Final Report

In Vitro Microwell Micronucleus Assay in Chinese Hamster Ovary (CHO) Cells with 2463608

Study:
Covance 7608-550
Genetic Toxicology Assay No. 29269-0-460GLP

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Quality Assurance Statement

In Vitro Microwell Micronucleus Assay in Chinese Hamster Ovary (CHO) Cells

This report has been reviewed by the Quality Assurance Unit of Covance Laboratories Inc. and accurately reflects the raw data. The following study specific inspections were conducted and findings reported to the study director (SD) and associated management.

Inspection Dates			Date Reported to SD and SD Management
From	To	Phase	
09 Jul 2007	09 Jul 2007	Protocol Review	09 Jul 2007
25 Jul 2007	25 Jul 2007	Test Article Administration	25 Jul 2007
09 Sep 2007	11 Sep 2007	Draft Report and Data Review	12 Sep 2007
17 Oct 2007	17 Oct 2007	Protocol Amendment Review	17 Oct 2007
17 Oct 2007	17 Oct 2007	Draft to Final Report Review	17 Oct 2007

Jeannie Nasca 18 Oct 07

Jeannie Nasca, Representative
Quality Assurance Unit
Covance Laboratories Inc.

Date

Compliance Statement

In Vitro Microwell Micronucleus Assay in Chinese Hamster Ovary (CHO) Cells

Compound: 2463608 Study: Covance 7608-550

This study, except as noted below, conformed to the following Good Laboratory Practice Standards in place at the time of study initiation.

United States Food and Drug Administration (CFR 21 - Part 58)
Organisation for Economic Co-operation and Development

Meeting the previously stated requirements satisfies the Bilateral Agreement with Japan.

Exceptions:
1. Concentrations of the dose preparations and the stability of the test article in the vehicle were not determined.
2. The historical controls presented in the report are not GLP compliant.
3. The end of study potency was not GLP compliant.

Hemalatha Murli 19 Oct. 2007

Hemalatha Murli, PhD
Study Director
Genetic and Molecular Toxicology
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Date

Testing Facility Management:

19 Oct. 2007

Timothy E. Lawlor, MA
Associate Director
Genetic and Molecular Toxicology
Covance Laboratories Inc.

Date

Sponsor Signature Page

I have reviewed the data and agree with the interpretation of the data presented.

Amy L. Gam
Study Monitor
Eli Lilly and Company

10 October 2007
Date

Summary

In Vitro Microwell Micronucleus Assay in Chinese Hamster Ovary (CHO) Cells

Study: Covance 7608-550

The objective of this study was to assess the capability of the test article, 2463608, to induce clastogenicity/aneugenicity in Chinese hamster ovary cells (CHO-WBL) by measuring the extent of micronucleus formation.

In the dose range-finding assay, the CHO-WBL cells were treated for ~4 hours in the presence and absence of S9 metabolic activation and ~23 hours in the absence of metabolic activation, and cultures were harvested ~24 hours after initiation of treatment. Cytotoxicity was assessed by the appearance of the cultures in each well and by cell cycle kinetics. Doses tested in the micronucleus assay were selected based on the results of a dose rangefinding assay.

In the micronucleus assay, the CHO-WBL cells were treated for ~4 hours in the presence and absence of S9 metabolic activation and ~23 hours in the absence of metabolic activation, and cultures were harvested ~24 hours after initiation of treatment. The doses selected for evaluation were 75.0, 150, 225, and 550 mcg/mL in the ~4-hour trial in the presence of S9 metabolic activation, 75.0, 150, 225, and 300 mcg/mL in the ~4-hour trial in the absence of S9 metabolic activation, and 12.5, 37.5, and 75.0 mcg/ml in the ~23-hour trial in the absence of S9 metabolic activation. Statistical significance was observed at test article concentrations of 150 mcg/mL in the ~4-hour exposure period in the presence of metabolic activation and 225 mcg/mL in the ~4-hour exposure period in the absence of metabolic activation. However, these responses were not dose dependent and were within the historical control range for the vehicle controls. Therefore, these responses are not biologically relevant.

The test article, 2463608, was considered negative for inducing micronuclei under the conditions of the assay.

Study Conduct

Objective

The objective of this i*n vitro* study was to assess the capability of 2463608 to induce clastogenicity/aneugenicity in Chinese hamster ovary cells (CHO-WBL) by measuring the extent of micronucleus formation. This methodology is consistent with recent recommendations of the *In Vitro* Micronucleus Working Group (IWGT) as described in the reference by Kirsch-Volders, *et al*. (2003) and has been used to formulate draft OECD guidance.

Study Timetable

Study Initiation Date	29 June 2007
Experimental Start Date	10 July 2007
Experimental Completion Date	04 September 2007
Study Completion Date	19 October 2007

Protocol Adherence

This study was conducted in accordance with the protocol and amendment(s) (Appendix A), with the exception of the deviation(s) noted in Appendix A. The deviation(s) did not affect the integrity or interpretability of the results of the study.

Regulatory Guidelines

The assay design was based on the proposed OECD Guideline 487, 2nd Version, 21 December 2006.

Major Computer Systems

System[a]	Application Function
EMCS	Monitors and documents facility storage conditions (e.g., refrigerators, freezers, constant room temperatures and humidity levels)
EMCSDR	Transfers data from EMCS for reporting purposes
Program Trend	Statistical analysis
MTTS	Test article accessioning and dispensing

a All version numbers of the applications are maintained by Covance. Definitions for the acronyms can be found in the Glossary.

Record Retention

All raw data, documentation, records, the protocol and the final report generated as a result of this study will be stored in the Covance-Vienna archives for at least three years following finalization of the report. The Covance archives staff will contact the sponsor after at least three years following the report finalization to determine disposition of the archived materials (except for raw data on durable media, study correspondence, protocol, and final report which will be kept by Covance-Vienna). The Sponsor will authorize the transport of the materials to their site (or that of their designee), or authorize the transport of the materials to the archive facilities of EPL Archives, Inc., Sterling, VA (EPL). Covance staff will have access to materials archived at EPL for continued research and/or regulatory audit. All raw data, documentation, records, original reports generated as a result of work performed at Eli Lilly as a part of this study will be archived by Eli Lilly.

Test and Control Articles

The test article was supplied by the Sponsor as a white crystalline powder on 03 July 2007 and identified as follows.

Test article	Lot No.	Storage	Theoretical Potency	Actual Potency
2463608	KD0-E01100-039-C	At room temperature	100.0%	>99%

In the assays without metabolic activation, negative controls were cultures which contained only cells and culture medium. Vehicle controls were cultures containing dimethlysulfoxide (DMSO), the vehicle for the test article, at 10.0 mcL/mL, the highest concentration used in test cultures. In the assays with metabolic activation, negative and vehicle controls were the same as described in the assays without metabolic but with the S9 (Sigma, Lot Nos. 2061 and 2090) and energy producing system (S9 activation mix) included. The vehicle control article was supplied as follows.

Control Article	CAS No.	Supplier	Lot Nos.	Storage	Purity	Expiration Date
Dimethylsulfoxide	67-68-5	Acros Organics	00437DH and 04934ME	At room temperature	NP	Jun 2009, Jan 2009

NP = Not provided

The positive control agents which were used in the assays were mitomycin C (MMC) for the assays without metabolic activation and cyclophosphamide (CP) in the assays with metabolic activation. Mitomycin C is a clastogen that does not require metabolic activation. Cyclophosphamide does not act directly but must be converted to active intermediates by microsomal enzymes. In the micronucleus assays, MMC (0.300 mcg/mL) and CP (5.00 mcg/mL) were used to induce micronuclei. The positive control articles were supplied as follows.

Control Article	CAS No.	Supplier	Lot No.	Storage	Expiration Date
Mitomycin C (MMC)	50-07-7	Sigma	075K0479	Frozen at -10 to -30°C	30 Apr 2009
Cyclophosphamide (CP)	6055-19-2	Sigma	036K1225	Frozen at -10 to -30°C	01 Aug 2008

Information on synthesis methods, stability, purity, composition, or other characteristics that define the test article and control article components is on file with the Sponsor or the respective manufacturer(s).

Test System Rationale

The assay is designed to establish whether the test article(s) or metabolites can interact with cells to inhibit the cell cycle and/or induce micronuclei. Cells grown in the presence of cytochalasin B are capable of undergoing a nuclear mitosis but the cytokinesis portion of cell division is inhibited (Fenech and Morley, 1985). Using this cytokinesis block method, the result of a single cell division is a binucleated daughter cell rather than a normal pair of daughter cells (each with one nucleus). In this way, the number of nuclei within a cell indicates the number of divisions the cell has undergone in the presence of cytochalasin B.

Scoring the frequency of cells with one, two or more nuclei allows the estimation of the average generation time of the cells. The cytokinesis block method used in this test system has demonstrated the ability to detect clastogenic agents in cells that have undergone one cell division by scoring binucleated cells for micronuclei (Ciaravino et al., 1993).

Chemically induced lesions may result in chromosome breaks that are either repaired by the cell or result in damage. If the induced lesions are acentric fragments (without a centromere), they fail to migrate to either of the daughter nuclei and persist in the cytoplasm as micronuclei. Numerical changes (aneuploidy) also can be induced and result in failed migration of entire chromosomes that can also present as micronuclei. Thus, micronuclei are indicators of chromosome damage (or mitotic apparatus damage) and when scored in binucleated cells represent clastogenic (or aneugenic) damage generated prior to or during the previous metaphase.

