Citrex, Inc.
TEST ARTICLE: Citrex 2

CAT-Tox (L)
Human Liver Cell Stress Gene Assay (13 constructs)

Study Number: CIT-0597aR
May, 1997

SPONSOR/Monitor

Dr. Fernando Tamames III
Citrex, Inc.
881 Belle Meade Island
Miami, FL 33138-5247

TESTING FACILITY

Client Research
Laboratory Services
Xenometrix, Inc.
2425 North 55th Street
Boulder, Colorado 80301
Telephone (303) 447-1773
Fax (303) 447-1758

TABLE OF CONTENTS

		Page
I.	SUMMARY	5
II.	INTRODUCTION	5
III.	TEST ARTICLE	6
IV.	METHODS	6
	I. *CAT-Tox (L) Assay*	6
	II. *Metabolic Activation*	7
	III. *Positive Controls*	8
	IV. *Test Article Preparation*	8
V.	DATA ANALYSIS	8
	I. *CAT-Tox (L) Calculations*	8
	II. *Determination of Fold Induction*	9
	III. *Archived Data*	9
VI.	RESULTS	10
VII.	DATA INTERPRETATION	14
Appendix 1. Definitions		16
Appendix 2. Stress Genes		18
Appendix 3. Quality Control Results		25

I. SUMMARY

The test article (Citrex 2) was assayed in:

CAT-Tox (L), a stress gene assay using a human liver cell line (HepG2), and 13 mammalian stress gene reporter constructs driving expression of the chloramphenicol acetyltransferase (CAT) gene.

The test article was soluble in sterile water. Individual stock solutions were made up in this solvent. Stock solution concentrations are given in section IV.

The cell line containing the XHF construct has been removed from the CAT-Tox (L) assay due to unsatisfactory performance.

In the CAT-Tox (L) Assay, test article Citrex 2 caused a significant reduction in viability between the top two doses tested (25.0 μg/ml - 50 μg/ml). An LC50 of 35 μg/ml was interpolated from the assay data.

Test article Citrex 2 showed only marginal levels of activity for one construct, GRP78, a response associated with protein perturbation. The cytotoxicity seen at higher doses of test article Citrex 2, is therefore unlikely to be as a consequence of activities detected in the CAT-Tox (L) assay. Any metabolites formed as a consequence of biotransformation in the liver were not tested in this study, which was performed in the absence of S9 fraction.

II. INTRODUCTION

The purpose of this study was to assess the gene activity induced by exposure of human liver cells to test article Citrex 2. One stress gene assay was performed: CAT-Tox (L). A 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 CAT-Tox (L) 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 3 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. The appendixes provide information on terminology used throughout the report, descriptions and references on each of the stress genes monitored, and quality control data for the cell lines used in this study.

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. *CAT-Tox (L) Assay Version 2.0*

General Description

The mammalian stress gene assay, CAT-Tox (L), is capable of measuring stress-induced differential gene expression in the human hepatoma cell line, HepG2. Thirteen different recombinant human liver cell lines were generated by creating stable transfectants of different stress promoter-CAT gene fusions (CAT = chloramphenicol acetyltransferase). The activity of a given promoter is measured simply by the accumulation of CAT protein, measured using a standard CAT ELISA detection system.

A broad range of promoters responsive to DNA damage, heavy metal ions, protein denaturants, aromatic hydrocarbons, retinoids, and changes in intracellular cyclic AMP levels have been included in the assay. In some cases specific response elements are monitored, permitting fine analysis of stress-regulated gene expression.

The CAT-Tox (L) assay has been designed to use a 96-well microtiter plate format. This system gives simultaneous dose-response information at five different concentrations of the test chemical for the 13 recombinant cell lines. CAT-Tox (L) requires only milligram quantities of compound for dosing and yields results in 24 to 48 hours. The results are displayed either numerically with appropriate statistical treatment or in histogram form as a XenoMatrix™. The assay also includes the parental HepG2 line for the measurement of cytotoxicity. The CAT-Tox (L) assay can distinguish subtle differences among closely related compounds, and can indicate molecular mechanisms of sub-lethal cellular injury.

Assay Procedure

A shortened version of the assay protocol is as follows:

1. The 13 recombinant cell lines and the parental HepG2 cell line are plated, one row each, over two 96-well microtiter plates.
2. The cell lines are dosed at five test chemical concentrations and incubated at 37°C, 5 % CO_2 for 48 hours.

3. After the exposure incubation period the cells are washed two times and lysed with a detergent based buffer to release total cellular protein.
4. An aliquot of the total protein is transferred to 96-well microtiter plates containing Bradford protein dye. Incubation of the protein with the protein dye creates a color change that can be measured at OD_{600}. This reading serves as a normalization factor for total cellular protein from well to well in the assay.
5. The remaining cellular protein is transferred to 96-well plates containing polyclonal anti-CAT antibodies. A standard sandwich ELISA is performed and in the final step horseradish peroxidase catalyzes a color change reaction that can be measured at OD_{405}.
6. The parental HepG2 cell line that was dosed in the same manner as the 14 recombinant lines is used to perform a MTT-based cellular viability assay. The results of this assay can be monitored at OD_{550}.
7. Xenometrix software uses the OD_{600} and OD_{405} readings to calculate the transcriptional fold induction for each recombinant cell line at each test chemical dose. The software also converts the OD_{550} to cellular viability percentages.

