control group and estrogen-treated group, respectively, as analyzed by single-factor ANOVA with $p < 0.05$. 

Figure 7. The Weight of Levator Ani Muscle.
Figure 7. 12 Testicular Sperm Counts (Million Sperm/Testis) or (Million Sperm/g tissue).

Intact, male Sprague-Dawley rats received daily subcutaneous doses of the indicated drugs for 69 days. Each bar represents the mean ± S.D. (n = 5 or 6/group). The letters “I” and “E” above each error bar represent a significant difference between the group and intact control group and estrogen-treated group, respectively, as analyzed by single-factor ANOVA with p < 0.05.
Table 7. 2 Effects of Drug-Treatments on Pituitary and Testicular Hormones.

Intact, male Sprague-Dawley rats received daily subcutaneous doses of the indicated drugs for 69 days. The letters “I” and “E” above each error bar represent a significant difference between the group and intact control group and estrogen-treated group, respectively, as analyzed by single-factor ANOVA with \( p < 0.05 \).

<table>
<thead>
<tr>
<th>Group</th>
<th>EB (µg/day)</th>
<th>C-31 (mg/day)</th>
<th>N</th>
<th>LH (ng/mL)</th>
<th>FSH (ng/mL)</th>
<th>Serum Testosterone (ng/mL)</th>
<th>Intratesticular Testosterone (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>--</td>
<td>--</td>
<td>6</td>
<td>0.21 ± 0.050</td>
<td>5.6 ± 1.2</td>
<td>2.2 ± 1.1</td>
<td>91.4 ± 50.3</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>--</td>
<td>5</td>
<td>&lt;0.04</td>
<td>5.2 ± 0.43</td>
<td>0.35 ± 0.37&lt;sup&gt;I&lt;/sup&gt;</td>
<td>22.8 ± 29.9&lt;sup&gt;I&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0.05</td>
<td>6</td>
<td>&lt;0.04</td>
<td>4.8 ± 1.4</td>
<td>0.092 ± 0.12&lt;sup&gt;I,E&lt;/sup&gt;</td>
<td>11.7 ± 14.5&lt;sup&gt;I,E&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.1</td>
<td>6</td>
<td>&lt;0.04</td>
<td>4.8 ± 0.90</td>
<td>&lt;0.0075</td>
<td>4.36 ± 6.57&lt;sup&gt;I,E&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0.3</td>
<td>6</td>
<td>&lt;0.04</td>
<td>4.3 ± 1.5</td>
<td>&lt;0.0075</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0.5</td>
<td>6</td>
<td>&lt;0.04</td>
<td>3.4 ± 0.30&lt;sup&gt;I,E&lt;/sup&gt;</td>
<td>&lt;0.0075</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>0.75</td>
<td>6</td>
<td>&lt;0.04</td>
<td>3.6 ± 0.46&lt;sup&gt;I,E&lt;/sup&gt;</td>
<td>&lt;0.0075</td>
<td>&lt;0.025</td>
</tr>
</tbody>
</table>

Data presented as mean ± s.d.; I: \( p < 0.05 \) vs intact control; E: \( p < 0.05 \) vs EB group
Figure 7. Mean Plasma Concentration-Time Profile of C-31 in Male Rats.

Plasma concentrations of C-31 were measured using HPLC. Data were expressed as microgram per milliliter, and each point represents the mean ± S.D. (N = 4 or 5/group).
Table 7. Mean Pharmacokinetic Parameters of C-31 in Male Rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>i.v. 10 mg/kg (n = 4)</th>
<th>p.o. 10 mg/kg (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{0→∞} (min *µg/L)</td>
<td>11.3 ± 0.33</td>
<td>10.8 ± 0.66</td>
</tr>
<tr>
<td>λ_{z} (min⁻¹)</td>
<td>0.00153 ± 0.00017</td>
<td>0.00140 ± 0.000156</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>751 ± 67</td>
<td>879 ± 61</td>
</tr>
<tr>
<td>CL (mL/min/kg)</td>
<td>0.87 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>V_{ss} (mL/kg)</td>
<td>655 ± 78</td>
<td></td>
</tr>
<tr>
<td>F_p.o. (%)</td>
<td></td>
<td>95.9</td>
</tr>
<tr>
<td>T_{max} (min)</td>
<td></td>
<td>252 ± 137</td>
</tr>
<tr>
<td>C_{max} (mg/mL)</td>
<td></td>
<td>9.4 ± 0.7</td>
</tr>
<tr>
<td>CL/F (mL/min/kg)</td>
<td></td>
<td>0.90 ± 0.06</td>
</tr>
<tr>
<td>V_{ss}/F (mL/kg)</td>
<td></td>
<td>649 ± 68</td>
</tr>
</tbody>
</table>
CHAPTER 8

