APPENDIX B

Model Protocol for the Conduct of the Hershberger with Stimulated Weanling Rats
INTRODUCTION

1. The overall aim of the validation program is to demonstrate that the Hershberger bioassay is a reliable and reproducible bioassay that can be considered as the basis for an OECD Test Guideline. This document provides the essential requirements for Phase-3 of the OECD program on the validation of the rodent Hershberger bioassay using the stimulated weanling male. Detailed laboratory protocols for the OECD validation program are to be built on the requirements, recommendations, and options contained in this document.

2. The precursor of the rodent Hershberger bioassay was first developed in the 1930s and included various tissues of the male reproductive tract (1)(2)(3), including the ventral prostate, the seminal vesicles with coagulating glands, the Cowper’s glands, the glans penis, and preputial glands. The measurement of the levator ani and bulbocavernosus muscles were subsequently investigated in the 1940s (3)(4). After publication of work with an extensive number of compounds by Hershberger et. al. in 1953 (5), the procedure has been commonly referred to as the Hershberger bioassay. The primary model for the Hershberger bioassay has been the surgically castrated pubertal male, and this model has been the basis for work in Phase-1 and Phase-2 of the Hershberger validation program. However, there are animal welfare concerns with the castration procedure, and an alternative is sought to avoid the castration step. This model protocol outlines the use of the uncastrated stimulated weanling as that possible alternative.

3. The Hershberger bioassay is an in vivo short-term assay whose conditions are designed to achieve low endogenous hormone levels and employ target tissues that are highly responsive to administration of exogenous hormones. The focus of the Hershberger bioassay is on the detection of compounds that may interfere with endogenous male sex hormones. This includes other androgens, androgen antagonists and 5α-reductase inhibitors. The objective in Phase-3 of the validation with this model protocol is to assess the potential of the stimulated weanling as reliable and relevant alternative to the surgical castration. For the stimulated weanling, the program encompasses both previous and current validation work on the surgical castrate.¹

4. The Hershberger is being validated by OECD as potential short-term screen (6). The information generated by the bioassay can be used to build on that information already available, e.g. from relevant in vitro screens, to narrow the field of chemicals that may need longer-term animal testing. This current stimulated weanling protocol is based largely on the standardisation and optimisation work performed in the Syngenta CTL laboratory.

BASIS FOR THE VALIDATION WORK WITH THE STIMULATED WEANLING

5. The OECD program on the Hershberger assay has previously demonstrated the response and the reliability of surgical castrate by measuring male sex accessory glands and tissues, i.e., ventral prostrate (VP), seminal vesicles with coagulating glands (SVCG), the levator ani and bulbocavernousus muscles

¹ For the surgical castrate, Phase-3 refers only to work with coded test substances including both positive and negative test substances.
(LABC), the Cowper’s (or bulbourethral) glands (COWS), and glans penis (GP). In the case of the stimulated weanling, the immature animals have not undergone preputial separation due to their sexual immaturity. This lack of preputial separation compromises the dissection of the GP, so that the reliability of this particular tissue with the stimulated weanling is in question and will be addressed in the validation program. Therefore, the mandatory target tissues in the stimulated weanling will be reduced to four: the VP, SVCG, LABC, and COWS. However, two tissues removed during castration are available for investigation in this model, the testes (T) and epididymes (EP). Both of these tissues will be measured and their utility assessed as part of the validation program.

6. Biological plausibility would suggest that the stimulated weanling and the surgically castrated versions of the Hershberger bioassay should respond in a qualitatively similar manner. In both versions, growth of the target mandatory tissues is controlled through the androgen receptor, and testosterone is converted to dihydrotestosterone by the 5α-reductase enzyme. The same male reproductive tract tissues can be employed, with the exception of the GP, T, and EP as noted above.

7. There are, however, differences to be noted between the stimulated weanling and the surgically castrated male. As a result, comparative work is needed to define the quantitative differences that may exist between the two versions. The key differences in the stimulated weanling are low prepubertal circulating levels of testosterone produced by the testes and the potential ability of the intact hypothalamic-pituitary-gonadal axis to respond and compensate to some degree to chemical insult. These may diminish the responsiveness of the stimulated weanling version of the assay. For further background, there are reviews and recent manuscripts that trace the development of the male rat for both hormonal levels and the growth of reproductive tract tissues prior to puberty (7)(8)(9)(10). It should be noted that the post natal day (pnd) 20-35 time period in the male is not fully analogous to the female. There are no dramatic surges in the male in hormonal levels or tissue growth in this time period. The change and rearrangement of the hypothalamic-pituitary-gonadal axis is more gradual in circulating FSH, LH, testosterone, and 5α-reduced steroids. At the same time, just as this is a period of rapid overall growth in the animal, the weights of the testes and the relevant tissues of the reproductive tract are also increasing in absolute terms. The increase relative to body weight for these tissues is rather modest and constant, indicating no disproportionate surge in growth.

