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No. 161, *Peer Review Report for the Validation of the Stably Transfected Transcriptional Activation Assay for the Detection Androgenic and Anti-Androgenic Activity of Chemicals (2011)*

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FOREWORD

This document presents the Peer Review Report (PRR) of the validation of the *Androgen Receptor Mediated Stably Transfected Transcriptional Activation (AR-STTA) assay to detect androgenic and anti-androgenic activities of chemicals*. It also includes a statement of the Working Group of National Coordinators of the Test Guidelines Programme on the follow-up to the PRR.

The project for developing a Test Guideline for an AR STTA assay was proposed by Japan and included in the work plan of the Test Guidelines Programme in 2008. A draft validation report was submitted to the Validation Management Group for non animal testing in December 2010. The PRR was endorsed by the Working Group of National Coordinators of the Test Guidelines Programme at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (Joint Meeting) agreed to its declassification on 5 October 2011.

This document is published under the responsibility of the Joint Meeting.

Agreement of the Working Group of the National Coordinators of the Test Guidelines Program on the Follow-up to the Validation Peer Review Report

The Validation Peer Review Report of the *Androgen Receptor Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities of Chemicals* was submitted for endorsement to the Working Group of National Coordinators of the Test Guidelines Program (WNT) at its April 2011 meeting.

Considering the major recommendations of the Peer Review Panel (summarized below), i.e.:

a) A dedicated inter-laboratory study should be carried out, using the final test protocol to test substances covering a broad range of activity, especially including non-active substances and weak agonists and antagonists. The number of substances already tested (five test chemicals) in the inter-laboratory validation, and the affinity range that they cover, is not sufficient;

b) The following discussion points should be added to the validation report:
   a. advantages of the AR-STTA assay over similar AR activation assays (i.e., lack of Glucocorticoid receptors in this cell line eliminates cross-talk with AR, and more discussion of positive results in AR-STTA that are negative in AR binding assays),
   b. potential interference of partial agonists with antagonist effects, and proposed solutions to elucidate such interference,
   c. potential impacts of differences between protocols used for the pre-validation and the inter-laboratory validation studies,
   d. the lack of a cytotoxicity measurement in the agonist assay, which masks identification of true negatives from false negatives;

- The protocol should be revised to:
   e. add acceptance and assessment criteria for the positive control (5α-Dihydrotestosterone (DHT)),
   f. precisely define the decision criteria for classification, especially considering cytotoxic effects (e.g. introduce the option of equivocal/not conclusive results, since cytotoxicity can interfere with the detection of androgenic and especially anti-androgenic responses),
   g. Explore the biological and statistical appropriateness of the PC10 in more detail,
   h. Include a list of proficiency chemicals for both the androgenic and the anti-androgenic assay,

the WNT agreed that, before finalizing the development of the draft Test Guideline for an AR-STTA assay,

- The Validation Management Group for non-animal testing should address the above recommendations as appropriate, in particular the recommendation to test more substances in a new inter-laboratory validation, while ensuring a good balance of substances with androgenic and anti-androgenic activity, negative and positive control substances;
- The cell line should be made freely available.

SUMMARY REPORT OF THE PEER REVIEW PANEL ASSESSING THE ANDROGEN RECEPTOR MEDIATED STABLY TRANSFECTED TRANSCRIPTIONAL ACTIVATION (AR-STTA) ASSAY TO DETECT ANDROGENIC AND ANTI-ANDROGENIC ACTIVITIES

Prepared for

Environment Directorate
Organisation for Economic Co-operation and Development (OECD) Paris

March 03, 2011

PREAMBLE

This document presents the summary report of the assessment of the validation of the Androgen Receptor Mediated Stably Transfected Transcriptional Activation (AR-STTA) in vitro assay to detect androgenic and anti-androgenic activities of chemicals by an independent Peer Review Panel.

The AR-STTA assay utilises the AR-EcoScreen™ cell line, which is derived from a Chinese hamster ovary derived cell line (CHO-K1). Three stable constructs have been inserted in this cell line. The constructs are a human AR expression construct (encoding the full-length human receptor), a firefly luciferase reporter construct bearing four tandem repeats of a prostate C3 gene-responsive element driven by a minimal heat shock protein promoter and a constitutive Renilla luciferase expression vector.

The assay is used to detect changes in the activity of the androgen receptor. The activated receptor translocates into the nucleus where it binds to specific DNA response elements and transactivates a luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer.

In order to better understand the purpose of the assay, the AR-STTA has to be considered in view of the OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals. This framework comprises the five levels

- sorting & prioritization based upon existing information (Level 1)
- *in vitro* assays providing mechanistic data (Level 2)
- *in vivo* assays providing data about single endocrine mechanisms and effects (Level 3)
- *in vivo* assays providing data about multiple endocrine mechanisms and effects (Level 4)
- *in vivo* assays providing data on effects from endocrine & other mechanisms (Level 5).