Materials

The experimental procedures used for this study are presented in further detail in Appendix A.

Dose Range-Finding Study

The dose range-finding assay was conducted with an ~4-hour exposure to the test article in the absence and presence of the S9 metabolic activation system. In addition, an ~23-hour exposure in the absence of S9 was also conducted. Eight single concentrations of the test article were used for each of the three exposure conditions. In the absence of any toxicity information, the high dose should have been above the observed solubility limit in culture medium or at 10 mM or 5 mg/mL (whichever is lower) for soluble test articles. Successively lower doses were determined by using 50% of the preceding dose.

Based upon the dose range-finding study results and after consultation with the Sponsor, seven to eleven test article concentrations (in quadruplicate) per exposure condition were selected for the definitive micronucleus assay.

Slide Scoring

All wells of each slide were assessed for scorability (sufficient number of cells) by low magnification microscopic examination of the amount of stained material present. The frequency of CHO-WBL cells in each scorable well on each slide containing one, two, or more than two nuclei in 500 cells (when possible) were assessed to determine the vehicle control, negative control, and test article effect on the cell cycle kinetics.

Assessment of Toxicity

The Cytochalasin B proliferation index (CBPI) in both treated and control cultures were determined by using the following formula and expressed in tabular fashion as a percent and as a percent of the vehicle control:

$$\frac{1 \times \text{the number of mononucleated cells} + 2 \times \text{the number of binuclueated cells} + 3 \times \text{the number of tri- and tetranucleated cells}}{\text{total number of cells scored}}$$

Since toxicity was observed, at least three analyzable test concentrations with no more than a $\sqrt{10}$-fold spacing (square root of 10-fold spacing) between the

concentrations were tested, with the highest concentration exhibiting approximately 50% toxicity (if achievable). The micronucleus assay was performed using quadruplicate cultures for each treatment condition and concentration (2 wells per concentration or control on each of two slides).

Micronucleus Assay

Without S9 Activation

Exponentially proliferating CHO-WBL cells were used to seed chamber slides. The cells were grown in a humidified incubator at 37 ± 2°C in an atmosphere of 5% ± 1.5% CO_2 in air. One day after culture initiation, the culture medium was aspirated and fresh complete medium containing the vehicle control, negative control or MMC at 0.300 mcg/mL, or a selected concentration range of test article was added to the wells. The cultures were then returned to the incubator for an ~4-hour exposure period. After the ~4-hour treatment period, the treatment medium was removed by aspiration and the cells rinsed with PBS and replenished with complete McCoy's 5A medium containing cytochalasin B (3 mcg/ml). The cells were then returned to the incubator for an additional ~19 hours. Approximately 1 hour prior to harvest, the cultures were rinsed with PBS, refed with McCoy's 5A medium, and returned to the incubator for an additional hour.

An additional group of cultures were treated in the presence of cytochalasin B (3 mcg/ml) without metabolic activation with the vehicle control, negative control, MMC at 0.300 mcg/mL or a selected concentration range of the test article for a period of ~23 hours. After the ~23-hour treatment time, the treatment medium was removed by aspiration. The cultures were rinsed with PBS, refed with McCoy's 5A medium, and returned to the incubator for an additional hour.

Assay with S9 Activation

Exponentially proliferating CHO-WBL cells were used to seed chamber slides. The cells were grown in an incubator at 37C ± 2°C in an atmosphere of 5% ± 1.5% CO_2 in air. One day after culture initiation, the culture medium were aspirated and either fresh complete medium containing the vehicle and untreated controls plus the S9 metabolic activation mixture, the test article plus the S9 metabolic activation mixture, or CP at 5.00 mcg/mL plus the S9 metabolic activation mixture were added to the appropriate wells. The cultures were then returned to the incubator for an ~4-hour exposure period. After the ~4-hour treatment period, the treatment medium was removed by aspiration and the cells rinsed with PBS and replenished with complete McCoy's 5A medium containing cytochalasin B (3 mcg/ml). The cells were then returned to the incubator for an additional ~19 hours. Approximately 1

hour prior to harvest, the cultures were rinsed with PBS, refed with McCoy's 5A medium, and returned to the incubator for an additional hour.

Slide Scoring

All wells of each slide were assessed for scorability (sufficient number of cells) by low magnification microscopic examination of the amount of stained material present. The frequency of CHO-WBL cells in each scorable well on each slide containing one, two, or more than two nuclei in 500 cells (when possible) were assessed to determine the vehicle control, negative control, test and positive control articles effect on the cell cycle kinetics. From these data, the Cytochalasin B Proliferation Index (CBPI) was calculated for each treatment concentration divided by 500 by classifying 500 consecutive cells as either mononucleated, binucleated or > binucleated. The CBPI was expressed as a percentage.

The highest dose level that demonstrated an approximate 50% reduction in the percent of binucleated cells relative to the vehicle controls and at least two lower dose levels spanning the range from moderately cytotoxic to noncytotoxic were selected for scoring when possible. In the absence of a 50% reduction in the percent of binucleated cells throughout the tested dose range, the highest dose level tested and the next two or three lower nontoxic dose levels were selected for scoring precluding excessive compound precipitate. If precipitate is excessive such that accurate scoring is precluded, the lower concentration showing signs of precipitate and 2-3 lower concentrations were scored in the initial definitive assay. One thousand binucleated cells/slide (500 per chamber/slide, when possible) from the dose levels selected for scoring are then scored in a blinded fashion by the microscopist for the presence of micronuclei. The criteria for scoring micronuclei are: 1.) the diameter of the micronuclei must be approximately 1/3 or less the diameter of the main nucleus, 2.) the micronuclei must be non-refractile and located in the cytoplasm and 3) the binucleates have non-overlapping nuclei with distinct borders.

Data

Data Analysis

The frequency of micronuclei per 2000 total binucleated cells (when possible) were generated for the specified test article concentration from all four of the quadruplicate wells.

Criteria for a Valid Test

Before assay data will be evaluated, all criteria for a valid assay must be met. The following criteria will be used to determine a valid assay.

Spontaneous Frequency of MN-BN

The frequency BN-MN for the vehicle control cultures must be within historical ranges.

Positive Control Values

The positive control articles must induce a statistically significant increase ($p < 0.05$) in the frequency of MN-BN, as compared to the concurrent vehicle controls.

Statistical Analyses

Statistical analysis will employ a Fisher-Irwin exact test for pair wise comparisons between each treated and vehicle control cultures (Thakur et al., 1985). One-sided tail probabilities will be used to evaluate statistical significance ($p < 0.05$). A chemical will be concluded to be positive if a statistically significant increase in the frequency of micronucleated cells is obtained from at least one test article concentration compared to the concurrent vehicle control and the linear trend test confirms a positive dose-response (alpha level = 0.05).

Assay Evaluation Criteria

Once criteria for a valid assay have been met, responses observed in the assay are evaluated as follows:

Criteria for a Positive Response

A test article will be considered to have produced a positive response if it induces a statistically significant, dose-dependent increase in the frequency of MN-BN.

Criteria for a Negative Response

A test article will be considered to have produced a negative response if no statistically significant or dose dependent increases in the frequency of MN-BN are observed.

Criteria for an Equivocal Response

Despite extensive testing, a test article may produce results that are neither clearly positive nor clearly negative. In those rare instances (e.g., statistically significant or dose dependent increases), the test article may be considered to have produced equivocal responses.

Other criteria also may be used in reaching a conclusion about the study results (e.g., comparison to historical control values, etc.). In such cases, the Study Director will clearly report and describe such any considerations.

Results

In the dose range-finding assay (Trial 1, Tables 1 and 2), the CHO-WBL cells were treated for ~4 hours in the presence and absence of S9 metabolic activation and ~ 23 hours in the absence of metabolic activation, and cultures were harvested ~24 hours after initiation of treatment. The doses tested under all three conditions were 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, and 500 mcg/mL. Cytotoxicity was assessed by the appearance of the cultures in each well and by cell cycle kinetics. Cytotoxicity was observed at ≥125 mcg/mL in the ~4-hour exposure period in the asence of metabolic activation, at ≥62.5 mcg/mL in the ~4-hour exposure period in the absence of metabolic activation, and at ≥15.6 mcg/mL in the ~23-hour exposure period in the absence of metabolic activation.

Doses tested in the micronucleus assay (Trial 2, Tables 3 and 4) were selected based on the results of the dose rangefinding assay. In the micronucleus assay the treatment period was for ~4 hours with and without metabolic activation and ~23 hours in the absence of metabolic activation, and cultures were harvested ~24 hours after initiation of treatment. The doses tested in the ~4 hour assay in the presence of metabolic activation were 5.00, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, and 75.0 mcg/mL. The doses tested in the ~4 hour assay in the absence of metabolic activation were 5.00, 10.0, 20.0, 30.0, 40.0, 50.0, and 60.0 mcg/mL. The doses tested in the ~23 hour assay in the absence of metabolic activation were 1.25, 2.50, 5.00, 10.0, 20.0, 30.0, 40.0, and 50.0 mcg/mL. Due to lack of toxicity, the micronucleus assay was repeated in Trial 3.