Comments

The CAT-Tox (L) assays performed as a part of this service:

- Were performed in triplicate.
- Were performed in the absence of S9.
- Used sterile water as solvent.
- Used quality controlled reagents and cells.
- Incorporated a dose-range finding pre-assay.
- Included a 48 hour exposure period.
- Followed CAT-Tox (L) Version 2.0 guidelines.

IV.II. *Metabolic Activation*

The Stress Gene assay performed in this report (CAT-Tox (L)) was done in the absence of S9 fraction and therefore did not include exogenous metabolic activation.

IV.III. *Positive Controls:* CAT-*Tox (L)*

Promoter	Control Chemical	Concentration
CYP1A1	3-methylcholanthrine	10 µM
GSTYa	3-methylcholanthrine	10 µM
XRE	3-methylcholanthrine	10 µM
HMTIIA	methyl methanesulphonate	100 µg/ml
FOS	methyl methanesulphonate	100 µg/ml
NFκBRE	Phorbol myristate acetate	400 ng/ml
HSP70	methyl methanesulphonate	100 µg/ml
CRE	methyl methanesulphonate	100 µg/ml
p53RE	methyl methanesulphonate	100 µg/ml
RARE	all trans retinoic acid	10 µM
GADD153	methyl methanesulphonate	100 µg/ml
GADD45	methyl methanesulphonate	100 µg/ml
GRP78	methyl methanesulphonate	100 µg/ml

For the CAT-Tox (L) Assay utilized in this study, all promoters responded to positive control exposures within acceptable parameters.

IV.IV. *Test Article Preparation*

The test article was prepared in sterile water to make a stock solution as listed below. The stock solution was prepared immediately before use.

Test Article	Stock Conc.
Citrex 2	500 µg/ml

V. DATA ANALYSIS

V.I. *CAT-Tox (L) Calculations*

$$Activity_n = \left[\frac{OD_{405n}}{OD_{600n}} \right]$$

Where:
$Activity_n$ = the activity of cells exposed to test article at concentration *n*
OD_{405n} = the OD_{405} (ELISA) value for cells exposed to test article at concentration n
OD_{600n} = the OD_{600} (total protein) value for cells exposed to test article at concentration *n*

NB: In cases where the OD_{600n} value is less than 0.05, this value is set to 0.05
In cases where the OD_{405n} value is less than 0.10, this value is set to 0.10
In either of these cases, the fold induction at concentration n will be set to 1.0

V.II. *Determination of Fold Induction*

The fold induction of construct activity compared to the background (zero dose) activity was determined by dividing the mean construct activity 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 1.0. 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 activity value for sample 1 (zero dose control)
$\bar{x}_2$ = the mean activity value for sample 2 (concentration X)
S_1^2 = the variance of activity value 1
S_2^2 = the variance of activity value 2
n_1 = the number of activity measurements taken for mean activity value 1
n_2 = the number of activity measurements taken for mean activity value 2

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.

