DRAFT HERSHBERGER BACKGROUND REVIEW DOCUMENT

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List of Abbreviations

The following abbreviations are used in this Background Review Document:

ADME	absorption, distribution, metabolism, and elimination
AFP	α-fetoprotein
AIS	Androgen Insensitivity Syndromes
AMH	anti-Műllerian hormone
AR	androgen receptor
AREs	androgen response elements
BMI	body mass index
BRD	background review document
CAB	combined androgen blockage
CALUX	chemically activated luciferase expression
CG	chorionic gonadotropin
CHO	Chinese hamster ovary cells
CNS	central nervous system
COS	cell lines derived from the CV-1 cell line
DES	diethylstibestrol
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
E2	17β-estradiol
EE	ethynyl estradiol
EACs	endocrine active compounds
ECVAM	European Center for Validation of Alternative Methods
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EDTA	Endocrine Disrupters Testing and Assessment
EGF	epidermal growth factor
EPA	U.S. Environmental Protection Agency
FLU	flutamide
FSH	follicle stimulating hormone
GD	gestation day
GnRH	gonadotrophin releasing hormone
GR	glucocorticoid receptor
H-P-G	hypothalamic-pituitary-gonadal axis
HRE	hormone response element
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods

List of Abbreviations (cont'd)

IC50	concentration of inhibitor necessary to reduce specific ligand binding by 50%
IGF-1	insulin like growth factor 1
LABC	levator ani plus bulbocavernosus complex
LBD	ligand binding domain
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LHRH-a	luteinizing hormone-releasing hormone analog
LNCaP	cell line established from a metastatic lesion of human prostatic
MIS	Műllerian inhibiting substance
MMTV	mouse mammary tumor virus promoter
mRNA	messenger ribonucleic acid
NADH	reduced form of nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NOAEL	no observed adverse effect level
NOEL	no observable effect level
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
PND	postnatal day
PO	"per os", by mouth (gavage)
PPS	preputial separation
PR	progesterone receptor
PTU	propylthiouracil
RAR	retinoic acid receptor
RBAs	relative binding affinities
RT-PCR	real-time polymerase chain reaction
SARs	Structure activity relationships
SBMA	Spinobulbar muscular atrophy
SC	subcutaneous
SF-1	steroidogenic factor-1
SHBG	steroid hormone binding globulin
StAR	steroidogenic acute regulatory protein
T	testosterone
T3	triiodothyronine
T4	thyroxin
Tfm	mouse testicular feminization gene
TGF	transforming growth factor

List of Abbreviations (cont'd)

THRH	thyroid hormone releasing hormone
TIS	Tier 1 Screening
TIS 1	tetradecanoyl phorbol acetate-inducible sequence gene
TP	testosterone propionate
TRPM-2	T-repressed prostatic message-2
TSH	thyroid stimulating hormone
VIN	vinclozolin

CHAPTER 1. EXECUTIVE SUMMARY

The purpose of this detailed background review document is to provide a critical summary of the published scientific literature available as of August 2006, in support of the standardization and validation of the rodent Hershberger assay (Hershberger *et al.*, 1953). The validation process of a test method includes:

- Standardization of the study design, protocol, and standard operating procedures (SOPs).
- Testing the export of the protocol to obtain reproducible results among qualified laboratories.
- Testing the intra- and interlaboratory variability of the endpoints over time.
- Regulatory acceptance.

The protocols under examination involve intact prepubertal (sexually immature), castrated prepubertal or adult castrated male rats exposed to the test chemical (and/or a reference androgen) for a number of consecutive days (typically 5-14), and measurement of accessory sex gland weights (and testes if the subjects are intact) at necropsy immediately following the last dose. The intended purpose of the Hershberger assay is to identify chemicals that act as androgen agonists (androgenic) or androgen antagonists (anti-androgenic) *in vivo*. It is considered a specific mechanistic screening assay, sensitive only to those chemicals that interact with the androgen receptor (AR). The assay, once validated, is intended for use as a short-term *in vivo* assay in an overall testing strategy for the detection (hazard) and assessment (risk) of potential endocrine disruptors (*Chapter 2, Introduction*). Depending on the outcome of the Hershberger assay, substances may then require additional assays/tests to evaluate their potential for causing adverse reproductive and/or developmental effects.

The Hershberger assay was developed initially to identify the drivers (hormones) for male sexual development, and it has been used in pharmaceutical development of androgen agonists and antagonists for many years (*Chapter 3, History of Assay*).

The basis of the Hershberger assay is the absolute requirement for testosterone (T; made in the testis) and/or dihydrotestosterone (DHT; converted from T by 5α-reductase in the testis and other end target organs) for the rapid growth and maturation of the accessory sex organs during puberty in intact males, for their maintenance postpuberty, for their rapid regression and involution after castration (removal of the source of T), and for their rapid regrowth in the castrate administered an exogenous androgen (typically T propionate; TP). The accessory sex organs of interest are predominantly the epididymides (if they are not removed at castration), the prostate (the ventral lobe or ventral plus dorsolateral lobes), and the levator ani plus bulbocavernosus complex (LABC) muscle, as well as the other accessory sex organs (seminal vesicles with

coagulating glands, Cowper's glands, and preputial glands). The mode of action for T, DHT, and other androgens is initiated by the entrance of the androgen into the target cell and binding to the AR (*i.e.*, the androgen is the ligand). The binding of the ligand to the AR creates a conformational change so that the altered AR complex binds to the specific DNA-binding domain, initiates (agonists) or blocks (antagonists) androgen-mediated transcriptional activation of a cascade of genes to produce mRNA, and translation of the messages into proteins directing mass molecular, biochemical, and physiological processes to induce growth of the accessory sex organs (*Chapter 4, Biological Basis of the Assay/Androgen Mode of Action*). This sequence is the reason why an intact adult male cannot be used in the Hershberger assay, since the presence of endogenous T (and DHT) for maintenance of the accessory sex organs and other male secondary and tertiary sexual characteristics (including sexual behaviors and reproduction) makes the intact male insensitive to exogenous androgens. The development of the male reproductive tract and other androgen-responsive tissues and their interactions with androgens (T and DHT) are presented in *Chapter 5, Male Reproductive Tract and Other Androgen Responsive Tissues*).

There are three versions of the Hershberger assay in rats in use or under discussion: the prepubertal intact male, the castrated adult male, and the peripubertal castrated male (*Chapter 6, Versions of the Assay/Procedural Variables*).

- For the prepubertal intact male version, after weaning on postnatal day (PND) 21, the males are exposed to the test chemical and vehicle during the postwean peripubertal period prior to acquisition of puberty (approximately PND 41-42 in rats and PND 26-28 in mice). The endpoints of interest are age at acquisition of puberty (preputial separation; PPS), weights of the testes and epididymides (since these animals are not castrated), and weights of the sex accessory organs. The timing and duration of treatment is therefore circumscribed to a maximum of approximately 14 days in rats (from PND 21-22 to PND 35 after which PPS begins to occur). These animals are intact, so the hypothalamic-pituitary-gonadal axis is intact. Therefore, this is an apical test and can detect effects and many mechanisms at various organization levels, as well as on synthesis, distribution, transport, metabolism, and elimination, including interactions with the AR, but it cannot identify a mechanism/mode of action.

- For the castrated adult male version, the male rats are castrated after puberty with a postsurgical recovery period (so the accessory sex organs involute/regress) or no recovery period (so the androgen-dependent accessory sex organs have not yet regressed). The test chemical (or reference androgen) is administered for typically 3-5 days by per os (po) or subcutaneous (sc) injection, and the animals are necropsied 24 hours after the last dose and the accessory sex organs weighed.

- For the peripubertal castrated male version, Gray *et al.* (2005) recommended castration of sexually immature rats on PND 42 (so the rats can acquire PPS with/without exogenous androgens), a 12-day recovery period, initiation of treatment on PND 53-54 for ten consecutive days, and necropsy (the day after the last dose on PND 64-65). The endpoints are acquisition of puberty and weights of

the accessory sex organs. For this version, the animal exhibits enhanced sensitivity during peripubertal sexual development to exogenous androgens. The weight of the glans penis (after PPS) is another endpoint, and there may be qualitative as well as quantitative differences in the pre-/peripubertal animal versus the adult. Since this is a castrated animal model, it is specific to androgen-dependent, AR-mediated mechanisms (Chapter 6).

The details, procedures, and variables for each version of the Hershberger assay are also discussed in Chapter 6. They include species/strain, group sizes, age at castration, recovery period (presence and duration), start and duration of treatment, route of administration, reference androgens and endpoints (such as tissue dissection, pre- and post-fixation weighing, statistical analyses), as well as animal husbandry differences such as caging, bedding, lighting, feed, housing, and drinking water.

The synopsis of the protocol selected to be validated by the OECD, the peripubertal castrated male rat, is fully described in *Chapter 7, Synopsis of Protocol*, and briefly described above.

It is important that a widely used assay is specific, robust, reproducible, uses the minimum number of animals, has known strengths, and has its limitations identified. There are a vast number of studies that have evaluated chemicals for androgenic/anti-androgenic activity in castrated rats, beginning in the 1930s, that are improved somewhat and standardized by Hershberger *et al.* in 1953. The so-called Hershberger assay has been proposed by both EDSTAC (1998) and OECD (1998) to be validated for use in a comprehensive screen to detect potential endocrine disruptors. The assay has also been used in the pharmaceutical industry to screen for these properties in drug candidates in development for therapeutic purposes. *Chapter 8, Example Compounds*, provides a compendium of chemicals, with information on protocol variables and results in terms of weights of the accessory sex organs. There are a very few inconsistencies (*e.g.,* the fungicide fenitrothione is an anti-androgen *in vitro* and positive in the Hershberger assay [Tamura *et al.,* 2001] but negative in the Hershberger assay [Sohoni *et al.,* 2001]). When tested in a relatively standardized Hershberger protocol, the vast majority of chemicals tested have produced consistent results across laboratories. The Hershberger assay appears to be robust, specific, and reproducible within and across capable laboratories and over time. It is also considered an efficient use of animals. The overall use of animals in any testing strategy can be further reduced by assessing the structure of unknown substance as possible ligands for the AR by conducting *in vitro* screens for AR binding affinity and transcriptional activation or inhibition, or other *in vitro* assays responsive to androgen ligands before conducting the Hershberger assay. It is important to note that *in vitro* assays do not incorporate such components as ADME (absorption, distribution, metabolism, and elimination), response of target organs/cells/organelles, etc., which are present in *in vivo* assays. As a result, false positives (defined in this document as a substance incorrectly identified as an androgen agonist/antagonist *in vitro* not supported by *in vivo* results), as well as false negatives (defined in this document as a substance incorrectly identified as not an androgen agonist/antagonist *in vitro* with positive *in vivo* results) can result. False positive and false negative results can also be obtained in *in vivo* assays due to variations in control weights of the accessory sex

organs, effects on non-accessory sex organs (*e.g.*, liver, kidneys, etc.), and subsequent indirect effects on the accessory sex organs. The toxico-pharmacodynamics and toxico-pharmokinetics of androgen metabolism support the necessity for an *in vivo* assay with animals in the overall assessment of possible androgen agonists and antagonists. The essential assumption (as with the estrogenic/anti-estrogenic compounds, Uterotrophic Assay Background Review Document ENV/JM/MONO, 2003) is that the active "factor" is the concentration in the serum (or plasma) of the free (not bound) bioavailable ligand in equilibrium with the target organ intracellular ligand concentrations. This portion of an administered substance is then the fraction available to bind the AR ligand. A number of considerations affect the serum/plasma concentration of the ligand and its *in vivo* activity in the Hershberger assay. These considerations include intestinal and liver metabolism, leading to active or inactive metabolites, intestinal and liver conjugation reactions, biliary, renal, fecal, and other routes of elimination, specific and nonspecific binding to serum proteins, sequestration in other body compartments, and the specific receptor concentrations in target tissues (*Chapter 9, Additional Screens and Markers*).

Also note that the Hershberger assay, involving castrated males (pre- or postpubertal) is specific and limited to AR-dependent actions.

An important question is whether the Hershberger assay results are predictive of adverse effects. This is especially true of weak androgen agonists/antagonists. There is also the recognition that there may be hazard (the intrinsic capacity of the test chemical to do harm), but there may not be risk (the capacity of the test chemical to cause adverse effects at environmentally relevant doses at relevant routes of administration during sensitive life stages).

A positive response in the Hershberger assay suggests the need for additional testing for adverse reproductive and developmental effects, and that performance of more definitive tests administering the test material chronically by gavage over a wide dietary dose range, including human exposure doses (*i.e.*, a two-generation reproductive toxicity study under OECD No. 416 [2001] or EPA OPPTS 870.3800 [U.S. EPA, 1998a] testing guidelines) would result in androgen-mediated, adverse results.

Although the endpoints in the Hershberger assay (specifically changes in the weights of the androgen-dependent accessory sex organs) are not necessarily considered adverse, per se, Gray *et al.* (2005) "have found that chemicals that are positive in the Hershberger assay often produce effects during puberty and after *in utero* exposure."

The data summarized in this document provide broad support for the validation and regulatory use of the Hershberger assay as a robust, specific, mechanism-based, *in vivo* screen to detect possible androgen agonists and antagonists. In addition:

- The evidence clearly supports the binding of ligand (endogenous or exogenous) to the AR as the initial step in the transactivation of androgen-dependent genes and the subsequent cascade of molecular, biochemical, and physiological events that results in increased growth of the accessory sex organs. Increased growth of these organs is measured by their weight.

- The extensive history of the Hershberger assay supports the ability of the assay to evaluate the androgenic/anti-androgenic potential of substances, even weak androgen agonists/antagonists which act as ligands and interact with the AR.

- Of the three major versions of the Hershberger assay, the castrated peripubertal male version appears to be the best of the three.

- The major procedural variables for the Hershberger assay are known, and their impact(s), if any, have been established.

- Many laboratories have the technical skill, equipment, facilities, and experience to conduct the Hershberger assay.

- The overall specificity, robustness, and reproducibility of the Hershberger assay appear to be adequate and appropriate to different classes of chemicals. False positive and negative results are relatively rare. Criteria for accepting data (*e.g.,* acceptable control ranges of weights for accessory sex organs) and guidance on interpretation are available and will be useful.

- Toxico-pharmacodynamics and toxico-pharmacokinetic factors in the castrated or intact animal can modify the activity of a test substance. This supports the need for *in vivo* testing in animals and a tiered hierarchical framework (*e.g.,* U.S. EPA [1998b] EDSTAC Report; OECD, 1998) with ultimately relevant routes, doses, and timing of administration.

- The Hershberger assay results in subsequent testing outcomes for adverse effects (both positive and negative).

- Structure activity relationships (SARs) and *in vitro* assays appear able to identify (prescreen) substances with a potential androgenic mode of action as candidates for the Hershberger assay, thereby minimizing the use of animals and resources.

- The results for the *in vivo* Hershberger assay are suitable to identify those endocrine-active substances that act through an AR mechanism/mode of action to be appropriate and suitable for further testing for androgenic (agonistic) or anti-androgenic (antagonistic) reproductive and/or developmental effects (*Chapter 10, Discussion and Conclusions*).

CHAPTER 2. INTRODUCTION

Background

Need for assays and tests to identify endocrine-active compounds

There has been accumulating scientific evidence that humans, domestic animals, fish, and wildlife species may have adverse health consequences from exposure to environmental chemicals that interact with the endocrine system. This evidence includes adverse effects observed in certain wildlife, fish, and ecosystems, the increased incidence of certain endocrine-related birth defects, changes in physiological parameters, and disease in human. Supporting evidence has been provided by experiments involving a variety of laboratory animals including rodents, fish, birds, and frogs. These observations have stimulated many national governments, international organizations, scientific societies, industry, and public interest groups to mobilize resources to better understand and respond to this concern.

Organisation for Economic Cooperation and Development (OECD) validation

In 1997, in response to the aforementioned health concerns, the OECD reviewed its Health Effects Testing Guidelines and concluded that the existing guidelines were insufficient to identify chemicals acting through certain endocrine mechanisms (oestrogen, androgen, and thyroid), and might not be adequate to fully characterize the hazards posed by such chemicals. Therefore, a *Special Activity on the Testing and Assessment of Endocrine Disrupters* was initiated as part of the OECD Health Effects Testing Guidelines Program. The purpose of this activity was to revise existing guidelines and to develop new OECD Test Guidelines in order to, first, screen chemicals to identify substances that could interact with the endocrine system, and second, to ensure that the tests could characterize the hazards of potential disrupters. An OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) was established to provide a focal point within the OECD to distinguish and recommend priorities for the development and validation of new and improved methods to identify and assess substances acting through endocrine mechanisms (OECD, 1998).

Since 1998, in the final report of the EPA Endocrine Disruptor Screening and Testing Advisory Committee, the rodent Hershberger bioassay was proposed for use as a screen for identifying potential androgenic or anti-androgenic substances (EDSTAC; U.S. EPA, 1998a). The Hershberger bioassay was given high priority for validation by several expert panels and workshops, involving both U.S. and European scientists, as a mechanistic screen for substances that would act through an androgenic or anti-androgenic mode of action (U.S. EPA, 1998a; Gray *et al.*, 1997; EC/EEA/OECD/WHO, 1997; SETAC-Europe, 1997). EDSTAC and other panels recognized that although the Hershberger bioassay has been in use since the 1930s for pharmaceutical discovery and evaluation of androgens, the bioassay would have to be validated for its use as a mechanistic screen for weak androgens and anti-androgens.

Validation is a scientific process designed to characterize the operational characteristics and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose. OECD Guidance Document 34 provides the principles of test validation and practical guidance for validation that are followed by OECD. These principles were introduced in the report from a workshop on validation in Solna (OECD, 1996) and are consistent with the approaches used in Europe by the European Center for Validation of Alternative Methods (ECVAM) (ECVAM, 1995) and the U.S. Interagency Coordinating Committee on Validation of Alternative Methods (ICCVAM) (ICCVAM, 1997).

The first stage of the validation process is *test development*, an applied research function which culminates in an initial protocol. As part of this phase, a detailed background review document (BRD) is prepared to explain the purpose of the assay, the context in which it will be used, and the scientific basis of the assay's protocol, endpoints, and relevance. The BRD reviews the scientific literature for candidate protocols and evaluates each with respect to a number of considerations, such as whether the candidate protocols meet the assay's intended purpose, the relative costs, and other practical considerations. The BRD also identifies the stage of development of each protocol, and questions related to its conduct and suitability. For example, the BRD addresses information necessary to answer the questions, and, when possible, recommends an initial protocol for the initiation of *prevalidation* laboratory studies in which the protocol is refined, optimized, and initially assessed for transferability and performance. Several different types of studies are conducted during the assay's prevalidation phase, depending upon the status of the method and the nature of the questions that the protocol raises. The initial assessment of transferability is generally a trial in a second laboratory to determine that another laboratory besides the lead laboratory can follow the protocol and execute the study. *Inter-laboratory validation* studies are conducted in multiple independent laboratories, after the protocol optimization during prevalidation. The results of inter-laboratory validation studies are used to determine inter-laboratory variability and to set or cross-check performance criteria. Inter-laboratory validation is followed by *peer review*, an independent scientific review by qualified experts, and by *regulatory acceptance*, adoption for regulatory use by regulatory authorities. The purpose of this background review paper is to provide a summary of the literature in support of the standardization and validation of the Hershberger assay for use in the detection and evaluation of endocrine-active compounds.

Hershberger Assay

While the EPA EDSTAC was deliberating (1996-1998), an OECD Task Force on EDTA was established to provide a focal point within the OECD to identify and recommend priorities for the development and validation of methods for identifying endocrine active compounds (EACs), including the Hershberger assay (Hershberger *et al.*, 1953) for males, and the uterotrophic assay for females (Kanno *et al.*, 2001, 2003).

Brief description

Historically, the Hershberger assay (Hershberger *et al.*, 1953) was based on a myotrophic test for the assay of protein anabolic activities of androgens that measures the levator ani muscle in castrated male rats receiving different androgens (Eisenberg and Gordan, 1950; Gordan *et al.*, 1951). In the initial myotrophic test, PND 21 rat weanlings were castrated and retained for 23 days postcastration until the test chemicals were administered. On the eighth day (postcastration), 22-26 consecutive hours after the last injection, the animals were sacrificed. The levator ani muscle, ventral prostate, and seminal vesicles (free of the coagulating glands) were dissected from each male and weighed. Dry weights of the levator ani muscle were also recorded after desiccation at 72°C. The results (organ weights at various doses of each of many test materials) provided information on the relative potency of each test material for androgenic activity and/or myotrophic activity. The response of the levator ani muscle was termed the myotrophic response, and the response of the sex accessory glands was termed the androgenic response. Hershberger *et al.* (1953) reported that TP was the most potent for both myotrophic and androgenic responses, but that over the wide range of compounds tested, there was a "distinct lack of parallelism" between myotrophic and androgenic activities. Preliminary screening indicated that 19-norT and other 19-nor analogs of androgens were effective anabolic agents (*i.e.*, causing a myotrophic response) and relatively weak androgenic agents. Androsterone exhibited strong androgenic and weak myotrophic activity, and T exhibited strong androgenic and strong myotrophic activity. Subsequent studies confirmed the usefulness (diagnostic, easy, inexpensive, and quick) of the assay (Deanesly and Parkes, 1936; Dorfman, 1962b; Dorfman and Dorfman, 1963; Dorfman and Kincl, 1963). [Hershberger *et al.* (1953) also castrated male rats on PND 21 and administered sc injections of test substances for seven days, beginning on the day of castration.]

OECD EDTA validation

The OECD EDTA selected the rodent Hershberger assay, in addition to the rodent uterotrophic assay, and the enhanced OECD 407 guideline study (28-day treatment study), to start international cooperative work (Gray *et al.*, 2002). The Hershberger assay was proposed by both EDTA and EDSTAC to test for chemicals that have the potential to act as androgens or anti-androgens and, indeed, it has long been widely used by the pharmaceutical industry for this purpose to evaluate drugs for potential therapeutic use (Hershberger *et al.*, 1953; Dorfman, 1962b; Dorfman and Dorfman, 1963; Dorfman and Kincl, 1963). Accessory sex glands/tissues require androgen stimulation to gain and maintain their weights during and after puberty. For example, if male rodents are castrated (*i.e.*, the endogenous testicular source of androgen is removed), an exogenous androgen is necessary if involution is to be avoided (treatment must begin immediately after castration) or reversed (treatment begins after involution has occurred). In the castrated male rodent, therefore, effects on these tissues are likely to be direct and not a result of pituitary or gonadal secretion. For assessment of androgenicity, test chemicals are administered to castrated males, however, for anti-androgenicity, test chemicals are given to castrated animals previously treated with TP.

Since there are many variations to the Hershberger assay protocol, an internationally recognized standard for the assay was developed, and validated for specificity, sensitivity, and reproducibility within the OECD framework (Gray *et al.*, 2002, 2005; Owens *et al.*, 2006). The OECD protocol for the Hershberger assay recommended ten days of treatment to peripubertal male castrated rats, and included weighing individual sex accessory tissues since little was known about the response of individual sex accessory tissues to exogenous chemicals that may have androgenic effects. The organs required for evaluation were the ventral prostate, seminal vesicles together with coagulating glands, glans penis, Cowper's glands, and the levator ani and bulbocavenosus muscles. In addition, determination of liver weight was highly recommended, as some test substances appear to exert anti-androgenic effects by increasing metabolism of TP by hepatocytes. Furthermore, the weights of the adrenal glands and kidneys, and the levels of serum luteinizing hormone and T were included as optional endpoints (Gray *et al.*, 2002).

Three versions of the Hershberger assay

Three versions of the Hershberger assay currently exist.

Castrated adult male

The initial Hershberger assay (Hershberger *et al.*, 1953), as it has evolved, employs adult castrated male rats. Seven to 14 days postcastration (so that T-dependent organs have involuted and regressed) or immediately after castration (so that T-dependent organs have not yet involuted or regressed), the rats are exposed to test chemicals or to vehicle control, usually by gavage, injection, or less commonly, dosed feed, daily for three to five days. Twenty-four hours after the last dose, the males are terminated and necropsied, and the sex accessory organs are weighed. If the sex accessory organs in the treatment groups are heavier than those in the vehicle control group, the test material is considered to be positive and is designated androgen-like. This version of the assay has clearly defined strengths and limitations. Its strengths are:

- With removal of the testes, the hypothalamic-pituitary-gonadal-end organ axis is disrupted, so the assay does not detect agents that act at the level of the hypothalamus, pituitary, or at nonreceptor-mediated levels. The accessory sex organs are dependent on T (or DHT) for their growth and maintenance, mediated by recognition of an androgen, and binding of the recognized molecule to the AR. Therefore, the positive response to a test material as evidenced by the increased weight of the sex accessory organs identifies the test material as not just androgen-like but as an androgen agonist. Thus, the test is very specific and identifies the single mechanism evaluated (AR receptor binding).

- An anti-androgen (*i.e.*, an androgen antagonist) can also be detected if the castrated male is presented with exogenous T (usually TP), with and without the test material. If the sex accessory glands weigh less in the presence of T and test material than they do in the presence of T alone, then the test material

and T compete for binding to the AR. The test material is recognized by the AR and binds to it but does not activate the down-stream genes to cause growth of the sex accessory glands. Thus, the test material is not just an anti-androgen, but is an androgen antagonist.

- Since the male is castrated, the start of administration of the test material can be delayed and/or the duration of administration can be prolonged, allowing for flexibility (since there is no endogenous source of T).

- This assay has been shown to be a robust and specific assay. Based on the major protocol variables independently evaluated (Ashby and Lefevre, 2000a), and the OECD validation of the assay (Owens *et al.*, 2006), the use of different rat strains, use of the mouse, use of different diets, bedding, caging, light cycles, and animal room temperature and relative humidity does not affect the outcome as long as all the animals on a particular study are the same species and strain and are exposed to the same environmental conditions.

Limitations to the castrated male version of the assay are as follows:

- The assay is limited to detection of androgen agonists or antagonists. It cannot, and does not, detect agents that act upstream of the testis, such as at the level of central regulation, in the hypothalamus or pituitary. It also does not detect agents that do not act through AR-mediated mechanisms, such as effects on androgen synthesis (*e.g.*, phthalates), transport, metabolism, elimination, etc.

- The castrated adult male assay also employs, by necessity, a postpubertal adult male. Given the increasing evidence that younger animals are more sensitive, both quantitatively (at lower doses) and qualitatively (different effects) to EACs, the assay may not be sensitive enough to detect weak agents or agents at low (environmentally relevant) doses and/or by different (environmentally relevant) routes of administration.

- The assay cannot be a stand-alone screen since it looks at only one mechanism. Other assays must be used to evaluate if other endpoints are affected.

Intact prepubertal male

An alternate study design for the evaluation of androgenic or anti-androgenic activity, described by Ashby and Lefevre (2000b), employs prepubertal, intact males. After weaning (PND 21 in rats and mice), the males are exposed to the test material or to the vehicle control in the postwean prepubertal period. The endpoints of interest are the age at acquisition of puberty (PPS) and the weights of reproductive (including testis and sex accessory organs) and possibly other organ systems (*e.g.*, liver). The timing and duration of the exposure period are critical and somewhat limited since natural puberty in the CD® rat begins soon after weaning, with control mean male CD® rat age at

acquisition of PPS approximately 41-42 days (with no PPS-positive animals at 35 days of age), and the CD-1 mouse mean male age at acquisition approximately 26-28 days. Since the young males are not castrated, the hypothalamic-pituitary-gonadal axis is intact, so it is considered an "apical" test (*i.e.*, multiple mechanisms are possible at various organizational levels, but no mechanism is specifically identified). The present performance and interpretation of this version of the assay provides recognition of its strengths and limitations. Its strengths are as follows:

- Since the hypothalamic-pituitary-gonadal-end organ axis is intact, this assay can detect agents that act centrally as well as peripherally, act at the neuroendocrine (CNS-endocrine) interface, act via synthesis, distribution/ transport, metabolism, elimination, which act at the level of carrier proteins, etc., as well as those that act through AR agonism or antagonism (but see the list of limitations below).

- The test involves young, prepubertal, intact animals so it can detect agents that act on developing systems, functions, and organs, providing greater sensitivity both quantitatively (at lower doses), and qualitatively (entirely different endpoints) at a sensitive developmental age (puberty; Stoker *et al.*, 2000). In the prepubertal intact uterotrophic assay, the female is maximally responsive to exogenous estrogens between PND 18 and 26 (at puberty). There is a surge in endogenous estrogen, so the animal is less sensitive if the uterus is already stimulated. *A prori*, the same relative sensitivity is expected for the peripubertal intact Hershberger assay.

- This assay can detect agents that act initially on nonreproductive targets that secondarily affect reproductive structures and functions (*i.e.*, liver damage that results in prolonged elevated hormone levels since hepatic metabolism is impaired), thyroid effects (Chowdury *et al.*, 1984) that impact on the maturation of the reproductive system (early exposure results in delayed differentiation of the testicular Sertoli cells with continued replication, resulting in "megatestis," with increased sperm counts [Cooke and Meisami, 1991; Cooke *et al.*, 1992, 1996] versus results from later exposure resulting in reduced sperm count), etc.

- This assay can detect androgenic as well as anti-androgenic test materials depending on whether puberty is accelerated or delayed, and by whether the weights of the accessory sex organs increase or decrease relative to the vehicle control group values.

- This is an excellent screening assay since, like the original Hershberger assay design, it evaluates a large number of endpoints, mechanisms/modes of action, and levels in one assay.

The limitations are:

- This is a test that evaluates multiple endpoints, mechanisms, and levels, but as an apical assay, it cannot, by itself, identify which mode(s) or mechanism(s) of action is (are) impacted.

- There is a relatively brief window of opportunity to evaluate effects on the developing reproductive system before natural puberty begins and confounds any effects from the test material. The animal must be weaned, so there is no confounding from the maternal organism (*e.g.*, nursing, behavior) or siblings, so the earliest start date is well defined. The tracking and recording of the exact ages in days at the start and end of dosing and at necropsy are imperative because of the rapid, natural changes in endpoints during this time of rapid growth and specific hormonal changes.

- The dissection of very small reproductive structures in a young prepubertal male is more difficult than the dissection of large reproductive structures in an adult male (requires technical practice and training and more sensitive and precise balances).

- Because the intact, young male is more sensitive than the castrated adult male to some agents, and because this assay involves developmental processes, the endpoints (age at acquisition of puberty, organ weights, etc.) can be affected by exogenous EACs in the bedding, feed, or water. For example, phytoestrogens in feed can cause delays in male acquisition of puberty and thus act like an anti-androgen and confound effects from the test material. Various phthalate esters interfere with T biosynthesis and could, as contaminants, also act as an anti-androgen and confound any effects from the test material. Characterization and analyses of the feed, water, and bedding, and/or use of the same environmental conditions for all groups can diminish the confounding of test compound-related effects. Perhaps the only real risks are the possibility that the presence of environmental confounders will produce a maximal response in the endpoints of interest, so no further response is possible and a false negative result will occur, or the possibility that endocrine effects from the contaminants in feed, water, bedding, etc., will be interpreted as a positive result from the test chemical (*i.e.*, a false positive).

Castrated prepubertal male

A third version of the Hershberger assay employs sexually immature male rats, castrated at peripuberty by removal of the testes and epididymides (*i.e.*, orchidoepididyectomized; Gray *et al.*, 2005 on PND 42). Gray *et al.* (2005) recommended initiation of treatment 12 days after castration (PND 53-54), dosing for ten consecutive days, and necropsy on the day after the last dose (PND 64-65). Castrated, immature males given TP (sc 0.2-0.4 mg/kg/day for ten days) will initiate PPS. The strengths of this version of the assay include:

- At this peripubertal stage of sexual development, the glans penis and other sex accessory organs are sensitive to androgens (or to anti-androgens in the

presence of androgens) since both ARs and appropriate steroidogenic enzymes are present at this stage.

- At this stage, the sex accessory tissues display high sensitivity and relatively small weights, both of which are considered to aid in minimizing variation in responses among individual animals.

- Since the testes and epididymides are removed, the hypothalamic-pituitary-gonadal axis is interrupted. As in Version 1 (adult castrated male), the response of the sex accessory glands is dependent on the recognition of the test chemical by the AR, the subsequent binding to the AR, and initiation of a downstream cascade of transcriptional activation of relevant genes (with an androgen agonist) or blockage of the initiation of the downstream transcriptional activation of relevant genes (with androgen antagonist).

- This is a powerful, specific assay, especially due to its use of peripubertal males.

- Since the males are castrated (*i.e.*, no endogenous source of T or DHT), the initiation and/or duration of dosing can be delayed and/or extended giving the assay more flexibility. Ten consecutive days of dosing are recommended by Gray *et al.* (2005).

The limitations are similar to the limitations for the adult castrated male (Version 1), although this version is considered much more sensitive with acquisition of PPS incorporated into the assay:

- Only test materials that act by an androgen-receptor mechanism will be detected.

- A test material that acts at the level of the hypothalamus pituitary and/or testis will not be detected.

- This assay evaluates just one mechanism and must be part of a multiple study screening program to detect EACs by all currently known mechanisms.

- In most laboratory rat strains, such as the Sprague-Dawley, Long Evans, or Wistar, peripuberty occurs within the age range of five to seven weeks. In the CD® Sprague-Dawley rat at RTI, the historical control grand mean of study means is 41.9 days of age for PPS in intact animals. This issue deserves additional consideration since the use of younger animals may capture additional effects not observed using the timing of castration and subsequent treatment(s) proposed by Gray *et al.* (2005) and employed by the OECD validation initiative (Owens *et al.*, 2006). Gray *et al.* (2005) strongly recommended castration on PND 42 to allow PPS to occur (in his experience, PPS commonly did not occur with rats castrated at younger ages).

Enhancements

Additions to initial assay

After evaluation with multiple chemicals, Gray *et al.* (2005) proposed including additional parameters in the initial study design (of the castrated/prepubertal male). These additions include weighing the T-dependent sex accessory glands separately (rather than as one unit), weighing paired organs separately or as a pair, and adding additional organs as follows:

- Testes (if present)
- Epididymides
- Seminal vesicles plus coagulating glands (with and/or without their fluids)
- Prostate (or as dorsolateral and ventral lobes separately)
- Bulbourethral (or Cowper's) glands
- Preputial glands
- LABC
- Glans penis

Identification of other targets or mechanisms

With the addition of other parameters, the Hershberger assay can be used to identify other targets or mechanisms:

- Liver weight and possible histopathology. Is the test material truly anti-androgenic, or are the effects on the accessory sex organs due to increased metabolism of TP (positive control material used in association with test material to detect androgen antagonistic or anti-androgenic responses) by the liver (resulting in increased liver weight and hepatocellular hypertrophy)?
- Adrenal weight and possible histopathology. Are there effects of the test material on the hypothalamus-pituitary-adrenal gland axis (which is intact in all three versions)?
- Kidney weight and possible histopathology. Are there effects of the test material on kidney structure or function? Are the effects direct or indirect?
- Obtain thyroid weight and possible histopathology. Are there effects of the test material on the hypothalamus-pituitary-thyroid axis that are intact in all three versions (Yamada *et al.*, 2004; Noda *et al.*, 2005)?

- Analysis of serum or plasma for T, DHT, follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), triiodothyronine (T3), and tetraiodothyronine (thyroxin; T4). A single blood collection at terminal necropsy will only provide the hormone status at that point in time. Hormone production and release are typically cyclical and change over time (*e.g.*, if T3/T4 levels drop, pituitary TSH levels increase to stimulate T3/T4 production once T3/T4 are at normal levels, TSH levels drop, etc.). It is suggested that blood samples be collected prior to castration (Versions 1 and 3) or prior to onset of puberty (Version 2), immediately prior to test material administration for all three versions and at terminal necropsy for all three versions.

- Ashby *et al.* (2001) have suggested replacement of surgical castration (in Versions 1 and 3) by GnRH inhibition, the gonadotrophin-releasing hormone from the hypothalamus that initiates the pituitary-gonad-end organ cascade. A second suggestion is to use Leydig cell ablation. These cells are located in the testicular interstitium and synthesize T and DHT. The Sertoli cells (within the seminiferous tubules) should not be insl3, which controls the formation and function of the gubernacular cords in males to result in testis descent in late gestation to the inguinal ring of the lower abdomen and in testis descent into the scrotal sacs in late lactation, and inhibin (a glycoprotein) which provides negative feedback on release of FSH from the anterior pituitary. FSH, in turn, binds to receptors on Sertoli cells which release factors for spermatogenesis. Sertoli and Leydig cells coordinate using FSH and T (both cell types have receptors for both hormones). These and other procedural variables will be further discussed in Chapter 6.

OECD Selection of Castrated Prepubertal Male Version of Hershberger Assay

Screening assays should be assessed against five appropriate attributes. They should:

- Be inexpensive, quick, and easy to perform.

- Be validated and standardized as soon as possible (ICCVAM, 1997; Zeiger and Stokes, 1998), defining characteristics such as sensitivity and specificity against a "gold" standard once it is identified.

- Be more "sensitive" than they are "specific," meaning that they should have as their primary objective the minimization of false negative (or Type II) errors, while permitting an as-of-yet undetermined but acceptable level of false positive (or Type I) errors.

- Capture multiple endpoints and reflect as many modes of endocrine action as possible.

- Be broadly predictive across species, gender, and age.

The castrated prepubertal male assay (Version 3) is the best of the three versions, in that it can detect androgen agonists, androgen antagonists, metabolic modulators (Ashby and Lefevre, 2000a,b), and thyroid agonists/antagonists/disruptors (Chowdury *et al.*, 1984; Noda *et al.*, 2005). It is not inexpensive or quick, but it is straightforward. Chapter 7 will provide a detailed description of the castrated prepubertal male Hershberger assay used by OECD in its standardization and validation program.

CHAPTER 3. HISTORY OF THE ASSAY

Development of the Mammalian Male Reproductive System

Primary sex determination (Y chromosome)

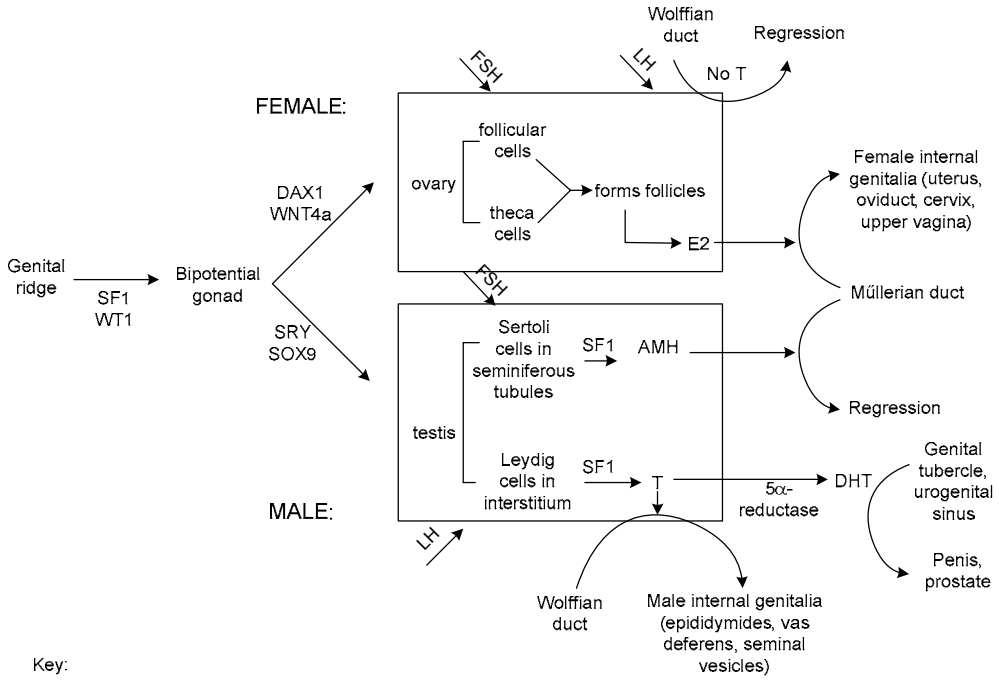
Primary sex determination is chromosomal; the male is XY and the female is XX. The Y chromosome carries a SRY gene that encodes a testis-determining factor. If the conceptus is XY, the indifferent gonad will form a testis in which T will be produced, and the prenatal infant will form male internal and external sexual organs. If the conceptus is XX, the indifferent gonad will form an ovary in which 17ß-estradiol (E2) will ultimately be produced, and the prenatal infant will form female internal and external sexual organs or structures. The female phenotype is, in fact, the default state. Internal and external female structures will form even in the absence of gonads (gonads removed when they are still indifferent or due to a genetic lesion). The female conceptus receives E2 prenatally from her mother through the placenta, and she cannot synthesize E2 in her ovaries *in utero*, or in her adrenal glands, because a number of P450 isoforms are missing (Greco and Payne, 1994). The T and DHT, made by the male conceptus, act on the development of his internal and external genitalia.

Secondary sex determination (phenotype)

Secondary sex determination involves the sex-specific structures (phenotype), excluding the gonad. A male mammal has a penis, epididymides, seminal vesicles and coagulating glands, a prostate gland, bulbourethral (Cowper's) glands, preputial glands, as well as sex-specific LABC muscle. A female mammal has oviducts, uterus, cervix, vagina, and mammary glands. Each sex has sex-specific size, vocal cartilage, and musculature. These secondary sex characteristics are determined, initiated, and maintained by hormone secretion from the gonads (but see above for structural females developing without gonads).

The scheme of mammalian sex determination is shown in **Figure 1**. If the Y chromosome is absent, the gonadal primordia will develop into ovaries, and E2 from the mother (and offspring) will cause the Műllerian duct from the mesonephric kidney duct (used as part of the renal system in water-dwelling animals) to form the upper portion of the female reproductive tract (oviducts, uterus, cervix, and upper end of vagina). If the Y chromosome is present, the testes form from the indifferent gonad and produce two major hormones, anti-Műllerian hormone (AMH), also known as Műllerian inhibiting substance (MIS), which destroys the Műllerian duct. The second hormone, T (and DHT), masculinizes the fetus, stimulating the formation of the internal and external genitalia, including the penis and scrotum, and inhibiting the development of breast primordia (Gilbert, 1997).

Figure 1. Mammalian sex determination



Key:

SRY = SRY gene (on Y chromosome - begins male-specific cascade)

SOX9 = SOX9 gene (autosomal)

AMH = Müllerian inhibiting substance; anti-Müllerian hormone (AMH; made in Sertoli cells, causes Müllerian duct to regress in males)

E2 = 17ß-estradiol; initially from mother, then made in ovary

DHT = 5α-dihydrotestosterone; made from T by 5α-reductase enzyme, predominantly in the testis (also in other end organs)

FSH = follicle-stimulating hormone

LH = luteinizing hormone

T = testosterone

Modified from S.F. Gilbert, 1997 (p. 775, Figure 20.2)

Embryologically, the genital ridge is bipotential; it can differentiate into an ovary or a testis, depending on the hormonal milieu. In humans, the genital ridge (or gonadal rudiment) forms in the intermediate mesoderm in the dorsal region on both sides of the developing central nervous system (CNS) during gestational weeks 4-7. During this indifferent stage, the epithelium of the genital ridge proliferates into the loose connective mesenchymal tissue. These cells form sex cords that will surround the germ cells that migrate into the gonad (from the yolk sac external to the body during week 6). During this period in XX and XY gonads, the sex cords remain connected to the surface epithelium.

If the fetus is XY, the sex cords continue to proliferate through the eighth week, extending deeply into the connective tissue. These cords fuse with each other to form a network of internal (medullary) sex cords and, at its distal end, the rete testis. The testis cords then lose contact with the surface epithelium and become separated from it by a thick extracellular matrix, the tunica albuginea. These cords remain solid during fetal life and childhood. At puberty, the cords hollow out to form seminiferous tubules, and the germ cells begin sperm production, supported by Sertoli cells (which also make AMH and are differentiated from the cells of the early testis cords), and triggered by T produced in the interstitial cells of Leydig (which are differentiated from mesenchymal cells). The sperm are transported from the lumina of the seminiferous tubules in the testes through the rete testis, which joins the efferent ducts (vas deferens remnants; of the mesonephric kidney), which link the testis to the Wolffian duct (which was the collecting tube of the mesonephric kidney). The Wolffian duct differentiates into the epididymides and vas deferens. The sperm from the testes and the seminal fluid, produced in the sex accessory glands (prostate, seminal vesicles, coagulating glands, Cowper's gland, etc.), move along the vas deferens to the caput (head), corpus (body), and cauda (tail) of the epididymides where they are stored (transit time from head to tail in rats is seven to ten days). The sperm acquire motility and fertilizing ability in the epididymides, and are ejaculated from the caudae epididymides through the vas deferens, the urethra and the penis. From puberty to death in male mammals, T maintains the structures and functions of the sex accessory glands (DHT, the more potent and more strongly bound androgen, plays a dominant role in *in utero* sexual development and less at and after puberty). T is responsible for development and maintenance of the testes, epididymides, vas efferens, vas deferens, seminal vesicles (plus coagulating glands), LABC muscle, testis descent, puberty, and most male secondary and tertiary sex characteristics. DHT is responsible for the development of the male urethra and prostate, the formation of the penis and scrotum from embryonic genital folds, and scrotal and penis growth and development.