Doses tested in the repeat of the micronucleus assay (Trial 3, Tables 5 and 6) were selected based on the results of Trials 1 and 2. In the repeat micronucleus assay the treatment period was for ~4 hours with and without metabolic activation and ~23 hours in the absence of metabolic activation, and cultures were harvested ~24 hours after initiation of treatment. The doses tested in the ~4 hour assay in the presence of metabolic activation were 4.70, 9.40, 18.8, 37.5, 75.0, 150, 225, 300, 400, 550, and 700 mcg/mL. The doses tested in the ~4 hour assay in the absence of metabolic activation were 4.70, 9.40, 18.8, 37.5, 75.0, 150, 225, 300, 400, 550, and 700 mcg/mL. The doses tested in the ~23 hour assay in the absence of metabolic activation were 3.75, 7.50, 12.5, 25.0, 37.5, 50.0, 75.0, 115, and 150 mcg/mL. The doses selected for evaluation in the ~4-hour definitive trial were 75.0, 150, 225, and 550 mcg/mL in the presence of metabolic activation, 75.0, 150, 225, and 300 mcg/mL in the ~4-hour trial in the absence of metabolic activation, and 12.5, 37.5, and 75.0 mcg/ml in the ~23-hour trial in the absence of metabolic activation. The high doses selected for analysis demonstrated an ~60 reduction in CBPI and/or were observed to have precipitate. Statistical significance was observed at test article concentrations of 150 mcg/mL in the ~4-hour exposure period in the

presence of metabolic activation and 225 mcg/mL in the ~4-hour exposure period in the absence of metabolic activation. However, these responses were not dose dependent and were within the historical control range for the vehicle controls. Therefore, these responses are not biologically relevant.

All criteria for a valid study were met.

Conclusion

The test article, 2463608, was considered negative for inducing micronuclei under the conditions of the assay with and without metabolic activation.

References

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OECD (2006) "*In Vitro* Micronucleus Test," *OECD Guidelines for Testing of Chemicals*, Draft Guideline 487, 2nd Version, 21 December 2006.

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Table 1 Results of the *In Vitro* Microwell Micronucleus Controls

Assay Number: 29269-0-460GLP	Trial: 1	Date: 11 July 2007			Lab Number: CY070907A	
Test Article: 2463608	Cell Cycle Kinetics					
Treatment	1N	2N	>2N	Total	CBPI[a]	Cytotoxicity[b]
S9 ACTIVATED (4 hour exposure)					Time in CYB (hours): 19.0	
Negative Control: McCoy's 5a						
	16	162	22	200	2.03	--
	24	150	26	200	2.01	2.42
	12	167	21	200	2.05	--
Vehicle Control: DMSO (10.0 mcL/mL)						
	11	170	19	200	2.04	--
	9	170	21	200	2.06	--
	18	163	19	200	2.01	2.90
Totals:	38	503	59	600	2.04	--
NONACTIVATED (4 hour exposure)					Time in CYB (hours): 19.0	
Negative Control: McCoy's 5a						
	23	164	13	200	1.95	--
	14	170	16	200	2.01	3.06
	22	159	19	200	1.99	--
Vehicle Control: DMSO (10.0 mcL/mL)						
	10	181	9	200	2.00	--
	17	175	8	200	1.96	2.55
	12	178	10	200	1.99	--
Totals:	39	534	27	600	1.98	--
NONACTIVATED (23 hour exposure)					Time in CYB (hours): 23.0	
Negative Control: McCoy's 5a						
	16	179	5	200	1.95	10.71
	10	178	12	200	2.01	4.57
	13	171	16	200	2.02	4.09
Vehicle Control: DMSO (10.0 mcL/mL)						
	9	170	21	200	2.06	--
	15	158	27	200	2.06	--
	8	173	19	200	2.06	--
Totals:	32	501	67	600	2.06	--

N = Nuclei BN = Binucleated cells McCoy's 5a = culture medium DMSO = Dimethylsulfoxide CYB = Cytochalasin B

a The Cytochalasin B proliferation index (CBPI) is calculated using the following formula: CBPI = ((Cells with 1N x 1) + (Cells with 2N x 2) + (Cells with greater than 2N x 3))/Total Cells

b Cytotoxicity = 100-[100*(CBPI Test Article - 1)/(CBPI Vehicle - 1)]

Table 2 Summary Results of the *In Vitro* Microwell Micronucleus Assay

Assay Number: 29269-0-460GLP		Trial: 1	Date: 11 July 2007		Lab Number: CY070907A		
Test Article: 2463608		Cell Cycle Kinetics					
Treatment		1N	2N	>2N	Total	CBPI[a]	Cytotoxicity[b]
S9 ACTIVATED (4 hour exposure)					Time in CYB (hours): 19.0		
Vehicle Control: DMSO (10.0 mcL/mL)							
(Totaled)		38	503	59	600	2.04	--
Test Article							
3.91	mcg/mL	22	159	19	200	1.99	4.83
7.81	mcg/mL	29	156	15	200	1.93	10.14
15.6	mcg/mL	54	133	13	200	1.80	23.19
31.3	mcg/mL	90	109	1	200	1.56	46.38
62.5	mcg/mL[c]	140	57	3	200	1.32	69.57
125	mcg/mL[c]	188	12	0	200	1.06	94.20
250	mcg/mL[c]	168	31	0	199	1.16	84.95
500	mcg/mL[d]	180	19	1	200	1.11	89.86
NONACTIVATED (4 hour exposure)					Time in CYB (hours): 19.0		
Vehicle Control: DMSO (10.0 mcL/mL)							
(Totaled)		39	534	27	600	1.98	--
Test Article							
3.91	mcg/mL	29	161	10	200	1.91	7.65
7.81	mcg/mL	37	156	7	200	1.85	13.27
15.6	mcg/mL	61	124	15	200	1.77	21.43
31.3	mcg/mL	130	64	6	200	1.38	61.22
62.5	mcg/mL[c]	168	27	5	200	1.19	81.12
125	mcg/mL[c]	176	24	0	200	1.12	87.76
250	mcg/mL[c]	181	19	0	200	1.10	90.31
500	mcg/mL[d]	189	11	0	200	1.06	94.39

N = Nuclei BN = Binucleated cells McCoy's 5a = culture medium DMSO = Dimethylsulfoxide CYB = Cytochalasin B

a The Cytochalasin B proliferation index (CBPI) is calculated using the following formula:

CBPI = ((Cells with 1N x 1) + (Cells with 2N x 2) + (Cells with greater than 2N x 3))/Total Cells

b Cytotoxicity = 100-[100*(CBPI Test Article - 1)/(CBPI Vehicle - 1)]

c Precipitate observed at wash and harvest.

d Precipitate observed at dose, wash, and harvest.

e Precipitate observed at harvest.

Table 2 (continued) Summary Results of the *In Vitro* Microwell Micronucleus Assay

Assay Number: 29269-0-460GLP		Trial: 1	Date: 11 July 2007			Lab Number: CY070907A	
Test Article: 2463608		Cell Cycle Kinetics					
Treatment		1N	2N	>2N	Total	CBPI[a]	Cytotoxicity[b]
NONACTIVATED (23 hour exposure)					Time in CYB (hours): 23.0		
Vehicle Control: DMSO (10.0 mcL/mL)							
(Totaled)		32	501	67	600	2.06	--
Test Article							
3.91	mcg/mL	43	149	8	200	1.83	22.05
7.81	mcg/mL	89	109	2	200	1.57	46.61
15.6	mcg/mL	155	40	5	200	1.25	76.38
31.3	mcg/mL	157	41	2	200	1.23	78.74
62.5	mcg/mL[e]	189	11	0	200	1.06	94.80
125	mcg/mL[e]	191	8	1	200	1.05	95.28
250	mcg/mL[e]	184	16	0	200	1.08	92.44
500	mcg/mL[d]	195	5	0	200	1.03	97.64

N = Nuclei BN = Binucleated cells McCoy's 5a = culture medium DMSO = Dimethylsulfoxide CYB = Cytochalasin B

[a] The Cytochalasin B proliferation index (CBPI) is calculated using the following formula:

CBPI = ((Cells with 1N x 1) + (Cells with 2N x 2) + (Cells with greater than 2N x 3))/Total Cells

[b] Cytotoxicity = 100-[100*(CBPI Test Article - 1)/(CBPI Vehicle - 1)]

[c] Precipitate observed at wash and harvest.

[d] Precipitate observed at dose, wash, and harvest.

[e] Precipitate observed at harvest.