V.III. *Archived Data*

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

Notebook	User	Pages
145/TRL	Sally Haugen	72-74

VI. RESULTS

VI.I. CAT-Tox (L): Citrex 2

Figure 1.
Toxicity Evaluation: Test Article Citrex 2. LC50 = 35 μg/ml

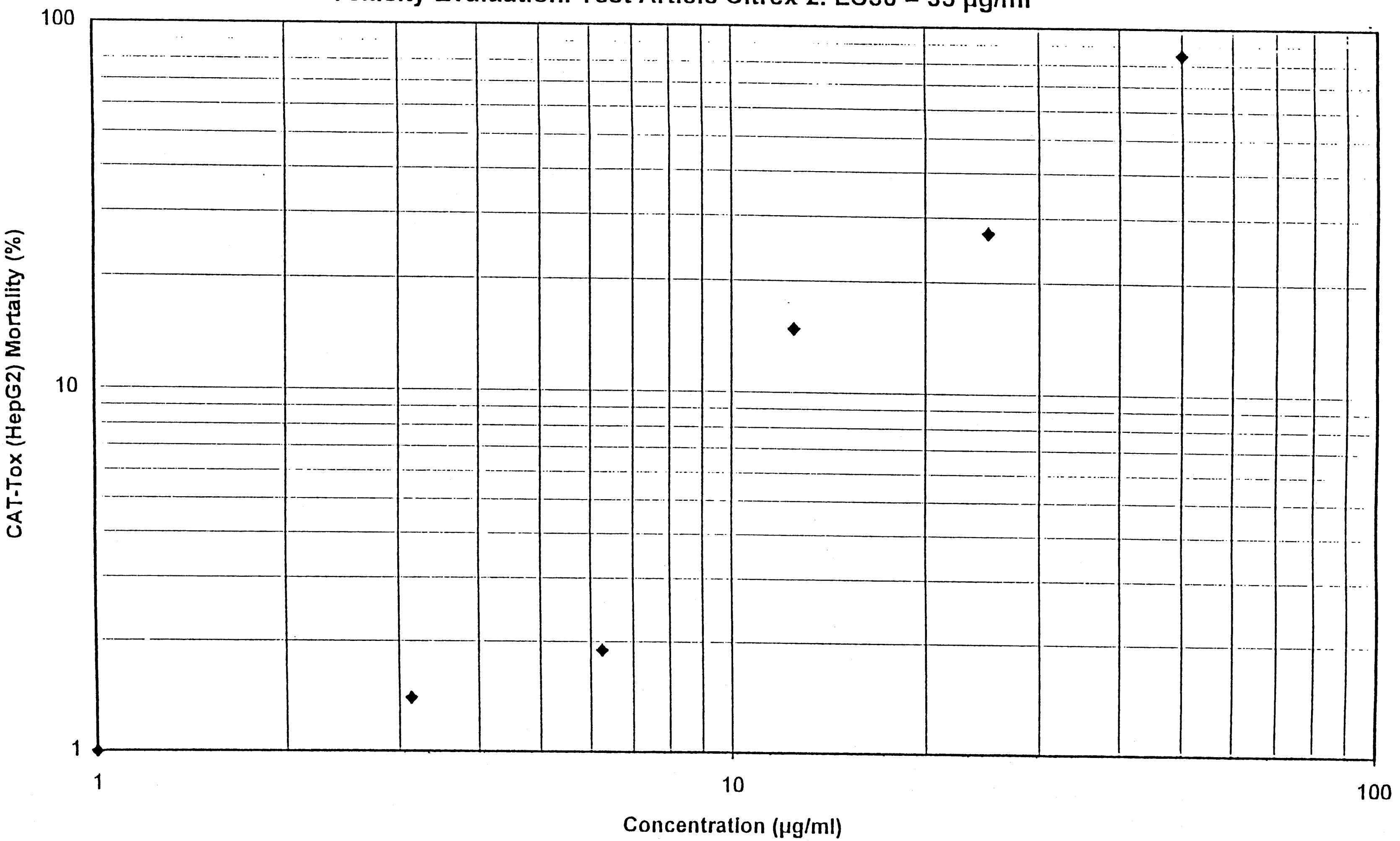


TABLE 1. TEST ARTICLE Citrex 2 EVALUATION WITH CAT-TOX (L) ASSAY.

LC50 = 35 µg/ml | Solvent: distilled water | Incubation: 48 hour exposure period

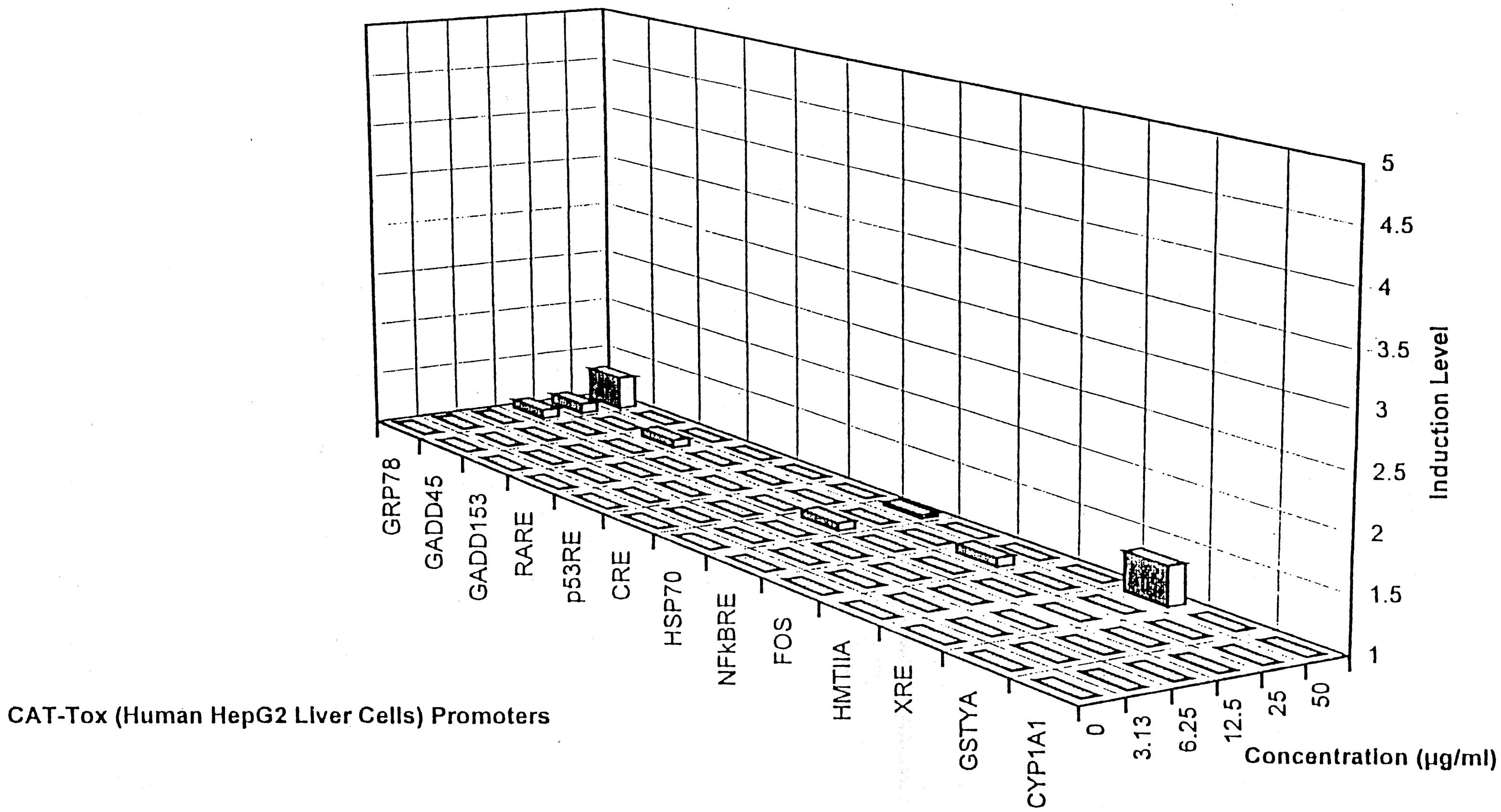
FOLD INDUCTION (n = 3); Student's t Test significant at 0.05 level if calculated values are greater than 2.92

