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Transformation of BALB/c-3T3 Cells: IV. Rank-Ordered Potency of 24 Chemical Responses Detected in a Sensitive New Assay Procedure

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This report introduces an improved method of detecting chemical-induced morphological transformation of A-31-1-13 BALB/c-3T3 cells. The new procedure uses an increased target cell population to assess chemical-induced damage by increasing the initial seeding density and by delaying the initiation time of chemical treatment. Furthermore, a newly developed co-culture clonal survival assay was used to select chemical doses for the transformation assay. This assay measured the relative cloning efficiency (RCE) of chemical treatments in high-density cell cultures. In addition, transformation assay sensitivity was enhanced through the use of improved methods to solubilize many chemicals. From a group of 24 chemicals tested in at least two trials, clear evidence of chemical-induced transformation was detected for 12 chemicals (aphidicolin, barium chloride-2H_2O, 5-bromo-2′-deoxyuridine, C.I. direct blue 15, *trans*-cinnamaldehyde, cytosine arabinoside, diphenylnitrosamine, manganese sulfate-H_2O, 2-mercaptobenzimidazole, mezerein, riddelliine, and 2,6-xylidine); 2 chemicals had equivocal activity [C.I. direct blue 218 and mono(2-ethylhexyl)phthalate], 9 chemicals were inactive [carisoprodol, chloramphenicol sodium succinate, 4-chloro-2-nitroaniline, C.I. acid red 114, isobutyraldehyde, mono(2-ethylhexyl)adipate, sodium fluoride, and 12-*O*-tetradecanoylphorbol-13-acetate), and 1 chemical had an indeterminate response (2,6-dinitrotoluene). All positive responses were detected in the absence of an exogenous activation system and exhibited significant activity at two or more consecutive doses. This report also presents a mathematical method that uses *t*-statistics for rank-ordering the potency of chemical-induced transformation responses. This model detects sensitivity differences in experiments used to evaluate chemical-induced transformation. Furthermore, it provides a method to estimate a chemical's transformation response in terms of the historical behavior of the assay, as well as its future activity. The most active of the 24 chemicals was mezerein, and the least active chemical was diphenylnitrosamine.

Introduction

The BALB/c-3T3 cell transformation assay design recommended by government agencies (*1*) and scientific committees (*2*) has remained essentially unchanged from the method first described by Kakunaga (*3*). Although this protocol has been demonstrated to detect some carcinogenic chemicals (*4,5*), it has a low sensitivity (*4*) for detecting the diverse group of chemicals screened in the NTP/NCI rodent bioassay (*6–9*).

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The low sensitivity of the standard BALB/c-3T3 transformation assay method has been reproduced in this laboratory. In 1983 and 1984, using the recommended method (*1–3*) we screened 55 coded chemicals on an interagency contract with the National Institute of Environmental Health Sciences and the Environmental Protection Agency. This group of chemicals contained 2 model chemicals and 53 other chemicals that were being considered at that time for evaluation in the rodent bioassay. The results of our investigations have been presented in abstract format (*10*), are summarized in Appendix B, and have confirmed the low sensitivity of the standard assay method.

At the onset of this program, we investigated many different experimental parameters to determine those that affected the sensitivity of the BALB/c-3T3 cells to detect chemical-induced transformation. This report summarizes our findings and describes a new assay procedure that was designed to enhance sensitivity for detecting

chemical-induced transformation. The present investigation reports the activities of 24 chemicals that have been tested using the new transformation protocol. This methodology has been also used to test the activities of an additional 166 test chemicals (*11,12*).

Materials and Methods

Cell Culture

The investigations in this report used the 1-13 clone of A31 BALB/c-3T3 cells (*13,14*). The materials and methods used to culture the cells have been previously reported in detail (*15*) and are summarized in part I of these investigations (*17*).

Standard Clonal Survival Assay

The standard clonal survival assay was used to *a*) estimate the cytotoxic activity of a test chemical, *b*) select treatment doses for the preliminary co-culture clonal survival assay described below, *c*) assess the reproducibility of the chemical-induced cytotoxic responses, and *d*) determine the relative shift in test chemical cytotoxic responses between high- and low-density cell cultures. The standard clonal survival assay used low-density cultures of BALB/c-3T3 cells and was conducted according to our modification (*15*) of the method first described by Kakunaga (*3*). Briefly, 200 WT cells were seeded in either 60-mm culture dishes (Corning Science Products, Corning, NY) or 25-cm^2 culture flasks (Corning). The test chemical treatment doses were applied to triplicate cultures for 48 hr beginning 2 days after seeding. Treatments were terminated by removal of the chemical treatment medium, washing the culture vessels twice with Hank's balanced salt solution (HBSS; Quality Biologicals, Gaithersburg, MD) and feeding with culture medium. After a total culture period of 8 days, the vessels were washed with HBSS, fixed with 100% methanol, stained with 10% Giemsa in tap water, and colonies of cells were hand tabulated using an illuminated light box.

Co-culture Clonal Survival Assay

The co-culture clonal survival assay was used to select chemical treatment doses for transformation assays, assess the reproducibility of chemical-induced cytotoxic responses, and verify that the test chemical and positive control treatment doses were cytotoxic in the transformation assay. The procedure used for the co-culture clonal survival assay has been previously reported in detail (*11,13*) and is summarized in part III of this series (*17*).

Calculation of Cytotoxic Response

The cytotoxic responses of chemicals were compared using the concentration in millimoles that resulted in 50% RCE of chemical-treated cells relative to untreated cultures. This LD_{50} treatment dose was extrapolated from graphs of dose-related changes in cytotoxic responses of the chemical detected in the co-culture and the standard clonal survival assays.

Transformation Assay

The BALB/c-3T3 cell transformation assay design in this study used our modification (*15*) of the method first described by Kakunaga (*3*). The transformation assay culture vessels were seeded with 3.2×10^4 WT cells/vessel. The positive control and test chemical treatment sets had 20 60-mm culture dishes (18 25-cm^2 culture flasks), and the negative control (NC-1) had 40–80 60-mm culture dishes (36–72 culture flasks). The positive control for each assay was benzo[*a*]pyrene (BaP; Sigma, St. Louis, MO), and it was tested at doses of 0.200 and 0.0633 μg/mL to assess the reproducibility of dose-related increases of BaP-induced cytotoxic and transforming activities (*17*). A total of three to six test chemicals were included in each transformation experiment, and each chemical was tested at four treatment doses in two or more independent trials. The four doses were chosen based on chemical-induced cytotoxic activities detected in the co-culture clonal survival assay. These doses attempted to cover a range of cytotoxic responses of 10–100% RCE. Test chemical, positive control, and solvent control treatments of cell cultures were performed as described for the standard clonal survival assay. Transformation assay culture vessels were fed biweekly with minimal culture media a total of seven times over 3.5 weeks, and the assay was terminated after a total culture period of 28 days.

The transformation assays in this investigation also included additional components to extend the information obtained from each experiment. For example, each transformation experiment had concomitant standard and co-culture clonal survival assays, and the purpose of using both assays is explained above. In addition, the transformation assay included seeding density controls (NC-2 and NC-3) of 1.0×10^4 cells/vessel and 3.2×10^3 cells/vessel, respectively. These controls were used to detect crowding effects and preexisting transformed variants that were occasionally detected in transformation assays using wild-type (WT) BALB/c-3T3 cells (*16*). Finally, because each chemical was tested in two or more trials, one active test chemical was used as a second positive control for each experiment and tested along with test chemicals of unknown activity.

Transformation Assay Acceptance and Evaluation Criteria

Single Transformation Experiment. In this investigation, a test chemical's activity in a single transformation experiment was evaluated as having one of four possible transformation responses: sufficient positive (SP), limited activity (LA), sufficient negative (SN), and limited negative (LN). An SP transformation response required that a test chemical response was statistically significant at two or more consecutive treatment doses. One of the two doses must have been significant at the 99% confidence level ($p \leq 0.01$), but the second dose could have been significant at either the 99% or the 95% confidence level ($0.05 \leq p \leq 0.01$). In addition, the SP response must have included a dose-related increase in activity relative to the experiment

solvent control. In contrast to the SP response, a LA transformation response required that a test chemical response was statistically significant at either one treatment dose alone at the 99% confidence level or at two consecutive doses at the 95% confidence level.

An SN transformation response required that a test chemical response did not have a statistically significant increase in transformation responses at any of the four treatment doses. Furthermore, one or more of the chemical treatment doses induced a significant cytotoxic response. A significant cytotoxic response is a test chemical treatment dose that resulted in 50% RCE detected in the co-culture clonal survival assay.

An LN transformation response occurred under two different circumstances. First, the four test chemical treatment doses did not induce a statistically significant transformation response; however, in contrast to an SN transformation response, the test chemical treatments did have significant cytotoxic responses. Therefore, higher concentrations of the test chemical could have induced a significant cytotoxic response, and this could have resulted in a statistically significant transformation response. Second, the test chemical had the equivalent of an SN transformation response; however, the positive control for the transformation experiment was inactive and did not induce a statistically significant response.

Two or More Transformation Experiments. When a test chemical is evaluated in two consecutive experiments, its cumulative response in these experiments is arbitrarily described in this investigation as either active, weakly active, inactive, or indeterminate. Test chemicals were evaluated as active when two different combinations of transformation responses were obtained: *a*) two SP responses and *b*) an SP and an LA response. A weakly active test chemical occurred when the two consecutive responses were an SP and an SN. Conversely, test chemicals were evaluated as inactive when three different combinations of transformation responses were obtained: *a*) two LA responses; *b*) two SN responses; and *c*) an LA and an SN response.

Test chemicals with indeterminate activity occurred under four different combinations of transformation responses: *a*) two LN responses; *b*) an LA and an LN; *c*) an SN and an LN response; and *d*) an SP and an SN response. Thus, test chemicals with a combined SP and an SN response in two consecutive trials could have been evaluated as either weakly active or indeterminate. If the mean *t*-statistics of the two experiments are not significantly different from one another, then the chemical was evaluated as having been weakly active in the two experiments (see "Statistical Analysis and Mathematical Model," below). Conversely, if the mean *t*-statistics of the two experiments are significantly different from one another, the test chemical was evaluated as having had an indeterminate activity.

Test chemicals evaluated as having an indeterminate activity must be tested in a third experiment before they can be reevaluated as either active or inactive in the BALB/c-3T3 transformation assay. However, a few test chemicals such as titanium dioxide (*12*) were noncytotoxic at treatment doses > 5-fold higher than their solubility limit in culture medium supplemented with pluronic F68. Because cytotoxic responses could not be achieved with this type of chemical at any reasonable treatment dose, the response of this test chemical was evaluated as having been inactive with an indeterminate activity.

Evaluation of Transformed Foci

Spontaneous and BaP-induced transformation responses of this clone of BALB/c-3T3 cells have been shown to include a continuum of type I, II, and III foci of different sizes (*16,18*). The number of type III foci were identified microscopically according to published criteria (*2–3,19,20*). Type III foci ≥ 2 mm in diameter had three phenotypic properties, including piling and overlapping of cells, disorientation of cells at the periphery of the focus, and invasion of transformed cells into a contact-inhibited monolayer of WT cells. Type I and II foci of BALB/c-3T3 cells were also recorded and appeared in many different sizes, but they lacked the combination of three phenotypic properties previously noted for the type III transformed focus. This report will present only the type III focus data for the test chemicals.

Handling of Test Chemicals

Many chemicals in this investigation had physicochemical properties that could have potentially interfered with them being adequately tested in the BALB/c-3T3 cell transformation assay (see Table 1). Therefore, procedures were developed to ensure that all test chemicals would be consistently and adequately evaluated.

pH. For example, all test chemicals were evaluated at a physiologic pH. Thus, stock solutions of test chemicals that altered the physiological pH range of culture medium (i.e., pH 7.2–7.4) were neutralized with concentrated stock solutions of either hydrochloric acid (1 or 5 *N* HCl) or sodium hydroxide (1 or 5 *N* NaOH) before their use as dosing solutions. In addition, all test chemical dosing solutions and controls were prepared in a 5-fold concentrated form and rapidly administered in 1 mL of medium to culture vessels containing 4 mL of medium. This procedure avoided wide fluctuations of the pH of medium in the culture vessels during dosing.