If the fetus is XX, the germ cells also migrate into the indifferent gonad but reside near the outer surface of the gonad. The initial intragonadal sex cords regress and a new set of sex cords forms clusters around each germ cell. The germ cells will become ova, the clusters of sex cords will form the granulosa cells, and the mesenchymal cells of the ovary will form the thecal cells. These granulosa and theca cells will combine to form a follicle around each ovum and synthesize E2. The Műllerian duct remains intact and forms the oviducts, uterus, cervix, and upper vagina, and the Wolffian duct, in the absence of T, degenerates (Gilbert, 1997).

The genes involved in the differentiation of sex-specific gonads (see **Figure 1**) are:

- The SRY gene on the Y chromosome, which is the testis-determining gene located to a region near the tip of the small arm of Y. The SRY gene acts in the genital ridge immediately before and during testis differentiation (expressed in the somatic cells of the indifferent gonads), and expression is lost after the testis is formed. SRY is necessary, but not sufficient, for development of the mammalian testis. A conformational change in the SRY gene DNA is required (to allow distantly bound proteins to interact). Since SRY encodes a transcription factor, the search began for activation or suppression of "downstream" genes expressed in the genital ridge.

- The first downstream gene, likely activated by SRY-encoded protein transcription factors, is SOX9, which is essential in humans for testis formation and is expressed in the genital ridge just after SRY. In addition, SF1 (steroidogenic factor 1) is a transcription factor which is activated by SRY and, in turn, activates several genes involved in steroidogenesis in the Leydig cells. SF1 also plays a role in development of the adrenal glands and the gonads (the gonads develop but then degenerate in its absence) and is involved directly with testis development, activating both the Sertoli AMH and Leydig T synthetic pathways.

Puberty in the male, triggered by a surge in T (preceded by a surge in LH), initiates the completion of spermatogenesis and development of tertiary sex characteristics (muscle mass, hair distribution, voice changes, behaviors, etc.). The production of T continues to maintain the testicular spermatogenesis and the structures and functions of the secondary and tertiary sex characteristics.

Removal of the testis when it is an indifferent gonad *in utero* results in a female phenotype. Removal of the testis after puberty (castration) results in regression of the sex accessory glands and loss of the male phenotype (including upper body strength, male pattern baldness, body hair, etc.). Administration of exogenous T *in utero* (in the absence of endogenous T) restores the development of the phenotype, and administration of exogenous T after castration postpuberty restores the secondary and tertiary structures and functions (Gilbert, 1997). This sequence of castration, regression, administration of exogenous hormone, and regrowth of the sex accessory structures forms the basis for development of assays to assess active androgens (and anti-androgens).

History of Androgenic (and Anti-androgenic) Assays

Comb growth in capons (castrated male chickens)

The earliest and most widely used assay for testicular hormone involved measuring the comb growth of capons (castrated male chickens) after injection of the test material, reported by many researchers (*e.g.*, McGee, 1927; McGee *et al.*, 1928; Dodds *et al.*, 1930; Freud, 1933; Funk and Harrow 1929, 1930; Koch and Gallagher, 1929;

Gallagher and Koch, 1930, etc.; cited in Korenchevsky, 1932), with details provided by Gallagher and Koch (1930). Upon castration, the male comb regresses in the absence of "testicular hormone." Injection of an endocrine-active material causes the comb to grow. The extent of the growth was used as a measure of potency of the test materials (all at the same dose).

The strengths of this test (Korenchevsky, 1932) include: 1) only a short time (five days) is necessary for its completion; 2) the results are precisely expressed as increases in length and height of the comb; and 3) the same birds can be used for several assays since the comb regresses to its initial size after injections cease. However, this test also has weaknesses. The comb is present in both sexes of chickens, although differing in size and appearance, and comb growth can be obtained in birds of both sexes after injection of the hormone (Freud, 1933; Champy, 1931; cited in Korenchevsky, 1932). This weakness could also be considered a strength. It is likely that comb growth in both sexes is sensitive to androgens just as vitellogenesis, the formation of yolk in females (which is under E2 control), can be induced by estrogenic compounds in male fish, amphibians, reptiles, and birds. Also, injections of yolk or ovarian extract cause similar comb growth to that produced by testicular extract (Champy, 1931; cited in Korenchevsky, 1932), so (in modern terms) estrogenic as well as androgenic compounds can cause comb growth. In fact, Champy (1930; cited in Korenchevsky, 1932) considered the hormone-producing comb growth assay to be specific and useful for both sexes. From a more modern perspective, Robaire *et al.* (1979) reported that exposure to E2 in the male rat reduced prostate, seminal vesicle, and testis weights and epididymal sperm counts, with the effects considered due to reduced serum luteinizing hormone (LH) and therefore reduced T from the inhibiting action of estrogens at the hypothalamus-pituitary level in males. Also, in support of the effects of E2 on males, Hess *et al.* (1997) identified E2 receptors in the testis, efferent ducts, epididymides, and prostate of the male mouse. Male mice carrying a null mutation in the E2α-receptor gene were infertile, due (at least in part) to the failure of the afferent ducts to resorb fluid (Hess *et al.*, 1997). The complexity of the feedback loops from sex steroid hormones to the hypothalamus-pituitary in mammals (and birds) created difficulties in interpretation of the results of the comb test in the absence of knowledge on the feedback from the hypothalamic-pituitary-gonadal (H-P-G) axis.

A second weakness is that an extract that is inactive on comb growth can be active on the growth of genital organs of castrated guinea pigs and vice versa (Champy, 1931; cited in Korenchevsky, 1932). From the perspective of 2006, this is not necessarily a disadvantage; different organs in different species may be differentially sensitive to different androgens. The comb test fell out of favor for a number of reasons, not the least of which was the development of the mammalian assay (see below).

A third weakness is that although low doses give satisfactory results in this assay, high doses of testicular preparations produce "exceedingly irregular results" (Gallagher and Koch, 1930; cited in Korenchevsky, 1932). For example, when 45 capons were injected with the same dose of the same testicular preparation, the results were 0% comb growth in 2.2%, 2 mm in 13.3%, 3 mm in 15.5%, 4 mm in 28.8%, 5 mm in 28.8%, 6 mm in 4.4%, and 7 mm in 6.6%. However, this is not necessarily a disadvantage since the

data sort into a bell-shaped curve, with an overall mean of ~3.8 mm, and individual variability in response is the rule, not the exception. These data probably imply that a large number of animals must be tested to obtain sufficient statistical power to detect an effect.

Mammalian assays

Mammalian assays were also being proposed or developed at this time (late 1920s, early 1930s) to identify testicular hormones. Korenchevsky (1932, with references therein) describes them as follows:

- In rats: assessment by sperm motility tests, prostate cytology, seminal vesicle cytology, vas deferens cytology, and Cowper's gland cytology;
- In mice: assessment by seminal vesicle cytology; and
- In guinea pigs: assessment by electroejaculation test.

These tests in the various species were viewed as satisfactory by researchers during this time (late 1920s to early 1930s) (*e.g.*, Korenchevsky, 1932, and others) to detect testicular hormones. However, Gallagher and Koch (1930; cited in Korenchevsky, 1932) criticized them as taking too long, lacking quantitative precision, and the need for operating on new animals for each assay. Two additional assays were proposed by Korenchevsky *et al.* (1932):

- Measurement of the size of the prostate and/or seminal vesicles in rats; this assay was discarded due to contradictory results (Freud, 1930; Dodds *et al.*, 1930; cited in Korenchevsky, 1932); and
- Weighing the prostate plus seminal vesicles and the penis in castrated rats.

Evolution of the Castrated/Intact, Immature/Mature Rat Assay (1930s-1950s)

In 1932, Korenchevsky described in detail, procedures for the assay in bullet #2 above, and provided data on organ weights (not linear measurements, which were considered unsuitable because of the difficulty in accurately measuring glands of irregular shape and the need for length and height or width to characterize the organ's size) of castrated rat males with and without injection of testicular hormone. Korenchevsky weighed the prostate with seminal vesicles, the penis, adrenal glands, thyroid gland, hypophysis (pituitary gland), and retroperitoneal fat. The selection of organs was based on the atrophy of the prostate, seminal vesicles, penis, and thyroid with castration; the hypertrophy of the adrenals and hypophysis with castration; and the increase in retroperitoneal fat in castrated male rats and their return to normal with injections of testicular hormone. He standardized the weights of the organs as relative to a rat body weight (200 g), used matched pairs of rats from the same litter (one with and one without injection), standardized the diet, and standardized the route and duration of injections (s.c., for six to seven days). However, he did not standardize the age at

castration or the time from castration to the start of injections, indicating only that "the pairs differed in age and in the period of time since castration," and also did not standardize the age at termination (Korenchevsky, 1932; p.421).

Korenchevsky and Dennison (1935a) evaluated the effects of the newly discovered androgen, transdehydroandrosterone (previously, only androsterone, T, and oestrone were known to be present in reproductive organs and urine), on gonadectomized male and female rats. For the males, there was no information on the age at castration or the time from castration to treatment. The duration of treatment was 7 or 21 days, with sc injections twice daily of the test material in sesame oil (at three different doses) or sesame oil alone (vehicle control). At necropsy (specified at 57-65 days or 68 and 86 days of age), body weights and the weights of the seminal vesicles, prostate, seminal vesicles plus prostate, penis, preputial glands, adrenal glands, hypophysis, thymus, liver, kidney, and heart were recorded. Korenchevsky and Dennison also recognized that the principle and approach of this male assay could be applied to a female assay to identify estrogens (and anti-estrogens). They used each assay to inform and enhance the other as they built the understanding of the hypothalamic-pituitary-gonadal axis.

For the females, the following information was provided. Ovariectomy occurred "before sexual maturity," and injections were begun 14-34 days after ovariectomy and continued daily for 21 days. The females were terminated at 61-77 days of age. The female body weights and weights of the following organs were recorded: uterus (in diestrus), cervix, vagina, preputial glands, adrenal glands, thyroid glands, hypophysis, thymus, liver, kidney, heart, and retroperitoneal fat. This study was important for two reasons:

- Korenchevsky and Dennison (1935a) defined a "rat unit" (a specific level of quantitative response on specific organs) as resulting from 8γ of T, 20γ of androsteronedial, 170γ of androsterone, and 940γ of transdehydroandrosterone to characterize relative potency. They also refined Korenchevsky's previous work on the comparison of capon units and rat units for relative potency of various androgenic/anti-androgenic compounds (Korenchevsky *et al.*, 1932, 1935).

- This work on ovariectomized female rats, with or without injections, was the first step toward the development of the uterotrophic assay in mature ovariectomized female rats for detection of estrogenic (and anti-estrogenic) compounds.

Korenchevsky *et al.* (1933, 1935b; Korenchevsky and Dennison, 1935a,b) continued to explore the potency of various testicular hormonal preparations, the effect of differing durations from castration to treatment, and of differing durations of treatment. He and his co-authors began to specify the age at castration (*e.g.*, 22-29 days of age), and the duration between castration and treatment (*e.g.*, 28-42, 32-75 days). Korenchevsky *et al.* (1937) also examined the response of castrated and ovariectomized rats to prolonged treatment with TP which had been shown to be the most effective of the T metabolites (esterification increased the androgenic activity of T) from short-term treatment studies.

TP also prolonged the duration of the effect of a single injection or two injections (five days apart), with the maximum effect observed in the 11th day after the first injection, with activity lasting two to four weeks, versus injection of T, with the maximum effect observed "a few days" after the injection and activity gone by the 11th day postinjection. They, therefore, dosed the male rats with TP or T for 23 days and terminated the males one or nine days after the last injection. TP was more effective than T, and the changes persisted (partially or completely) out to nine days after the last dose.

Korenchevsky *et al.* (1937) also dosed ovariectomized females with TP, T, estrone, or TP plus estrone. The age of the females at castration was not provided, nor was the interval from castration to start of treatment. The duration of treatment was 21 consecutive days, and the females were terminated at 56, 61, 82, or 106 days of age. At the necropsy, the typical list of organs were weighed, plus the addition of vaginal smears to detect vaginal cornification (*i.e.*, persistent estrus) and examination of the uterus for distension (at least) to enlarged and thickened horns. The authors reported that the vaginal cornification test was "unreliable and insufficient;" organ weights plus histopathology were both "satisfactory and accurate." TP caused more profound estrogenic effects in the female than estrone or T. TP plus estrone resulted in abnormally large vaginas and female preputial glands, with histological changes in the uterus and vagina "reminiscent of the changes observed during pregnancy in normal rats," and the peripheral end of the female uterus (with TP or T injections) reportedly developed into a structure "having the appearance of a clitoris" (Korenchevsky *et al.*, 1937; p. 484).

Additional researchers in the 1930s continued to evaluate both the chemicals in the assay and the castrated male assay itself in the rat (early work did not specify the rat strain), varying the numbers per group, number of groups, the age at castration, the duration between castration and the start of treatment, duration of the treatment, duration from end of treatment to termination, which organs were weighed, and organ weights fresh or fixed (usually in Bouin's fixative). Organ weight data were presented as absolute weight in mg or gram, and/or in mg or g per gram body weight, per 200 g body weight, or per kg body weight (*e.g.*, David *et al.*, 1934; Callow and Deanesly, 1935; Bülbring and Burn, 1935; Dingemanse *et al.*, 1935; Deanesly and Parkes, 1936).

Deanesly and Parkes (1936) used rats castrated at 40-50 g (likely on PND 21 at weaning, based on the weight) and used "not less than one month later," with administration of one of 11 androgen-like compounds by sc injection for ten days. At termination, prostate and seminal vesicles, fixed in Bouin's, were weighed. Several compounds were administered at different doses, with dose-response curves presented. Responses were specific for the organs weighed. Dingemanse *et al.* (1935) used rats castrated at 21-25 days of age and not used until 6-6.5 months of age. There were five rats/group, dosed with urine or testicular extracts for 24 days by twice daily sc injections. At termination (age not specified), the prostate, seminal vesicles, and penis were weighed.

Greene and Burrill (1940) castrated immature rats at 32-38 days of age. Twenty rats/group were administered TP by sc injection once 24 hours after castration. At termination, 24 hours after the single dose, some organs did respond significantly at

higher doses. The prostate weight increased by 30%, and seminal vesicle weight increased by 16.5%. Considerable variability in organ weight was present within groups. In a second paper, Greene *et al.* (1941) used immature rats castrated at 22-24 or 33-38 days of age. Castrated rats were administered TP by sc injection 48 hours postcastration and terminated 48 hours after injection, with seminal vesicles weighed. Organ weight variability was greater at 33-38 days of age than at 22-24 days of age. There were statistically significant differences between organs from treated versus nontreated castrates evaluated at 22-24 days of age.

Mathieson and Hays (1945) used immature Wistar male rats as well as a series of castrations at different ages, recovery times, and dosing times. The animals were treated with TP by sc injection during several time periods for two- to five-day dosing durations. At necropsy, only seminal vesicles were weighed. Their results indicated that atrophy of the seminal vesicles was a slow process once systemic growth had been stimulated after days 35-38. They concluded that rats castrated at 50 days of age were unsuitable due to slow regression of the seminal vesicles after castration and inconsistent responses to TP (a very potent androgen). They strongly recommended the juvenile castrate.

In the late 1940s and early 1950s, Eisenberg and Gordan (1950) and others published a number of papers on the "myotrophic assay" in rats. This assay evaluated the protein anabolic activities of androgens by measuring the oxygen uptake and/or the weight of the androgen-dependant levator ani muscle in castrated rats receiving different androgens. In 1949, Eisenberg *et al.* used the Long-Evans and Slonaker-Wistar rats (four to five rats per group), castrated them at 30 days of age (prepubertal) with no apparent recovery period, and dosed them by sc injection once daily for 30 days with one of nine compounds plus pituitary growth hormone. They reported the oxygen uptake and weights of the liver and levator ani muscle (the weights of other sex accessory glands were not reported; the focus was on anabolic activity). Eisenberg and Gordan (1950) used the Long-Evans rat, castrated at approximately 30 days of age (prepubertal), with a 23-day recovery. The rats were then dosed by sc injection for seven consecutive days with one of nine compounds, including estradiol dipropionate. The authors concluded that the levator ani weight was a reasonable indicator for anabolic activity from androgenic substances, but its growth was not wholly androgen dependent. Seminal vesicles were also weighed. The seminal vesicle response was androgen dependent, although estradiol also stimulated seminal vesicle weight. As the assay evolved, the response of the levator ani muscle (wet or dry weight) was termed the myotrophic response, and the response of the sex accessory glands (initially weighed as one unit, then separately) was termed the androgenic response. Gordan *et al.* (1951) reported that different androgens expressed different myotrophic and/or androgenic potencies. For example, methylandrostenediol was a potent protein anabolic steroid with little androgenic activity using this assay.

The Hershberger assay

Hershberger *et al.* (1953) improved and standardized the prepubertal castrated male assay as follows.

- Male rats were castrated on PND 21 (day of weaning).
- The castrated rats were dosed with the test material by sc injection for seven consecutive days, beginning on the day of castration (no recovery period).
- On the eighth day postcastration, 22-26 hours after the last injection, the animals were terminated.
- The levator ani muscle, ventral prostate, and seminal vesicles (free of the coagulating glands) were dissected from each male and weighed.
- Dry weights of the levator ani muscle were also recorded after desiccation at 72°C.
- The results, expressed as organ weights versus a range of doses of each of many test materials, provided information on the relative potency of each test material for androgenic activity (from ventral prostate and seminal vesicle weights) and/or for myotrophic activity (from the levator ani muscle weight).

In 1954, Eisenberg and Gordan reasoned that: (1) since the growth of the levator ani muscle of castrated, immature male rats was induced by various anabolic agents, (2) since the amount of growth paralleled the anabolic potency of these agents, and (3) since purified pituitary growth hormone extract also stimulated growth of the levator ani, then it is likely that the muscle could be used as an index of changes in nitrogen balance induced by steroids and other growth-promoting substances. Therefore, they studied whether the levator ani muscle changes also reflected the loss of protein that accompanied growth-inhibiting conditions, such as hypothyroidism. Since small doses of thyroid hormone are known to be anabolic in hypothyroid rats and large doses are catabolic, the weight of the levator ani muscle was measured after induction of hypothyroidism, after hypothyroidism treatment with low doses of thyroid hormone, and after induction of severe hyperthyroidism. The effect of the catabolic steroid, cortisone, was also studied. Long-Evans rats were divided into seven groups:

- Normal controls
- Hypothyroid rats with intact gonads (made hypothyroid by 0.1% propylthiouracil in the drinking water during the second month of life)
- Hyperthyroid rats with intact gonads (made hyperthyroid by feeding each rat 30 mg of thyroid substance every other day from the 35^{th} to the 60^{th} day of life; the thyroid-dependent oxygen consumption in liver slices for these rats was two-fold the value of liver slices from normal animals)

- Castrate controls (castrated on PND 30; prepubertal)
- Castrate hypothyroid animals
- Castrate hypothyroid rats treated with 2 mg desiccated thyroid substance daily from the 53rd to the 60th day of life
- Castrate animals treated with cortisone (0.1 mg of cortisone in sesame oil) by sc injection for seven days preceding necropsy

All animals were terminated by decapitation on the 60th day of life, and the levator ani and seminal vesicles were removed and weighed. Their results were as follows:

- Relative to the normal control values, terminal body weights were reduced in the hypothyroid and hyperthyroid rats with intact gonads.
- In the hypo- and hyperthyroid animals with intact gonads, the weights of the levator ani muscle and the seminal vesicles were reduced relative to the normal control values (but see #1 result).
- In the hyperthyroid group, body weight and weight of the levator ani were both lower than those of castrate rats (the hyperthyroid group values were approximately normal for rats at 30 days of age, the age at castration).
- The seminal vesicles in the hyperthyroid group weighed 2.5 fold more than in the castrate animals.
- In the castrate hypothyroid group, body weight was statistically equivalent to that in the hypothyroid group with intact gonads but lower than that of the intact or castrate controls.
- In the castrate-hypothyroid group, the levator ani muscles weighed only slightly less than those in the castrate control rats, but only 50% of the weight of the levator ani in the hypothyroid group with intact gonads.
- Administration of thyroid substance to the castrate hypothyroid group resulted in restoration of the weight of the levator ani muscle and gain in body weight, but there was no effect on the weight of the seminal vesicles.
- Injection of cortisone for seven days prior to termination caused further atrophy of the already atrophic levator ani muscle ($p<0.01$) but did not affect the weight of the seminal vesicles.

The authors concluded that the weight of the seminal vesicles was completely dependent on the presence of androgen, but that the growth of the levator ani was not solely an androgenic phenomenon since the levator ani muscle in the castrate rat grew

with treatment with thyroid hormone, with pituitary growth hormone, with nonandrogenic doses of methylandrostenediol (Gordan *et al.*, 1951), or with nonandrogenic molecules. In all of these cases, the weight of seminal vesicles remained unchanged, so the assumption was that there was no androgenic activity.

In addition, Hershberger-like protocols for the pharmaceutical industry (*e.g.*, Dorfman, 1969a,b) and a regulatory screen for steroidal androgens (Hilgar and Vollmer, 1964) have been published. More recently, Ashby and Lefevne (2000a), Yamada *et al.* (2000), and Yamasaski *et al.* (2001) have investigated protocol variables with weak anti-androgens.

The Hershberger assay, employing peripubertal castrated male rats, was introduced in Chapter 2 and described in detail in Chapter 7. Its strengths are its usefulness, its specific nature (it can identify mechanism), its relative speed (the males are castrated at 42 days of age, treated for ten days starting 12 days postcastration from PND 53-54 to 64-65, and terminated immediately after treatment ceases; Gray *et al.*, 2005), and relatively low cost (Hershberger *et al.*, 1953; Dorfman, 1962a,b; Dorfman and Dorfman, 1963; Dorfman and Kincl, 1963).

Molecular enhancments for the Hershberger assay

Recent work has exploited the Hershberger bioassay to obtain AR binding data, to develop ancillary transcriptional reporter gene assays, and to characterize the global response to castration and TP supplementation using gene arrays. In 1996, Yamashita *et al.*, reported on the effects of various interferences with androgen action in the rat by a two-week treatment with leuprolelin (an LH-releasing hormone analog), finasteride (a 5α-reductase inhibitor), diethylstilbestrol (DES), or by physical castration. The focus was on the effects on the coagulating gland, ventral prostate, lateral type 1 prostate, lateral type 2 prostate, and dorsal prostate (considered by some researchers and anatomists as all part of the prostate complex), assessed morphologically and quantitatively by DNA content, wet weight, protein content, and zinc concentrations. Castration produced the most profound reductions in the above endpoints, with DES and leuprolelin causing intermediate effects, and finasteride causing the smallest reductions. Morphological changes from all treatments included reductions in epithelial height, relative increase of connective tissue, and reduction in ductal diameter, length and number. Different lobes of the prostate responded differently to the different treatments (*e.g.*, regressive morphological changes were striking in the coagulating gland and in the ventral prostate, and indistinct in the lateral type 2 prostate). Prostatic zinc concentration in control and treated rats was highest in the lateral type 2 prostate and was reduced, after treatment, to the greatest extent in the dorsolateral prostate (lateral type 1 and 2 and dorsal prostatic lobes). The zinc distribution paralleled the metallothionein (zinc carrier protein) expression found only in the lateral type 2 prostate of control and treated rats. Intraprostatic regional heterogeneity of zinc and metallothionein expression likely indicates differences in lobular function within the rat prostate (Yamashita *et al.*, 1996).

Nishi *et al.* (1996) investigated the changes in steady-state levels of mRNAs coding for several growth factors and their receptors (by Northern blot analysis) during castration-induced involution and subsequent regrowth induced by an androgen in the ventral and dorsolateral lobes of the rat prostate. The changes in growth factor systems and typical secretory proteins in the ventral lobe were similar to (but more prominent than) those in the dorsolateral lobe, consistent with greater androgen dependency in the ventral lobe. The growth factors and their receptors investigated were prostatein, probasin, dorsal protein 1, H4 histone, epidermal growth factor (EGF) rat, EGF mouse, EGF receptor, transforming growth factor (TGF-α, TGF-ß1, TGF-ß), type II receptor, methionine (c-met) and its receptor, basic fibroblast growth factor (bFGF), FGF receptor 1, keratinocyte growth factor (KGF), and hepatocyte growth factor (HGF).

EGF showed apparent positive androgen dependence: EGF mRNA in the ventral lobe dropped to 30% of normal within 24 hours after castration and increased to 200-300% of normal within three to five days after androgen administration to the castrated rats. mRNAs coding for all of the other factors examined increased after castration and after a brief leveling or decrease, some of them (TGF, EGF, KGF, and c-met) increased again to peak levels by three to five days after hormone replacement. The authors concluded that multiple growth factor receptor systems participate in both the regression of castrated male prostates and their androgen-dependent regrowth (Nishi *et al.*, 1996).

Yuasa *et al.* (1997) studied the changes in tissue localization of nuclear estrogen receptor by immunohistochemistry in the seminal vesicles of immature castrated rats without or with postcastration treatment with E2 and or DHT. In rats castrated at six weeks of age, the percentage of nuclear ER-positive cells increased greatly in the periglandular stromal region but not in the epithelium or peripheral stromal region of the seminal vesicles. E2 administration to the castrated rats dramatically increased the percentage of nuclear ER-stained cells in both the epithelium and peripheral stromal region, with significant percentile decreases in ER-stained cells after E2 was withdrawn. The treatment with both E2 and DHT completely inhibited an ER-mediated ER expression in the epithelium and stroma. The authors concluded that ER-stained cells increase only in the presence of E2 and in the absence of DHT in the seminal vesicle of castrated rats (Yuasa *et al.*, 1997).

Gray *et al.* (1997) conducted a workshop on endocrine screening methods in 1996, including hazard identification methods to detect androgenic and anti-androgenic activity *in vitro* and *in vivo*. The discussion on screening for anti-androgens that act through the AR discussed three variations and/or additions to the Hershberger *in vivo* assay:

- To detect AR-mediated toxicants, specifically anti-androgens, individual sex accessory gland weights (dorsolateral and ventral prostate or seminal vesicle) are measured in castrated T or TP treated (injections or implants) prepubertal or adult male rats after four to seven days of treatment with the test chemical. Other endpoints that could be evaluated include AR distribution, TRPM2/C3 (T-repressed prostatic message 2/prostate-specific binary protein polypeptide

C3) gene expression, ODC (ornithine decarboxylase), and/or 5α-reductase activity in the prostate.

- In addition, on a case-by-case basis, use of AR binding (competitive binding assays using isolated receptor or whole cell binding assays) can be somewhat instructive.

- As needed, reporter gene assays can be used with transiently transfected cell assays to distinguish between AR agonist/antagonist activity.

- To detect androgenic action, TP or T hormone administration is deleted from variations 1 and 3 (Gray *et al.*, 1997).

The workshop participants also discussed sex accessory gland weight and alterations of gene expression *in vivo*. It was noted that the use of castrated males can distinguish between direct or indirect effects on sex accessory gland weights from toxicant exposure as follows. In intact males, alterations in prolactin, growth hormone, gonadotropin (LH and FSH) secretion, or hypothalamic lesions can affect pubertal maturation due to the intact hypothalamus-pituitary-gonad axis, so changes in sex accessory gland weight can be due to any number of potential mechanisms. Even with castration, the growth and maintenance of sex accessory glands can be influenced by thyroid hormone, growth hormone, prolactin and/or EGF (epithelial growth factor), as well as the predominant T and DHT. Even exposure to estrogenic compounds (*e.g.*, pesticides) can reduce sex accessory gland size. Other useful endpoints to identify the mechanisms of action include serum levels of T, DHT, and LH, AR distribution, TRPm2/C3 gene activities (also discussed in the EDSTAC Report, 1998), ODC, and 5α-reductase activities. There is a differential dependence on various hormones in the various sex accessory glands. For example, the prostate depends on enzymatic activation of T to DHT by 5α-reductase, and in contrast, the seminal vesicles are less dependent on this conversion. Therefore, effects on 5α-reductase (which converts T to DHT) can be distinguished from AR-mediated mechanisms by determining whether the prostate is preferentially affected. Growth of the levator ani muscle is T dependent, with little capacity to convert T to the more potent DHT, so weight of this muscle is useful to identify anabolic androgens and anti-androgens. This assay is used extensively by the pharmaceutical industry, with identification of androgens done in the absence of T or TP hormone administration, and identification of anti-androgens done in the presence of T or TP administration (Gray *et al.*, 1997).

There are also cell-free "simple" binding assays used to determine the ability of environmental chemicals to compete with endogenous ligand for binding to AR isolated from castrated rat reproductive tissue. The assay can be run with increasing concentrations of radiolabeled ligand at different fixed concentrations of inhibitor or with a fixed concentration of radiolabeled androgen, with increasing concentrations of unlabeled competitors (Freyberger and Ahr, 2004).

Separation and measurement of protein-bound ligand from free ligand is plotted versus competing inhibitor concentration. The data yield apparent equilibrium binding

affinity constants for the inhibitor (Ki) which reflects the affinity of the chemical for the AR. Ki values can be used to rank chemicals for their ability to bind AR and therefore their potential to induce adverse effects. IC_{50} (chemical concentration at 50% inhibition) values can be used to calculate Ki and the relative binding affinity of the toxicant to AR as compared to DHT or T. The disadvantages of this assay are that radiolabeled ligands are needed, there is no information whether AR binding results in agonist or antagonist activity, and there is no information on AR stabilization, degradation, or rates of association and disassociation from the AR. There is also no metabolic capability in the assay, so parent compound and/or metabolites would have to be individually examined to determine what the active moiety is for AR binding. Interestingly, Sato *et al.*, (2001) have reported that several environmental pollutants have binding affinities for both AR and ERα; some researchers consider the sex steroid receptors to be a bit "promiscuous".

AR whole-cell binding assays use mostly kidney COS cells transiently transfected with a cDNA encoding the human AR (hAR), resulting in high level expression of hAR. The transfected cells are incubated in the presence of a single saturating concentration of radiolabeled ligand and increasing concentrations of the toxicant/competing ligand. After the incubation, the bound ligand is separated from the free ligand, and the bound ligand is assessed by liquid scintillation counting. The dose-response data are typically presented as IC_{50} values (concentration of inhibitor necessary to reduce specific ligand binding by 50%).

AR transcriptional activation (cis-trans) is evaluated in monkey kidney CV1 cells. Transcriptional activation evaluates not just AR binding but whether the chemical acts as an AR agonist or antagonist. These cells are also transiently transfected with a hAR expression vector, together with a reporter construct (*e.g.*, ß-galactosidase, firefly luciferase, or CAT) containing an AR-dependent promoter such as the mouse mammary tumor virus promoter. Transfected cells are cultured for 48 hours in the presence (for antagonistic activity) or absence (for agonist activity) of a single concentration of androgen (*e.g.*, 0.1 nM DHT), together with increasing concentrations of inhibitor. After incubation, luciferase activity is measured in the solubilized cell extract as an estimate of AR-induced transcriptional activity in the presence of an androgenic chemical (agonist activity), or to detect inhibition of transcription with androgen + test chemical versus the activity of androgen alone (antagonistic activity).

AR DNA binding assays, using Sf9 insect cells and a baculovirus AR expression system (with hAR), provide information on mechanism of action for AR antagonists. Chemicals that bound to the StAR, and blocked endogenous ligand binding (*i.e.*, antagonists), reduced the intensity of the DNA mobility bands (the androgen response element DNA labeled with P^{32}) in a dose-response manner.

Finally, yeast-based AR assays with permanently transfected hAR and reporter gene can detect AR binding and transcriptional activation, but chemicals that bind to AR, activate transcription, and induce reporter gene activity can be either agonists or antagonists (the assay does not differentiate them).

Franck-Lissbrant *et al.* (1998) studied the castration-induced regression and T-stimulated regrowth of the vasculature in the rat ventral prostate lobe by stereological techniques. Seven days after castration of "adult" male Sprague-Dawley rats (age not given, but body weights were 285-430 g), the endothelial cell proliferation rate (bromodeoxyuridine labeling index), the total weights of the blood vessel walls, blood vessel lumina, endothelial cells, glandular epithelial cells, and total organ weight were all decreased. Within two days after the start of sc injection with T, the total weights of blood vessel walls, endothelial cells, and vascular lumina, as well as endothelial cell proliferation rate, had all returned to normal. In contrast to the rapid response of the vasculature, the total weight of the glandular epithelium and total organ weight did not return to normal levels during the four days of T treatment. Growth of the vasculature clearly preceded the growth of the glandular epithelium. The T-dependent factors stimulating the vasculature are unknown, but factors derived from epithelial cells, mast cells (which accumulate in the prostate during the first day of T treatment) and tissue macrophages could all be involved. This experimental model was therefore viewed as useful to study factors regulating angiogenesis and organ growth in the developing and/or castrated prostate.

Deckers *et al.* (2000) investigated the effects of mono- and di-substitutions of 11-methylene, Δ^{15}, and/or 18-methyl groups on the function and potency of unsubstituted norethisterone by *in vitro* binding transactivation assays on AR as well as progesterone receptors (PR), and by sc injections in *in vivo* animal models including a McPhail test for progestational activity in immature rabbits, an ovulation inhibition test in cycling rats, and the Hershberger assay for androgenic activity in immature castrated males. The results of the *in vitro* receptor binding transactivation assays agreed with the Hershberger assay results in terms of androgenicity (increase in ventral prostate weight), progestagenicity with the immature rabbit (endometrium proliferation), and inhibition of ovulation in the rat.

The translation of results from preclinical assays and tests to the clinical treatment of prostate cancer by androgen ablation typically includes surgical castration or administration of a luteinizing hormone-releasing hormone analog (LHRH-a). This latter treatment interferes with androgen production on the level of the hypothalamus (the hypothalamus produces LHRH, to stimulate pituitary LH release and testicular T production). However, although castration ablates androgen release from the testes, androgen biosynthesis in the adrenal glands (producing 8-10% of total circulating androgens) is not affected. Because of this, a widely used management strategy for advanced prostate cancer is combined androgen blockage (CAB) in which surgical or chemical blockade is combined with administration of an anti-androgen. Monotherapy with an anti-androgen is not yet a standard management option, but its benefit in conserving sexual potency and comparable efficacy between high-dose pure anti-androgen therapy at castration may speed its acceptance. The first anti-androgen used clinically was Cyproterone acetate (but it causes progestogenic side effects and liver toxicity). Several pure nonsteroidal anti-androgens have since been developed. These nonsteroidal anti-androgens have been shown to work through competitive inhibition of the binding of T, and its more potent and more strongly binding metabolite, DHT, to the nuclear AR. The *in vitro* and *in vivo* animal models were used to establish relative

affinities and potencies of various anti-androgens and to bracket likely effective doses in humans (Kolvenbag *et al.*, 1998). Again, preclinical data have been critical to transition drugs into clinical practice. These data have identified subtle differences between agents under development in terms of affinity, potency, pharmacokinetics, bioavailability, distribution, elimination and tolerability. This information is essential (not just useful) to appropriate dosing in first-time-in-man studies, but they are not absolute determinants since these parameters can differ for a specific agent between animal model(s) and humans (Kolvenbag *et al.*, 1998).

The Hershberger assay, as originally conceived, as currently employed and as enhanced with additional endpoints and evaluations, is a robust, specific assay to detect androgenic and anti-androgenic compounds, with a long successful history of use.

CHAPTER 4. BIOLOGICAL BASIS OF THE ASSAY/ANDROGEN MODE OF ACTION

Testosterone (T)

The reproductive system steroid hormones are produced primarily in the gonads, with some steroidogenic enzymative steps also found in peripheral tissues. In the male, the primary steroidogenic pathway is in the testes in the Leydig cells found in the interstitial spaces outside of the seminiferous tubules (and to a much lesser extent in the adrenal glands). In the Leydig cell, the steroidogenic pathway begins in the cytoplasm, with intermediate steps in the mitochondria and then in the cytoplasmic smooth endoplasmic reticulum (Chen *et al.,* 1996).

Synthesis

For this chapter, the steroidogenic pathway to T will be considered as those processes occurring in the testes after stimulation of the hypothalamic-pituitary-testis axis. The pathway begins in the Leydig cell with: (1) intracellular signal transduction, (2) continues with cholesterol production in the cytoplasm and its transport to the mitochondrial inner membrane, and (3) ends with a series of enzymatic conversions from cholesterol to T in the smooth endoplasmic reticulum of the testicular Leydig cell.

Signal transduction

LH from the anterior pituitary enters the Leydig cell by binding to the membrane-bound LH receptor. The LH receptor is coupled with a G-protein and, when stimulated (by LH binding), interacts with adenylate cyclase to form cyclic adenosine 3',5'-cyclic monophosphate (cAMP). Increased cAMP (termed the second messenger) stimulates protein kinase A, which initiates cholesterol biosynthesis and synthesis of the cholesterol transport protein (Cooke, 1996; Stocco, 1999). In order for maximal stimulation of steroidogenesis, intracellular calcium (Ca++) levels must increase after LH binding (Janszen *et al.*, 1976). The increase in intracellular Ca++ is due to release of Ca++ from intracellular storage depots and/or importation of extracellular Ca++ through cell membrane-bound calcium channels. Calmodulin (a calcium binding protein) is also involved (Hall *et al.*, 1981). This series of events also enhances cholesterol transport into the mitochondrion. Chloride (Cl-) is also involved in steroidogenic signal transduction in the Leydig cell mitochondrion (Choi and Cooke, 1990). Chloride channels are present in the Leydig cell membrane, and chloride conductance is stimulated by LH and cAMP.

LH stimulation increases the release of arachidonic acid in the Leydig cell (Naor, 1991; Cooke, 1996). Arachidonic acid appears to act as an intracellular mediator with direct inhibition and indirect stimulation on steroidogenesis. Steroid hormone production is inhibited when arachidonic acid activates protein kinase C. It is enhanced by the metabolites of arachidonic acid (*e.g.*, leukotrienes) stimulating cholesterol transport into the mitochondria.

Other intracellular substances also affect steroidogenesis, including free radicals (*i.e.*, superoxide anion and hydroxyl-free radical), hydrogen peroxide, nitric oxide, molecular oxygen, and hydrogen peroxide (H_2O_2), which affects cholesterol transport and inhibits 3ß-hydroxysteroid dehydrogenase, an enzyme that converts pregnenolone to progesterone (see below and **Figure 2**; Clark *et al.*, 1994) and nitrous oxide (Davidoff *et al.*, 1995).

Cholesterol synthesis and transport

Cholesterol from the serum is the common precursor to the synthesis of all gonadol steroid hormones. Cholesterol is transported to the Leydig cell by serum protein carriers (*e.g.*, high- or low-density lipoprotein). Once inside the cell, cholesterol is immediately utilized or stored in lipid droplets as cholesterol esters. A second minor source of cholesterol is *de novo* synthesis after hormone stimulation of the Leydig or follicle cells, beginning with acetate, which is converted to malonate, then squalene, and then lantosterol which is converted to cholesterol. LH-stimulated mobilization of stored or newly synthesized cholesterol occurs with cholesterol transported out of the Leydig cell cytoplasm into the mitochondria and from the outer to the inner mitochondrial membrane with a transport protein. LH stimulation of steroidogenic cells activates rapid synthesis of the cholesterol transport protein (requires *de novo* protein synthesis and has a short t ½ half-life). This essential protein mediates the rate-limiting step of steroid hormone production and is therefore designated as the steroid acute regulatory (StAR) protein. The StAR protein is synthesized in the cytoplasm as an inactive precursor molecule. It is transported into the mitochondrion where it is cleaved to its active form. In the mitochondrion, StAR protein transports cholesterol to the inner mitochondrial membrane where the series of enzymatic conversions of cholesterol to T take place. Steroidogenesis is therefore regulated by StAR protein production; the StAR gene is regulated by steroidogenic factor-1 (SF-1). SF-1 regulates the basal and hormone stimulated expression of the StAR gene. The effect of SF-1 is modulated by cAMP (so the signal transduction phase is linked to the control of the carrier protein). Other regulators of the StAR gene include estrogen, growth hormone, IGF-1, and calcium (which also up-regulates the StAR gene).

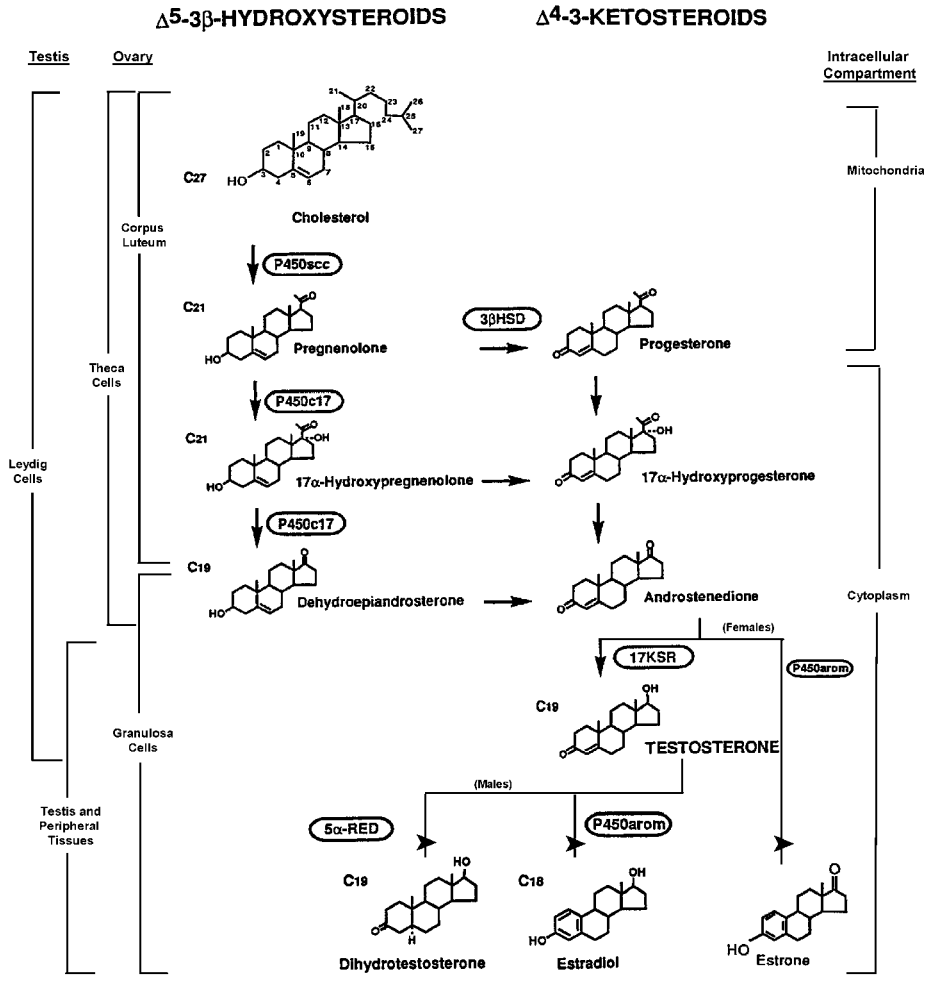
Enzymatic conversions

The StAR protein transports cholesterol from the outer to the inner mitochondrial membrane where the first cytochrome P450 enzymatic conversion takes place (see **Figure 2**).

The first enzyme reaction is the conversion of cholesterol to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc). This is also considered a rate-limiting step in the production of gonadal steroid hormones. This reaction on the inner mitochondrial membrane involves three oxidation reactions, each requiring molecular oxygen and NADPH. The reactions add two hydroxyl groups to cholesterol (one each at C_{22} and C_{20}), followed by cleavage between the added hydroxyl groups. Cholesterol (a 27-carbon steroid) is thereby cleaved of its 6-carbon group (the

"side chain"), resulting in pregnenolone, a 21-carbon steroid (Kagawa and Waterman, 1995).