OUTLINE OF THE WORK FOR THE VALIDATION OF THE STIMULATED WEANLING

8. The program to assess the stimulated weanling as an alternative will proceed in the same fashion as that taken by the preceding surgical castrate model. Due to the low circulating testosterone levels and the intact HPG axis in the weanling, testosterone propionate (TP) (CASRN 57-85-2) and Flutamide (FLU) (CASRN 1311-84-7) standardization curves with the stimulated weanling male are necessary to ensure the proper TP dose is selected for the antagonist studies. Therefore, the first two stages, analogous to Phase-1A and Phase-1B with the surgical castrate (11) are:

• In the first stage, a dose response curve with TP will be generated as the first step to select optimum candidates for the TP coadministration dose with antiandrogens. The basis for selection is intended to be the same as with the surgical castrate: to identify ED50-ED70 doses of TP that are applicable to all the responsive tissues. The doses to be employed are 0.4, 0.8, 1.0, 1.2, and 1.6 mg/kg/d in order to provide a complete comparison with the surgical castrate data set. This dose series should adequately characterize the dose response for each of the four tissues (VP, SV, LABC, and COWS), its relative maximum response, and provide insight on the CVs that labs can achieve with the smaller weanling tissues. With the vehicle control, this comprises a total of six dose groups per laboratory (36 animals based on n = 6). This will allow the selection of two TP doses to proceed into the second stage, the FLU dose series experiments.
• In the second stage, two selected doses of TP will be used as the reference dose for coadministration to assess the response of stimulated weanling tissues to a dose series of FLU. The doses of FLU to be employed are 0.3, 1.0, and 3.0 mg/kg/d FLU. Higher doses are judged to be wasteful of animals, based on the available data. With the two TP reference dose groups, this comprises a total of eight dose groups per laboratory (48 animals based on n = 6). Two additional groups are voluntary: a vehicle control and a fourth dose of FLU at 10 mg/kg/d. This will allow the selection of the optimum TP reference dose in order to proceed into the third stage, the dose response experiments to weak agonists and antagonists.

9. The next two stage for the stimulated weanling comprise studies analogous to the completed Phase-2 dose response studies for the surgical castrate and to the current Phase-3 coded studies for the surgical castrate:

• In the third stage, the stimulated weanling will be used to assess its capability to detect a weak androgen agonist, trenbolone, and to detect two weak androgen antagonists, linuron and \( p,p' \)-DDE. All three compounds challenged the surgical castrate in one or more laboratories where one or more of the five castrate mandatory tissues did not achieve statistical significance. In order to make a comparison, the same dose series will be used, but the lowest dose will be omitted in the interests of animal welfare and conserving resources:
  - Trenbolone (TREN) (CASRN 10161-33-8):
    1.5, 8, and 40 mg/kg/d (rationale: no lab reached statistical significance with the surgical castrate at 1.5 mg TREN/kg/d; therefore, the 0.3 mg/kg/d dose is not an apparent sound use of animals or resources)
  - Linuron (LIN) (CASRN 330-55-2):
    10, 30, 100 mg/kg/d (rationale: no lab reached statistical significance with the surgical castrate at 10 mg LIN/kg/d; therefore, the 3 mg/kg/d dose is not an apparent sound use of animals or resources)
  - \( p,p' \)-DDE (DDE) (CASRN 72-55-9):
    16, 50, 160 mg DDE /kg/d (rationale: no lab reached statistical significance with the surgical castrate at 10 or 16 mg/kg/d – doses inside and outside of Japan, respectively; therefore, the 5 mg/kg/d or similar dose is not an apparent sound use of animals or resources)

• The fourth stage is analogous to final Phase-3 of the surgical castrate validation. The weanling version’s reliability will be assessed by its response to and ability to correctly identify coded positive and negative substances in both agonist and antagonist studies. In these studies, the doses of certain positive substances will be identical to that in stage 3, so that the reproducibility of the bioassay over time may be assessed. In addition, the relative sensitivity and effectiveness of the different sex accessory tissues and glands in the assay will continue to be assessed.

