Appendix I

BG1LUC4E2 ER TA TA Phase I Protocol Amendments

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Amendment I

BG1LUC4E2 ER TA Assay Validation Study Design and Work Plan
Assessment of Cell Viability

Amendment:

The amendment modifies the current Validation Study Design Work Plan that requires the comparison of two methods for assessing cell viability (i.e., visual observation and CellTiter-Glo®). This amendment will determine the feasibility of limiting the assessment of cell viability to the visual observation method only. If all three labs demonstrate proficiency with the visual observation method during Phase I, CellTiter-Glo® testing, which is currently scheduled for Phase IIa, will no longer be necessary (see Rationale outlined below). This would result in testing 24 fewer plates than are currently proposed for Phase II. This proposal was discussed and agreed to in principle during the 15 May 2007 BG1LUC4E2 ER TA Assay Validation Study Management Team (SMT) teleconference. If the above is confirmed, reference to the CellTiter-Glo® method will be deleted from BG1LUC4E2 ER TA Assay protocols.

Rationale for Amendment:

A comparison of the visual observation and CellTiter-Glo® methods for assessing cell viability was conducted during the BG1LUC4E2 ER TA Assay Protocol Standardization Study. Results of the comparison were evaluated and presented in a supplementary report entitled "Quantitative versus Qualitative Assessment of Cell Viability in the BG1LUC4E2 ER TA Assay". Following a discussion during its 16 November 2006 teleconference, the SMT concluded that the qualitative visual observation method was similar to that of the quantitative CellTiter-Glo® method for establishing a cytotoxic dose of a test substance. The SMT agreed that the visual observation method was both an accurate and practical method for assessing cell viability in the BG1LUC4E2 ER TA Assay, particularly considering that using the CellTiter-Glo® method necessitates running concurrent parallel plates.

Assessment of Proficiency with the Visual Observation Method:

Each laboratory will demonstrate proficiency with the visual observation method by conducting three independent tests with bisphenol A during Phase I. Double serial dilutions will be made from a stock solution of 10 mg/mL bisphenol A to yield a series of six final concentrations ranging from 3.125 to 100 µg/mL. The general procedures described in **Section 9** (Plating of BG1Luc4E2 Cells) and **Section 10** (Preparation of Test Substance) of the BG1LUC4E2 ER TA Assay Agonist Protocol will be followed. These concentrations of bisphenol A have previously been shown to induce a complete range of cytotoxicity (i.e., Categories 1, 2, 3, and 4 as defined in the BG1LUC4E2 ER TA Assay Visual Observation Cell Viability Manual). These concentrations would be tested independently in triplicate wells of a 96-well plate during three separate experiments, and following the general procedures in **Section 13** (Comprehensive Testing) of the BG1LUC4E2 ER TA Assay Agonist Protocol.

Exposed BG-1 cells from each test plate will be assessed for cell viability with an inverted phase contrast microscope at 100X as per the procedures in the BG1LUC4E2 ER TA Assay Visual Observation Cell Viability Manual. Digital photomicrographs of all exposed cells will be recorded and provided with the cytotoxicity scoring information. Photomicrographs and corresponding cytotoxicity scores will be reviewed by NICEATM and the validation study "Lead Laboratory" to determine if proficiency with the visual observation method has been demonstrated by each laboratory.

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Amendment II

BG1LUC4E2 ER TA Assay Validation Study Modifications of Test Plate Designs for Agonist and Antagonist Range Finder and Comprehensive Testing

Amendment:

The amendment modifies the current BG1LUC4E2 ER TA Assay validation study test plate designs to use all wells of 96 well agonist and antagonist test plates for range finder and comprehensive testing. The modifications are based on the BG1LUC4E2 ER TA Assay Validation Study Management Team (SMT) review of results from testing conducted at Xenobiotic Detection Systems, Inc. (the validation study Lead Laboratory) that demonstrated that differences in values between inner and outer wells in range finder testing did not impact selection of the appropriate starting concentration for comprehensive testing and did not impact calculation of EC_{50} values in comprehensive testing.

Rationale for Amendment:

Range finder testing in the BG1LUC4E2 ER TA agonist assay is used to select the starting concentration for the comprehensive testing of substances being evaluated for estrogenic activity. The plate layout for reference standards and the different controls used for agonist range finder testing in the Protocol Standardization Study limited testing to logarithmic (log) serial dilutions for five substances, with each concentration tested in a single well only. However, this methodology resulted in studies where the selection of the starting concentration to be used for comprehensive testing was problematic. To minimize this problem in future studies, the study design for agonist range finder testing was made more robust by testing duplicates of each test substance concentration. However, this change resulted in a reduction in the number of substances that could be tested on a single plate when using the standard plate configuration which excluded using outer wells. In order to increase efficiency, the plate designs were modified to use all 96 wells to run reference standards, controls and test substances. To evaluate whether using the outer wells would bias the data due to so-called "edging effects"[1], bisphenol A (Chemical Abstract Services Registry Number [CASRN] 80-05-7 [BPA]) was tested over a seven-point logarithmic serial dilution concentration range (100 µg/mL – 1 $x10^{-4}$ µg/mL) in each plate column using the modified plate design. Results of this testing demonstrated that, although there are statistical differences between the level of relative light unit values in the outer and inner wells, these differences do not impact selection of the appropriate starting concentration for comprehensive testing.

To increase the efficiency of comprehensive testing, it was proposed that the plate layouts for agonist comprehensive testing also be revised to use all 96 wells. To evaluate whether using the outer wells would bias the data due to edging effects, serial dilutions of BPA were tested in plate columns using inside and outside wells and BPA EC_{50} values derived from replicates using outside wells were compared to EC_{50} values derived from replicates using inside wells. The comparison demonstrated that there were no significant differences between EC_{50} values derived from replicates using inside wells and those derived from outside wells. This allows for the testing of 11-point double serial dilutions of two substances in triplicate, instead of only one substance as would occur if the original plate configuration was used.

Following a discussion during its 25 July 2007 teleconference, the SMT agreed to modify the BG1LUC4E2 ER TA Assay validation study test plate designs to use all wells of 96 well agonist and antagonist test plates for range finder and comprehensive testing.

[1] "Edging" or "edge" effects refer to differences in the RLU detected on a plate between the outer (n = 36) and inner (n = 60) wells of a 96-well plate. These differences are thought to result from differences in vapor pressure between the two sets of wells (Nagy 2002, Oliver 1989).

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