Figure 2. Enzymatic conversions of cholesterol and intermediate/end-product hormones



The second enzymatic reaction results in the conversion of pregnenolone to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase Δ^5-Δ^4-isomerase (3β-HSD). This reaction also occurs on the inner mitochondrial membrane. 3β-HSD catalyzes dehydrogenation and isomerization to convert a Δ^5-3β-hydroxy steroid (pregnenolone) to a Δ^4-3-ketosteroid (progesterone), the active form of steroid hormone. Pregnenolone may also be converted to progesterone by 3β-HSD in the cytosol. Therefore, the steroidogenic pathway bifurcates into a Δ^5-hydroxysteroid pathway, starting with pregnenolone and a Δ^4-3-ketosteroid pathway, starting with progesterone. Even though the same enzymes participate in both pathways, using different substrates along parallel pathways, both pathways ultimately converge to form androstenedione. 3β-HSD converts the Δ^5-3β-hydroxysteroid pathway substrates, 17α-hydroxypregnenolone and dehydroepiandrosterone (DHEA) into their respective Δ^4-ketosteroids, 17α-hydroxyprogesterone, and androstenedione, respectively (see **Figure 2**).

The third enzymatic reaction involves cytochrome P450 17α-hydroxylase/C_{17-20} lyase (P450c17). This enzyme catalyzes two chemical reactions, hydroxylation and cleavage (to convert a 21-carbon steroid to a 19-carbon molecule), requiring molecular oxygen and NADPH. The products after the initial hydroxylation step are considered intermediates. Therefore, in the Δ^5-hydroxysteroid pathway, P450c17 initially converts pregnenolone to 17α-hydroxypregnenolone and then to DHEA. DHEA is then converted to androstenedione by 3β-HSD. In the Δ^4-ketosteroid pathway, P450c17 converts progesterone to 17α-hydroxypregnenolone which is then converted to androstenedione. The lyase activity of P450c17 differs among species for the intermediate substrates. For example, in humans P450c17 converts 17α-hydroxypregnenolone to DHEA (Δ^5-hydroxy steroid pathway) but not 17α-hydroxyprogesterone to androstenedione (Δ^4-ketosteroid pathway), while in rats, P450c17 converts the intermediates of both the Δ^5-hydroxy steroid and Δ^4-ketosteroid pathways equally.

The fourth enzymatic reaction involves the conversion of androstenedione to T by 17-ketosteroid reductase (17KSR), also designated as 17β-hydroxysteroid dehydrogenase (17β-HSD). In the female, in the follicle cells, androstenedione is then converted to estrone by aromatase. The conversion of androstenedione to T is reversible, dependent on product concentrations. 17β-HSD (17KSR) can catalyze both the reduction (forward) or the oxidation (reverse) reactions. NADH/NAD+ are co-factors in this interconversion. In the male, T is converted to DHT by 5α-reductase (see section below).

The fifth and last enzyme in the steroidogenic pathway is aromatase. Aromatase converts T into E2 (in males and females) and androstene into estrone (in females). Aromatase is present in many peripheral tissues and in male and female gonadal tissue. It is an enzyme complex (two cytochrome P450 enzymes: a reductase and an aromatase) bound to the endoplasmic reticulum. This complex catalyzes two hydroxylation steps and the aromatization of Ring A of the steroid nucleus, which results in the loss of the C-19 carbon atom, producing a C-18 molecule characteristic of estrogens. The reaction requires molecular oxygen and NADPH.

Production and activity of these steroidogenic enzymes are under hormonal control. First, the gonadotrophins: P450 scc and P450c17 enzymes, are regulated by pituitary LH. In males, pituitary FSH stimulates release of a Sertoli cell factor that increases the effect of LH on 3β-HSD activity to increase T production. In females, FSH increases the activity of aromatase to increase estrone production. FSH binds to receptors on Sertoli cells that release and metabolize factors required for spermatogenesis. FSH increases the number of LH receptors in the testis, which in turn increases T induction and testis growth. Sertoli cells have receptors for FSH and T for coordination between the Sertoli and Leydig cell population in the testis. Sertoli cells also produce a glycoprotein, inhibin, which provides negative feedback on the release of FSH from the anterior pituitary. Gonadal hormones also provide negative feedback to the steroidogenic enzymes. T inhibits P450c17 activity by acting on the second messenger cAMP pathway. T also suppresses 3β-HSD through inhibitory effects on cAMP-mediated 3β-HSD mRNA.

The endproducts in steroidogenesis are considered T, DHT, E2, and estrone.

Locations

Sites of steroidogenesis

Reproductive system steroid hormones are produced primarily in the gonads (testes and ovaries), although some of the steroidogenic reactions are also found in peripheral tissues (*e.g.*, the adrenal glands, placenta). Other active androgenic hormones are produced in the testes and in peripheral tissues. Other peripheral tissues are also involved in T's role as a prohormone. For example, T is converted into E2 in the liver, testis, and brain (hypothalamus, to determine male-specific behaviors), converted to DHT in the testis, liver, brain, prostate, and external genitalia. T is converted to androsterone or etiocholanolone in the liver; it is glucuronidated in the liver for excretion and converted to androstenedione in the testis and liver (Federman, 1981). Aromatase, which converts T into E2 in males and females, is found not only in male and female gonads but also in many different peripheral tissues.

Sites of androgenic/anti-androgenic activity

Steroid hormones are carried in the circulatory system by steroid hormone binding globulin (SHBG), α-fetoprotein, albumin, etc., in perinatal rats and humans. Sites (*e.g.*, cells) with androgen-dependent activity have ARs that recognize and bind T and translocate it to the nucleus where the receptor with bound T interacts with genes to turn on or turn off specific gene expression (see later in this chapter). The cells of the embryonic anlagen (precursors) of the male reproductive tract (*e.g.*, Wolffian ducts) and the embryonic external genital folds have receptors for T and/or DHT that appear at the appropriate times to initiate hormone-dependent gene activation and structural and functional developmental changes.

At puberty in males, T and its receptors are ubiquitous in the testes, accessory sex organs, muscles, hair follicles on the face, back, underarms, pubic area, etc., skin

(adolescent acne), voice box (male voice changes), growth zones in the long bones (male growth spurt), sweat glands, external genitalia, adrenal glands (glucocorticoid pathway), etc. Interference at the AR will impact all subsequent androgen-dependent activities (see later in this chapter). Interference with steroidogenesis by genetic, endogenous, or exogenous (environmental) chemical exposure will interfere with T biosynthesis and, depending on the timing of the interference, affect male internal and external sexual development, onset of puberty, etc. Effects at the level of the CNS hypothalamus and pituitary can also impact T biosynthesis.

Functions

Androgens and estrogens are essential for the initial development of the reproductive system *in utero,* for the maturation of the reproductive system and accessory sex organs, and secondary and tertiary sex characteristics at puberty, and for the maintenance of the reproductive system and accessory sex organ structures and functions until death (in males) or menopause (in females). Androgens and estrogens are also needed for feedback regulation of the hypothalamus-pituitary-gonadal axis and spermatogenesis in males (and oogenesis in females). T is responsible for testis development and descent, maturation of the epididymides, vas efferens, vas deferens, seminal vesicles and coagulating glands, LABC muscle, and PPS at puberty in rodents. DHT is responsible for development of the male urethra and prostate, formation of the penis and scrotum, and male secondary sex characteristics such as scrotal growth, development of scrotal rugae and pigmentation, and penis growth and development. There are obvious consequences in structures and functions from a reduction or increase in T production; the type of change and extent and location of the change are all dependent on the timing and direction (increase/decrease) of the alteration in hormone levels.

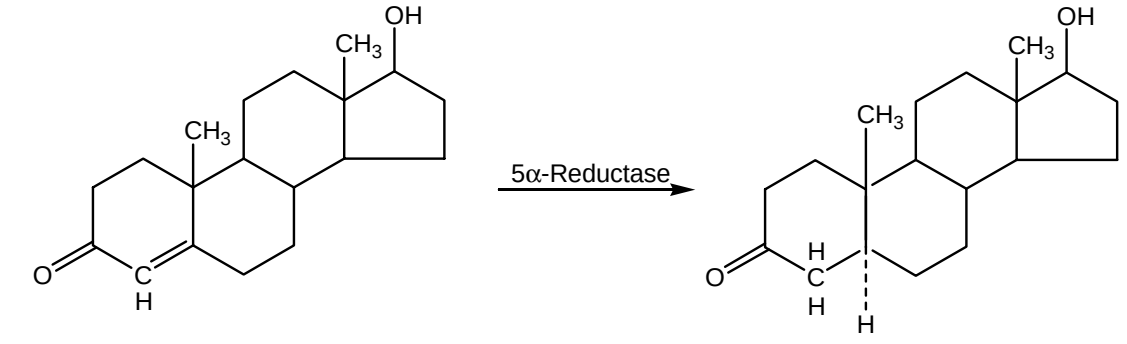
Dihydrotestosterone (DHT)

In pioneer experiments on rabbit fetuses, Jost (1953) demonstrated that even with surgical removal of the gonads in male and female rabbit fetuses *in utero*, female organogenesis (*i.e.*, Müllerian duct stimulation to form oviducts and uterus, and Wolffian duct inhibition to prevent development of male internal reproductive structures) still occurred in all fetuses regardless if they were XX or XY, in the absence of T, DHT, or E2. Jost (1953) inferred that male sexual differentiation is imposed upon the natural tendency of the fetus toward femaleness. Normal male sexual differentiation requires the production and secretion of two factors by the testis; T (and DHT) to stimulate development of male structures, and Müllerian inhibiting substance (MIS) to prevent formation of female structures. In human male fetuses, at the time of sexual differentiation *in utero*, DHT formation occurs in the urogenital sinus, urogenital tubercle, and urogenital swellings. However, DHT formation does not occur in the Wolffian anlagen until after differentiation has occurred, which suggests that DHT and T have specific roles in male reproductive development (Siiteri and Wilson, 1974).

Locations

In the mammal (rat, mouse, rabbit, and human), fetal androgen production during gestation is required for normal male sexual differentiation. T is necessary for proper development of the testes (which is the site of T synthesis in the interstitial cells of Leydig), as well as the stabilization and differentiation of the Wolffian ducts into the epididymides, vasa efferentia, deferentia, and seminal vesicles. DHT, locally produced from T by the enzyme Δ^4-steroid 5α-reductase (**Figure 3**), stimulates normal differentiation and development of the urogenital tubercle, urogenital sinus, and urogenital swellings *in utero* into the urethra, prostate, and external genitalia. The growth of the perineum (anogenital distance) and apoptosis and regression of the ventral nipple anlagen in males *in utero* are also DHT-dependent processes.

Figure 3. Transformation (reduction) of T to DHT by 5α-reductase



Testosterone (17β–hydroxy-4-androsten-3-one)

5α-Dihydrotestosterone (17β-hydroxy-5α-androstan-3-one)

Functions

In a fascinating human "accidental experiment," 24 males (46, XY) in 13 families, in the small, isolated village of Salinas (population 4300) in the Dominican Republic, were born with ambiguous external genitalia and raised as girls. At birth, they had bilateral testes, presenting as inguinal or labial masses, a labial-like scrotum, a urogenital sinus with a blind vaginal pouch, and a clitoral-like phallus. There were no Müllerian structures present (Imperato-McGinley *et al.*, 1974).

At puberty, the voices of these girl-like boys deepened, they developed a typical male phenotype with increased muscle mass, no breast development, and the phallus enlarged to form a functional penis. The change was so striking that the townspeople referred to these individuals as "guevedoces" (penis at 12 [years of age]). In addition, the

scrotum became rugated and hyperpigmented, the testes descended, and there was an ejaculate. The prostate remained small (to absent), beard growth was scanty, there was no temporal recession of the hairline (*i.e.*, no male-patterned baldness), and no acne. Their psychosexual orientation was male, and testicular biopsy indicated complete spermatogenesis with normal Leydig cells. The epididymides and vasa deferentia were normal. These males (termed pseudohermaphrodites) had normal internal male reproductive structures, incomplete differentiation of the male external genitalia at birth, and partial to complete recovery at puberty. T biosynthesis and androgen activity were therefore normal; the abnormality was likely due to a "downstream" defect in metabolism of T (*i.e.*, biotransformation of T to 5α-DHT by 5α-reductase). Analyses of the T and DHT plasma concentrations, and other 5α-reduced metabolites of T, indicated that the affected males had approximately 1/40 the DHT of unaffected males, with obligate male carriers exhibiting an intermediate concentration. Pseudohermaphrodite males are considered to be homozygous recessive for the inherited disorder of steroid metabolism; females homozygous recessive for the error are normal.

Obligate carriers of both sexes are heterozygous for the autosomal (not x-linked) gene mutation, with normal phenotypes. In 12 of the 13 families, the trait could be traced back seven generations to a woman who married four different men. The isolation of the town and common ancestors (*i.e.*, consanguinity) suggest that the increase in gene frequency was a consequence of founder effect. It is not known if the heterozygotes have a selective advantage. In 1974, the location of the biochemical error was unknown. The deficiency could have been related to the synthesis, structure, or metabolism of Δ^4-steroid 5α-reductase.

This genetic effect on 5α-reductase is not unique to the Dominican Republic or to isolated inbreeding communities. In Japan, 81 male Japanese patients (age 0-14 years) with micropenis were evaluated for the SRD5A2 gene that encodes 5α-reductase-2 (Sasaki *et al.*, 2003). The authors found mutations in the SRD5A2 gene, especially at R227Q, which resulted in only 3.2% normal enzyme activity; it is relatively frequent in Asian populations (and is likely the cause of the micropenis phenotype). In contrast, a polymorphism V89L (valine to leucine substitution at the 89th codon) at exon 1 of the gene, which results in an ~30% reduction in 5α-reductase-2 activity, is considered unlikely to be the cause of development of micropenis. Again, interference with the function of the 5α-reductase, especially during *in utero* development, results in incomplete development of the male external genitalia controlled by DHT. As seen by the males in Salinas, the T surge at puberty causes completion of most (but not all) of the initially incompletely developed reproductive structures.

Synthesis of DHT

5α-Reductase

In the male, T is converted to DHT by 5α-reductase, which is found in cellular membranes, nuclear membranes, and endoplasmic reticulum. DHT is produced primarily in peripheral tissues (at target organs), but it is also found in the testis. The activity of 5α-reductase in the Leydig cells and testes varies with age; the highest activity occurs

around puberty. Genetic differences in 5α-reductase occur. In this defect, abnormal masculinization is localized *in utero* to the urogenital sinus and external genitalia (since their differentiation and growth are mediated by DHT, not T). T and E2 blood levels are normal, because aromatase directly converts T to E2 (DHT is not involved). This deficiency in the male is characterized by a blind vaginal pouch, testes, and upper tract accessory sex organs (under T control), no enlarged breasts, no internal female genitalia, and masculinization at puberty when a surge in T results in differentiation and growth of external genitalia (Androgen Insensitivity Syndromes (AIS), later in this chapter).

Locations and functions

Using a 5α-reductase inhibitor, Imperato-McGinley *et al.* (1985) developed a male pseudohermaphrodite rat which was subsequently used to examine development of various sex-specific structures and functions, such as nipple retention in males (Imperato-McGinley *et al.*, 1986), testis descent (Spencer *et al.*, 1991a; Turner *et al.*, 2002), and prostate and genital differentiation (Imperato-McGinley *et al.*, 1992). Other workers have used finasteride, a specific 5α-reductase inhibitor, to distinguish between T-mediated and DHT-mediated effects (Blohm *et al.*, 1986; Bowman *et al.*, 2003), and determined critical developmental periods for effects of low or no DHT on male rat genitalia (Clark *et al.*, 1990, 1993), on the rat gubernaculum (George, 1989), on androgen physiology in the immature rat (George *et al.*, 1989), and on embryogenesis of the rat prostate (George and Peterson, 1988). Early on, it was noted that there were differences in the amount and level of conversion of T to DHT in the prostate (now known to be DHT dependent), versus the epididymis (now known to be T dependent) (Gloyna and Wilson, 1969). The role of T and DHT in sexual ducts and the genital tubercle of rabbit fetuses was assessed during sexual organogenesis, with and without fetal decapitation (to assess the role of the hypothalamus and pituitary in *in utero* sexual development (Veyssiere *et al.*, 1982). Additional work on T vs DHT effects and targets has been done in the rat fetus (Berman *et al.*, 1995), and in both rat and rabbit fetuses (Wilson and Lasnitzki, 1971).

Androgen Receptor (AR)

There are three major classes of receptors to which hormones bind: (1) receptors found on the surface of cells (to which peptide hormones bind); (2) receptors found in the cytoplasm of cells (to which the steroid hormones bind); and (3) receptors found in the nuclei of cells (to which the thyroid hormones bind). Moreover, there are two major mechanisms of hormone action: (1) activation of plasma membrane receptors by hormone binding to transport the hormones into the cell (used by catecholamines, peptides, and protein hormones); and (2) activation of intracellular receptors to transport the hormones into the nucleus for DNA binding (used by steroid and thyroid hormones).

A vast array of receptor proteins and genes are associated with cells that may contain ~10,000 protein receptors for a single steroid hormone. As many as ~50-100 genes within a cell may be controlled by the binding of a single type of hormone to the various cellular receptors Some genes are also affected by more than one receptor hormone complex.

Role of the AR in fetal masculinization

Differentiation of the testis from the indifferent gonadal ridge begins at approximately six weeks of gestation in humans of a 39- to 40-week pregnancy, and on GD 14 in rodents with a 19- (mouse) to 22- (rat) day pregnancy. This differentiation is directed by testis-determining factor, a DNA-binding protein transcription factor encoded by a gene (SRY) on the short arm of the Y chromosome, the sex determining region of the Y chromosome, probably in concert with other factors encoded by autosomal or X-chromosomal genes. Testis differentiation is not an androgen-dependent process; however, androgens mediated by the AR do play an indispensable role in the induction of male sex differentiation and development of the male phenotype.

After development of the testis (sex determination), the events of male sex differentiation follow two paths: one inhibiting and one stimulating. The function of the inhibitory path is to cause regression of the Műllerian ducts and therefore to prevent the development of female internal genitalia (oviducts, uterus, and upper vagina) in the male. This process occurs between gestational weeks 6 and 8 in humans and is mediated by the anti-Műllerian hormone, also known as the MIS, a glycoprotein member of the transforming growth factor β family, which is secreted by Sertoli cells in the embryofetal testis.

The stimulating events of male sex differentiation require high levels of androgen and a functional AR. Androgens are required to stabilize the Wolffian duct system to prevent its involution/regression and to induce the differentiation of the Wolffian ducts into the epididymides, vasa deferentia, and seminal vesicles during gestational weeks 9 through 13, in humans. This sequence of duct differentiation is induced by the action of T itself, probably by a paracrine effect due to its high local concentrations in the vicinity of the Wolffian ducts, which is in close proximity to the testes (source of T) (Quigley *et al.*, 1995). T secretion by fetal Leydig cells begins at eight weeks gestation and peaks between 11 and 18 weeks of gestation in humans. Maternal chorionic gonadotropin (CG) (Quigley *et al.*, 1995) is considered to be the major controller of T secretion during this period. Some researchers (*e.g.*, Word *et al.*, 1989) have reported that T synthesis and adenylate cyclase activity in the early human fetal testis appear to be independent of human CG control, so other factors may be involved.

The potent metabolite of T (DHT) is not involved in the process of developing internal male structures, since the enzyme 5-α reductase 2, required for the conversion of T to DHT (**Figure 2**), is not expressed in these tissues until approximately 13 weeks gestation in humans, at which time the process of internal masculinization is complete. Development of the prostate and prostatic urethra from the urogenital sinus, and masculinization of the external genital primordia, the genital tubercle, urethral folds, and labiosacral swellings into the penis, penile urethra, and scrotum also occur between 9 and 13 weeks gestation, and require the more potent fetal androgen DHT; 5-α reductase 2 is also expressed in these tissues at this time.

A functional AR is absolutely required to mediate the actions of both T and DHT in inducing the expression of androgen-dependent genes necessary for internal and

external male genitalia. Disturbance of the production or action of androgens during this critical period of male differentiation interrupts the ordered sequence of events and results in failure of complete masculinization.

The AR gene

The genes for the AR for both T and DHT are localized on the X chromosome, with the human genes homologous to the mouse testicular feminization (Tfm) gene (Migeon *et al.*, 1981), in a highly conserved region of the X chromosome, found in monotremes, marsupials, and eutherian mammals (Spencer *et al.*, 1991b), and localized to Xq11-12 on the human X (Brown *et al.*, 1989). The absence of T, or of appropriate receptors that recognize T or DHT, can result in full or partial AIS in humans and many other mammals (Quigley *et al.*, 1995, 2004). The newborn genetic male (XY) with AIS presents as an external female with mid-abdominal testes (triggered by the SRY gene [male determining] on the Y chromosome) and no accessory sex organs (T is made in the testis, but it is not recognized by the altered ARs in the target structures, so male accessory sex organs are not induced). Partial AIS males present with a range of external genital phenotypes that vary from near-normal female to normal or near-normal male, with or without gynecomastia, and relatively "mild" signs of under virilization (Gottlieb *et al.*, 1998). Somatic mutations in the AR have been found in advanced prostate cancer (Culig *et al.*, 1993); the somatic mutations in the AR on the X chromosome are found in cells of specific tissues (*i.e.*, the prostate), but are not in all tissues from a germline mutation (in the X chromosomes of either the sperm or ovum). The tissue distribution of the AR message or protein is presented in **Table 1**.

Table 1. Tissue distribution of AR[a]

What	Method of Detection	Where Localized	Species
AR mRNA	Northern blot analysis	Testis, prostate	Human
		Genital skin fibroblasts	Human
		Prostate and human breast cancer cell lines	Human
		Liver	Human
		Kidney, brain, epididymis	Rat
		Antipituitary and other renal tissues	Rat
		Lacrimal gland	Rat
		Smooth muscle cells of penis	Rat
		Kidney and liver	Mouse
		Larynx of male	Xenopus
AR protein	Immunoblot (Western blot) analysis	Genital skin fibroblasts	Human
		Penis	Rat
AR protein	Sucrose density gradient analysis	Gubernaculum	Rat
		Urogenital sinus	Rat

(continued)

Table 1 (continued)

What	Method of Detection	Where Localized	Species
AR protein	Immunohistochemistry	Nuclei of glandular epithelial cells of prostate	Human and rat
		Epididymis	Rat
		Ductus deferens	Rat
		Ventral prostate	Rat
		Seminal vesicles	Rat
		Hypothalamic nuclei	Rat
		Anterior and posterior pituitary	Rat
		Epithelial cells of lacrimal gland	Rat
		Somatostatin-producting neurons of hypothalamus	Rat
		Cultured foreskin fibroblasts	Normal and AIS men
		Testis	Rat, mouse, guinea pig
		Prepubertal testis	Human
AR protein	Immunohistochemistry	Ovary	Human, monkey, rat
		Testis: Sertoli cells, peritubular myoid cells, interstitial cells, and elongated spermatids	Rat
		Ovary: granulosa, theca, and luteal cells	Rat
AR	Immunohistochemistry of paraffin-embedded tissues	In nuclei of sweat glands, hair follicles, cardiac muscle, vascular and gastrointestinal smooth muscle cells, thyroid follicular cells, renal cortical cells	Human

[a] From Quigley *et al.*, 1995

The most recent AR mutational database (Gottlieb *et al.*, 1998) contains 309 entries, representing over 200 different AR mutations from over 360 patients with AIS, over 35 patients with advanced or metastatic prostate cancer, and one case of laryngeal cancer. The mutation types include insertions, substitutions, deletions, duplications, and splice-junctional deletions/substitutions. The deletions, to date, have included up to five base pairs (bp) or deletion of the entire gene. These changes can occur in the N-terminal region (amino acids numbered 1-538), the DNA binding domain (amino acids numbered 539-627), introns, exons, termination codons, etc. See **Table 2** for an overview of the types and incidences of genetic changes detected to date in the human AR. Single amino acid insertions or deletions will result in frameshift mutations, including the garbling of all subsequent three-base amino acid codes in the mRNA and consequent abnormal

amino acid sequences in the specified protein, and the possibility of creation or loss of a stop codon. These changes can result in altered binding specificity, oligospermia, etc. Splice-junction site deletions or substitutions also result in complete or partial AIS.

Table 2. AR mutations in the database (modified from Table 2, Gottlieb *et al.*, 1998)

		Mutation Type	
	Phenotype	Germline	Somatic
Structural Defects			
Complete gene deletions	Complete AIS[a]	3	
Partial gene deletions	Complete AIS	8	
Partial gene deletions	Mild AIS	1	
Partial gene deletions	Prostate cancer		1
Partial gene deletions	Laryngeal cancer		1
1-4 bp deletions	Complete AIS	8	
1-4 bp deletions	Partial AIS	1	
Intron deletions	Partial AIS	1	
Splice-junction deletion	Complete AIS	1	
Insertions	Complete AIS	4	
Bp Duplications	Complete AIS	1	
Subtotal:		**28**	**2**
Single Base Mutations			
Amino acid substitution	Complete AIS	119	
Amino acid substitution	Partial AIS	95	
Amino acid substitution	Mild AIS	4	
Amino acid substitution	Prostate cancer		26
Multiple amino acid substitutions	Complete AIS	3	
Multiple amino acid substitutions	Prostate cancer		7
Splice-junction substitution	Complete AIS	5	
Splice-junction substitutions	Partial AIS	1	
Premature termination codons	Prostate cancer		1
Premature termination codons	Complete AIS	18	
Subtotal:		**245**	**34**
	Grand Total:	**309**	
Trinucleotide Repeat Polymorphisms in Exon 1	**See Text**		

[a] AIS = androgen insensitivity syndrome

Two trinucleotide repeat polymorphisms in the AR have also been identified. They encode a series of variable length glutamine (CAG) and glycine (GGC) repeats in exon 1. The CAG repeat has implications for other diseases. Its expansion causes the motor neuron disease (Spinobulbar muscular atrophy; SBMA), and similar CAG expansions in a variety of unrelated genes cause a number of other neurodegenerative diseases (Gottlieb *et al.*, 1998). Gottlieb *et al.* (1998) also noted that there is a shift in the distribution of CAG-repeat sizes in the hAR gene of breast cancer tissue, that CAG-repeat sizes may act as molecular markers for prostate cancer risk, and that codon-usage variants and GGN-tract sizes may be markers for (associated with) particular diseases.

The AR is a member of a group of four closely related steroid receptors (the "GR-like receptors"), including the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), and the PR. This group is related by sequence homology and by the ability to activate target gene transcription by the same hormone response element (HRE). It comprises a subfamily of a larger family of nuclear transcription factors that includes the ER, thyroid hormone receptors (TRα and TRβ), vitamin D receptor, retinoic acid receptor (RAR), and a number of other related receptors. The AR, as a hormone-receptor complex, interacts directly with its target genes to regulate their transcription (as do the other members of this receptor family). Failure of the receptor to activate its target genes in the presence of hormones results in target organ resistance to the hormone.

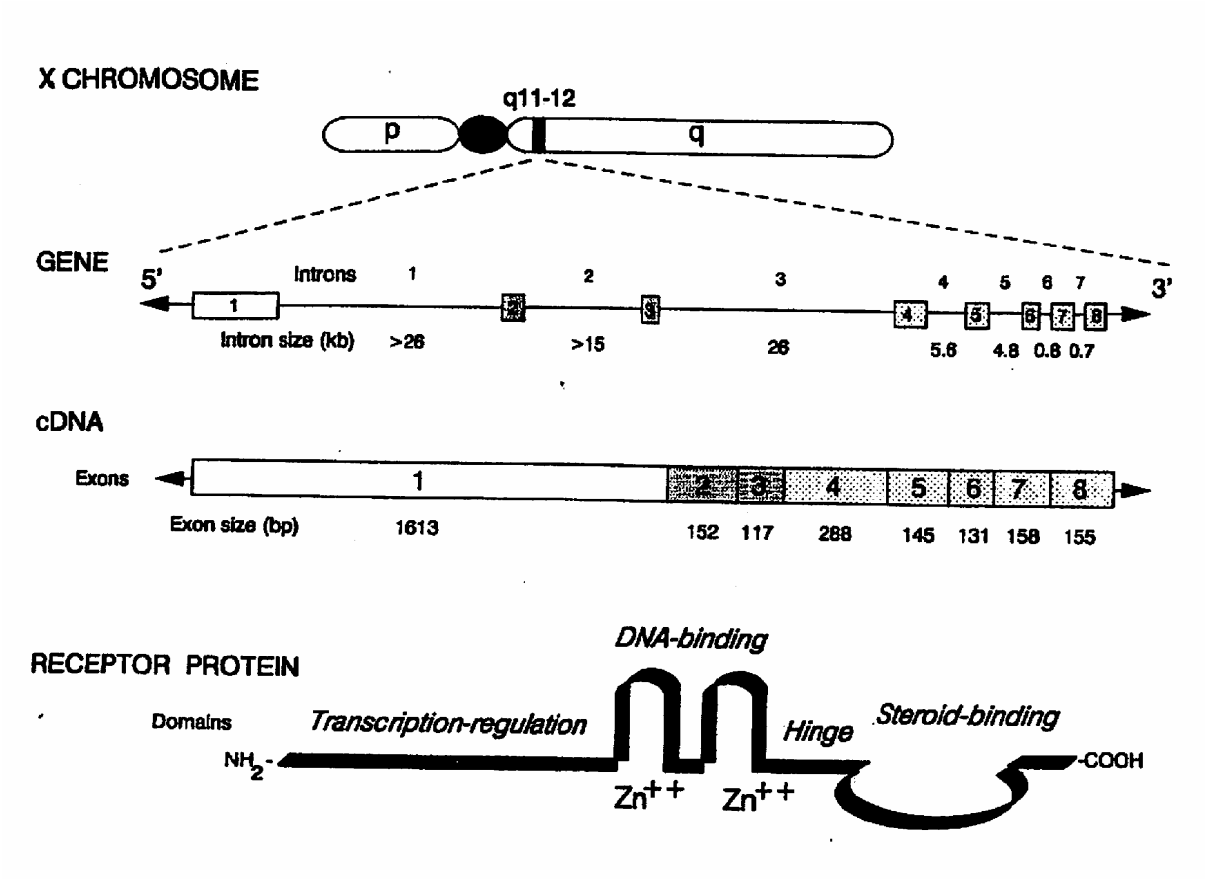
Structure

The AR gene and its encoded protein are structurally and functionally similar to those of the other steroid receptors. The AR gene is a single copy X-chromosomal gene that spans 75-90 kilobases (Kb) of genomic DNA in the pericentromeric region of the long arm of the chromosome at Xq11-12. Its protein coding region (~ 2757 base pairs [bp] open reading frame) comprises eight exons (designated 1 through 8 or A through H), separated by introns from 0.7 to more than 26 Kb. At the 5' end of the gene is exon 1, containing a CAG triplet repeat region of average length of 21±2 repeats, with a range of 11-31 in a normal mixed-sex population, and a range of 14-35 in a group of unselected males. This repeat region is highly polymorphic; there are 20 different allele sizes, with 90% of females heterozygous for the size of this repeat at this locus. There are also racial differences, with the most frequent allele size (mode) equal to 18 in the African American population and 21 in whites.

The principal site of transcription initiation (designated TIS 1) in the AR gene is an adenosine residue located approximately 1100 bp from the 5' end of the translation-initiating methionine codon. A second transcription start site (TIS II) is 11 nucleotides downstream from TIS I). TIS I is used in all tissues and cell lines to date; the role of TIS II is not yet known (Quigley *et al.*, 1995). The AR promoter is like that for the PR gene promoter in that it does not contain a typical TATA or CAAT box, but does contain GC-rich elements, including a binding site for the common mammalian transcription factor SPI (Tilley *et al.*, 1990), a homopurine sequence (Baarends *et al.*, 1990), and a cAMP response element (Lindzey *et al.*, 1993), as well as binding sites for a number of other

transcription factors. The minimal region of the human AR gene promoter necessary for activity includes nucleotides -74 to +84 surrounding the TIS 1 (Takane and McPhaul, 1994), but Song *et al.* (1993) have identified a more distal 5'-promoter/enhancer region of the rat AR gene at nucleotides 96-940, upstream of the transcription start site, which is critical for the activity of the rat AR gene promoter, and binds another (yet unidentified) transcription factor. A bipartite promoter element is also present, which binds two protein factors and may confer age sensitivity of hepatic AR expression (Supakar *et al.*, 1993). Another region in the rat AR gene promoter at -754 to -551 nucleotides has been identified as a repressor element by binding nuclear factor KB, which in this context functions as a negative regulator of AR gene transcription (Supakar *et al.*, 1994). A number of half-palindromic potential HRE-binding sites for AR, GR, and PR have been found within the rat AR promoter region (Song *et al.*, 1993), and one half-site for ER has also been reported (Song *et al.*, 1993). Such sequences may be important for steroid hormone regulation of AR mRNA expression. The structure of the AR gene is presented in **Figure 4**.

Figure 4. Structure of the AR gene



Modified from Quigley *et al.*, 1995; see text for explanations

Regulation of the AR is age-, time-, and cell- or tissue-type dependent, and occurs at both the transcriptional (DNA to RNA) and translational (RNA to protein) levels. In addition, AR protein and mRNA have contrasting responses to androgen(s), even within the same cell type. At the protein level, androgens stabilize the receptor in transfected cells in culture by reducing protein degradation. This may account for the higher receptor levels in male versus female fetal rats and in rats exposed perinatally to high androgen levels. In contrast, the overall effects of androgens at the mRNA level are down regulation.

Androgen withdrawal by chemical or physical castration results in an increase in rat AR mRNA, which is reversed by androgen replacement. Down regulation of other steroid receptor mRNAs results from a decrease in transcription rate and/or decreased half-life of the receptor mRNA. By analogy with the mechanism of GR gene repression, it is likely that in the AR, sequences within the DNA-binding domain are required for receptor-mediated gene repression and mediate AR autoregulation. The Tfm (testicular feminization) rat (caused by a single amino acid change in the steroid binding domain of the AR) exhibits disrupted AR function, but not by the normal androgen-dependent down regulation of AR mRNA as in the wild-type animal. Hormones other than androgens have also been found to regulate AR in various cell or tissue types (*e.g.*, FSH up regulates AR mRNA and protein in Sertoli cells through cAMP, and epidermal growth factor [EGF] down regulates AR mRNA in LNCaP prostate cancer cells).

Mechanism of action

Androgens are transported in the blood supply, largely bound to carrier proteins (*e.g.*, SHBG, α-fetoprotein [AFP], albumin). When they reach target tissues, they dissociate from the carrier proteins, diffuse into target cells, and bind to the intracellular AR protein. Androgen binding induces a conformational change in the AR that facilitates receptor dimerization, translocation to the nucleus, interaction with target DNA, and then regulation of target gene transcription. A variety of androgens and other steroids bind to AR. ARs have the highest affinity for DHT; less for T (primarily because T dissociates from the AR more rapidly than DHT; Wilson and French, 1976).

Androgen binding induces a change in the AR that converts the inactive receptor into its active DNA-binding state. As with other steroid receptors, hormone binding results in the removal of certain receptor-associated proteins, such as the 90 DKa (kilodaltons) heatshock protein. Removal of these proteins unmasks functional domains and initiates the conformational changes necessary for nuclear import, dimerization, and DNA binding. Receptor activation is ligand specific; functional activity of the receptor correlates with the binding activity of the ligand. Androgen binding stabilizes the receptor, which results in higher levels of receptor and higher levels of binding receptor phosphorylation, a post-translational process common to steroid receptors and other transcriptionally active proteins. It is enhanced in the presence of androgens (because of the higher AR protein levels; see above). However, the AR is phosphorylated in both the presence and absence of hormone, and both the phosphorylated and unphosphorylated

forms of the AR bind androgens with high affinity; the precise role of (reason for) receptor phosphorylation is not yet clear.

Binding of androgen to the AR is necessary for DNA binding and subsequent transcriptional activity. In the absence of androgens, the steroid binding domain acts as a repressor of AR transactivation regions, likely due to an inhibitory conformation adopted by the unliganded AR protein (Quigley *et al.*, 1995).

The AR is believed to be cytoplasmic when there is no androgen bound. Once bound by androgens, the AR is clearly nuclear. Intranuclear, hormone-activated AR binds as a homodimer to the hormone response elements (HREs) of target genes and their flanking DNA. The binding of the receptor to its HRE regulates the rate of transcription of target genes through interactions with other components of the transcription complex near the transcription start site of the gene. *In vitro*, AR binds to a simple HRE consensus sequence (a 15 pb partial palindrome 5'-AGNACAnnn-TGTNCT-3'), which is also bound by GR and PR. This overlap in activity among AR, PR, and GR at certain HREs may allow for synergistic actions of androgens and glucocorticoids. In addition to acting through the simple consensus HRE, the AR also acts through selective androgen-specific complex androgen response elements (AREs). These AREs are comprised of a number of interacting elements, including the HRE itself, and recognition sequences for other transcriptional control factors. Enhancement of AR and GR binding to HREs occurs in the presence of an accessory protein identified as insulin degrading protein. This interaction may represent some type of relationship between insulin and androgen signaling.

The AR Protein

Structure

The AR gene encodes a receptor protein of 110-114 KDa molecular weight, comprising 910-919 amino acids. It is a single polypeptide (like other steroid receptors) with discrete functional domains: a transcription-regulating, amino-terminal domain (corresponding to the 5' end of the gene), a DNA-binding domain, a hinge region, and a steroid-binding domain ending at the carboxy-terminal end of the protein (corresponding to the 3' end of the gene) (see **Figure 4**).

The amino-terminal domain

This large domain encoded by exon 1 comprises more than half of the AR protein (amino acid residues 1-537). This domain is the least homologous in sequence and the most variable in size among members of the steroid receptor family. It contains a transactivation (transcription activation) domain between amino acid residues 141 and 338. There may also be other subregions for regulation of target gene transcription. This domain may also be involved in the establishment and maintenance of the three-dimensional structure of the AR molecule through interactions with other regions of the protein. This domain (in humans, rats, and mice [so far]) also contains a number of homopolymeric amino acid stretches not present in other members of its subfamily of steroid receptors. The initial amino acid stretch (at the amino terminus of the protein) is a

polyglutamine stretch (approximate amino acid residues 58-79 encoded by the polymorphic CAG triplet repeat region in the gene) of average length of 21±2 glutamine residues. A shorter stretch of nine proline residues at amino acid residues 372-379 is further downstream and there is another polymorphic stretch of approximately 24 glycine residues at amino acid residues 449-472 closer to the DNA binding domain (see **Figure 4**). The polyglutamine and polyproline stretches are likely important in transcriptional regulation by protein-protein interactions with other transcription factors (Gerber *et al.*, 1994), or to maintain the three-dimensional conformation of the protein.

The DNA-binding domain

This central cysteine-rich region of the AR (amino acid residues 538-627), encoded by exons 2 and 3, contains the DNA-binding domain (amino acid residues 559-624). In GR and ER, this region is arranged as a pair of looped structures folded to form a single structural unit made up of two zinc-binding motifs (see **Figure 4**). Four cysteine residues, present in all steroid receptors, coordinately bind a zinc cation in each of the two motifs ("zinc fingers"). The first zinc finger (residues 559-579) is encoded by exon 2, and the second zinc finger (residues 595-619) is encoded by exon 3. The amino acid sequence of this domain is the most highly conserved region among the members of the steroid receptor family, with approximately 80% homology (amino acid identity) with those of MR, PR, and GR (Freedman, 1992). The DNA binding domain determines the specificity of AR (and of other steroid receptor(s)) interaction with DNA. Three amino acids at the base of the first zinc finger (glycine 577, serine 578, and valine 581) are identical with those in GR, PR, and MR and interact with transcriptional enhancer nucleotide sequences (HREs) present in or near target genes, usually in the 5'-flanking region, and appear critical for recognition. The second highly basic zinc finger stabilizes DNA-receptor interactions by contact with the DNA phosphate backbone and mediates receptor dimerization. At the carboxy-terminal end of this AR domain is an arginine-lysine pair that is part of the nuclear targeting sequence (see *The hinge region* below).

The hinge region

The hinge region is located between the DNA-binding domain (*vide supra*) and the steroid binding domain (*vide infra*). It has low sequence homology between AR and other steroid receptors. This hinge region is encoded by the 5'-portion of exon 4 and contains the major part of the AR nuclear targeting signal, comprised of a cluster of basic residues at positions 629-633: arg (arginine), lys (lysine), leu (leucine), lys, lys. This region mediates the transfer of AR from the cytoplasm to its site of action in the nucleus.

The steroid-binding domain

The carboxy-terminal third of the AR, encoded by the 3'-portion of exon 4 and exons 5 through 8, is the steroid-binding domain at residues 670-919. Sequence homology in this region is approximately 50% among the AR, GR, MR, and PR. This region has four functions: (1) specific, high affinity binding of androgens (a principal function); (2) the binding site for inhibitory proteins such as the 90 DKa heatshock

protein; (3) role in other receptor functions, including dimerization; and (4) a transactivation region (to support transcriptional activation) within this domain.

Mechanism of action

See Mechanism of Action, of the AR gene, which includes the function of the AR protein itself.

Androgen Insensitivity Syndromes (AIS)

The syndromes of androgen insensitivity are the most common, identifiable causes of male pseudohermaphroditism. Affected individuals have a male (46, XY) karyotype, normally developed, but incompletely descended testes, and an external genital phenotype from fully female (normal breast development and no or full pubic hair), to partially masculinized. AIS is classified into clinical subtypes, based on the external genital phenotype (complete AIS, partial AIS, Reifenstein syndrome, infertile male syndrome), or into biochemical subgroups, based on the presence or absence of specific high-affinity androgen binding in cultured genital skin fibroblasts. Molecular analysis of the AR gene in individuals with AIS reveals that the various AIS clinical forms and their associated AR defects form a continuum resulting from AR gene mutations of variable type and severity. The two subtypes and their clinical features are presented below.

Complete AIS

Complete AIS is a relative rare X-linked disorder. Prevalence estimates range from as low as one in 64,200 males to as high as one in 2,000 males; although, these figures are confounded (either positively or negatively) by ascertainment bias and by some of the studies utilizing only hospitalized cases.

Individuals with complete AIS are diagnosed at various life stages, with age at detection changing as the "index of suspicion for the diagnosis has increased" (Quigley *et al.*, 1995). Individuals are diagnosed before or soon after birth, from the discrepancy between a 46, XY karyotype (male) by prenatal amniocentesis and the presence of a female phenotype by prenatal ultrasound examination or at birth. More are diagnosed with the development of inguinal hernias (often bilateral) during infancy. Phenotypically female children are also present with inguinal hernias. Phenotypically female children are diagnosed after puberty with primary amenorrhea.

Karyotypic male individuals with classic complete AIS have unambiguously female external genitalia, with a blind-ending vagina and no uterus. The assumption has been that there are no Müllerian structures. However, in up to one third of the 43 human cases evaluated histopathologically, there were Műllerian structures such as very small fallopian tubes (Rutgers and Scully, 1991). Remnants of Wolffian structures such as vestigial vas deferens or epididymides have also been found (Bale *et al.*, 1992).

Individuals with complete AIS have excellent feminization at puberty, with normal or increased breast development and clear, smooth, acne-free complexions. The

feminization of breasts and body contours is from estrogen, produced by testicular and peripheral aromatization of androgens (see **Figure 5** and **Table 3**), that is unopposed by the effects of androgens (since the mutated AR cannot recognize T, and T biosynthesis is reduced in abdominal testes). Phenotypic women with AIS are taller than average for females and are reported to have an eunuchoid build (long arms and legs).

Smith *et al.* (1994) described the first reported case of a man (from consanguineous parents), homozygous for an ER gene mutation (a premature termination codon), that caused complete estrogen resistance. At 29 years of age, he was 204 cm tall (over 6 foot 7 inches) and still growing, with open epiphyses and severe osteoporosis. Observations from the people with complete AIS, and from this man with complete estrogen resistance, suggest that androgens alone have little direct action on epiphyseal fusion and prevention of osteoporosis, and that conversion to estrogens (which is active in epiphyseal fusion and protection against osteoporosis) is required. The normal bone maturation reported in phenotypic females with complete AIS supports the interpretation that effects of sex hormones on growth are mediated by the ER. However, delayed onset of puberty in 46, XY girls with complete AIS (and some 46, XX female carriers [heterozygous]) argues for a direct role of androgens in induction of pubertal hypothalamic-pituitary-gonadal axis activity (Quigley *et al.*, 1995).