INITIAL CONSIDERATIONS AND PRINCIPLE OF THE ASSAY

10. The rodent Hershberger assay has been based historically on changes in the weights of androgen-responsive male sex accessory tissues largely in peripubertal, castrated male rats. Accessory sex tissues and glands depend upon androgen stimulation to gain and maintain weight during and after puberty. When endogenous sources of androgen are low (the weanling before puberty), the biological activity of exogenous substances can be assayed by the increase (agonist response) in the weights of these sex accessory tissues or by blocking (antagonist response) the activity of administered androgens and by preventing an increase in the weights of these sex accessory tissues. The rodent Hershberger assay modified to use the stimulated weanling then evaluates the ability of a chemical to show biological
activities consistent with the agonism or antagonism of androgens (e.g., testosterone and dihydrotestosterone).

11. The available data indicates that the androgen-dependent sex accessory tissues of the weanling are sensitive to androgens. This is plausible as these animals have both androgen receptors and appropriate steroidogenic enzymes necessary for agonist, antagonist, and $5\alpha$-reductase inhibitor responses. In addition to sensitivity, the weanling rodent sex accessory tissues have a small relative weight.

12. Primary objectives of Phase-3 of the validation program are to demonstrate:

- In stage 1, the dose response of the stimulated weanling version of the Hershberger bioassay to a series of Testosterone Propionate (TP) doses for comparisons with the surgical castrate and as the basis for selecting two doses for coadministration studies with Flutamide (FLU)
- In stage 2, the dose response of the stimulated weanling version of the Hershberger bioassay to a series of FLU doses using two selected TP coadministration doses for comparisons with the surgical castrate and as the basis for selecting a single TP dose for coadministration with antagonists in dose response studies
- In stage 3, the dose response of the stimulated weanling version of the Hershberger bioassay to a series of doses with a weak agonist, trenbolone, and two weak antagonists, linuron and DDE
- In stage 4, to assess the capability of the stimulated weanling version of the Hershberger bioassay to identify coded positive and negative substances.
- The relative effectiveness of the different sex accessory tissues and glands in the assay.
- The reproducibility of the stimulated weanling version of the bioassay over time by comparing appropriate data from stage 4 to that generated in stages, 1, 2 and 3.
- Continue the investigation of the value of the different accessory tissues and glands

13. The test substances will be coded and administered to groups of six animals (n = 6) for 10 consecutive days in all studies in all stages. The animals will then be necropsied approximately 24 hours later on the 11th day (24 hours after the last test substance administration). After dissection, the weights of the mandatory sex accessory tissues will be measured as well as the T and EP weights.

14. In addition to the sex accessory tissues, daily body weights, including at necropsy, are mandatory measures to allow precise dose administration, to provide information on the general health and well being of the animals, and so that body weight can be used as a statistical covariable. The liver, adrenal and kidney weights are optional measurements that may provide supplementary information about the systemic toxicity, target organs and other effects of the test substance.

**Androgen agonists**

15. Biological activity consistent with androgen agonists is tested by administering a test substance to intact, weanling male rats for 10 consecutive days. The positive control for the tissue responses is TP. The vehicle is the negative control. The weights of the sex accessory tissues of the test chemical groups are compared to the vehicle group for a statistically significant increase in weight.

**Androgen antagonists**

16. Biological activity consistent with androgen antagonists and $5\alpha$-reductase inhibitors is tested by administering the test substance to intact, weanling male rats for 10 consecutive days together with a reference androgen agonist, TP. Administration of TP alone is the negative control. The weights of the sex accessory tissues after co-administration of the test chemical and the reference androgen TP together are compared with the weights of tissues of the reference androgen TP alone for a statistically significant decrease in weight. FLU may be coadministered with TP to another group as a positive control.
DESCRIPTION OF METHOD/PREPARATIONS FOR THE TEST

Animal Species and Strain
17. This protocol allows laboratories to select the strain of rat to be used in the validation of the assay. The selection should be the strain used historically by the participating laboratory. Where the screening assay may be preliminary to a repeated dose oral study, a reproductive and developmental study, or a long-term study, preferably animals from the same strain and source should be used in all studies. If a laboratory is planning to use an unusual rat strain, or one unique to their own facility, they should determine whether the sexual development criteria noted under the section, INITIAL CONSIDERATIONS AND PRINCIPLE OF THE ASSAY, are met.

Age and Acclimatisation
18. Young weanling animals are to be employed in a relatively small time window between weaning and before puberty, i.e. postnatal days 20 to 34. Animals will be observed daily, and any animals with evidence of disease or physical abnormalities will be removed. The treatment with initiation of dosing (on study) may commence as early as pnd 21 days of age, but preferably not later than pnd 24. This allows a laboratory some flexibility to schedule the experimental work efficiently.