As a transcriptional activation (TA) assay providing mechanistic data, the AR-STTA is a Level 2 assay.

While several TA and receptor binding assay are currently about to enter or are undergoing validation exercises, only the "Stably Transfected Transcriptional Activation (TA) using HeLa-9903 cell line for detecting estrogenic activity of chemicals", the AR-STTA assay counterpart for estrogenic activities, has been adopted as an OECD test guideline (TG 455) in 2009. Indeed, the AR-STTA assay is the first androgenic activity detecting assay undergoing OECD peer review.

Summary Report of the Peer Review Panel assessing the Androgen Receptor Mediated Stably Transfected Transcriptional Activation (AR-STTA) assay to detect androgenic and anti-androgenic activities of chemicals

The peer review process

1. The Peer Review Panel (Panel) was formed in December 2010 in order to provide an independent review of the validation of the Androgen Receptor Mediated Stably Transfected Transcriptional Activation (AR-STTA) assay to detect androgenic and anti-androgenic activities of chemicals.

The work of the Panel was coordinated by a panel chair contracted by the OECD. In addition to experts invited by the Secretariat, potential Panel members were nominated by the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) and then approached by the chair. The members of the Panel are listed in Annex 1.

Furthermore, Dr. Masahiro Takeyoshi, from the Chemicals Evaluation Research Institute (CERI), Japan, who is familiar with the AR-STTA, was nominated to support the panel in case of open issues needing clarification.

2. The Panel was asked to evaluate the data collected on the test method, and to answer specific charge questions. These questions addressed the eight OECD validation criteria set out in the OECD Guidance Document. Panel members were asked to base their review on the document 'Draft Report of Pre-validation and Inter-laboratory Validation For Androgen Receptor (AR) Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities', provided as the file '100903_AR_Validation_Report_FINAL.doc'.

As background information, they were also provided with the OECD Guidance Document on the Validation and International Acceptance of New or Updated Methods for Hazard Assessment, Series on Testing and Assessment, Number 34, 2005 (last access on February 14, 2011, under http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2005)14&doclanguage=en).

The charge to the Panel was to assess to what extent the eight OECD validation criteria set out in the OECD Guidance Document had been met. The charge questions as provided to the Panel are listed in Annex 2. A summary of the Panel's responses to the individual questions is presented in paragraphs 6 to 44. For transparency, the individual comments from the Panel members are provided anonymously and in an edited form in Annex 3.

3. During the review process, the Panel held two teleconferences (December 16, 2010, and January 28, 2011) which were organised and coordinated by the chair. Furthermore, Dr. Masahiro Takeyoshi was contacted twice (January 17 and 28, 2011) in order to provide clarifications on issues raised by members of the Panel. The reply to the first inquiry, which comprised three issues, is provided in Annex 4a. This reply was discussed during the second teleconference. In this conference, further clarification on two aspects was requested. Again, Dr. Masahiro Takeyoshi was asked to provide help. The questions and the respective reply are provided in Annex 4b.
Each Panel member provided written responses on the charge questions to the Panel chair by February 11, 2011. Based on these responses, a draft report taking into account all individual comments was compiled by the chair and provided to the Panel for review and comments (February 15, 2011). Furthermore, it contained some clarifications on specific issues, which were added by the chair. The Panel commented on the draft report until February 22. Accounting for this feedback and resolving remaining open issues, the

final report was drafted by the chair and send to the PRP for approval on February 24. This report presents the resulting approved responses of the Panel to each of the charge questions.

General Panel responses

4. The reviewers acknowledged the work which has been carried out in order to validate the AR-STTA. The following general editorial issues were raised:

- The report should be updated to the current general situation, e.g. REACH came into force in 2007 (see paragraph 18).
- Tests under Reprotect are pre-validated only (see paragraph 18).
- Unnecessarily repetitions should be removed (see e.g. paragraph 30).
- The report should be edited (e.g. paragraph 78 is incomplete; see also the replies to charge question 3 in Annex 3).
- The report title indicated that the report is a draft, while the name of the provided file suggested a final version. It was assumed, that the report title was not up-to-date.

Panel responses to the charge questions: The eight OECD principles and criteria for test method validation

5. The PRP reached consensus on all eight charge questions.

Charge question 1: A rationale for the test method should be available, including a clear statement of scientific basis and the regulatory purpose and need for the test method

6. The Panel agreed that this criterion had been met.
The rationale for the test method is clearly stated with regard to the scientific basis and regulatory purpose. In addition, it was commented that the assay has been well-established and that the approach (using the Renilla luciferase reporter) is especially suitable.