Figure 5. Conversion of T to 17β-estradiol

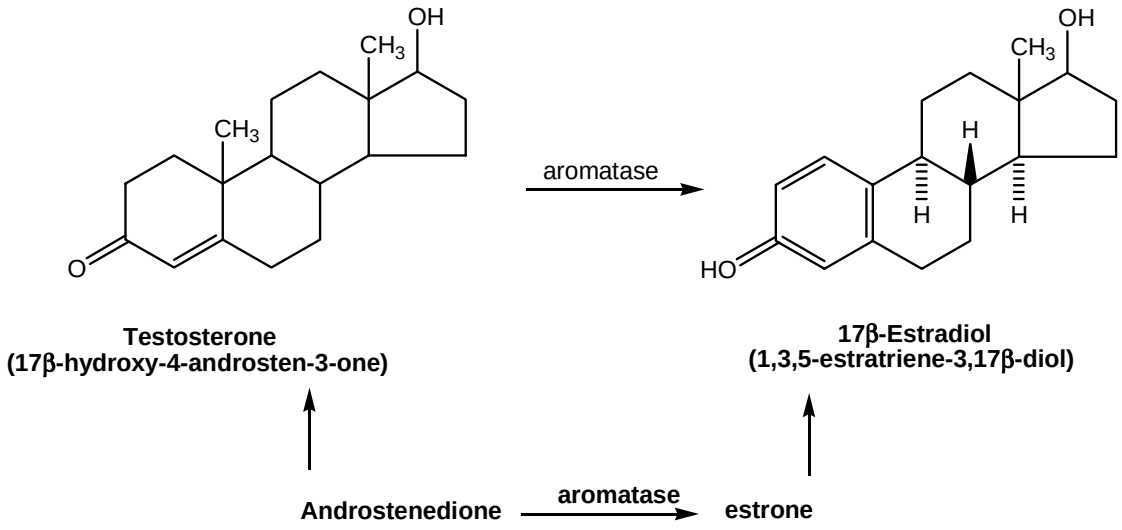


Table 3. Sites of estradiol synthesis

Location	Local Function
Ovaries: granulosa and theca cells of the follicles	Necessary to maintain oocytes in the ovary (role in estrous cyclicity, feminization, etc.)
Brain: from steroid precursors (aromatized from T in male brain; Figure 3), T crosses blood-brain barrier; E2 cannot, so it is made locally from T	E2 masulinizes male brain, male-specific behaviors in rodents (not known if in humans)
Testis: interstitial cells of Leydig (aromatizes T; Figure 2)	Prevents apoptosis of male germ cells
Placenta: by aromatization of prehormones (*e.g.*, androstenedione; Figure 3) produced in the fetal adrenal glands (neither fetal ovaries nor fetal adrenal glands can produce E2; missing some P450 isoforms; Greco and Payne, 1994)	Not yet known
Fat	Responsive to environmental conditions to trigger puberty for reproduction when food is plentiful (likely responsible for precocious puberty in obese, young girls)

Partial AIS

The partial or incomplete forms of AIS express a wide spectrum of clinical (genital) phenotypes. Because of the variability of the clinical manifestation and the presence of subtle or atypical forms of androgen resistance, such as the infertile male syndrome, the prevalence of partial AIS is unknown, but is considered to be at least as common as complete AIS (Quigley *et al.*, 1995). Individuals with the most severe form of partial AIS may have a complete female phenotype with mild clitoromegaly and/or slight labial fusion, while others have significant genital ambiguity at birth, resulting in delayed or inappropriate sexual assignment. In some cases, especially those with Reifenstein syndrome, there is more extensive masculinization with the affected individuals essentially male, but with a severely undermasculinized phenotype with micropenis, perineal hypospadias, and cryptorchidism. In its mildest forms, partial AIS may be expressed only by uncomplicated hypospadias, by infertility in a phenotypically normal male, or by only gynecomastia and androgen binding abnormalities in a fertile male. In fact, affected individuals with widely different phenotypes can be found within a single family.

Since androgen induces stabilization and differentiation of the Wolffian ducts in an embryonic male, Wolffian structures may also develop to a variable extent in partial AIS, depending on the degree of responsivity/resistance to androgens. Epididymides, vasa deferentia, and seminal vesicles may therefore vary from absent, to rudimentary, to fully formed.

At puberty, virilization and/or feminization may occur depending on the hormonal milieu of the affected individual. If the testes are present, the T secreted can induce a degree of virilization proportional to the degree of masculinization which occurred *in utero*. Feminization of breasts and body curves occur because of relatively high estrogen levels in the presence of androgen resistance (as in complete AIS) (Quigley *et al.*, 1995).

Quigley *et al.* (2004) reported on partial androgen insensitivity, with sex phenotype variation in two unrelated families. This was associated with missense mutations in the AR that disrupted the NH_2-terminal/carboxy-terminal interaction. Each mutation caused a single amino acid change within the region of the ligand-binding domain that performs activation function 2 (AF2). In one family, the mutation designated 1737T was in alpha helix 4, and in the other family, the mutation designated F725L was between helices 3 and 4. Neither mutation altered androgen binding, but transactivation (transcription activation) was impaired. In the family with AR 1737T, sex phenotype varied from severely defective masculization in the proband (the identified patient) to a maternal great uncle whose only manifestation of AIS was severe gynecomastia (breast development). He was fertile and passed the mutation to this two daughters (obligate heterozygote carriers). The proband with AR F725L was also incompletely masculinized, but was raised as a male, while his affected half sibling by a different father was raised as a female. It is clear that the function of an AR AF2 mutant and male sexual development can vary greatly, depending on the genetic background (Quigley *et al.*, 2004).

CHAPTER 5. MALE REPRODUCTIVE TRACT AND OTHER ANDROGEN RESPONSIVE TISSUES

Introduction

In general, androgens promote protein synthesis and growth of tissues that possess ARs. The resulting effects in humans are classified as virilizing or anabolic. Anabolic effects include growth of muscle mass and strength, increased bone density and strength, and stimulation of linear growth and bone maturation. Virilizing effects include maturation of sex organs including, for example, the penis and formation of the scrotum in fetuses. Moreover, at puberty, the virilizing effects are responsible for producing a deepening of the voice and growth of facial and auxillary hair.

Relatively high levels of AR have been measured in the tissues of the male urogenital tract and in the adrenal glands and gonads of both sexes. Conversely, in other tissues such as the male LABC muscles, preputial gland, scrotal skin, and vagina, there are lower levels of AR. Using an immunoblot assay technique, one study reported that levels of AR were undetectable in the uterus, kidney, spleen, liver, gut, heart, lungs, pituitary, or hypothalamus (Bentvelsen *et al*., 1996); however, receptors have been found at a detectable level in the pituitary and hypothalamus by other techniques.

In some target tissues, such as the penis, exogenous androgens decrease the AR concentration in the tissue; whereas, exogenous androgens increase the AR concentration in the ventral prostate.

Castration can also have varying effects on the AR levels of tissues. Castration seems to cause a profound decrease in the AR levels in the ventral prostate, but no detectable effect on levels in the adrenal gland. However, by seven days after hypophysectomy, AR levels decrease in both the ventral prostate and adrenal gland (Bentvelsen *et al.*, 1996). Hormones, such as prolactin (PRL), augment T-mediated growth of the prostate in a permissive manner (Prins, 1987) by increasing nuclear AR levels to promote lateral prostatic growth.

Prostate gland development

At the end of the human fetal period, (*i.e.*, the seventh week of gestation), the human male and female urogenital systems are identical. The paramesonephric (Mullerian) ducts, the mesonephric (Wolffian) ducts, and the primordial ureters all lead into a rudimentary urinary bladder, which has developed from the urogenital sinus. In the male embryo, the paramesonephric ducts degenerate, leaving behind the vestiges of the uterovaginal primordium, the prostatic utricle. The ureters separate from the mesonephric ducts, which become incorporated into the urinary bladder and effectively migrate distally to the proximal part of the urethra. The mesonephric ducts differentiate to form the ejaculatory ducts.

The first indication of the formation of the prostate is an increase in cellularity of the splanchnic mesoderm surounding the proximal part of the urethra. During gestational week 10, the androgen responsive urogenital sinus mesenchyme induces epithelial buds in the presumptive prostatic urethra. Initially, 14 to 20 solid epithelial buds, in at least five groups, grow into the surrounding mesoderm; this is the presumptive peripheral glandular area. Shortly thereafter, a second phase of budding gives rise to the internal glandular area. The solid buds extend and branch under mesodermal control. By the 11th week, lumens form within the epithelial cords and cellular end buds form primitive acini. Mesenchymal cells differentiate into smooth muscle, fibroblasts, and blood vessels. During the 12th week, the epithelium continues to proliferate, while connective tissue septae extend into the acini and the stroma of the gland thins as the ducts and acini expand.

By 13 to 15 weeks, T concentrations have reached their peak embryonic levels. Androgen mediated epithelial mesenchyme interactions cause the simple cuboidal epithelial cells to differentiate, at first in the proximal regions of the larger ducts then progressing distally. By the end of the 15th week, the secretory cells are functional, the basal cell population has developed, and scattered neuroendocrine cells are present. Maturation of the gland continues, while embryonic T levels are high; however, as T levels fall during the third trimester, the gland enters a quiescent state.

The prostate at puberty

The quiescent state persists until puberty, when T levels again increase and the epithelium proliferates, giving rise to the complex folding seen in the mature gland. The prostate doubles in size during this phase of development, ARs are expressed by the epithelial cells, and the full secretory phenotype is established. By 45 to 50 years, T levels are in decline again, and the prostate undergoes a period of involution. With increasing age, atrophication of the gland may continue, though commonly, benign prostatic hypertrophy occurs.

The prostate gland is an example of sex-specific, androgen-regulated development in many species. The above describes human prostate development, though most of the genetic data has been developed using the rodent prostate as a model. Great care must be exercised when comparing late prostatic developmental events between species, though the early events during branching are sufficiently similar to be relevant. The timing of developmental events is imprecise and changes are progressive; the figures given are approximations only and may vary by +/- 10%

The prostate is a fibromuscular exocrine gland. It is a male accessory reproductive gland which expels a complex proteolytic solution into the urethra during ejaculation. The proteolytic enzymes liquify the semen after ejaculation, and the phosphatases and salts modify the vaginal environment to enhance sperm survival, though the exact function of many of the components of prostatic secretion have yet to be determined.

In humans, the prostate gland surrounds the first 3 cm of the urethra (prostatic urethra) as it leaves the urinary bladder. The ejaculatory ducts enter dorsally and join the urethra within the gland, either side of the prostatic utricle. Anatomically, the most caudal aspect of the gland, which apposes the urinary bladder, is referred to as the base of the gland. The walls of the prostatic urethra are highly convoluted and lined with transitional epithelium. In its resting (not distended) state, the ureter has a longitudinal ridge (the urethral crest) running the length of the gland. The majority of the ductal glands secrete into longitudinal grooves (the urethral sinuses) formed on either side of the ridge. Near the junction of the ejaculatory tubes and the urethra is a short diverticulum in the urethral crest. This is the prostatic utricle, the male vestigial remnants of the female uterus and vagina.

The prostate is covered by a thin vascularized fibrous sheath which surrounds a fibromuscular layer continuous with the smooth muscle surrounding the bladder. The fibromuscular layer extends within the organ as septae, dividing the gland into ill-defined lobules and functional areas.

Secretory Development of the Prostate

The secretory components of the gland are divided into three concentric layers. The innermost area is comprised of mucosal glands which are concentrated around and secrete into the upper region of the prostatic urethra. The middle or internal area contains submucosal glands which secrete via short ducts into the urethral sinuses. The outer or peripheral area constitutes the majority of the gland and secretes via long ducts into the urethral sinuses. The anterior isthmus is an area of the gland ventral to the urethra, relatively free of glands and rich in fibromuscular tissue.

The prostate is a compound tubuloacinar gland. Within the acini and tubules, the epithelium forms complex folds and papillae supported by a thin, highly vascularized, loose connective tissue. The secretory epithelium is mainly pseudostratified, comprising tall columnar cells and basal cells which are supported by a fibroelastic stroma containing randomly orientated smooth muscle bundles. The epithelium is highly variable, and areas of low cuboidal or squamous epithelium are also present, with transitional epithelium in the distal regions of the longer ducts. Densely packed basal nuclei are characteristic of the prostatic epithelium. The tall columnar secretory cells have an extensive basal golgi complex, apical lysosomes, and secretory granules. The epithelium contains scattered neuroendocrine cells, which partly control release and expulsion of prostatic secretions during ejaculation.

The fluid secreted by the prostate gland is rich in acid phosphatase and citric acid. It contains the proteases fibrinolysin and prostate specific antigen (PSA), the enzyme amylase, kallikreins, semenogelin, fibronectin, phospholipids, cholesterol, zinc, calcium, and many proteins of unknown function such as beta- microseminoprotein.

Developmental genetics of the prostate

The development of the prostate is controlled by steroid hormones that in turn induce and maintain a complex and little understood cross talk between the various cell types making up the gland. The result of this intercellular communication can be either new growth or growth quiescence, depending upon the differentiation state of the cell type being stimulated. Secretory function of the prostate is dependent upon direct stimulation of fully differentiated prostatic epithelial cells by androgens. Thus, the prostate seems to be regulated in a similar manner to other organs of the male and female genital tract, with proliferative control mediated by cell-cell interactions; whereas, differentiated function is determined by direct steroid action on the parenchymal cells.

Although the androgen-dependent nature of prostate development has been well established, it is not clear how androgens regulate prostate differentiation and growth. Identification of androgen-dependent gene pathways present during prostate development is essential in order to differentiate between normal and disease processes of this male-specific organ. The use of high-density cDNA hybridization arrays and subtractive hybridization techniques can aid in the identification of these gene pathways. These can be further studied by analysis in transgenic mouse models. A normalized cDNA library derived from neonatal mouse prostate has been developed. This library has been screened to identify overexpressed clones in the developing male prostate compared to female reproductive tissues. Genes of interest were sequenced, their expression characterized by whole-mount *in situ* hybridization, and subjected to functional analysis using *in vitro* organ cultures, transgenic, and/or targeted mutagenesis in the mouse. Genes identified in this manner may provide useful prognostic markers for early prostate cancer.

Seminal Vesicles and Coagulating Gland Development

Using an electron microscope, the development of the seminal vesicle from the Wolffian duct and the prostate from the urogenital sinus has been studied in rat fetuses from gestational day 14 to birth. Prior to the onset of androgen secretion, the cells of the urogenital sinus and the caudal part of the Wolffian duct have a simple, undifferentiated appearance. After the onset of androgen secretion by the fetal testes at day 15, "intracytoplasmic confronting cisternae" of the granular reticulum appear in both the urogenital sinus and Wolffian duct. Portions of the granular endoplasmic reticulum of the urogenital sinus become distended with a finely granular, moderately dense material. In the urogenital sinus, many hemidesmosomes are formed at the basal surface of the epithelium. Specializations of the extracellular materials are present opposite the hemidesmosomes. The formation of the seminal vesicles and the prostate begins at day 18–19 of gestation. The cells of the seminal vesicle are taller than the Wolffian duct cells from which they arise, the granular endoplasmic reticulum is moderately increased, and a patent lumen is formed. The cells of the fetal prostate do not differ greatly from those of the urogenital sinus from which they arise, except that the prostatic cells initially lack hemidesmosomes.

Development of the seminal vesicle is elicited by androgens and is dependent on epithelial-mesenchymal interactions. Androgenic signal transmission from the androgen-receptor-positive mesenchyme to the epithelium has been postulated to involve paracrine factors. Keratinocyte growth factor (KGF), a member of the fibroblast growth factor family, is produced by stromal/mesenchymal cells and acts specifically on epithelial cells. The KGF transcript was detected by reverse transcription-polymerase chain reaction in newborn mouse seminal vesicle s and by Northern blot analysis of RNA from cultured neonatal seminal vesicle mesenchymal cells. Newborn seminal vesicles placed in organ culture undergo androgen-dependent growth and differentiation. Addition of a KGF-neutralizing monoclonal antibody to this system caused striking inhibition of both seminal vesicle growth and branching morphogenesis. This inhibition was due to a decline in epithelial proliferation and differentiation, as the mesenchymal layer was not affected by anti-KGF treatment. When KGF (100 ng/ml) was substituted for T in the culture medium, seminal vesicle growth was approximately 50% that observed, with an optimal dose of T (10^{-7} M). All of these findings suggest that KGF is present during a time of active seminal vesicle morphogenesis and functions as an important mediator of androgen-dependent development.

Cowper's Glands Development

Cowper's glands are pea-sized glands present inferior to the prostate gland in the male reproductive system. They produce thick, clear mucus prior to ejaculation that drains into the spongy urethra. Though it is well established that the function of the Cowper's gland secretions is to neutralize traces of acidic urine in the urethra, knowledge regarding the various lesions and associated complications of this gland is scarce. Cowper's glands (also known as the bulbourethral glands) were named after the 17th century English surgeon William Cowper. Their relation to the prostate gland is, perhaps, both anatomical and metaphorical.

During the 10th week of human development, the pelvic urethra gives rise to the paired bulbourethral glands. Whereas the prostate develops from the prostatic urethra, the bulbourethral glands develop from the membranous urethra under the influence of many endocrine and paracrine signals. Moreover, Cowper's glands are most significantly under the control of DHT.

As the prostate develops, the paired bulbourethral glands sprout from the urethra just below the prostate. During the 10th week, the seminal vesicles arise from the distal mesonephric ducts in response to T, whereas the prostate and bulbourethral glands develop from the urethra in response to DHT. The vas deferens and seminal vesicles derive from the mesonephric ducts and the prostate from the cranial urogenital sinus. The bulbourethral glands develop from the intermediate urogenital sinus, which differ in the inductive capacity of its mesenchyme.

Cowper's gland secretory development

Sex accessory tissues include the prostate, seminal vesicles, ampullae of vas deferens, and bulbourethral glands and are believed to play an important role in the reproductive process. They are homologues of the greater vestibular glands in the female. Cowper's glands are accessory sexual organs that contribute to urethral lubrication. It has also been demonstrated that Cowper's gland secretion has a role in semen coagulation, which has been demonstrated in rodents (Beil and Hart, 1973). The two main Cowper's glands are situated within the urogenital diaphragm, with a second pair of accessory glands situated in the bulbospongiosal tissue. The main Cowper's ducts enter the ventral surface of the bulbourethra near the midline by piercing the spongiosum. The accessory ducts can enter the urethra directly or drain into the main duct. The ducts of the Cowper's gland empty into the bulbourethra. The bulbourethra extends from the inferior urogenital diaphragm to the suspensory penile ligament superiorly and penoscrotal junction inferiorly. The Cowper's gland consists of well-demarcated lobules of small, compact tubuloalveolar glands radiating from a central excretory duct lined by pseudostratified epithelium, and entrapped within fascicles of muscle. The glands have a thin connective tissue capsule composed of simple columnar epithelium. The glands histologically stain positively for mucin, smooth muscle actin (periphery of acini), and negatively for prostatic acid phosphatase (PAP), S100, carcinoembryonic antigen (CEA), and are variable for prostate-specific antigen (PSA) and CK903 (Elgamal *et al.*, 1994).

Functions of Cowper's glands

During sexual excitement, these glands secrete clear glycoproteins into the bulbous urethra. The male sex accessory tissues require the continued function of the testes for their development, growth, and maintenance of secretions that form the major components of the ejaculate.

Cowper's glands secrete glycoproteins during sexual stimulation, which functions as a lubricant for the semen. In response to sexual stimulation, the bulbourethral glands secrete an alkaline mucus-like fluid. This fluid neutralizes the acidity of the urine residue in the urethra, helps to neutralize the acidity of the vagina, and provides some lubrication for the tip of the penis during intercourse. Cowper's gland secretions contain no sperm. These findings are confirmed in a study which demonstrated smears of pre-ejaculatory Cowper's gland secretion obtained during foreplay from at least two different occasions and semen samples after masturbation. None of the pre-ejaculatory samples contained sperm, while all the patients had sperm in routine sperm analyses. It has been shown that pre-ejaculatory fluid secreted at the tip of the urethra from Cowper's gland during sexual stimulation does not contain sperm (Zukerman *et al.*, 2003).

Cowper's glands are involved in the immune defense of the genitourinary tract and secrete many glycoproteins, including Prostate Specific Antigen (PSA) (Pedron *et al.*, 1997). Immunohistochemical studies on whole-mount cadaveric Cowper's gland and cystoprostatourethrectomy samples showed that although, PSA and PSAP are mostly produced by prostatic tissue, it was not exclusive (Elgamal *et al.*, 1994). These findings

support the hypothesis of extraprostatic sources of PSA and may impact on the specificity and sensitivity of PSA serum levels after radical prostatectomy.

Glans Penis Development

By eight weeks of human gestation, the Leydig cells of the developing testis are capable of producing T under the stimulation of human chorionic gonadotropin (hCG). Circulating T causes development of tissues with T receptors. Normally, T is taken into the cell and bound by the AR. In the cytoplasm, it is converted to DHT by the enzyme 5-alpha-reductase. DHT is at least four times more potent than T.

The genital eminence, an external mound arising between the umbilicus and the tail, is made up of the genital tubercle and the genital swellings. The urogenital sinus opens at the base of the genital tubercle between the genital swellings. These structures form identically in male and female embryos up to week 7 of gestation.

At nine weeks of gestational age, and under the influence of T, the genital tubercle starts to lengthen. In addition, the genital swellings (also called the labio-scrotal folds) enlarge and rotate posteriorly. As they meet, they begin to fuse from posterior to anterior. As the genital tubercle becomes longer, two sets of tissue folds develop on its ventral surface on either side of a developing trough, the urethral groove. The more medial endodermal folds will fuse in the ventral midline to form the male urethra. The more lateral ectodermal folds will fuse over the developing urethra to form the penile shaft skin and the prepuce. As these two layers fuse from posterior to anterior, they leave behind a skin line: the median raphe.

By 13 weeks, the urethra is almost complete. A ring of ectoderm forms just proximal to the developing glans penis. This skin advances over the corona glandis and eventually covers the glans entirely as the prepuce or foreskin.

Male Penile Urethra Development

The human penis goes through a natural state of hypospadias as it develops from a primitive, undifferentiated structure into a fully differentiated penile urethra (Baskin *et al*., 2001). The urogenital system of the male embryo develops during weeks 8 to 14 following ovulation (Moore *et al*., 2003). At 8 weeks, the external genitalia of both male and female embryos are indistinguishable. Both have a midline genital tubercle just above a urogenital membrane flanked on each side by outer genital swellings and inner urethral folds (Baskin *et al*., 2001).

Masculinization is an androgen-driven process, beginning under the influence of hCG, which stimulates the Leydig cells of the fetal testes to produce T (Moore *et al*., 2003). Testosterone is then converted to the more active DHT by the enzyme 5-α reductase type II. For DHT to effect masculinizing action on the developing genitalia, it must bind to ARs located in the genital tissues (Yamada *et al*., 2003). Cellular signaling through the ARs must be intact (Cunha and Baskin, 2004).

The effects of T occur early and include an increase in the distance between the anus and genital structures, followed by elongation of the genital tubercle, which will become the penile shaft and glans (Baskin, 2000). The genital swellings, also called the labioscrotal folds, migrate caudally and start to fuse, forming the scrotum (Moore *et al*., 2003). A urethral groove developing on the underside of the penis becomes the urethra (Baskin, 2000). Folds of tissue, called urethral folds, that frame the lateral walls of this groove, have inner (endodermal) and outer (ectodermal) edges (Moore *et al*., 2003; Baskin, 2000).

As the urethral groove develops from the posterior to anterior surface, it is soon enclosed by fusion of the endodermal folds to form a tubular penile urethra (Cunha and Baskin, 2004; Hynes and Fraher, 2004). The fusion of the endodermal edges creates an epithelial seam that is subsequently reabsorbed (Baskin *et al*., 2001). The ectodermal edges then fuse over the urethra to fashion the penile shaft skin, leaving behind the median raphe (Baskin, 2000). The distal, or glanular urethra, develops last by one of two possible mechanisms (Cunha and Baskin, 2004). The classic theory is that the distal portion of the urethra develops as an ingrowth from the tip of the penis until it joins the proximal tubular urethra (Moore *et al*., 2003; Kurzrock *et al*., 1999). Recent evidence however suggests that the entire urethra, from base to tip, is formed by continuous extension and fusion of the endodermal urethral groove (Hynes and Fraher, 2004; Belman, 2002).

The main defect of hypospadias, the abnormally located urethral opening, is considered a failure of some stage in the orderly process of development outlined above. A normal penile urethra, with a meatus at the tip of the glans, requires proper formation of the urethral groove, urethral folds, and fusion of the folds with seam formation and seam removal. Failed seam formation during fusion of the urethral folds results in hypospadias (Baskin *et al*., 2001) and the site of failure dictates the final position of the urethral meatus (Yamada *et al*., 2003).

The two features that commonly accompany hypospadias, penile curvature and incomplete foreskin, also represent normal stages of embryologic development. The developing fetal penis is curved ventrally because the penis and the shaft skin grow faster on the dorsal (upper) than on the ventral aspect (Snodgrass *et al*., 2002). Concurrent with urethral development, at about 8 weeks following ovulation, the prepuce (foreskin) arises from the base of the glans, growing primarily on the dorsal surface of the penis. As the prepuce advances distally, it also grows ventrally to completely cover the glans (Baskin, 2000). If the urethral folds fail to fuse, as in hypospadias, the preputial growth is also interrupted (Baskin, 2000). Hypospadias at more distal locations (*e.g.*, distal glanular hypospadias) is associated with fusion of the urethral folds to the base of the glans, at least. Thus, in these very minor degrees of hypospadias, normal preputial development is possible (Hynes and Fraher, 2004).

Preputial Glands

The preputial glands of rodents are highly modified, paired sebaceous glands lying anterior to the male prepuce between the body wall and the skin. The rodent glands

are not homologous with the glandular developments of the preputial skin found in other mammalian groups, but are unique to the order Rodentia (Mallick, 1991). Early studies demonstrated that preputial glands of hypophysectomizd rats grew in response to exogenous steroids such as T (Noble and Collip, 1955) and 4-androstene-3,17-dione (Selye and Clarke, 1943). The preputial glands were highly responsive to steroids which induced androgenic effects in animals but not to steroids of the phenolic estrogen type.

Preputial glands are sebaceous glands and their sebocytes, which have AR, are stimulated to proliferate and produce secretions by androgens. The AR gene expression increases as the sebocytes begin to differentiate (Miyake, 1994).

Adrenal Glands

From birth until 75 days, both in male and female rats, the weight of the animals and that of the adrenal glands demonstrate a linear growth with time. The area of the zona glomerulosa (ZG) increases in size from birth until ~40 days of age. After that, growth has a much smaller slope (females, $r=0.84$, $P<0.001$; males, $r=0.81$, $P<0.001$). Aldosterone secretion has a marked increase until 20 days of age and thereafter shows a tendency to decrease (females, $r=-0.19$, $P<0.02$; males $r=-0.26$, $P<0.001$). Plasma renin activity follows a trend parallel to that of aldosterone. However, the steroid precursor 18-OH-deoxycorticosterone (18-OH-DOC) demonstrates an increase progressively with age, especially in the females (females, $r=0.57$, $P<0.001$; males, $r=0.40$, $P<0.001$). The expression of the enzyme 3-β-hydroxysteroid dehydrogenase (3-β-HSD) was also studied by immunohistochemistry, and it was shown to be very low at birth and then starting to increase by 10 days of age. After 30/40 days of age, the amount of this enzyme existing in the ZG was comparable with that of the outer zona fasciculata (ZF). It was concluded that the development of the ZG in the rat has particularities that make it different from that of the rest of the cortex (Burmnn *et al*., 1998).

Gonadal steroids have been shown to decrease adrenal weight in the rat by many investigators. In 1996, Bentvelson *et al*. (1997), measured high levels of AR in the adrenal by immunoblot assay techniques. Castration appears to have no effect on the amount of detectable AR in the adrenal gland.

Striated Muscle

It was noted by Bowman in 1840, that striated muscles are more developed in males than in female mammals and since that time that the growth of a number of striated muscles is increased by androgens in different species. Castration of males produces regression of many striated muscles and the injection of androgens reverse this process (Kochakian *et al*., 1948 and 1956).

Michel and Baulieu (1974) reported the presence of AR in the rat quadriceps femoris and later groups confirmed this finding. There have also been demonstrated *in vitro* effects of androgens on cultured myoblasts.

Demonstration of Androgen Receptor in Tissue

ARs are present in most tissues, as demonstrated by a variety of methods. 3H-androgen binding assays were the initial approach used to determine tissue distribution of AR. By injecting (3H)-T into rats, followed by quantitation of (3H)-androgen uptake in various tissues, Gustafsson and Pousette (1975) were able to identify target organs of androgens. The development of anti-AR antibodies has complemented the autoradiographic methods of AR detection (Husmann *et al*., 1990; Tilley *et al*., 1994; Takeda *et al.*, 1990; and Chang *et al*., 1989). Using anti-AR antibodies, Takeda *et al*. (1990) evaluated AR distribution in various rat tissues. They were able to demonstrate that all male sexual organs in the rat showed a strong, positive nuclear staining for AR, whereas several other tissues, including hepatic, renal, neuronal, muscular and female reproductive organs had weak, albeit positive, nuclear staining. In fact, the only tissue which did not demonstrate staining for AR was the spleen. The use of microwave-based antigen retrieval for AR (Janssen *et al*., 1994) enhanced the immunohistochemical detection of AR in paraffin sections allowing for evaluation of archival tissue sections.

In addition to identification of AR protein, detection of AR mRNA in tissues has been accomplished by several methods. Cloning of human AR (hAR) and rat AR (rAR) cDNA (Chang *et al*., 1988; Lubahn *et al*., 1988) allowed for development of probes which were used to detect in various tissues the AR mRNA by Northern blotting or by *in situ hybridization*. In addition to confirming previous immunohistochemical staining data, the analysis of AR mRNA by Northern blot resulted in identification of two isoforms in the A/B domain of AR mRNA in the vocal organ of *Xenopus* (Fischer *et al*., 1993) and the brain of rodents (Burgess and Handa, 1993). The sensitivity of detection of AR mRNA was greatly improved by competitive RT-PCR (Young *et al*., 1994). **Table 4** summarizes their results for the relative expression (compared to prostate) of AR mRNA in various tissues of male and female Sprague-Dawley rats based on competitive RT-PCR. These results demonstrate agreement between mRNA levels and immunohistochemical staining intensity (Takeda and Chang, 1991).

AR expression is modified during fetal development, sexual development, aging, and malignant transformation. Regulation of AR levels may occur anywhere along the path from AR gene transcription to post-translational modification. A variety of factors, including androgens, have been implicated in modulating the AR protein and mRNA expression.

In the case of mouse fetal development, AR mRNA, based on *in situ* hybridization, was not found in the urogenital sinus at 13.5 days of gestation, whereas at 15.5 days of gestation, both AR mRNA and protein levels were detectable (Takeda and Chang, 1991).

Table 4. Relative abundance of androgen receptor mRNA in various organs to the level in the prostate gland (prostate = 100)

Organ	Male	Female
Hypothalamus	42	217
Adrenal gland	141	186
Epididymis	115	ND
Thyroid gland	68	ND
Harderian gland	58	ND
Pituitary gland	56	9
Preputial gland	44	38
Quadriceps muscle	35	ND
Levator ani muscle	30	ND
Kidney	27	7
Coagulating gland	25	ND
Seminal vesicle	25	ND
Testis	20	ND
Liver	18	9
Submaxillary gland	17	ND
Bulbocavernosus muscle	16	ND
Vagina	ND	9
Heart	8	7
Ovary	ND	4
Uterus	ND	2

Table 4: The relative abundance of AR mRNA in rat tissues (Young *et al*., 1994). Total RNA from the indicated tissues was subjected to competitive reverse transcription-polymerase chain reaction for quantitation of AR mRNA levels. The data are reported as percentage of AR mRNA relative to the prostate AR mRNA levels (averaged from 5 male rats). The data represent mean values from 3-5 rats. ND = not determined.

If, as discussed below, one subscribes to the idea that both T and DHT mediate their effects by interacting with a single nuclear receptor protein, then how can we account for the requirement of both T and DHT for sexual development? The answer may be found by examining the ability of these androgens to interact with the AR. Several investigators have demonstrated that T has approximately three times faster association and dissociation rates than DHT on both the rat (Wilson and French, 1976) and human (Grino *et al*., 1990; Zhou *et al*., 1995) AR. In agreement with these binding kinetics, Zhou *et al.* (1995) have demonstrated that T is less effective at stabilizing AR than DHT. These observations suggest that DHT, by enhancing the stabilization of AR and its action, amplifies the T signal in those tissues which contain 5 alpha-reductase. Perhaps in these tissues, the AR expression is not sufficient for T to mediate a physiologic response, but due to the ability of DHT to enhance AR activity, a response is observed.

In contrast to the inability of T to mediate various androgen-dependent events during sexual development, the biology of hair growth offers an example of the different effects that androgens exert on the proliferation of similar populations of epithelial cells. Specifically, T can stimulate facial hair growth but causes the regression of scalp hair in aging individuals (Randall, 1994). Hair follicles are intimately associated with the mesenchymally-derived dermal papilla, which is believed to provide an important influence on the follicular proliferation.

Several lines of evidence support the role of androgens in controlling growth of hair follicles through modulation of the dermal papilla activity: 1) AR has been identified by both ligand binding assay (Hodgins *et al*., 1991) and by immunohistochemical staining in the dermal papilla (Choudhry *et al*., 1992); 2) dermal papillae from androgen-dependent hair follicles contain a greater number of AR than those in nonbalding areas (Randall *et al*., 1992); 3) in primary dermal papilla cell lines from either androgen-dependent (*i.e.*, beard) or -independent (*i.e.*, nonbalding scalp) hair follicles, only the beard dermal papilla-derived cells were able to synthesize DHT when T was added to the media (Randall, 1994); 4) the level of 5 alpha-reductase varied between hair follicles from frontal and occipital sites (Sawaya, 1994); and 5) dermal papilla can produce extracellular matrix components and mitogenic factors (Randall, 1994). Taken together, these findings suggest that androgen, via the AR, can indirectly mediate an effect on hair follicle proliferation through modulating dermal papilla activity. Though these data do not explain how T modulates cell proliferation in hair follicles, the differences in androgen metabolism and AR expression may, in part, account for the opposite proliferative responses observed in various epithelial tissues. The effect of androgens on cell proliferation in hair follicles may be regulated through regulation of expression of growth factors. Androgens can modulate expression of a variety of growth factors in the prostate stroma.

Mesenchymal-epithelial interactions are critical in the development of prostate tissue [reviewed in (Cunha *et al*., 1992)]. Prostate mesenchyme, when coincubated with Sertoli cells, synthesizes extracellular matrix in an androgen-independent manner; however, when exposed to an androgen, it produces a diffusible substance similar in action to P-Mod-S (Verhoeven *et al*., 1992). P-Mod-S, a protein secreted by peritubular

cells, can activate a variety of responses such as inhibin production and aromatase activity in Sertoli cells. The effect of P-Mod-S on the prostate epithelia is currently unknown; however, it may also stimulate the activity similar to that observed in Sertoli cells, thus accounting for one mechanism of mesenchymal-epithelial interaction within the prostate. Gleave *et al*. (1994) demonstrated that prostate fibroblasts secrete a diffusible substance which can stimulate the growth of LNCaP prostate carcinoma cells. Androgen deprivation mediated by castration resulted in expression of tenascin in the rat prostate (Vollmer *et al*., 1994).

T and DHT mediate their effects by altering gene expression in target tissues via a single receptor, the AR. Despite the binding of T and DHT to a single receptor, the effect of these hormones on a single gene may be quite distinct. For example, differential regulatory effects of T and DHT on expression of several genes, including Far-17a (Seki *et al*., 1991) and the cytokines interleukin-4 (IL-4), IL-5 and g-interferon (gIFN), has been reported (Araneo *et al*., 1991). The observation that both T and DHT can differentially regulate the expression of the androgen responsive genes has led to the controversial idea that more than one AR may exist. This hypothesis is supported by the following observations [reviewed in (Sheridan, 1991)]: (1) (3H)T, when injected into rats, concentrates in the hypothalamic nuclei, and 100x unlabeled DHT does not inhibit this localization; (2) hypothalamic localization is not observed after injection of (3H)DHT; and (3) the ability of T, but not DHT, to induce neuronal proliferation or male sexual behavior in castrated male rats. These data are consistent with two different possibilities. One possibility is that two different ARs indeed exist. On the other hand, these data may be due to different metabolic interactions of the androgens on a single AR. As discussed above, T dissociates from the AR 3 times faster than DHT and is less effective in stabilizing the AR (Zhou *et al*., 1995). This difference in the dissociation rate has been directly related to the androgens' ability to stimulate transcription of an androgen responsive gene (Deslypere, 1992). These observations could account for the differential effects of these androgens mediated by one receptor.

Perhaps the strongest evidence for the presence of one AR is derived from the observation that genotypic XY mice and rats with testicular feminization syndrome (Tfm) do not have a fully functional AR (Deslypere, 1992; Yamamoto *et al*., 1983; Aarden, 1989; Yarbrough *et al*., 1990), and even though they express both T and DHT, they develop into phenotypic females. This experiment of nature demonstrates that loss of one AR can result in loss of action of both androgens.

The presence of two androgens acting on one receptor may serve several possible functions. Cells which contain steroid 5 alpha-reductase can convert T into DHT. Thus, the overall effect of this system may be to amplify the action of T via conversion to DHT within these cells, thus providing a mechanism of local regulation.

Another method of studying the multi-hormonal nature of the developmental process has been to treat the animal with an antagonist to the developmental signal for the duration of the critical period and then discontinue treatment. Higgins *et al*. (1981) used an estrogen as a probe to identify critical periods of development of the male accessory glands and treated rats for five days with estradiol benzoate [50 µg in oil (20µl)] during

either the neonatal, prepubertal, or pubertal stages or as adults. Neonatal rats treated at birth for five days show several abnormalities in the male reproductive system development. These include smaller testes, epididymides (caput affected more than the cauda, with DNA content decreased to approximately one half off the control value), seminal vesicles and ventral prostate. The seminal vesicles and ventral prostate weights were approximately one quarter that of the controls. The DNA content for both was decreased, but not proportionately to the tissue weight, indicating that the estradiol affected cell size as well as cell proliferation. When animals were allowed to go out to 120 days beyond treatment, some compensatory growth occurred in the seminal vesicles, but not in the ventral prostate, and full secretory activity was not restored in the seminal vesicles; therefore, delayed maturity may not be the only explanation.

Rats treated for five days during the prepubertal period (aged 20-25 days) had normal sized testes. Tissue weights and DNA contents for the seminal vesicles and ventral prostates were near control values. Caput and cauda epididymides were smaller with proportionally less DNA, but no different than the amounts noted in the animals treated as newborns. Thus, this developmental process seems to be insensitive to estrogen treatment during the prepubertal period. However, when rats were treated during the pubertal period, 40-45 days of age, they had slightly smaller testes and underdeveloped seminal vesicles and ventral prostate, but less marked than when treated in the neonatal period. This suggests that the estrogen had affected cell proliferation and not cell size. The effects on the epididymides were the same as in the two earlier periods.

Rats treated in the adult period of life caused a small decrease in seminal vesicle weight and a 40% decrease in ventral prostate weight.

Conclusions

Many types of substances can have an influence on the reproductive tissues of both males and females. For instance, it has been demonstrated that ascorbic acid is essential for maintaining the physiological integrity of the androgen target reproductive organs in male guinea pigs. Androgenic, estrogenic, anti-androgenic, and anti-estrogenic compounds all have been shown to have effects on the target organs that are responsive to androgens. The Hershberger assay is one attempt to detect these types of compounds, based on their relative influence on the target tissues: ventral prostate, LABC, seminal vesicles, glans penis, and Cowper's glands.

CHAPTER 6. VERSIONS OF THE ASSAY/PROCEDURAL VARIABLES

Versions of the Assay

Prepubertal intact male

This study design is similar to the weanling male version (see I.D. below). Ashby and Lefevre (2000a) employed prepubertal, intact male rats. After weaning on PND 21, the males were exposed to the test chemical or vehicle control during the postwean prepubertal period. The start of exposure is not necessarily immediately after weaning (on PND 21 or 22), but can be later during the prepubertal period, depending on the duration. The endpoints of interest are the age at acquisition of puberty (PPS), the weights of the reproductive organs (including the testes and epididymides since these are intact animals), and other organs of interest, such as the liver. The timing and duration of treatment are critical and somewhat circumscribed since natural puberty in the CD (SD) rat begins after PND 35 (no CD males have acquired PPS on or before PND 35 in the authors' laboratory), with the control overall mean approximately 41-42 days of age. The CD-1 mouse control mean male age at acquisition is on PND 26-28, which is even earlier than in the rats.

Since these males are intact (not castrated), the hypothalamic-pituitary-gonadal axis is intact, so it is considered an "apical" assay; *i.e.*, multiple mechanisms of interference are possible (and detected) at various organizational levels, but no mechanism is specifically identified. This version's strengths include its apical nature and the range of mechanisms of disruption it can detect (*e.g.*, central versus peripheral, CNS-endocrine interface, synthesis versus transport, metabolism, elimination, effects on carrier proteins, as well as at the AR). Also, the time prior to puberty is a time of increasing sensitivity to exogenous androgens. At puberty, there is a surge of T, so the animal and its organs are less sensitive to exogenous androgens (they may be maximally stimulated by the endogenous T). This version can also detect agents that act initially on nonreproductive targets, such as liver damage which results in slow degradation and elimination of T, or liver stimulation, which accelerates T degradation and elimination, or effects on the thyroid which are known to affect the reproductive organs, especially early in development (Noda *et al*. 2005).

The liabilities are lack of mechanism identification and a brief prepubertal window of opportunity.

Castrated adult male

The Hershberger assay has been shown to be more sensitive and specific to AR-mediated alterations in the castrated adult male (or the other versions) than the assessment of endocrine activity in the intact adult male rat, which does not consistently detect the anti-androgenic activity of severe weak anti-androgenic pesticides (*e.g.*, 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene [*p,p'*-DDE]) and linuron (Cook *et al*.,

1993; O'Connor *et al*., 1999, 2002). The above toxicants are easily detected in the Hershberger assay (Lambright *et al*., 2000; Parks *et al*., 2000; Yamasaki *et al*., 2003a). The assay is also capable of discriminating between T- versus DHT-dependent changes. Finasteride, a 5α-reductase inhibitor (which converts T to DHT), dramatically reduces the weight of DHT-dependent structures, such as the male accessory sex organs, but has little or no effect on the LABC muscle, which has low levels of 5α-reductase and is T dependent (Gray *et al*., 2004).

The procedures for this version include castration postpuberty with a recovery period, so T-dependent (and DHT-dependent; see below) organs have involuted/regressed or no recovery period (so the male hormone-dependent organs have not yet regressed). Administration of the test chemical or reference androgen is by gavage or sc injection, typically for three to five days. Twenty-four hours after the last dose, the animals are terminated and necropsied and the accessory sex organs weighed.

- If there is a recovery period, so organ involution occurs (in the vehicle castrate control group), and the groups administered the test chemical exhibit increased growth and organ weights, then the test chemical is an androgen-agonist. The potency can be determined by the increase in organ weights in the treatment group versus the organ weights in the reference androgen group. Another comparison is between the organ weights in the noncastrated controls versus the organ weights in the castrated animals administered test chemical.

- Androgen antagonists can be detected if a castrated male group is dosed with the reference androgen and test chemical. If the accessory sex glands weigh less in the presence of both the reference androgen and the test chemical than they do in the presence of the reference androgen alone, then the test chemical and the reference androgen must be competing for binding to the AR. They are both recognized by the AR, but the anti-androgenic test chemical does not activate the downstream genes to simulate the growth of the accessory sex organs.

- If there is no recovery period, the endpoint of interest is whether or not the accessory sex organs can maintain their precastration weight (compared to intact controls) in the presence of the test chemical. If so, the test chemical is an androgen agonist and the degree of maintenance (versus the group treated with the reference androgen) will determine the potency.

- If there is no recovery, and the castrate group treated with the test chemical and the reference androgen has lower accessory sex organ weights than in the castrate group treated with reference androgen alone, and lower organ weights than in the intact control group, then the test chemical is an androgen antagonist. The potency is based on the comparison of the accessory sex organ weights in the test chemical group versus the test chemical plus reference androgen group, and versus the intact control group.