Table 3 Results of the *In Vitro* Microwell Micronucleus Controls

Assay Number: 29269-0-460GLP	Trial: 2	Date: 25 July 2007			Lab Number: CY072607A
Test Article: 2463608	Cell Cycle Kinetics				
Treatment	1N	2N	>2N	Total	CBPI[a]
S9 ACTIVATED (4 hour exposure)					
Negative Control: McCoy's 5a	318	1255	430	2003	2.06
Vehicle Control: DMSO 10.0 mcL/mL	232	2803	965	4000	2.18
Positive Control: CP 5.00 mcg/mL	497	1395	108	2000	1.81
NONACTIVATED (4 hour exposure)					
Negative Control: McCoy's 5a	136	1368	496	2000	2.18
Vehicle Control: DMSO 10.0 mcL/mL	234	2752	1014	4000	2.20
Positive Control: MMC 0.300 mcg/mL	468	1384	148	2000	1.84
NONACTIVATED (23 hour exposure)					
Negative Control: McCoy's 5a	68	1431	501	2000	2.22
Vehicle Control: DMSO 10.0 mcL/mL	198	2785	1017	4000	2.20
Positive Control: MMC 0.300 mcg/mL	440	1407	153	2000	1.86

N = Nuclei BN = Binucleated cells McCoy's 5a = culture medium DMSO = Dimethylsulfoxide CP = Cyclphosphamide MMC = Mitomycin C CYB = Cytochalasin B

[a] The Cytochalasin B proliferation index (CBPI) is calculated using the following formula:

CBPI = ((Cells with 1N x 1) + (Cells with 2N x 2) + (Cells with greater than 2N x 3)) /Total Cells

Table 4 Summary Results of the *In Vitro* Microwell Micronucleus Assay

Assay Number: 29269-0-460GLP		Trial: 2	Date: 25 July 2007			Lab Number: CY072607A	
Test Article: 2463608		Cell Cycle Kinetics					
Treatment		1N	2N	>2N	Total	CBPI[a]	Cytoxicity[b]
S9 ACTIVATED (4 hour exposure)					Time in CYB (hours): 19.0		
Vehicle Control: DMSO (Totaled)		232	2803	965	4000	2.18	--
Test Article							
5.00	mcg/mL	57	720	223	1000	2.17	--
10.0	mcg/mL	54	712	234	1000	2.18	--
20.0	mcg/mL	60	700	240	1000	2.18	--
30.0	mcg/mL	55	709	236	1000	2.18	--
40.0	mcg/mL	77	721	202	1000	2.13	--
50.0	mcg/mL	72	727	201	1000	2.13	0.98
60.0	mcg/mL	87	708	205	1000	2.12	1.49
75.0	mcg/mL	123	673	199	995	2.08	3.42
NONACTIVATED (4 hour exposure)					Time in CYB (hours): 19.0		
Vehicle Control: DMSO (Totaled)		234	2752	1014	4000	2.20	--
Test Article							
5.00	mcg/mL	77	664	259	1000	2.18	0.82
10.0	mcg/mL	76	671	253	1000	2.18	1.05
20.0	mcg/mL	100	658	242	1000	2.14	2.64
30.0	mcg/mL	67	661	272	1000	2.21	--
40.0	mcg/mL	75	685	240	1000	2.17	1.59
50.0	mcg/mL	98	710	192	1000	2.09	4.82
60.0	mcg/mL	141	665	194	1000	2.05	6.68
NONACTIVATED (23 hour exposure)					Time in CYB (hours): 23.0		
Vehicle Control: DMSO (Totaled)		198	2785	1017	4000	2.20	--
Test Article							
1.25	mcg/mL	48	694	258	1000	2.21	--
2.50	mcg/mL	77	666	257	1000	2.18	0.09
5.00	mcg/mL	70	682	248	1000	2.18	0.18
10.0	mcg/mL	66	684	250	1000	2.18	--
20.0	mcg/mL	34	686	280	1000	2.25	--
30.0	mcg/mL	27	697	283	1007	2.25	--
40.0	mcg/mL	122	661	217	1000	2.10	3.99
50.0	mcg/mL	598	358	44	1000	1.45	33.73

N = Nuclei BN = Binucleated cells McCoy's 5a = culture medium DMSO = Dimethylsulfoxide CP = Cyclphosphamide MMC = Mitomycin C CYB = Cytochalasin B

[a] The Cytochalasin B proliferation index (CBPI) is calculated using the following formula: CBPI = ((Cells with 1N x 1) + (Cells with 2N x 2) + (Cells with greater than 2N x 3))/Total Cells

[b] Cytotoxicity = 100-[100*(CBPI Test Article - 1)/(CBPI Vehicle - 1)]

Table 5 Results of the *In Vitro* Microwell Micronucleus Assay Controls

Assay Number: 29269-0-460GLP Trial: 3 Date: 15 August 2007 Lab Number: 081507 Test Article: 2463608

Treatment	Cell Cycle Kinetics 1N	2N	>2N	Total	CBPI[a]	MNBN 1 Slide	MNBN 2 Slide	MNBN Total	Total BN Scored	% MNBN[b]
S9 ACTIVATED (4 hour exposure)									Time in CYB (hours): 19.0	
Negative Control:	McCoy's 5a									
	97	1542	361	2000	2.13	8	15	23	4000	0.58
Vehicle Control:	DMSO									
10.0 mcL/mL	151	2997	852	4000	2.18	32	53	85	8000	1.06
Positive Control:	CP									
5.00 mcg/mL	234	1604	162	2000	1.96	148	184	332	4000	8.30*
NONACTIVATED (4 hour exposure)									Time in CYB (hours): 19.0	
Negative Control:	McCoy's 5a									
	91	1576	333	2000	2.12	16	25	41	4000	1.03
Vehicle Control:	DMSO									
10.0 mcL/mL	188	2992	820	4000	2.16	41	48	89	8000	1.11
Positive Control:	MMC									
0.300 mcg/mL	230	1621	149	2000	1.96	231	229	460	4000	11.50*

N = Nuclei BN = Binucleated cells MNBN = Binucleated cells with micronuclei CP = Cyclophosphamide
MMC = Mitomycin C McCoy's 5a = culture medium DMSO = Dimethylsulfoxide

[a] The Cytochalasin B proliferation index (CBPI) is calculated using the following formula:
CBPI = ((Cells with 1N x 1) + (Cells with 2N x 2) + (Cells with greater than 2N x 3))/Total Cells

[b] * The %MNBN is statistically increased compared to the concurrent vehicle control at $p \leq 0.05$.

Table 5 (continued) Results of the In Vitro Microwell Micronucleus Assay Controls

Assay Number: 29269-0-460GLP Trial: 3 Date: 15 August 2007 Lab Number: 081507 Test Article: 2463608

	Cell Cycle Kinetics					MNBN	MNBN	MNBN	Total BN	%
Treatment	1N	2N	>2N	Total	CBPI[a]	1 Slide	2 Slide	Total	Scored	MNBN[b]
NONACTIVATED (23 hour exposure)									Time in CYB (hours): 23.0	
Negative Control:	McCoy's 5a									
	51	1229	720	2000	2.33	23	16	39	4000	0.98
Vehicle Control:	DMSO									
10.0 mcL/mL	122	2478	1400	4000	2.32	42	64	106	8000	1.33
Positive Control:	MMC									
0.300 mcg/mL	312	1576	112	2000	1.90	307	215	522	4000	13.05*

N = Nuclei BN = Binucleated cells MNBN = Binucleated cells with micronucleiCP = Cyclophosphamide
MMC = Mitomycin C McCoy's 5a = culture medium DMSO = Dimethylsulfoxide

[a] The Cytochalasin B proliferation index (CBPI) is calculated using the following formula:
CBPI = ((Cells with 1N x 1) + (Cells with 2N x 2) + (Cells with greater than 2N x 3))/Total Cells

[b] * The %MNBN is statistically increased compared to the concurrent vehicle control at $p \leq 0.05$.

Table 6 Summary Results of the *In Vitro* Microwell Micronucleus Assay

Assay Number: 29269-0-460GLP		Trial: 3			Date: 15 August 2007		Lab Number: 081507			Test Article: 2463608	
		Cell Cycle Kinetics					%CBPI	MNBN	MNBN	MNBN	BN	%
Treatment		1N	2N	>2N	Total	CBPI[a]	Reduction	1 Slide	2 Slide	Total	Scored	MNBN[b]
S9 ACTIVATED (4 hour exposure)										Time in CYB (hours): 19.0		
Vehicle Control:												
(Totaled)		151	2997	852	4000	2.18	--	32	53	85	8000	1.06
Test Article												
4.70	mcg/mL	38	781	181	1000	2.14	1.83					
9.40	mcg/mL	47	781	172	1000	2.13	2.29					
18.8	mcg/mL	54	787	159	1000	2.11	3.21					
37.5	mcg/mL	109	758	133	1000	2.02	7.34					
75.0	mcg/mL	401	544	55	1000	1.65	24.3	7	14	21	2000	1.05
150	mcg/mL	566	392	42	1000	1.48	32.1	15	27	42	2000	2.10*
225	mcg/mL	656	322	22	1000	1.37	37.2	9	15	24	2000	1.20
300	mcg/mL[c]	876	122	2	1000	1.13	48.2					
400	mcg/mL[c]	876	124	1	1001	1.13	48.2					
550	mcg/mL[c]	910	90	0	1000	1.09	50.0	17	12	29	2000	1.45
700	mcg/mL[c]	938	62	0	1000	1.06	51.4					

N = Nuclei BN = Binucleated cells MNBN = Binucleated cells with micronuclei VC = Vehicle control
DMSO = Dimethylsulfoxide

[a] The Cytochalasin B proliferation index (CBPI) is calculated using the following formula:
CBPI = ((Cells with 1N x 1) + (Cells with 2N x 2) + (Cells with greater than 2N x 3))/Total Cells

[b] * The %MNBN is statistically increased compared to the concurrent vehicle control at $p \leq 0.05$.

[c] Precipitate observed at dose, wash and harvest.