Charge question 2: The relationship between the test method endpoint(s) and the biological effect and to the toxicity of interest should be described, addressing also limitations of the test methods

7. The Panel agreed that this criterion has been partly met.

8. Potential advantages of the AR-STTA assay as compared to similar assays should be described. In particular, more information on the lack of Glucocorticoid receptor (GR) in the cell line should be provided, especially in view of GR cross talk interference with AR, which has been described for other cell lines developed for the similar purposes. It is proposed that the AR-STTA may therefore be more robust favouring it over similar assays. Potential consequences when comparing AR-STTA assay results with results of other AR assays are addressed in paragraph 40.

9. Potential interference of partial agonists with antagonist effects has not been addressed and should be discussed, eventually proposing solutions to elucidate such interference.

10. As the transcriptional activation of endogenous genes *in vivo* might involve additional factors and mechanisms, an important limitation of the AR-STTA is that not necessarily all transcriptional responses *in vivo* are reflected. This aspect should be described.

11. The fact that the assay will not detect effects mediated by AR mechanisms that are independent of direct interactions with DNA / AREs, e.g. activation of intracellular signalling pathways by membrane-associated AR, should be addressed. In this context, the Renilla system should be discussed in the light of recent findings published in peer-reviewed literature. Firefly luciferase specific inhibitors that affect the catalytic activity of the enzyme have been described. In addition, chemicals might stimulate post-transcriptional effects, especially on protein stability, as has been shown for the "superinduction" of luciferase luminescence by genistein. In particular, AR-independent effects might have played a role in the effects observed for fluoranthene and medroxyprogesterone.

12. The robustness of viral promoter, such as SV40, should be discussed. As a potential consequence, mild cytotoxic effects might not be detected in the Renilla or firefly Luciferase cytotoxicity control.

13. A major limitation of the agonist assay is the lack of cytotoxicity measurements. As a consequence, true negative chemicals can hardly be discriminated from false negative due to masking by cytotoxicity, i.e. cytotoxicity is observed at concentrations below the concentration needed to elicit an agonistic response. A discussion of this aspect should be added, especially focusing on hydroxyflutamide and the use of the cytotoxicity data from the antagonist assay for the interpretation of the agonist assay.

14. Finally, limitations related to the substance Methyltrienolone (R1881) were identified. The availability of such an anabolic steroid might be very restricted due to potential import restrictions. Therefore, it does not qualify as a control, reference or proficiency chemical. As a potential replacement the substance mestanolone (MDHT) could be considered. Furthermore, the activity of R1881 might be discussed in view of the work of Hanson *et al.* (2007) in 'Frontiers in Bioscience 12, (1387-1394)'.

Charge question 3: A detailed protocol for the test method should be available

15. The Panel agreed that this criterion had been partly met.

16. Assessment/Acceptance criteria for the positive control DHT are lacking and should be added. In this context, it is suggested to include a graph of the expected DHT concentration-response curve in the protocol.

17. Regarding the data analysis, the applied approaches are likely to be unfamiliar to test laboratories. More guidance on the data analysis, e.g. by making a validated data analysis template publicly available, should be provided. In addition, it was recommended that in case of incomplete concentration-response curves, approximations of inhibiting and effective concentration should be clarified and improved. Furthermore, it should be clarified if other software besides GraphPad can be used.

18. The protocol used in the between-laboratory study differed from the protocol used in the pre-validation study. Nevertheless, the data have been combined for analysis. The potential impacts of protocol differences should be discussed, especially demonstrating, possibly supported by data, why these differences allow data combination. In particular, an explanation why the Renilla reporter was not used in the pre-validation should be provided.

19. The AR-EcoScreen cell line and especially the construct that was stably transfected to allow constitutive expression of the Renilla luciferase should, if possible, be described in more detail.

20. As substances were dissolved in ethanol, ethanol should be included in the protocol as a potential solvent.

21. It is recommended to propose proficiency chemicals for both the androgenic and the anti-androgenic assay.

22. The decision criteria to conclude on the classification should be more clearly defined. In this respect, cytotoxic effects should be considered in more detail. Since cytotoxicity can interfere with the detection of androgenic and especially anti-androgenic responses (see e.g. results of ketoconazole, DDT and methoxychlor) it is proposed to account for this, e.g. by introducing the option of equivocal/not conclusive results or by considering such substances as being outside of the applicability domain of these assays.

23. Testing concentration up to 10μM may not allow detection of weakly active compounds. Therefore testing up to higher concentrations, up to the solubility limit and up to the onset of cytotoxicity should be considered.