Housing and feeding conditions
19. Temperature in the experimental animal room should be 22 °C (± 3°). The relative humidity should be 50 to 60%, but should not exceed limits of 30 to 70% except during room cleaning. Lighting should be artificial, the photoperiod being 12 hours light, 12 hours dark.
20. Laboratories participating in the validation should use the laboratory diet normally used in their chemical testing work. In previous phases, no effects or variability were observed that were attributable to the diet. The diet used will be recorded and a sample of the laboratory diet will be retained for possible future analysis. Both diet and drinking water will be supplied ad libitum.
21. Weanling animals should be caged in groups of no more than 6 similarly treated rats per cage, giving a minimum of 1 cage of 6 rats/cage per treatment group and a maximum of 2 cages of 3 rats/cage per treatment group. When cages are properly sized (~2000 square centimeters), six animals or less per cage avoids crowding. Cages should be thoroughly cleaned to remove possible contaminants and arranged in such a way that possible effects due to cage placement are minimised.
22. Each animal will be identified individually (e.g., ear mark or tag). The method of identification will be recorded.

Body Weight and the selection of animals for the study
23. Increasing differences in body weight may be a source of variability in the weight of tissues of interest within and among groups of animals. Variations in body weight should be both experimentally and statistically controlled, and the statistical analysis should be done both with and without body weight as a covariate. As toxicity may also impact the body weight, the body weight on the first day of administration can be used as the covariate in those cases where significant reductions in body weights has occurred.
24. Experimental control of body weight is accomplished in two steps. The first step involves selection of animals with relatively small variation in body weight for the study cohort from the larger population of animals that have been supplied. Unusually small or large animals should be avoided and should not placed in the study cohort. A reasonable level of body weight variation within the study cohort should be tolerated. Here, ± 20% of the mean body weight for the cohort population is judged to be reasonable (e.g. 50g ± 10 g). The second step involves the assignment of animals to different treatment groups (n = 6) by a
randomised complete block approach. Under this approach animals are randomly assigned to treatment groups so that each group has the same mean and standard deviation in weight at the beginning of the study. The procedure used for block randomization should be recorded.

Non-routine health and safety requirements

25. The test substances are possible reproductive and developmental toxicants and, therefore, appropriate precautions should be taken to protect personnel during the validation work, e.g. necessary training, labeling and storage procedures, and protective handling procedures during dose preparation and dose administration.

26. Appropriate precautions such as wearing protective gloves, protective clothing and eye protection will be taken when handling the animals, diets, cages, and wastes (e.g. remaining test solutions, faeces, and carcasses). Waste disposal will be in accordance with good practice and existing regulations applicable to a given laboratory.

PROCEDURE - VALIDATION OF THE STIMULATED WEANLING

Administration of doses

27. TP will be administered by subcutaneous (sc) injection in all stages of the validation program.

28. All other test substances will be administered by oral gavage.

29. Sc injections will be on the dorsal surface of the animal after shaving or trimming of fur. Multiple injections sites may be used. The maximum limit on the volume administered per animal is approximately 1.0 ml/kg body weight per day.

30. Oral gavage will be the delivery of the test substance in vehicle by means such as intubation with an oral gavage syringe. The maximum limit on the volume administered per animal will be 5 ml/kg/day. As the weanling animals are small, the technical staff conducting the gavage should be experienced in order to avoid gavage errors that might lead to morbidity or mortality.

31. 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 daily based on the concurrent daily measures of body weight. The volume of dose and time that it is administered will be recorded on each day of exposure.

Good Laboratory Practice

32. Work should be conducted according to the principles of Good Laboratory Practice (OECD Good Laboratory Practice and Compliance Monitoring (12)). In particular, data should have a full audit trail and be retained on file. Data will be collected in a manner that will allow independent peer review and written records maintained.

Vehicle

33. All participating laboratories should use a vehicle, such as stripped corn oil, that is not easily disposed to potential microbial degradation of the vehicle or the reference and test substances. If the dosing samples are not made daily, care should be taken to preserve and to avoid contamination and spoilage of the samples.

34. The following procedures are to be conducted in four consecutive stages.
STAGE 1 (Analogous to the completed Phase-1A with the surgical castrate)

Test substance and doses in stage 1 of the stimulated weanling validation

35. The test substance for stage 1 of the stimulated weanling validation will be TP.

36. The reference androgen agonist, TP, will be administered in a series of doses comprising 0.4, 0.6, 1.0, 1.2, and 1.6 mg/kg/d.

Test groups in stage 1 of the stimulated weanling validation

37. 6 animals of the same age and cohort will be used for the vehicle, the TP doses, and any other control group or treatment used by the laboratory.

38. The response of the sex accessory tissues and glands to a reference agonist, Testosterone Propionate (TP), will be studied. This work will involve five test groups for increasing doses of TP and one vehicle control group. The test groups are illustrated in Table 1.