Volatility. All liquid chemicals have a vapor pressure at 37°C and are volatile at this temperature. Therefore, all liquid chemicals were tested in closed culture flasks to ensure that the chemical treatment doses remained constant throughout the 48-hr treatment period and were in equilibrium with the aqueous culture medium environment.

Chemical Reactivity. Some test chemicals react with strong acids or strong bases (i.e., *trans*-cinnamaldehyde, 2,6-dinitrotoluene, and sodium fluoride) or with hypochorites (2,6-xylidine; Table 1). These problems were of no concern because these reactive chemicals were not used in the assay procedures. In addition, 21 test chemicals that were not included in this investigation were observed to pit, or etch, the plastic culture vessels at the same range of treatment doses used to assess cytotoxic activity (*12*). This

Table 1. Summary of cytotoxic responses of 24 test chemicals detected in a co-culture clonal survival assay.

Chemical	CAS no.	Molecular weight	Physiochemical properties			Cytotoxic responses, mM[a]
			PS	SV	TP	
Aphidicolin	38966-21-1	338.5	S	DCF	ts	0.000414
Barium chloride-2H_2O	10326-27-9	244.0	S	C	sp	1.70
5-Bromo-2'-deoxyuridine	59-14-3	307.4	S	DCF	ls, ts	0.0612
Carisoprodol	78-44-4	260.0	S	DCF		3.33
Chloramphenicol sodium succinate	982-57-0	445.2	S	C		5.62
4-Chloro-2-nitroaniline	89-63-4	172.5	S	DCF		0.638
C. I. Acid red 114	6459-94-5	830.0	S	DCF		0.719
C. I. Direct blue 15	2429-74-5	996.9	S	CF	ls,	2.68
C. I. Direct blue 218	28407-37-6	1087.9	S	CF		0.448
trans-Cinnamaldehyde	14371-10-9	132.2	L	DCF	ls, ts, oa, rab, vol	0.0535
Cytosine arabinoside	147-94-4	279.7	S	DC	ts	0.000601
2,6-Dinitrotoluene	606-20-2	150.0	S	DCF	ts, rab	2.03
Diphenylnitrosamine	86-30-6	198.22	S	DCF	ls	0.479
Isobutyraldehyde	78-84-2	72.10	L	DCF	ts, oa, vol	4.37
Manganese sulfate-H_2O	10034-96-5	246.5	S	CF	apH	0.100
2-Mercaptobenzimidazole	583-39-1	150.0	S	DCF	ts	3.25
Methdilazine-HCl	1229-35-2	296.0	S	DCF	ls	0.0314
Mezerein	34807-41-5	654.0	S	DCF		0.0306
Mono(2-ethylhexyl)adipate	4337-65-9	258.42	L	DCF	bpH, ts	1.12
Mono(2-ethylhexyl)phthalate	4376-20-9	278.4	S/L	DCF	bpH, ts	1.04
Riddelliine	23246-96-0	349.0	S	CF	bpH, hac, ts, oa	4.78
Sodium fluoride	7681-49-4	42.0	S	C	apH, ls, rab	2.31
12-*O*-Tetradecanoylphorbol-13-acetate	16561-29-8	616.0	S	DC	ts	0.0145
2,6-Xylidine	87-62-7	121.2	L	DCF	vol, ts, rhc	4.86

Abbreviations: CAS no., Chemical Abstract Service registry number; LD_{50}, lethal dose for 50% of the cells; PS, physical state, S, solid, L, liquid; SV, solvent vehicle; D, dimethyl sulfoxide; C, culture medium; F, pluronic F68; A, acetone, E, ethanol; TP, technical problems; ls, light sensitive; ts, temperature sensitive, oa, oxidized in air; vol., volatile at 37°C; rac, reacted with acids; rab, reacted with bases; apH, caused acid pH, bpH, caused basic pH, hac, hydrolyzed under alkaline conditions; rhc, reacted with hypochlorites; and sp, solubility problem.

[a]The co-culture clonal survival assay design used to detect the cytotoxic response of the test chemical is described in Materials and Methods. The cytotoxic responses of chemicals in individual experiments are summarized in terms of the millimolar LD_{50} treatment dose that resulted in 50% survival of the chemically treated cells relative to the survival of untreated or solvent control treated cell cultures. The LD_{50} cytotoxic response is an average of two or more experiments with the chemical. The molecular weight of each chemical is provided so that treatment doses can be converted from mM to μg/mL. For example, based on the molecular weight of 338.5 for aphidicolin, the LD_{50} detected for aphidicolin was about 0.000414 mM or 0.146 μg/mL.

chemical reaction occurred within minutes after the soluble chemical treatment doses were added to culture vessels; thus, the amount of test chemical in the culture medium was time dependent. Furthermore, the relative time the chemical had to interact with the target cell was reduced to minutes compared to the standard 48-hr treatment time. These chemicals had to be tested in a chemical resistant culture vessel. In this investigation, isobutyraldehyde reacted with plastic; however, this problem was not observed to occur when the chemical was completely dissolved in culture medium supplemented with pluronic F68.

Solubility. Approximately 65% of 200 chemicals tested in this program were relatively insoluble in water. Although many chemicals that were insoluble in water were soluble in one or more organic solvents such as acetone, dimethylsulfoxide (DMSO), and ethanol, these organic solvents did not help increase the relative solubility of the test chemicals in culture medium. To overcome this problem, the test chemical was dissolved in the appropriate organic solvent at a high concentrations and than dispersed in medium supplemented with a noncytotoxic, nonionic surfactant pluronic F68 at 1.25% w/v (*11,21*). The final concentration of the solvent vehicles applied to cell cultures was low and limited to $\leq$ 0.2% v/v organic solvent and 0.25% w/v pluronic F68. Using this procedure, many test chemicals were completely soluble, or they formed a stable emulsion or a fine particulate suspension.

Test Chemicals

The following test chemicals in this investigation were supplied by Radian Corporation (Houston, TX), which maintained the chemical repository for the National Toxicology Program: barium chloride-2H_2O; carisoprodol; chloramphenicol sodium succinate; 4-chloro-2-nitroaniline; C.I. acid red 114; C.I. direct blue 15; C.I. direct blue 218; trans-cinnamaldehyde; isobutyraldehyde; manganese sulfate-H_2O; 2-mercaptobenzimidazole; methdilazine-HCl; mezerein; mono(2-ethylhexyl)adipate; mono(2-ethylhexyl)phthalate; riddelliine; sodium fluoride; and 2,6-xylidine. Aphidicolin and 5-bromo-2'-deoxyuridine were purchased from Sigma (St. Louis, MO), and cytosine arabinoside and diphenylnitrosamine were purchased from Aldrich (Milwaukee, WI). 12-*O*-Tetradecanoylphorbol-13-acetate was purchased from Consolidated Midland Corporation. 2,6-Dinitrotoluene was obtained from Midwest Research Institute.

Statistical Analyses and Mathematical Models

Mathematical Transformation of Focus Data. In a typical transformation experiment, the normal expression

of the transformed cell phenotype included a few vessels that appeared randomly with large numbers of type I, II, and III foci in control and in chemical-treated culture vessels (*15–16,18,22*). These vessels resulted in a distribution of foci/vessel that was abnormal (*15–16,22*), and they were not statistical outliers relative to the historical behavior of the assay (*23*). Several mathematical transformations (*24*) were investigated, and the historical database for the assay was found not to deviate significantly from a normal distribution of foci/vessel when the data were transformed to the $\log_{10}$ (*16*). Thus, before any statistical analyses of the data, one was added to the number of all scored vessels to avoid computational instabilities with the log transformation, and the resulting total was converted to its $\log_{10}$ equivalent value.

Significance of Transformation Responses. The statistical significance of chemical transformation responses was determined by computer in three steps using SAS software (*25*). First, an analysis of variance of test chemical and control transforming activities was performed on $\log_{10}$ data using the *F*-test (*18,24*). Second, the significance of differences of control and chemical-induced transformation responses was calculated using modifications of the Student's *t*-test, one assuming equal variance (EV) between the control and the chemical response and the other assuming unequal variance (UV) (*25*). Use of the EV or UV model was distinguished by an *F*-test for heterogeneous variance; significant departure (i.e., $p<0.05$) suggested use of the UV model. Third, the probability of the individual test chemical treatment transformation response exhibiting a significant departure from no effect was determined using the appropriate UV or EV *t*-statistic.

Methods for Rank-Ordering Test Chemical Transformation Responses. Test chemical transformation responses were rank ordered on the basis of the significance of their activity in the transformation assay. The significance of the test chemical response varied proportionally to the magnitude of the *t*-statistic, and the *t*-statistic was independent of the absolute spontaneous transformation response of the solvent control (refer to Appendix A for the *t*-statistics of 24 chemical transformation responses). Variability among the mean spontaneous control transformation responses in individual experiments precluded the use of mean chemical-induced transformation responses for this purpose. The average significance of each chemical transformation response, or mean *t*-statistic, was calculated by averaging the *t*-statistics of the four test chemical (or two positive control) treatment doses. Treatment doses with <5% RCE and incomplete monolayers were deleted, and negative *t*-statistics were arbitrarily assigned the value of zero. This mean *t*-statistic was used to rank order chemical transformation responses in individual experiments. A similar method has been employed using *z*-statistics from nonparametric statistical tests (*26*). The test chemical activity in two or more experimental trials was assessed using a weighted rank *t*-statistic. It was calculated using all the *t*-statistics for test chemical treatments in two or more experimental trials. Examples of these calculations are provided in the Results.

Statistical Sensitivity versus Spontaneous Transformation Responses. The median spontaneous transformation response for 110 experiments conducted over a 2-year period has been reported (*16*), and it was highly variable. Variability of spontaneous responses was correlated with the use of different ampules of cells from the same cryopreserved pool (*16*), and variable responses directly affected the ability of transformation assays to discriminate significant positive control BaP-induced responses (*18*). Experiments with a spontaneous response lower than the median activity had a lower statistical sensitivity to detect test chemical responses. The statistical sensitivity was estimated by calculating the ratio of $t^{exp.}/t^{med.}$. This is the *t*-statistic of an individual experiment [$t^{exp.}$] divided by the *t*-statistic of the median experiment [$t^{med.}$]. Thus, the statistical sensitivity was equal to the ratio of the $X^{exp.}/SE^{exp.}$ divided by $X^{med.}/SE^{med.}$. The $X^{exp.}$ and the $X^{med.}$ are the mean experimental and median spontaneous responses, and the $SE^{exp.}$ and the $SE^{med.}$ are the standard errors of the mean experimental and median spontaneous transformation responses. Using the magnitude of this ratio, 110 independent experiments were rank-ordered from the highest to the lowest in terms of statistical sensitivity (*16*).

The ranking of spontaneous transformation responses revealed that *a*) 10.9% (12/110) of the experiments had a significantly high statistical sensitivity, *b*) 89/110 (80.9%) of the experiments had statistical sensitivities that were not significantly different from the median experiment statistical sensitivity, and *c*) 8.2% (9/110) of the experiments had a significantly low statistical sensitivity (*16*). An experiment with a significantly low statistical sensitivity had a spontaneous response that was less than about 0.20 type III foci/vessel, and an experiment with a significantly high statistical sensitivity had a spontaneous transformation response greater than about 2.5 type III foci/vessel (*16*).

Detection Sensitivity versus BaP Transformation Responses. BaP induced highly significant transformation responses in 109/110 experiments in this investigation (*18*). Nevertheless, the magnitude of the BaP response was variable among these experiments, and a portion of this variability correlated with the serum lot used and with the aliquot of cells used to initiate the transformation experiments (*18*). Because certain serum lots reduced both the BaP transformation response and the cytotoxicity of BaP, it was not considered to be a serious problem. The serum effect could be overcome by adjustment of the BaP treatment dose concentrations and testing BaP at comparable levels of cytotoxicity. Thus, the serum-dependent effect on cytotoxic responses should not have affected the activities of test chemicals tested at cytotoxic treatment doses in this investigation. In contrast, variability among BaP responses was not correlated to the source of BaP or with the passage level of cultures (*18*).

