Test Summary

The test article, Citrex 2, was analyzed at Xenometrix in May, 1997. The following is a brief summary of the data obtained:

- Citrex 2 inhibited bacterial growth by 50% (at the individual cell level) at a concentration of 9 ppm (9 µg/ml).

- By monitoring the activity of 16 bacterial genes during exposure of these bacteria to Citrex 2, it was seen that a gene which corresponds to changes in bacterial cell membranes became active. This may indicate that the bacteria are experiencing membrane changes or damage when exposed to Citrex 2, and that this is the mechanism for the reduction in bacterial growth.

- Among bacteria, Ames strains of *Salmonella* appeared to be more sensitive than *E. coli* upon exposure to Citrex 2.

- Citrex 2 was also tested in the Ames II *Salmonella* Mutational Spectra assay over two orders of magnitude dosing. At no dose tested did this test article indicate any mutagenic activity in any of the eight Salmonella strains tested.

In conclusion: Citrex 2 appears to be effective at killing two different species of test bacteria. The mechanism of bacterial cell death may be concurrent with membrane changes induced by Citrex 2. From the data obtained, the test article does not cause mutations (a fundamental cancer-causing event) in the absence of exogenous metabolism.

Sincerely,

John Schneider
Client Research Laboratory

XENOMETRIX
Molecular Toxicology
& Information Assays

Citrex, Inc.
TEST ARTICLE: Citrex 2

Ames II Mutational Spectra
6 Ames II Salmonella *Strains + TA98, TA1537 & Mix*

Study Number: CIT-0597c (R)
May, 1997

SPONSOR/Monitor

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XENOMETRIX CLIENT RESEARCH LABORATORY SERVICES
QUALITY ASSURANCE

I hereby certify that test components (including media, reagents and cell lines) produced by Xenometrix Inc. were tested and found to be satisfactory according to criteria established by Xenometrix's Standard Operating Procedures (SOPs).

Quality Assurance: Christopher H. Sommers, Ph.D.
Head, Quality Control

Signed: C H Sommers

Date: 6-3-97

XENOMETRIX CLIENT RESEARCH LABORATORY SERVICES
LABORATORY PRACTICES

The assays reported in this document were performed according to Xenometrix's Quality Assurance SOPs, but do not meet formal Principles of Good Laboratory Practice.

Study Director: John Schneider, M.S.
Manager, Client Research Laboratory

Signed: J Schneider

Date: 6-3-97

CLIENT RESEARCH LABORATORY REPORT

CITREX, INC.
Test Article: Citrex 2

Distribution List:
Copy 1 - Citrex, Inc.
Copy 2 - Citrex, Inc.
Copy 3 - Xenometrix (on file)

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I. SUMMARY

Test article Citrex 2 was assayed in one system:

The Ames II Mutational Spectra bacterial mutagenesis assay, using a *Salmonella typhimurium* host, and 6 unique genetic reversion targets in the bacterial genome. This assay also includes the traditional Ames tester strains TA98 and TA1537. The assay performed also included *Salmonella* strains TA7001, TA7002, TA7003, TA7004, TA7005, and TA7006 in an equimolar mix. The strains are all histidine auxotrophs, and mutagenesis at specific bases in each strain will lead to reversion of the strain (and therefore of the Mix) to histidine prototrophy. Details of each strain (genotype) are given in section IV.

A 100 µg/ml stock of the test article was prepared in sterile water.

Assays for each *Salmonella* strain were performed in triplicate. Therefore, for each strain 3 independent cultures were exposed to the test article. Doses of test article in the Ames II Assay (Mix + TA98) were: 0, 0.06, 0.125, 0.25, 0.5, 1, 2, and 4 µg/ml, with concurrent solvent (water) negative controls which gave revertant numbers and frequencies within expected ranges for each strain (for expected ranges see Table 1).

In the Ames II Mutational Spectra Assay performed in May of 1997, test article Citrex 2 demonstrated no mutagenic activity.

II. INTRODUCTION

The purpose of this study was to assess the mutagenic potential of test article Citrex 2. One mutagenesis assay, Ames II Mutational Spectra, was performed. A brief description of the assay can be found in section IV.