<table>
<thead>
<tr>
<th>Test Groups for Stage 1 of the Stimulated Weanling Validation: Testosterone Propionate Dose Series</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agonist response</strong></td>
</tr>
<tr>
<td>Group A Vehicle Control</td>
</tr>
<tr>
<td>Group B 0.4 mg/kg/d Testosterone Propionate</td>
</tr>
<tr>
<td>Group C 0.8 mg/kg/d Testosterone Propionate</td>
</tr>
<tr>
<td>Group D 1.0 mg/kg/d Testosterone Propionate</td>
</tr>
<tr>
<td>Group E 1.2 mg/kg/d Testosterone Propionate</td>
</tr>
<tr>
<td>Group F 1.6 mg/kg/d Testosterone Propionate</td>
</tr>
</tbody>
</table>

STAGE 2 (Analogous to the completed Phase-1B with the surgical castrate)

Test substance and doses in stage 2 of the stimulated weanling validation

39. The test substances for stage 2 of the stimulated weanling validation will be TP and FLU.

40. Two doses of the reference androgen agonist, TP, will be selected based upon stage 1 data, and these selected doses will be coadministered with a series of FLU doses. The FLU doses are 0.3, 1.0, and 3.0 mg/kg/d. Laboratories may voluntarily extend the FLU dose series by adding test groups with the selected TP dose plus 10 mg/kg/d.

Test groups in stage 2 of the stimulated weanling validation

41. 6 animals of the same age and cohort will be used for the vehicle, the two selected TP doses, and two series of three groups each where a selected TP dose is coadministered with an increasing series of FLU doses.

42. The response of the sex accessory tissues and glands to selected doses of a reference agonist, Testosterone Propionate (TP), coadministered to a series of FLU doses will be studied. This work will involve TP control groups for each selected dose and three test groups for each selected dose where that TP
dose is coadministered with FLU having a prescribed dose of the test substance and one vehicle control group. The required and voluntary test groups are illustrated in Table 2.

Table 2. Test Groups for Stage 2 of the Stimulated Weanling Validation:
Two Selected Testosterone Propionate Doses Coadministered with Flutamide Dose Series

<table>
<thead>
<tr>
<th>Group</th>
<th>Antagonist response</th>
<th>Antagonist response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Selected TP dose #1</td>
<td>Selected TP dose #2</td>
</tr>
<tr>
<td>Group A</td>
<td>Vehicle Control</td>
<td>Vehicle Control</td>
</tr>
<tr>
<td>Voluntary</td>
<td>Vehicle Voluntary</td>
<td>Vehicle Voluntary</td>
</tr>
<tr>
<td>Group B</td>
<td>Selected Dose of TP #1</td>
<td>Selected Dose of TP #2</td>
</tr>
<tr>
<td>Negative Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group C</td>
<td>Selected Dose of TP #1 + 0.3 mg/kg/d FLU</td>
<td>Selected Dose of TP #2 + 0.3 mg/kg/d FLU</td>
</tr>
<tr>
<td>Group D</td>
<td>Selected Dose of TP #1 + 1.0 mg/kg/d FLU</td>
<td>Selected Dose of TP #2 + 1.0 mg/kg/d FLU</td>
</tr>
<tr>
<td>Group E</td>
<td>Selected Dose of TP #1 + 3.0 mg/kg/d FLU</td>
<td>Selected Dose of TP #2 + 3.0 mg/kg/d FLU</td>
</tr>
<tr>
<td>Group F</td>
<td>Selected Dose of TP #1 + 10 mg/kg/d FLU</td>
<td>Selected Dose of TP #2 + 10 mg/kg/d FLU</td>
</tr>
<tr>
<td>Voluntary</td>
<td>Voluntary</td>
<td>Voluntary</td>
</tr>
</tbody>
</table>

STAGE 3 (Analogous to the completed Phase-2 with the surgical castrate)

Test substances in stage 3 of the stimulated weanling validation

43. The reference androgen agonist will be TP. The reference androgen antagonist will be FLU. The appropriate doses of these substances will be selected based upon stage 2 data.

44. The test substances for Phase 3 stage 3 dose response studies for the stimulated weanling will be:

- Trenbolone (17β-Hydroxyestra-4,9,11-trien-3-one) CAS No 10161-33-8
- Linuron CAS No 330-55-2
- p,p’-DDE (4,4’Dichlorodiphenyldichloroethylene) CAS No 72-55-9

45. The agonist test substance doses for Phase 3 stage 3 dose response studies for the stimulated weanling will be:

- Trenbolone 1.5, 8, and 40 mg/kg/d

The two weak antagonists will be administered along with a dose of TP selected from the stage 2 studies with each of the following doses:

- Linuron 10, 30, 100 mg/kg/d
- p,p’-DDE 16, 50, 160 mg/kg/d
Test groups in stage 3 of the stimulated weanling validation

46. 6 animals of the same age and cohort will be used for the vehicle, TP, and any other control group and for each treatment or test substance group.