Because all cells in this investigation were obtained from one cryopreserved pool of cells, ampule-dependent BaP transformation responses demonstrated that capacity to detect chemical-induced transformation was not equal in each experiment (*15*). Therefore, BaP responses were sta-

tistically compared to the median experiment, and the 110 experiments were rank ordered according to their detection sensitivity for BaP (*18*). The ranking of the experiments revealed that 25.5% (28/110) had a significantly high detection sensitivity for BaP, 48.2% (53/110) of the experiments had a detection sensitivity comparable to the median experiment, and 26.4% (29/110) of the experiments had a significantly low detection sensitivity.

Effect of Statistical Sensitivity on Detection Sensitivity for BaP. Experiments with normal or significantly high statistical sensitivity had normal detection sensitivity for BaP (*18*). Thus, these two groups of experiments were predicted to have a normal capacity to detect chemical-induced transformation responses, and they had actual rank *t*-statistics that were accurate estimates of the test chemical's activity. Therefore, these two groups of experiments had actual rank *t*-statistics that were equal to the estimated rank *t*-statistic.

In contrast, experiments with significantly low statistical sensitivity have been demonstrated to affect the detection sensitivity for BaP (*18*). For the nine experiments with significantly low statistical sensitivity, no experiments had significantly high detection sensitivity for BaP and six of nine experiments had significantly low detection sensitivity for BaP. Therefore, these experiments had a high probability of underestimating the activity of test chemicals, as well as underestimating the rank *t*-statistic.

To compensate for the diminished sensitivity to detect chemical-induced transformation, the rank *t*-statistic is multiplied by a correction factor to obtain an estimated rank *t*-statistic. The correction factor was equal to the sum total rank order numbers for the test chemical experimental statistical sensitivity and detection sensitivity for BaP divided by the median number of experiment (i.e., 55). For example, the most active test chemical, mezerein, had statistical sensitivities for spontaneous transformation responses of 75 and 8*/110 for experiments 59 and 95, respectively, and detection sensitivities for BaP of 76 and 29/110 for the same experiments (Table 2). Therefore, the average rank order of the two experiments was 47.0 (i.e., 75 + 8 + 76 + 29/4 = 47.0). For a total of 110 experiments, the median experiment has an automatic average rank order of 55.0 (i.e., 110/2 = 55.0). Thus, the correction factor for the experimental sensitivity to detect chemical-induced transformation was 47.0/55.0 or 0.855. Experiments 59 and 95 had a combined statistical sensitivity and detection sensitivity that were slightly above the median of 55.0. Because the correction factor is less than 1, the actual rank *t*-statistic is not corrected and was left equal to the estimated rank *t*-statistic. If the correction factor had been more than one, the actual rank *t*-statistic would have been multiplied by the correction factor to obtain the estimated rank *t*-statistic. A justification for this correction factor has been reported (*18*).

Results

Cytotoxic Responses of 24 Test Chemicals

The cytotoxic responses of 24 test chemicals were assessed using standard and co-culture clonal survival assays. The cytotoxic response data derived from the co-culture assay was most important because these data measured clonal survival of chemical-treated cells in the high-density cell cultures used in the transformation assay. Thus, this assay was used not only to select treatment doses for the transformation assay, but also to verify that the test chemical treatments had an acceptable

Table 2. Test chemical transformation responses in experiments with different statistical sensitivities to detect spontaneous transformation and different detection sensitivities for BaP-induced transformation.

		Transformation response[a]					
Chemical	Trial no. (exp. no.)[b]	Spontaneous,[c] type III foci/vessel:	rank order[d]	BaP,[e] call/	rank order[d]	Test chemical,[d] call/	mean *t*-statistic
Active chemicals							
Aphidicolin	1 (DRI1)	0.939	~56	SP	ND	LA	1.88
	2 (97)	0.414	54	SP	44	SP	7.68
Barium chloride-2H_2O	1 (42)	0.861	55	SP	8***	SP	5.67
	2 (53)	2.78	11*	SP	39	LA	0.663
5-Bromo-2′-deoxyuridine	1 (IP3)	0.149	~102*	SP	ND	SP	20.5
	2 (IP7)	0.992	~52	SP	ND	SP	5.43
	3 (95)	2.84	8*	SP	29	LA	1.93
C. I. Direct blue 15	1 (64)	0.291	92	SP	71	SP	5.61
	2 (69)	0.288	85	SP	102***	SP	3.64
trans-Cinnamaldehyde	1 (79)	5.12	4**	SP	1***	LA	4.85
	2 (94)	1.52	18	SP	31	SP	2.51
Cytosine arabinoside	1 (IP4)	0.278	~90	SP	ND	SP	19.3
	2 (IP7)	0.992	~52	SP	ND	SP	4.46
	3 (95)	2.84	8*	SP	29	SP	5.09
Diphenylnitrosamine	1 (75)	0.882	21	SP	21**	SP	1.83
	2 (91)	0.322	56	LA	109***	SN	0.983
Manganese sulfate-H_2O	1 (49)	0.433	65	SP	32	SP	4.41
	2 (57)	0.278	86	SP	74	SP	2.19
2-Mercaptobenzimidazole	1 (47)	0.579	61	SP	47	SP	2.82
	2 (56)	0.260	84	SP	84*	SP	2.76

(Continued on next page)

Table 2. *Continued.*

Chemical	Trial no. (exp. no.)[b]	Transformation response[a]: Spontaneous,[c] type III foci/vessel: rank order[d]		BaP,[e] call/rank order[d]		Test chemical,[d] call/mean *t*-statistic	
Mezerein	1 (59)	0.297	75	SP	76	SP	13.9
	2 (95)	2.84	8*	SP	29	SP	13.1
Riddelliine	1 (48)	0.537	64	SP	84*	SP	4.03
	2 (66)	0.056	108**	SP	99***	LA	1.84
2,6-Xylidine	1 (84)	0.511	44	SP	65	LA	2.02
	2 (100)	0.268	73	SP	49	SP	2.68
Chemicals with equivocal activity							
C. I. Direct blue 218	1 (49)	0.433	65	SP	65	LA	1.38
	2 (58)	0.189	97	SP	102***	LA	2.58
Mono(2-ethylhexyl)phthalate	1 (79)	5.12	4**	SP	1***	LA	3.05
	2 (94)	1.52	18	SP	31	LA	1.31
Inactive chemicals							
Carisoprodol	1 (46)	0.384	88	SP	77	SN	0.000
	2 (52)	1.09	34	SP	45	SN	1.53
Chloramphenicol sodium succinate	1 (49)	0.433	65	SP	32	SN	0.000
	2 (58)	0.189	97	SP	94**	SN	0.580
4-Chloro-2-nitroaniline	1 (46)	0.384	88	SP	77	SN	0.053
	2 (53)	2.78	11*	SP	39	SN	0.113
C. I. Acid red 114	1 (64)	0.291	92	SP	72	SN	0.868
	2 (69)	0.288	85	SP	102***	SN	0.670
Isobutyraldehyde	1 (78)	3.28	9*	SP	37	LA	4.01
	2 (106)	1.30	28	SP	15***	SN	0.000
Methdilazine-HCl	1 (47)	0.579	61	SP	47	SN	0.000
	2 (55)	0.129	104*	SP	90***	SN	1.22
Mono(2-ethylhexyl)adipate	1 (82)	8.01	1***	SP	3***	LA	1.08
	2 (94)	1.52	18	SP	31	SN	0.127
Sodium fluoride	1 (70)	0.526	47	SP	43	SN	0.000
	2 (101)	0.260	62	SP	48	LA	1.67
12-*O*-Tetradecanoylphorbol-13-acetate	1 (70)	0.526	47	SP	43	SN	0.000
	2 (105)	0.581	29	SP	105***	SN	0.000
Chemicals with an indeterminate activity							
2,6-Dinitrotoluene	1 (91)	0.322	56	SP	105***	LN	0.000
	2 (105)	0.581	29	SP	97***	SN	0.000

Abbreviations: exp. no., experiment number; ND, not determined; SP, sufficient positive; LA, limited activity, SN, sufficient negative; LN, limited negative.

[a]The assay design and procedures used in the standard transformation assay are described in Materials and Methods. The transforming activities of individual chemical treatment doses (*i.e.*, focus data), as well as the individual transformation responses (*i.e.*, type III foci/vessel), are provided in detail in Appendix A.

[b]Most of the chemicals were tested in 110 sequential experiments using a standard procedure (*16,18*); however, certain chemicals were tested in experiments that compared the standard protocol to an alternative method (e.g., experiments labeled DRI, IP). Only the results of the standard method are presented here.

[c]The method used to calculate the spontaneous response, as well as the positive control and test chemical responses, is explained in Materials and Methods. The transformation responses are expressed as type III foci/vessel and were calculated using a $\log_{10}$ mathematical transformation procedure. The arithmetic value for foci/vessel in this table is the antilog of the $\log_{10}$ mean transformation minus one. The procedure for rank ordering the spontaneous responses from 110 experiments is based on the different statistical sensitivities of transformation experiments with different spontaneous responses is explained in Materials and Methods. Experiments with high spontaneous responses have a high statistical sensitivity and relatively low rank-order numbers. For example, *trans*-cinnamaldehyde had a high spontaneous response of 5.12 foci/vessel in experiment 79, which had a high statistical sensitivity and rank order number of 4/110. Conversely, experiments with a low statistical sensitivity have high rank-order numbers. For example, riddelliine had a low spontaneous response of 0.056 foci/vessel in experiment 66, which had a low statistical sensitivity with a high rank-order number of 108/110.

[d]The method used to call individual experiments is described in detail in Materials and Methods. The significance of the transformation responses of individual chemical treatment doses were calculated using SAS software (*25*). The mean *t*-statistic represents the average of the *t*-statistics of the four test chemical treatment doses in the experiment. The *t*-statistics used in these calculations are provided in Appendix A.

[e]The method used to call individual transformation experiments is described in detail in Materials and Methods. The method used to rank-order the BaP transformation responses from the 110 experiments is based on statistical comparison of the BaP transformation at the two treatment doses detected in an individual experiment with the mean historical activity of the assay (*18*). The rational for rank-ordering the experiments is analogous to that described for the spontaneous transformation responses (*16*).

*Significant spontaneous or BaP transformation response, $0.01 < p \leq 0.05$.

**Significant spontaneous or BaP transformation response, $0.001 < p \leq 0.01$.

***Significant spontaneous or BaP transformation response, $p \leq 0.001$.

cytotoxic effect on the target cells. The standard clonal survival assay was shown to be inaccurate for both of these purposes.

The results of co-culture clonal survival assay experiments for 24 chemicals tested in two or more experiments are summarized Table 1. The chemicals are listed in alphabetical order along with a summary of the physicochemical properties that influenced the methods by which the chemicals were handled and tested. The first chemical, aphidicolin, was cytotoxic to the BALB/c-3T3 cells and had an average cytotoxic response, or LD_{50}, of 0.000414 mM. Based on a molecular weight of 338.5, this concentration of aphidicolin was equivalent to 0.146 μg/mL. In contrast, the least cytotoxic chemical was chloramphenicol sodium succinate, which had an LD_{50} of 5.62 mM and was equivalent to 2315 μg/mL.

The methods by which test chemical technical problems related to different physicochemical properties were handled in this investigation are discussed in Materials and Methods. While most of the technical problems, such as volatility, could be adequately overcome through the use of a consistent methodology, two problems were insurmountable in this investigation and could have influenced the results in these experiments. First, three test chemicals were oxidized when exposed to air (*trans*-cinnamaldehyde, isobutyraldehyde, and riddelliine). This problem could be partially avoided by storing the chemicals under an inert atmosphere, but it could not be avoided in an assay that has a 48 hr, 37°C, treatment period in a 95% air atmosphere. Second, one of the test chemicals, barium chloride, only exhibited significant cytotoxic activity (< 50% RCE) at treatment doses above its solubility limit in medium. Thus, this chemical was tested at treatment doses both above and below its solubility limit.