Charge question 4: Within- and between-laboratory reproducibility of the test method should be demonstrated

24. The Panel agreed that this criterion had been partly met.

25. Regarding the between-laboratory reproducibility, the amount of substances and the affinity range covered by these substances was not appropriate. Assuming that potential candidate substances are available, more substances should be tested. Additional substances should cover a broad affinity range, especially including weak agonists and antagonists as well as negative substances, and substances with incomplete concentration-response curves. Some reviewers signalled willingness to support the selection of such substances.

26. An explanation should be provided why data on cytotoxicity for the substance R1881 are lacking.

27. Different concentrations of test substances have been tested in the pre-validation and the inter-laboratory study. An explanation for this difference should be provided.

Charge question 5: Demonstration of the test method's performance should be based on testing of representative, preferably coded reference chemicals

28. The Panel agreed that this criterion had been partly met.

29. Too few substances that did not appropriately cover the entire range of possible responses of the AR-STTA assay were tested for between-laboratory reproducibility. For example, in the agonist assay only the structurally similar substances DHT, i.e. the positive control, and R1881 have been tested in several laboratories. Re-enforcing the recommendations of paragraph 24, the panel is of the opinion that it will be inevitable to test additional carefully selected substances in a dedicated inter-laboratory study. In this regard it should be noted that this potential shortcoming has already been identified in paragraph 15 of the reviewed validation report '... though the number of chemicals used under multi-laboratory validation study may not be sufficient.' Furthermore, additional testing is considered necessary in order to allow proposing a list of proficiency chemicals, as proficiency chemicals should be supported by a sound and broad database.
In regard of the number of chemicals to be tested, the general issue of the in practice less stringent requirements for *in vivo* assays than *in vitro* methods was raised. For example, while for the inter-laboratory reproducibility assessment of an *in vivo* assay a limited number of chemicals might be

considered sufficient, usually a higher number of chemicals is expected for the inter-laboratory reproducibility assessment of an *in vitro* assay. This difference is considered to be driven essentially by animal welfare consideration and, if at all, only marginally by scientific reasoning. As the scientific requirements for in vitro and in vivo test should be similar, but in practice are not, this constitutes a fundamental obstacle for an objective review of validation studies in a general framework that equally addresses *in vivo* as well as *in vitro* tests.

Charge question 6: The performance of test methods should have been evaluated in relation to existing relevant toxicity data as well as information from the species of concern

30. The Panel agreed that this criterion had been partly met.

31. AR-binding data should be discussed in more detail, especially focusing on substances with positive responses in the AR-STTA assay that are either not detectable in the *in vitro* binding assay for technical reasons (comparison with published binding data), e.g. for vinclozolin or nonylphenol, or mediated by other mechanisms than direct AR-binding. The presented discussion is not convincing due to differences in maximum concentrations used in these assays.

32. Inconsistencies in the presented data have been found, e.g. in Paragraph 116 and Table 14 it is stated that 4-Hydroxytamoxifen was, despite significant binding to the AR receptor, not detected as an AR agonist or AR antagonist. However in Table 8, it is listed as an antagonist in the AR-STTA assay.

33. The ICCVAM list is several years old and consequently not necessarily up-to-date. Furthermore, some classifications are questionable, especially for antagonists. It is recommended, if possible, to update the results presented here, e.g. when the ICCVAM list is revised.

34. The assay is not discussed in relation to the Hershberger assay. Comparison with the Hershberger assay and/or a discussion of problems in the comparison to the Hershberger is proposed.

35. The biological relevance of PC10, especially in combination with no concentration-response, as well as its statistical significance is questioned. The use of a more pronounced PC that potentially better balances biological relevance and statistical significance should be explored in more detail, taking into account that comparison with the ICCVAM list and with AR binding data needs further discussion (see paragraphs 8, 31, 33 and 41). .

36. The approach taken to calculate EC50 and IC50, i.e. irrespective of the completeness of the concentration-response curve, is questioned. Calculation based on comparison to the concentration-response of the positive control (i.e. DHT) expressed as percentage of control should be explored.

37. For the antagonist assay lin.IC30 was used in the validation study. The reason for using lin.IC30 is not clear, as, e.g. in Table 11, no differences between lin.IC50 or lin.IC30 are apparent.

38. The presentation of the data, data analyses and interpretation should be improved and be consistent throughout the report. In particular, the information if data are based on single or several experiments should be provided. Furthermore, it should be clarified if the agonist and antagonist experiments were performed in parallel or independent of each other.

39. Negative classification in the agonist assay because of insufficient induction of the luciferase activity, although EC50 values can be calculated, should be discussed in more detail considering that low inductive activities at low concentrations could still be biologically significant.