FINAL REPORT

Study Title

AMES II™ Mutagenicity Assay

Test Article

GB67B and GB594

Authors

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Study Completion Date

03 April 2008

Testing Facility

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AC13JU-JV.850.BTL

Sponsor

Emory University
1515 Dickey Drive
Atlanta, GA 30322

AMES II™ Mutagenicity Assay

STUDY INFORMATION

Sponsor:	**Emory University** **1515 Dickey Drive** **Atlanta, GA 30322**
Sponsor Representative:	**Gregory Bluemling**
Testing Facility:	**BioReliance** **9630 Medical Center Drive** **Rockville, Maryland 20850**
Test Article Receipt and Login:	**04 March 2008**
Study Initiation:	**11 March 2008**
Experimental Start Date:	**11 March 2008**
Experimental Completion Date:	**13 March 2008**
Laboratory Manager:	**Shannon Wilson Bruce, M.F.S.**

Test Article ID	BioReliance Study Number	Test Article Description	Test Article Purity	Storage Conditions
GB67B	AC13JU.850.BTL	white powder	99%	Refrigerated (2 to 8°C) in the dark with desiccant
GB594	AC13JV.850.BTL	white powder	99%	

Study Director: Kamala Pant 03 Apr 2008

Kamala Pant, M.S. Date

AMES II™ Mutagenicity Assay

SOLUBILITY INFORMATION

Maximum test article solublility in Dimethyl Sulfoxide (DMSO)	**25.0 mg/mL**
Highest concentration of test article tested on microplate	**1000 µg/mL**
Test article concentrations tested on microplate	**1000, 250, 62.5, 15.6, 3.90, and 0.975 µg/mL**
Concentration of positive control without S9 activation	**4-nitroquinoline N-oxide (4-NQO) 1.0 µg/mL**
Concentration of positive control with S9 activation	**2-aminoanthracene (2AA) 5.0 µg/mL**

EXPERIMENTAL DESIGN AND METHODOLOGY

Test System

The tester strains will include the Ames II™ mixed strains (TA7001 to TA7006) and TA98 as described by Gee *et al.* (1994).

Strain	Mutation	Type	Target	Cell Wall	Repair	pKM101
TA98	hisD3052	Frameshift	GC	rfa	*uvrB*	Yes
Mixed Strains Contain						
TA7001	hisG1775	b.p. sub.	A:T>G:C	rfa	*uvrB*	Yes
TA7002	hisC9138	b.p. sub.	T:A>A:T	rfa	*uvrB*	Yes
TA7003	hisG9074	b.p. sub.	T:A>G:C	rfa	*uvrB*	Yes
TA7004	hisG9133	b.p. sub.	G:C>A:T	rfa	*uvrB*	Yes
TA7005	hisG9130	b.p. sub.	C:G>A:T	rfa	*uvrB*	Yes
TA7006	hisC9070	b.p. sub.	C:G>G:C	rfa	*uvrB*	Yes

The *rfa* mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The deletion in the *uvrB* gene results in a deficient DNA excision-repair system. The tester strains also contain the pKM101 plasmid (carrying the R-factor).

Experimental Design

The test system were exposed to the test article via the microplate liquid culture modification of that reported by Gee *et al.* (1994). The test articles were tested at a maximum of six dose levels along with appropriate negative and positive controls with mixed Ames II™ strain and tester strain TA98 with and without S9 activation. All dose levels of test article, negative controls, and positive controls were tested in triplicate.

Scoring Procedures

The plates were scored by placing them on a light box and counting the number of positive wells (those which were yellow or had a bacterial colony at the bottom of the well). The number of positive wells were counted in each 48 well section of a 384 well plate.

Evaluation of Results

The test article will be considered non-mutagenic, if the mean number of positive wells for all tester strains is within the historical negative control range under all experimental conditions. Historical control data is given in Appendix II.

In the experiments where the average vehicle control value for the positive wells is less than one, a value of one (1.0) will be substituted for the vehicle control for calculating the fold increase with the test article and the positive control treated doses.