Transforming Activities of 24 Test Chemicals

The variable spontaneous and BaP-induced transformation responses detected in these experiments have been reported as part of a total of 110 experiments (*15,16*). Due to the variability among spontaneous transformation responses, these experiments had different statistical sensitivities to detect chemical-induced transformation responses. Likewise, variability among BaP responses showed that individual experiments had different detection sensitivities for BaP and could have had different sensitivities to measure test chemical-induced transformation responses. Therefore, test chemical transformation responses were evaluated in terms of the rank-ordered sensitivity of individual experiments in terms of their spontaneous and BaP-induced transformation responses.

In this investigation, 24 chemicals were tested in two or more experiments in a modified standard BALB/c-3T3 cell transformation assay. The results of these experiments are summarized in Tables 2 & 3, and the experimental data are provided in detail in Appendix A. Explanations of the different acceptance and evaluation criteria for the transformation assay response calls is provided in Materials and Methods. The activities of the individual chemicals is described below, and the actual and estimated rank orders of the individual test chemical transformation responses are provided in Table 3.

Aphidicolin. Aphidicolin was a very cytotoxic chemical with an average LD_{50} of 0.000414 mM and no insurmountable technical problems (Table 1). The statistical sensitiv-

Table 3. Actual and estimated rank-ordered potency of the responses of 24 chemicals detected in a standard transformation assay.

	Transformation response, rank *t*-statistic[a]	
Chemical	Actual	Estimated
Active chemicals		
Mezerein	13.6	13.6
Cytosine arabinoside	8.35	8.35
5-Bromo-2′-deoxyuridine	7.35	7.35
C. I. Direct blue 15	4.62	7.35
Aphidicolin	5.13	5.38
Riddelliine	2.94	4.73
Manganese sulfate-H_2O	3.66	4.28
trans-Cinnamaldehyde	3.85	3.85
2-Mercaptobenzimidazole	2.79	3.49
Barium chloride-$2H_2O$	3.17	3.17
2,6-Xylidine	2.35	2.47
Diphenylnitrosamine	1.40	1.40
Chemicals with equivocal activity		
C. I. Direct blue 218	2.07	3.11
Mono(2-ethylhexyl)phthalate	2.08	2.08
Inactive chemicals		
C. I. Acid red 114	0.77	1.23
Isobutyraldehyde	1.05	1.05
Carisoprodol	0.92	0.98
Sodium fluoride	0.84	0.84
Methdilazine-HCl	0.61	0.84
Mono(2-ethylhexyl)adipate	0.61	0.61
Chloramphenicol sodium succinate	0.29	0.38
4-Chloro-2-nitroaniline	0.08	0.08
12-*O*-Tetradecanoylphorbol-13-acetate	0.00	0.00
Chemical with indeterminate activity		
2,6-Dinitrotoluene	0.00	0.00

[a]The method used to calculate the significance of test chemical transformation responses used SAS software (*24*) and is described in detail in Materials and Methods. The correct *t*-statistics of each treatment dose of the test chemical in a single experiment are presented in the Appendix A, and these *t*-statistics were averaged to determine the mean *t*-statistic of the test chemical for the experiment (see Table 2). The mean *t*-statistic for two or experiments for each chemical were weighted according to the number of treatment doses evaluated and averaged to determine the actual rank *t*-statistic presented in this table. For example, the actual rank *t*-statistic of mezerein transformation responses in experiments 59 and 95 is equal to 13.6 [4.01 + 9.05 + 17.0 + 25.7 (experiment 59) + 5.63 + 20.3 + 13.5 (experiment 95)/7 = 13.6].

[b]The estimated rank *t*-statistic is used to estimate both the historical activity of the test chemical in the transformation assay, as well as to predict its activity in additional tests. It is calculated by correcting the actual rank *t*-statistic using a correction factor. The data presented in Table 2 show that individual experiments had different rank-ordered sensitivities to detect chemical-induced transformation. Therefore, the estimated rank *t*-statistic modified the actual rank *t*-statistic to correct for differences in the sensitivities of individual experiments. The method uses the rank-ordered sensitivity of individual experiments to detect spontaneous and BaP-induced transformation (see Materials and Methods for an example).

ities of trials 1 and 2 were 56 and 54/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were not determined (ND) and 44/110, respectively (Table 2). In a preliminary trial 1, the test chemical was tested at widely spaced treatment doses, and it had an LA transformation response. In trial 2, the chemical had an SP transformation response. Aphidicolin was evaluated as very active in the transformation assay, and its actual and estimated rank t-statistics were 5.13 and 5.38, respectively (Table 3).

Barium chloride-2H_2O. Barium chloride-2H_2O was a moderately cytotoxic chemical with an average LD_{50} of 1.70 mM and one technical problem (Table 1). It had a solubility limit of about 400 μg/mL in medium; thus, it was tested for cytotoxic and transforming activities at treatment doses both below and above this limit. The statistical sensitivities of trials 1 and 2 were 55 and 11/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 8 and 39/110, respectively (Table 2). In a preliminary trial 1, the chemical was tested at two doses above the solubility limit and had an SP transformation response. In trial 2, the chemical was tested with only one dose above the solubility limit, and it had an LA transformation response. Barium chloride-2H_2O was evaluated as active in the transformation assay, but only at doses above its solubility limit in culture medium. Its actual and estimated rank t-statistics were both 3.17 (Table 3).

5-Bromo-2'-deoxyuridine. 5-Bromo-2'-deoxyuridine was a very cytotoxic, light-sensitive chemical with an average LD_{50} of 0.0612 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1–3 were 102, 52, and 8/110, respectively; the detection sensitivities for BaP of trials 1–3 were ND, ND, and 29/110, respectively (Table 2). In a preliminary trials 1 and 2, the chemical had an SP transformation response. In trial 3, the chemical had an unexpectedly low LA transformation response. 5-Bromo-2-deoxyuridine was evaluated as very active in the transformation assay, and its actual and estimated rank t-statistics were both 7.35 (Table 3).

Carisoprodol. Carisoprodol was a moderately cytotoxic chemical with an average LD_{50} of 3.33 mM with no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 88 and 34/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 77 and 45/110, respectively (Table 2). In trials 1 and 2, the chemical had SN transformation responses. Carisoprodol was evaluated as inactive in the transformation assay, and its actual and estimated rank t-statistics were 0.92 and 0.98, respectively (Table 3).

Chloramphenicol Sodium Succinate. Chloramphenicol sodium succinate was a moderately cytotoxic chemical with an average LD_{50} of 5.62 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 65 and 97/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 32 and 94/110, respectively (Table 2). In trial 1, the chemical had an SN transformation response, and in trial 2, which had a relatively low sensitivity to detect chemical-induced transformation, the chemical also had an SN transformation response. Chloramphenicol sodium succinate was evaluated as inactive in the transformation assay, and its actual and estimated rank t-statistics were 0.29 and 0.38, respectively (Table 3).

4-Chloro-2-Nitroaniline. 4-Chloro-2-nitroaniline was a cytotoxic chemical with an average LD_{50} of 0.638 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 88 and 11/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 77 and 39/110, respectively (Table 2). In trials 1 and 2 the chemical had SN transformation responses. 4-Chloro-2-nitroaniline was evaluated as inactive in the transformation assay, and its actual and estimated rank t-statistics were both 0.08 (Table 3).

C. I. Acid Red 114. C. I. Acid red 114 was a cytotoxic chemical with an average LD_{50} of 0.719 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 92 and 85/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 72 and 102/110, respectively (Table 2). Thus, trials 1 and 2 had relatively low sensitivities to detect chemical-induced transformation. In trials 1 and 2, the chemical had SN transformation responses; therefore, C.I. acid red 114 was evaluated as inactive in the transformation assay. The chemical's actual rank t-statistic was 0.77, and due to the low sensitivities of the two experiments, the estimated rank t-statistic was 1.23 (Table 3). Thus, C.I. acid red 114 had the highest probability of the nine inactive chemicals of exhibiting activity in a third experiment.

C. I. Direct Blue 15. C.I. Direct blue 15 was a light-sensitive, relatively noncytotoxic chemical with an average LD_{50} of 2.68 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 92 and 85/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 71 and 102/110, respectively (Table 2). Thus, trials 1 and 2 had a relatively low sensitivities to detect chemical-induced transformation. In trials 1 and 2 the chemical had SP transformation responses; therefore, C.I. direct blue 15 was evaluated as very active in the transformation assay. The chemical's actual rank t-statistic was 4.62, but the estimated rank t-statistic was 7.35 due to the relatively low sensitivities of the experiments (Table 3).

C. I. Direct Blue 218. C. I. Direct blue 218 was a cytotoxic chemical with an average LD_{50} of 0.448 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 65 and 97/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 65 and 102/110, respectively (Table 2). In trial 1, the chemical had an LA transformation response. In trial 2, which had a relatively low sensitivity to detect chemical-induced transformation, the chemical also had an LA transformation response. Thus, C.I. direct blue 218 was evaluated as having equivocal activity in the transformation assay. The chemical's actual rank t-statistic was 2.07; however, the estimated rank t-statistic was 3.11 (Table 3). Thus, the low sensitivity of the second trial makes it highly probable that this test chemical could exhibit activity in a third experiment.

Trans-*Cinnamaldehyde.* *Trans*-cinnamaldehyde was a very cytotoxic chemical with an average LD_{50} of 0.0535

mM and many physicochemical properties that required special attention (Table 1). Although most of these technical problems could be overcome, the chemical was reported to be oxidized in air. Due to the long treatment period of 48 hr at 37°C in a 95% air atmosphere, it is likely that the chemical tested in this investigation included both the parent chemical and some of its oxidative by-products. The statistical sensitivities of trials 1 and 2 were 4 and 18/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 1 and 31/110, respectively (Table 2). In a preliminary trial 1 which had a relatively high sensitivity to detect chemical-induced transformation, the chemical had an LA transformation response. In trial 2, which also had a relatively high sensitivity to detect chemical-induced transformation, the chemical had an SP transformation response. Therefore, *trans*-cinnamaldehyde was evaluated as active in the transformation assay. Its actual and estimated rank *t*-statistics were 3.85 (Table 3).

Cytosine Arabinoside. Cytosine arabinoside was a very cytotoxic chemical with an average LD_{50} of 0.000601 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1, 2, and 3 were 90, 52, and 8/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were ND, ND, and 29/110, respectively (Table 2). In trials 1–3 the chemical had an SP transformation response. Therefore, cytosine arabinoside was evaluated as very active in the transformation assay, and its actual and estimated rank *t*-statistics were both 8.35 (Table 3).

2,6-Dinitrotoluene. 2,6-Dinitrotoluene was a moderately cytotoxic chemical with an average LD_{50} of 2.03 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 56 and 29/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 105 & 97/110, respectively (Table 2). In a preliminary trial 1, which had a relatively low sensitivity to detect chemical-induced transformation, the chemical did not induce significant cytotoxic activity and was evaluated as having an unacceptable LN transformation response. Trial 2 also had a relatively low sensitivity, but the chemical treatments had significant cytotoxic activity. Trial 2 was evaluated as an SN transformation response. Therefore, 2,6-dinitrotoluene was evaluated as having an indeterminate activity in the transformation assay, and it has to be tested in a third experiment before its activity in the BALB/c-3T3 cell transformation can be clearly defined. The actual and estimated rank *t*-statistics were both 0.00 (Table 3).

Diphenylnitrosamine. Diphenylnitrosamine was a cytotoxic chemical with an average LD_{50} of 0.479 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 21 and 56/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 21 and 109/110, respectively (Table 2). In a preliminary trial 1, which had a relatively high sensitivity to detect chemical-induced transformation, the chemical had an SP transformation response. In contrast, trial 2 had a low sensitivity to detect chemical-induced transformation, and the chemical had an SN transformation response. Although this chemical had disparate responses in two transformation experiments, there was no significant difference in the mean *t*-statistic of the two experiments. Furthermore, the chemical exhibited increased transformation responses in the same range of doses in trial 2 that were statistically significant in trial 1. Therefore, diphenylnitrosamine was evaluated as weakly active in the transformation assay, and its actual and estimated rank *t*-statistics were both 1.40 (Table 3). Therefore, this chemical was the least active of the 12 active chemicals, and it had the lowest probability of the 12 active chemicals of being active in a third experiment.