Table 6 (continued) Summary Results of the *In Vitro* Microwell Micronucleus Assay

Assay Number: 29269-0-460GLP	Trial: 3	Date: 15 August 2007	Lab Number: 081507	Test Article: 2463608

Treatment	Cell Cycle Kinetics 1N	2N	>2N	Total	CBPI[a]	% CBPI Reduction	MNBN 1 Slide	MNBN 2 Slide	MNBN Total	BN Scored	% MNBN[b]
NONACTIVATED (4 hour exposure)									Time in CYB (hours): 19.0		
Vehicle Control:											
(Totaled)	188	2992	820	4000	2.16	--	41	48	89	8000	1.11
Test Article											
4.70 mcg/mL	28	759	213	1000	2.19	--					
9.40 mcg/mL	47	764	189	1000	2.14	0.926					
18.8 mcg/mL	70	848	82	1000	2.01	6.94					
37.5 mcg/mL	372	586	42	1000	1.67	22.7					
75.0 mcg/mL	505	466	29	1000	1.52	29.6	13	16	29	2000	1.45
150 mcg/mL	722	247	33	1002	1.31	37.4	19	11	30	2000	1.50
225 mcg/mL	576	379	45	1000	1.47	31.9	20	16	36	2000	1.80*
300 mcg/mL[c]	888	112	0	1000	1.11	48.6	7	19	26	1796	1.45
400 mcg/mL[c]	969	31	0	1000	1.03	52.3					
550 mcg/mL[c]	935	65	0	1000	1.07	50.5					
700 mcg/mL[c]	941	59	0	1000	1.06	50.1					

N = Nuclei BN = Binucleated cells MNBN = Binucleated cells with micronuclei VC = Vehicle control
DMSO = Dimethylsulfoxide

[a] The Cytochalasin B proliferation index (CBPI) is calculated using the following formula:
CBPI = ((Cells with 1N x 1) + (Cells with 2N x 2) + (Cells with greater than 2N x 3))/Total Cells

[b] * The %MNBN is statistically increased compared to the concurrent vehicle control at $p \leq 0.05$.

[c] Precipitate observed at dose, wash and harvest.

Table 6 (continued) Summary Results of the *In Vitro* Microwell Micronucleus Assay

Assay Number: 29269-0-460GLP		Trial: 3		Date: 15 August 2007		Lab Number: 081507		Test Article: 2463608			
		Cell Cycle Kinetics					% CBPI	MNBN	MNBN	MNBN	BN	%
Treatment		1N	2N	>2N	Total	CBPI[a]	Reduction	1 Slide	2 Slide	Total	Scored	MNBN[b]
NONACTIVATED (23 hour exposure)										Time in CYB (hours): 19.0		
Vehicle Control:												
(Totaled)		122	2478	1400	4000	2.32	--	42	64	106	8000	1.33
Test Article												
3.75	mcg/mL	12	643	345	1000	2.33	--					
7.50	mcg/mL	119	726	155	1000	2.04	12.1					
12.5	mcg/mL	442	522	36	1000	1.59	31.5	20	14	34	2000	1.70
25.0	mcg/mL	763	229	8	1000	1.25	46.1					
37.5	mcg/mL	778	225	1	1004	1.23	47.0	11	23	34	2000	1.70
50.0	mcg/mL	746	228	26	1000	1.28	44.8					
75.0	mcg/mL	834	155	11	1000	1.18	49.1	12	15	27	2000	1.35
115	mcg/mL	905	94	1	1000	1.10	52.6					
150	mcg/mL	884	114	2	1000	1.12	51.7					

N = Nuclei BN = Binucleated cells MNBN = Binucleated cells with micronuclei VC = Vehicle control
DMSO = Dimethylsulfoxide

[a] The Cytochalasin B proliferation index (CBPI) is calculated using the following formula:
CBPI = ((Cells with 1N x 1) + (Cells with 2N x 2) + (Cells with greater than 2N x 3))/Total Cells

[b] * The %MNBN is statistically increased compared to the concurrent vehicle control at $p \leq 0.05$.

Appendix A: Protocol, Protocol Amendment and Protocol Deviation

COVANCE
THE DEVELOPMENT SERVICES COMPANY

Protocol

Test Facility: Covance Laboratories Inc. 9200 Leesburg Pike Vienna, VA 22182	Sponsor: Eli Lilly and Company Lilly Corporate Center Indianapolis, IN 46285
Study Type:	In Vitro Microwell Micronucleus Assay in Chinese Hamster Ovary (CHO) Cells
Compound:	2463608 (Test Article: 2463608)
Lot Number:	KD0-E01100-0390C
Theoretical Potency:	100.0%
Covance Study Number:	7608-550
GT Assay Number	29269-0-460GLP
Protocol ID:	460GLP, Edition 1
Proposed Experimental Start Date:	10 July 2007
Proposed Experimental Termination Date:	10 August 2007
Proposed Audited Draft Report Date:	24 August 2007
Proposed Final Report Date:	24 September 2007

Compliance

This study conforms to the US Food and Drug Administration (CFR 21 - Part 58) and Organisation for Economic Cooperation and Development (OECD) Good Laboratory Practice (GLP) standards in place at the time of study initiation with the following exceptions: the concentrations of the test article preparations and the stability and homogeneity of the test article in the vehicle are not determined. Conformance to these GLP standards satisfies the Bilateral Agreement with Japan.

This protocol, at least one critical phase of the work in progress, and the final report will be subject to audit by Quality Assurance in accordance with Standard Operating Procedures maintained at Covance. The study will be conducted by Covance at 9200 Leesburg Pike, Vienna, Virginia 22182 (Covance-Vienna).

Test Article Analysis

Determination and documentation of the identity, strength, purity, stability and uniformity of the test article, as defined in the Good Laboratory Practice (GLP) regulations, is the responsibility of the Sponsor. The Sponsor should provide these test article characterization data (a Certificate of Analysis or equivalent) for review by the Study Director and inclusion in the final report.

Notification of Regulatory Submission

In order to comply with GLP regulations, consulting laboratories must be notified if all or part of a study is intended for regulatory submission. Covance maintains a master schedule of studies which fall under regulatory review. Please indicate which agency, if any, might receive the results of this study:

☐ Undetermined ☒ FDA ☐ EPA-TSCA ☐ EPA-FIFRA
☐ MHLW ☒ OECD (proposed)

Meeting the previously stated requirements satisfies the Bilateral Agreement with Japan.

Approval of Study Protocol

Study Director

Hemalatha Murli 29 June 2007

Hemalatha E. Murli, PhD
Study Director
Covance Laboratories Inc.

Date

Testing Facility Management

29. JUN. 2007

Timothy E. Lawlor, MA
Associate Director
Covance Laboratories Inc.

Date

The final version of the protocol was approved by the Study Monitor for Study Director signature on 25 June 2007.

Study Monitor

Amy L. Garn
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Objective

The objective of this study is to assess the capability of a test article to induce clastogenicity/aneugenicity in Chinese hamster ovary cells (CHO-WBL) by measuring micronucleus formation. This methodology is consistent with recent recommendations of the *In Vitro* Micronucleus Working Group (IWGT; Kirsch-Volders, *et al.*, 2003), as well as OECD Draft Guideline 487 (OECD, 2006).

Test System Rationale

The assay is designed to establish whether the test article(s) or metabolites can interact with cells to inhibit the cell cycle and/or induce micronuclei. Cells grown in the presence of cytochalasin B are capable of undergoing a nuclear mitosis but the cytokinesis portion of cell division is inhibited (Fenech and Morley, 1985). Using this cytokinesis block method, the result of a single cell division is a binucleated daughter cell rather than a normal pair of daughter cells (each with one nucleus). In this way, the number of nuclei within a cell indicates the number of divisions the cell has undergone in the presence of cytochalasin B.

Scoring the frequency of cells with one, two or more nuclei allows the estimation of the average generation time of the cells. The cytokinesis block method used in this test system has demonstrated the ability to detect clastogenic agents in cells that have undergone one cell division by scoring binucleated cells for micronuclei (Ciaravino et al., 1993).

Chemically induced lesions may result in chromosome breaks that are either repaired by the cell or result in damage. If the induced lesions are acentric fragments (without a centromere), they fail to migrate to either of the daughter nuclei and persist in the cytoplasm as micronuclei. Numerical changes (aneuploidy) also can be induced and result in failed migration of entire chromosomes that can also present as micronuclei. Thus, micronuclei are indicators of chromosome damage (or mitotic apparatus damage) and when scored in binucleated cells represent clastogenic (or aneugenic) damage generated prior to or during the previous metaphase.

Materials

Test System

The CHO-WBL cell line was derived from an ovarian biopsy of a female Chinese hamster. Cells to be used in this assay were originally obtained from Dr. S. Wolff, University of California (San Francisco, CA). The cells were subsequently subcloned in this laboratory, and stock cultures are stored in liquid nitrogen.

Media and Cell Culture Conditions

CHO-WBL cells will be grown in complete McCoy's 5A culture medium [McCoy's 5A supplemented with 10 % (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL

penicillin G, and 100 µg/mL streptomycin], under standard conditions (37°C ± 2°C in a humidified atmosphere of 5% ± 1.5% CO_2 in air). Cultures for use in the micronucleus assay will be prepared in microwell slides by seeding approximately 5 – 10 x 10^3 exponentially growing CHO-WBL cells in complete McCoy's 5A medium (on Day -1; the actual number seeded will be determined by the laboratory) and incubating overnight prior to treatment.

Test For Mycoplasma Contamination

Mycoplasma tests are performed by a commercial laboratory or in-house. Stock cultures are maintained for up to eight weeks after thawing from a frozen stock. Testing will be performed on cells twice during this period, to verify that there is no mycoplasma during the life of the culture. The direct culturing method and the indirect Hoechst staining method are used by the commercial laboratory, while the latter is performed in-house. Mycoplasma-contaminated cells are discarded and not used for the assay.

