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May 23, 1996

Mr. Fernando Tamames, President
Citrex, Inc.
881 Belle Meade Island
Miami, FL 33138

Dear Mr. Tamames,

Enclosed please find a copy of our most recent Xenometrix Toxicology Reference Laboratory reports for assays performed with test article Citrex Liquid in May, 1996. The data included in this report are from a single Cyto-Tox Assay of test article Citrex Liquid.

The six lines tested were: KC - Human keratinocyte skin cell line
NT2 - Human Teratocarcinoma CNS precursor cell line
MES - Human squamous cell lung carcinoma cell line
RKO - Human colon carcinoma cell line
ACHN - Human renal adenocarcinoma cell line
HepG2 - Human hepatocellular carcinoma cell line

The HepG2 liver cell line, tested previously in CAT-Tox (L) assays with Citrex Liquid is the most resistant of the cell lines, with significant cytotoxicity not seen until 35 µg/ml. These data are very consistent (similar) with the CAT-Tox (L) assays obtained in our first set of tests (August 1995), where 30 µg/ml led to significant cytotoxicity.

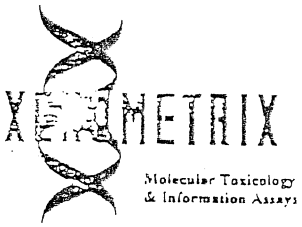
Importantly, we believe that these data show consistent performance of Citrex Liquid in these assays.

The other lines were somewhat more sensitive to the test article. But none was more than 7 times more sensitive. The sensitivities therefore all fell within a narrow range of concentration. All six lines followed the general same trend with increasing dose, with none showing sharp reductions in viability at then lower doses.

These reductions in cell viability have not been correlated with carcinogenicity in this assay. Data from the CAT-Tox (L), Pro-Tox (C) and Ames II assays performed on Citrex Liquid previously, did not indicate any obvious carcinogenic potential.

Sincerely,

Mark B. Benjamin, Sc.D.
Director of Scientific Affairs



CITREX, INC.
TEST ARTICLE: CITREX LIQUID

Cyto-Tox
Human Cytotoxicity Assay (6 cell lines)

Study Number: Cit-0596
May, 1996

SPONSOR/Monitor

Fernando Tamames III
President
Citrex Inc.
881 Belle Meade Island
Miami, FL 33138

TESTING FACILITY

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TABLE OF CONTENTS

I. SUMMARY

Test article Citrex Liquid was assayed in:

Cyto-Tox, a cytotoxicity assay using 6 human cell lines.

The test article was soluble in sterile, distilled water. Individual stock solutions were made up in this solvent. The stock solution concentration is given in section IV.

For the test article, simultaneous exposure to 6 cell lines in culture yielded data sets for dose-dependent viability changes. Two values are used to define the relationship between exposure level (dose) and viability in this assay: NOEL and LC50. Both of these terms are defined in appendix 1. A NOEL is determined for each cell line exposed to the test article. The NOEL (No observed effect level) gives an indication of the highest exposure level at which no statistically significant change in viability was recorded for each cell line. The NOEL therefore is an indicator of size of the shoulder of a survival curve. An LC50 (the exposure level at which viability of a given cell line is reduced to 50 % of the control value) is also recorded. The LC50 is an indicator of the slope of the curve, showing how rapidly viability declined at exposure levels above the NOEL. The two tables below list NOELs and LC50s for each line exposed to test article Citrex Liquid, ranked in ascending order. Lower NOELs and LC50s indicate that a test article was exerting an impact on the cell line at a lower concentration.

The dose range used was 0.2 to 100.0 µg/ml in doubling increments for all cell lines.

Citrex Liquid

Rank	Cell Line	NOEL (µg/ml)
1	MES	0.2
2	HepG2	0.4
3	KC	0.8
	RKO	0.8
4	NT2	3.1
	ACHN	3.1

Citrex Liquid

Rank	Cell Line	LC50 (µg/ml)
1	KC	5.5
2	NT2	8.0
3	RKO	17.0
4	ACHN	21.0
5	MES	29.5
6	HepG2	35.5

Key to cell lines: RKO:Human colon carcinoma cell line; NT2: Human teratocarcinoma CNS precursor line; MES: Human squamous cell lung carcinoma line; KC: Human keratinocyte skin cell line; ACHN: Human renal adenocarcinoma line; HepG2: Human hepatocellular carcinoma line.