Conc. (ng/ml)	CYP1A1	GSTYA	XRE	HMTIIA	FOS	NFKBRE	HSP70	CRE	p53RE	RARE	GADD153	GADD45	GRP78
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Standard. Dev.	0.25	0.21	0.09	0.06	0.16	0.19	0.31	0.07	0.15	0.05	0.08	0.12	0.07
Aver. Activity	0.10	0.10	0.10	0.10	0.30	0.20	0.96	0.47	0.20	0.10	0.48	0.63	1.22
Act. Std. Dev.	0.03	0.02	0.01	0.01	0.05	0.04	0.30	0.03	0.03	0.01	0.04	0.07	0.08
Coeff. Var. (%)	25.05	21.21	9.31	6.30	15.75	18.94	30.67	7.39	14.72	5.28	8.44	11.66	6.54
3.13	1.0	1.0	1.0	1.0	0.9	1.0	0.8	0.9	0.8	1.0	0.9	0.8	1.0
Standard. Dev.	0.0	0.0	0.0	0.0	0.2	0.1	0.1	0.1	0.2	0.0	0.1	0.1	0.0
Coeff. Var. (%)	0.0	0.0	0.0	0.0	19.7	8.1	7.6	12.5	19.3	0.0	15.4	9.1	4.1
t Test	0.00	0.00	0.00	0.00	0.97	0.34	1.11	0.98	1.27	0.00	1.06	1.96	0.45
6.25	1.0	1.0	1.0	1.0	0.8	1.0	0.9	0.9	1.0	1.0	0.8	0.8	0.9
Standard. Dev.	0.0	0.0	0.0	0.0	0.1	0.0	0.2	0.2	0.1	0.0	0.1	0.1	0.0
Coeff. Var. (%)	0.0	0.0	0.0	0.0	15.0	0.0	16.4	18.6	5.8	0.0	5.9	11.6	0.4
t Test	0.00	0.00	0.00	0.00	1.92	0.00	0.46	0.65	0.54	0.00	3.35	2.35	3.18
12.5	1.0	1.0	1.0	1.0	0.9	1.0	1.1	0.9	0.9	1.0	1.0	0.9	1.1
Standard. Dev.	0.0	0.0	0.0	0.0	0.1	0.0	0.3	0.1	0.1	0.0	0.2	0.1	0.1
Coeff. Var. (%)	0.0	0.0	0.0	0.0	13.4	0.0	23.3	9.7	6.3	0.0	16.0	5.0	9.9
t Test	0.00	0.00	0.00	0.00	1.26	0.00	0.31	1.34	0.65	0.00	0.00	0.82	1.08
25	1.0	1.0	1.0	1.0	1.1	1.0	1.0	0.9	1.0	1.0	1.1	0.9	1.1
Standard. Dev.	0.0	0.0	0.0	0.0	0.4	0.0	0.2	0.1	0.2	0.0	0.1	0.3	0.2
Coeff. Var. (%)	0.0	0.0	0.0	0.0	32.0	0.0	19.6	15.1	17.3	0.0	12.0	28.2	15.6
t Test	0.00	0.00	0.00	0.00	0.36	0.00	0.10	1.74	0.40	0.00	0.78	0.63	0.86
50	1.0	1.0	1.4	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.3
Standard. Dev.	0.0	0.0	0.6	0.0	0.0	0.0	0.2	0.1	0.0	0.0	0.0	0.0	0.1
Coeff. Var. (%)	0.0	0.0	45.0	0.0	0.0	0.0	15.7	14.7	0.0	0.0	0.0	0.0	7.0
t Test	0.00	0.00	0.98	0.00	0.00	0.00	0.15	0.88	0.00	0.00	0.00	0.00	4.98

MTT ASSAY RESULTS

Conc. (µg/ml)	**0**	**3.13**	**6.25**	**12.5**	**25**	**50**
Viability:	100.0	98.6	98.1	85.1	72.7	14.8
Standard Dev.	0	5.4	5.6	4.9	3.8	2