For the test article to be evaluated mutagenic, it must cause a dose-related increase (at least doubling compared to the negative/vehicle control) in the mean number of positive wells per dose over a minimum of two increasing concentrations of test article.

Criteria for a Valid Test

The mean number of spontaneous positive wells per assay (yellow wells and wells containing a bacterial colony at the bottom regardless of the color) of the negative control must be within the following range: TA98 $\leq$ 8 positive wells; TAMix $\leq$ 5 positive wells. The mean number of positive wells (yellow wells and wells containing a bacterial colony at the bottom regardless of the color) must be within the following ranges: TAMix – 4NQO $\geq$ 25 positive wells; TAMix – 2AA $\geq$ 15 positive wells; TA98 – 4NQO $\geq$ 20 positive wells; TA98 – 2AA $\geq$ 25 positive wells.

The values for the mean number of spontaneous positive wells in the negative control must be within historical control data range. The values for the mean number of positive wells in the positive control must be within historical control data range. In instances where the number of revertant (positive) wells in the negative control is less than 1.0, that number will be substituted with 1.0 for the purposes of calculations.

Archives

Upon issue of the final report, all raw data for the procedures performed at BioReliance will be returned to the Sponsor.

Deviations

No known deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

RESULTS FOR GB67B (AC13JU)

TA98	Without S9 Activation			With S9 Activation		
Dose (µg/mL)	Mean Number of Positive Wells	Standard Deviation	Fold Induction as Compared to Negative Control	Mean Number of Positive Wells	Standard Deviation	Fold Induction as Compared to Negative Control
Negative Control (DMSO)	1.0*	0.6	NA	1.7	1.2	NA
0.975	0.0	0.0	0.0	3.7	2.9	2.2
3.9	0.3	0.6	0.3	2.3	0.6	1.4
15.6	1.7	2.1	1.7	4.7	2.5	2.8
62.5	0.7	0.6	0.7	1.7	1.5	1.0
250	0.3	0.6	0.3	1.7	0.6	1.0
1000	0.0	0.0	0.0	0.0	0.0	0.0
Positive Control	48.0	0.0	48.0	48.0	0.0	28.8

TAMix	Without S9 Activation			With S9 Activation		
Dose (µg/mL)	Mean Number of Positive Wells	Standard Deviation	Fold Induction as Compared to Negative Control	Mean Number of Positive Wells	Standard Deviation	Fold Induction as Compared to Negative Control
Negative Control (DMSO)	1.7	2.1	NA	1.0	1.0	NA
0.975	0.0	0.0	0.0	1.7	1.5	1.7
3.9	1.0	1.0	0.6	1.0	1.0	1.0
15.6	3.3	2.9	2.0	1.7	0.6	1.7
62.5	1.7	1.5	1.0	0.0	0.0	0.0
250	2.0	1.0	1.2	1.0	0.0	1.0
1000	0.0	0.0	0.0	0.3	0.6	0.3
Positive Control	30.7	1.2	18.4	44.0	1.7	44.0

* = Because the mean revertant value was less than 1.0, 1.0 was substituted for the actual control mean value for purposes of calculations.

RESULTS FOR GB594 (AC13JV)

TA98	Without S9 Activation			With S9 Activation		
Dose (µg/mL)	Mean Number of Positive Wells	Standard Deviation	Fold Induction as Compared to Negative Control	Mean Number of Positive Wells	Standard Deviation	Fold Induction as Compared to Negative Control
Negative Control (DMSO)	1.0	0.0	NA	1.0*	0.6	NA
0.975	2.0	2.0	2.0	0.3	0.6	0.3
3.9	1.3	2.3	1.3	2.3	1.5	2.3
15.6	0.7	1.2	0.7	0.0	0.0	0.0
62.5	2.0	1.7	2.0	1.7	0.6	1.7
250	1.7	2.1	1.7	2.0	2.0	2.0
1000	1.7	0.6	1.7	1.3	1.2	1.3
Positive Control	38.0	8.7	38.0	48.0	0.0	48.0