Isobutyraldehyde. Isobutyraldehyde was a moderately cytotoxic chemical with an average LD_{50} of 4.37 mM and several technical problems (Table 1). Although most of the technical problems could be overcome, the chemical was reported to be oxidized in air. Due to the long treatment period of 48 hr at 37°C in a 95% air atmosphere, it is likely that the chemical tested in this investigation included both the parent chemical and some of its oxidative by-products. The statistical sensitivities of trials 1 and 2 were 9 and 28/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 37 and 15/110, respectively (Table 2). In trials 1 and 2, which had a relatively high sensitivities to detect chemical-induced transformation, the chemical had an LA and SN transformation responses. Therefore, isobutyraldehyde was evaluated as inactive in the transformation assay, and its actual and estimated rank *t*-statistics were both 1.05 (Table 3).

Manganese Sulfate-H_2O. Manganese sulfate-H_2O was a very cytotoxic chemical with an average LD_{50} of 0.100 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 65 and 86/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 32 and 74/110, respectively (Table 2). The chemical had SP transformation responses in both trials 1 and 2. Therefore, manganese sulfate-$2H_2O$ evaluated as very active in the transformation assay, and its actual and estimated rank *t*-statistics were 3.66 and 4.28, respectively.

2-Mercaptobenzimidazole. 2-Mercaptobenzimidazole was a moderately cytotoxic chemical with an average LD_{50} of 3.25 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 61 and 84/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 47 and 84/110, respectively (Table 2). In a preliminary trial 1, the chemical had an SP transformation response. In trial 2, which had a relatively low sensitivity to detect chemical induced transformation, the chemical also had an SP transformation response. Therefore, 2-mercaptobenzimidazole was evaluated as very active in the transformation assay, and its actual and estimated rank *t*-statistics were 2.79 and 3.49, respectively (Table 3).

Methdilazine-HCl. Methdilazine-HCl was a very cytotoxic chemical with an average LD_{50} of 0.0314 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 61 and 104/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 47 and 90/110, respectively (Table 2). In a preliminary trial 1, the chemical had an SN transformation response. In trial 2, which had a low sensitivity to

detect chemical-induced transformation, the chemical had an SN transformation response. Therefore, methdilazine was evaluated as inactive in the transformation assay, and its actual and estimated rank *t*-statistics were 0.61 and 0.84, respectively (Table 3).

Mezerein. Mezerein was a very cytotoxic chemical with an average LD_{50} of 0.0306 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 75 and 8/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 76 and 29/110, respectively (Table 2). In trials 1 and 2 the chemical had an SP transformation response. Therefore, mezerein was evaluated as very active in the transformation assay. The actual and estimated rank *t*-statistics were both 13.6 (Table 3); thus, mezerein is one of the most active chemicals in the BALB/c-3T3 cell transformation assay.

Mono(2-ethylhexyl)adipate. Mono(2-ethylhexyl)-adipate was a moderately cytotoxic chemical with an average LD_{50} of 1.12 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 1 and 18/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 3 and 31/110, respectively (Table 2). In a preliminary trial 1, which had a very high sensitivity to detect chemical-induced transformation, the chemical had an LA transformation response. In trial 2, which had a relatively high sensitivity to detect chemical-induced transformation, the chemical had an SN transformation response. Therefore, mono(2-ethylhexyl)-adipate was evaluated as inactive in the transformation assay, and the actual and the estimated rank *t*-statistics were both 0.61 (Table 3).

Mono(2-ethylhexyl)phthalate. Mono(2-ethylhexyl)-phthalate was a moderately cytotoxic chemical with an average LD_{50} of 1.04 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 4 and 18/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 1 and 31/110, respectively (Table 2). In a preliminary trial 1, which had a very high sensitivity to detect chemical-induced transformation, the chemical had an LA transformation response. In trial 2, which also had a relatively high sensitivity to detect chemical-induced transformation responses, the chemical also had an LA transformation response. Therefore, mono(2-ethylhexyl)phthalate was evaluated as having equivocal activity in the transformation assay, and its actual and estimated rank *t*-statistics were both 2.08. Thus, of the two test chemicals with equivocal activity in this investigation, this chemical had a lower probability of being active in a third transformation experiment than C. I. direct blue 218 (Table 3).

Riddelliine. Riddelliine was a moderately cytotoxic chemical with an average LD_{50} of 4.78 mM and several technical problems (Table 1). Although most of these problems could be surmounted, the chemical was reported to be oxidized by air. Because the chemical is exposed to a long treatment period of 48 hr at 37°C in a 95% air atmosphere, it is highly likely that the chemical tested in this investigation included both the parent chemical its and oxidative by-products. The statistical sensitivities of trials 1 and 2 were 64 and 108/110, respectively. The detection sensitivities for BaP of trials 1 and 2 were 84 and 99/110, respectively (Table 2). In a preliminary trial 1, the chemical had an SP transformation response. In trial 2, which had a very low sensitivity to detect chemical-induced transformation, the chemical had an LA transformation response. Therefore, riddelliine was evaluated as active in the transformation assay. Due to the relatively low sensitivity of the second trial, the estimated rank *t*-statistic was 4.73 and much higher than the actual rank *t*-statistic of 2.94 (Table 3).

Sodium Fluoride. Sodium fluoride was a moderately cytotoxic chemical with an average LD_{50} of 2.31 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 47 and 62/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 43 and 48/110, respectively (Table 2). In a preliminary trial 1, the chemical had an SN transformation response, and in trial 2 the chemical had an LA response. Therefore, sodium fluoride was evaluated as inactive in the transformation assay, and its actual and estimated rank *t*-statistics were both 0.84 (Table 3).

12-O-Tetradecanoyl-Phorbol-13-Acetate. 12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA) was a very cytotoxic chemical with an average LD_{50} of 0.0145 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 47 and 29/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 43 and 105/110, respectively (Table 2). In trials 1 and 2, the chemical had SN transformation responses. Therefore, TPA was evaluated as inactive in the transformation assay, and its actual and estimated rank *t*-statistics were both 0.00 (Table 3).

2,6-Xylidine. 2,6-Xylidine was a moderately cytotoxic chemical with an average LD_{50} of 4.86 mM and no insurmountable technical problems (Table 1). The statistical sensitivities of trials 1 and 2 were 44 and 73/110, respectively; the detection sensitivities for BaP of trials 1 and 2 were 65 and 49/110, respectively (Table 2). In a preliminary trial 1, the chemical had an LA transformation response. In trial 2, the chemical had an SP transformation response. Therefore, 2,6-xylidine was evaluated as active in the transformation assay, and its actual and estimated rank *t*-statistics were 2.35 and 2.47, respectively (Table 3).

Discussion

This investigation reports the results of using a new sensitive method to detect cytotoxic (*17*) and transformation responses (*10,11*) of 24 test chemicals using a clone A31-1-13 of BALB/-3T3 cells (*13–15*). The new method has enhanced sensitivity for detecting chemical-induced transformation compared to published procedures (*2,4,5,27–29*), and the enhanced sensitivity of the transformation assay procedure is due to three procedural changes from published procedures. First, the published method of detecting chemical-induced cytotoxic effects on cells in culture uses a standard clonal survival assay (*1–3*) employing 200 WT cells, and this method inaccurately measures the cytotoxic responses of chemicals in high-density cell cultures (*17*). For most chemicals, the clonal survival assay using 200 WT cells overestimates the chem-

ical's cytotoxic activity when it is applied to transformation assays (*17*), as well as assays for detecting cell to cell communication activities in cultured mammalian cells (*30*). This investigation used a co-culture clonal survival assay that quantitatively and accurately measured the RCE of chemical-treated cells at the same cell densities used to measure the induction of the transformed cell phenotype (*14*). The LD_{50} cytotoxic responses of the 24 test chemicals are presented in Table 1.

Second, the standard BALB/c-3T3 and C3H10T1/2 transformation assay methods have been reported to have a low sensitivity for detecting chemical-induced transformation (*4,27*). Although many investigators have assumed this problem relates to deficiencies in host cell metabolism (*4*), this report introduces an assay method with high sensitivity to detect the chemical-induced transformation of BALB/c-3T3 cells in the absence of any exogenous activation system (*11*). The enhanced sensitivity of this new method arose in part through the use of an increased seeding density of the target cells from 1×10^4 to 3.2×10^4 and a delay in the treatment of the cell cultures on day 1 to day 2 (see Materials and Methods). This increased the total number of treated cells from approximately 0.3×10^6 to 2.0×10^6 cells in a treatment set of 20 culture vessels.

Third, traditional methods of solubilization of test chemicals for *in vitro* genotoxicity assays have relied on the use of a single organic solvent. This procedure rarely offers any improvement in the inherent solubility of the test chemical in culture medium, because the chemical solubilized in the preferred organic solvent usually precipitated when dispersed into the polar culture medium environment. This technical problem was overcome for many test chemicals in this investigation by using a two-step solubilization procedure. The test chemical was first dissolved in an appropriate organic solvent, and then the test chemical in the organic solvent was further diluted with medium supplemented with a noncytotoxic, nonionic surfactant, pluronic F68 (*21*). The presence of the pluronic F68 expanded the relative solubility range of many chemicals, as well as forming a stable emulsion or fine-particulate suspension for other chemicals (*11*).

This investigation has also studied the problem of establishing criteria for the evaluation activity of chemical-induced transformation responses in a single experiment. Previous reports have considered a chemical-induced transformation response from a single experiment to be adequate for evaluating a chemical in the BALB/c-3T3 transformation assay (*1–2,4*). Furthermore, some investigators have argued that a single chemical treatment dose with statistically significant activity was sufficient evidence for a positive response in a transformation assay (*4–5,28,29*). Our investigation has led to what we believe are more adequate methods for analysis of chemical-induced transformation response data obtained in the BALB/c-3T3 cell transformation assay. New assay evaluation criteria were developed for a chemical tested in a single trial (See Materials and Methods), as well as a method to analyze data obtained from replicate trials for one chemical (Tables 2 and 3).

The transformation assay evaluation criteria for a single transformation assay trial are explained in Materials and Methods. These four criteria were modeled after the rodent bioassay evaluation criteria used U. S. Environmental Protection Agency (EPA) Gene-Tox Program Report (*8*), and consist of sufficient positive, limited activity, sufficient negative, and limited negative responses. These four responses can be used to evaluate either the type III focus transformation response or transformation responses that include type I and II foci. The majority of test chemicals that induced SP transformation responses in this investigation had their highest activity detected at treatment doses that were moderately cytotoxic to the cells (*11*); therefore, it was necessary to examine all test chemicals at treatment doses that resulted in significant cytotoxic responses (i.e., at the LD_{50} dose level detected in the co-culture clonal survival assay). The difference between the SP and LA transformation responses related to the appearance of statistically significant transformation responses at consecutive treatment doses. The SP response had a statistically significant response at two or more consecutive treatment doses, whereas the LA response had only one dose with significant ($p<0.01$) activity. If test chemical treatments were not cytotoxic and significant transformants were not observed, then the experiment was unacceptable and evaluated as having an LN response. The SN response was assigned to inactive test chemicals that had a significant cytotoxic response.

Nevertheless, the most important criterion for determining the presence or absence of activity in a BALB/c-3T3 cell transformation assay is not the result of a single transformation assay trial, but rather the results obtained in two or more independent experiments (*11*). Although some chemicals induced transformation responses at two or more treatment doses that were 10-fold above the spontaneous transformation response, most active chemicals had less obvious responses. Therefore, relatively small increases in the frequency of chemical-induced transformants had to be determined statistically, and the repeatability of chemical responses in two experiments was considered as the most convincing evidence for activity in the BALB/c-3T3 transformation assay.