Figure 2.
Molecular Responses: Test Article Citrex 2. LC50 = 35 μg/ml



VII. DATA INTERPRETATION

Data Interpretation

Test Article Citrex 2

CAT-Tox (L) (Table 1, Figures 1 and 2)
The test article was dosed up to 50 µg/ml in sterile water. No precipitate was detected at any dose in this range. The dose range used was 0, 3.13, 6.25, 12.5, 25 and 50 µg/ml.
A reduction in HepG2 cell viability was seen at all concentrations of test article, with viability reduced to 15 % of control values at the highest dose. The LC50 (the concentration at which HepG2 cell viability is reduced to 50% of control values) for test article Citrex 2 was interpolated as 35 µg/ml.
Only one construct showed an induction to a statistically significant (at the $\alpha = 0.05$ level) level in the assay. The GRP78 construct, responsive to possible protein perturbations, was marginally induced to 1.3 fold at 50 µg/ml, the highest dose tested.
The induction NOEL (highest dose at which no observable gene effect was seen) for this test article was 25 µg/ml, with viability reduced to 73 % of control values at this dose.
The LOEL for gene induction (lowest observable effect level) was recorded as 50 µg/ml (GRP78).

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. Usually NOELs are analyzed in relation to cytotoxicity.

Lowest Observed Effect Level (LOEL)
The lowest exposure level at which there is a statistically significant increase above background in the end point of interest.

In the Stress Gene assay CAT-Tox (L), LOELs are recorded for gene induction, and represent the exposure level at which any construct in the assay is induced to a level which is statistically significantly different to its basal activity value (negative control) in that assay.

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

In the Stress Gene Assay CAT-Tox (L) , 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.

Fold induction
The level of gene induction in CAT-Tox (L), expressed as multiples of basal or background values. In the Stress Gene assays, background activity values are normalized and represented as 1.0 if fold induction is utilized.

Basal activity
In the stress gene assays, most (but not all) constructs produce reporter protein in the absence of any exposure (at the zero dose). This level of activity is referred to as a basal activity. Although each construct will exhibit a unique basal activity, for a fold induction expression, all basal activities are normalized to 1.0 (see above).

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).

Appendix 2: Stress Genes

CAT-Tox (L) Constructs

1. CYPIA1. Cytochrome P450 IA1 promoter. The CYPIA1 promoter is involved in the oxidative metabolism of many exogenous chemicals and drugs (Fujii-Kuriyama et al. 1992). Induction of this gene is controlled by the xenobiotic response (XRE) element (Neuhold et al. 1989). Inducing agents bind to the cytoplasmic Ah (aryl hydrocarbon) receptor which is present not only in the liver but also in other extrahepatic tissues such as lung, skin and kidney. The Ah receptor-ligand complex translocates to the nucleus and induces transcription by binding to the XREs in the CYPIA1 gene. This gene responds to polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene (B[*a*]P), 7-ethoxycoumarin, and 3-methylcholanthrene (3-MC). The CYPIA1 promoter can also respond to other chemicals such as b-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and polychlorinated biphenyls (PCBs).

2. GST Ya. Glutathione *S*-transferase *Ya* subunit promoter. The GSTs are a family of enzymes that add a thiol of reduced glutathione to a variety of electrophiles. GSTs can also directly bind to other compounds including heme, bilirubin, polycyclic aromatic hydrocarbons (PAHs), and dexamethasone. The active loci of the GSTs contains two binding sites, one site for glutathione (G-site), and a substrate binding (H-site) site (Rushmore and Pickett 1993). GST Ya inducible transcription is controlled by two distinct response elements, the xenobiotic response element (XRE) and the antioxidant-responsive element (ARE) (Rushmore et al. 1990). The XRE is identical to and transcriptionally activates GST Ya in a similar fashion as the XRE in the CYPIA1 gene. The ARE can work in an Ah (aryl hydrocarbon) receptor dependent or independent manner (Rushmore and Pickett 1991). The ARE contributes to induction of GST Ya mediated by PAHs through interaction with the Ah receptor. The ARE is also activated by compounds that do not require the Ah receptor. These compounds include the phenolic antioxidants *tert*-butylhydroquinone and 3,5-di-*tert*-butylcatechol.

3. XRE. Xenobiotic response element. Two copies of the XRE from the CYPIA1 gene have been placed upstream of the minimal thymidine kinase (TK) promoter. The arrangement allows segregation of multiple responses through the CYPIA1 and/or GST Ya promoters to isolate mechanistic differences. Inducing agents bind to the cytoplasmic Ah (aryl hydrocarbon) receptor which is present not only in the liver but also in other extra-hepatic tissues (e.g., lung, skin and kidney). The Ah receptor-ligand complex translocates to the nucleus and induces transcription by binding to the XREs in this construct. This promoter fusion responds specifically to polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene (B[*a*]P), 7-ethoxycoumarin, and 3-methylcholanthrene (3-MC). XRE can also respond to other chemicals such as b-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and polychlorinated biphenyls (PCBs).