Peripubertal castrated male

This version of the Hershberger assay employs sexually immature rats castrated at peripuberty by removal of the testes and epididymides on PND 42 (Gray *et al*., 2005). Gray *et al*. (2005) also recommended a 12-day recovery period, with initiation of treatment on PND 53-54 for ten consecutive days and necropsy the day after the last dose on PND 64-65. The late age at castration, plus administration of the reference androgen, allows the males to initiate PPS since in Gray's experience, males castrated at a younger age do not initiate PPS with or without exogenous androgen.

The strengths include:

- The enhanced sensitivity of the peripubertal stage of sexual development to exogenous androgens, since both the AR and appropriate steroidogenic enzymes are present at this stage;

- The glans penis and other accessory sex organs display high sensitivity and small weights at this stage, which should minimize variation in responses across animals within groups;

- Since the animal is castrated, the hypothalamic-pituitary-gonadal axis is interrupted and the response of the accessory sex organs is absolutely dependent on the recognition of the test chemical by the AR, subsequent binding to the AR, and initiation of the downstream cascade of transcriptional activation of relevant genes, resulting in accessory sex gland growth (if the test chemical is an androgen agonist) or blockage of the initiation of the downstream transcription activation of relevant genes, resulting in no growth of the sex accessory glands or lesser growth in the presence of both the test chemical and the reference androgen (if the test chemical is an androgen antagonist); and

- The use of younger animals (versus adults) may enable identification of the same effects at lower doses (*i.e.*, a quantitative difference) or additional or different effects (*i.e.*, a qualitative effect), such as effects on PPS (Gray *et al*., 2005).

Other versions

Castrated weanlings

The Hershberger assay, as originally presented (Hershberger *et al*., 1953) used male rats castrated at weaning on PND 21, with treatment beginning on the day of castration (*i.e.*, no recovery period). Other researchers have also used animals castrated as weanlings (*e.g.*, Wakeling *et al*., 1981; Snyder *et al*., 1989) with variable lengths of recovery before beginning treatment.

Intact weanlings

Intact weanlings have also been used as an assay for endocrine disruptors, where the window of opportunity is after weaning and before the onset of puberty, which is initiated by a burst of endogenous T (Ashby and Lefevre, 1997). There is at least two weeks (PND 21-35) after weaning before any male rat exhibits PPS (the hallmark of puberty), for the CD (SD) rat (Tyl *et al*., unpublished observations), and the Wistar rat (Gray *et al*., 2005).

Chemical castration

Ashby *et al*. (2001) suggested replacement of surgical castration (in Versions B and C) by GnRH (the gonadotrophin-releasing hormone) inhibition from the hypothalamus which is responsible for production and release of LH and FSH from the anterior pituitary, which in turn stimulates T (and DHT) production. A second possibility is to use Leydig cell ablation (these cells in the interstitium of the testes synthesize T and DHT). The Sertoli cells inside the seminiferous tubules should not/must not be affected.

Procedural Variables

Species/Strain

The Hershberger assay was initially introduced in rats (Hershberger *et al*., 1953), and the early papers (1920s-1950s) usually did not provide strain information. Mice were also evaluated for use for androgenic (Dorfman, 1969a) and anti-androgenic (Dorfman, 1969b) activity. Since then, the rat has been the species of choice. Yamasaki *et al*. (2001b) evaluated strain sensitivity differences in the Hershberger assay based on the presence and degree of alteration in the weights of accessory sex organs in castrated males from three strains. The rats were castrated at 49 days of age, and eight days later began the ten-day treatment (from 56 days of age through 65 days of age). The rats were terminated approximately 24 hours after the last dosing by ether anaesthesia and exsanguination. The negative control group for each strain was comprised of intact rats given vehicle, and a second vehicle control group was comprised of castrated rats given vehicle. The positive control group was comprised of castrated rats given TP. The treated group for each strain received both TP and flutamide (FLU). At necropsy, the authors measured terminal body weight, ventral prostate, seminal vesicle, bulbocavernosus/levator ani muscle, glans penis, and Cowper's glands in castrated Fischer 344 (F344; inbred), Sprague-Dawley (SD; outbred), and Wistar (outbred) rats. The rats were orally administered one dose level of FLU (a known androgen antagonist) at 3.2 mg/kg and, for the positive control group, TP by sc injection at 0.4 mg/kg. There were no abnormalities noted in clinical signs *in vivo*, body weights, or gross findings at necropsy in any strain or group. In all three strains evaluated, there were no effects on the weights of any of the accessory sex organs in the two vehicle control groups and in the positive control group (castrated rats given TP only). The TP plus FLU group in all three strains exhibited significant reductions in the weights of the ventral prostate, seminal vesicles, bulbocavernosus/levator ani muscle, and Cowper's glands. The SD and Wistar rats in this group also exhibited a significant reduction in the weight of the glans

penis. Analysis of covariance (ANCOVA) between absolute organ weights and body weights across strains indicated significant effects between the SD and F344 rats and between the Wistar and F344 rats. Specifically, the interaction in the ventral prostate, seminal vesicles, and glans penis weights and strain by two-way ANOVA was significant between SD and F344 rats and between Wistar and F344 rats, but not between SD and Wistar rats. In short, the SD and Wistar reacted similarly, and the F344 responses were different from both the SD and Wistar strains. The authors concluded that "these findings demonstrate that F344 rats are less suitable than SD or Wistar rats for detecting FLU-induced changes." They suggested that, at least for the Hershberger assay, outbred strains may be more sensitive, and that the difference in sensitivity may be due to the smaller size of the F344 rat, and lower body weights and concomitantly lower weights of the accessory sex glands. It is not clear whether the implication by the authors is that it is a detection problem rather than a sensitivity problem (Yamasaki *et al*., 2001b).

O'Connor *et al*. (1999) compared the CD (SD) and Long-Evans rats in detection of the environmental anti-androgen, *p,p'*-DDE, using the EPA (EDSTAC) screening battery and Hershberger assay, and found both strains to be similarly sensitive. Both strains exhibited reduced weights of the seminal vesicles and prostate, with reduction of the seminal vesicles weight statistically significant in both strains. The reduced prostate weight was statistically significant only in the Long-Evans rat. You *et al*. (1998) reported strain differences in sensitivity to DDE for the SD versus the Long-Evans hooded rats after *in utero* and lactational exposure. Ashby and Lefevre (2000a) employed the Alpk:APf SD (AP) rat from Astra Zeneca Pharmaceuticals, with comparable sensitivity.

Age at castration

Hershberger *et al*. (1953) castrated their rats at weaning (PND 21), long before acquisition of puberty, as did Eisenberg and Gordan (1950) and Gordan *et al*. (1951). Yamada *et al*. (2001) castrated their rats at three weeks (*i.e.*, weaning; Experiment 4), six weeks (Experiments 2 and 3), and ten weeks of age (Experiments 1, 2, and 3) after one week of quarantine (so the rats two weeks old arrived with their dams). The authors concluded that castrated rats at all three ages could detect the anti-androgenic effects of FLU and *p,p'*-DDE (Yamada *et al*., 2001). Rats were also castrated at weaning by Wakeling *et al*. (1980) and Snyder *et al*. (1989) for use in the Hershberger assay. Gray *et al*. (2005) castrated their rats at "peripuberty" on PND 42 (removing both the testes and epididymides), with the rationale that earlier castration interfered with subsequent PPS. This timing was adopted for the OECD Hershberger assay validation. Ashby and Lefevre (2000a), Kang *et al*. (2004), and Charles *et al*. (2005) castrated their rats at six weeks of age (PND 42). Young adults (less than four months old) were also castrated for use in the Hershberger assay by Raynaud *et al*. (1980) and Shao *et al*. (1994). Kelce and Wilson (1997) castrated rats at 125 days old. Ashby and Lefevre (2000a) also used rats castrated at 126-127 days of age. Adult animals were castrated for this assay at a number of ages (*e.g.*, O'Connor *et al*., 1999; also see Chapter 3, History of the Assay, for the early adult Hershberger assay procedures). For animals castrated and immediately treated, the endpoint is whether or not the accessory sex organs regressed or continued to grow normally in the presence of the test chemical (if castrated prior to puberty). For animals castrated with a delay before initiation of treatment (to allow the accessory sex

organs to regress/involute), the endpoint is whether the organs of interest have grown in animals exposed to test chemical relative to the organs in the castrated negative control group.

As an aside, a number of researchers have suggested intact (noncastrated) prepubertal males (*e.g.*, Kelce and Wilson, 1997; Monosson *et al*., 1999; Stoker *et al*., 2000; Ashby and Lefevre, 2000b). Since these rats have an intact hypothalamic-pituitary-gonadal axis and have not yet achieved puberty, they can detect androgens or anti-androgens by accelerated or inhibited sex organ growth and development of androgen (T, DHT)-dependent accessory sex organs and by accelerated or delayed puberty.

Recovery period between castration and start of treatment

The recovery period between castration and the start of treatment is also variable in the Hershberger assay. Hershberger *et al*. (1953) and other workers (Kelce *et al*., 1997) used no recovery period, instituting treatment on the day of or the day after castration. Yamada *et al*. (2000) inserted capsules containing T (as the positive control) subcutaneously through a dorsal incision immediately after castration. Subsequent administration of test chemicals began seven days later (Yamada *et al*., 2000). Seven days for recovery from the surgery and to allow involution of the accessory sex organs (in the absence of T) is relatively common (*e.g.*, Snyder *et al*., 1989; Shao *et al*., 1994). Ashby and Lefevre (1999) used eight days of recovery ("to insure complete recovery and healing from the operation," p. 93). Charles *et al*. (2005) used ten days, and O'Connor *et al*. (1999) recommended 11 days of recovery. Gray *et al*. (2005) recommended 12 days of recovery; this duration was adopted for the OECD Hershberger assay validation. Prior to Hershberger's assay, as presented in 1953, Eisenberg and Gordan (1950) waited 23 days postcastration before beginning treatment.

Start and duration of treatment

Based on the length of the recovery period (see above), the age of the rats at the start of treatment range from 21 days of age (at weaning immediately after castration) to 54-55 days of age (Gray *et al*., 2005; adopted for the OECD Hershberger assay validation). The duration of treatment has been from three days (Peets *et al*., 1973), four days (Rittmaster *et al*., 1991; Ashby and Lefevre 2000a), five days (Kelce *et al*., 1997; Yamada *et al*., 2000, 2001), seven days (Hershberger *et al*., 1953; Ashby and Lefevre, 2000a), ten days (Synder *et al*., 1989; Gray *et al*., 2005), 11 days (Ashby and Lefevre, 2000a), to 14 days (Shao *et al*., 1994; Ashby and Lefevre, 2000a). Dorfman (1969b) suggested that a longer period of treatment is required for oral administration versus sc injection. In fact, he recommended 20 days of treatment by the oral route and ten days by the sc route (see below for routes of administration).

Routes of administration

The sc injection route for the test chemicals (dose volume ~ 1 ml/kg) appears to be very common (*e.g.*, Hershberger *et al*., 1953; Dorman, 1969b; Rittmaster *et al*., 1991; Ashby and Lefevre, 1999; Gray *et al*., 2005). The oral route (~ 5 ml/kg dosing volume) has also been widely used (*e.g.*, Peets *et al*., 1973; Snyder *et al*., 1989; Kelce *et al*., 1997; Yamada *et al*., 2000). As indicated above, Dorfman (1969b) suggested that a longer treatment period is required for oral versus sc injection administration (he recommended ten days by sc injection and 20 days by oral administration). Silicon capsules, placed subcutaneously, have also been used (*e.g.*, Yamada *et al*., 2000), usually for the positive control chemical (see below).

The oral route provides direct, first-pass metabolism/elimination through the liver (the hepatic portal system carries the blood from the GI tract directly to the liver). Therefore, if the parent compound is the active moiety, it will most likely be sequestered/metabolized/cleared rather rapidly by this route. If a metabolite is the active moiety, again it will be formed, likely in the liver, rapidly by the oral route of administration. The sc injection route of administration provides systemic exposure (once it gets into the circulatory system) without first-pass metabolism/elimination directly to the liver. If the parent compound is the active moiety, it will persist longer by non-oral routes of administration and only gradual access to the liver. If the active moiety is a metabolite, it will be formed more slowly (versus oral administration) and over time as the chemical reaches the liver from the systemic circulation.

Reference androgens

T has been administered both as a silastic capsule implant (Kelce *et al*., 1997; Yamada *et al*., 2000) and by sc injection at doses up to 3 mg/kg/day to adult rats and total dose of 2.4 mg to weanlings (Dorfman, 1969b). TP has also been widely used at daily sc doses of 200 μg/kg (0.2 mg/kg; Wakeling *et al*., 1981; Ashby and Lefevre, 2000a), 0.25 mg/kg/day (Yamada *et al*., 2000), 0.4 mg/kg/day (Kang *et al*., 2004), and to 1 mg/kg (Shao *et al*., 1994). Other reference androgens that have been used include T enanthate (at ~ 2 mg/kg; Sunahara *et al*., 1987) and DHT (at 8 mg/kg/day; Snyder *et al*., 1989). MethylT (a potent androgenic pharmaceutical) has also been used by injection by Gray *et al*. (2004) and by oral administration (twice daily) by Sloan *et al*. (2002). The implants are inserted once and are present for the duration of the treatment period. The sc injections and the oral route are usually administered once daily for the duration of the period.

The reference androgens are used for two reasons: (1) as a standard against which the response of the accessory sex organs to the test chemical is compared; if there is growth, the test chemical is androgenic and the potency can be determined from comparison of the results in the test chemical group with the results from the reference androgen group; and (2) the reference androgen and test chemical are administered to the same animals simultaneously to measure the ability of the test chemical to compete with the reference androgen and block its androgenic action. If the weights of the accessory sex organs are lower in the group exposed to both and reference androgen and test

chemical (less than maximal response) than in the group exposed to the reference androgen alone, then the test chemical is acting as an anti-androgen (antagonizing the effects of the reference androgen). The degree of inhibition can be determined from the comparison of accessory sex organ weights, in the presence of both the test chemical and the reference androgen, versus the accessory sex organ weights of castrated males in the presence of only the reference androgen.

Endpoints

Hershberger *et al*. (1953) recorded terminal body weight, ventral prostate weight, seminal vesicle weight, and levator ani weight at terminal necropsy, and calculated the ratio of increase in levator ani weight to increase ventral prostate weight (if levator ani increase was highly significant, $p<0.01$, as compared to controls). These organs were also monitored in the studies reviewed by Dorfman (1969a,b) for assessment of androgens and anti-androgens. More recent studies have also monitored the same organs (Snyder *et al*., 1989), weighed only the prostate (Raynaud *et al*., 1980; Rittmaster *et al*., 1991; Shao *et al*., 1994), or weighed the prostate plus the seminal vesicles (Wakeling *et al*., 1981; Kelce *et al*., 1997). Ashby and Lefevre (2000a) weighed the prostate, seminal vesicles (including the coagulating glands), LABC muscle complex, and Cowper's glands. Gray *et al*. (2004) weighed the ventral prostate, Cowper's glands, seminal vesicles (with coagulating glands and fluids), glans penis and LABC muscles. For the pubertal intact male rat assay, Gray *et al*. (2004) recommended weighing the same organs as above plus the testes and epididymides. Yamada *et al*. (2004) weighed the ventral prostate, dorso-lateral prostate, seminal vesicles with coagulating glands, LABC muscles, glans penis, Cowper's glands, and pituitary.

Nonreproductive organ weights included liver and kidneys (Ashby and Lefevre, 2000a; Yamada *et al*., 2004) and adrenal and pituitary glands (Gray *et al*., 2004). If the Hershberger assay is used to detect thyroid hormone modulators, then the thyroid is also weighed (Yamada *et al*., 2004; Noda *et al*., 2005).

Additional data collected included mortality, clinical observations, regular body weights (predominantly to calculate dosing volume based on the most recent body weight), feed consumption, and blood at termination for subsequent determination of circulating serum hormones (*e.g.*, T, LH, T3, T4, TSH, etc.). The organ weights are presented as absolute and relative to terminal body weight. The organs to be weighed were dissected free, trimmed, and weighed either fresh or postfixation (*e.g.*, thyroid, adrenal glands, pituitary), wet (fresh or in fixative), or dry (after dessication).

Histopathology after fixation, embedment, and sectioning at ~5 μm thickness, and staining with H&E (hematoxylin and eosin) is routine for the thyroid (if evaluated; Yamada *et al*., 2004; Noda *et al*., 2005) and is sometimes employed for accessory sex organs. When peripubertal animals are used, age at PPS is also recorded (note that the rationale for castration on PND 42 by Gray *et al*. [2005] was to allow acquisition of PPS). A number of investigators have noted that the early-castrated males (even with androgenic administration) cannot acquire PPS (Ashby and Lefevre, 2000a,b; Gray *et al*., 2005).

Although the endpoints in the Hershberger assay (specifically changes in androgen-dependent accessory sex organ weights) are not necessarily considered adverse, Gray *et al*. (2004, p. 429) "have found that chemicals that are positive in the Hershberger assay often produce adverse effects during puberty and after *in utero* exposure."

Potential Additions/Enhancements

This section presents discussion on three potential modifications to the Hershberger assay: additions, enhancements, and replacements. The potential additions, measuring relevant hormone levels and changes in gene expression, should improve our understanding of the action of androgens (T and DHT) and anti-androgens in various target tissues, and perhaps lead to identification of gene expression "footprints" to distinguish between exposure to androgens versus anti-androgens, between exposure to an effective dose versus an ineffective (NOAEL) dose, and for prediction of type and/or degree of outcome(s).

The addition of thyroid endpoints to the castrated rat Hershberger assay will allow evaluation not just of AR receptor-mediated androgens/anti-androgens, but also thyroid hormone modulators with an intact hypothalamic-pituitary-thyroid axis (so thyroid hormone modulators will be detected which act at the level of the hypothalamic thyroid hormone releasing hormone [THRH], the anterior pituitary thyroid stimulating hormone [TSH], AR receptor, and the synthesis, transport, circulation, metabolism, and excretion of T3 and T4).

The potential enhancements and replacements include *in vitro* assays using intact and transfected cell lines and comparison of *in vitro* versus *in vivo* (Hershberger) assays to determine effects, identify the strength of the *in vitro* versus *in vivo* correlations, and whether *in vitro* assays would enhance or could replace the *in vivo* Hershberger assay, considered the "gold standard."

Additions for more information on androgenicity

Two major additions are suggested for the Hershberger assay: 1) measuring hormone levels and/or 2) determining changes in gene expression of androgen-responsive genes.

Measurement of T and DHT (and/or pituitary LH) over time during this assay provides confirmation that the levels of these androgens drop over time in the castrate-only group, that the levels of these androgens remain high and consistent in the intact group, and that the levels of T (and DHT) in the castrate plus TP administration are (or are not) comparable to the levels in the intact group. This will help explain any differences in accessory sex organ weights in the intact control group versus the castrate plus TP group. 5α-reductase, which converts T to DHT, is present locally in many end target organs and peripheral tissues, and so continues to function in the presence of exogenous T in the absence of endogenous T in the castrate groups. The demonstration of and the nature of the relationship between the T (and DHT) levels and the prevention of involution of (if no recovery period) or the regrowth of the accessory sex organs (after

a recovery period) in the presence of exogenous T are also very useful to characterize the interaction. The measurement of circulating LH should also confirm the increase in LH over time in those groups with no endogenous or exogenous T or DHT and the lack of increases in LH in those groups with endogenous or exogenous T or DHT. The discussion on whether or not to measure T and DHT (and/or LH) encompasses the advantages (see above) and the main disadvantage of these assays, which is the high variability of the assay results within and between laboratories, which makes them less sensitive and/or less reliable then the accessory sex organ weights and diminishes the usefulness of the comparisons between the hormone levels (and regulation of pituitary LH by T and DHT) and the organ weights.

Nellemann *et al*. (2001) presented results from a real time RT-PCR (real-time polymerase chain reaction) method using LightCycler technology for quantitative determination of gene expression. This technology combines rapid thermocycling with on-line fluorescence detectors of PCR product formation. Sixty-day-old castrated Wistar rats were treated daily with T, with or without current administration of FLU or vinclozolin (VIN; both androgen antagonists), for seven days. They investigated prostate specific-binding protein polypeptide C3 (PBP C3) and T-repressed prostatic message-2 (TRPM-2) in the ventral prostate and correlated quantification of levels of gene expression with systemic (liver, kidney, adrenals) and reproductive organ weights (ventral prostate, seminal vesicles and LABC muscle) and hormones (*e.g.*, LH).

Nellemann *et al*. (2001) reported that there was a significant decrease ($p < 0.05$) in the TRPM-2 expression in the animals dosed with T alone, and a significant increase ($p < 0.05$) in the expression of TRPM-2 gene in the animals treated with T plus FLU, or T plus a low dose of VIN versus in the animals treated with T alone. They also detected a significant increase in PBPC3 expression in the castrated animals plus T, and a significant decline in the expression of PBPC3 in the same two groups, castrated with T plus FLU or VIN. Exposure of castrated males to T resulted in a decrease in LH versus the control (castrated) group values; LH was increased in castrated males also treated with T and FLU ($p < 0.05$), but not with VIN (at 50 mg/kg/day, sc).

Body weights were significantly increased in the castrate plus T group versus the castrate control group. Ventral prostate, seminal vesicle and LABC muscle weights were all significantly increased in the castrate plus T or plus vinclozolin, and significantly reduced in castrate or castrate plus T plus FLU versus castrate plus T animals. Although the effects on organ weights and LH levels were similar in direction to the effects on gene expression for the four groups, the gene expression data were the most sensitive endpoint (Nellemann *et al*., 2001).

Addition of thyroid endpoints to screen for thyroid hormone modulators

Yamada *et al*. (2004) evaluated the reliability of the "enhanced" Hershberger assay to detect thyroid hormone modulating activity without confounding the evaluation of (anti-) androgenic activity. Castrated CD (SD) rats or castrated rats subcutaneously injected with TP at 0.2 or 0.25 mg/kg/day were dosed for ten days by oral gavage (six animals/group) with vehicle (corn oil) or propylthiouracil (PTU; a potent inhibitor of

thyroid hormone synthesis; 2.5 mg/kg/day), Phenobarbital (PB); hepatic P450 inducer that accelerates thyroid hormone (TH) degradation and clearance; 125 mg/kg/day), or *p,p'*-DDE (another hepatic enzyme inducer, 100 mg/kg/day), all at only the one dose level. One day after final administration, the rats were necropsied, and body and organ weights and serum hormone levels of T4 (μg/dl), T3 (ng/dl), and TSH (ng/ml) were determined. Results included:

- PTU markedly increased thyroid weights, decreased serum T3 and T4, increased serum TSH, and caused major microscopic alteration (consistent with TSH stimulation) of the thyroid gland;

- PB and *p,p'*-DDE significantly reduced serum T4 (with no significant effects on T3 or TSH) and caused similar histopathologic findings in the thyroid gland with lower incidence and severity than from PTU;

- Histopathologic alterations in the thyroid appeared more "robust" in the presence of TP;

- *p,p'*-DDE exhibited anti-androgenic effects: decreased weights of the seminal vesicles, ventral and dorso-lateral prostate, LABC, glans penis, and Cowper's glands; liver weight was significantly increased; and the thyroid gland and kidney weights were unaffected;

- PTU and PB had little or no effects on the weights of the androgen-sensitive accessory sex glands/tissues (listed above); and

- The LABC muscle was decreased by PB in TP-treated, castrated rats.

The authors concluded that the enhanced Hershberger assay, with evaluation of thyroid weights, histopathology, and hormone levels, "appeared to be reliable" for screening for both (anti-) androgenic chemicals and thyroid hormone modulators. The authors noted that whether the sensitivity and specificity of the thyroid assessment is adequate (and whether it interferes with the androgenic/anti-androgenic component of the assay) for routine screening purposes will require additional dose-response studies using lower doses (Yamada *et al*., 2004).

Enhancements and/or replacements

This section on enhancements and/or replacements includes a number of *in vitro* assays, including binding assays, transcriptional activation (transactivational) assays, and cell lines in culture with transiently or permanently transfected AR and a reporter system (typically an AR-dependent luciferase receptor gene). These assays are proposed to examine the correlation between *in vitro* assays and the *in vivo* assay (Hershberger) for androgenicity/anti-androgenicity using a variety of test chemicals. As with the *in vivo* assays, there is a reference androgen (*e.g.*, R-1881, methyltrienolone, an AR agonist) and a reference anti-androgen (*e.g.*, RU486, mifepristone, FLU, etc.). In the binding assays, the endpoint is binding to the AR (but one cannot distinguish an androgenic response

from an anti-androgenic response; both types of compounds bind to the AR). In the transactivational assays, the endpoints are both binding (or not) to the AR and subsequent transcriptional activation (or not), so one can identify both androgenic and anti-androgenic chemicals. The reference androgen and the test chemical are added to the culture to determine whether the test chemical inhibits the androgenic response to the reference androgen (*e.g.*, R-1881), *i.e.*, acts as an anti-androgen. Relative potency can be assigned relative to binding or inhibition from T or R-1881. The possible usefulness of these *in vitro* screens has been proposed as follows:

- To be performed before the Hershberger assay (as an initial prescreen);
- To be performed in conjunction with the Hershberger assay (simultaneously or after);
- To provide additional confirmation of androgenicity or anti-androgenicity; or
- To be performed in place of the Hershberger assay.
- High throughput screening assays have been developed, especially by pharmaceutical companies, to identify endocrine-active compounds (acting via the AR [or ERα or β] receptor). These are sensitive, fast, high capacity, low cost, and responsive to concerns about animal usage. These are mechanism-based assays and considered ideal for first-line screening (Sonneveld *et al*., 2005).

The *in vitro* assays may be falsely positive for compounds that do not, in fact, bind to the AR *in vivo*, because they are never "seen" by the testicular AR receptors since they are metabolized to an inactive metabolite (due to ADME) in the whole animal. The *in vitro* assays may be falsely negative for chemicals that are metabolized to an active metabolite in the intact animal (ADME) but not in culture and for chemicals with a more complex interaction with the AR *in vivo* (Charles *et al.*, 2005).

Molecular analyses have also been used to evaluate endocrine-active environmental pollutants. Tamura *et al*. (2001) used HepG2 human hepatoma liver cells in culture, transiently transfected with human AR and an AR-dependent luciferase receptor gene, to detect that fenitrothion (O,O-dimethyl O-(4-nitro-m-tolyl) phosphorothioate) blocked DHT-dependent AR activity in a concentration-dependent and competitive manner. *In vivo*, seven-month-old SD rats were castrated and treated with TP (50 μg/day, sc), with or without fenitrothion (15 or 30 mg/kg/day) or TP and FLU (50 mg/kg/day), *i.e.*, a Hershberger assay (Tamura, 2001). The binding assays *in vitro* and the *in vivo* organ weights demonstrate that fenitrothion is a competitive AR antagonist, comparable in potency to FLU and more potent (based on the *in vitro* assays) than the environmental anti-androgens linuron and *p,p'*-DDE (Tamura *et al*., 2001).

Dr. Marty's laboratory (Charles *et al*., 2005) employed both the *in vivo* Hershberger assay (CD® [SD] rats, castrated on PND 42, administered the test chemicals by gavage beginning on PND 52 for ten days) and two *in vitro* assays (AR transactivation

assay using AR (+) LNCaP prostate carcinoma cell line transfected with an inducible luciferase reporter gene construct and an AR binding assay using the recombinant ligand-binding domain [LBD] of the human AR and detection of binding by fluorescence polarization). The objective was to examine the correlation between the *in vitro* and *in vivo* anti-androgenicity assays using a variety of test chemicals. They were *p,p'*-DDE, FLU, spironolactone, procymidone, RU486, methoxychlor (an estrogenic and anti-androgenic pesticide), benzo(a)pyrene, and selected metabolites.

In the prostate carcinoma cell line (using R-1881, an AR agonist, as a reference androgen), all of the test chemicals, including the hydroxylated metabolites of benzo(a)pyrene and methoxychlor, were positive (*i.e.*, they inhibited the androgenic response to R-1881). In the AR binding assay, both RU486 and 9-hydroxy-benzo(a)pyrene resulted in IC50s comparable to that of T. Other parent compounds and their metabolites exhibited lesser binding affinity. In the *in vivo* Hershberger assay, neither benzo(a)pyrene nor methoxychlor caused significant reductions on accessory sex organ weights relative to TP (the reference androgen). However, at the highest doses tested, benzo(a)pyrene significantly decreased body weight, and methoxychlor significantly increased absolute and relative liver weights. RU486 significantly decreased ventral prostate, seminal vesicle, and Cowper's gland weights without affecting body weight. FLU decreased all accessory sex organ weights measured. The anti-androgenic properties of *p,p'*-DDE, spironolactone, and procymidone had been demonstrated in previous Hershberger assays. Five of the seven anti-androgenic test chemicals were therefore positive in all three assays, but the results differed for methoxychlor and benzo(a)pyrene. The authors concluded that "Overall, there was a reasonably good correlation between the results of *in vitro* and *in vivo* anti-androgenicity screening assays" (Charles *et al*., 2005), although they did indicate that other compounds with complex interactions with the endocrine system may not be structurally categorized into a single mode of action, and that the category, interaction with ARs, is too simplistic to explain these more complex interactions *in vivo*. The authors concluded that "These data indicate the importance of including *in vivo* results in assessing the endocrine activity of test materials" and further stress "the importance of utilizing a weight of evidence approach in assessing endocrine activity of test materials" .

CHAPTER 7. SYNOPSIS OF PROTOCOL

Animal Requirements

The protocol, which was validated by OECD, used sexually immature male rats. As mentioned in Chapter 2, this model involved rats castrated at peripuberty by the removal of testes and epididymides (orchidoepididyectomized). In most laboratory rat strains (such as Sprague-Dawley, Long Evans, or Wistar), peripuberty is expected to take place within five to seven weeks of age. Peripuberty is marked by separation of the prepuce from the penile shaft (glans penis). Chemicals such as TP will initiate PPS so that it is possible to weigh the glans penis. At this peripubertal stage of sexual development, the glans penis and other androgen-sensitive tissues exhibit their sensitivity to androgens, since both ARs and appropriate steroidogenic enzymes are present during this time. The advantage of using rodents of this age is that the sex accessory tissues (SAT) display high sensitivity and relatively small weights, both of which aid in minimizing the variability in responses between individual animals.

Principles

The Hershberger assay is based on changes in the weight of male SAT in these sexually immature castrated male rats. The sex accessory glands and tissues used for this protocol are the ventral prostate, seminal vesicles, coagulating glands, LABC muscles, glans penis, and Cowper's (or bulbourethral) glands.

In addition to the weight of the SAT, body weight gain should be determined to provide information on the general health of the animals. Liver weight can be used as an endpoint to determine if a test substance is truly anti-androgenic or simply appearing that way due to inducing an increased metabolism of the test material (*e.g.*, TP) by the liver, which would be indicated as increased liver size (cell hypertrophy). Necropsy of the adrenals and kidneys as optional endpoints may provide supplementary information about the effect of the test substance on other related biochemical pathways. Similarly, measurement of serum T and LH may also be determined in this context.

To test for androgen agonists, the test substance is administered to immature, castrated rats for ten consecutive days. The vehicle is the negative control, and a substance such as TP (a potent androgen) can be used as a positive control.

For androgen antagonists, the test substance is administered to immature, castrated rats for ten consecutive days, together with a reference androgen agonist such as TP. Administration of TP alone can be used as the positive control, to which treatments are compared for anti-androgenic activity. The weights of the SAT after co-administration of the test substance and reference androgen are compared to the weights of tissues from this control group.

Conditions

The immature, male rats may be singly housed, or housed 2-3/cage, with food and water *ad libitum* and a 12/12 light cycle.

Procedure

Animals should be uniquely identified and weighed upon receipt. At 42 days of age, the animals are castrated using the testing laboratory's Institutional Animal Care and Use Committee (IACUC) approved methods that eliminate pain and distress. Approximately one day prior to dosing, at PND 53 to 54, the rats should be weighed to the nearest 0.1 g, and a homogeneous population of study animals should be created by eliminating the highest and lowest outliers. All unhealthy animals (or those not gaining weight) should also be removed from the study. The animals should be assigned to dose groups using a randomized, complete block design. The body weight means and standard errors should be calculated for each treatment group to ensure they are as similar as possible.

Testing for Androgenic Activity

The administration of vehicle or test substance in vehicle by gavage should be performed daily for ten days, starting at 53-54 days of age, using a separate 16-gauge, 2-inch gavage needle for each group. The rats should be weighed daily during treatment. The test substance should be prepared at the selected doses in a vehicle, such as corn oil at 2.5 ml/kg body weight, and administered between 7:00 a.m. and 10:00 a.m. daily. The volume administered should be adjusted daily for body weight changes. The body weight and dose volume should be recorded for each animal. Monitoring of feed consumption should also be performed.

Testing for Anti-androgenic Activity

Testosterone proprionate in corn oil should be administered orally on a 0.2 to 0.4 mg/kg body weight/day basis, or alternatively at 0.1 mg/rat/day, to the appropriate groups by sc injections at the same time of day on the dorsal surface, caudal to the nape of the neck, but anterior to the base of the tail, with a 25-G x 5/8-inch needle using a separate 1 cc glass tuberculin syringe for each treatment condition. The test compound should be administered by gavage at the appropriate dose in 2.5 ml corn oil per kg/body weight. Dosing should be repeated for ten days, and the body weight and dose volume should be recorded daily.

Necropsy of Animals

Overview

Animals should be necropsied by block design across dose groups. Approximately 24 hours after the last day of treatment, the animals should be assessed for PPS, necropsied, the data collected, collated, and analyzed, and the report prepared.

The rats at 64-65 days old are necropsied to isolate organs and tissues for the study of androgenic or anti-androgenic effects. The endpoints evaluated at necropsy are:

- Body weight
- Seminal vesicles plus coagulating gland with fluid weight
- LABC muscle weight
- Glans penis weight
- Cowper's glands (bulbourethral glands) weight
- Liver weight

Optionally, the following may be determined:

- Serum T and luteinizing hormone levels
- Paired kidney weight
- Paired adrenal weight

Procedure

- Weigh the rats.
- Anesthetize the animals.
- Collect blood by cardiac puncture if hormone levels are to be measured.
- Euthanize the anesthetized animals humanely.
- Dissect and weigh the SAT (ventral prostate, seminal vesicle with coagulating glands and fluid, Cowper's glands, LABC muscles, and glans penis), as well as other organs of interest such as the liver, kidneys, and adrenal glands if desired, to the nearest 0.1g.
- Determine if the prepuce of the penis has separated from the glans penis. If it has, retract the prepuce, remove the glans penis, and weigh to the nearest 0.1 mg. If it has not separated, note this and do not weigh the glans penis.
- Remove the abdominal skin and muscle layers to expose the viscera.
- Optionally, remove and weigh the liver, the paired kidneys, and the paired adrenal glands each to the nearest 0.1 mg.
- Expose the seminal vesicles plus coagulating glands and bladder.

- Dissect the **ventral prostate** by separating the bladder from the ventral muscle layer by cutting the connective tissue along the midline with microdissecting scissors. Displace the bladder anteriorly towards the seminal vesicles, revealing the left and right lobes of the ventral prostate covered by a layer of fat. Carefully tease away the fat layer from the right and left lobes of the ventral prostate with microdissecting forceps. Displace the right lobe of the ventral prostate from the urethra and dissect this lobe of the ventral prostate from the urethra with scissors. While holding the right lobe of the ventral prostate with forceps, displace the left lobe away from the urethra and then dissect this lobe from the urethra with microdissecting scissors and weigh in tared weigh boat to the nearest 0.1 mg.

- Displace the bladder caudally, expose the vas deferens and right and left lobes of the **seminal vesicles plus coagulating glands**, and clamp a hemostat at the base of the seminal vesicle where the vas deferens joins the urethra to prevent leakage from the seminal vesicle. Using the microdissecting scissors, dissect the seminal vesicle from the urethra. Trim the fat and adnexa and remove the hemostat. Place the seminal vesicles plus coagulating glands with fluid in a tared weigh boat and weigh to the nearest 0.1 mg.

- Expose the **LABC** and the base of the penis with penile bulbs by removing the skin and adnexa from the perianal region extending from the base of the penis to the anterior end of the anus. With forceps and microdissecting scissors, remove the fat from these tissues until the muscles can be identified. Grasp the bulbocavernosus muscle with blunt forceps and dissect the muscle from the penile bulb so that the white connective tissue and reddish corpus spongiosum are detached from the bulbocavernosus muscles on each side. Lift the bulbocavernosus muscles upward and away from the body. Cut the colon in two with the scissors, pull the LABC further upward, and pull the fat and adnexa off with forceps. Remove the LABC, trim the fat, and weigh it to the nearest 0.1 mg.

- Remove the round **Cowper's or bulbourethral glands** at the base of, and slightly dorsal to, the penile bulbs with dissecting scissors. Avoid nicking the thin capsule to void leakage. Weigh the paired glands to the nearest 0.1 mg.

Expected Outcomes

The weights of the androgen-dependent tissues will increase with exposure to androgenic compounds. This response in the castrated, immature male rat will generally be dose dependent. Anti-androgenic compounds will inhibit the TP-induced growth of the SAT in a dose-related manner.

Considerations

- Doses should be carefully selected so that the lowest dose shows no or minimal effects and the highest dose should not produce signs of toxicity. Therefore, the animals should also not show a loss of ≥ 10% body weight over the course of the assay, so that the toxicity of the compound does not become part of the evaluation (*i.e.*, confounder).

- The individuals involved in the conduct of this assay should be well trained and consistent in their dissections and observations.

- The entire assay, with all test and control groups, should be run at one time.

- This version of the Hershberger protocol, used in the OECD standardization and validation initiative (Owens *et al.*, 2006) and described by Gray *et al.* (2005), specifies castration of the "prepubertal" male rat on PND 42, with dose administration commencing on PND 53-54. In the authors' laboratories, with the CD® (SD) rat, the grand mean of the individual study control group means for age at PPS is 41.9 days, with the range of individual study means from 41.1 days to 43.6 days. This means that on average, at castration on PND 42, 50% of the prepubertal males are, in fact, postpubertal, having acquired PPS prior to castration. The data to be collected at scheduled necropsy (after the treatment period) is the percentage of males with PPS (Gray *et al.*, 2005); therefore, the authors make two recommendations for future use of this protocol:

    1. That the evaluation of males for PPS should also be made at castration (so the baseline of animals already with PPS is known); and

    2. That the males be castrated much earlier, *e.g.* the latest on PND 35 (when no control CD® SD males have acquired PPS in the authors' laboratories), or the earliest on PND 22 (the day after weaning on PND 21). Hershberger *et al.* (1953) castrated their males on PND 21 and began treating them on the same day.

An example basic protocol is located in Appendix A.

CHAPTER 8. EXAMPLE CHEMICALS TESTED IN THE HERSHBERGER ASSAY

Introduction

Testing of chemicals for determination of andogenic properties in castrated rats began in the 1930s with the experiments of Tschopp, 1935; Ruzicka *et al.*, 1934; Korenchevsky and Dennison, 1934, 1935a; and Korenchevsky *et al.*, 1935b testing androsterone and androsterone-diol. They found that the secondary sex organs (prostate, seminal vesicles, and penis) became enlarged after the injection of androsterone and even more enlarged with androsterone-diol. Korenchevsky *et al.* (1937) tested TP in this bioassay, and it also produced increased weight in these organs. Wainman and Shipounoff (1941) also demonstrated the stimulating effect of TP upon the perineal complex and thus the appropriateness of the muscles (LABC) as an index of myotrophic activity. Variations of this early assay occurred over the next 15-20 years, with scientists trying different ages for castration, different time periods between castration and testing, various amounts of potent androgens, and determination of the weight of various organs.

In 1950, Eisenberg and Gordan further used the levator ani weight and showed that some of the increased muscle weight produced by TP over T was retention of water, and that it actually produced slightly less weight increase in the tissue over T. Still, even based on dry weight, unesterified T, TP, and methyltestosterone were the most potent group of steroids tested for myotrophic activity. Progesterone showed a moderate response, and no significant increase in weight was seen with estradiol dipropionate, desoxycorticostrone, cis-testosterone, or ethinyl testosterone. A distinct lack of parallelism was seen between the degree of andogenic effect (growth of seminal vesicles) and the degree of myotrophic activity.

In 1953, Hershberger *et al*., improved the assay and made the first attempt to standardize it for prepubertal castrated males. Rats were castrated at weaning on PND 21, and sc injections were given for seven consecutive days, beginning on the day of castration. On the eighth day after castration, approximately 22-26 hours after the last injection, the animals were terminated. The levator ani muscle, ventral prostate, and seminal vesicles (without the coagulating glands) were dissected from each animal and weighed. The dry weight was also determined for the levator ani muscle after it was desiccated at 72ºC. Results from this assay, expressed as organ weights versus a range of doses of each test material, provided information on their relative potency for androgenic (from the ventral prostate and seminal vesicle weights) and/or for myotrophic activity (from the levator ani muscle weight). The Hershberger assay is also sometimes called the Hershberger anti-androgen assay.

This *in vivo* bioassay has been proposed by both EDSTAC and OECD to test chemicals that have the potential to act as androgens or anti-androgens, and it has been

widely used by the pharmaceutical industry for screening drugs with these potentials designed to be used therapeutically. For assessing androgenicity, chemicals that act as agonists are identified in the Hershberger assay if they produce statistically significant increases in the weight of the target, androgen-dependent tissues in the castrated animal. For assessing anti-androgenicity, chemicals that act as antagonists cause decreases in the stimulated target tissues weights when they are co-administered with a potent androgen such as TP. Inhibitors of androgen synthesis and anti-androgens may cause male reproductive tract malformations, and thus a screen for these chemicals is necessary to attempt to reduce the number of these occurrences.

The assay variables are currently being standardized and assay validation in multiple laboratories is being established. TP (commonly 0.4 mg TP/kg-bw/day) is generally used as the reference androgen agonist and FLU (0.1-10 mg/kg-bw/day) as the reference androgen antagonist. Corn oil is the vehicle used most often; peanut or olive oil have been found to cause a significant increase in body weight (Yamasaki *et al.,* 2001c). Issues still exist as to the comparison of this assay with the use of the weanling male rat assay (Ashby, 2004) and whether the castration by surgery can be eliminated by using a GnRH antagonist (Ashby *et al.*, 2001).

Results for some chemicals have been inconsistent in the assay. For instance, the fungicide fenitrothione, which acts as an anti-androgen *in vitro*, was positive in the Hershberger assay performed by Tamura *et al*. (2001), but negative in one performed by Sohoni *et al. (*2001). However, the majority of chemicals tested have given fairly consistent results across laboratories when a more standardized protocol is used. **Table 5** lists the results of some chemicals that have been tested in the Hershberger assay and the variables that were used when they were available from the publications.