TAMix	Without S9 Activation			With S9 Activation		
Dose (µg/mL)	Mean Number of Positive Wells	Standard Deviation	Fold Induction as Compared to Negative Control	Mean Number of Positive Wells	Standard Deviation	Fold Induction as Compared to Negative Control
Negative Control (DMSO)	1.0*	0.0	NA	2.0	1.0	NA
0.975	0.7	0.6	0.7	1.7	1.2	0.8
3.9	0.3	0.6	0.3	1.3	1.2	0.7
15.6	0.3	0.6	0.3	1.7	0.6	0.8
62.5	0.7	0.6	0.7	0.7	0.6	0.3
250	0.0	0.0	0.0	0.3	0.6	0.2
1000	0.3	0.6	0.3	1.0	1.0	0.5
Positive Control	48.0	0.0	48.0	40.7	3.5	20.3

* = Because the mean revertant value was less than 1.0, 1.0 was substituted for the actual control mean value for purposes of calculations.

Discussion

Test article GB67B (AC13JU) had a doubling in the number of positive wells with one dose in tester strain TAMix without metabolic activation and had a doubling in the number of positive wells with two doses in tester strain TA98 with metabolic activation. However, in these doses the doublings were within historical negative control range and there was no dose response trend. Thus, the test article was determined to be non-mutagenic in those test conditions. The test article was non-mutagenic with tester strains TA98 without metabolic activation and TAMix with metabolic activation.

Test article GB594 (AC13JV) had a doubling in the number of positive wells with two doses in tester strain TA98 with and without metabolic activation. However, in these doses the doublings were within historical negative control range and there was no dose response trend. Thus, the test article was determined to be non-mutagenic in those conditions. The test article was non-mutagenic with tester strain TAMix with and without metabolic activation.

References

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Piegorsch, W.W., Simmons, S.J., Margolin, B.H., Zeiger, E., Gidrol, X.M., and Gee, P. (2000). Statistical modeling and analyses of a base-specific Salmonella mutagenicity assay. Mutation Research 467:11-19.

APPENDIX I

Data Tables

Raw Data Tables

Study Number: AC13JU.850.BTL B1

Test Article: GB67B

Date Plated: 3/11/08

Date Read: 3/13/08

Activation: -S9

TA98 Plate 1

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	0
2	0.975	0
3	3.9	0
4	15.6	1
5	62.5	1
6	250	1
7	1000	0
8	Positive Control	48

TAMix Plate 1

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	1
2	0.975	0
3	3.9	0
4	15.6	0
5	62.5	0
6	250	1
7	1000	0
8	Positive Control	32

TA98 Plate 2

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	0
2	0.975	0
3	3.9	0
4	15.6	4
5	62.5	1
6	250	0
7	1000	0
8	Positive Control	48

TAMix Plate 2

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	0
2	0.975	0
3	3.9	2
4	15.6	5
5	62.5	2
6	250	2
7	1000	0
8	Positive Control	30

TA98 Plate 3

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	1
2	0.975	0
3	3.9	1
4	15.6	0
5	62.5	0
6	250	0
7	1000	0
8	Positive Control	48

TAMix Plate 3

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	4
2	0.975	0
3	3.9	1
4	15.6	5
5	62.5	3
6	250	3
7	1000	0
8	Positive Control	30

Raw Data Tables

Study Number: AC13JU.850.BTL B1

Test Article: GB67B

Date Plated: 3/11/08

Date Read: 3/13/08

Activation: +S9

TA98 Plate 1

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	1
2	0.975	7
3	3.9	2
4	15.6	2
5	62.5	2
6	250	2
7	1000	0
8	Positive Control	48

TAMix Plate 1

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	2
2	0.975	0
3	3.9	0
4	15.6	1
5	62.5	0
6	250	1
7	1000	0
8	Positive Control	43

TA98 Plate 2

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	3
2	0.975	2
3	3.9	3
4	15.6	7
5	62.5	0
6	250	2
7	1000	0
8	Positive Control	48