4. HMTIIA. Metallothionein-II_A promoter. Metallothioneins (MTs) are a family of low-molecular weight heavy metal binding proteins. MT-IIA is classically induced by a variety of heavy metals including cadmium, zinc, copper, and silver (Richards et al. 1984) through the metal responsive element (MRE). In mammals MT-II_A is also induced by glucocorticoids. The precise functions of MTs are not known but it is hypothesized that they may act as intracellular storage sites for zinc and as sequestration sites for cadmium (Cherian et al. 1994). MTs do

mediate resistance to the toxic effects of metals, and they may also play a role in cellular resistance to alkylating agents and ionizing radiation. The alkylating agents methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS), as well as heavy metals, can transcriptionally induce MT-IIA.

5. FOS. *c-fos* promoter. *c-fos* is one of many nuclear proto-oncogenes. It may form an integral part of the AP-1 transcriptional complex along with the nuclear proto-oncogene, *c-jun* (Angel and Karin 1991). The AP-1 complex in turn binds to the TPA (12-*O*-tetradecanoylphorbol 13-acetate) response element (TRE). The TRE is present in several genes including collagenase, MT-IIA, stromelysin, interleukin-2, and *c-fos*. TPA, a phorbol ester tumor promoter, is a potent activator of the protein kinase C pathway. Other agents which lead to the activation of protein kinase C, such as serum and growth factors, can also upregulate these genes through the TRE. The *c-fos* promoter is regulated by a variety of response elements including the TRE, serum response element (SRE), and a cAMP response (CRE) element (Sassone-Corsi et al. 1988). The *c-fos* has also been implicated in the mammalian UV response pathway which appears to have an important role in the response to DNA damaging agents such as methyl methanesulfonate (MMS) and 4-nitroquinoline N-oxide (4-NQO) (Hollander et al. 1989).

6. NFκBRE. Nuclear factor of k enhancer (B site) response element. NFkB is a multi-subunit, transcription factor that can rapidly activate the expression of genes involved in inflammatory, immune, and acute phase responses (Baeuerle 1991). The protein is constitutively regulated in B cells but is inducible in many other tissue types. When exposed to activating agents, NFkB is released from its inhibitor IkB in the cytoplasm, translocates to the nucleus, and then binds the NFkB RE present in several different genes. For this construct, one NFkB RE has been placed upstream of the SV40 minimal promoter. Many different agents can activate the DNA binding activity of NFkB including viruses, bacterial lipopolysaccharide, protein synthesis inhibitors, cytokines (TNF-a, TNF-b and interleukin-1), phorbol esters, lectins, and calcium ionophores (Nelsen et al. 1988). NFkB is also hypothesized to be part of the UV response pathway and can therefore respond to certain classes of DNA damage agents as well (Angel and Karin 1991).

7. HSP70. 70 kd heat shock protein promoter. Cells react to hyperthermia and other protein-damaging conditions by increasing the synthesis of a group of stress induced or heat shock proteins (Hartl et al. 1992). Under physiologic conditions, these proteins play an essential role in protein-protein interactions. Members of the HSP70 family participate in the folding, assembly, and translocation of intracellular proteins. HSP70 also binds to proteins unfolded as a consequence of metabolic or exogenous stress. The inducible activity of the HSP70 gene is regulated by the heat shock element (HSE) located in the promoter (Hunt and Morimoto 1985) which binds the cloned heat shock factor (HSF). This element appears to be sufficient for all of HSP70's inducible responses. The HSP70 gene can respond to a variety of agents and conditions which affect protein conformation. These include heat, cadmium, amino acid analogs, reduced pH, nonionic detergents, urea, and alkylating agents (Mosser et al. 1988).

8. CRE. cyclic adenosine monophosphate (cAMP) response element. There are two major signaling pathways that utilize cAMP (Borrelli et al. 1992). One involves protein kinase C and

ultimately controls genes that contain a TRE (12-*O*-tetradecanoylphorbol 13-acetate response element). The other involves protein kinase A and alters gene activation through the CRE. Up-regulation of the cAMP pathway indicates that a compound activates cellular signaling specifically through protein kinases. Kinases are enzymes that catalyze the transfer of phosphate groups from ATP, ADP or AMP to a substrate. The fusion construct used in the CAT-Tox Assay contains two copies of the consensus CRE upstream of the SV40 minimal promoter. This construct responds to any agent that increases the level of cAMP in cells.

9. p53RE. p53 response element. The p53 wild-type protein is involved in transcriptional activation of genes that negatively control growth and/or invasion through the p53RE (Vogelstein and Kinzler 1992). Mutant forms of p53 have been implicated in deregulation of gene expression in many tumor types. The p53RE construct contains 10 copies of the p53RE from the GADD45 gene (Zahn et al. 1993) fused to the minimal thymidine kinase promoter. The p53 response element construct can be upregulated by DNA damaging agents (Kastan et al. 1991). It is hypothesized that this may be due to p53's role in the inhibition of replicative DNA synthesis. The p53 response element construct can also be induced by growth arresting agents.