Karyotype Stability

Karyotype stability is under constant scrutiny because the endpoint of the assay is cytogenetic analysis. Cell stocks will be thawed periodically, recultured twice a week, then eliminated for use in assays after about 8 weeks of continuous culture.

Test Article

The test article is identified in Part 1 of this protocol. Storage conditions will be specified by the Sponsor.

Stability

The test article will be re-assayed for potency by the Sponsor's designated lab. A sample of test article, collected at the time of test article weighing for the micronucleus assay, will be assayed for potency of 2463608 after completion of dosing. A test article sample (at least 200 mg) will be shipped at ambient temperature to:

John Masters (Principal Investigator for assay of potency)
Eli Lilly and Company
Lilly Corporate Center
Indianapolis, IN 46285
Tel: 317-277-7969
Fax: 317-277-6778
eMail: jjim@lilly.com

The test article sample will be identified at least by study number, and substance name or number. The study monitor and recipient will be notified of the shipment by facsimile. The Principal Investigator for the potency assay will send results to the study director for inclusion in the final report. The potency information will be used for this study and 7608-549.

Control Articles

Negative Control Article(s)

The vehicle control article will be evaluated concurrently under each treatment condition, at the same dose volume as is used to administer the test article. If the vehicle used is atypical, untreated control cultures also will be included to ensure that the vehicle has no deleterious effect upon cell growth or the endpoints measured.

Positive Control Articles

Mitomycin C (MMC; CAS #50-07-7), a clastogen that does not require metabolic activation, will be evaluated concurrently at a concentration of 0.3 µg/mL without S9. Cyclophosphamide (CP; CAS #6055-19-2), an agent that does require metabolic activation to its clastogenic form, will be evaluated concurrently at a concentration of 5.00 µg/mL with S9.

Metabolic Activation System (S9)

Liver Homogenate

Liver homogenate (S9) will be purchased commercially. It is prepared from male Sprague-Dawley rats that have been injected (i.p.) with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, five days before sacrifice (Ames *et al.*, 1975).

S9 Mix

S9 mix will be freshly prepared on the day of use and will contain the components indicated below (final concentrations during treatment).

Component	Concentration in Cultures
NADP (sodium salt)	1.5 mg/mL (1.8 mM)
Isocitric acid	2.7 mg/mL (10.5 mM)
Homogenate (S9 fraction)	15.0 µL/mL (1.5%, v/v)

Experimental Design

Test Article Handling

A correction for potency will not need to be made as the concentrations in this assay will be based off of the theoretical potency of 100.0%.

The test article will be prepared in dimethylsulfoxide (DMSO). Routinely, vortex mixing, sonication and heating to 37 ± 2°C may be employed to aid in solubilization.

In the absence of solubility information from the Sponsor (or other sources), a solubility test will be conducted with the two vehicles most commonly used with this test system: de-ionized water (di-H_2O) and dimethylsulfoxide (DMSO; CAS #67-68-5). If suitable solubility is not obtained, additional vehicles may be evaluated on a case-by-case basis.

Aqueous vehicles may be used at concentrations up to 10% (v/v, final), while organic solvents typically are limited to ≤1.0% (v/v, final).

The solubility and dispersion characteristics of the test article will be determined, and the vehicle selected will be the one that gives the best solubility or dispersion. If the test article is insoluble in vehicles that are compatible with the test system, the vehicle chosen will be that which produces a homogeneous (evenly dispersed) suspension. Routinely, vortex mixing, sonication and heating to 37 ± 2°C may be employed to aid in solubilization.

A preliminary solubility test will be performed to determine the solubility and dispersion characteristics of the test article in the vehicle as well as after dosing into treatment medium. The pH and osmolality of the culture medium will be measured after addition of the highest possible test article dose, to ensure these parameters are not affected deleteriously. If marked pH changes are observed during the solubility test (i.e., color changes in medium), HEPES buffer will be included in the medium to attempt to counteract the pH change. Further neutralization (e.g., with HCl or NaOH) will be performed to maintain a normal culture pH range (approximately 6.8 to 7.4) only after consultation with the Sponsor.

Dose Range-finding Assay

The dose range-finding assay will be conducted using ~4-hour exposures with and without S9, and an ~24-hour exposure without S9 only. At least eight concentrations of the test article will be evaluated in single cultures for each exposure condition. In the absence of any toxicity information, the highest concentration evaluated will be 5.00 mg/mL (or 10 mM, whichever is lower). However, if limited by solubility, the maximum concentration evaluated will be the highest able to be administered, or not expected to interfere with scoring the assay. Each successive lower concentration will be equal to 50% of the preceding one.

Treatment

Four-hour (approximate) treatments will be initiated (on Day 0) by aspirating the culture media from each well and replacing it with fresh complete McCoy's 5A medium containing the test or control articles at the appropriate concentrations (with or without S9, as appropriate). The cultures will be incubated under standard conditions for ~4 hours. After the ~4-hour incubation, the treatment medium will be aspirated, the cells will be rinsed with serum free McCoy's 5A medium, and the cultures will be incubated for an additional 20 hours in fresh complete McCoy's 5A medium containing 3 µg/mL cytochalasin B.

Continuous treatments (–S9 only) will be performed in a similar manner, except the cultures will be incubated with the test and control articles for ~24 hours in the presence of 3 µg/mL cytochalasin B.

Slide Preparation

Approximately 24 hours after the start of treatment (on Day 1), the medium will be decanted and the slides will be blotted on absorbent paper. Cold hypotonic buffer (1% Na citrate) will be added for 3 to 5 minutes, and the cells will be fixed in methanol for twenty minutes and air dried. Slides will be stained with acridine orange and air dried (in the dark).

Slide Scoring

All wells of each slide will be assessed for scorability (sufficient number of cells) by low magnification microscopic examination of the amount of stained material present. The frequency of CHO-WBL cells containing one, two or more nuclei, in 500 total cells when possible, will be assessed in each scorable well on each slide to determine cell cycle kinetics.

Evaluation of Results

The Cytochalasin B proliferation index (CBPI) will be determined for all scorable cultures using the following formula. It will be expressed in tabular fashion as the absolute percent, as well as relative to the solvent control.

$$\text{CBPI} = \frac{1 \times \text{the number of mononucleated cells} + 2 \times \text{the number of binuclueated cells} + 3 \times \text{the number of tri- and tetranucleated cells}}{\text{total number of cells scored}}$$

Micronucleus Assay

Design

The micronucleus assay will be performed using quadruplicate cultures for each test and control article concentration (using duplicate wells on each of two duplicate slides).

If no toxicity is observed, the highest test article concentration evaluated in the subsequent micronucleus assay will be the limit dose (5.00 mg/mL or 10 mM, whichever is lower). However, if limited by solubility, the maximum concentration evaluated will be the highest able to be administered, or not expected to interfere with scoring the assay. For test articles that are toxic, the highest concentration evaluated will be that expected to produce 60% toxicity (60% decrease in CBPI, if possible). In all situations, at least two lower concentrations of test article will be evaluated, with no more than $\sqrt{10}$-fold intervals between.

Treatment and Slide Preparation

Treatments and slide preparation will be performed as described for the dose range-finding assay.

Slide Scoring

All wells of each slide will be scored as described above to determine scorability and CBPI. Once the relative CPBI is determined for reach culture, the concentrations of test article to be scored will be selected.

The highest test article concentration to be scored will be the limit dose (for non-toxic, freely soluble test articles), the highest concentration able to be administered or scored (for insoluble materials), or that producing a decrease in CBPI of ~60%. At least two or three lower concentrations (spanning the range of moderately cytotoxic to noncytotoxic, if applicable) will be scored. A total of 2000 binucleated cells (1000 binucleated cells/slide, 500 binucleated cells/well; when possible) will be scored for each test and control article concentration chosen for scoring of micronucleated binucleate cells (MN-BN). Scoring for micronuclei will be done in a blinded fashion.

The criteria for scoring micronuclei are: 1) the diameter of the micronuclei must be approximately 1/3 or less the diameter of the main nucleus; 2) the micronuclei must be non-refractile and located in the cytoplasm; and 3) the binucleate cells must have non-overlapping nuclei with distinct borders.

Data

The frequency of MN-BN per 2000 total binucleated cells, when possible, will be calculated for each test and control article concentration for all replicate wells.

Criteria for a Valid Assay

Before assay data will be evaluated, all criteria for a valid assay must be met. The following criteria will be used to determine a valid assay.

Spontaneous Frequency of MN-BN

The frequency BN-MN for the vehicle control cultures must be within historical ranges.

Positive Control Values

The positive control articles must induce a statistically significant increase ($p < 0.05$) in the frequency of MN-BN, as compared to the concurrent vehicle controls.

Statistical Analyses

Statistical analysis will employ a Fisher-Irwin exact test for pair wise comparisons between each treated and vehicle control cultures (Thakur et al., 1985). One-sided tail probabilities will be used to evaluate statistical significance ($p < 0.05$). A chemical will be concluded to be positive if a statistically significant increase in the frequency of micronucleated cells is obtained from at least one test article concentration compared to the concurrent vehicle control and the linear trend test confirms a positive dose-response (alpha level = 0.05).

Assay Evaluation Criteria
Once criteria for a valid assay have been met, responses observed in the assay are evaluated as follows:

Criteria for a Positive Response
A test article will be considered to have produced a positive response if it induces a statistically significant, dose-dependent increase in the frequency of MN-BN.

Criteria for a Negative Response
A test article will be considered to have produced a negative response if no statistically significant or dose dependent increases in the frequency of MN-BN are observed.