The assay was performed with concurrent negative and positive control exposures. Positive control chemicals for the assay are given in section IV. The assays all performed within acceptable limits for both positive and negative controls.

This report consists of 5 main sections, and 2 appendixes. The main sections cover the details of test article preparation, methodologies employed in the performance of the assays, tools employed in data analysis and results and interpretation. Appendix 1 provides positive control data and Appendix 2 lists the raw data.

III. TEST ARTICLE

Test article Citrex 2 arrived in a polypropylene dropper bottle. The bottle was labeled with a white printed label:

CITREX 2
MFG DATE 4/4/97

The test article came as a light brown, viscous liquid, and was stored at room temperature in the dark.

IV. METHODS

IV.I Ames II Mutational Spectra Assay Version 2.0

General Description:
The Ames II Assay consists of six new *Salmonella typhimurium* tester strains, each of which detects only one of the six possible base pair substitution mutation types, allowing the generation of mutational spectra data without the need for DNA sequence analysis. Because these strains revert only by specific mutational events, spontaneous reversion frequencies are approximately 10 to 100-fold lower than those typically observed with traditional *Salmonella* tester strains such as TA100 or TA102 (see table below). The tester strains TA98 and TA1537 are included in the Ames II Mutational Spectra Assay for the detection of frameshift-inducing mutagens. The new *Salmonella* tester strains TA7001-TA7006 have been modified for maximum sensitivity to test compound. These modifications include:

- Deletion of the excision repair gene u*vrB*. This mutation prevents the removal of bulky adducts from DNA.
- Mutation of a gene required for synthesis of the bacterial cell wall (*rfa*) which increases permeability of *Salmonella* to test compound.
- Introduction of the episome pKM101, which carries the u*muDC* homologues *muc A/B*. These gene products increase the cells ability to perform mutagenic lesion bypass repair during DNA replication.

IV.II. Bacterial Strains

Table 1 shows the Ames II Assay (Complete) strains.

Table 1. AMES II Strains

Strain	Target	Mutation [a]	rfa	uvrB	pKM101
TA7001	bp subst.	A:T→G:C	✓	✓	✓
TA7002	bp subst.	T:A→A:T	✓	✓	✓
TA7003	bp subst.	T:A→G:C	✓	✓	✓
TA7004	bp subst.	G:C→A:T	✓	✓	✓
TA7005	bp subst.	G:C→T:A	✓	✓	✓
TA7006	bp subst.	C:G→G:C	✓	✓	✓
Mix	bp.subst.	All above	✓	✓	✓
TA98	frameshift	+GC	✓	✓	✓
TA1537	frameshift	+GC	✓	✓	×

[a] Base change detected by this strain

IV.III. Assay Procedure

Because of the low spontaneous reversion frequencies for the TA7000 strains, the assay has been converted to a modified fluctuation test using 384 well (48 wells per sample, per dose) microtiter plates. The frameshift tester strains (TA98 and TA1537) are assayed concurrently in microtiter plates.

The modified Ames II fluctuation test, in brief, is as follows:

1. Pre-growth of tester strains overnight in oxoid broth.
2. A 90 minute incubation at 37 °C (10^8 cells x 1 generation) in Exposure Medium with limiting histidine (1.0 mg/ml) in the presence of toxicant and S9 if employed.
3. Dilution and plating of cells in medium which selects for revertants. This medium contains a pH indicator dye which turns color from purple to yellow upon colony growth.
4. Incubation of the microtiter plates for 48 hours at 37 °C to allow for growth of revertant colonies.
5. Scoring of microtiter plates for positive (yellow) wells, data entry, and evaluation of mutagenic potential

Dosing Protocol

In one well of a 24 well plate (one well/strain/dose/replicate) 0.190 ml of Exposure Medium was aliquoted. To this was added 0.050 ml of culture, giving a total volume of 0.240 ml. To each of these cultures, 0.010 ml of test article, diluted to the appropriate concentration was added, to give a total of 0.250 ml. This mixture was then incubated for 90 minutes at 37°C with agitation at 250 rpm.