Table 5. Examples of Chemicals tested in the Hershberger assay

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Phthalic acid di-*n*-hexyl ester	84-75-3	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	40,200,1000/ Pnd 56 for 10 days/0.2TP sc	Gavage/ olive oil	-	-	-	-	-	-
Phthalic acid di-*n*-amyl ester	131-18-0	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	40,200,1000/ Pnd 56 for 10 days/0.2TP sc	Gavage/ olive oil	-	↓ 1000 +TP	↓ 1000 +TP	↓ 200, 1000 +TP	-	↓ 40, 2000 +TP
Phthalic acid di-*n*-propyl ester	131-16-8	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	40,200,1000/ Pnd 56 for 10 days/0.2TP sc	Gavage/ olive oil	-	-	-	-	-	↑ 200 +TP
Diethylstilbestrol	56-53-1	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	0.002,0.01,0.05/Pnd 56 for 10 days/0.2TP sc	Gavage/ olive oil	↓ 0.05; 0.05 +TP	↓ 0.05 +TP	↑ 0.05	-	-	-
17 β-Estradiol	50-28-2	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	0.1,0.4,2.0/ Pnd 56 for 10 days/0.2TP sc	Gavage/ olive oil	↓ 2.0; 2.0+TP	-	-	↓ 2.0	↓ 2.0 +TP	↑ 0.4

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Tamoxifen	10540-29-1	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	0.004, 0.02,0.1/Pnd 56 for 10 days/0.2TP sc	Gavage/ olive oil	↓ 0.1; 0.1+TP	-	-	-	-	-
5α-Dihydrotestosterone	521-18-6	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	8,40,200/Pnd 56 for 10 days/0.2TP sc	Gavage/ olive oil		↑ 40,200	↑ 200	↑ 200	↑ 200	↑ 200
Dichlorodiphenyl dichloroethane	72-54-8	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	8,40,200/Pnd 56 for 10 days/0.2TP sc	Gavage/ olive oil	-	-	↓ 200 +TP	↓ 200 +TP	-	-
Cyproterone acetate	427-51-0	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	0.4,2,10/Pnd 56 for 10 days/0.2TP sc	Gavage/ olive oil	↓ 10 +TP	↓ All +TP	↓ 2,10 +TP	↓ 2,10; All +TP	↓ All +TP	↓ 10; All + TP
6α-Methyl-17α-hydroxy-progesterone	520-85-4	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42	20,100,500/ Pnd 56 for 10 days	Gavage	↓ 100, 500 +TP	↓ 100; 100,500 +TP	↓ 500 +TP	↓ 500; 100, 500 +TP	↓ 500 +TP	↓ 100, 100, 500 +TP

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Atrazine	1912-24-9	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS) / Pnd 42/6 rats/group	3.2,16,80/Pnd 56 for 10 days/0.2 TP sc	Gavage/ olive oil	↓ 80	-	-	-	-	-
Spirolactone	52-01-7	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	8,40,200/Pnd 56 for 10 days/0.2TP sc	Gavage/ Olive oil	-	↓ All +TP	↓ 40,200 +TP	↓ 40, 200 +TP	↓ 200 +TP	↓ 200 +TP
4-n-Amylphenol	14938-35-3	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600 (400) Dose reduced due to toxicity/Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 600 (400); 600 (400) +TP	-	-	↓ 600 (400) +TP	-	-
p-Dodecyl-phenol	104-43-8	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	10,30,100/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	-	-	-	↓ 100	-	-

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
p-(Tert-pentyl)phenol	80-46-6	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600 (400) reduced due to toxicity/Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	-	-	↑ 50 +TP	-	-	-
4-Cyclohexylphenol	1131-60-8	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600 (400) Reduced due to toxicity/Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 600 (400)	-	-	-	-	-
4-(1-Adamantyl)phenol	29799-07-3	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	10,50,200/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 200, 200 +TP	-	↑ 200 +TP	-	-	↑ 200 +TP
4.4'-Thiobis-phenol	2664-63-3	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	10,50,200/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 200	↑ 10,200 +TP	↑ 10,200 +TP	-	-	-

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Diphenyl-p-phenylenediamine	74-31-7	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,800/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	-	↑ 50,800 +TP	↑ 800	-	↑ 50	-
4-Hydroxy azobenzene	1689-82-3	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	10,30,100/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	-	-	-	↓ 30	↑ 10 +TP	-
4-(Phenylmethyl) phenol	101-53-1	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600 (400) reduced due to toxicity/Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 600 (400)	-	↑ 50 +TP	-	↑ 50,200	-
4,4'-(Hexafluoroisopropylidene)diphenol	1478-61-1	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200, 600 (400) reduced due to toxicity/Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 200,600 (400); 200,600 (400) +TP	–	↑ 50, 600 (400) +TP	–	↑ 50, 600 (400) +TP	↑ 600 (400) +TP

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Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
2,2-bis(4-Hydroxyoxyphenyl)-4-methyl-n-pentane	6807-17-6	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	10,50, 200 (100) reduced due to toxicity/Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 50,200 (100); 200 (100)+TP	↑ 10,200 (100)+TP	-	-	-	-
4,4'- (Octahydro-4,7-methano-5H-ubden-5-ylidene)bisphenol	1943-97-1	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	2,10,50/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 10,50+TP	-	-	-	↑ 10,50 +TP	-
4,4'-Dihydroxybenzo-phenone	611-99-4	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 600+TP	↑ 50 ↓ 200+TP	-	↓ 200; 200 +TP	↑ 600+TP	-
2,2',4,4'-Tetrahydroxybenzo-phenone	131-55-5	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600 (400) reduced due to toxicity/Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 200,600 (400)	-	-	-	-	-

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Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
4-Hydroxybenzo-phenone	1137-42-4	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	-	↓ 600	-	-	-	-
2,4,4'-Trihydroxybenzo-phene	1470-79-7	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 600	-	-	-	-	-
2,4,4'-triOH-BP	1470-79-7	Suzuki et al., 2005	F344/PND 28/6 rats per group	100,300/ PND For 10 days 0.5TP sc	Sc/DMSO	NA	↑ 300+TP	↑ 300+TP	NA	NA	NA
Testosterone enanthate	315-37-7	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	-	↑ 50,200, 600; 50,200, 600+TP	↑ 50,200, 600;50, 200,600 +TP	↑ 50,200 ,600; 200, 600 +TP	↑ 50,200 ,600; 600 +TP	↑ 50,200,600; 200,600 +TP
Methyltestosterone	58-18-4	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	0.5,5,50/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	-	↑ 0.5,5,50; 50+TP	↑ 0.5,5,50; 50+TP	↑ 50; 50+TP	↑ 5,50; 50+TP	↑ 0.5,5,50; 50+TP

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Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
17 Alpha estradiol	57-91-0	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	0.5,2,10/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 2,10 +TP	-	-	-	-	-
Estrone	53-16-7	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	0.5,2,10 (6) reduced due to toxicity/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 2,10(6); 10(6) +TP	↑ 10(6)	↑ 10(6); 0.5,2, 10(6) +TP	-	↑ 2,10(6) +TP	-
Equilin	474-86-2	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	0.5,2,10(6) reduced due to toxicity/ PND 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 0.5,2,10 (6);0.5, 2,10(6) +TP	↑ 2,10(6)	↑ 2,10(6); 0.5,2,10 (6) +TP	-	↑ 2,10(6) ; 10(6) +TP	↑ 0.5,2,10(6) +TP
Norethindrone	68-22-4	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	0.5,2,10/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 10; 10 +TP	-	-	-	↑ 10+TP	-

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Norgestrel	797-63-7	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	10,30.100/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 100; 100+TP	↑ 30,100; 100+TP	↑ 30,100; 100+TP	↑ 30,100 :100 +TP	↑ 100; 100+TP	
Ethynyl estradiol	57-63-6	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	10,50,200/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 50,200; 200+TP	↑ 50	↑ 50; 50,200 +TP	-	↑ 50,200 ;200+TP	-
Bisphenol A	80-05-7	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	-	-	-	-	↑ 600	
Bisphenol A	80-05-7	Nishino, et.al, 2006	Wistar/ NA /13 rats per group	3,50,200,500/ NA for 7 days	Gavage/ propylene glycol	↓ 200,500 ; 500 +FL	-↓ Rel wt. 200,500	-↓ Rel.wt. 200,500	NA	NA	NA

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Bisphenol A	80-05-7	Kim *et al*., 2002	Sprague-Dawley Crl:CD/PND 35 or 42/ NA	10,100,1000 or 50,100,250, 500/Pnd 42or 49 for 7 days/ 0.4 T sc	Gavage/ Corn oil	-	-	-	-	NA	NA
Bisphenol B	77-40-7	Yamasaki *et al*., 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600 / Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 600; 600+TP	↑ 200,600 +TP	↑ 200,600+ TP	↑ 200, 600; 600 +TP	↑ 600+TP	↑ 600+TP
Bisphenol F	620-92-8	Yamasaki *et al*., 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,1000/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 1000; 200, 1000 +TP	-	-	-	-	↑ 200+TP
4-Tert-octylphenol	140-66-9	Yamasaki *et al*., 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600 (400) reduced due to toxicity/Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 200	-	↑ 50	-	-	↑ 200+TP

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
p-cumyl phenol	599-64-4	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	50,200,600/ Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 600;600 +TP	-	-	-	-	-
nonylphenol	25154-52-3	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS)/ Pnd 42/6 rats/group	10,50,200/Pnd 56 for 10 days 0.2TP sc	Gavage/ Olive oil	↓ 200	-	↓ 200+TP	-	-	-
fenitrothion	122-14-5	Sohoni *et al.*, 2001	Alp-k:ApfSD/PND 42/5 rats per group	15/Pnd 50 for 10 days 0.2 or 0.4 TP sc with 10/15 fenitrothion	1 gavage and 1 sc dose/day/ HPMC	-	-	-	-	-	↑ 10+TP
deltamethrin	52918-63-5	Andrade *et al.*, 2002	Wistar/8 or 9 per group	2.0,4.0/ for 7 days	Gavage/ distilled water was used for the test chemical, canola oil for others/ vehicle	NA	-	-	NA	NA	NA

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
d-phenothrin	26002-80-2	Yamada *et al.*, 2003	Crj:CD(SD)IGS/PND 42/6 rats/group	100,300,1000 /PND 56 for 10 days/0.2TP sc	gavage/ corn oil	-			↑ 100		
methyltestosterone	58-18-4	Yamada *et al.*, 2003	Crj:CD(SD)IGS/PND 42/6 rats/group	100/PND 56 for 10 days	Gavage/ corn oil		↑	↑	↑	↑	↑
methyltestosterone	58-18-4	Kennel *et al.*, 2004	Sprague-Dawley/Pnd 46/ 6 rats per group	0.5,2,10,40/ Pnd 53-57 for 10 days	Gavage/ MC 500		↑ 10,40	↑ 10,40	↑ 10,40	↑ 40	↑ 40
p,p'-DDE	72-55-9	Yamada *et al.*, 2003	Crj:CD(SD)IGS/PND 42/ 6 rats/group	100/ PND 56 for 10 days	Gavage/ corn oil		↓	↓	↓	↓	↓
p,p'-DDE	72-55-9	Kang, *et al.*, 2004	Sprague-Dawley/ PND 42/ 6 rats/group	25,50,100/ PND 50 for 10 days/ 0.4TP	Gavage/ corn oil	-	↓ 100+TP	↓ 50,100 + TP	↓ 100 +TP	-	↓ 100+TP

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Flutamide (Positive Control)	13311-84-7	Kang, *et al*., 2004	Sprague-Dawley/ PND 42/ 6 rats/group	1,5,10,20/ PND 50 for 10 days/ 0.4TP	Gavage/ corn oil	-	↓ All doses +TP	↓ All doses+TP	↓ All doses +TP	↓ 5,10,20 +TP	↓ 5,10,20+TP
vinclozolin	50471-44-8	Kang, *et al*., 2004	Sprague-Dawley/ PND 42/ 6 rats/group	25,50,100/ PND 50 for 10 days/ 0.4TP	Gavage/ corn oil	-	↓ 50,100 +TP	↓ 50,100 +TP	↓ 50,100 +TP	↓ 100 +TP	↓ 50,100+TP
procymidone	32809-16-8	Kang, *et al*., 2004	Sprague-Dawley/PND 42/6 rats/group	25,50,100/ PND 50 for 10 days/0.4TP	Gavage/ corn oil	-	↓ All doses+TP	↓ All doses+TP	↓ All doses +TP	↓ 100+TP	↓ All doses +TP
procymidone	32809-16-8	Kennel *et al*., 2004	Sprague-Dawley/Pnd 46/ 6 rats per group	3,10,30,100/ Pnd 53-57 for 10 days/0.4 TP	Gavage/ MC 500	-	↓ 30,100 +TP	↓ 30,100 +TP	↓ 100 +TP	-	↓ 100+TP
linuron	330-55-2	Kang, *et al*., 2004	Sprague-Dawley/PND 42/6 rats/group	25,50,100/ PND 50 for 10 days/0.4TP	Gavage/ corn oil	-	↓ 50,100 +TP	↓ 50,100 +TP	↓ 100 +TP	↓ 100+TP	↓ 50,100+TP

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Testosterone propionate (TP)	57-85-2	Kang, *et al.*, 2004	Sprague-Dawley/PND 42/6 rats/group	0.1,0.2,0.4,0.8,1.6/ PND 50 for 10 days/	Sc/corn oil	-	↑ 0.2,0.4,0.8,1.6	↑ All doses	↑ 0.2,0.4,0.8,1.6	↑ 0.2,0.4,0.8,1.6	↑ 0.2,0.4,0.8,1.6
fenarimol	60168-88-9	Vinggaard, *et al.*, 2005	Wistar/PND 28 /6 rats per group	200/PND 42 for 7 days/0.5TP	gavage/ peanut oil			↓ 200 +TP	↓ 200 +TP		
Butylated hydroxyanisole (BHA)	25013-16-5	Kang *et al.*, 2005	Crj:CD (SD)/PND 42/8 rats per group	50,100,250, 500/PND 51 for 10 days/0.4TP sc	gavage/ corn oil	↓ 250,500	↑ 250+TP	↑ 250+TP	↑ 50;250 +TP	↑ 250+TP	↑ 250+TP
finasteride	98319-26-7	Kennel *et al.*, 2004	Sprague-Dawley/Pnd 46/ 6 rats per group	0.2,1,5,25/ Pnd 53-57 for 10 days/0.4TP sc	Gavage/ MC 500	-	↓ 1,5,25 +TP	↓ All doses +TP	↓ 25 +TP	-	↓ 1,5,25 +TP
benomyl	17804-35-2	Yamada *et al.*, 2005	Crj:CD(SD)IGS /PND 42/6 rats per group	100,300,1000 /PND 49 for 10 days/0.2-0.4TP sc	gavage/ corn oil	-	↓ 100,1000 +TP	↓ 1000 +TP	↓ 1000 +TP	-	-
prochloraz	67747-09-5	Vinggaard *et al.*, 2002	Wistar/PND 28/6 rats per group	250/PND 42 for 7 days /0.5 TP	Gavage/	-	↓	↓	↓	NA	NA

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
prochloraz	67747-09-5	Vinggaard *et al.*, 2002	Wistar/PND 28/6 rats per group	50,100,200/ PND 42 for 7 days /0.5 TP	Gavage/ Peanut oil	-	↑ All doses +TP	↑ All doses+TP	↑ All doses+ TP	NA	NA
DE-71		Stoker *et al.*, 2005	Wistar/PND 42/6 rats per group	30,60,120, 240/PND 53-61/0.4 TP sc	gavage/ corn oil	-	↓ All doses	↓ 60,120,240	↓ 240	↓ 240	↓ 120,240
fenthion	55-38-9	Kitamura *et al.*, 2003	F344/PND35/ 7 rats per group	25,50/PND 42 for 7 days 0.5TP sc	sc/panacete	↓ 50	↑ 25,50+TP	↑ 25,50+TP	NA	NA	NA
fenthion	55-38-9					↓					
Flutamide (positive control)	13311-84-7	Ashby *et al.*, 2004	Alpk:APfSD/ PND 42 /6 rats per group	3/PND 50 for 10 days/0.4TP	gavage/ stripped corn oil	-	↓	↓	↓	↓	↓
Finasteride	98319-26-7	Ashby *et al.*, 2004	Alpk:APfSD/ PND 42 /6 rats per group	0.2,1,5,25/ PND 50 for 10 days/0.4TP	gavage/ stripped corn oil	-	↓ All doses +TP	↓ All doses +TP	↓ 1,5,25 +TP	↓ All doses +TP	↓ 1,5,25 +TP
Finasteride	98319-26-7	Ashby *et al.*, 2004	Alpk:APfSD/ PND 42 /6 rats per group	0.008,0.04,5/ PND 50 for 10 days/0.4TP	gavage/ stripped corn oil	-	↓ All doses +TP	↓ All doses + TP	↓ 5 +TP	↓ All doses +TP	↓ 0.04,5+TP

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Testosterone propionate (TP)	57-85-2	Ashby *et al.*, 2004	Alpk:APfSD/ PND 42 /6 rats per group	/PND 50 for 10 days/0.4TP	Sc/ stripped corn oil						
DDE	3547-04-4	Ashby *et al.*, 2004	Alpk:APfSD/ PND 42 /6 rats per group	5,16,50,160/ PND 50 for 10 days/0.4TP	gavage/ stripped corn oil	-	↓ 50,160 +TP	↓ 50,160 +TP	↓ 50,160 +TP	↓ 160 +TP	↓ 50,160 +TP
Procymidon	32809-16-8	Ashby *et al.*, 2004	Alpk:APfSD/ PND 42 /6 rats per group	3,10,30,100/ PND 50 for 10 days/0.4TP	gavage/ stripped corn oil	-	↓ 10,30,100 +TP	↓ All doses+TP	↓ All doses+ TP	↓ 10,30, 100 +TP	↓ All doses +TP
17 alpha methyltestostrone	58-18-4	Ashby *et al.*, 2004	Alpk:APfSD/ PND 42 /6 rats per group	/PND 50 for 10 days/0.4TP	gavage/ stripped corn oil						
trenbolone	10161-33-8	Ashby *et al.*, 2004	Alpk:APfSD/ PND 42 /6 rats per group	/PND 50 for 10 days/0.4TP	gavage/ stripped corn oil						
vinclozolin	50471-44-88	Ashby *et al.*, 2004	Alpk:APfSD/ PND 42 /6 rats per group	/PND 50 for 10 days/0.4TP	gavage/ stripped corn oil						
linuron	330-55-2	Ashby *et al.*, 2004	Alpk:APfSD/ PND 42 /6 rats per group	/PND 50 for 10 days/0.4TP	gavage/ stripped corn oil						

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
fluoxymesterone	76-43-7	Dorfman, 1962b	Charles River/PND 25-28/	4,8,16,32,64/ from day of castration for 10-30 days	Gavage/ aqueous	NA	↑	↑	↑	NA	NA
TP	57-85-2	Edgren, 1963	Charles River/PND 26/ 6 rats per group	1/PND 28 for 7 days/	sc/ corn oil	↑ 1	↑ 0.3,1	↑ 0.3,1	↑ 0.3,1	NA	NA
Testosterone (T)	58-22-0	Edgren, 1963	Charles River/PND 26/ 5 rats per group	/PND 28 for 7 days/	sc/ cornoil					NA	NA
Methyltestosterone	58-18-4	Edgren, 1963	Charles River/PND 26/ 10 rats per group	0.625/PND 28 for 7 days/	sc/ corn oil	↑	↑	↑	↑	NA	NA
4,5α-dihydrotestosterone	521-18-6	Edgren, 1963	Charles River/PND 26/ 5 rats per group	/PND 28 for 7 days/	sc/corn oil					NA	NA

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Δ^1- Testosterone		Edgren, 1963	Charles River/PND 26/ 5 rats per group	/PND 28 for 7 days/	sc/corn oil					NA	NA
Δ^1-Methyltestosterone		Edgren, 1963	Charles River/PND 26/ 6 rats per group	3.0/PND 28 for 7 days/	sc/corn oil	-	↑	↑	↑	NA	NA
2-Hydroxymethylene-17-α-methyl-4,5α-dihydroT		Edgren, 1963	Charles River/PND 26/ 10 rats per group	3.0/PND 28 for 7 days/	sc/corn oil	-	↑	↑	↑	NA	NA
Stanazol		Edgren, 1963	Charles River/PND 26/ 10 rats per group	2.0/PND 28 for 7 days/	sc/corn oil	↑				NA	NA
1-Methyl-Δ^1-4,5α-dihydrotestosterone acetate		Edgren, 1963	Charles River/PND 26/ 5 rats per group	/PND 28 for 7 days/	sc/corn oil					NA	NA
1-Methyl-Δ^1-4,5α-dihydroT enanthate		Edgren, 1963	Charles River/PND 26/ 5 rats per group	/PND 28 for 7 days/	sc/corn oil					NA	NA

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/ days given/TP dose	Route for Test Chemical/ Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
19-Nortstosterone	434-22-0	Edgren, 1963	Charles River/PND 26/ 10 rats per group	1.5/PND 28 for 7 days/	sc/corn oil		↑	↑	↑	NA	NA
17α-Ethyl-19-nortestosterone	52-78-8	Edgren, 1963	Charles River/PND 26/ 5 rats per group	1.2/PND 28 for 7 days/	sc/corn oil					NA	NA
19-NorT. B-phenylpropionate		Edgren, 1963	Charles River/PND 26/ 6 rats per group	0.5/PND 28 for 7 days/	sc/corn oil	-/↑	↑	↑	↑	NA	NA
Wy 3475		Edgren, 1963	Charles River/PND 26/ 6 rats per group	0.3/PND 28 for 7 days/	sc/corn oil	↑ 0.3	-		↑	NA	NA
Propylthiouracil (PTU)	51-52-5	Yamada *et al*., 2004	Crj:CD(SD)IGS/PND 42/6 rats per group	2.5/PND 49 for 10 days/ 0.2 TP	Gavage/ corn oil	-	-	-	-	-	-
phenobarbital	50-06-6	Yamada *et al*., 2004	Crj:CD(SD)IGS/PND 42/6 rats per group	125/ PND 49 for 10 days/ 0.2 TP	Gavage/ corn oil	-	-	-	↓ +TP	-	-

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Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/days given/TP dose	Route for Test Chemical/Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
p,p'-DDE	72-55-9	Yamada *et al.*, 2004	Crj:CD(SD)IGS /PND 42/6 rats per group	100/ PND 49 for 10 days/ 0.2 TP	Gavage/ corn oil	-	↓ +TP	↓ +TP	↓ +TP	↓ +TP	↓ +TP
3-amino-1,2,4-triazole (AT)	61-82-5	Noda *et al.*, 2005	BrlHan WIST@Jcl (GALAS)/PND 42/6 rats per group	0,40,200,1000/ PND 63 for 10 days/ 0.2 TP	Gavage/ olive oil	-	-	↑ 1000; 200, 1000 +TP	↑ 40,200 +TP	-	-
Nomegestrol acetate	58652-20-3	Duc *et al.*, 1995	Sprague-Dawley/ PND 21	2.5 – 20/ PND21/0.5ml/ rat/day sc	Gavage/ olive oil		↓ 2.5,5,10 +T	↓ 2.5,5,10 +T			
raloxifene	84449-90-1	Nubauer et al., 1993	Immature males castrated 3 days before dosing/7 rats per group	0.05,0.5,5.0/ for 7 days/0.2 TP	Corn oil	-	↓ All doses +TP				

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/days given/TP dose	Route for Test Chemical/Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
androstrone		Korenchev-sky and Dennison, 1935	NA/PND 20-28/4-7 per group	67,200,450,600,900,13501800 rat U/28-42 days after castration(PND 53-66)	Injection 2x per day for 7 days/oil	-	↑ All doses	↑ All doses			
androsterone	53-41-8	Korenchevsky et al., 1935	NA/PND 21-26/4-10 rats per group	1993,3986,7971,11957 rat units/30-38 days post castration	Sc injection 2-3x per day/oil or water	-	↑	↑		↑	
Androsterone-diol		Korenchevsky et al., 1935	NA/PND 21-26/2-9 rats per group	97,194,389,777,1555,3109 rat units/30-38 days post castration	Sc injection 2-3x per day/ oil or water	-	↑	↑		↑	

(continued)

Table 5 (continued)

Chemical	CAS No.	Citation	Strain/Age at Castration/# per Dose Group	Doses Given (mg/kg/day)/days given/TP dose	Route for Test Chemical/Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight (mg)				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
Transdehydroandrosterone		Korenchev-sky and Dennison, 1936	NA/NA	0.8, 1,2.4/1,2,3/ necropsied at PND 68/86/injected for 7 or 21 days	Sc/ sesame oil		↑	↑		↑	

Table 6. Chemicals Tested in the Hershberger Assay By Mode of Action

Androgens

T propionate

T

methylT

T 17-dichloroacetate

T benzoate

trans-androstanediol

cis-androstanediol

17β-hydroxy-5α-androstan-3-one

5α-androst-2-en-17β-ol

dihydroT

Trenbolone-steroid not affected by aromatase-feedlot contaminant

Progestins

cyproterone acetate-partial androgenic activity

megestrol acetate

nomegestrol acetate (progesterone derivative)

6α-methyl-17α-hydroxy-progesterone

spironolactone

Antiandrogens

flutamide

DE-71 (a commercial polybrominated diphenyl ether (PBDE) mixture

Fungicides

fenarimol

vinclozolin

prochloraz

Table 6 (continued)

Pesticides

methoxychlor-pesticide

procymidone

linuron (herbicide?)

iprodione

chlorolinate

p,p'-DDE (AR antagonist)

ketoconazole

Organophosphate insecticides

fenitrothion

butylated hydroxyanisole (BHA)

finasteride- 5-alpha reductase inhibitor

Reduced LABC weight only

4-n-amylphenol

p-dodecyl-phenol

Estrogens

17 β-estradiol

methoxychlor

tamoxifen

diethylstibesterol

Increased only seminal vesicle weight when combined with TP

p-(tert-pentyl)phenol

Table 6 (continued)

<u>Thyroid Modulators</u>

PTU

PB

p,p'-DDE

3-amino-1,2,4-triazole (AT)

Chemicals That Tested Negative in Hershberger Assay

Bisphenol-A

tamoxifen

atrazine

4-cyclohexylphenol

Pyrethroids

Esfenvalerate

fenvalerate

permethrin

Table 7. Examples of Chemicals That Appear to Bind to the AR but Have Not Been Tested in the Hershberger Assay

AA560-antiandrogen

Allylestrenol-antiandrogen

Androstanolone

Androstenedione

Casodex-antiandrogen

Cimetidine-antiandrogen

dehydroepiandrosterone

Dexamethasone

Drospirenone-antiandrogen (progestogen)

ICI 176,334-antiandrogen

Medroxyprogsterone acetate (MAP)

megestrol acetate

Methylandrostenediol

Nomegestrol acetate

19-nortestosteronr cyclopentylpropionate

Permethrin (insecticide)-pyrethroid

RU 58642-antiandrogen

T undeconoate-androgen

TZP-4238-antiandrogen

WIN 49596-antiandrogen (AR antagonist)

Conclusions

The Hershberger assay is valuable as an *in vivo* androgenic screen for AR antagonists, AR agonists, and 5α-reductase inhibitors that prevent T to DHT conversion. This bioassay furnishes the natural targets for androgens, a relatively rapid growth response yielding quantifiable weight changes, and no requirement for specialized equipment unless hormone determinations are added as an enhancement.

These attributes of the assay become important in various fields such as risk assessment, toxicology/teratology, and drug development. For instance, the aim of prostate cancer therapy is to block the androgens. The ideal drug would have potent anti-androgenic activity and yet be devoid of androgenic, glucocorticoid, progestational, estrogenic or any other hormonal or antihormonal action. Thus, appropriate screening for the various classes of androgenic and anti-androgenic compounds becomes vital in these roles.

CHAPTER 9. ADDITIONAL SCREENS AND MARKERS

Introduction and Background

Identification of nuclear receptor-mediated endocrine activities is important in many fields, ranging from pharmacological and clinical screening of potential therapeutics, to food and feed safety, toxicological monitoring, and risk assessment. Traditionally, animal studies such as the Hershberger assay (Hershberger *et al.*, 1953) are used for the assessment of AR-mediated androgenic potencies. To enhance and increase the speed of analyses of the activities of new chemicals, food additives, and pharmaceutical compounds, a number of additional markers, screening strategies, etc., have been developed. These include:

- Use of *in vitro*, high-throughput, cell-line screens, as well as other *in vitro* screening assays measuring receptor binding or reporter gene activation. These can be compared with the data obtained from other *in vitro* or *in vivo* bioassays for correlation, variances, robustness, reproducibility, and specificity. If the *in vitro* assays are found to be good predictors of *in vivo* androgenic (or anti-androgenic) activity of a range of compounds, they can be used to pre-screen chemicals to remove nonresponders and reduce the number of chemicals going into *in vivo* screens and tests in animals, thereby likely reducing the number of animals and even the number of animal studies.

- Markers (biochemical and molecular) included in the *in vivo* studies, to compare with (enhance) the sensitivity, specificity, and robustness of the current endpoints, accessory sex organ weights.

- -Omics (genomics, proteomics, and metabolomics), physiological markers, to use with *in vitro* and/or *in vivo* studies: (1) to identify the initiating steps of activation or inactivation of genes for a given test chemical (transcription to mRNAs [messenger RNAs] by examining the "footprint" of mRNAs) from genes that are activated (androgen agonists) or inactivated (androgen antagonists) from the exposure to a test chemical; *i.e.*, genomics; (2) to identify translational changes (from mRNA to protein) by examining changes in protein levels from specific genes, *i.e.*, proteomics; and (3) most recently, subtle, sensitive markers have been identified by metabolomics, which examines the differences in concentrations of endogenous metabolites (in blood, urine, milk, sweat, amniotic fluid, expired air, etc.). These physiological markers can be used to distinguish untreated versus treated males, animals administered test material at a no-effect-level versus those dosed at an effect level, and in animals with a response versus those without a response (and comparisons in animals with more severe versus less severe outcomes), etc.

These –omics evaluations may be predictive, identifying sensitive susceptible subpopulations (to prevent adverse outcomes), identifying individuals early in the development of the adverse outcome, or tracking progression of the adverse outcome/disease state and/or tracking results of treatment (*i.e.*, amelioration and/or curse).

This chapter will examine the screens and markers currently available or proposed to prioritize the chemicals for animal studies, to further enhance the animal studies, to possibly replace the animal studies, and to provide important predictive information in animal models and in humans.

***In Vitro* Screens**

Sonneveld *et al.* (2006), on behalf of the commission of the European Communities, examined a large number of predominantly steroidal compounds ($\geq$ 26) in cell free and cell culture *in vitro* assays and in an *in vivo* assay in two different laboratories. They measured receptor binding in MCF-7 cytosolic preparations, reporter gene activation in U2-OS-based AR (and ER α) CALUX (chemically activated luciferase expression) reporter gene assays (AR CALUX and ER CALUX), and CHO (Chinese hamster ovary) cell line-based steroid reporter gene assays (CHO-AR). The receptors used in all of the *in vitro* assays are of human origin. The *in vitro* assays used luciferase reporter genes; *e.g.*, multimerized responsive elements for the cognate receptors coupled to a minimal promoter element (the TATA box) and luciferase for CALUX, the mouse mammary tumor virus promoter (MMTV) for AR in CHO cells. The MCF-7 receptor binding assays obtain relative binding affinities (RBAs) of the compounds with the reference compound DHT but cannot distinguish between AR agonists and antagonists. The reporter gene activation assays (CALUX and CHO) can distinguish between AR agonists and antagonists since they evaluate both binding and activation/repression of the reporter gene. The authors also used the Hershberger assay for the *in vivo* test in immature male orchidectomized rats, with six animals/chemical/dose and administration of the test chemical or reference androgen (T at 160 μg/kg) twice daily for seven consecutive days. At necropsy, the weights of the ventral prostate, seminal vesicles, and levator ani muscle were recorded. The authors examined correlation of the same chemical tested in different *in vitro* assays, comparisons of receptor binding assays with reporter gene assays, and correlations between the *in vitro* reporter gene assay and the *in vivo* assay (Sonneveld *et al.*, 2006).

The range in EC_{50} values measured with different ligands over time, including the positive control DHT (in AR CALUX), was "small," reflected by an interassay CV (coefficient of variation) of 22% for the AR CALUX. The AR CALUX cell line showed high sensitivity toward all androgens tested with the following range of potencies (EC50 values): DHT (110 μM), T (657 pM), its 19-nor derivatives nandrolone (19-nor-T; 301 pm), 19-nor-11-keto-T (2845 pM), and 11-methylene-19-nor-T (98 pM). The selectivity of the AR CALUX cells is high since representative steroids for other hormone receptors (E2 and EE for ER, progesterone for PR, and dexamethasome for GR) showed no

"substantial" agonistic response with relative agonistic activities below 0.001, except for dexamethasone (0.003) which also had a relative transcriptional activity (RTA) of 8% versus DHT (Sonneveld *et al.,* 2006).

The CHO-hAR cell lines were exposed to relatively high potency compounds and excluded known AR antagonists, FLU, cyproterone acetate, and RU486, since they will bind to their cognate receptor but generally fail in transcriptional activity (Sonneveld *et al.,* 2006).

"Surprisingly good correlations" were obtained between data obtained with the same compounds in the different reporter gene assays in the two laboratories (with different setups). The correlation between data from the AR CALUX cells and the data from the CHO-AR cells, with 47 compounds tested, was r^2=0.75, $p<0.0001$. These correlation coefficients were very similar to the correlation coefficients found in an unbiased approach (with no correlation exclusions) between the two AR reporter gene assays (n=60, r^2=0.76). There were notable differences between the AR CALUX and CHO-AR reporter gene assays (defined as > 10-fold difference between relative antagonistic activities [RAAs]) for 17α-(2-propenyl)-19-nor-T, 11β-ethyl-NET, Δ15-NET, and T-17,17'-(2,2'-oxybisacetate) for all comparisons AR CALUX > CHO-AR (Sonneveld *et al.,* 2006).

Some notable exceptions were found that showed relatively high *in vitro* reporter gene activity coupled with low *in vivo* activity: nandrolone (*in vitro* 6x higher), 11-keto-19-nor-T (7x), (14β-17α, 2OS)-20-OH-19-norpregna-4,9-diene-3-one (8x), and 7α-methyl-17α-(2-propenyl)-estr-5(10)-en-17β-01 (8x). Exceptions were also found that showed relatively high *in vivo* activity coupled with low *in vitro* reporter gene activity: T (*in vivo* 7x higher). Looking at cross-assay bioactivity of androgens (n=26), the RAAs of androgenic compounds with 11β-substitutes, 5α-hydrogen, or 7α-methyl substitutes tended to cluster separately.

Small substitutions in norethisterone (as seen above with T) also affected *in vitro* receptor binding and transactivation assays and biological activity in *in vivo* animal models (Deckers *et al.,* 2000).

In both *in vitro* assays in both of the laboratories employed, the response of induced chemicals *in vitro* was almost without exception able to predict the *in vivo* activity with an uncertainty of a factor of ten or less in either direction, suggesting that the correlation between the *in vivo* versus *in vitro* tests will not be very different with an expanded data set. The authors also felt that with the high sensitivity of the *in vitro* screens, there should be a very low level of false negatives, even when taking an uncertainty factor of 10-100 into account (Sonneveld *et al.,* 2006).

Charles *et al.* (2005; from Marty's laboratory at Dow) have also compared the results of the *in vitro* and *in vivo* EDSTAC test battery for detecting anti-androgenic activity. The test chemicals were p,p'-DDE, FLU, spironolactone, procymidone, RU486, methoxychlor (MXC), benzo(a)pyrene (BAP), and selected hydroxylated metabolites. The *in vitro* AR binding experiments used the recombinant ligand-binding domain of the

human AR and fluorescence polarization methodology and produced IC_{50}s from RU486 and 9-OH-BAP comparable to that of T. Other parent compounds and metabolites exhibited lower binding affinity. For the AR transactivation assay, AR(+)LNCaP prostate carcinoma cells were transfected with an inducible luciferase reporter construct (pGudLuc7ARE) and exposed for 24 hours to the test materials ($\leq$ 10 μM) in the presence or absence of 1 nM of the AR agonist R-1881 (reference androgen). All of the test chemicals, including the hydroxylated metabolites of BAP and MXC, produced significant anti-androgenic activity *in vitro*, based on their inhibition of the response of the transfected cell line to R-1881 (Charles *et al.,* 2005).

The *in vivo* anti-androgenic evaluation used the Hershberger assay, with castrated CD rats dosed by gavage for ten days with MXC (10, 50, 100, 200 mg/kg/day), BAP (1, 10, 50, 100 mg/kg/day), RU486 (1, 5, 10, 25 mg/kg/day), or FLU (10 mg/kg/day) in the presence of sc injection of TP at 0.4 mg/kg/day as the reference androgen. Interestingly, neither BAP nor MXC significantly reduced the weights of the accessory sex organs relative to the organ weights in the TP-only controls. MXC significantly reduced body weight at 200 mg/kg/day, and BAP significantly reduced absolute and relative liver weights at 100 mg/kg/day. RU486 at 25 mg/kg/day significantly reduced weights of the ventral prostate, seminal vesicles, and Cowper's glands without affecting body weight. FLU at 10 mg/kg/day reduced all the accessory sex organ weights measured. The anti-androgenic activity of p,p'-DDE, spironolactone, and procymidone *in vivo* had been previously demonstrated. These authors stressed the importance of a weight of evidence approach and the importance of including *in vivo* assessments. This can be interpreted to mean that in their capable hands, the *in vitro* results did not adequately predict the *in vivo* results (Charles *et al.,* 2005).

In contrast, Sonneveld *et al.* (2006) determined relationships between compounds and relationships between various bioassays. Although correlation of AR CALUX data with those of the Hershberger assay was lower than that found with other AR *in vitro* assays (with 34 compounds, r^2=0.46; $p<0.0001$), the overarching conclusion was that the *in vitro* assays were found to be good predictors of *in vivo* androgenic (or estrogenic) activity of a range of compounds "allowing pre-screen and/or possible reduction of animal studies."

As an alternative to the typical strategy for detection of anti-androgens in the Hershberger assay, Hwang *et al.* (2005) presented the use of an *in vivo* screen using humanized, double-transgenic mice co-expressing tetracycline-controlled transactivator (tTA) and the human cytochrome P450 (CYP) enzyme CYP1B1. The tTA-driven hCYP1B1 was expressed at high levels in neonates and at low levels in adults. Upon castration, the patterns of tTA-driven hCYP1B1 expression were reduced to those of female mice. Treatment of castrated mice with T restored the adult male patterns. After treatment with FLU (a representative anti-androgen), the levels of tTA-driven hCYP1B1 were increased. This is the basis of the assay, whereby adult, humanized, transgenic mice co-expressing tTA-driven human CYP1B1 were exposed to each of the five different anti-androgens at a number of doses: di-(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DBP), diethylphthalate (DEP), linuron (3,-(4-dichlorophenyl)-methoxy-1-methylurea),and 2,4'-DDE (1,1-dichloro-2-(p-chlorophenyl)-2-(o-chlorophenylethylene).

The tTA-driven hCYP1B1 expression of the transgenic mice was analyzed by real-time polymerase chain reaction (RT-PCR) and/or Western blot and for O-debenzylation activity at transcript, protein, and activity levels. Exposure to all five anti-androgens resulted in dose-related, increased expression of the hCYP1B1 transgene in the livers of the adult males. This effect was "almost identical to that due to castration" (Hwang *et al.,* 2005, p. 161). The authors concluded that this novel *in vivo* bioassay, using humanized transgenic mice, is useful to detect anti-androgens and is a more relevant bioassay than the Hershberger since it is related to human response to exposure (Hwang *et al.,* 2005).

Additional Markers

Biochemical

Vinclozolin, a known androgen antagonist (*i.e.*, binds to AR; Kelce *et al.*, 1997), may have another effect mediated by a completely different mechanism. Dr. M.K. Skinner's laboratory (at Washington State University, Pullman, WA) has reported on possible epigenetic, transgenerational, male reproductive effects of vinclozolin (and methoxychlor, an estrogen agonist and androgen antagonist, and likely procymidone).

In 2003, Cupp *et al.* (also from Skinner's laboratory) exposed timed-pregnant Sprague-Dawley rats to methoxychlor by intraperitoneal injection from GD 7 to 15 (GD 0 = plug/sperm date) at 0, 50, or 150 mg/kg/day. Testes were collected from GD 16 fetuses and from postnatal male pups on PND 4, 10, 17-20, and 60. At 50 mg/kg/day, the GD 16 testes exposed *in utero* to methoxychlor did form seminiferous cords, but there was a decrease in the area of the cords and an increase in interstitial area. The number of cells within the tubules was also reduced by 20%. There were no differences in the number of seminiferous cords in control versus treated GD 16 testes. Also at 50 mg/kg/day, the testes taken from pups on PND 4, 10, 17, and 60 did not differ between treatment and control animals in the area of seminiferous cords/tubules, the interstitial area in the number of seminiferous cords, or the number of intratubular Sertoli cells or interstitial Leydig cells. There were no effects from *in utero* exposure in prepubertal or adult testis weights or in a number of different reproductive organ weights. Exposed males did exhibit reduced germ cell numbers on PND 17. There were nonstatistically significant trends for reductions in the number of seminiferous tubules in PND 17 exposed testes and in the number of germ cells on PND 10 and 60 in treated testes.

Cupp *et al*. (2003) also found that in addition to reduced germ cell numbers on PND 17, there were reduced numbers of mitotic germ cells/testis sections and an increase in germ cell apoptosis on PND 17-20 testes (testes collected from younger males did not exhibit increased apoptosis). Pnd 60 testes exhibited an increase in germ cell apoptosis in elongating spermatids associated with Stage X-XI of the spermatogenic cycle, and the number of apoptotic cells in tubules other than Stage X-XI were higher, but not statistically significantly. The number of tubules with apoptotic cells doubled in males exposed *in utero* to 150 mg/kg/day methoxychlor. Exposure to 50 mg/kg/day did not have consistent effects on apoptotic germ cell numbers at PND 60. Serum T levels were equivalent in PND 20 and 60 males for control and treated animals. PND 60 males (four

treated and four controls) were bred to PND 60 control females. All females became pregnant, and litter size and pup weights did not differ between groups. Preliminary analysis of caudal epididymal sperm in adults indicated no change in sperm number, a slight (15%) decrease in percent mobile sperm, and a small increase in sperm with abnormal morphology. The authors speculated whether the male reproductive effects of methoxychlor were due to anti-androgenic metabolites. They concluded that reduction in germ cells in cell division on PND 17 and increase in germ cell fragmentation on PND 17-20 indicate that a brief *in utero* exposure to methoxychlor results in reduced germ cell survival and abnormal development during puberty. Effects were also seen in PND 60 testes, with the increase in apoptotic cells possibly due to reduced T (Cupp *et al*., 2003).

A follow-on paper from the same laboratory (Uzumcu *et al*., 2004) tested the hypothesis that transient embryonic exposure to an anti-androgenic endocrine disruptor at the time of testis determination alters testis development and subsequently influences adult male spermatogenic capacity and reproduction. To that end, the authors evaluated the effects of vinclozolin on embryonic testicular cord formation *in vitro*. The testes were removed from untreated embryonic males on GD 13 (sperm smear = GD 0) and cultured in the absence or presence of vinclozolin at 50, 100, 200, or 500 μM or to FLU at 1 μM (positive control). The authors also exposed timed-pregnant females to vinclozolin at 100 mg/kg/day, injected intraperitoneally from GD 8 to GD 14 or GD 15 to 21. The testes from male offspring from exposed dams were collected on PND 0 (day of birth), 20, and 60. The PND 60 males were evaluated for epididymal cauda sperm motility and number. Serum T was measured. Testes for PND 20 and 60 males were weighed, embedded, sectioned, stained, and evaluated for histopathology and the presence of apoptotic cells.

Exposure of the GD 13 embryonic testes in culture to 50-500 μM vinclozolin resulted in no effects on seminiferous cord formation at 50-200 μM vinclozolin. The number and morphology of the cords were also unaffected at 50-200 μM vinclozolin. At 500 μM vinclozolin, the number of seminiferous cords was reduced and enlarged or "swollen." At 1 μM FLU, there were no or a minimal number of cords, only "patchy cords in limited areas."

Pregnant females were exposed to vinclozolin by intraperitoneal injection on GD 8-14 (the time of male sex determination and testicular cord formation) at 0 or 100 mg/kg/day, and the testes from F1 male offspring were examined on PND 0, 20, or 60. This scenario resulted in no effects on body or absolute or relative (to body weight) testes weights at any age. Anogenital distance on PND 0, testes descent, gross morphology of the testes, and external genitalia were also unaffected. There were no effects of *in utero* exposure to vinclozolin on apoptosis in PND 0 testes. However, PND 20 and 60 testes from exposed males exhibited significantly higher numbers of apoptotic germ cells (a two-fold increase), with Stages XI and XII tubules exhibiting $\geq$10 apoptotic germ cells/tubule (a three-fold increase). Exposure of rat dams to vinclozolin on GD 15-21 (versus GD 8-14 above) did not result in increases of F1 male testicular apoptosis on PND 20 or 60. Serum T was unaffected. Epididymal cauda sperm motility was significantly reduced in exposed PND 60 males, with the slight reduction in epididymal

sperm number from exposed males not statistically significant. Interestingly, when treated males were mated with treated proestrous females, 9 of 12 matings resulted in sperm-positive vaginal smears. Of the three unsuccessful pairings, two of the three treated females had irregular estrous cycles. The authors concluded that there was no "dramatic" reduction in fertility in the adult males exposed *in utero* (Uzumcu *et al*., 2004).