Criteria for an Equivocal Response
Despite extensive testing, a test article may produce results that are neither clearly positive nor clearly negative. In those rare instances (e.g., statistically significant **OR** dose dependent increases), the test article may be considered to have produced equivocal responses.

Other criteria also may be used in reaching a conclusion about the study results (e.g., comparison to historical control values, etc.). In such cases, the Study Director will clearly report and describe such any considerations.

References

Ames, B. N., J. McCann and E. Yamasaki (1975) "Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test," *Mutation Res.,* 31:347-364.

Ciaravino, V., M.J. Suto and J.C. Thiess (1993) High capacity *in vitro* micronucleus assay for assessment of chromosome damage: results with quinolone/naphthyridone antibacterials, *Mutation Res.*, 298:227-236.

Fenech, M. and A. Morley (1985) Measurement of micronuclei in lymphocytes, *Mutation Res.* , 147:29-36.

Kirsch-Volders, M., T. Sofuni, M. Aardema, S. Albertini, D. Eastmond, M. Fenech, M. Ishidate, S. Kirchner, E. Lorge, T. Morita, H. Norppa, J. Surrallés, A. Vanhauwaert and A. Wakata (2003) Report from the *in vitro* micronucleus assay working group, *Environ. Mol.Mutagen.*, 540:153-163.

OECD (2006) "*In Vitro* Micronucleus Test," *OECD Guidelines for Testing of Chemicals*, Draft Guideline 487, 2nd Version, 21 December 2006.

Thakur, A.K., K.J. Berry and P.W. Mielke, Jr. (1985) A Fortran program for testing trend and homogeneity in proportions. *Comp. Progr. Biomed.* 19:229-233.

Report Format

Reports will be issued individually by type of test and by test article. Each final report will provide the following information:

Covance employs a standard report format for each assay design. The final report will provide the following information:

Sponsor identification.
Quality Assurance statement.
Statement of GLP compliance.
Study Director signature.
Test article identification, a physical description of the test article, CAS number (if known), and date of receipt.
Type of study, protocol number and Covance Study Number.
Dates of study initiation and completion.
Identification of Study Director and Laboratory Supervisor.
Method information, per the draft OECD Guideline 487.
Assay evaluation and acceptance criteria.
Interpretation of results.
Conclusions.
References.
Test results presented in tabular form.
Historical control data summarized in tabular form.

Major Computer Systems

System[a]	Application Function
Environmental Monitoring and Control System (EMCS)	Monitors and documents facility storage conditions (e.g., refrigerators, freezers, constant room temperatures and humidity levels)
Environmental Monitoring and Control System Data Reporting (EMCSDR)	Transfers data from EMCS for reporting purposes
Material Tracking and Testing System (MTTS)	Test article accessioning and dispensing

[a] All version numbers of the applications are maintained by Covance.

Changes or Revisions

The Sponsor will be notified of any changes or revisions. Any changes or revisions of this approved protocol will be documented by an amendment that will be signed by the Study Director, dated, and maintained with this protocol.

Records To Be Maintained

All raw data, documentation, records, the protocol and the final report generated as a result of this study will be stored in the Covance-Vienna archives for at least three years following finalization of the report. The Covance archives staff will contact the sponsor after at least three years following the report finalization to determine disposition of the archived materials (except for raw data on durable media, study correspondence, protocol, and final report which will be kept by Covance-Vienna). The Sponsor will authorize the transport of the materials to their site (or that of their designee), or authorize the transport of the materials to the archive facilities of EPL Archives, Inc., Sterling, VA (EPL). Covance staff will have access to materials archived at EPL for continued research and/or regulatory audit. All raw data, documentation, records, original reports generated as a result of work performed at Eli Lilly as a part of this study will be archived by Eli Lilly.

Protocol Amendment 1

Page 1

COVANCE
THE DEVELOPMENT SERVICES COMPANY

STUDY TITLE: In Vitro Microwell Micronucleus Assay in Chinese Hamster Ovary (CHO) Cells

PROTOCOL NO.: 460GLP, Edition 1

GENETIC TOXICOLOGY ASSAY NO.: 29269-0-460GLP

COVANCE STUDY NO.: 7608-550

1. **Page 2; Notification of Regulatory Submission**
   Effective Date: 11 July 2007

   The word "proposed" is removed from the OECD listing under Notification of Regulatory Submission.

   Reason: Proposed was listed in error as the OECD Testing Guidelines are proposed and not the OECD GLP Guidelines.

2. **Page 7; Micronucleus Assay**
   Effective Date: 25 July 2007

   The following doses will be tested in the Micronucleus Assay:

   S9 Activated (4 hour): 75.0, 60.0, 50.0, 40.0, 30.0, 20.0, 10.0, and 5.00 µg/mL.

   Nonactivated (4 hour): 60.0, 50.0, 40.0, 30.0, 20.0, 10.0, and 5.00 µg/mL.

   Nonactivated (23 hour): 50.0, 40.0, 30.0, 20.0, 10.0, 5.00, 2.50, and 1.25 µg/mL.

   Reason: To provide doses for the micronucleus assay.

Compound: 2463608 (Test Article: 2463608)
Study: Covance 7608-550

Protocol Amendment 1

Page 2

COVANCE
THE DEVELOPMENT SERVICES COMPANY

STUDY TITLE: In Vitro Microwell Micronucleus Assay in Chinese Hamster Ovary (CHO) Cells

PROTOCOL NO.: 460GLP, Edition 1

GENETIC TOXICOLOGY ASSAY NO.: 29269-0-460GLP

COVANCE STUDY NO.: 7608-550

3. **Page 7; Micronucleus Assay**
   Effective Date: 08 August 2007

   Due to a lack of toxicity in the micronucleus assay, the assay will be repeated with the following doses:

   S9 Activated (4 hour): 700, 550, 400, 300, 225, 150, 75.0, 37.5, 18.8, 9.40, and 4.70 µg/mL.

   Nonactivated (4 hour): 700, 550, 400, 300, 225, 150, 75.0, 37.5, 18.8, 9.40, and 4.70 µg/mL.

   Nonactivated (23 hour): 150, 115, 75.0, 50.0, 37.5, 25.0, 12.5, 7.50, and 3.75 µg/mL.

   Reason: To provide doses for the repeat of the micronucleus assay.

Compound: 2463608 (Test Article: 2463608)
Study: Covance 7608-550

Protocol Amendment 1

Page 3

COVANCE
THE DEVELOPMENT SERVICES COMPANY

STUDY TITLE: In Vitro Microwell Micronucleus Assay in Chinese Hamster Ovary (CHO) Cells

PROTOCOL NO.: 460GLP, Edition 1

GENETIC TOXICOLOGY ASSAY NO.: 29269-0-460GLP

COVANCE STUDY NO.: 7608-550

Approval of Protocol Amendment

Study Director

Hemalatha Murli — 12 October 2007

Hemalatha Murli, PhD
Study Director
Covance Laboratories Inc.

Date

Testing Facility Management

12-Oct-2007

Timothy E. Lawlor, MA
Associate Director
Covance Laboratories Inc.

Date

Compound: 2463608 (Test Article: 2463608)
Study: Covance 7608-550

Protocol Deviation

Two hundred cells per dose level were analyzed for the Cytochalasin B Proliferation Index for the dose range finding portion of the assay (Trial 1). This deviation had no impact on the integrity of the study as analyzing 200 cells is the current laboratory procedure.

The end of study potency presented in this study was performed under Covance study number 7608-544 and not as a part of this study by Eli Lilly.

Appendix B: Test Article Characterization and Potency

www.lilly.com

Lilly Research Laboratories
A Division of Eli Lilly and Company
Lilly Corporate Center
Indianapolis, Indiana 46285 U.S.A.

Phone 317 276 2000

CHEMICAL PROCESS RESEARCH AND DEVELOPMENT

CERTIFICATE OF ANALYSIS

TITLE: Lilly CB-1 Antagonist

COMPOUND NUMBER: 2463608
LOT NUMBER: KD0-E01100-039-C
DOCUMENT PREPARATION DATE: August 6, 2007
DATE OF MANUFACTURE March 30, 2007
ITEM CODE N/A
RETEST DATE N/A
STORAGE CONDITIONS: Ambient Temperature

TEST AND METHOD	METHOD	SPECIFICATIONS	RESULT
Purity	HPLC at 215 nm	NLT 95 (area %)	>99%
Identity	^{1}H NMR (DMSO-d_6)	Conforms to structure	Conforms to structure
Appearance	Visual	White to off- white solid No Visible contaminants	White solid No visible contaminants

NLT=Not Less Than

Lot KD0-E01100-039-C was manufactured under Non-GMP conditions

The following signatures indicate that the results listed above have been generated in accordance with local Standard Operating Procedures (SOP) and local Operating Procedures (OP).

Prepared By: _____ Quality Assurance Representative 06 Aug 07 Date

Verified By: Robert A. Jasper Quality Assurance Representative 06-Aug-07 Date

ATPortal ID: IND-REQ3216-INNV-CY4-KD0-E01100-039-C-13-Jul-2007-16:23:51
Instrument: IND_REPORT_INST
Report Type: FINAL REPORT
NotebookRef: KD0-E01100-039-C
Open Access: 1
Test Type: REPORT
Test Name: Structure Problem Solving
TimeDate: 13 Jul 2007 16:23:51

NMR Purity Check of KD0-E01100-039-C

Robert Boyer

Analytical Technologies Group
Discovery Chemistry Research and Technologies
Lilly Research Laboratories
A Division of Eli Lilly and Company
Indianapolis, IN 46285

Submitted by: J. Mullaney

Date received: July 9, 2007 Date started: July 10, 2007
Date oral results: July 10, 2007 Date written report: July 13, 2007
Project: CB-1 Antagonist Effort 4

Problem: Perform quantitative NMR.