10. RARE. Retinoic acid response element. Retinoids have a broad range of normal biological activities in growth and differentiation (Allenby et al. 1993). Retinoids have also been implicated in disturbing fetal development through aberrant gene activation. Retinoic acid analogs interact differentially with the retinoic acid receptors (a, b, and g) and the retinoid X receptors (a, b, and g) which can then bind to RAREs in downstream genes (Hoffmann et al. 1990). The RARE construct in the CAT-Tox Assay contains 2 copies of the RARE from the retinoic acid b receptor fused to the minimal thymidine kinase promoter. This construct responds to retinoic acid analogs including all-*trans*-retinoic acid and 9-*cis*-retinoic acid.

11. GADD153. 153 kd growth arrest and DNA damage promoter. GADD153 is a CCAAT/enhancer binding protein related gene whose expression is regulated by growth arrest and DNA damage (Fornace et al. 1989). Although the GADD153 promoter has been cloned, the specific sequences responsible for the DNA damage response have not been well characterized (Park et al. 1992). GADD153 has been shown to respond to a variety of DNA damaging agents including UV-mimetic, DNA-cross-linking, and alkylating agents (Leuthy and Holbrook 1992). This response appears to be directly related to altered DNA structure.

12. GADD45. 45 kd growth arrest and DNA damage promoter. The GADD45 gene was cloned at the same time as GADD153 (Fornace et al. 1989) and these genes were originally thought to be coordinately regulated. More recently, subtle differences in their regulation have been noted (Zahn et al. 1993) including the finding that the GADD45 gene contains a p53 response element in its third intron. The GADD45 promoter has been analyzed and it has been determined that a large stretch of promoter is necessary for the DNA damage response (Hollander et al. 1993). GADD45 can be induced by many, if not all, of the same agents as GADD153.

13. GRP78. 78 kd glucose regulated protein promoter. GRP78 is a major endoplasmic reticulum (ER) protein that functions as a chaperone. It associates with a variety of other proteins in the ER that are transmembrane targeted and may also be involved in the assembly of

oligomeric proteins (Wooden et al. 1991). Proteins that are malfolded because of mutagenesis, under-glycosylation, or other stress conditions in the ER associate with and upregulate the expression of GRP78. GRP78 appears to be the ER counterpart of HSP70 which resides in the cytoplasm. GRP78 can also be induced by depleted intracellular calcium stores and alkylating agents.

General Reference

Lewin, B. (1990) Genes IV. Cell Press, Cambridge, MA. Chapter 29:543-574.

Promoter References

Allenby, G., M.T. Bocquel, M. Saunders, S. Kazmer, J. Speck, et al. (1993) Retinoic acid receptors and retinoid X receptors: Interactions with endogenous retinoic acids. Proc. Natl. Acad. Sci. USA. 90:30-34.

Angel, P., and M. Karin (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta. 1072:129-157.

Baeuerle, P.A. (1991) The inducible transcription activator NFkB: regulation by distinct protein subunits. Biochim. Biophys. Acta. 1072:63-80.

Borrelli, E., J.P. Montmayeur, N.S. Foulkes, and P. Sassone-Corsi (1992) Signal transduction and gene control: the cAMP pathway. Crit. Rev. Onco. 3:321-338.

Cherian, M.G., S.B. Howell, N. Imura, C.D. Klaassen, J. Koropatnick, et al. (1994) Contemporary issues in toxicology: role of metallothionein in carcinogenesis. Tox. Appl. Pharm. 126:1-5.

Fornace, Jr., A.J., D.W. Nebert, M.C. Hollander, J.D. Luethy, M. Papathanasiou, et al. (1989) Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. Mol. Cell. Biol. 9:4196-4203.

Fujii-Kuriyama, Y., H. Imataka, K. Sogawa, K.I. Yasumoto, and Y. Kikuchi (1992) Regulation of CYPIA1 expression. FASEB 6:706-710.

Hartl, F.U., J. Martin and W. Neupert (1992) Protein folding in the cell: the role of molecular chaperones hsp70 and hsp60. Annu. Rev. Biophys. Biomol. Struct. 21:293-322.

Hoffmann, B., J.M. Lehmann, X.K. Zhang, T. Hermann, M. Husmann, et al. (1990) A retinoic acid receptor-specific element controls the retinoic acid receptor-b promoter. Mol. Endocrinology 4:1727-1736.

Hollander, M.C., and A.J. Fornace, Jr. (1989) Induction of fos RNA by DNA-damaging agents. Cancer Res. 49:1687-1692.

Hollander, M.C., I. Alamo, J. Jackman, M.G. Wang, O. W. McBride, et al. (1993) Analysis of the mammalian gadd45 gene and its response to DNA damage. J. Biol. Chem. 268:24385-24393.

Hunt, C. and R.I. Morimoto (1985) Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. Biochemistry 82:6455-6459.

Kastan, M.B., O. Onyekwere, D. Sidransy, B. Vogelstein, and R.W. Craig (1991) Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51:6304-6311.

Luethy, J.D. and N.J. Holbrook (1992) Activation of the gadd153 promoter by genotoxic agents: a rapid and specific response to DNA damage. Cancer Res. 52:5-10.

Mosser, D.D., N.G. Theodorakis, and R.I. Morimoto (1988) Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. Mol. Cell. Biol. 8:4736-4744.

Mosser, D.D., J. Duchaine, and B. Massie (1993) The DNA-binding activity of the human heat shock transcription factor is regulated in vivo by hsp70. Mol. Cell. Biol. 13:5427-5438.