SUMMARY

Physical methods (e.g., condoms) remain the sole contraceptive choice for men. Clinical trials of testosterone-base hormonal male contraception represent the most advanced stage of research in male contraception. Nonsteroidal selective androgen receptor modulators (SARMs) discovered in our laboratory offer potential applications in androgen replacement, including hormonal male contraception. The advantages of SARMs over testosterone are: AR specificity, tissue selectivity, and better pharmacokinetic properties. The long-term goal of this research is to develop a safe, reversible, orally active, and affordable male contraceptive through an iterative and step-wise experimental paradigm. Studies completed during this dissertation project included:

1. Determination of the AR binding affinity of novel AR ligands with multiple substituents in the aromatic B-ring using a radiolabeled competitive binding assay in vitro.
2. Examination of the AR-mediated functional activity of novel AR ligands with multiple substituents in the B-ring using a cotransfection assay in CV-1 cells.
3. Testing the androgenic and anabolic activity of AR ligands, which demonstrated high AR binding affinity and/or in vitro functional activity, in castrated male rats as a means to identify AR ligands as SARMs.
4. Analysis of the in vitro and in vivo structure activity relationships of these AR ligands.
5. Elucidation of the pharmacokinetics and metabolism of a potent SARM (C-3) in the rat.
6. Characterization of the pharmacology of a potent SARM (C-6) in castrated and intact male rats for hormonal contraception.
7. Evaluation of the effects of **C-6** on masculine sexual behavior in male rats alone or in the combination with estradiol benzoate (EB).

8. Design of a novel SARM (**C-31**) with integrated knowledge of structure-activity relationships, pharmacokinetics, and metabolism of SARMs.

9. Examination of the effects of **C-31** plus EB regimens in male rats for hormonal male contraception.

Conclusions and major findings of the current studies are as following:

1. The inhibition constant (Ki) values of AR ligands varied from 1 to 51 nM. Compounds with significantly improved AR binding affinity were designed by incorporation of multiple substituents in the aromatic B-ring of a known SARM (e.g., **S-1**).

2. Most AR ligands examined in the current studies were identified as full agonists. Several compounds with high AR binding affinity were identified as partial agonists using an *in vitro* functional assay.

3. Compounds with halogen substituents at the *para* and *meta* positions of the B-ring demonstrated the highest *in vivo* pharmacologic activity, which was more potent and efficacious than that of a known SARM (e.g., **S-1**) in castrated male rats. Partial agonists were inactive *in vivo*, suggesting that both AR ligand binding assay and the *in vitro* functional assay are important screening assays to select candidates for *in vivo* tests. The pharmacokinetics, metabolism and pharmacology of two potent SARMs (i.e., **C-3** and **C-6**) were further studied in intact male rats as a prelude to male contraception studies.

4. When compared with **S-1**, no improvement was observed in terms of the pharmacokinetic properties of **C-3**. The major metabolism pathways of **C-3**, a potent SARM with di-fluoro groups in the B-ring, included nitro reduction in the A-ring, oxidation in the B-ring, and sulfate conjugation. Changing the cyano group in the A-ring to a nitro group may increase the *in vivo* stability of this series of compounds.

5. The introduction of a *para*-Cl group and a *meta*-F group to the B-ring resulted a unique SARM (**C-6**), which was as efficacious (E_{max}) in the prostate and levator ani muscle, but much more potent (E_{50}) in terms of anabolic activity than
androgenic activity in castrated male rats. Additionally, C-6 potently inhibited LH and FSH production in castrated male rats and suppressed testosterone biosynthesis and spermatogenesis in intact male rats.

6. C-6 alone failed to induce masculine sexual behavior in sexually naïve, castrated male rats, which was in agreement with the concept that androgen alone is not sufficient to induce or maintain sexual behavior in castrated male rats. However, masculine sexual behavior was fully restored by C-6 plus 5 µg/day of EB in sexually naïve rats. In sexually experienced male rats, C-6 alone partially maintained sexual behavior.

7. C-31 was identified as a potent and efficacious SARM, which was more potent than C-6 in castrated male rats. Either azoospermia or oligozoospermia was successfully achieved in the EB plus 0.1 mg/day C-31 combination regimen with a 100% infertility rate observed in the efficacy study. C-31 and EB combination therapy selectively decreased weights of prostate, seminal vesicles, testis, and epididymis, retained muscle weight, decreased fat and increased lean mass, and increased BMD. Furthermore, C-31 demonstrated high oral bioavailability (97%).

In summary, introduction of multiple substituents in the aromatic B-ring of the SARM pharmacophore is a novel approach to discover new SARMs with high AR binding affinity, AR-mediated transcriptional activation, and in vivo pharmacologic activity. Additionally, such structural modifications provided candidates with profound CNS effects for hormonal male contraception. The identification of EB plus C-31 combination therapy represents an important step forward toward the “male pill”.

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