The MES and HepG2 lines appear at the top of the NOEL table, indicating that they showed the more rapid changes in viability at the lower doses. In both cases, these changes are *increases* in viability and therefore although these two lines appear to be the most sensitive to low doses of Citrex Liquid, this low dose sensitivity is not cytotoxicity. In contrast, the NT2 and ACHN lines are the least sensitive, requiring higher doses of Citrex Liquid to reach statistically significant reductions in viability.

The MES and HepG2 lines also appear at the bottom of the LC50 table, indicating that they required higher doses of Citrex Liquid to reach 50 % viability than the other 4 lines. These lines were therefore more resistant than most at the higher concentrations of Citrex Liquid, perhaps as a consequence of elevated viability at the lower doses.
The LC50 for HepG2 cells with Citrex Liquid when tested previously in the CAT-Tox (L) assay (see Xenometrix study number: Cit-0895) was 30 µg/ml which compares favorably with the current data.

II. INTRODUCTION

The purpose of this study was to assess the cytotoxicity caused by exposure of human cell lines to the test article Citrex Liquid. One cytotoxicity assay was performed: Cyto-Tox. Complete description of this assay is provided upon request, and a brief description can be found in section IV.

The assay was performed with concurrent negative controls, and assayed separately with known positive control exposures. Positive control chemicals for the Cyto-Tox 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 1 appendix. 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. The appendix provides information on terminology used throughout the report.

III. TEST ARTICLE

The test article arrived in a white plastic container, with a white label. This label was printed with the following information (in addition to the Citrex commercial logo):

Citrex Liquid
Batch # 200
MFG. DATE: 5 12 95

The test article arrived at Xenometrix in August, 1995, and was tested at that time in the Pro-Tox (C), CAT-Tox (L) and Ames II (Complete) assays. These data are compiled in Xenometrix Toxicology Reference Laboratory study number: Cit-0895

IV. METHODS

IV.I. *Cyto-Tox Assay Version 1.0*

General Description

The Cyto-Tox Assay provides, in a single plate, the ability to test the toxicity of a test article using six different human cell lines in culture. The assay incorporates human colon, liver, lung, neuronal, kidney and skin cell lines. Within one assay, all six lines are exposed to the test article over a range of up to ten doses, including a zero dose control. Three plates are tested concurrently, to generate triplicate data points.

Culture viability is measured at each dose by the application of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT). MTT is a hydrogen acceptor, which can be taken up by viable cells and reduced by mitochondria to yield a purple formazan salt. By solubilizing these formazan salt crystals in DMSO, this turnover can be measured spectrophotometrically. Only viable cells can carry out this reaction.

The Cyto-Tox Assay has been designed to use a 96-well microtitre plate format. The assay requires only milligram quantities of compound for dosing and yields results in 24 hours. The results are displayed either numerically with appropriate statistical treatment or in graphical form.

Assay Procedure

A shortened version of the assay protocol is as follows:

1. Pre-plated cell lines are inspected for to ensure appropriate confluences (optimal confluences are between 50 and 80%).
2. Growth medium is replenished
3. Using a separate 96-well plate, serial dilutions of the test article are prepared.
4. Test article dilutions (in an appropriate solvent) are added to the pre-plated cells.
5. The plates are incubated for 24 hours at 37°C, 5.0 % CO_2.
6. MTT solution is added to each well, and the plates are incubated for 30 minutes.
7. Culture medium is carefully aspirated from each well.
8. DMSO is added to each well to lyse the cells and solubilize the formazan salt formed.
9. The plates are read using a microplate reader, at 550 nm light.
10. Xenometrix software uses the OD_{550} readings to calculate cellular viability at each test article dose. The software also converts the OD_{550} changes, relative to those seen at the zero dose to an estimate of cellular viability percentages.

Comments

The Cyto-Tox assays performed as a part of this service:

- Were performed in triplicate, (n = 3).
- Were performed in the absence of S9.
- Used distilled water as solvent.
- Used quality controlled reagents and cells.
- Incorporated a range-finding pre-Assay.
- Followed Cyto-Tox Version 1.0 guidelines.

IV.II. *Metabolic Activation*

The assays performed in this report were done so in the absence of S9 fraction and therefore did not include exogenous metabolic activation.