The most recent research paper from Skinner's group (Anway *et al*., 2005), built on the previous papers from this group that indicated transient *in utero* exposure to vinclozolin on GD 8-15 by intraperitoneal injection at 100 or 200 mg/kg/day, resulted in increased spermatogenic cell apoptosis and decreased sperm number and motility in adult F1 male offspring. In this study, pregnant outbred Sprague-Dawley and inbred Fischer 344 (F344) rats (the F0 generation) were administered vinclozolin at 0 or 100 mg/kg/day by daily intraperitoneal injection on GD 8-15 (during the time of gonadal sex determination). F1 generation male rats were bred to F1 generation females from different litters within groups. Subsequent breeding continued for F2, F3, and F4 generations with no sibling pairings. Adult males from the F1, F2, F3, and F4 generations were terminated between PND 60 and 180. The testes were removed and processed for histopathologic examination, and epididymal cauda sperm were evaluated for motility and numbers. The adult F1, F2, F3, and F4 male offspring exposed *in utero* to vinclozolin exhibited a greater than two-fold increase in spermatogenic cell apoptosis, a "minimal" 20% reduction in sperm number, and a 25-35% reduction in sperm forward motility. More than 90% of all males analyzed from all offspring generations exhibited the increased spermatogenic cell apoptosis (the "phenotype"), and the frequency or severity did not decline across the generations. F1 through F4 male offspring examined on PND 60 exhibited normal testicular morphology. However, periodically, some males older than PND 90 (~20%) developed small testes, severely reduced spermatogenesis, and complete infertility, associated with abnormal seminiferous tubule morphology. The F1 through F4 fertile males had normal absolute and relative (to body weight) testes weights and sired litters with normal numbers and body weights of pups. However, nearly all of the treated male offspring had the minimal phenotype of a two-fold increase in spermatogenic cell apoptosis, and the majority had a decrease in epididymal sperm number (Anway *et al*., 2005). Methoxychlor, administered by intraperitoneal injection daily on GD 8-15 at 200 mg/kg/day, also caused a similar phenotype in the F1 and F2 offspring examined (Anway *et al*., 2005).

The authors also bred second generation F2 vinclozolin males (whose grandmothers were injected) with a normal (control) female, producing male offspring with the same phenotype, while an F1 female (from a treated vinclozolin mother) bred to a normal (control) male did not produce offspring males with the "vinclozolin phenotype." The authors (Anway *et al*., 2005) indicated that the high and unchanging incidence of effects (>90%) on males over four offspring generations suggested an epigenetic mechanism. Given that the only epigenetic mechanism currently known to influence germline transmission involves the methylation pattern of imprinted genes, the authors looked at the methylation pattern of DNA in testes from PND 6 male offspring from control versus vinclozolin-treated F344 (not SD) F0 dams. They presented evidence for a change in the methylation pattern of DNA from PND 6 testes and

specifically for the region of the gene for cytokine-inducible SH2 protein in F2 and F3 offspring male epididymal sperm from two different litters per generation, with 4 of 8 F2 males and 2 of 5 F3 animals exhibiting the changes.

The Anway *et al*. (2005) paper on transgenerational effects of vinclozolin in *Science* prompted a Science Scope commentary in the same issue (Kaiser, 2005). Kaiser indicated that the "startling results… support the idea that… endocrine disrupters could be causing population-wide reproductive problems such as lowered sperm counts in men." Researchers interviewed by Kaiser indicated that if the results are "reproducible," they will have a "large impact on how we look at these kinds of chemicals," and that "we don't have a clue to what's really happening." There was concern that the changes observed in methylation may correlate with (be the result of) declining fertility and not the cause. Skinner is quoted as indicating that "we are describing a new phenomenon" and that "the hazards of environmental toxins are much more pronounced than we realized." EPA researchers believed that this single study will not change regulations for vinclozolin and similar anti-androgens, but "it's going to be very important for other people to look at this" (Kaiser, 2005).

Skinner and Anway (2005) followed up their transgenerational study (Anway *et al*., 2005) with a literature review of the molecular and cellular controls of embryonic testis development through analysis of the embryonic testis transcription (genes that are transcribed to produce mRNA) to identify potential regulatory factors for male sex determination and testis morphogenesis. One critical factor identified was neurotropin 3 (NT3). At the onset of male sex determination, Sertoli cells in the embryonic testis initiate differentiation and express NT3 to act as a chemotactic factor for mesonephros cells to migrate and associate with Sertoli germ cell aggregates to promote cord formation. Promotor analysis suggested that NT3 may be an initial downstream gene to SRY (the sex determining gene on the Y chromosome that initiates testis formation prior to hormonal influences) and helps promote testis morphogenesis. The authors' previous work (Anway *et al*., 2005) had established that transient gestational exposure to vinclozolin (or methoxychlor) at the time of male sex determination caused a transgenerational phenotype of spermatogenic cell apoptosis and subfertility. Their explanation was that this was an apparent epigenetic mechanism involving altered DNA methylation and permanent reprogramming of the male germline. Therefore, their current work involves identifying a series of genes with altered methylation and imprinting. Observations reviewed support the authors' hypothesis (at least to them) that a transient embryonic *in utero* exposure to an endocrine disruptor does influence the embryonic testis transcriptome, and through epigenetic effects (*e.g.*, DNA methylation) results in abnormal germ cell differentiation, which subsequently affects adult male spermatogenic capacity and male fertility, and that this phenotype is transgenerational through the male germ line. The authors concluded that their literature review provides insight into the molecular and cellular control of the embryonic testis development, male sex determination and programming of the male germ line, and that their observations "critically impact the potential hazards of these compounds as environmental toxins" (Skinner and Anway, 2005).

The work of Skinner's laboratory employed vinclozolin at 100 mg/kg/day, administered to the mothers by intraperitoneal injection much earlier in gestation (GD 8-15; at the time of male sex determination and early testicular cord formation). They reported no major changes in F1 male offspring reproductive system organ weights and no effects on anogenital distance. They did report reduced cauda epididymal sperm counts and motility, increased apoptotic germ cells in the testes, and increased incidence of abnormal seminiferous tubule morphology. Their initial paper on vinclozolin in the series (Uzumcu *et al*., 2004) reported these effects in the F1 offspring (as had many others). It is the paper by Anway *et al*. (2005) that resulted in such attention. In that paper, they used the Uzumcu *et al*. (2004) F0 dam dosing paradigm, but they mated F1 x F1 (within 0 or 100 mg/kg/day groups), F2 x F2, and F3 x F3 to produce F1, F2, F3, and F4 offspring. They reported the same testicular and epididymal effects at the same incidence ($\geq$90%) in all four male offspring generations. Although these matings were with males and females from the same group, crossover matings indicated that the "phenotype" occurred in the male offspring of an affected male (x control female), not from an affected female (x control male). The high and unchanging incidence of effects on males over the four offspring generations suggested an epigenetic mechanism, and the only epigenetic mechanism currently known to influence germline transmission involves the methylation pattern of imprinted genes.

Sperm are a highly specialized cell type developed to deliver the paternal haploid genome to the oocyte. The epigenetic (or gene regulatory) properties of the sperm prepare the paternal genome to contribute to the genome of the oocytes, thereby restoring the diploid genome and to contribute to embryogenesis. Informational damage to the sperm prior to fertilization includes DNA damage, chromosomal aneuploidy, reduced telomere length, malformation of the centromere, genomic imprinting errors, altered messenger RNA (mRNA) profiles, and abnormal nuclear condensation and packaging. Any or all of these effects on the sperm have been shown to affect embryogenesis in animal models and humans (Emery and Carrell, 2006).

The acquisition of genomic DNA methylation patterns, including those important for development, begins in the germ line. In particular, imprinted genes are differentially marked in the developing male and female germ cells to ensure parent-of-origin-specific gene expression which is a critical mechanism in development and adult life (Trasler, 2006). One of the key elements of the imprinting mechanism is DNA methylation (Swales and Spears, 2005); the other is histone acetylation. The presence or absence of a methyl group on specific cytosine DNA bases in CpG-rich regions of the male or female heptoid genome determines whether that allele of the gene is active (transcribed to mRNA and translated into protein) or not active (no transcription). Current views are that alleles (within intron sites) with methylated bases are silent, and alleles (within intron sites) with unmethylated bases are active. DNA methylation is controlled by DNA methytransferase enzymes. Germ cells undergo reprogramming to ensure that sex-specific genomic imprinting (to be based on the sex of the offspring, no longer from the sex-specific gametes) is initiated, allowing normal embryonic development to progress after fertilization. Errors in genomic imprinting can lead to embryolethality, perturbation of growth, altered placental function, neurobehavioral effects, developmental disorders, and disease. Recent studies have suggested a link between the use of assisted

reproductive technologies (ART) and an increase in normally rare imprinting disorders in children conceived by these methods; *e.g.*, Prader Willi syndrome (Swales and Spears, 2005; Horsthemke and Ludwig, 2005).

The Anway *et al*. (2005) paper apparently looked at "DNA" for differences in methylation patterns in treated versus control testes. The authors indicate that they did "not investigate specific known imprinted genes" but rather focused "on the effects of the endocrine disruptor on the total genome (Anway *et al*., 2005; p. 1468). They also used F344 PND 6 testes (although the *in vivo* multigeneration portion apparently was performed with Sprague-Dawley rats). Their subsequent paper (Skinner and Anway, 2005) indicated that "a series of genes with altered DNA are being identified," but the paper itself relies on a review of the literature.

Chang *et al*. (2006), also from Skinner's laboratory, recently reported on 15 candidate sequences with altered methylation patterns in F1, F2, and F3 germ lines. The original paper (Amway *et al*., 2005) reported on only one gene (cytokine-inducible SH2 protein).

There is also the possibility that if there are specific, relevant genes with altered methylation patterns, then the altered methylation may not be the cause of the increased apoptosis and subfertility, but the result or consequence of the reproductive effects. In this case, the changes in methylation may be downstream markers of exposure or of effect. The authors (Anway *et al*., 2005; p. 1468) also reported that "the epigenetic alterations observed involve both hypermethylation and hypomethylation," with no further comments.

This "seminal" (pun intended) work has to be replicated. If the phenomenon is real (inherited transgenerational effects in the absence of continued exposure), then there should be increased concern on the long-term effects and multiple mechanisms of specific endocrine disruption. Long-term studies with continued exposure (*e.g.*, the current testing guideline two-generation reproductive toxicity studies) may still be necessary, but it may no longer be sufficient (although the reproductive effects would be detected, it would be during continual exposure, not in subsequent unexposed generations).

Zoeller (2006) has asked the major scientific question after the confirmation of the Skinner laboratory papers: Do family lines carry an epigenetic record of previous generations' exposures?

Physiological

Physiological markers can be defined as in-life parameters which provide information on health or biological status of the individual. As such, they can include body weight (body mass index, BMI), body core temperature, circulating hormones, hematology, blood pressure, clinical chemistry, urinalysis, as well as molecular markers of susceptibility/vulnerability, disease state, progression, amelioration, or cure of a

disease state, etc. These latter molecular markers include evaluation of the new "-omics" markers: genomics, transcriptomics, proteomics, and metabolomics.

Genomics (and transcriptomics)

The universe of genes is designated "the genome;" presumably all diploid cells in the body have the same genome. What is important is which genes are active; *i.e.*, transcribed to mRNAs (transcriptomics) and ultimately translated into proteins (proteomics).

In 2002, the National Institute of Environmental Health Sciences, the National Institutes of Health, Office of Rare Diseases, the EPA, and the American Chemistry Council held a conference aimed at planning the future for the integration of emerging technologies in developmental and reproductive toxicology research (Mirkes *et al*., 2003). This conference provided a framework for government agencies to begin the needed investment in scientific endeavors that have helped to establish the framework of a systems biology approach for developmental and reproductive biology. Since that time, the application of genomics tools in the study of reproductive and developmental biology has rapidly emerged, and ground-breaking research in genomic analysis for reproductive and developmental toxicology has surfaced in the peer-reviewed literature.

As examples, studies have demonstrated that gene expression analysis can be used to identify:

- How specific chemicals influence highly sensitive processes in early mammalian development (Clausen *et al*., 2005).

- A potential role of gene expression experiments in defining the shape of the dose-response curve at low doses (Daston and Naciff, 2005)

- The effect of estrogens or estrogen agonists on gene expression related to the embryonic and fetal development of the rat testis and epididymis (Naciff *et al*., 2005).

- How drugs induce alterations in the expression of genes known to be expressed during development of the craniofacies (Gelineau-van Waes *et al*., 1999).

- Genes that are induced in the embryo following exposure to teratogens (Mikheeva *et al*., 2004; Kultima *et al*., 2004).

- Genes and mechanisms involved in estrogen-induced organ growth with comparison to conventional toxicology endpoints (Moggs *et al*., 2004; Naciff and Daston, 2004).

- Genes involved in the development of the uterus and ovary (Daston and Naciff, 2005).

- The use of Gene Ontology and pathway analysis to provide insights into the molecular mechanisms of estrogens (Currie *et al*., 2005).

Dr. George Daston's laboratory at Procter and Gamble Company (Daston and Naciff, 2005; Naciff *et al*., 2005; Naciff *et al*., 2002a,b) has been evaluating and detecting changes in gene expression in response to estrogens in the female reproductive tract during embryofetal development (Naciff *et al*., 2002a,b), in the juvenile prepubertal female rat reproductive tract, and in the male rat testes from transplacental exposure to low and high doses of a potent estrogen (17α-ethinylestradiol), a phytoestrogen (genistein), and a weak xenoestrogen (bisphenol A) (Naciff *et al*., 2005). This group has used microarrays to evaluate thousands of changes in gene expression in a single experiment and has presented the data as "heat" maps, with increases in expression of specific genes in red and reductions in expression of specific genes in green. Naciff *et al*. (2005) determined the effects of 0.1 to 10 mg/kg/day of 17α-ethynyl estradiol (EE) given by sc injection to 20-day-old Sprague-Dawley rats for four days (PND 20-23). The authors (Naciff and Daston, 2004) reported the changes in expression of 1,394 genes (with respect to control) to at least one of the EE dosages. However, the tissues from rats exposed to the lowest EE dose showed changes in the expression of only 33 genes. Approximately 600 genes were shown to exhibit a significant dose-dependent relationship. Further studies were conducted to evaluate the dose dependency of the gene pattern that is responsive to EE, genistein, and bisphenol A (BPA) during fetal development of the rat testis and epididymis (Naciff *et al*., 2002a, 2005). These studies demonstrated that gene expression profile of target tissues was modified in a dose-responsive manner, even when morphological changes were not observed, and that 50 genes were expressed in a common direction across the three chemicals, pointing to cellular pathways that are affected by exposure to estrogenic compounds (Naciff *et al*., 2005).

In the Daston laboratory, the current microarrays include transcript evaluation of almost 9000 genes. Their results indicate that a number of genes (from dozens to hundreds) are changed in a reproducible, dose-related manner in response to estrogens. They have then identified many of these responsive genes, begun to assign potential roles of selected genes on processes of proliferation and differentiation, and to suggest plausible relationships among these genes in eliciting subsequent responses at the tissue or organ level. They have also suggested the utility of and performed gene expression experiments in determining the shape of the dose-response curve at low doses. Daston and Naciff (2005) interpreted their data to mean that dose-response pattern for gene expression in the juvenile rat uterus is monotonic down to doses "a few orders of magnitude" below the no observable effect level (NOEL) for the uterotrophic response (*i.e.*, gene changes but no subsequent morphological/biochemical response), suggesting that gene expression (and by inference higher order responses) does follow patterns that are predictable, based on responses at higher doses, and that a transcript profile characteristic of chemicals with estrogenic activity (Naciff and Daston, 2004) may replace other *in vitro* studies and ultimately replace *in vivo* studies (*e.g.*, developmental or reproductive toxicity studies). Their work is critical for the following two reasons:

- Although Daston's work is predominantly in females and looks at uterine response at various developmental stages (but see Naciff *et al*., 2005 for information on the testis), the same approach is being used to identify changes in gene expression in male rat reproductive systems at various developmental stages, to identify early events (markers) and gene changes from doses below those which result in biochemical and/or morphological changes in male reproductive organs. Huang *et al*. (2004) have recently reviewed the estrogenic regulation of signalling pathways and homeobox genes during rat prostate development.

- This approach can be (and is being) very useful in determining the gene effects and dose response for those effects below the morphological response, to inform the current public, scientific, and regulatory concerns about the presence, nature, and slope of low-dose effects (*e.g.*, Calabrese's publications on hormesis and the dose-response curve, and Dr. vom Saal's data on non-monotonic, dose-response patterns and very low dose effects, etc.).

Berquin *et al*. (2005) examined gene expression patterns in the normal, sexually mature mouse prostate from two normal mouse strains and a prostate-specific knock-out mouse strain. Genome-wide mRNA expression was measured by DNA microarrays from both the epithelium and stroma from the ventral, dorsolateral, and anterior prostate lobes. Their analysis indicated that the gene expression pattern in the mouse dorsolateral lobe was closest to that of the human prostate peripheral zone, providing support for the hypothesis that these prostate compartments are functionally equivalent. Stroma from a given lobe had more similar gene expression patterns with stroma from other lobes than with epithelium from the same lobe. Stroma appeared to have higher expression complexity than epithelium (*i.e.*, stromal cells had higher expression levels of genes involved in cell adhesion, muscle development and contraction, and in components such as sarcomere and extracellular matrix collagen). Epithelial cells had higher expression levels of genes for secretory proteins, including seminal vesicle secretion proteins 2 and 5. Surprisingly, stroma cells expressed genes for many osteogenic molecules which may predispose prostate cells for survival in bone. Osteogenic chemokine Cxl12, but not its receptor, Cxcr4, was expressed in normal prostate. In prostate tumors, Cxl12 expression was up regulated in epithelial cells with concomitant expression of its receptor, Cxcr4. The authors suggest that expression of both the receptor and ligand in the tumor may provide an autocrine mechanism for tumor-cell migration and invasion. The expression of these osteogenic ligands and receptor molecules may determine the preferential metastasis of prostate cancer cells to bone (Berquin *et al*., 2005).

Recently developed genomics technology has the potential to improve our understanding of an organism's response to stressors, including endocrine-active chemicals (U.S. EPA, 2004). Yamada *et al*. (2005) linked gene expression profiling to the Hershberger assay evaluation of benomyl, a fungicide that has been shown to adversely affect male reproduction in rats. Yamada *et al*. (2005) included *in vitro* reporter gene assays, as well as the *in vivo* Hershberger assay with enhancements, since the mechanisms of action of benomyl's reproductive effects do not appear to involve the endocrine system. The authors performed microarray analysis of the gene expression profile in the ventral prostate in the Hershberger assay, from castrated rats treated with

benomyl (1000 mg/kg/day, po) and TP (0.2 mg/kg/day, sc), and from rats treated with FLU (10 mg/kg/day, po) or with p,p'-DDE (100 mg/kg/day, po) after one, four, or ten days of treatment. p,p'-DDE is considered a weak anti-androgen and FLU a potent anti-androgen, both *in vitro* and *in vivo* (both are AR antagonists). The authors reported the number of probes with different expressions (as both total and those with more than two-fold difference for each chemical and each time point). After four and ten days of treatment, a large number of identified genes exhibited similar up regulation and down regulation from exposure to either p,p'-DDE or FLU, but not from benomyl. Genes changed in common by both p,p'-DDE and FLU numbered 100 (after four days) and 39 (after ten days) for up regulation and 99 (four days) and 28 (ten days for down regulation. Of these genes, only 1-15% were also altered by benomyl treatment. Benomyl does not affect serum androgen levels in intact rats and is considered a testicular toxicant. The authors therefore suggested that benomyl does not interfere with androgen synthesis and that it may act by interfering with the assembly and disassembly of microtubules in the spindle fibers during mitosis and meiosis in spermatogenesis. The authors concluded that genomics analysis may allow for the development of rapid (effects within four days of treatment), sensitive, and cost-effective methods for screening endocrine-active chemicals.

Proteomics

Although gene expression profiling has demonstrated utility in providing mechanistic insight into cellular mechanisms of drug and chemical-induced effects, proteomics provides advantages in areas not directly obtainable through gene studies. For example, proteins and post-translational modifications are known to be directly involved in the generation of congenital malformations (Barrier *et al*., 2005), activities that cannot be assessed or identified through DNA sequencing or measurement of mRNA levels. In the last five years, the application of proteomics in toxicology studies has rapidly grown (Wetmore and Merrick, 2004), as well as the field of toxicogenomics that involves the integration of conventional toxicological examinations with gene, protein, or metabolite expression profiles (Stierum *et al*., 2005). Papers on proteomic applications in reproductive and developmental toxicology have rapidly increased in the literature in recent years (*e.g.*, Barrier and Mirkes, 2005; Chu *et al*., 2006; Stein *et al*., 2006; Lalancette *et al*., 2006; Tsangaris *et al*., 2006; Roth *et al*., 2006), reproductive toxicology studies that incorporate metabolomic analysis are not readily found.

Proteomics involves collection of complex protein mixtures and identification of the proteins within these mixtures. For example, proteomic analysis of amniotic fluid in pregnancies with and without Down's syndrome can identify proteins unique to or differentially expressed in Down's syndrome fetuses. These identified proteins can then be used as potential markers for prenatal diagnosis (Tsangaris *et al*., 2006). Another study (Chu *et al*., 2006) collected chromatin from meiotic cell types undergoing spermatogenesis in *C. elegans* to identify evolutionarily conserved fertility factors (proteins). They isolated the sperm proteins based on abundance while eliminating general chromatin proteins. They started with 1099 proteins co-purified with spermatogenic chromatin and ended up with 132 proteins for functional analysis. Their studies identified conserved spermatogenesis-specific proteins vital for DNA compaction,

chromosome segregation, and fertility. Unexpected roles in spermatogenesis were also detected for factors initially identified as involved in other processes. To evaluate evolutionary conservation of these fertility factors from *C. elegans* to mammals, they created mouse gene knockouts corresponding to the nematode proteins; 37% (7 of 19) of these knockouts caused male sterility in the mice. The authors concluded that their list provides significant opportunity to identify causes of male infertility and targets for male contraceptives (Chu *et al*., 2006). Chengalvala *et al*. (2006) have also identified several factors (proteins) that are functionally critical for the regulation of mammalian female fertility. Stein *et al*. (2006) subjected proteins on the sperm surface and within the acrosome to proteomics analysis to generate a proteome of the sperm head subcellular compartments which interact with oocytes. Of the proteins identified, for which a gene knockout had been tested, one third were shown to be essential for reproduction *in vivo*. They identified over 100 proteins expressed in mature sperm at the site of the sperm-oocyte interactions, with 25% of these proteins previously uncharacterized (Stein *et al*., 2006).

Proteomic profiling has also been used to identify the proteins up-/down-regulated in the epididymides and vas deferens during *in utero* rat genital tract development (Umar *et al*., 2003). AR expression in the developing male genital tract occurs in a strict temporal and cranial to caudal sequence. Mesenchymal AR is expressed in the rat genital tract as early as embryonic day 14 (E14; GD 14), while epithelial AR expression in the epididymis starts at E18 in the caput (head) epididymis and after birth in the cauda (tail) epididymis and vas deferens. The epididymis and vas deferens are dependent on androgens for their growth and differentiation. Umar *et al*. (2003) therefore generated protein expression profiles (using two-dimensional gels) from the fetal rat epididymides and vas deferens on E17-E21. Proteins differentially expressed between E17 and E21 were cut from the gels, digested into tryptic peptides, and analyzed on a matrix-assisted laser, desorption/ionization time-of-flight mass spectrometer. Twenty proteins were identified that were regulated over time. They were categorized into cytoskeletal proteins, nuclear proteins, transport proteins, chaperone proteins, and enzymes (mainly glycolytic). When epididymides and vasa deferentia, isolated on E19, were cultured *in vitro* ± R1881 (a synthetic androgen; at 10 nM) for 9, 24, or 48 hours, regulation and post-translational modifications were observed for glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase, heterogeneous nuclear ribonucleoprotein A2/B, and heterogeneous nuclear ribonucleoprotein A3, similar to the observed changes *in vivo*. The authors concluded that androgen-induced post-translational modification of glycolytic enzymes may be an important functional link between signaling pathways and cytoskeletal rearrangements in the control of growth and differentiation of the male internal genital tract. These same links may be critical to the regrowth of the regressed accessory sex organs in castrated males in the presence of androgens (*e.g.*, in the Hershberger assay).

Although these studies are not directly relevant to enhancing/replacing the Hershberger assay, the proteomics methodology is very important. It can identify critical proteins related to subsequent morphological and functional activities and effects. It can measure the relative dynamic protein turnover in a cell, and it can develop sensitive biological fluid- and tissue-based proteomics that are predictive of a sensitive/vulnerable

subpopulation and of the onset and progression (amelioration or cure) of adverse effects. Proteomics can also provide mechanistic detail to *in vitro* or *in vivo* assays (*e.g.*, the Hershberger assay) to identify the protein(s) essential to the processes and to hormonally mediated (or other mediated) effects.

Metabolomics

Metabolomics involves the study of the low molecular weight endogenous complement in cells, tissues, and biological fluids. The basis for this approach is that when an environmental chemical or drug interacts at the gene or protein level, perturbations in endogenous metabolomic profiles are expected to occur and to correlate with phenotypic anchors (*e.g.*, hormone levels, tissue pathology, blood pressure) in order to provide examples of phenotypic anchors.

NMR and chromotagraphy coupled mass spectrometry methods are the most frequently applied tools for metabolomics studies. Metabolomics has been applied to the analysis of urine, serum, plasma, seman, milk, and a variety of tissue extracts. These studies have demonstrated the utility of metabolomics (or metabolite profiling) in differentiating metabolomics profiles of urine based on gender, age, and ethnicity (Stanley *et al*., 2005; Plumb *et al*., 2005). Additional studies (Odunsi *et al*., 2005) have demonstrated that metabolomics can be used to differentiate serum obtained from pre- and postmenopausal women, as well as to differentiate patients based on the stage of ovarian cancer. Earlier studies have demonstrated the use of NMR analysis of seminal fluid (Tomlins *et al*., 1998) to investigate the changes in biochemical composition of whole human seminal fluid and an artificial mixture of prostatic and seminal vesicle fluid. Lynch and Nicholson (1997) demonstrated the use of NMR to differentiate prostatic fluid samples from healthy subjects and subjects with either benign prostatic enlargement or prostatic cancer. A more recent study (Rantalaenen *et al*., 2006) showed the power of combining proteomic and metabolomic approaches in the study of prostate cancer. In this investigation, using a mouse model for prostate cancer, multiple correlations between metabolites and proteins were found, including associations between serotransferrin precursor, tyrosine, and 3-D-hydroxybutyrate and an increased presence of gelsolin. Rudolph and co-workers (Rudolph *et al*., 2006) have recently demonstrated the use of metabolomics in the study of lactation. In this study, mammary glands from lactating mice were evaluted at day 17 of pregnancy and day 2 of lactation, indicating a significant change in carbohydrate and amino acid metabolism, with an increase in osmolytes in the lactating gland. A recent study was also conducted that showed the differentiation of plasma samples from women who developed preeclampsia during pregnancy from women who did not have pregnancy complications (Kenny *et al*., 2005). In addition, metabolomics have been used in a number of dose-response studies conducted in animal models to investigate target organ toxicity from compounds that are known reproductive or developmental toxicants, including, for example, effects of methyl mercury of cadmium chloride to the kidney or testis.

Many metabolomic studies conducted to date have demonstrated the use of the technology to differentiate or classify samples based on patterns of endogenous metabolites. The significance of these findings is developed through the assignment of metabolites and interpretation through mapping metabolites to biochemical pathways.

Involvement of Other Genes and Ligands in the Response of the Castrated Male Rat

It has been shown that inhibitors of prostaglandin synthesis suppress the proliferative effect of TSH on the thyroid and of estradiol on the pituitary, suggesting possible involvement of prostaglandins in the hormonal control of cell proliferation. Testosterone and DHT are the mitogenic factors for the male accessory sex glands, so Lysón and Pawlikowski (1983) investigated whether indomethacin or aspirin (both inhibitors of prostaglandin synthesis) would affect the proliferative response of rat seminal vesicles and prostate to T or DHT. Using both SD and Wistar rats, castrated on PND 20 and injected sc beginning ten days later (with ip injection of colchicine 120 minutes before decapitation to arrest mitoses in metaphase), they (Lysón and Pawlikowski, 1983) showed that the proliferative reaction of the seminal vesicles to T (0.25 mg/rat) was suppressed by indomethacin (1.25 mg/kg) and aspirin (20 mg/kg), with indomethacin being more potent than aspirin. In the ventral prostate, neither of the prostaglandin inhibitors affected the mitogenic response to test strain. DHT-induced proliferation was not inhibited by indomethacin in either the seminal vesicles or the ventral prostate. The authors suggest the existence of different mechanisms of proliferation reactions in the seminal vesicles versus the ventral prostate to T. They also suggest an involvement of prostaglandins in the mitogenic response of T in the seminal vesicles rather than a non-specific property of the drugs tested. Androgens are known to increase the concentrations of prostaglandin F in the seminal vesicles, the epididymides, and the ductus deferens of the rat, but they do not affect the concentration of prostaglandin F in the prostate (the prostate does not contain the androgen-dependent phospholipase A, an enzyme involved in the first step of prostaglandin synthesis). Testosterone and DHT are mitogens for both the seminal vesicles and the prostate, whereas estrogens exert a significant mitogenic effect on the seminal vesicles, with the effect on the ventral prostate very weak. Testosterone is rapidly converted to DHT (and androstanediol) by 5α-reductase in male accessory sex organs, with the enzyme levels in the prostate much higher than in the seminal vesicles. The rate of conversion from T to DHT is inversely proportional to the mitotic response to T in different rat accessory sex organs, which may explain the specificity of response in these two sex accessory organs.

Lee *et al*. (1999) have evaluated the possibility that the protooncogene, c-myc, plays a role in the rat ventral prostate after castration, by examining the levels of mRNA from c-myc, as well as the androgen-regulated genes Cl and TRPM-2 by Northern blot hybridization. Cl is a subunit for prostate binding protein, and T-repressed protein modulator-2 is a marker for prostate involution after androgen withdrawal. They identified two stages of induction (early and late). Levels of c-myc mRNA in the ventral prostate reached maximum levels at 6 and 48 hours postcastration. The level of Cl mRNA did not change, and TRPM-2 mRNA was not detected in the early phase. The induction of c-myc mRNA was increased in the ventral prostate by cycloheximide (a

protein synthesis inhibitor) but was diminished by actinomycin D (a DNA-dependent RNA transcriptional inhibitor). Administration of androgen at the time of castration prevented the early induction of c-myc mRNA. It is clear that c-myc is differentially regulated in the ventral prostate after castration, and its induction does not require protein synthesis.

Rat $\alpha_{2\mu}$-globulin is a male rat-specific protein appearing in the kidney, serum, and urine, and it is known to be induced by androgens (it is not observed in female rats, male or female mice, or in humans). Takeyoshi *et al*. (2005) investigated the usefulness of measuring $\alpha_{2\mu}$-globulin in the serum of rats as a marker for androgenic activity of chemicals tested in the Hershberger assay. CD male rats were castrated at six weeks of age, given a two-week recovery period, and then administered TP (a common reference androgen) by sc injection for ten consecutive days at 0, 20, 100, or 500 μg/kg/day. At scheduled necropsy, the ventral prostate, LABC, glans penis, and Cowper's gland were collected and weighed. All of the androgen-dependent organ weights were significantly increased (versus the castrated males with no TP) at 100 and 500 μg/kg/day (with no effects at 20 μg/kg/day), while the serum $\alpha_{2\mu}$-globulin level was significantly increased only at 500 μg/kg/day. The authors concluded that the serum $\alpha_{2\mu}$-globulin level may be a useful biomarker for detecting androgenic activity, but it is less sensitive than the weights of the androgen-sensitive organs in the Hershberger assay.

A number of laboratories are working on the structural requirements of a molecule to bind to the AR in order to increase information on structure activity relationships (SARs), on the influence (if any) of the molecular size and shape on the interaction of the molecule with the AR, and on the nature of the interaction, agonist, or antagonist. A second aim is to provide information to more efficiently design nonsteroidal anti-androgen pharmaceuticals. In a very sophisticated approach, Waller *et al*. (1996) used three-dimensional quantitative SARs (*i.e.*, 3DQSARs) to look at the interactions between the AR and its ligands.

Tamura and collaborators (Tamura *et al*., 2001, 2003) have identified several environmental chemicals capable of binding to the AR. These chemicals interfere with normal AR function (they are antagonists). These chemicals are nonsteroidal, environmental organophosphate pesticides and related compounds (such as fenitrothion and its analogs). The demonstration of their interaction with the AR has heightened public, scientific, and regulatory concern about possible adverse endocrine effects across a broad spectrum of environmental chemicals. Hirosumi *et al*. (1995a,b) have also identified a novel nonsteroidal inhibitor (FK143) of steroid 5α-reductase (which converts T to DHT), with *in vitro* effects on human and prostatic enzymes (Hirosumi *et al*., 1995a) and *in vivo* effects on rat and dog prostates (Hirosumi *et al*., 1995b). di Salle *et al*. (1998) reported on another novel dual type I and II 5α-reductase inhibitor, designated PNU 157706. They (di Salle *et al*., 1995) demonstrated that 5α-reductase inhibitors (so T is not converted to DHT) severely reduced the levels of prostatic androgens. Kelce *et al*. (1995) first reported that the persistent DDT metabolite, p,p'-DDE is, in fact, a potent AR antagonist, yet another nonsteroidal AR ligand. Other new nonsteroidal anti-androgens can act to reduce androgens by different mechanisms at the level of steroid biosynthesis

or at the level of the hypothalamus. Ideyama *et al*. (1998) reported that a novel nonsteroidal inhibitor of cytochrome $P450_{17\alpha}$ (17α-hydroxylase/C17-20 lyase), designated YM1116, decreased prostatic weight by reducing serum concentrations of T and adrenal androgens in rats. Lamberts *et al*. (1988) administered a depot formulation of an analog of gonadotropin-releasing hormone (GnRH; found in the hypothalamus) and then co-administered ketoconazole, cyprotenone acetate, or RU23908 to rats, and they reported prostatic weight regression from these agents (in the presence of GnRH which stimulates FSH and LH to increase production of T).

Indications Relative to Current Endpoints and Accessory Sex Organ Weights

Two possible major confounders to the accessory sex organ weights (the primary endpoint in the Hershberger assay) are: (1) reduced terminal body weights from toxicity of the test chemical (or from feed restriction; Marty *et al*., 2003), which may confound effects or lack there of on the accessory sex organ weights, and/or (2) natural variability and the influence of the concurrent control values on the detection and interpretation of low-dose or weak endocrine toxicities (Ashby *et al*., 2004).

The Hershberger assay and the male EDSP intact pubertal onset assay rely on changes in reproductive and/or accessory sex organ and systemic organ weights in young animals. Exposure to test chemicals may affect growth rate (*i.e.*, systemic toxicity) which may in turn affect organ weights. Therefore, Marty *et al*. (2003) examined the relationship between body weights and accessory sex organ weights using feed restriction to produce a target 10% decrease in body weight. In the male intact pubertal onset assay, 23-day-old rats (12/group) were fed *ad libitum* or with feed restriction until euthanized at 45, 49, 52, 56, or 59 days of age. There was a 10% body weight differential between the *ad libitum* and feed restriction groups, but acquisition of puberty was not affected and testis weights were conserved. Absolute weights of the prostate, ventral prostate, seminal vesicles, epididymides, and liver were decreased in the feed restriction group. Relative weights of the prostate, ventral prostate, and seminal vesicles were similar to *ad libitum* controls, but relative weights of the epididymides and liver still exhibited differences from the *ad libitum* group. In the Hershberger assay, male rats (12/group) were castrated at 36 days of age, with a ten-day recovery period, and given *ad libitum* feed or restricted feed in the presence of or absence of TP (the reference androgen) from 46-55, 50-59, or 56-65 days of age. At 56, 60, or 66 days of age, the rats were euthanized and organs weighed. In untreated animals (castrated, no TP), feed restriction did not alter the absolute weights of the ventral prostate, seminal vesicles, or Cowper's gland. However, absolute and relative weights of the LABC were affected. In the TP-treated castrated animals, absolute organ weights (ventral prostate, seminal vesicles, LABC, and glans penis) were relatively insensitive to feed restriction. The weight of the Cowper's gland was affected by feed restriction only at 66 days of age.

In summary, reproductive and accessory sex organ weight endpoints in the Hershberger (and male pubertal onset) assay can be influenced by feed restriction levels that result in a 10% decrease in terminal body weight. The authors concluded that: (1) the establishment of objective criteria for a positive or negative result is problematic due to the confounding effect of body weight on some endpoints; (2) a 10% decrease in body

weight may be excessive as a requirement for high-dose toxicity in these assays due to the risk of false positives from toxic agents which are not endocrine active, and/or the risk of false negatives from agents that cause endocrine-active effects coincident with body weight changes; and (3) caution must be used in interpreting results in the presence of a 10% body weight depression due to the possible confounding effects on accessory sex organ weight from this degree of growth suppression (Marty *et al*., 2003).

John Ashby's laboratory at Syngenta CTL (Central Toxicology Laboratory) investigated unanticipated and nonreproducible effects in the Hershberger and uterotrophic assay as follows. While they were defining the no-effect-level for the 5α-reductase inhibitor, finasteride, in the rat Hershberger assay, they encountered an inverted-U, low dose pattern, with increased weight ("trophic effect") of the prostate gland. Two attempts to confirm this observation were unsuccessful, and they concluded that the positive effect initially observed was associated with the normal biological variability of the prostate gland weight (Ashby *et al*., 2004). They also attempted, unsuccessfully, to repeat their observation of weak uterotrophic activity in the rat for the sunscreen 3-(4-methylbenzylidene) camphor (4 MBC). They concluded that 4 MBC is uterotrophic only when the control uterine weights were at the low end of their normally encountered range. These two nonreproducible studies led to their re-evaluation of the earlier uterotrophic assay data for bisphenol A (BPA). They had originally concluded that BPA resulted in irreproducible evidence of weak uterotrophic activity. When they examined the weight studies they performed, ordered by the control uterine weights, they confirmed reproducible, weak uterotrophic activity of BPA only when the control uteri were at the low end of their normal weight range. Ashby *et al*. (2004) then also reanalyzed data from various laboratories reporting weak or low-dose endocrine effects (which were not confirmed in other laboratories), including effects of BPA on the CF1 mouse prostate, the activities of BPA, octylphenol, and nonylphenol on the rat testis, the effect of BPA on daily sperm production in the rat, and the effect of polycarbonate (a polymer of BPA) caging on control mouse uterine weight. In all of these cases, variability among control values was a major confounder and obstacle to data interpretation and confirmation. These failures to confirm endocrine toxicities between laboratories ostensibly using the same test protocol, are distinctly different from endocrine-mediated toxicities specific to the conditions of the test, such as strain (*e.g.*, F344, SD, Wistar, Long-Evans in rats; CD-1, C57BL6 in mice) or species (rat or mouse) specific susceptibilities/resistance, endpoints, etc.

To resolve the differences observed among laboratories using the same chemical(s) and test protocol and to avoid them in the future, Ashby *et al*. (2004) recommended:

- For the endpoints studied, it is useful to have a historical database with which to compare current assay performance and test results. Inclusion of this database in publications would aid resolution of these irreproducible results.

- Most laboratories experience variability in the endpoints measured. Understanding the origin of these variations is a necessary precursor to concluding that a test agent does or does not have weak endocrine activity.

- When low-dose or weak endocrine effects are apparently present, it is important to confirm the observations before publication.

- Unadjusted data from separate experiments should be presented as opposed to pooled or adjusted (relative weights, percent of control, etc.) data. Adjusted data are subsidiary to the original data.

- A distinction should be drawn between effects that lie within recent historical control ranges and those that exceed those ranges. Different mechanisms may operate in these two situations (*e.g.*, direct action on the target organ versus a centrally mediated effect), leading to different approaches to data "extrapolation."

- Use of appropriate statistical methods enables objective, qualitative (as well as quantitative) judgements to be made. Subjective discussion of statistically nonsignificant effects should be avoided" (Ashby *et al*., 2004; p. 852).

In addition to the confounders of reduced body weights, the impact of natural variability, and the influence of control values, is the level of phytoestrogens, even in the Hershberger assay, which evaluates androgens and anti-androgens (rather than estrogens). Natural compounds with estrogenic properties are present in many plants (phytoestrogens), including a number of food crops: soybean and derivative products, peas, alfalfa, and beans. Some rodent diets contain soybean meal (a major source of daidzein and genistein), two well-known estrogenic isoflavones**.** These estrogenic isoflavones are present as natural ingredients of rodent diets at concentrations which could have marked effects on the results of studies evaluating the sexual hormonal activity of various test chemicals. Stroheker *et al*. (2003) therefore evaluated the effects of three rodent diets differing in soybean meal content (L5, a semi-synthetic phytoestrogen-free diet; D04 containing 8.5% weight/weight soybean meal and yeast; and D03 containing 22.5% weight/weight of soybean meal and yeast) on the response of the seminal vesicles, prostate, and bulbocavernosus/levator ani (BC/LA) muscle to androgenic and anti-androgenic chemicals in the Hershberger assay. They determined the effects of dietary soy isoflavones after ten days of exposure and in animals fed L5 or D03 diets throughout their lifetime (including the period of treatment with the androgenic or anti-androgenic chemicals). The rats were Wistar, castrated on PND 21 with one week of recovery. TP was the reference androgen, and vinclozolin was the reference anti-androgen. Exposure to TP (sc injection; 0.1, 0.2, 0.4, or 0.8 mg/kg body weight/day) or vinclozolin (gavage; 25, 50, or 100 mg/kg/day) was administered once daily for ten consecutive days. After ten days of exposure, there were no effects of diet, per se, on the accessory sex organs of the male rats. Diet did affect the androgenic response to TP. TP caused significant dose-dependent increases in the relative weights of the seminal vesicles ($\geq$ 0.2 mg/kg/day), BC/LA, and prostate ($\geq$ 0.1 mg/kg/day). The effects on the seminal vesicles and prostate were significantly lower in rats fed D03 than in rats fed L5. Di*et al*so affected the anti-androgenic response to vinclozolin (plus TP). Vinclozolin caused significant, dose-dependent decreases in the relative weights of the prostate, seminal vesicles, and BC/LA at vinclozolin $\geq$ 25 mg/kg/day (and TP, 0.4 mg/kg/day).

Diet had no effect on the responses of the seminal vesicles or prostate, but the BC/LA weights were significantly greater with vinclozolin plus TP in animals fed D03 versus those fed L5 (or the weights were significantly lower with vinclozolin plus TP in animals fed L5 versus those fed D03). The authors concluded that rodent diets containing phytoestrogens, such as daidzein and genistein, can alter the results of androgen/anti-androgen assessments. They recommended using a standardized, open formula diet devoid of phytoestrogens, such as L5, in the Hershberger assay (Stroheker *et al*., 2003).