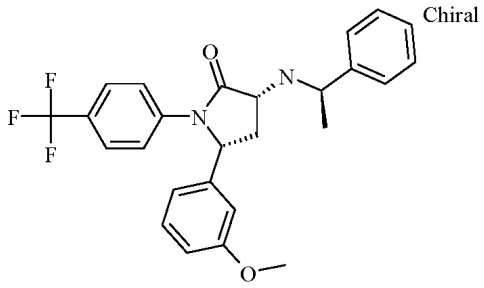
Experimental: An aliquot, 7.77 mg, of the sample was dissolved in 700 μl of dimethyl sulfoxide-d_6 and the resulting solution transferred to a 5 mm NMR tube. NMR experiments were performed on a Varian INOVA 500 spectrometer (blackcomb) equipped with a 5 mm inverse cold probe and the sample temperature maintained at 25 degrees Celsius.

Quantitation was performed using an external reference [9.96 mM 1,4-Bis(trimethylsilyl)-benzene] and the qadd macro created by Krish Krishnamurthy. The ^{1}H NMR spectra for the sample and the reference were performed with a 45 degree pulse, relaxation delay of 25 seconds, 4 steady state pulses, and the spinner turned off. The qadd macro adds the reference and the sample spectra and generates a new spectrum such that the reference is at a 2 mM concentration. Normal integration, followed by calculation, generates the weight of the desired compound in the sample.

Quantitation by NMR
General equations used
1) A x B x C x D x E x F = wt of desired compound
Where A = volume of sample solution
B = volume conversion factor, if needed
C = concentration of reference
D = reported purity of reference compound
E = ratio of desired compound to reference (from integration)
F = molecular weight of desired compound

2) wt % = weight desired compound / weight sample used

Results and discussion: The ^{1}H NMR spectrum is consistent with the structure shown below. No impurities were readily observable in the spectrum. Quantitation using the qadd macro indicates that this material is highly pure.



Calculations:

$$0.7\ \text{ml} \times \frac{1\ \text{liter}}{1000\ \text{ml}} \times \frac{2\ \text{mmole ref}}{1\ \text{liter}} \times (0.96) \times \frac{100.00\ \text{mmole comp}}{7.65\ \text{mmole ref}} \times \frac{454.49\ \text{mg comp}}{\text{mmole comp}} = 7.98\ \text{mg comp}$$

$$\frac{7.98\ \text{mg comp}}{7.77\ \text{mg sample}} = 103\ \text{wt\%}$$

Report filename: KD0-E01100-039-C.pdf

Notebook: CY4-A10657-172

Spectral filenames:

KD0-E01100-039-C_20070710_01	PROTON_01, PROTON_02 (with reference added)
KD0-E01100-039-C-REF_20070710_01	PROTON_01

ATPortal ID: IND-REG4630-INNV-CY4-KD0-E01100-039-C-16-Aug-2007-17:45:12
Instrument: IND_REPORT_INST
Report Type: FINAL REPORT
NotebookRef: KD0-E01100-039-C
Open Access: 1
Test Type: REPORT
Test Name: Specific Tech NMR
TimeDate: 16 Aug 2007 17:45:12

NMR Purity Check of KD0-E01100-039-C (Post testing)

Robert Boyer

Analytical Technologies Group
Discovery Chemistry Research and Technologies
Lilly Research Laboratories
A Division of Eli Lilly and Company
Indianapolis, IN 46285

Submitted by: J. Masters

Date received: August 14, 2007 Date started: August 15, 2007
Date oral results: August 15, 2007 Date written report: August 16, 2007
Project: CB-1 Antagonist Effort 4

Problem: Perform quantitative NMR of sample after GLP study. See CY4-A10657-172 for analysis of this material before the study.

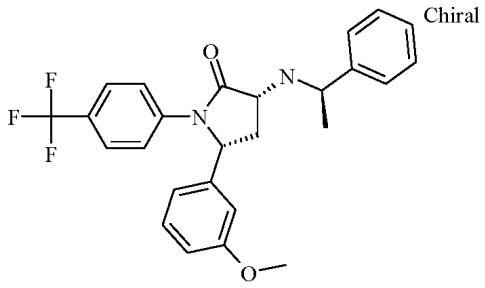
Experimental: An aliquot, 8.28 mg, of the sample (out of 859 mgs submitted) was dissolved in 700 μl of dimethyl sulfoxide-d_6 and the resulting solution transferred to a 5 mm NMR tube. NMR experiments were performed on a Varian INOVA 500 spectrometer (blackcomb) equipped with a 5 mm inverse cold probe and the sample temperature maintained at 25 degrees Celsius.

Quantitation was performed using an external reference [9.96 mM 1,4-Bis(trimethylsilyl)-benzene] and the qadd macro created by Krish Krishnamurthy. The ^{1}H NMR spectra for the sample and the reference were performed with a 45 degree pulse, relaxation delay of 25 seconds, 4 steady state pulses, and the spinner turned off. The qadd macro adds the reference and the sample spectra and generates a new spectrum such that the reference is at a 2 mM concentration. Normal integration, followed by calculation, generates the weight of the desired compound in the sample.

Quantitation by NMR
General equations used
1) A x B x C x D x E x F = wt of desired compound
Where A = volume of sample solution
B = volume conversion factor, if needed
C = concentration of reference
D = reported purity of reference compound
E = ratio of desired compound to reference (from integration)
F = molecular weight of desired compound

2) wt % = weight desired compound / weight sample used

Results and discussion: The ^{1}H NMR spectrum is consistent with the structure shown below and matches the spectrum of this material obtained previously (see CY4-A10657-172/Boyer). No impurities were readily observable in the spectrum. Quantitation using the qadd macro indicates that this material is highly pure.



Calculations:

$$0.7\ \text{ml} \times \frac{1\ \text{liter}}{1000\ \text{ml}} \times \frac{2\ \text{mmole ref}}{1\ \text{liter}} \times (0.96) \times \frac{100.00\ \text{mmole comp}}{7.47\ \text{mmole ref}} \times \frac{454.49\ \text{mg comp}}{\text{mmole comp}} = 8.17\ \text{mg comp}$$

$$\frac{8.17\ \text{mg comp}}{8.28\ \text{mg sample}} = 99\ \text{wt\%}$$

Report filename: KD0-E01100-039-C-post.pdf

Notebook: CY4-A10657-196

Spectral filenames:

KD0-E01100-039-C_20070815_01	PROTON_01, PROTON_02 (with reference added)
KD0-E01100-039-C-REF_20070815_01	PROTON_01

Appendix C: Historical Control Data, Key Personnel, and Glossary

Historical Control Data (Non GLP)

In Vitro Microwell Micronucleus Screening Assay - 2004 through 2005

	Activation		% MNBN Cells
Negative Controls			
~4 Hour Exposure	With	Minimum	0.42
		Maximum	3.92
		Average ± SD	1.84 ± 0.83
		N	25
~4 Hour Exposure	Without	Minimum	1.33
		Maximum	3.75
		Average ± SD	1.99 ± 0.69
		N	22
~23 Hour Exposure	Without	Minimum	0.83
		Maximum	4.17
		Average ± SD	1.82 ± 0.82
		N	23
Vehicle Controls			
~4 Hour Exposure	With	Minimum	0.83
		Maximum	4.33
		Average ± SD	2.04 ± 0.93
		N	25
~4 Hour Exposure	Without	Minimum	1.08
		Maximum	3.17
		Average ± SD	1.87 ± 0.58

		N	22
~23 Hour Exposure	Without	Minimum	0.83
		Maximum	3.42
		Average ± SD	1.94 ± 0.70
		N	23
Positive Controls			
~4 Hour Exposure	With	Minimum	12.25
		Maximum	28.50
		Average ± SD	20.09 ± 4.31
		N	25
~4 Hour Exposure	Without	Minimum	11.00
		Maximum	35.25
		Average ± SD	23.31 ± 6.26
		N	22
~23 Hour Exposure	Without	Minimum	6.13
		Maximum	32.00
		Average ± SD	19.73 ± 6.98
		N	23

SD = Standard Deviation

N = Number of Trials

MNBN = Micronucleated binucleated cells

Key Personnel

Study Monitor	Amy L. Garn Eli Lilly and Company
Study Director	Hemalatha Murli, PhD Covance-Vienna
Study Toxicologist	Veronica Freerks, BS Covance-Vienna
Laboratory Supervisor	Daksha N. Patel, BS, MT(ASCP), CLSp(CG) Covance-Vienna

Major Computer Systems

EMCS	Environmental Monitoring and Control System
EMCSDR	Environmental Monitoring and Control System Data Reporting
MTTS	Material Tracking and Testing System

Comments on the Data

Various models of calculators, computers, and computer programs were used to analyze data in this study. Because different models round off or truncate numbers differently, values in some tables (*e.g.*, means, standard deviations, or individual values) may differ slightly from those in other tables, from individually calculated data, or from statistical analysis data. Neither the integrity nor the interpretation of the data was affected by these differences.