TAMix Plate 2

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	0
2	0.975	2
3	3.9	2
4	15.6	2
5	62.5	0
6	250	1
7	1000	1
8	Positive Control	43

TA98 Plate 3

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	1
2	0.975	2
3	3.9	2
4	15.6	5
5	62.5	3
6	250	1
7	1000	0
8	Positive Control	48

TAMix Plate 3

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	1
2	0.975	3
3	3.9	1
4	15.6	2
5	62.5	0
6	250	1
7	1000	0
8	Positive Control	46

Raw Data Tables

Study Number: AC13JV.850.BTL B1

Test Article: GB594

Date Plated: 3/11/08

Date Read: 3/13/08

Activation: -S9

TA98 Plate 1

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	1
2	0.975	0
3	3.9	0
4	15.6	0
5	62.5	0
6	250	0
7	1000	1
8	Positive Control	48

TAMix Plate 1

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	0
2	0.975	1
3	3.9	0
4	15.6	0
5	62.5	1
6	250	0
7	1000	0
8	Positive Control	48

TA98 Plate 2

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	1
2	0.975	2
3	3.9	4
4	15.6	0
5	62.5	3
6	250	4
7	1000	2
8	Positive Control	34

TAMix Plate 2

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	0
2	0.975	0
3	3.9	1
4	15.6	1
5	62.5	1
6	250	0
7	1000	1
8	Positive Control	48

TA98 Plate 3

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	1
2	0.975	4
3	3.9	0
4	15.6	2
5	62.5	3
6	250	1
7	1000	2
8	Positive Control	32

TAMix Plate 3

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	0
2	0.975	1
3	3.9	0
4	15.6	0
5	62.5	0
6	250	0
7	1000	0
8	Positive Control	48

Raw Data Tables

Study Number: AC13JV.850.BTL B1

Test Article: GB594

Date Plated: 3/11/08

Date Read: 3/13/08

Activation: +S9

TA98 Plate 1

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	0
2	0.975	0
3	3.9	2
4	15.6	0
5	62.5	1
6	250	0
7	1000	2
8	Positive Control	48

TAMix Plate 1

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	1
2	0.975	1
3	3.9	2
4	15.6	2
5	62.5	0
6	250	1
7	1000	1
8	Positive Control	44

TA98 Plate 2

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	0
2	0.975	0
3	3.9	1
4	15.6	0
5	62.5	2
6	250	4
7	1000	2
8	Positive Control	48

TAMix Plate 2

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	2
2	0.975	3
3	3.9	2
4	15.6	2
5	62.5	1
6	250	0
7	1000	2
8	Positive Control	41

TA98 Plate 3

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	1
2	0.975	1
3	3.9	4
4	15.6	0
5	62.5	2
6	250	2
7	1000	0
8	Positive Control	48

TAMix Plate 3

Dose	Concentration (µg/mL)	Number of Positive wells
1	Solvent Control	3
2	0.975	1
3	3.9	0
4	15.6	1
5	62.5	1
6	250	0
7	1000	0
8	Positive Control	37

APPENDIX II

Historical Control Data

HISTORICAL CONTROL DATA DECEMBER 2006 TO PRESENT

AMES II MUTAGENICITY ASSAY (TA98)

	Negative Control		Positive Control	
Positive Wells	-S9	+S9	-S9	+S9
Mean	1.1	1.5	29.9	47.3
Minimum	0.0	0.0	20.3	26.0
Maximum	4.0	4.7	48.0	48.0

AMES II MUTAGENICITY ASSAY (TAMix)

	Negative Control		Positive Control	
Positive Wells	-S9	+S9	-S9	+S9
Mean	0.9	1.0	47.3	42.8
Minimum	0.0	0.0	30.7	24.0
Maximum	4.0	4.0	48.0	48.0

Negative Control: DMSO
Positive Controls: 4-Nitroquinoline-N-oxide (-S9)
2-Aminoanthracene (+S9)