At the conclusion of the 90 minute incubation, each well received 2.75 ml of *Salmonella* Indicator Medium and was mixed briefly before being distributed in 0.05 ml aliquots to 48 wells of a 384-well microtiter plate. One plate was used/strain/replicate.

Plates were then incubated at 37°C for 48 hours, after which yellow (positive) wells were counted:

IV.IV. Metabolic Activation

The Ames II Mutational Spectra assay performed in this report was done in the absence of S9 fraction and therefore did not include exogenous metabolic activation.

IV.V. Ames II Mutational Spectra Positive Controls

Table 2. Positive Controls

Ames II Strain	Control Chemical	Conc. (µg/ml)
TA7001	N4-aminocytidine	100, 50, 25, 12.5
TA7002	methylmethanesulfonate	1000, 500, 250, 125
TA7003	streptonigrin	10, 5, 2.5, 1.25 (ng/ml)
TA7004	4 nitroquinoline N-oxide	1, 0.5, 0.25, 0.125
TA7005	4 nitroquinoline N-oxide	1, 0.5, 0.25, 0.125
TA7006	4 nitroquinoline N-oxide	1, 0.5, 0.25, 0.125
TA1537	9-aminoacridine	2, 1, 0.5, 0.25
TA98	2-nitrofluorene	1, 0.5, 0.25, 0.125
Mix	4 nitroquinoline N-oxide & 2-nitrofluorene	1, 0.5, 0.25, 0.125

IV.VI. Test Article Preparation

A 100 µg/ml stock of the test article was prepared in sterile water.

Comments

The Ames II Mutational Spectra assay performed as a part of this service:

- Was performed in triplicate.
- Was performed in the absence of S9.
- Used a 384 well format (48 wells/strain/dose).
- Used sterile water as solvent.
- Used quality controlled reagents and cells.

Followed Ames II Mutational Spectra Version 2.0 guidelines.

V. DATA ANALYSIS

V.I. Determination of Colony Number

For the Ames II assay, the mean number of positive wells for each strain at each dose was determined. From this number, the number of bacterial colonies was determined. Since the 48 well format was used in this assay, no Poisson distribution was employed.

V.II. Determination of Fold Increase

The fold increase of bacterial revertant colonies compared to the background (zero dose) revertant colony number was determined by dividing the mean number of colonies at each dose by that at the zero dose. Student's *t*-tests were used to determine significance (at the $\alpha = 0.05$ level) for fold inductions greater than 3.0. The Student's t-test was performed to test the null hypothesis for every concentration of compound in comparison to the zero-dose control. Because the samples have different variance, the following formula was used:

$$t = \frac{\left[\bar{x}_1 - \bar{x}_2\right]}{\sqrt{\left[\frac{S_1^2}{n_1}\right] + \left[\frac{S_2^2}{n_2}\right]}}$$

where:

$\bar{x}_1$ = the mean colony count for sample 1 (zero dose control)
$\bar{x}_2$ = the mean colony count for sample 2 (concentration X)
S_1^2 = the variance of colony count 1
S_2^2 = the variance of colony count 2
n_1 = the number of replicate colony counts for count 1 ($n = 3$)
n_2 = the number of replicate colony counts for count 2 ($n = 3$)

Since the t-test was used to evaluate means *greater* than the control value, reference was made to a table of critical values for the Student's t-test which records an area of α in the right hand tail only.

The degrees of freedom value was determined by the smaller of the values n_1-1 or n_2-1, both of which equal 2.

Although statistical analyses have been applied to all data collected, fold increases in revertant numbers in the Ames II Assay are not classified as positive if less than 3.0. Below this fold increase value, the data are unreliable with respect to determining mutagenicity. To be classified as a mutagen, a compound is therefore required to yield a reproducible fold increase of greater than 3.0, or to show a clear dose response.

V.III Archived Data

Raw data for this study are located in Xenometrix laboratory notebooks as follows:

Table 3. Archived Data

Notebook	User	Pages
145/TRL	Sally Haugen	75-77