BG1Luc ER TA Test Method: Results of an International Validation Study and Proposed Performance Standards

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Summary

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently convened an international peer review panel to assess the validation status of the BG1Luc ER TA test method (also known as the LUMI-CELL assay). The BG1Luc ER TA test method uses transactivation of an estrogen-responsive luciferase reporter gene in human ovarian cancer cells to assess compounds for in vitro estrogen agonist and antagonist activity. This test is intended to be used as one component of a multi-test screening strategy described in US EPA's Endocrine Disruptor Screening Program (EDSP), and it offers potential benefits over the existing method, OPPTS 890.1300. BG1Luc ER TA is the only method validated to assess ER TA in vitro activity up to the 1 mM limit currently required in the US EPA's EDSP, and it is the only ER TA method to be validated for the detection of anti-estrogenic substances. We will provide an overview of the validation report and discuss performance standards that may be applicable to the development of similar test methods.

Keywords: validation, BG1Luc ER TA, LUMI-CELL assay, endocrine disruptor

1 Introduction

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has completed its evaluation of the validation status of the LUMI-CELL BG1Luc4E2 ER TA test method (hereafter, BG1Luc ER TA test method) as a screening test to identify substances with *in vitro* estrogen receptor agonist and antagonist activity. A test method evaluation report has been published (ICCVAM, 2011a), providing ICCVAM's recommendations for the BG1Luc ER TA test method based on the results of an international validation study and the demonstrated validity (usefulness and limitations). The report includes (1) protocols recommended by ICCVAM for future data collection and evaluation for the BG1Luc ER TA test method, (2) a background review document (BRD) describing the validation status of this test method, (3) recommendations for future studies, and (4) performance standards to evaluate functionally and mechanistically similar test methods.

Xenobiotics Detection Systems, Inc. (XDS, Durham, NC) nominated the BG1Luc ER TA test method to ICCVAM for an interlaboratory validation study. This test method uses BG-1 cells (a human ovarian carcinoma cell line) that are stably transfected with an estrogen-responsive luciferase reporter gene to measure whether and to what extent a substance induces or inhibits TA activity via ER mediated pathways. ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) recommended that the BG1Luc ER TA should be considered a high priority for interlaboratory validation studies, given the lack of adequately validated test methods and the regulatory and public health need for such test methods. The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) subsequently led and coordinated an international validation study with its counterparts in Europe (the European Centre for the Validation of Alternative Methods) and Japan (the Japanese Center for the Evaluation of Alternative Methods) to assess the accuracy and reliability of the BG1Luc ER TA test method for the qualitative detection of substances with *in vitro* ER agonist or antagonist activity. Following completion of this study, NICEATM, ICCVAM, and the ICCVAM Endocrine Disruptor Working Group (EDWG) prepared a draft BRD and draft test method recommendations. The drafts were provided to an independent international scientific peer review panel (hereafter Panel) and to the public for comment. The Panel met in public session on March 29-30, 2011 to discuss its peer review of the ICCVAM draft BRD and to provide conclusions and recommendations regarding the validation status of the BG1Luc ER TA test method. The Panel also reviewed how well the information contained in the draft BRDs supported ICCVAM's draft test method recommendations (ICCVAM, 2011b).

In finalizing this test method evaluation report and the BRD, ICCVAM considered the conclusions and recommendations of the Panel, comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and public comments.

2 Results

The BG1Luc ER TA was evaluated for its ability to correctly identify *in vitro* estrogen receptor agonists and antagonists. For this analysis, test substance classification (positive or negative for ER agonist/antagonist activity) obtained during the validation study was compared to the ICCVAM reference classification of the same substance based on a preponderance of available data.

Test method accuracy – agonist assay
Test method accuracy was evaluated based on several different analyses, but the primary evaluation of the BG1 Luc ER TA accuracy is based on two comparisons: (1) the extent to which the BG1 Luc ER TA result corresponds to the ICCVAM reference classification for each substance and (2) the comparative accuracy of the BG1 Luc ER TA and the EPA's OPPTS 890.1300/OECD TG 455 assay.

A total of 35 substances (28 positive, 7 negative) were used for the accuracy evaluation of the BG1 Luc ER TA agonist assay. When using the consensus classification obtained from all BG1 Luc ER TA tests for these 35 substances, the following statistics were obtained: concordance of 97% (34/35), sensitivity of 96% (27/28), specificity of 100% (7/7), a false positive rate of 0% (0/7), and a false negative rate of 4% (1/28). Similar results were obtained when all results from each laboratory were used instead of the consensus classification.

The EPA's OPPTS 890.1300/OECD TG 455 is the only test guideline published by a U.S. regulatory agency for generating ER TA data. Therefore, BG1 Luc ER TA concordance with the EPA OPPTS 890.1300/OECD TG 455 was also evaluated using the 26 reference substances for which both BG1 Luc ER TA and EPA OPPTS 890.1300/OECD TG 455 data are available, and identical accuracy statistics were calculated: concordance of 96% (25/26), sensitivity of 95% (21/22), specificity of 100% (4/4), a false positive rate of 0% (0/4), and a false negative rate of 5% (1/22).

Test method accuracy – antagonist assay
A total of 25 substances (3 positive, 22 negative) were used to evaluate the accuracy of the BG1 Luc ER TA antagonist assay. When using the consensus classification obtained from all BG1 Luc ER TA tests for these 25 substances, the following statistics were obtained: concordance of 100% (25/25), sensitivity of 100% (3/3), specificity of 100% (22/22), a false positive rate of 0% (0/22), and a false negative rate of 0% (0/3). Because there currently is not a valid EPA OPPTS 890.1300/OECD TG 455 antagonist protocol, no comparison to the BG1 Luc ER TA antagonist results was conducted.