47. The response of the sex accessory tissues and glands to a weak agonist, trenbolone, will be studied. This work will involve three test groups for each agonist having a prescribed dose of the test substance, and one vehicle control group used as the negative control. The required test groups are illustrated in Table 3.

48. The response of the sex accessory tissues and glands to two weak antagonists, linuron and \( p,p' \)-DDE, and will be studied. This work will involve three test groups for each antagonist and the coadministration of a dose of the reference agonist TP to each group. Each test substance will have a prescribed dose for each of its groups. The positive TP control group for the antagonist series and the dose of coadministered TP (___ mg/kg/d TP) will be the same and will be selected based upon stage 2 results. The required test groups are illustrated in Table 3.

Table 3. Test Groups for Stage 3 of the Stimulated Weanling Validation: Agonist and Antagonist Dose Responses

<table>
<thead>
<tr>
<th></th>
<th>Trenbolone response</th>
<th>Linuron response</th>
<th>DDE response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A</strong></td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
</tr>
<tr>
<td>Vehicle Control</td>
<td><strong>Mandatory</strong> for agonist</td>
<td><strong>Voluntary</strong> for antagonist</td>
<td><strong>Voluntary</strong> for antagonist</td>
</tr>
<tr>
<td><strong>Group B</strong></td>
<td>Provided by vehicle control (no additional group needed for agonist)</td>
<td>Selected TP dose from stage 2</td>
<td>Selected TP dose from stage 2</td>
</tr>
<tr>
<td>Negative Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group C</strong></td>
<td>1.5 mg/kg/d Trenbolone</td>
<td>Selected TP dose from stage 2 + 10 mg/kg/d linuron</td>
<td>Selected TP dose from stage 2 + 16 mg/kg/d DDE</td>
</tr>
<tr>
<td><strong>Group D</strong></td>
<td>8 mg/kg/d Trenbolone</td>
<td>Selected TP dose from stage 2 + 30 mg/kg/d linuron</td>
<td>Selected TP dose from stage 2 + 50 mg/kg/d DDE</td>
</tr>
<tr>
<td><strong>Group E</strong></td>
<td>40 mg/kg/d Trenbolone</td>
<td>Selected TP dose from stage 2 + 100 mg/kg/d linuron</td>
<td>Selected TP dose from stage 2 + 160 mg/kg/d DDE</td>
</tr>
<tr>
<td><strong>Group F</strong></td>
<td>TP dose selected from stage 2</td>
<td>TP dose selected from stage 2 + FLU dose selected from stage 2</td>
<td>TP dose selected from stage 2 + FLU dose selected from stage 2</td>
</tr>
<tr>
<td>Positive Treatment</td>
<td>Voluntary</td>
<td>Voluntary</td>
<td>Voluntary</td>
</tr>
<tr>
<td>Group Voluntary</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

STAGE 4 (Analogous to the current Phase-3 with the surgical castrate)

Doses in stage 4 of the stimulated weanling validation

49. 6 animals of the same age and cohort will be used for the vehicle, TP, and any other control group and for each treatment or test substance group.

50. All participating laboratories will use the coded test substances supplied and follow the instructions for the preparation of the proper doses in order to achieve specified dosages that can be compared data generated in stage 3. There are two coded series. One series is for androgen agonists, and this series requires a positive vehicle control. A second series is for androgen antagonists and requires a reference TP dose group using a ___ TP mg/kg/d dose to be selected based upon the results from stage 2. In the latter antagonist series, a vehicle control group and a positive control group of FLU + the selected TP dose are voluntary. The required test groups are illustrated in Table 4.
Table 4. Test Groups for Stage 3 of the Stimulated Weanling Validation: 
Coded Dose Studies