In the process of considering the traditional methods of analysis of chemical-induced transformation of BALB/-3T3 (*1–5*) and C3H10T1/2 (*19*) cells, as well as genotoxic effects of chemicals in other *in vitro* systems, a critical flaw was observed when data were combined from multiple trials. These methods assumed that independent trials had identical sensitivities to measure phenotypic changes. However, a historical analysis of the BALB/c-3T3 cell transformation assay revealed that the frequency of spontaneous and positive control observations were highly variable (*15,18*). Furthermore, when the phenotypic frequency of the spontaneous control varied, it affected the inherent statistical sensitivity of the experiment (*16*). Experiments with high spontaneous responses had relatively high sensitivities to resolve significant (or fold) increases in activity. In contrast, experiments with low spontaneous responses had relatively low sensitivities to

resolve significant activities. Similarly, variability among positive control responses demonstrated that the target cells had inherently different sensitivities to detect chemical-induced transformation in different experiments. If one ignored this variability among the positive and negative controls, one imposed a bias on the interpretation of the test chemical responses in the same experiments. For example, when test chemical responses in two trials were disparate, the differences were automatically associated with the behavior of the test chemical and not to potential differences in the sensitivities of the experiments to detect the phenotypic change in the WT cells.

The above-mentioned problems were overcome in this investigation through the use of statistical ranking procedures for chemical-induced transformation responses. Three new methods were introduced to facilitate the analysis of chemical-induced transformation responses in the BALB/c-3T3 cell transformation assay. First, the potency of the chemical transformation responses was determined by calculating the mean t-statistics (Table 2). The greater the combined significance of the four t-statistics of the four chemical transformation responses/experiment, the greater the mean t-statistic. Second, the rank-ordered potency of the test chemical response was determined by calculating the actual rank t-statistic (Table 3). The actual rank t-statistic was the average t-statistic for all of the acceptable experiments for the test chemical. The greater the average rank t-statistic, the higher the potency of the test chemical transformation response. Third, the estimated rank t-statistic was used to predict the future response of the test chemical in the assay (Table 3). It used rank-ordered historical spontaneous and BaP-induced transformation responses of 110 experiments to determine the statistical sensitivities and detection sensitivities for BaP in each experiment. This information permitted the correction of the actual rank t-statistic for experiments with low statistical sensitivity (see Materials and Methods).

The t-statistic was selected for this purpose for two reasons. First, the absolute value of the t-statistic varied in proportion to the significance of observations. For example, significant observations with confidence levels of $p<0.001$, <0.01 and <0.05 have t-statistics at or above approximately 3.65, about 2.70, and 1.96. Furthermore, the significance of the observation is not biased by high or low spontaneous transformation responses in the individual experiments when the underlying requirements for use of the t-distribution are met. Second, the absolute value of the t-statistic took into account the variability or variance of observations in different experiments and different treatment sets within a single experiment.

Rank-ordered transformation data sets with rank t-statistics may be used to solve three problems that are not easily resolved when data are merely classified into two groups of active and inactive chemicals. First, the mean t-statistics for a chemical's response in two independent experiments provide an unbiased means of predicting whether a third experimental trial for any test chemical is warranted. For example, the two experimental trials for diphenylnitrosamine had SP and SN transformation responses. Although these results were disparate, the explanation for the difference in activities was apparent in the different sensitivities of the two experiments and the overall weak activity of the test chemical. If the mean t-statistics of the two experiments are compared, there is no significant difference in the two experiments (Appendix A). In contrast, there have been several test chemicals in which the activities detected in two individual experiments were SP and SN and the mean t-statistics were significantly different from one another. Although there were no examples of this problem in this investigation, this result occurred with other test chemicals, and it demanded that the test chemical be examined in a third trial. The results of the third trial were pooled with the results of the other two trials to calculate the rank t-statistic.

Thus, the transformation assay response calls of SP, LA, SN, and LN should only be used to evaluate the activity from a single experiment. The data from two experiments should be evaluated by examining the relative significance of activities in replicate trials. If these activities in two experiments are comparable, then test chemicals can be judged as having been highly active, active, equivocal activity, and inactive in the assay. If the results of the two experiments are disparate, then a third trial may be required to determine which of the first two experiments represents the activity of the test chemical.

Second, the use of mean t-statistics permits an assessment of the relative reproducibility of these observations with different types of chemicals. For example, one could compare the reproducibility of mean t-statistics of test chemicals with different solubilities in culture medium, different volatilities, and different inherent cytotoxic activities to the target cells. Third, rank-ordered responses for active chemicals detected in two experimental trials permits one to predict the relative probability of each chemical being active in a third trial. Although the third trial is not required to determine the activity of the chemical in the assay, it could be conducted in a different laboratory to determine the reproducibility of the results obtained in the current investigation. An active chemical with a high estimated rank t-statistic, like mezerein (rank t-statistic = 13.7), would have a high probability to have repeatable positive response in the assay (Table 3). Conversely, a chemical with a low estimated rank t-statistic, like diphenylnitrosamine (rank statistic = 1.40), would have a far lower probability of being active in a third trial and could even be inactive in that experiment. Likewise, test chemicals with equivocal responses, such as C. I. direct blue 218 and mono(2-ethylhexyl)phthalate, had different probabilities of being active in the third trial (Table 3). Finally, the rank t-statistics permitted the ranking of chemicals that were inactive in the assay in terms of their probability of being detected in a third experiment. Among the nine inactive chemicals in this study, C.I. acid red 114 had an estimated rank t-statistic of 1.23, and it had the highest probability of being detected in a third trial (Table 3).

The mechanism by which 12 different chemicals induced significant transformation of BALB/c-3T3 cells is best understood by comparing these data with the data pres-

ented in reports on detection of spontaneous (*16*) and BaP-induced transformation (*18*) under identical experimental conditions. The data presented in both of these investigations suggested, but did not prove, that spontaneous and BaP-induced transformation were the result of mutagenic change in the WT cells. According to this theory, the WT cells were genetically altered, and a permanent phenotypic change occurred that allowed the mutated or transformed cell to grow within a contact-inhibited monolayer of cells. Nevertheless, the genetic lesion and the gene product are not identified. The hypothesis was based totally on theoretical considerations of data obtained in many experiments that were conducted under several different experimental conditions.

In conclusion, this report presents mathematical methods using *t*-statistics that may be used to interpret and analyze the biological effects and activities of chemicals in assays using cultured mammalian cells. The report also summarized the cytotoxic and transformation responses of 24 test chemicals in a new experimental protocol for the BALB/c-3T3 cell transformation assay. In two or more independent experiments, 12 chemicals were active, 2 chemicals had equivocal evidence of activity, 9 chemicals were inactive, and 1 chemical had an indeterminate activity in the assay (Table 3). Using *t*-statistics, the relative potency of chemical transformation responses in groups of chemicals with different activities was determined. Although the data in this report are limited to a relatively small group of 24 chemicals, the same methods have been used to interpret the cytotoxicity and transformation responses >200 test chemicals (*11,12*).

The opinions expressed in this paper are solely those of the author and do not necessarily reflect the positions of the U.S. Food and Drug Administration.

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Appendix A.

Table A1. Summary of the transformation responses of 24 test chemicals.

Treatment Condition[a]		Cytotoxic Activity[b] RCE (%)		Transforming Activity[c]		Transformation Response[d]		
				Focus Data Type	Vessels	Foci/Vessel Focus Type	Significance[e]	
Drug	Conc. mM	S.A.	CC.A.	III	(N)	III	*t*-statistic	
Aphidicolin [APH, M.W. =338.5]								
Trial 1 [Exp. DRI1]								
B(a)P	.000791		ND					
B(a)P	.000250		ND					
MNNG	.0203	1.93	21.6	151	(15)	8.83***	+ 9.63	
MNNG	.0102	3.33	96.9	73	(15)	4.48***	+ 6.56	
MNNG	.00508	10.6	91.5	58	(15)	2.42**	+ 3.33	
APH	.000295	12.0	73.0	73	(15)	4.12***	+ 5.64	
APH	.0000934	70.0	103.	16	(15)	.803	0.00	(-0.42)
APH	.0000295	83.0	89.6	15	(15)	.755	0.00	(-0.59)
NC	Control	100.	100.	20	(30)	.939	Control	
							Mean t = 1.88	
Trial 2 [Exp. #97]								
B(a)P	.000791	4.74	78.0	118	(20)	5.02***	+ 12.5	
B(a)P	.000250	17.4	104.	52	(20)	2.26***	+ 7.26	
APH		8.70	7.94	61	(19)	2.78***	+ 8.25	
APH		3.16	47.1	94	(20)	4.42***	+ 12.3	
APH		29.2	93.1	50	(20)	2.13***	+ 6.83	
APH		53.8	98.8	26	(20)	1.08**	+ 3.32	
NC	Control	100.	100.	47	(80)	.414	Control	
							Mean t = 7.68	
Barium Chloride [BACL, M.W. 244.]								
Trial 1 [Exp. #42]								
B(a)P	.000791	2.41	35.7	280	(20)	13.7***	+ 20.2	
B(a)P	.000250	5.72	65.4	161	(19)	7.44***	+ 9.55	
BACL	1.84	1.51	4.73	142	(19)	7.04***	+ 12.2	
BACL	1.39	25.3	51.3	93	(20)	4.18***	+ 6.87	
BACL	.922	80.1	88.0	51	(20)	2.06**	+ 3.25	
BACL	.461	98.8	88.0	22	(19)	.965	+ 0.36	
NC-1	Control	100.	100.	52	(40)	.861	Control	
							Mean t = 5.67	
Trial 2 [Exp. #53]								
B(a)P	.000791	4.78	58.9	182	(20)	8.30***	+ 7.68	
B(a)P	.000250	7.97	76.8	195	(20)	8.74***	+ 7.74	
BACL	1.50	10.4	63.7	88	(20)	4.10**	+ 2.65	
BACL	1.00	69.3	73.5	33	(16)	1.82	0.00	(-2.25)
BACL	.500	101.	83.9	56	(20)	2.63	0.00	(-0.36)
BACL	.250	101.	98.7	52	(18)	2.62	0.00	(-0.35)
NC-1	Control	100.	100.	128	(40)	2.78	Control	
							Mean t = .663	
5-Bromo-2'-Deoxyuridine [BUDR, M.W. = 307.4]								
Trial 1 [IP3]								
B(a)P	.000791		ND					
B(a)P	.000250		ND					
BUDR	.179	30.1	ND	228	(20)	10.2***	+ 20.7	
BUDR	.0895	41.2	ND	369	(20)	17.4***	+ 31.3	
BUDR	.0447	75.7	ND	69	(20)	2.91***	+ 9.56	
NC	Control	100.	100.	8	(40)	.149	Control	
							Mean t = 20.5	

(Continued on next page)