IV.III. *Test Article Preparation*

The test article provided was mixed with sterile, distilled water to make a stock solution as noted below. This stock solution was made up immediately prior to use and remaining stock was discarded after use.

Test Article	Stock Conc.	Solvent
Citrex Liquid	1.0 mg/ml	H_2O

V. DATA ANALYSIS

V.I. *Cyto-Tox Calculations*

Determination of Percentage Viability

Cytotoxicity is determined by conversion of MTT to the formazan salt chromophore, measured by optical density at 550 nm. The viability of cells at a given dose compared to the control (zero dose) viability was determined by dividing the zero dose OD_{550} reading, by the OD_{550} reading obtained at each dose level. The OD_{550} values are linear up to 2.0, which is higher than the 0.2 to 1.0 maximum normally reached with confluent, healthy cells.

$$\%V_n = \frac{\left[\frac{\sum_{i=1}^{nD} OD_{550}}{n_D}\right]}{\left[\frac{\sum_{i=1}^{n0} OD_{550}}{n_0}\right]} \times 100\%$$

where:

$\%V_n$ = percentage viability at dose n

n_D = number of replicates taken for dose D (3)

n_0 = number of replicates taken for dose 0 (control, 3)

OD_{550} = the OD_{550} value

In some cases more or fewer replicate wells will be employed in this calculation.

Student's *t*-tests were used to determine significance (at the $\alpha = 0.05$ level) for percentage viabilities less than or greater than 100 %. 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 viability value for sample 1 (zero dose control)
$\bar{x}_2$ = the mean viability value for sample 2 (concentration *n*)
S_1^2 = the variance of viability value 1
S_2^2 = the variance of viability value 2
n_1 = the number of viability measurements taken for mean viability value 1
n_2 = the number of viability measurements taken for mean viability value 2

The degrees of freedom value was determined by the smaller of the values n_1-1 or n_2-1. In this case the control viability mean is a product of three replicates, and therefore 2 degrees of freedom were applied.

V.III. *Archived Data*

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

Notebook	User	Pages
117/TRL	Shannon Beard	15
117/TRL	Shannon Beard	18

VI. RESULTS

VI.I. Test Article Citrex Liquid

TABLE 1. TEST ARTICLE CITREX LIQUID EVALUATION WITH Cyto-Tox, MAY 1996

Solvent: Water — 24 h exposure to Test Article

Student's t Test significant at $\alpha = 0.05$ if calculated values greater than 2.92

HUMAN COLON CELLS: RKO					LC50 = 17.0 µg/ml						
Conc. (µg/ml)	0	0.2	0.4	0.8	1.6	3.1	6.3	12.5	25	50	100
	1.84	1.79	1.86	1.75	1.71	1.59	1.54	1.05	0.54	0.1	0.03
	1.78	1.88	1.86	1.92	1.64	1.51	1.52	1.08	0.56	0.1	0.04
	1.78	2.12	2	1.89	1.72	1.51	1.43	1.11	0.63	0.13	0.13
Average	1.80	1.93	1.91	1.85	1.69	1.54	1.50	1.08	0.58	0.11	0.07
Std. Dev.	0.035	0.171	0.081	0.091	0.044	0.046	0.059	0.030	0.047	0.017	0.055
Viability (%)	100.00	107.22	105.93	102.96	93.89	85.37	83.15	60.00	32.04	6.11	3.70
t Test		1.55	2.26	1.04	3.44	7.98	7.97	27.29	36.60	79.68	47.33

HUMAN LIVER CELLS: HepG2					LC50 = 35.5 µg/ml						
Conc. (µg/ml)	0	0.2	0.4	0.8	1.6	3.1	6.3	12.5	25	50	100
	2.01	1.96	2.07	2.86	2.39	1.9	1.89	1.51	1.11	0.62	0.05
	2.11	2.45	2.49	3	2.74	2.09	2.14	1.65	1.32	0.53	0.05
	1.87	2.11	2.52	2.35	2.21	2.6	2.22	2.11	1.4	0.51	0.05
Average	2.00	2.17	2.36	2.74	2.45	2.20	2.08	1.76	1.28	0.55	0.05
Std. Dev.	0.121	0.251	0.252	0.342	0.270	0.362	0.172	0.314	0.150	0.059	0.000
Viability (%)	100.00	108.85	118.20	137.06	122.54	110.02	104.34	87.98	63.94	27.71	2.50
t Test		1.16	2.39	3.92	2.83	1.02	0.73	1.35	6.53	19.74	39.56