Concordance with other ED assays
Although the primary goal of the BG1Luc ER TA is to provide a qualitative assessment of estrogenic/anti-estrogenic activity, quantitative measures of activity (i.e., EC_{50} and IC_{50} values) are usually obtained for positive results. EC_{50} and IC_{50} values obtained from BG1Luc ER TA test results were compared to median values from other ER TA test methods reported in the literature, and this comparison produced a high correlation. There was 97% (33/34) concordance between the BG1Luc ER TA and ER binding data. The only discordant substance (medroxy-progesterone acetate) was positive in BG1 Luc ER TA and negative based on ER binding data. Similarly, based on a comparison with available data in the *in vivo* uterotrophic assay, there was 92% (12/13) concordance between the BG1Luc ER TA and ER binding data. The only discordant substance (butylbenzyl phthalate) was positive in BG1 Luc ER TA and negative based on uterotrophic data.

Test method reliability
Intralaboratory reproducibility of the BG1Luc4E2 agonist and antagonist test methods was assessed by comparing reference standard and control results for all plates tested within each laboratory during the course of the validation study, as well as results from Phase 2a and 2b testing, during which 12 substances were tested in at least three independent experiments in each of the three laboratories.

The resulting classifications for each of the 12 substances that were tested at least three times at each laboratory were used to evaluate the extent of intralaboratory agreement. Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the three repeat tests for both agonist and antagonist protocols.

Interlaboratory reproducibility was determined for the 12 substances that were tested at least three times for agonist and antagonist activity during Phase 2, as well as classifications for each of the 41 substances that were tested once for agonist and antagonist activity at all three laboratories during Phase 3.

For each of the 12 Phase 2 substances, agreement among the three laboratories was determined based on the consensus classification assigned by each laboratory for each of the 12 substances. The three laboratories agreed on 67% (8/12) of the substances tested for agonist activity. There was 100% agreement among the three laboratories for all 12 substances tested for antagonist activity.

Five of the 41 Phase 3 substances produced inadequate results that could not be considered in the evaluation. Among the 36 remaining substances that produced a definitive agonist test result in at least two laboratories, there was 100% agreement. All 41 substances produced definitive results for antagonist activity; the three laboratories agreed on 93% (38/41) of these substances.

Performance standards
Based on the results of this study, NICEATM and the ICCVAM Interagency Endocrine Disruptors Working Group (EDWG) developed performance standards, available at http://iccvam.niehs.nih.gov/methods/endocrine/BG1-PS/PerfStnds-27Mar2011v2.pdf, which are applicable to methods that are functionally and mechanistically similar to the BG1Luc ER TA. These performance standards can also be used by naïve laboratories to demonstrate technical proficiency in performing the BG1Luc ER TA.

In order to be considered functionally and mechanistically similar to the BG1Luc ER TA, a modified ER TA test method protocol must include the following components to ensure that the same biological effect is being measured:

- The test method should be based on a cell line that endogenously expresses ER.
- Reference standards, controls, and test substances should be dissolved in a solvent that is miscible with cell culture media at concentrations that are not cytotoxic and that do not otherwise interfere with the test system.
- The maximum test substance concentration should be 1 mM for ER TA agonist testing and 10 μM for ER TA antagonist testing unless otherwise limited by solubility, cytotoxicity, or other mechanisms that interfere with assay performance.
- A minimum of seven concentrations spaced at logarithmic (log10) intervals, up to the limit concentration, should be tested.
- An evaluation of cytotoxicity should be included and only data from concentrations at or above 80% viability should be used for data analyses.
- A reference estrogen and a reference anti-estrogen should be used to demonstrate the adequacy of the test method for detecting ER TA agonist and antagonist activity.
- The ability of the reference estrogen to induce ER TA activity and the reference anti-estrogen to inhibit ER TA activity should be demonstrated by generating a full concentration-response curve in each experiment that provides a minimum 3-fold estrogenic induction and a minimum 3-fold anti-estrogenic reduction.
- A set of concurrent controls should be included. For agonist assays, this would include the vehicle control and a weak agonist. For antagonist assays, this would include the vehicle control, weak antagonist, and reference estrogen.
- Test method protocols should incorporate the essential components listed above. Modifications should be detailed and scientifically justified, and the modified test method should perform as well as or better than the BG1Luc ER TA.

When evaluated using the reference substances listed in the performance standards document (http://iccvam.niehs.nih.gov/methods/endocrine/BG1-PS/PerfStnds-27Mar2011v2.pdf), the reliability and accuracy (i.e., sensitivity, specificity, false positive rate, and false negative rate) of a proposed ER TA test method should have accuracy and reliability characteristics that are equivalent to or exceed those of the BG1Luc ER TA test method. Although it is not realistic to expect identical results, any differences should be discussed in terms of the test method's ability to detect a similar range of potencies and chemical/product classes.

3 Discussion

The ICCVAM evaluation process incorporates a scientific peer review and a high level of transparency. The evaluation process for the BG1Luc ER TA test method included a public review meeting by an independent scientific peer review panel, multiple opportunities for public comments, and comments from the SACATM. ICCVAM and the Endocrine Disruptor Working Group considered the Panel report, SACATM comments, and all public comments before finalizing the ICCVAM test method evaluation report and final BRD for the BG1Luc ER TA test method. Based on all of the above, ICCVAM concluded that the accuracy and reliability of the BG1Luc ER TA test method protocols support their use as screening tests to identify substances with *in vitro* estrogen receptor agonist and/or antagonist activity. ICCVAM also concluded that the accuracy of this assay is at least equivalent to the current ER TA included in regulatory testing guidance, i.e., EPA's OPPTS 890.1300/OECD TG 455.

References

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