<table>
<thead>
<tr>
<th>Group</th>
<th>Agonist Coded Samples</th>
<th>Antagonist Coded Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Vehicle Control</strong></td>
<td><strong>Vehicle</strong></td>
</tr>
<tr>
<td><strong>Group A</strong></td>
<td><strong>Mandatory</strong> in agonist series</td>
<td><strong>Voluntary</strong> in antagonist series</td>
</tr>
<tr>
<td><strong>Vehicle Control</strong></td>
<td>Vehicle</td>
<td>Vehicle</td>
</tr>
<tr>
<td><strong>Group B</strong></td>
<td>Not applicable - provided by vehicle control (no additional group needed for agonist series)</td>
<td>Selected TP from stage 2</td>
</tr>
<tr>
<td><strong>Group C</strong></td>
<td>Coded Agonist Test substance #1*</td>
<td>Selected TP from stage 2 + Coded Antagonist Test substance #5*</td>
</tr>
<tr>
<td><strong>Group D</strong></td>
<td>Coded Agonist Test substance #2*</td>
<td>Selected TP from stage 2 + Coded Antagonist Test substance #6*</td>
</tr>
<tr>
<td><strong>Group E</strong></td>
<td>Coded Agonist Test substance #3*</td>
<td>Selected TP from stage 2 + Coded Antagonist Test substance #7*</td>
</tr>
<tr>
<td><strong>Group F</strong></td>
<td>Coded Agonist Test substance #4*</td>
<td>Selected TP from stage 2 + Coded Antagonist Test substance #8*</td>
</tr>
<tr>
<td><strong>Group G</strong></td>
<td>Not applicable</td>
<td>Selected TP from stage 2 + Coded Antagonist Test substance #9*</td>
</tr>
<tr>
<td><strong>Group H</strong></td>
<td>Not applicable</td>
<td>Selected TP from stage 2 + Coded Antagonist Test substance #10*</td>
</tr>
<tr>
<td><strong>Group F Positive Treatment Group</strong></td>
<td>TP dose selected from stage 2 <strong>Voluntary</strong></td>
<td>TP dose selected from stage 2 + FLU dose selected from stage 2 <strong>Voluntary</strong></td>
</tr>
</tbody>
</table>

* The doses of each test substance will be prescribed in order to have comparable data for the analysis of variability among labs and for comparison to previous data. The test substances in the agonist and antagonist coded samples may or may not be identical. Therefore, a sequential numbering is used across the series to avoid any suggestion that they might be the same.

OBSERVATIONS FOR ALL STAGES IN THE STIMULATED WEANLING VALIDATION

Clinical observations

51. Animals will be evaluated at least once daily for mortality, morbidity, and signs of injury as well as general appearance and signs of toxicity. Any animals in poor health will be identified for further monitoring.

52. 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 any apparent reasons for mortality.

Body weight and food consumption

53. Individual body weights will be recorded prior to start of treatment (to the nearest 1 g or to the nearest 0.1 g if that is normal practice of the laboratory with smaller animals), on each day of administration period and prior to necropsy. Group means and standard deviations will be calculated.
54. Food consumption should be generally observed and any significant changes recorded. It may be voluntarily recorded per cage, and an average value per animal calculated based upon the number of animals per cage.

**Necropsy**

55. Approximately 24 hours after the last administration of the test substance, the rats will be euthanized according to the normal procedures of the participating laboratory, and necropsy carried out. The method of humane killing will be recorded in the laboratory report.

56. The order in which the animals are necropsied will be designed such that one or two animals from each of the groups (e.g., one per cage if there are three animals per cage) are necropsied to achieve a randomization of the groups. In this way, all the animals in the same treatment group are not necropsied at once and any variation in the procedure over time will not unduly impact any particular group.

57. The four sex accessory tissues (VP, SV, LABC, COW) are mandatory measurements. Two additional tissue from the male reproductive tract, T and E, are optional measurements. All mandatory and optional tissues will be excised, carefully trimmed of excess adhering tissue and fat, and their fresh (unfixed) weights determined. Each tissue should be handled with particular care to avoid the loss of fluids and to avoid desiccation, which may introduce significant errors and variability by decreasing the recorded weights.

58. Several of the tissues may be very small or difficult to dissect, and this will introduce variability. Previous work has indicated a range of coefficient of variations that appears to differ based upon the proficiency of the laboratory. In a few cases, large differences in the absolute weights of the tissues such as the VP and COWS have been observed within a particular laboratory. Therefore, it is important that persons carrying out the dissection of the sex accessory tissues are familiar with standard dissection procedures for these tissues. A standard operating procedure (SOP) manual for dissection has been provided by the Lead Laboratory and was used in Phase-1 and Phase-2. This manual will remain the SOP reference for Phase-3. Careful training according to the SOP guide will minimize a potential source of variation in the study.

59. Each of the four mandatory sex accessory tissues as well as the paired testes and paired epididymes will be weighed without blotting to the nearest 0.1 mg, and the weights recorded for each identified animal.