HUMAN SKIN CELLS: KC					LC50 = 5.5 µg/ml						
Conc. (µg/ml)	0	0.2	0.4	0.8	1.6	3.1	6.3	12.5	25	50	100
	1.24	1.16	1.26	1.26	1.04	0.83	0.59	0.64	0.13	0.02	0.03
	1.2	1.36	1.17	1.1	0.94	0.73	0.57	0.6	0.13	0.02	0.02
	1.38	1.27	1.2	1.06	0.9	0.72	0.6	0.59	0.06	0.02	0.02
Average	1.27	1.26	1.21	1.14	0.96	0.76	0.59	0.61	0.11	0.02	0.02
Std. Dev.	0.095	0.100	0.046	0.106	0.072	0.061	0.015	0.026	0.040	0.000	0.006
Viability (%)	100.00	99.21	95.03	89.53	75.39	59.69	46.07	47.91	8.38	1.57	1.83
t Test		0.13	1.11	1.63	4.61	8.10	15.32	13.43	21.18	32.49	30.54

Averages and standard deviations are for OD_{550} readings listed under each dose level. Student's *t*-tests were performed on OD_{550} averages.

TABLE 1 CONTINUED. TEST ARTICLE CITREX LIQUID EVALUATION WITH Cyto-Tox, MAY 1996

Solvent: Water — 24 h exposure to Test Article

HUMAN NEURONAL CELLS: NT2

Student's t Test significant at $\alpha = 0.05$ if calculated values greater than 2.92

Conc. (µg/ml)	0	0.2	0.4	0.8	1.6	3.1	6.3	12.5	25	50	100
	1.6	1.57	1.52	1.53	1.41	1.29	0.84	0.44	0.05	0.05	0.04
	1.27	1.3	1.37	1.44	1.51	1.24	0.82	0.45	0.02	0.03	0.03
	1.26	1.25	1.27	1.23	1.33	1.12	0.67	0.35	0.02	0.02	0.03
Average	1.38	1.37	1.39	1.40	1.42	1.22	0.78	0.41	0.03	0.03	0.03
Std. Dev.	0.193	0.172	0.126	0.154	0.090	0.087	0.093	0.055	0.017	0.015	0.006
Viability (%)	100.00	99.76	100.73	101.69	102.91	88.38	56.42	30.02	2.18	2.42	2.42
t Test		0.02	0.08	0.16	0.35	1.40	5.13	9.50	15.65	15.77	16.52

HUMAN LUNG CELLS: SK-MES-1 — LC50 = 29.5 µg/ml

Conc. (µg/ml)	0	0.2	0.4	0.8	1.6	3.1	6.3	12.5	25	50	100
	1.1	1.06	1.01	1.1	1.19	1.09	1.06	0.88	0.65	0.13	0.03
	1.06	1.2	1.02	1.16	1.26	1.16	1.03	0.88	0.63	0.12	0.03
	1.11	1.3	1.05	1.22	1.32	1.09	1.05	0.91	0.64	0.11	0.04
Average	1.09	1.19	1.03	1.16	1.26	1.11	1.05	0.89	0.64	0.12	0.03
Std. Dev.	0.026	0.121	0.021	0.060	0.065	0.040	0.015	0.017	0.010	0.010	0.006
Viability (%)	100.00	108.87	94.19	106.42	115.29	102.14	96.02	81.65	58.72	11.01	3.06
t Test		1.61	3.28	1.98	4.46	0.85	2.54	11.19	30.24	65.19	80.32

HUMAN KIDNEY CELLS: ACHN — LC50 = 21.0 µg/ml

Conc. (µg/ml)	0	0.2	0.4	0.8	1.6	3.1	6.3	12.5	25	50	100
	1.33	1.25	1.07	1.33	1.43	1.32	1.05	0.82	0.52	0.32	0.06
	1.26	1.6	1.11	1.28	1.59	1.14	1	0.88	0.56	0.32	0.05
	1.56	1.55	1.28	1.42	1.2	1.31	1.11	1.01	0.61	0.35	0.06
Average	1.38	1.47	1.15	1.34	1.41	1.26	1.05	0.90	0.56	0.33	0.06
Std. Dev.	0.157	0.189	0.112	0.071	0.196	0.101	0.055	0.097	0.045	0.017	0.006
Viability (%)	100.00	106.02	83.37	97.11	101.69	90.84	76.14	65.30	40.72	23.86	4.16
t Test		0.59	2.10	0.43	0.16	1.20	3.81	4.63	9.94	14.81	19.97

Averages and standard deviations are for OD_{550} readings listed under each dose level. Student's *t*-tests were performed on OD_{550} averages.