Another concern is collecting only the organ weights of interest, which is viewed as providing little or no information on specificity of the effects. Nishino *et al*. (2006) state that although the Hershberger assay is a valid quantitative method for evaluating androgenic or anti-androgenic properties of substances by measuring the weights of seminal vesicles and prostate, the findings obtained by this assay provide only limited information on the specificity of the observed effects when only the differences in organ weights are evaluated. For example, the growth of the seminal vesicles can be stimulated not only by androgens but also by estrogens, termed a paradoxical effects of estrogens (Nishino *et al*., 2006). Therefore, Nishino *et al*. (2006) evaluated BPA (a weak xenoestrogen with conflicting studies) in the Hershberger assay over a very broad range of doses ("ultralow," "pharmacological," to "suprapharmacological"), with additional androgen-sensitive parameters in the prostate and seminal vesicles. These included immunohistochemistry (to detect ARs and proliferation markers detected by densitometry) and morphometry (epithelial height and luminal areas of the glandular ducts). The authors concluded that morphologic and functional analysis of cellular parameters in male accessory sex organs may allow a more "subtle" and reliable assessment of the (anti-) androgenicity of substances. Although they conclude "that BPA does not exert major androgenic effects" (Nishino *et al*., 2006, p. 155; *i.e.*, there were no effects on absolute weights of the prostate or seminal vesicles, and the increased relative weights were likely due to reduced body weights), they did report that the staining intensity for AR immunoreactivity was increased at low (but not at higher) doses of BPA versus the castrated controls, and that epithelial height and glandular luminal areas were increased at low BPA doses and decreased at higher BPA doses. As such, they state (p. 162) that, based on their data, "the densitometric analysis of AR immunoreactivity and the assessment of both cell morphology and cell proliferation proved to be independent and sensitive parameters for the evaluation of androgen effects on prostates and seminal vesicles," and that "the combined application of these parameters may provide an additional tool to test the broad spectrum of endocrine-active substances…"

Another concern with the Hershberger assay is whether it really is specific (*i.e.*, does it only detect AR-mediated responses, and how can it be enhanced to detect and define other androgen-mediated activities?). To address this point, Yamada *et al*. (2005) therefore examined benomyl and its metabolite, carbendazim, in the *in vivo* Hershberger (and uterotrophic) assay and in *in vitro* reporter gene assays for AR (and hERα). Benomyl is a benzimidazole fungicide whose activity is based on the ability to interfere with the assembly of fungal microtubules. Both benomyl and carbendazim have been shown to adversely affect male reproduction in rats, but the mechanisms of action do not appear to involve the endocrine system. In this study (Yamada *et al*., 2006), neither benomyl nor carbendazim caused androgen or estrogen agonist or antagonist activity in

the *in vitro* transfected Hela reporter gene assays for AR or ER at 0, 10, 100, and 500 nM and 1, 5, and 10 μM; and carbendazim at 0, 100, 100, 500 nM and 1, 5, and 10 μM. The Hershberger assay used Crj:CD(SD) IGS rats (six/group), castrated, with a seven-day recovery and a ten-day dosing period of benomyl by oral gavage at 0, 100, 300, and 1000 mg/day. The reference androgen was methyl T for androgenicity at 100 mg/kg/day, po, and TP by sc injection. In this assay, there was no evidence of agonist effects. Benomyl at the highest doses (with 0.2 mg/kg/day TP) caused decreased weights of some androgen-related organs (ventral prostate, seminal vesicles, and LABC) but not others (dorsolateral prostate, glans penis, or Cowper's glands). At 0.4 mg/kg/day TP, the effects of benomyl were less but still present. Benomyl had no effects on serum androgen levels. Microarray analysis of the gene expression profile in the ventral prostate, with TP (0.2 mg/kg/day) injected into castrated rats treated with benomyl (1000 mg/kg), indicated clear differences from the patterns observed with p,p'-DDE (100 mg/kg/day, po; a weak anti-androgen *in vitro* and *in vivo*) and FLU (10 mg/kg/day, po; a potent antiandrogen *in vitro* and *in vivo*) after one, four, and ten days of exposure. They reported the number of probes (genes) with differences (both total and those with more than two-fold difference) for each chemical at each time. There were changes over time within chemicals and among chemicals at a given time and over time. After four and ten days of treatment, gene activity (which changed in common with p,p'-DDE and FLU) included 110 (four days) and 39 (ten days) up regulated and 99 (four days) and 28 (ten days) down regulated genes. The numbers and identities of gene changes were similar for both p,p'-DDE and FLU (both AR antagonists) but not for benomyl. Since benomyl also does not affect serum androgen levels in intact rats, the authors concluded that benomyl does not interfere with androgen synthesis, although it is a testicular toxin. The authors suggested that benomyl may act by interfering with the assembly and disassembly of microtubules during the mitosis and meiosis steps in spermatogenesis in the testis. The authors also concluded that genomics analysis may allow the development of rapid, sensitive, and cost-effective methods for screening endocrine-active chemicals.

CHAPTER 10. DISCUSSION AND CONCLUSIONS

Discussion

There has been an increasing concern regarding the potential adverse effects of various environmental contaminants. Colburn *et al.* (1993) hypothesized that prenatal and postnatal exposure to endocrine disrupting chemicals could result in damage to wildlife and humans. Since then, many incidences of decreased reproduction in wildlife have been found. These include morphologic abnormalities, egg shell thinning, population declines, sex reversal, impaired offspring viability, altered hormone levels, and changes in socio-sexual behavior (Fox, 2001).

Assays that are capable of identifying the ability of chemicals to act as EDCs have been suggested by OECD and EDSTAC and are now being standardized and validated by OECD and EPA EDSP. Validation can be defined as the determination of the reliability and relevance of a test for a specific purpose (Clode, 2006). Although *in vitro* assays can identify substances with endocrine altering modes of action, the pharmacodynamics and pharmacokinetics of endocrine metabolism indicate the need for *in vivo* assays in the overall assessment of possible EDC. Hershberger *et al.* (1953) analyzed the response of the ventral prostate, seminal vesicles and coagulating glands, and the levator ani without the bulbocavernosus muscle to a number of active chemicals, including estrogens and progesterones. In the 1970s and 1980s, the discovery of the AR and the first compounds that acted as antagonists of the receptor, such as cyptotone acetate, was followed by modification of the assay to address antagonistic activity. A fixed dose of a reference agonist was administered to several groups of animals that also received a set of doses of the purported antagonist. The reliability of studies can be confirmed by showing that the weights of the accessory sex organs of rats given TP are increased over those of rats given the vehicle alone, and the organ weights of rats receiving flutamide plus TP are lower than those given TP alone.

The Hershberger and uterotrophic assays are part of the Tier 1 screening battery recommended by the EDSTAC. These assays are the most widely used short-term, *in vivo* assays designed to screen for chemicals that may be endocrine disruptors. **Table 8** gives a comparison of the two assays.

Table 8. Comparison of uterotrophic assay versus the Hershberger assay

Parameter	Uterotrophic Assay	Hershberger Assay
Current versions	Intact, sexually immature female Ovariectomized, sexually mature female	Intact, sexually immature male Castrated, sexually immature male Castrated, sexually mature male
Evaluation species	Rat, mouse	Rat, mouse
Initial developer(s)	Fellner (1913); Allen and Doisy (1923, 1924); Bülbring and Bern (1935)	Korenchevsky (1932); Eisenberg and Gordan (1950); Hershberger *et al*. (1953)
Endogenous sex steroid	17β-estradiol (E2)	Testosterone (T) and dihydrotestosterone (DHT) from T by 5α-reductase
Site of synthesis	Thecal and granulosa cells of the follicle in the ovaries	Interstitial cells of Leydig in the testes
Receptor	Estrogen receptor α and β (ER)	Androgen receptor α, β, γ (AR)
Response organs	Weight of uterus usually without luminal fluid (weighed wet and/or dry) Absolute weight (mg or g) or relative weight (mg or g per 100 g or kg terminal body weight)	Accessory sex organs: Weight of intact prostate (now usually ventral and dorsolateral prostate lobe weights separately) Paired seminal vesicles and coagulating glands; paired Cowper's (bulbourethral) glands; glans penis; levator ani bulbocavernosus (LABC) muscle Absolute weight or relative to terminal body weight
Procedure	A. Intact immature female Prior to puberty (for mouse, begin on PND 21/22; for rat, begin on PND 25), daily administrations of test chemical sc, po, iv, ip, diet, etc.) for 3-5 consecutive days. Necropsy 24 hours after last dose and weigh the uterus. Group sizes vary by author.	A. Intact immature male Prior to puberty (for mouse, begin on PND 24-25; for rat, begin on PND 32-38), daily administration of test chemical (sc, po, iv, ip, diet, etc.) for 5-7 days. Necropsy 24 hours after last dose and weigh the specific accessory sex organs.

Table 8 (continued)

Parameter	Uterotrophic Assay	Hershberger Assay
Procedure	B. Ovariectomized immature female (OECD method of choice; OECD series on Testing Assessment No. 38, 2003) Prior to puberty, ovariectomize females (no need to wait before administration of test material). Administer test material for 3-5 consecutive days, necropsy at last dose, and weigh the uterus.	B. Castrated immature male (OECD method of choice; Owens *et al*., 2006) Prior to puberty, castrate males (for OECD validation [Owens *et al*. 2006], castrate the males on PND 42 since castration prior to PND 40 prevents complete PPS). Wait 12 days for T/DHT-dependent tissues to regress. Then, administer test material for 10 consecutive days. Necropsy 24 hours after last dose and weigh the specific accessory sex organs.
	C. Ovariectomized, mature female Postpubertal adult female is ovariectomized at >60 days old. Wait 0-14 days post-ovariectomy for E2-dependent tissues to regress. Then, administer test material for 3-5 consecutive days. Necropsy 24 hours after last dose and weigh the uterus.	C. Castrated, mature male Postpubertal adult male (>70 days old) is castrated. Wait 14-28 days post-castration for T/DHT-dependent tissues to regress. Then, administer test material for 10 consecutive days. Necropsy 24 hours after last dose and weigh the specific accessory sex organs.

Table 8 (continued)

Parameter	Uterotrophic Assay	Hershberger Assay
To detect	Estrogenic activity: Compare the weight of the uterus after ovariectomy or prepuberty (with no treatment) to the weight of the uterus after repeated administration of test material If the uteri from treated females are significantly heavier than the uteri from untreated ovariectomized or prepubertal females, then the test material is estrogenic	Androgenic activity: Compare the weights of the sex accessory organs after castration or prepuberty (with no treatment) to the weights of the specified organs after repeat administration of test material If the weights of the specified organs from treated males are significantly higher than the specified organ weights from the untreated castrated or prepubertal males, then the test material is androgenic
To detect	Anti-estrogenic activity: Same as above except dose the prepubertal or ovariectomized female with a potent estrogen (E2 or ethinyl estradiol [EE]) and the test material concurrently Necropsy 24 hours after last dose Weigh the uteri from vehicle control (no treatment), positive control (E2), test material, and test material plus E2 only groups If the test material is an anti-estrogen, then the increase in uterine weight with E2 plus test material is significantly less than with E2 alone; uteri from the group treated with test material only are compared with uteri from the vehicle control group and should be equivalent (i.e., no growth), and the E2 only group should exhibit the maximal response	Anti-androgenic activity: Same as above except dose the prepubertal or castrated males with a potent androgen (e.g., testosterone propionate [TP], methyl-testosterone [mT], etc.) and the test material concurrently Weigh the accessory sex organs 24 hours after last dose, from the vehicle control (no treatment), positive control (TP, mT, etc.), test material only, or test material plus TP (or mT) If the test material is an anti-androgen, then the specified organ weights with T and test material will be significantly lower than the organ weights with T alone; the organ weights from the group treated with test material are compared with the weights for the vehicle control group and should be equivalent (i.e., no growth); the specified organ weights from the TP (or mT) only group should exhibit the maximal response.

Table 8 (continued)

Parameter	Uterotrophic Assay	Hershberger Assay
Background review document (BRD)	OECD Series on Testing and Assessment No. 38, detailed background review of the uterotrophic bioassay, March 2003	BRD in Progress
Phase 1:	Kanno *et al*., 2001	Owens *et al*., 2006
Phase 2:	Kanno *et al*., 2003	In preparation (draft report)
Phase 3:	Publication in preparation	In preparation (draft report)
Potent agonists	Ethinyl estradiol, DES, estriol, estrone, clomifene (selective estrogen receptor modulator; SERM)	Trenbolone acetate, methyl T, flutamide, TP, androstanolone
Weak agonists	Methoxychlor, genistein, diadzein, octylphenol, nonylphenol, bisphenol A, o,p'-DDT, zearalanol	Mesterolone, flyoxymesterone
Weak antagonists	Tamoxifen, raloxifene (SERMs)	Linuron, vinclozolin, flutamide, procymidone, p,p'-DDE, cyproterone
Agonist mechanism	Pre- and postpubertal uterus has ER (estrogen receptors) When an estrogenic compound (i.e., an estrogen agonist) is administered, it is recognized by and binds to the ER The presence of the agonist ligand on the ER initiates a cascade of gene transcriptional activation, resulting in inhibition and growth of the organ	The pre- and postpubertal accessory sex organs have ARs When an androgenic compound (i.e., an androgen agonist) is administered, it is recognized by and binds to the AR The presence of the agonist ligand on the AR initiates a cascade of gene transcriptional activation resulting in the growth of the accessory sex organs

Table 8 (continued)

Parameter	Uterotrophic Assay	Hershberger Assay
Antagonist mechanism	When an anti-estrogenic compound (i.e., an estrogen antagonist) is administered, it is also recognized by and binds to the ER The presence of the antagonist ligand on the ER blocks the cascade of gene transcriptional activations and also blocks any available endogenous estrogen (depending on the relative concentrations) from binding to the ER and triggering the cascade	When an anti-androgenic compound (i.e., an androgen antagonist) is administered, it is also recognized by and binds to the AR The presence of the antagonist ligand on the AR blocks the cascade of gene transcriptional activations and also blocks any available endogenous androgen (depending on the relative concentrations) for binding to the AR and triggering the cascade
Strengths of the ovariectomized/castrated animal model	The ovariectomized prepubertal or adult female is a robust specific assay with only one mechanism: through the ER It is standardized and validated by OECD (Kanno *et al*., 2001, 2003) It detects both strong and weak estrogen agonists and antagonists It works in various strains of mice and rats, with various environmental conditions (feed, bedding, caging, temperature, relative humidity, housing, etc.), as long as all groups are exposed to the same conditions	The castrated prepubertal or adult male is a robust, specific assay with only one mechanism: through the AR It is standardized and validated by OECD (Owens *et al*., 2006; Gray *et al*., 2005) It detects both strong and weak androgen agonists and antagonists It works in various strains of mice and rats, with various environmental conditions (feed, bedding, caging, temperature, relative humidity, housing, etc.), as long as all groups are exposed to the same conditions

Table 8 (continued)

Parameter	Uterotrophic Assay	Hershberger Assay
Weaknesses of the ovariectomized/castrated animal model	It does not detect estrogenic or anti-estrogenic compounds which do not act through the ER (e.g., those that affect steroidogenesis, that act through altered metabolism, ADME, etc.) It cannot act as a "decision node" since it evaluates only one mechanism	It does not detect androgenic or anti-androgenic compounds which do not act through the AR (e.g., those that affect steroidogenesis, that act through altered metabolism [ADME] etc.) It cannot act as a "decision node" since it evaluates only one mechanism
Strengths of the intact animal model	This assay is a robust, apical test that evaluates many mechanisms Use of the intact pubertal animal allows detection of agents that act "anterior" to the ovary (e.g., hypothalamus, pituitary), act on the ovary, or act "posterior" to the ovary (e.g., end-organ responses, distribution, metabolism, elimination, etc.) It can be used as a "decision node" since it evaluates many (all?) possible mechanisms	This assay is a robust, apical test that evaluates many mechanisms Use of the intact pubertal animal allows detection of agents that act "anterior" to the testis (e.g., hypothalamus, pituitary), act on the testis, or act "posterior" to the testis (e.g., end-organ responses, distribution, metabolism, elimination, etc.) It can be used as a "decision node" since it evaluates many (all?) possible mechanisms
Weaknesses of the intact animal model	Many mechanisms are evaluated but are not identified; additional assays/tests must be run to identify mechanism, if necessary	Many mechanisms are evaluated but are not identified; additional assays/tests must be run to identify mechanism, if necessary

Table 8 (continued)

Parameter	Uterotrophic Assay	Hershberger Assay
Window of Responsiveness	In the immature, intact female rat, there is a window of maximum sensitivity and responsiveness to estrogen between PND 18 and 26 Prior to PND 18, the uterus is insensitive to maternal estrogens, and there is a high circulating level of α-fetoprotein (AFP) AFP declines rapidly after birth with the nadir reached on PND 16-17 The window of sensitivity closes at puberty when there is a burst of E2 from the ovaries and a consequent increase in baseline uterine weight (and loss of sensitivity to exogenous estrogens) Approaching puberty, variability in mean group uterine weight increases Loss of optimal conditions without surgical (or chemical) intervention begins ~ PND 26 in the female rat (OECD, 2003) In the ovariectomized prepubertal or adult female, the tissues are responsive and sensitive to estrogens but (except for minor steroid synthesis [~8%] in the adrenal glands) there are no endogenous estrogens Therefore, the uterus is sensitive and responsive to exogenous estrogens prior to puberty and after surgical or chemical ovariectomy	The same situation exists in the intact, immature male, with sensitivity and responsivity optimum (maximum) prior to puberty (which begins in the rat after PND 35 and in the mouse after PND 25) In the intact male, puberty is the time of rapidly increased T from the testes and consequent increase in baseline weights of the accessory sex glands (and loss of sensitivity to exogenous androgens) Approaching puberty, variability in the mean weights of these organs is increased Loss of optimal conditions occurs at puberty without surgical (or chemical) intervention (~ PND 38 in the male rat) In the castrated prepubertal or adult male, the tissues are responsive and sensitive, but (except for minor steroid biosynthesis [~8%] in the adrenal glands) there are no endogenous androgens Therefore, the accessory sex organs are sensitive and responsive to exogenous androgens prior to puberty and after surgical or chemical castration

There are some chemicals, for instance technical-grade and formulated deltamethrin, which are not able to elicit antiestrogenic and anti-androgenic responses in either of these assays but do test positive in *in vitro* transcriptional assays (Andrade *et al.*, 2002).

The Hershberger assay has been found to be informative with chemicals that have an effect on the AR. However, there have been studies which indicate that the (anti)androgen potency, according to receptor binding assays, does not completely correspond to potency according to the Hershberger assay (Yamasaki *et al*., 2004). Charles *et al*. (2005) tested seven chemicals in the *in vitro* assays of AR transactivation, AR binding, and the *in vivo* Hershberger assay. Five of the seven anti-androgenic test materials produced positive results in all three assays. Two of the chemicals (MXC and BAP) differed in their responses, but it has not been established firmly whether anti-androgenicity is the primary mechanism of action for these chemicals. For instance, in the case of MXC, its profile of hormonal effects differs from both estrogenic and anti-androgenic materials (Gray *et al*., 1999). It is presumed that other compounds will have complex interactions with the endocrine system that can't be categorized into a single mode of action. Thus, the negative Hershberger assay results suggest that the interaction with the AR is too simplistic to explain the more complex interactions seen *in vivo*. Metabolism and other factors, such as pharmaco/toxicokinetics present in *in vivo* tests, are thought to account for some of these discrepancies.

Atrazine inhibits testosterone production in male rats following peripubertal exposure (Friedmann, 2002). Atrazine affects the pituitary/hypothalamic axis, and no endocrine disruptor properties are found using the Hershberger assay, since the AR ligand and receptor are not involved.

The results of *in vitro* and *in vivo* screens, such as the Hershberger assay, do not always agree. For instance, progesterone is negative *in vitro* but active in the Hershberger assay. This may be due to the conversion to active androstenedione/testosterone. Estrogens may also be active in the Hershberger assay, and ERβ is highly expressed in the prostate. Since hormones other than androgens have effects in the Hershberger assay, it is not entirely specific for androgens (Zacharewski, 1998).

Sonneveld *et al*. (2005) have shown that the Allen-Doisy (uterotrophic) assay requires a much lower amount of estrogenic compound administered to the animal to activate the estrogen receptor than for androgens activating the AR in the Hershberger assay. In fact, for the weaker androgens, the activating dose might not be reached. This may mean that for assessing chemical risk analysis of chemicals, using relatively weaker compounds, the Hershberger assay might not be the best test system (Charles *et al*., 2005).

Similarities and Differences Between Human and Rodent Male Reproductive Physiology

The basic physiological systems are similar for humans and rodents. There are no major differences between the circulatory, respiratory, endocrine, urinary, skeletal, and central nervous systems of the human and rodent male. The gall bladder is not present in the rat, but this does not appear to influence the reproductive system to produce any differences. The major difference is the timing of the events in the development of the human versus the rodent. The development of the endocrine system organs and their hormone production, release, and secretion are intrinsically different between species and even strains.

Similarities in Male Reproductive Physiology Between Humans and Rats (Modified from Gray et al., 2004, Table 1a)

- T and DHT, made in the embryofetal testis, control the initiation of the other male reproductive structures *in utero*.
- Central nervous system (CNS) – hypothalamic secretion of gonadotrophin-releasing hormone (GnRH) controls anterior pituitary synthesis and release of FSH and LH.
- LH in the male induces and regulates interstitial cells of Leydig outside the seminiferous tubules in the testes to produce T and DHT. These androgens, in the rat, initiate spermatogenesis in the early postnatal period. *In utero* production of T is initiated by the SRY gene on the Y chromosome. FSH in the male induces the Sertoli cells in the seminiferous tubules *in utero* to initiate nourishment and support of the developing gonocytes.
- At puberty, LH triggers a surge of T, which initiates completion of the process of spermatogenesis in boys (from spermatogonia to spermatids to spermatozoa).
- Both FSH and LH regulate germ cell development after puberty. T (and DHT) is required to maintain male spermatogenesis and initiate and maintain secondary sex characteristics in both boys and rodents.
- Some sexually dimorphic behaviours (e.g., “rough and tumble” play and mating behaviours) are imprinted by early exposure to androgens and maintained by estrogen made locally in the brain by conversion of T to estradiol (E2) by aromatase (the sexually dimorphic nucleus of the pre-optic area [SDN-POA] in the brain is the likely location; E2 cannot cross the blood brain barrier, but T can, so E2 is made in the brain).
- Puberty in males (and females) is initiated by dramatic endocrine changes resulting from CNS-HPG (hypothalamic-pituitary-gonadal) axis. Males generally acquire puberty (external indication is acquisition of PPS, separation of the glans penis from the prepuce in rodents) later than females (acquisition of vaginal patency in rodents) for both humans and rodents. The most

important androgen in the human and rat prostate is DHT, made locally by conversion of T to DHT by 5α-reductase in the prostate.

Differences in Male Reproductive Strategies Between Humans and Rats (Modified from Gray *et al*., 2004, Table 1b)

- In the rat, sexual differentiation of the reproductive tract is perinatal, with CNS sexual differentiation a postnatal event, regulated predominantly by local aromatization of T to E2 (play behaviour, an exception, is androgen dependent in both rats and humans).
- In nonhuman primates (and presumably humans), more CNS events are prenatal, and androgens are more important than in rats.
- Male rat sexual behaviour can be induced by estrogens and involves multiple series of ejaculations in a single mating. Mating involves approximately ten mounts, with intromission before each ejaculation, followed by a postejaculatory interval before the onset of the next series. The male rat responds to a female displaying sexual receptivity (only during estrus after E2 and then progesterone surges). In nonhuman primates (and humans), male sex behaviour is androgen mediated and does not depend on female behavioural or physiological estrus.
- Spermatogenesis begins at approximately five days of age in the rat. The postpubertal spermatogenic cycle (from spermatogonia to spermatozoa) is approximately 70 days in the rat (and approximately 56 days duration in mice). Sperm first appear in the rat epididymides at approximately 55 days of age. In humans, spermatogenesis begins during puberty at 10-14 years of age, and the entire spermatogenic cycle is approximately 75 days in duration.
- Puberty in male rats (measured by PPS, an androgen-dependent event) occurs at approximately 42 days of age in CD® (SD) and LE rat strains.
- Spontaneous male reproductive malformations are very rare in the rat. In humans, some male reproductive malformations, such as cryptochidism (undescended testes; under INSL3 [transabdominal descent] and DHT [inguinoscrotal descent] control), and hypospadias (when the opening of the urethra occurs along the ventral aspect of the penis, not at the tip), are considered the most frequent complications in newborn boys (~ 3% incidence).

Research for the past 15 years has started to focus on the development of the male reproductive tract at a cellular and molecular level. This approach may lead to biomarkers which enhance *in vivo* and *in vitro* assays that are under consideration for EDC and help to reduce some variability between species.

Ashby (2002) emphasized the importance of the resolution of study design and data interpretation issues before human hazard assessment for exposure to EDC can be approached with confidence.

Conclusions

Advantages

The Hershberger assay has been undergoing validation and has been shown to be robust, reliable and reproducible as a screening assay for the detection of androgen and anti-androgen effects. It appears to be sensitive and specific to androgen-mediated alterations and capable of detecting weak androgen antagonists such as *p,p'*-DDE and linuron (Lambright *et al*., 2000; O'Connor *et al*., 1999). The assay appears to function as an i*n vivo* androgenic screen (i.e., AR agonists, AR antagonists, and preventing the T conversion to the more potent DHT).

The tissues evaluated in the Hershberger assay are the natural targets for androgens, the tissue response in the assay is relatively rapid, the tissue weights provide quantitative endpoints, a limited number of animals is necessary, and no special facilities or equipment are required. These advantages, combined with the fact that the assay has been shown to measure relevant biological responses and be sufficiently sensitive, robust, and reproducible to detect androgenic and anti-androgenic activity, provide support for the use of this assay as an EDC screen.

The Hershberger assay is also being examined for its ability to detect thyroid function modulators (Noda *et al*., 2005; Yamada et. al., 2004). Because of the known presence of ARs in the thyroid gland of mammals (Pellietier, 2000; Banu *et al*., 2002), the thyroid is speculated to be a target of androgenic compounds. Testosterone also has a stimulatory effect on the expression of the TSH mRNA (Ross, 1990), and testosterone administration results in a significant decrease in serum T3 and T4 levels in the 15-day intact male assay (O'Connor *et al*., 2000). By enhancing the Hershberger assay with the addition of thyroid hormone determinations and thyroid histopathology, the assay appeared to be reliable for screening not only (anti)androgenic chemicals but also thyroid modulators. It has also been shown that the reliability of the Hershberger assay for assessing (anti)androgenicity is not confounded by alteration of thyroid homeostasis (Yamada *et al*., 2004).

Problems

One of the main problems with *in vivo* mammalian test methods has been the lack of relevant, reliable, and reproducible data due to different testing strategies, reference chemicals, and data interpretation.

There is an intrinsic variability for the developmental parameters used in the Hershberger assay and an absence of a universal rodent control database for these

parameters. This, coupled with the problems of variability of protocols and extrapolation of rodent effects to humans, creates the obvious pitfalls to overcome as much as possible before acceptance of the assay for screening EDC. This means that standardization and validation are vital to the future use of the Hershberger assay being used as a screening assay. These actions are currently underway.

Ashby (2002) also suggested establishing a range of sensitivity hierarchies for each of the EDC screening assays. He felt this will help to decrease the apparent discrepancies seen in the literature for some chemicals that have been tested.

Since the male endocrine system is so complex (including components such as the hypothalamus, pituitary, testis, thyroid, adrenal and pancreas), the Hershberger assay may not be sufficient to completely characterize the absence of endocrine action of some chemicals. There are numerous ways in which chemicals can produce interference in addition to effects on the AR. Short-term assays may not be able to detect interference mechanisms such as those affecting the hypothalamic-pituitary-gonadal axis. Also, the available established assays use a limited number of end points, and significant information gaps exist for other potential targets in the endocrine system.

A complimentary battery of *in vitro* and *in vivo* assays would appear to be valuable to fully characterize the absence of endocrine action of some chemicals. The data seem to indicate the need for utilizing a weight of evidence approach when assessing anti-androgenicity (Charles *et al*., 2005).

There is also a need to detect additive and/or synergic effects of these chemicals since most are not found singly in the environment but in mixtures. The Hershberger assay needs to be further tested for screening of mixtures.

Furthermore, the strongest response seen in the EDC screening assay may not turn out to be the most relevant response for human or wildlife assessment purposes (Ashby, 2002).

There is truly a difficulty present in attempting to represent the complexity of the endocrine system in either cell-based assays or animal models to detect possible endocrine disruptors in humans (Baker, 2001).

CHAPTER 11. REFERENCES

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APPENDIX A

EXAMPLE PROTOCOL

TITLE: **Hershberger Assay: Testosterone Proprionate Dose Response Curve**

SPONSOR:

TESTING FACILITY:

PROPOSED STUDY DATES (in-life):

AMENDMENTS:

No.	Date	Section(s)	Page(s)
1			
2			
3			

APPROVED BY:

Name Date
Study Monitor
Company

Name Date
Study Director
Company

Name Date
Sponsor's Representative
Company

Name Date
Quality Assurance Manager
Company

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I. RATIONALE, PURPOSE, AND BACKGROUND

Myotropic activity of anabolic steroids and androgenic activity of anabolic steroids can be differentiated with the Hershberger bioassay (Hershberger et al., 1953).

Testosterone supplementation in castrated male rats has been shown to increase bulbocavernous/levator ani weight ratio (BL/LA) and seminal vesicle, prostate, and Cowper's gland absolute organ weight (Ashley et al., 2000). Antiandrogens including flutamide, dibutyl phthalate, finasteride, and vinclozolin have been shown to reduce the absolute weight of androgen-sensitive organs such as the Cowper's gland, seminal vesicles, and prostate. Taken together, these data suggest that the Hershberger bioassay can be used to detect inhibitors of male sex hormone anabolism and antiandrogens, as well as androgens.

The rodent Hershberger assay was first described in 1953 (Hershberger et. al., 1953). Since that time, it has been used primarily in the pharmaceutical industry. The Hershberger assay is an *in vivo* short-term assay for chemicals that have the potential to act like endogenous sex hormones. It measures, as endpoints, changes in specific tissues that normally respond to endogenous hormones. The information generated by use of the assay can be used to build on that already available (e.g., from relevant *in vitro* screens that are used to narrow the field of chemicals that may need longer-term animal testing).

The rodent Hershberger assay evaluates the ability of a chemical to show biological activity consistent with the agonism or antagonism of natural hormones that have masculinizing effects. These hormones are known as androgens (e.g., testosterone). Accessory sex glands and accessory sex tissues are dependent upon androgen stimulation to gain and maintain weight during and after puberty. If endogenous sources of androgen are removed, exogenous sources of androgen are necessary to increase or maintain the weights of these sex accessory tissues. The sex accessory glands and tissues for this protocol are the: ventral prostrate (VP), seminal vesicles (SV) plus coagulating glands (CG), levator ani plus bulbocavernosus muscles (LABC), glans penis (GP), and Cowper's (or bulbourethral) glands (CP).

This protocol uses sexually immature male rats, castrated at peripuberty by removal of testes and epididymides (orchidoepididyectomized). In most laboratory strains, such as the Sprague-Dawley, Long Evans, or Wistar rats, peripuberty is expected to take place at approximately 6 weeks of age, within an expected age range of 5-7 weeks. Peripuberty is marked by prepuce separation. Testosterone proprionate (TP) will initiate prepuce separation so that the GP can be weighed. At the peripubertal stage of sexual development, the GP and other androgen-dependent sex accessory tissues are sensitive to androgens, having both androgen receptors and appropriate steroidogenic enzymes. The advantage of using this age of rodent is that the sex accessory tissues have a high sensitivity and small relative weight which both help to minimize variation in responses between individual animals.

Little is known about the response of individual sex accessory tissues to exogenous chemicals that may cause androgenic effects, although it has been shown that the male sex accessory tissues have different sensitivities to androgens and other steroid hormones (Ashby and Lefevre, 2000). This differential sensitivity has been used historically and continues to be used to this day in the pharmaceutical industry by companies searching for chemicals that are anabolic but not androgenic or estrogenic. One example of differential sensitivity is the LABC muscles that lack the 5-alpha reductase enzyme. These muscles lack the ability to convert testosterone to its active form, dihydrotestosterone. Weight increases of the LABC, without concomitant weight increases in the VP, CG, and SV glands (which contain 5-alpha reductase), may reflect a myogenic rather than an androgenic response.

A. Principle of Test

The rodent Hershberger assay is based on changes in weight of male sex accessory tissues in sexually immature castrated male rats. Test substances may stimulate or, in the presence of a reference androgen, inhibit the stimulated development of sex accessory tissues.

The test substance is administered in graduated doses to several groups of male rodents for a number of consecutive days. Measurement of the weight of sex accessory tissues provides information on the androgenic nature of a chemical, however it can also provide additional information on whether effects are due to the effects on the androgen hormone receptor *in vivo* or on other relevant biochemical mechanisms (e.g., effect on other enzymes involved in the production of sex hormones such as 5-alpha reductase).

In addition to the sex accessory tissues, body weight gain is a mandatory measurement to provide information on the general health and wellbeing of the animals. In the initial validation work, liver weight at necropsy is also a mandatory endpoint, as some test substances may appear to be anti-androgenic by inducing an increased metabolism of TP by the liver. This may be indicated by an increase in liver size.

B. Androgen Agonists

To test for androgen agonists, a test substance is administered to immature castrated rats for ten consecutive days. TP is administered by daily subcutaneous injection. TP provides the positive control in studies with substances of unknown androgenic activity. The vehicle provides the negative control.

C. Androgen Antagonists

To test for androgen antagonists, the test substance is administered to immature castrated rats for ten consecutive days together with TP. Administration of TP alone is used as the negative control where treatments are compared to for antiandrogenic activity. The weights of the sex accessory tissues after co-administration of the test chemical and reference androgen are compared with the weights of tissues from this control group.

II. GENERAL

A. Sponsor and Personnel

Sponsor: Name and address

Study Monitor: Name and address

Sponsor's Representative: Name and address

Testing Facility: Name and address

Personnel:

Study Director:
Animal Research Facility Veterinarian:
Study Coordinator:
Laboratory Animal Sciences:
Materials Handling Facility:
Quality Assurance Unit:

Additional personnel may be added and will be included in the final report.

B. Proposed Calendar

Dates are approximate; actual calendar will be included in the project records.

Starting Date of Acclimation (animals arrive at RTI):
Starting Date of Test Chemical Administration:
Starting Date of Mating Period:
Proposed Date for Completion of In-Life Phase (last pnd 21):
Proposed Date for Submission of the Draft Report (data audited):
Proposed Date for Return of Sponsor’s Comments to RTI:

C. Alteration of Design

Alterations of this protocol by the Study Director may be made with agreement of the Sponsor as the study progresses and documented by protocol amendment.

Examples of such alterations are dates of necropsy, length or timing of exposure to the drug, drug dose level, additional organs for weighing, collection of blood for hormone analyses, and additional laboratory studies.

D. Institutional Animal Care and Use Committee (IACUC) Approval

Approval from the IACUC will be obtained for this study prior to any animal testing (see Section III.C below).

E. Quality Assurance

This study will be conducted under OECD GLP Standards.

III. MATERIALS AND METHODS

A. Test Animals

Species/Strain: Rat; CD (Crl:CD®[SD]BR)

Supplier: Charles River Laboratories, Raleigh, NC. All animals will be castrated at approximately 42 days of age. All animals will be delivered by truck at least seven days before the study. Transport containers will be ventilated, escape-proof rodent shipping crates.

Rationale: The Sponsor has requested the use of live animals. Alternative test systems are not available for assessing the effects of a test substance in the Hershberger assay. The CD® rat is the species of choice by the Sponsor.

Number and Sex: Fifty (50) immature male rats will be ordered for the study. Thirty-six males will be used for the dose response curve. This number includes 14 extra males to provide sufficient animals to obtain animals within the mean ± 20% body weight requirement of the Sponsor. Arrival at the Animal Research Facility (ARF) will occur in one shipment.

Age and Weight: The males will be approximately six to seven weeks of age on receipt date. Actual weights will be recorded in the study records

within two days of arrival. Animals with body weights outside the ± 20% range may be removed from the study at the discretion of the Study Director.

Quarantine: Animals will be quarantined for approximately 7 days prior to the start of treatment. They will be observed daily for general health status, ability to adapt to the husbandry conditions, and will be released from quarantine by the ARF Veterinarian or designate prior to placement on study.

B. Husbandry Conditions

Housing: Test animals during the study will be housed individually in solid-bottom, polycarbonate cages with stainless steel wire lids (Laboratory Products, Rochelle Park, NJ) and Sani-Chips® cage litter (P.J. Murphy Associates). All animals will be housed in the ARF for the duration of the study. The animal rooms are air-conditioned, and temperature and relative humidity are continuously monitored by the Barber-Colman Network 8000 System VER 4.4.1 (Barber-Colman Company, Loves Park, IL). The target environmental ranges will be 22ºC (± 3º C) for temperature and 30-70% (ideally 50-60%) relative humidity, with 12 hours of light per day and 12 hours of dark.

Diet: Pelleted Purina Certified Rodent Chow® #5002 (Purina International [PMI], Richmond, IN) will be available *ad libitum*. Feed will be stored at approximately 60-70°F prior to use and will be used for no longer than 120 days after the milling date. Samples of the diet will be retained for possible analysis by the Sponsor.

Water: Water (tap water; source: City of _____ Department of Water Resources, City, State) will be available *ad libitum* by plastic water bottles with butyl rubber stoppers and stainless steel sipper tubes. Contaminant levels of the water are measured at regular intervals by the supplier per EPA specifications. Documentation of these analyses will be inspected by the Study Director and maintained in the study records. It is anticipated that contaminant levels will be below the maximal levels established for potable water and will not affect the design, conduct, or conclusions of this study.

C. Identification, Quarantine, and Animal Care Approval

Identification: Animals will be uniquely identified, prior to initiation of dosing, by individual ear tags. Animals will be given random numbers and assigned to treatment based upon body weight and random numbers in a randomized complete block approach (SOP _____).

Limitation of Discomfort: Surgery will be done under isoflurane gas anesthesia. Discomfort or injury to animals will be limited in that if any animal becomes severely debilitated or moribund, it will be terminated by CO_2 asphyxiation. All necropsies will be performed after terminal anesthesia with CO_2. Animals will not be subjected to undue pain or distress.

Culled Animals: Extra animals that arrive with the shipment that will not be used in the study will be euthanized or reassigned. Records will be kept documenting the fate(s) of all animals received for the study.

D. Test Substance and Vehicle

1. Testosterone Proprionate

Identity, purity, stability, and composition of the test substance have been determined by the manufacturer (see Certificate of Analysis for data regarding identity, purity, and composition, which will be provided with the chemical by the Supplier/Sponsor).

Test Substance Trade Names:	Testosterone proprionate
Vehicle:	Stripped corn oil
Supplier:	_____
CAS No.:	57-85-2
Molecular Weight:	420.6
Lot No.:	To be provided by Supplier or Sponsor
Description:	To be provided by Supplier or Sponsor
Formula:	$C_{28}H_{36}O_3$

Solubility:	Insoluble in water; soluble in alcohol ether, pyridine, other organic solvents, and in vegetable oils
Bulk Storage Conditions:	Desiccated at room temperature
Estimated Quantity:	10 grams
Dosing Solution Stability:	To be provided by Supplier or Sponsor
Dosing Solution Storage:	Room temperature
Safety Precautions:	Human exposure to the dosing solution will be restricted by the use of gloves, disposable laboratory clothing (coats or jumpsuits, booties), and safety glasses or goggles. When handling the neat chemical, protective clothing, gloves to prevent skin contact, and safety glasses or goggles are minimum required equipment.

2. Vehicle: Stripped Corn Oil

CAS No.:	8001-30-7
Supplier:	Sigma Chemical Company
Lot Number:	To be determined

E. Dose Formulation and Analysis

1. Preparation of Formulations and Storage

Dosage formulations for injection will be prepared by _____. The test compound to be used in this study will be TP (0.1, 0.2, 0.4, 0.8, and 1.6 mg/kg/day). The vehicle control will be stripped corn oil and will be prepared by _____.

2. Analysis of Formulations

Dosing formulations will be saved for possible analyses for concentration from aliquots of doses used in the study. Samples will be stored at room temperature until analysis.

IV. EXPERIMENTAL DESIGN

A. Study Design

The goal is to characterize the dose response of the sex accessory tissues in castrated male rats to the reference androgen, TP (Groups A-E, n=6 per group, Table 1). Animals will be dosed with TP by subcutaneous injection on the dorsal surface of the animal. The maximum limit on the volume administered per animal is approximately 0.5 ml/kg body weight per day. The animals will be dosed in the same manner and time sequence for ten consecutive days at approximately 24-hour intervals. The dosage level will be adjusted for changes in body weight.

The volume of dose and time that it is administered will be recorded on each day of exposure. Animals will be assigned to treatment groups by means of randomized complete block approach and stratified such that treatment groups have equivalent mean body weights.

Table 1. Experimental Design: Agonist Response

Group	Treatment
Group 1 - Vehicle Control	Vehicle
Group 2	TP: 0.1 mg/kg/day
Group 3	TP: 0.2mg/kg/day
Group 4	TP: 0.4 mg/kg/day
Group 5	TP: 0.8 mg/kg/day
Group 6	TP: 1.6 mg/kg/day

B. Experimental Procedures: Treatments

1. General Condition and Symptoms

Clinical examinations will be conducted and recorded at least once daily throughout the course of the study. Mortality checks will be done once daily. These cage-side observations will include, but not be limited to, changes in skin and fur, eyes, mucous membranes, respiratory system, circulatory system, autonomic and central nervous system, somatomotor activity, and behavior. Any animal found dead will be removed and disposed of without further data analysis. Any mortality of animals prior to necropsy will be included in the study record together with the reasons.

2. Body Weight

Body weights will be recorded once during quarantine (for randomization), at time of surgery, at daily treatments (for calculation of dosing volume), and at necropsy to

monitor body health and verify lack of overt general toxicity. A body weight loss of greater than 10% or reduced body weight gains will be considered as an indicator that general toxicity has resulted. Group means and standard deviations will be calculated.

3. Feed and Water Consumption

Feed consumption will be generally observed and any significant events recorded (e.g., animals not eating or drinking). Water and food consumption will not be measured. A sample of the diet will be collected for possible future analysis at the request of the Sponsor.

4. Administration of Compound

The males will be injected subcutaneously with TP or vehicle once daily for ten consecutive days.

C. Experimental Procedures

1. Necropsy

Animals will be used to evaluate the effect(s) of TP on sex accessory tissues in castrated male rats. Approximately 24 hours after the last administration of the test substance, blood will be collected by cardiac puncture (optional) and the rats will be euthanized by CO_2 inhalation and then exsanquinated.

The order in which the animals are necropsied will be a block design, such that one animal from each of the groups is necropsied (in a random, within-block fashion) before necropsy of the second animal from each group. In this way, all the animals in the same treatment group are not necropsied at once.

If the necropsy of each animal requires more total time than is reasonable for a single day, necropsy will be conducted on consecutive days. In this case, the work would be divided so that necropsy of two to three animals per treatment per day takes place on the first day, with dosing and necropsy being delayed by one day in the other animals.

The sex accessory tissues will be excised and their weights determined (see #3 below) for comparison with the weights of sex accessory tissues from the vehicle control group.

Carcasses will be disposed of in an appropriate manner following necropsy.

2. Histology

The sex accessory tissues will be preserved in 10% neutral buffered formalin and stored. Histology may be done at a later date at the request of the Sponsor for additional compensation.

3. Sex Accessory Tissue Weights

The sex accessory tissues will be excised and their weights determined, for comparison with the weights of sex accessory tissues from the vehicle control group or reference TP group (in the case of antagonist response). The sex accessory glands and tissues to be weighed are the: VP, SV plus CG, LABC, GP, and CP. The liver will also be excised and weighed. After excision and weighing of the ventral prostate, it will be fixed for 24 hours in 10% neutral buffered formalin (4% formaldehyde) and weighed again. The LABC will be weighed separately and then added. This allows the calculation of ratios, as these tissues are purportedly affected differently by different androgens (Hershberger et al., 1953).

D. Statistical Analyses

The sex accessory tissues will be excised and their weights determined (see above) for comparison with the weights of sex accessory tissues from the vehicle control group or reference TP group (in the case of antagonist response). Weights and other information will be placed in proper blanks on electronic spreadsheets furnished to the Sponsor.

V. RETENTION OF SPECIMENS AND RECORDS

_____will be responsible for data management until the end of the study. Study record data and reports will be sent to the Sponsor at the completion of the study, where they will be retained according to OECD retention requirements. Specimens will be maintained in formalin for one year from acceptance of the final report. If histology is not requested by the Sponsor within that year, the tissues will be discarded with the permission of the Sponsor's Representative.

VI. RECORDS TO BE MAINTAINED

Receipt of Chemical, Certificate of Analysis and other Chemistry Records
Dosing Formulation and Dose Administration Records
Animal Receipt Records, Animal Room Log Sheets
Temperature and Humidity Records for Animal Rooms
Quarantine Animal Health Surveillance Records
Randomization and Assignment to Study
Male Body Weights
Clinical Signs
Necropsy-Male Sex Accessory Tissue Weights and Gross Pathology Findings
Tissue Samples

VII. REPORTING

A. Status and Interim Reports

Informal status reports will be given via e-mail or telephone during the course of the study and documented in the study record. Time points of interest include initiation of dosing and completion of necropsy.

B. Draft and Final Reports

The Study Director, prior to the issuance of the final report, will issue a draft final report to the Study Monitor. The final report format will correspond to that specified for data reporting of drugs to the extent possible for this study. The final report, including individual data tables and complete statistical analyses of data, will be submitted to the Sponsor's Representative following acceptance of the draft report. The final report will include:

Abstract
Introduction
Experimental Design
Materials and Methods
Narrative Discussion of Parameters Evaluated
Discussion
Conclusions
References

Summary Data (means ± standard errors) by Dose Group and Time Points

Individual Data Tables

Statistical Analysis

Appendices: Protocol and Amendments, if any

Feed and Water Analysis

VIII. REFERENCES

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