Table A1. *Continued.*

Treatment Condition[a]		Cytotoxic Activity[b] RCE (%)		Transforming Activity[c]		Transformation Response[d]	
				Focus Data Type	Vessels	Foci/Vessel Focus Type	Significance[e]
Drug	Conc. mM	S.A.	CC.A.	III	(N)	III	*t*-statistic
Trial 2 [1P7]							
B(a)P	.000791		ND				
B(a)P	.000250		ND				
MNNG	.0170	19.9	ND	59	(15)	3.72**	+ 6.88
BUDR	.104	12.5	ND	110	(15)	6.40**	+ 7.87
BUDR	.052	78.3	ND	91	(15)	5.29**	+ 6.81
BUDR	.026	80.9	ND	32	(15)	1.66	+ 1.61
NC	Control	100.	ND	40	(30)	.97	Control
							MEAN t = 5.43
Trial 3 [Exp. #95]							
B(a)P	.000791	16.0	ND	152	(20)	7.35***	+ 9.48
B(a)P	.000250	33.3	ND	115	(20)	5.27***	+ 3.42
BUDR	.130	16.4	ND	79	(19)	3.78	+ 1.67
BUDR	.0976	27.9	ND	118	(19)	5.85***	+ 6.00
BUDR	.0651	39.0	ND	62	(17)	2.87	+ 0.05
BUDR	.0325	68.3	ND	22	(17)	1.08	0.00 (-4.40)
NC	Control	100.	ND	263	(77)	2.84	Control
							Mean t = 1.93
Carisoprodol [CARP, M.W. = 260.]							
Trail 1 [Exp. #46]							
B(a)P	.000791	5.52	35.7	127	(20)	6.14***	+ 12.4
B(a)P	.000250	22.4	81.1	75	(20)	3.32***	+ 8.63
CARP	6.15	.000	.000	6	(12,20)	.381	0.00 (-0.02)
CARP	4.62	.000	.000	4	(9,20)	.361	0.00 (-0.10)
CARP	3.08	41.7	71.3	7	(20)	.275	0.00 (-0.67)
CARP	1.54	75.5	84.5	24	(20)	.838	0.00 (-1.95)
NC-1	Control	100.	100.	24	(20)	.384	Control
							Mean t = .00
Trial 2 [Exp. #52]							
B(a)P	.000791	4.04	39.8	150	(20)	6.63***	+ 9.35
B(a)P	.000250	11.1	75.4	126	(20)	5.61***	+ 8.18
CARP	4.00	6.40	2.63	13	(18)	.562	0.00 (-1.96)
CARP	2.00	47.8	76.4	29	(20)	1.26	+ 0.56
CARP	1.00	77.8	89.2	34	(18)	1.60	+ 1.43
CARP	.500	94.6	94.6	55	(20)	2.14*	+ 2.60
NC-1	Control	100.	100.	55	(38)	1.09	Control
							Mean t = 1.53
Chloramphenicol Sodium Succinate [CHSU, M.W. 463.]							
Trial 1 [Exp. #49]							
B(a)P	.000791	.357	57.9	188	(19)	9.26***	+ 18.5
B(a)P	.000250	3.21	88.2	87	(20)	3.94***	+ 11.3
CHSU	5.83	.000	60.6	8	(19)	.339	0.00 (-0.64)
CHSU	3.89	2.86	42.2	5	(18)	.212	0.00 (-1.57)
CHSU	2.92	16.4	64.7	3	(20)	.110	0.00 (-3.02)
CHSU	1.94	50.7	104.	8	(20)	.275	0.00 (-1.07)
NC	Control	100.	100.	22	(40)	.433	Control
							Mean t = .000
Trial 2 [Exp. #58]							
B(a)P	.000791	3.92	34.3	128	(20)	6.17***	+ 22.5
B(a)P	.000250	14.7	60.6	71	(20)	1.83***	+ 4.78

(Continued on next page)

Table A1. *Continued.*

Treatment Condition[a]		Cytotoxic Activity[b] RCE (%)		Transforming Activity[c] Focus Data Type	Vessels (N)	Transformation Response[d] Foci/Vessel Focus Type	Significance[e]
Drug	Conc. mM	S.A.	CC.A.	III		III	*t*-statistic
CHSU	6.00	.000	52.2	10	(20)	.414	+ 1.97
CHSU	4.50	.327	94.4	6	(20)	.214	+ 0.23
CHSU	3.00	15.7	101.	2	(20)	.072	0.00 (-1.37)
CHSU	1.50	65.0	86.8	4	(15)	.203	+ 0.12
NC	Control	100.	100.	10	(40)	.189	Control
							Mean t = .580
4-Chloro-2-Nitroaniline [4C2NA, M.W. 172.5]							
Trial 1 [Exp. #46]							
B(a)P	.00791	5.52	35.7	127	(20)	6.14***	+ 12.4
B(a)P	.00250	22.4	81.1	75	(20)	3.32***	+ 8.63
4C2NA	.870	.000	19.1	5	(20)	.189	0.00 (-1.45)
4C2NA	.652	23.8	59.5	9	(20)	.320	0.00 (-0.37)
4C2NA	.435	43.1	84.8	10	(20)	.366	0.00 (-0.10)
4C2NA	.217	65.1	91.7	11	(20)	.423	+ 0.21
NC	Control	100.	100.	24	(40)	.384	Control
							Mean t = .053
Trial 2 [Exp. #53]							
B(a)P	.00791	4.78	58.9	182	(20)	8.30***	+ 7.68
B(a)P	.00250	7.97	76.8	195	(20)	8.74***	+ 7.74
4C2NA	.580	13.5	67.7	48	(20)	2.21	0.00 (-1.49)
4C2NA	.435	33.1	70.3	65	(20)	2.98	+ 0.45
4C2NA	.290	37.8	71.6	53	(20)	2.26	0.00 (-1.18)
4C2NA	.145	56.2	83.9	50	(20)	1.98	0.00 (-1.71)
NC	Control	100.	100.	128	(40)	2.78	Control
							Mean t = .113
C. I. Acid Red 114 [CIAR114, M.W. 830.]							
Trial 1 [Exp. #64]							
B(a)P	.000791	6.45	26.6	98	(20)	4.47***	+ 12.7
B(a)P	.000250	16.9	69.1	32	(20)	1.37***	+ 5.13
CIAR114	.964	1.61	28.1	4	(20)	.149	0.00 (-1.13)
CIAR114	.482	55.2	73.4	9	(20)	.366	+ 0.52
CIAR114	.241	87.9	81.3	13	(20)	.503	+ 1.28
CIAR114	.120	106.	92.1	21	(20)	.665	+ 1.67
NC	Control	100.	100.	17	(40)	.291	Control
							Mean t = .868
Trial 2 [Exp. #69]							
B(a)P	.000791	1.72	50.1	63	(20)	2.67***	+ 9.34
B(a)P	.000250	11.3	68.1	33	(20)	1.17***	+ 3.53
CIAR114	1.00	.000	30.9	5	(14)	.255	0.00 (-0.23)
CIAR114	.500	33.3	90.1	9	(18)	.370	+ 0.58
CIAR114	.250	84.2	97.7	12	(12)	.550	+ 1.67
CIAR114	.125	93.8	95.4	9	(9)	.347	+ 0.43
NC	Control	100.	100.	15	(40)	.288	Control
							Mean t = .670
C. I. Direct Blue 15 [DB15, M.W. 993.]							
Trial 1 [Exp. #64]							
B(a)P	.000791	6.45	26.6	98	(20)	4.47***	+ 12.7
B(a)P	.000251	16.9	69.1	32	(20)	1.37***	+ 5.13
DB15	3.52	.000	54.7	32	(19)	1.37***	+ 4.74
DB15	1.76	1.21	76.3	56	(20)	2.28***	+ 9.25

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Table A1. *Continued.*

Treatment Condition[a]		Cytotoxic Activity[b] RCE (%)		Transforming Activity[c] Focus Data Type	Vessels (N)	Transformation Response[d] Foci/Vessel Focus Type III	Significance[e] *t*-statistic
Drug	Conc. mM	S.A.	CC.A.	III			
DB15	.881	92.7	77.7	41	(20)	1.62***	+ 5.44
DB15	.441	112.	87.1	21	(20)	.844**	+ 2.99
NC	Control	100.	100.	17	(40)	.291	Control
							Mean t = 5.61
Trial 2 [Exp. #69]							
B(a)P	.000791	1.72	50.1	63	(20)	2.67***	+ 9.34
B(a)P	.000250	11.3	68.3	33	(20)	1.17**	+ 3.53
DB15	3.52	.000	47.1	33	(20)	1.39***	+ 5.61
DB15	2.64	3.78	51.5	36	(20)	1.43***	+ 4.55
DB15	1.76	65.3	65.4	18	(20)	.737**	+ 2.82
DB15	.881	99.7	85.0	17	(20)	.588	+ 1.54
NC	Control	100.	100.	15	(40)	.288	Control
							Mean t = 3.64
C. I. Direct Blue 218 [DB218, M.W. = 1088.]							
Trial 1 [Exp. #49]							
B(a)P	.00791	.357	57.9	188	(19)	9.26***	+ 18.5
B(a)P	.00250	3.21	88.2	87	(20)	3.94***	+ 11.3
DB218	2.00	.000	.000	0	(0,20)	.000	NA
DB218	1.00	.000	1.38	15	(20)	.489	+ 0.31
DB218	.500	1.79	4.13	15	(20)	.443	+ 0.05
DB218	.250	58.9	67.5	31	(20)	1.26***	+ 3.79
NC	Control	100.	100.	22	(40)	.433	Control
							Mean t = 1.38
Trial 2 [Exp. #58]							
B(a)P	.000791	3.92	34.3	128	(20)	6.17***	+ 22.5
B(a)P	.000250	14.7	60.6	71	(20)	1.83***	+ 4.78
DB218	.500	.000	45.2	21	(20)	.877***	+ 4.73
DB218	.375	5.23	72.5	5	(20)	.189	+ 0.00
DB218	.250	66.7	89.2	17	(20)	.726***	+ 4.05
DB218	.125	90.5	101.	10	(20)	.374	+ 1.53
NC	Control	100.	100.	10	(40)	.189	Control
							Mean t = 2.58
Trans-Cinnamylaldehyde [CINAL, M.W. 132.2, DENSITY = 1.05]							
Trial 1 [Exp. #79]							
B(a)P	.000791	22.7	79.6	279	(13)	20.8***	+ 13.9
B(a)P	.000250	39.3	94.2	241	(18)	12.9***	+ 9.34
CINAL	.0605	.000	75.7	430	(18)	23.6***	+ 19.4
CINAL	.0303	.000	100.	62	(18)	3.14	0.00 (-2.95)
CINAL	.0151	8.26	101.	79	(18)	3.76	0.00 (-1.81)
CINAL	.00756	47.1	105.	67	(18)	2.80	0.00 (-2.98)
NC	Control	100.	100.	430	(72)	5.12	Control
							Mean t = 4.85
Trial 2 [Exp. #94]							
B(a)P	.000791	.000	75.7	122	(18)	5.92***	+ 6.26
B(a)P	.000250	17.4	114.	81	(18)	3.88***	+ 4.08
CINAL	.0756	.000	.000	2	(8,18)	.189	0.00 (-3.28)
CINAL	.0567	.000	28.5	72	(18)	2.97*	+ 2.58
CINAL	.0378	.000	109.	62	(18)	3.13***	+ 4.07
CINAL	.0189	1.03	101.	39	(18)	1.90	+ 0.87
NC	Control	100.	100.	150	(71)	1.52	Control
							Mean t = 2.51

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Table A1. *Continued.*

Treatment Condition[a]		Cytotoxic Activity[b] RCE (%)		Transforming Activity[c] Focus Data Type III	Vessels (N)	Transformation Response[d] Foci/Vessel Focus Type III	Significance[e] t-statistic
Drug	Conc. mM	S.A.	CC.A.				
2,6-Dinitrotoluene [26DNT, M.W. = 150.]							
Trial 1 [Exp. #91]							
B(a)P	.000791	28.9	73.6	60	(20)	2.00***	+ 5.11
B(a)P	.000250	58.1	89.9	14	(20)	.503	+ 1.31
26DNT	1.333	29.8	84.4	6	(20)	.214	0.00 (-0.94)
26DNT	.667	59.6	87.8	5	(20)	.149	0.00 (-1.55)
26DNT	.333	79.1	92.1	3	(19)	.116	0.00 (-1.93)
26DNT	.167	79.3	94.2	4	(20)	.149	0.00 (-1.61)
NC	Control	100.	100.	31	(75)	.322	Control Mean t = .00
Trial 2 [Exp. #105]							
B(a)P	.000791	5.67	63.1	59	(20)	2.43***	+ 6.70
B(a)P	.000250	18.2	86.5	40	(19)	1.93***	+ 5.54
26DNT	8.00	.000	.000	0	(4,20)	.000	0.00 (-8.86)
26DNT	4.00	.000	.792	0	(20)	.000	0.00 (-8.86)
26DNT	2.00	.000	51.5	10	(20)	.354	0.00 (-1.36)
26DNT	1.00	1.21	74.1	9	(20)	.327	0.00 (-1.56)
NC	Control	100.	100.	58	(77)	.581	Control Mean t = .00
Cytosine Arabinoside [ARAC, M.W. = 279.68]							
Trial 1 [Exp. IP4]							
B(a)P	.000791		ND				
B(a)P	.000250		ND				
ARAC	.000322	.000	ND	206	(20)	9.66***	+ 20.3
ARAC	.000161	5.9	ND	263	(20)	12.52***	+ 23.5
ARAC	.0000805	39.4	ND	170	(20)	6.83***	+ 14.1
NC	Control	100.	ND	16	(40)	.278	Control Mean t = 19.3
Trial 2 [Exp. IP7]							
B(a)P	.000791	Not Available					
B(a)P	.000250	Not Available					
MNNG	.0170	19.9	ND	59	(15)	3.72***	+ 6.88
ARAC	.000358	.4	ND	73	(15)	4.45***	+ 8.61
ARAC	.000113	41.5	ND	49	(15)	3.04***	+ 4.69
ARAC	.000358	97.4	ND	17	(15)	1.02	+ 0.08
NC	Control			40	(30)	.97	Control Mean t = 4.46
Trial 3 [Exp. #95]							
B(a)P	.000791	16.0	ND	152	(20)	7.35***	+ 9.48
B(a)P	.000250	33.3	ND	115	(20)	5.27***	+ 3.82
ARAC	.00143	.000	ND	65	(17)	3.35	+ 0.89
ARAC	.000715	.000	ND	86	(20)	3.73	+ 1.59
ARAC	.000358	.000	ND	121	(20)	5.46***	+ 4.03
ARAC	.000179	3.55	ND	184	(20)	8.66***	+ 7.30
NC	Control	100.	ND	263	(77)	2.84	Control Mean t = 5.09
Diphenylnitrosamine [DPN, 198.22]							
Trial 1 [Exp. #75]							
B(a)P	.000791	7.10	66.5	149	(20)	6.35***	+ 10.9
B(a)P	.000250	28.4	85.4	67	(20)	3.10***	+ 6.56