Figure 1.
Cyto-Tox Assay: Citrex Liquid

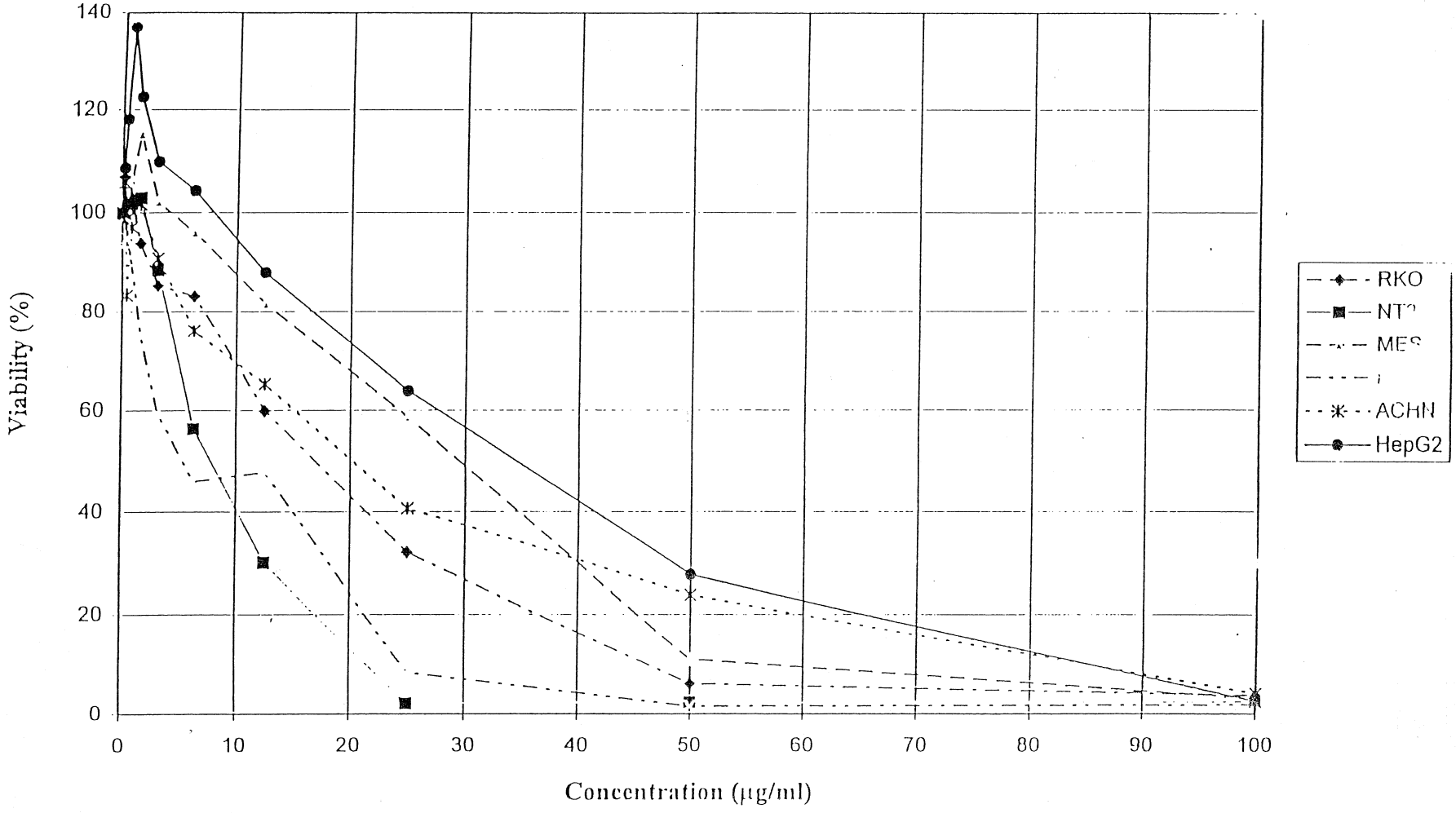
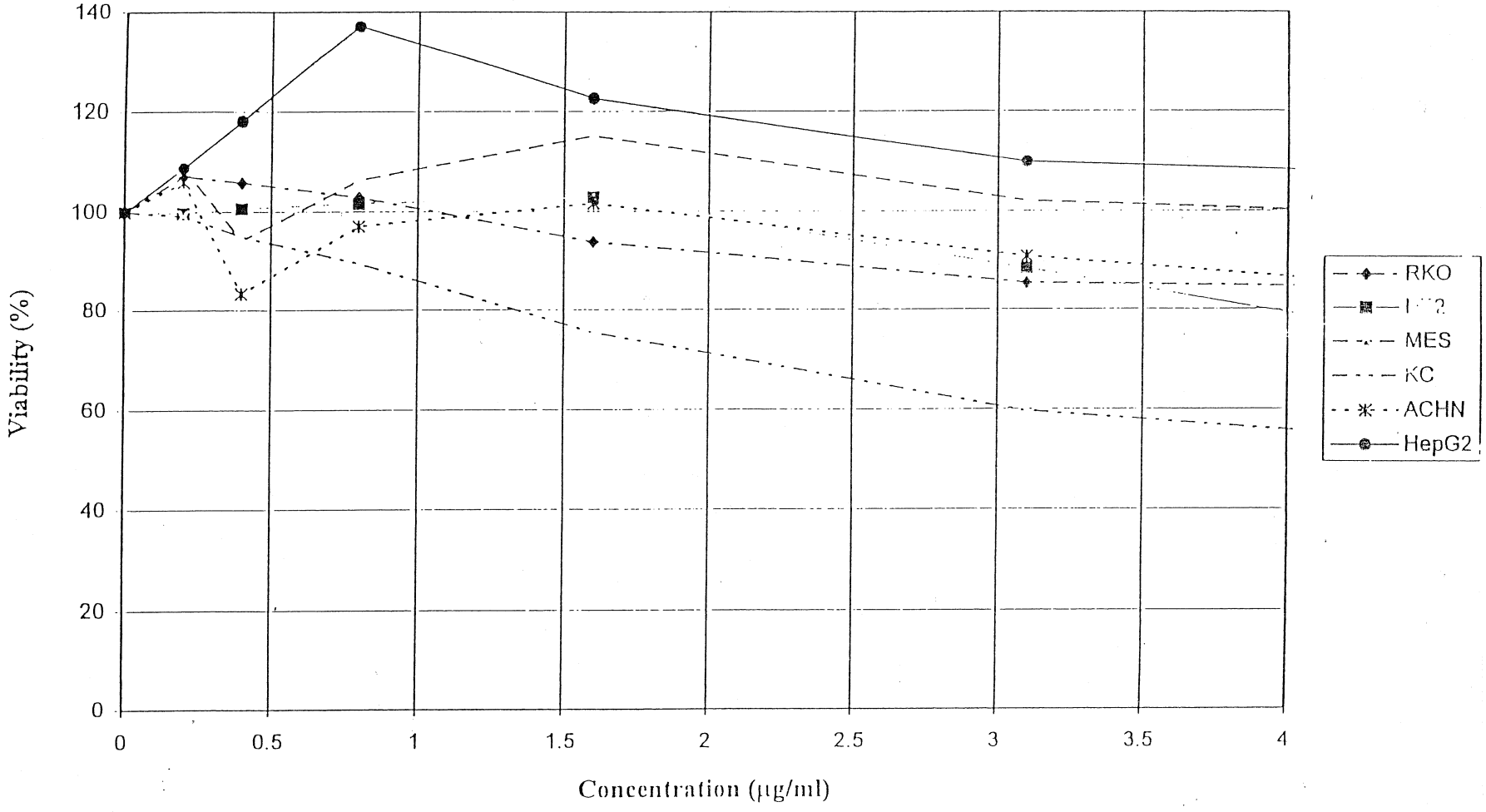


Figure 2.
Cyto-Tox Assay: Citrex Liquid
Magnified Scale



VII. DATA ANALYSIS

Data Analysis

VII.I. Citrex Liquid

Cyto-Tox Assay

The test article was soluble up 100 µg/ml in sterile, distilled water. No precipitate was seen at any dose. The dose range was from 0.2 to 100 µg/ml in doubling increments. Data can be found in Table 1, and Figures 1 and 2, and is summarized in the table below.