60. Liver, paired kidney, and paired adrenal weights are other optional measurements. Again, tissues should be trimmed free of any adhering fascia and fat. The liver will be weighted to the nearest 0.1 g, and the paired kidneys and paired adrenals will be weighted to the nearest 0.1 mg. All weights will be recorded for each identified animal.

61. If the evaluation of each chemical requires necropsy of more animals than is reasonable for a single day, the starting date may be staggered on two consecutive days so that the necropsy can be staggered and the work burden of a single day reduced. In this case the work could be divided so that necropsy of 3 animals per treatment per day (1 cage) takes place on the first day with the dosing and necropsy being delayed by one day in the second half of the animals. That is, each group should be split so that half of the animals are necropsied on each day in order to control variability among the groups.

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

**REPORTING**

**Data**

63. Data will be reported individually (i.e. body weight, accessory sex tissue weights, optional measurements and other responses and observations) and for each group of animals (means and standard deviations). The data will be summarised in tabular form. The data will show the number of animals at the start of the test, the number of animals found dead during the test or found the test number of animals
found showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration and severity.

64. To assist data reporting and compilation, a standardised electronic spreadsheet will be used by participating laboratories to report and transmit data during the validation work to the Secretariat so that it may be easily exchanged and compiled with the Lead Laboratory and independent statisticians. This spreadsheet will be provided by the OECD Secretariat.

Test report

65. The test report must include the following information:

   Laboratory identification
   - Name of laboratory, location
   - Principal investigator and other personnel and their roles in the study
   - Dates study began and ended

   Test substance:
   - Physical nature and, where relevant, physicochemical properties;
   - Identification data and source
   - Purity

   Vehicle identity and supplier:

   Test animals and procedures:
   - Species/strain used;
   - Source or supplier of animals, including full address;
   - Number, age and sex of animals;
   - Housing conditions (temperature, lighting, and so on), diet used, lot of diet, source of diet, bedding and source of bedding;
   - Caging conditions and number of animals per cage;
   - Age at receipt, age at start of test substance administration and time of acclimatization;
   - Individual weights of animals at the start of the study (to nearest 0.1 g);
   - Randomization process and a record of the assignment to vehicle, reference, and test substance groups;
   - Mean and standard deviation of the body weights for each group at the start of the study;
   - Necropsy procedures, including means of exsanguination and any anesthesia; and

   Results:
   - Daily observations during administration, including:
     - Daily body weights (to the nearest 1 g),
     - Clinical signs (if any),
     - Test substance treatment (Yes or No) and the identify of that test substance,
     - Dose level and volume administered each day,
     - Time of dosing each day, and
     - Notes on food consumption or measurement of actual food consumption each day.
   - On the day of necropsy, individual necropsy data on each animal including absolute sex accessory tissue weights, liver and body weights including the following:
     - Date of necropsy,
– Animal ID,
– Home Cage Number or ID,
– Prosector,
– Time of day necropsy performed,
– Animal age, and
– Order of animal killing and dissection at necropsy,
– Weights of all four mandatory sex accessory tissues and glands.
  – Ventral prostate (fresh weight – to the nearest 0.1 mg),
  – Seminal vesicles plus coagulating glands, including fluid (fresh weight – paired, to nearest 0.1 mg),
  – Levator ani plus bulbocavernosus muscle (fresh weight - to nearest 0.1 mg),
  – Cowper’s glands (fresh weight – paired, to nearest 0.1 mg).
– Weights of additional male reproductive tissues.
  – Testes (fresh weight, paired, to nearest 0.1 mg),
  – Epididymes (fresh weight, paired, to nearest 0.1 mg),
– Weights of optional tissues, if performed.
  – Liver (optional – to nearest 0.1 g),
  – Kidney (optional – paired, to nearest 0.1 mg), and
  – Adrenal (optional – paired, to nearest 0.1 mg).

– General remarks and comments

Discussion

Conclusions

Statistics and Interpretation of results

66. Statistical comparisons will be made for the different mandatory sex accessory tissues, the optional male reproductive tissues, and other optional tissues. Statistical significance will be considered as present with p < 0.05. For androgen agonism, the test substance groups will be compared to the vehicle control. A statistically significant increase in tissue weight of the mandatory sex accessory tissues with the same tissue in the vehicle control will be considered consistent with the finding of a positive androgen agonist result. For androgen antagonism, the test substance with co-administered reference androgen groups will be compared to the reference androgen control. A statistically significant decrease in tissue weight of the mandatory sex accessory tissues versus the same tissue in the positive control TP group will be considered consistent with a positive antagonist result. Statistically significant changes, positive or negative, in the tissues other than the mandatory sex accessory tissues will be noted and considered to be characteristic for the test substance, but not evidence for androgen agonism or antagonism.
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