Nelsen, B., L. Hellman, and R. Sen (1988) The NF-kB-binding site mediates phorbol ester-inducible transcription in nonlymphoid cells. Mol. Cell. Biol. 8:3526-3531.

Neuhold, L.A., Y. Shirayoshi, K. Ozato, J.E. Jones, and D.W. Nebert (1989) Regulation of mouse CYPIA1 gene expression by dioxin: requirement of two *cis*-acting elements during induction. Mol. Cell. Biol. 9:2378-2386.

Park, J.S., J.D. Luethy, M.G. Wang, J. Fargnoli, A.J. Fornace, Jr., et al. (1992) Isolation, characterization and chromosomal localization of the human GADD153 gene. Gene 116:259-267.

Richards, R.I., A. Heguy, and M. Karin (1984) Structural and functional analysis of the human metallothionein-I_A gene: differential induction by metal ions and glucocorticoids. Cell 37:263-272.

Rushmore, T.H., R.G. King, K.E. Paulson, and C.B. Pickett (1990) Regulation of glutathione S-transferase Ya subunit gene expression: identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. Proc. Natl. Acad. Sci. USA. 87:3826-3830.

Rushmore, T.H. and C.B. Pickett (1991) Xenobiotic responsive elements controlling inducible expression by planar aromatic compounds and phenolic antioxidants. Meth. Enzy. 206:409-420.

Rushmore, T.H. and C.B. Pickett (1993) Glutathione *S*-transferases, structure, regulation, and therapeutic implications. J. Biol. Chem. 268:11475-11478.

Sassone-Corsi, P., J.C. Sisson, and I.M. Verma (1988) Transcriptional autoregulation of the proto-oncogene *fos*. Nature 334:314-319.

Vogelstein, B. and K.W. Kinzler (1992) p53 function and dysfunction. Cell 70:523-526.

Wooden, S.K., L.J. Li, D. Navarro, I. Qadri, L. Pereira, et al. (1991) Transactivation of the grp78 promoter by malfolded proteins, glycosylation block, and calcium ionophore is mediated through a proximal region containing a CCAAT motif which interacts with CTF/NF-I. Mol. Cell. Biol. 11:5612-5623.

Zahn, Q., F. Carrier, and A.J. Fornace, Jr. (1993) Induction of cellular p53 activity by DNA-damaging agents and growth arrest. Mol. Cell. Biol. 13:4242-4250.

Appendix 3. Quality Control Results

CAT-Tox (L) QCTS

Effective Date: 01 Jan 1997

Cell Plate Lot #: 970511

Sample Number: N/A

Assayer: AWN/CS

Computer Program Version: CAT-Tox (L) Version 2.0

Cell Line	Neg. Cont.	31.6% Conc.	42.2% Conc.	56.3% Conc.	75% Conc.	100% Conc.	Compound	Limit	Pass/Fail	% Conf.
CYP1A1	1	17.8	22.82	29.77	37.21	48.48	1.5 ug 3-MC	>3.0	P	60-70
GSTYA	1	14.29	24.02	28.14	26.45	31.29	100ug MMS	>2.0	P	60-70
XRE	1	14.28	12.79	15.95	19.03	22.61	0.75 ug 3-MC	>10.0	P	60-70
HMTIIA	1	4.61	2.15	7.98	14.7	49.17	1.0 ug CdSO4	>5.0	P	60-70
FOS	1	1.78	2.36	5.35	9.97	17.45	100ug MMS	>10.0	P	60-70
NFkBRE	1	11.79	10.65	13.84	18.38	32.35	400 ng PMA	>10.0	P	60-70
XHF	1	1	1	1	1	1	N/A	N/A	P	N/A
HSP70	1	1.46	1.75	3.23	4.7	8.97	2.5ug CdSO4	>2.0	P	60-70
CRE	1	2.18	1.88	4.23	6.25	8.22	150ug MMS	>1.0	P	60-70
p53RE	1	1.62	1.41	3.68	10.21	17.46	100ug MMS	>3.0	P	60-70
RARE	1	4.36	7.31	6.57	6.56	7.71	20ug RA	>3.0	P	60-70
GADD153	1	2.8	2.64	9.89	12.87	19.02	100ug MMS	>5.0	P	60-70
GADD45	1	1.23	1.11	3.43	6.9	17.72	150ug MMS	>2.0	P	60-70
GRP78	1	1.83	1.79	3.4	3.71	5.57	100ug MMS	>2.0	P	60-70

Item	Manufacturer	Stock #	Lot #
Cell Culture Medium	Xenometrix	1007.001	1001618
5X Protein Dye	Xenometrix	N/A	49245A5
Cell Wash Solution	Xenometrix	1107.001	51N2047
MTT	Xenometrix	1210.001	97-6
Elisa Kit	Xenometrix	N/A	1618M-9
MMS	Sigma	M-4016	26H3655
CdSO4	Sigma	C-3266	34H03041
3-MC	Sigma	M-6501	75H2510
PMA	Sigma	N/A	36H0816
RA	Sigma	R-2625	84H0658

Done By: AWN/CS

Checked By: C Sommers