Cell Line[1]	NOEL[2]	LC50
RKO (Colon)	0.8 µg/ml	17.0 µg/ml
NT2 (Neuronal)	3.1 µg/ml	8.0 µg/ml
MES (Lung)	0.2 µg/ml	29.5 µg/ml
KC (Skin)	0.8 µg/ml	5.5 µg/ml
ACHN (Kidney)	3.1 µg/ml	21.0 µg/ml
HepG2 (Liver)	0.4 µg/ml	35.5 µg/ml

NB. *MTT data generated in these assays give an index of cell viability, but is, in reality a measure of cells with respiratory competence. In the descriptions below viability is therefore used as a quantitative measure derived from MTT data, but may not be equivalent to cloning efficiency.*

[1] key to cell lines: RKO = human colon, NT2 = human neuronal, MES = human lung, KC = human skin, ACHN = human kidney, HepG2 = human liver.

[2] NOELs are determined as the highest dose resulting in no statistically significant change (at the $\alpha = 0.05$ level) in viability.

[3] LC50s are determined directly from dose-response curves seen in Figure 1.

All 6 cell lines showed viability reductions below 50 % over the dose range employed. All six lines also showed viability reductions in a dose-response manner, for at least part of the dose-range. Two lines (HepG2 and MES) showed statistically significant *increases* in viability at the lower doses used (HepG2 to 137.06 % of control values at 0.8 µg/ml and MES to 115.29% of control values at 1.6 µg/ml). No line showed an overall increase in viability across the entire dose range. With the exception of the initial viability increases mentioned above, the six lines responded in a similar qualitative manner. Figure 1 shows the dose-dependent changes in viability for all six lines across the entire dose range. From approximately 12.5 µg/ml and higher, the lines respond very similarly, with viabilities determined by the dose-responses seen at the lower doses. The HepG2 liver line appears to

be the least sensitive to the cytotoxic effects of Citrex Liquid. The NT2 cell line appears to be the most sensitive overall, dropping to marginal levels of viability at 25 µg/ml. The four other lines are arrayed in dose-response patters between these two extremes, which remain largely in the same relative orientation at all doses above 12.5 µg/ml. All six lines showed only marginal levels of viability (less than 5.0 %) at the highest dose.

Appendix 1: Definitions

No Observed Effect Level (NOEL)
The highest exposure level at which there is no statistically significant increase above background for gene induction, and at which there is no reduction in cell viability.

Lethal Concentration $_{50}$ (LC50)
The dose of test article that is estimated to cause 50 % mortality in the exposed population.

In CAT-Tox (L) and CAT-Tox (D) Stress Gene Assays and the Cyto-Tox Assay, LC50s are recorded based on the results of MTT viability assays performed concurrent with the assay. LC50s represent those concentrations which therefore reduce the MTT value to 50 % of that of the negative control.

In the Genetic Toxicology Assays (Ames II, Yeast DEL and *E.coli Trp*), LC50s are determined from viability platings performed either as an integral part of the assay (Yeast DEL) or as an option (Ames II and *E.coli Trp*). In each case, LC50s will be determined from the mean number of surviving colonies at each dose, relative to the mean number of surviving colonies at the zero dose (negative control).

Statistical significance
Two criteria are imposed on the determination of statistical significance in Xenometrix *in vitro* assays:
1. Significance is determined at the $\alpha = 0.05$ level
2. The assays are performed in triplicate (n = 3) giving 2 degrees of freedom (df).

Using these criteria a Students *t*-test is employed in order to reject or accept the null hypothesis (H_o) that the means compared (e.g. mean fold induction at dose n and mean fold induction at the zero dose (1.0)) are the same. Using these criteria a *t*-test value of 2.92 or greater permits rejection of H_o and, at the $\alpha = 0.05$ level (95 %), a determination of statistically significant increase (e.g. in fold induction over basal level).

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