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Table A1. *Continued.*

Treatment Condition[a]		Cytotoxic Activity[b] RCE (%)		Transforming Activity[c] Focus Data Type	Vessels	Transformation Response[d] Foci/Vessel Focus Type	Significance[e]
Drug	Conc. mM	S.A.	CC.A.	III	(N)	III	*t*-statistic
DPN	.948	.000	.000	16	(20)	.619	0.00 (-1.22)
DPN	.631	.000	33.4	30	(20)	1.24	+ 1.38
DPN	.315	46.8	76.8	43	(19)	2.04***	+ 3.88
DPN	.158	76.4	83.5	35	(20)	1.44*	+ 2.04
NC	Control	100.	100.	89	(78)	.882	Control
							Mean t = 1.83
Trial 2 [Exp. #91]							
B(a)P	.000791	28.9	73.6	60	(20)	2.00***	+ 5.11
B(a)P	.000250	58.1	89.9	20	(20)	.503	+ 1.31
DPN	.757	.000	4.46	11	(18)	.423	+ 0.73
DPN	.568	.000	52.4	3	(20)	.110	0.00 (-2.04)
DPN	.378	8.36	71.3	12	(20)	.452	+ 0.99
DPN	.189	62.6	90.6	17	(20)	.644*	+ 2.21
NC	Control	100.	100.	31	(75)	.322	Control
							Mean t = .983
Isobutyraldehyde [IBA, 72.1, Density = 0.794]							
Trail 1 [Exp. #78]							
B(a)P	.000791	8.09	60.6	116	(18)	6.11***	+ 4.93
B(a)P	.000250	14.9	84.2	184	(18)	9.84***	+ 9.74
IBA	3.85	5.53	78.5	119	(18)	6.45**	+ 6.53
IBA	2.89	7.23	70.7	62	(18)	2.95	0.00 (-0.52)
IBA	1.93	19.1	78.5	42	(18)	2.02	0.00 (-2.34)
IBA	.964	33.2	70.8	103	(18)	4.43	+ 1.49
NC	Control	100.	100.	296	(72)	3.28	Control
							Mean t = 2.11
Trial 2 [Exp. #106]							
B(a)P	.000791	24.8	56.9	134	(18)	6.88***	+ 8.00
B(a)P	.000250	40.7	77.9	91	(18)	4.53***	+ 5.56
IBA	3.85	.000	.000	7	(18)	.240	0.00 (-4.50)
IBA	2.89	.000	10.3	12	(18)	.587	0.00 (-3.08)
IBA	1.93	.000	55.1	25	(18)	1.20	0.00 (-0.29)
IBA	.964	9.79	54.0	15	(18)	.671	0.00 (-2.04)
NC	Control	100.	100.	74	(43)	1.30	Control
							Mean t = .00
Manganese Sulfate-H_2O [MNSU, M.W. = 169.01]							
Trial 1 [Exp. #49]							
B(a)P	.00791	.357	57.9	188	(19)	9.26***	+ 18.5
B(a)P	.00250	3.21	88.2	87	(20)	3.94***	+ 11.3
MNSU	.272	.000	24.3	28	(20)	1.10**	+ 3.18
MNSU	.204	2.86	41.9	35	(20)	1.50***	+ 4.89
MNSU	.136	11.5	58.8	55	(20)	2.37***	+ 7.34
MNSU	.068	61.8	78.1	22	(20)	.872*	+ 2.24
NC		100.	100.	22	(40)	.433	Control
							Mean t = 4.41
Trial 2 [Exp. #57]							
B(a)P	.000791	3.55	30.1	162	(20)	7.55***	+ 18.7
B(a)P	.000250	5.32	69.9	37	(20)	1.63***	+ 6.91
MNSU	.272	.000	1.81	20	(19)	.804**	+ 2.92
MNSU	.204	.000	13.8	24	(20)	.892**	+ 2.84
MNSU	.136	5.67	60.0	21	(18)	.918**	+ 3.85
MNSU	.0680	57.4	84.4	19	(20)	.692*	+ 2.41
NC	Control	100.	100.	15	(40)	.278	Control
							Mean t = 3.03

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Table A1. *Continued.*

Treatment Condition[a]		Cytotoxic Activity[b] RCE (%)		Transforming Activity[c]		Transformation Response[d]	
				Focus Data		Foci/Vessel	
Drug	Conc. mM	S.A.	CC.A.	Type III	Vessels (N)	Focus Type III	Significance[e] *t*-statistic
2-Mercaptobenzimidazole [2MBZ, M.W. = 150.]							
Trial 1 [Exp. #47]							
B(a)P	.000791	.351	28.1	173	(20)	8.10***	+ 14.6
B(a)P	.000250	7.72	75.6	88	(20)	3.89***	+ 8.73
2MBZ	5.00	81.4	.000	27	(20)	1.11*	+ 2.20
2MBZ	3.75	82.1	9.76	45	(20)	2.04***	+ 5.36
2MBZ	2.50	103.	78.1	60	(20)	1.77**	+ 2.82
2MBZ	1.25	95.8	99.0	40	(20)	.869	+ 0.90
NC	Control	100.	100.	31	(39)	.579	Control
							Mean t = 2.82
Trial 2 Exp. #56]							
B(a)P	.000791	2.55	32.7	122	(20)	5.54***	+ 16.5
B(a)P	.000250	2.19	61.0	33	(20)	1.47***	+ 6.97
2MBZ	4.0	1.09	28.0	27	(20)	1.09***	+ 3.91
2MBZ	3.0	38.0	58.2	40	(20)	1.41***	+ 4.23
2MBZ	2.0	57.3	79.6	14	(19)	.583*	+ 2.21
2MBZ	1.0	65.4	86.8	9	(20)	.347	+ 0.69
NC	Control	100.	100.	13	(39)	.260	Control
							Mean t = 2.76
Methdilazine-HCl [MEDL, M.W. = 296.]							
Trial 1 [Exp. #47]							
B(a)P	.000791	.351	28.1	173	(20)	8.10***	+ 14.6
B(a)P	.000250	7.72	75.6	88	(20)	3.89***	+ 8.73
MEDL	.0541	.000	.000	0	(1,20)	.000	0.00 (-0.95)
MEDL	.0405	.000	15.6	8	(20)	.256	0.00 (-1.81)
MEDL	.0270	33.7	90.9	12	(20)	.452	0.00 (-0.66)
MEDL	.0135	77.9	99.2	7	(20)	.275	0.00 (-1.80)
NC	Control	100.	100.	31	(39)	.579	Control
							Mean t = .00
Trial 2 [Exp. #55]							
B(a)P	.000791	2.26	23.9	133	(20)	5.49***	+ 15.2
B(a)P	.000250	4.07	62.4	48	(20)	1.88***	+ 7.12
MEDL	.0507	.000	.784	0	(2,20)	.000	0.00 (-2.88)
MEDL	.0380	1.81	41.2	9	(20)	.347	+ 2.08
MEDL	.0253	53.8	70.6	5	(20)	.189	+ 0.68
MEDL	.0127	62.0	89.0	6	(20)	.214	+ 0.89
NC	Control	100.	100.	7	(40)	.129	Control
							Mean t = 1.22
Mezerein [MEZ, M.W. = 654.]							
Trial 1 [Exp. #59]							
B(a)P	.000791	1.35	36.5	165	(20)	7.31***	+ 17.9
B(a)P	.000250	7.41	74.3	33	(19)	1.34***	+ 4.28
MEZ	.0245	.000	71.9	29	(20)	1.18***	+ 4.01
MEZ	.0183	40.7	104.	50	(20)	2.22***	+ 9.05
MEZ	.0122	84.2	112.	140	(20)	6.33***	+ 17.0
MEZ	.0061	96.0	104.	228	(20)	11.0***	+ 25.7
NC	Control	100.	100.	15	(40)	.297	Control
							Mean t = 13.9
Trial 2 [Exp. #95]							
B(a)P	.000791	16.0	ND	152	(20)	7.35***	+ 9.48
B(a)P	.000250	33.2	ND	115	(20)	5.27***	+ 3.82

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Table A1. *Continued.*

Treatment Condition[a]		Cytotoxic Activity[b] RCE (%)		Transforming Activity[c]		Transformation Response[d]	
Drug	Conc. mM	S.A.	CC.A.	Focus Data Type III	Vessels (N)	Foci/Vessel Focus Type III	Significance[e] *t*-statistic
MEZ	.0245	.000	ND	0	(0,20)	.000	ND
MEZ	.0122	59.9	ND	128	(16)	7.45***	+ 5.63
MEZ	.00610	96.2	ND	256	(17)	14.9***	+ 20.3
MEZ	.00305	99.3	ND	201	(15)	12.9***	+ 13.5
NC	Control	100.	ND	263	(77)	2.84	Control
							Mean t = 13.1
Mono(2-ethylhexyl)adipate [M2EHA, M.W. = 258.42]							
Trial 1 [Exp. #82]							
B(a)P	.000791	46.8	47.2	371	(18)	19.4***	+ 7.45
B(a)P	.000250	56.3	51.6	288	(18)	15.5***	+ 7.97
M2EHA	2.32	.000	.000	1	(5,18)	.149	0.00 (-10.4)
M2EHA	1.16	8.45	49.0	229	(18)	11.9**	+ 3.25
M2EHA	.580	62.4	92.3	146	(18)	7.39	0.00 (-0.63)
M2EHA	.290	75.0	98.0	146	(18)	7.80	0.00 (-0.22)
NC	Control	100.	100.	649	(72)	8.01	Control
							Mean t = 1.08
Trial 2 [Exp. #94]							
B(a)P	.000791	.000	75.7	122	(18)	5.92***	+ 6.26
B(a)P	.000250	17.4	114.	81	(18)	3.88***	+ 4.08
M2EHA	1.93	.000	.000	1	(11,18)	.065	0.00 (-8.76)
M2EHA	1.45	.000	22.7	6	(18)	.240	0.00 (-6.18)
M2EHA	.967	.000	56.4	5	(18)	.136	0.00 (-6.56)
M2EHA	.484	.000	96.5	22	(9)	1.75	+ 0.38
NC	Control	100.	100.	150	(71)	1.52	Control
							Mean t = .127
Mono(2-ethylhexyl)phthalate [M2EHP, M.W. = 278.38]							
Trial 1 [Exp. #79]							
B(a)P	.000791	22.7	79.6	279	(13)	20.8***	+ 13.9
B(a)P	.000250	39.3	94.2	241	(18)	12.9***	+ 9.34
M2EHP	1.44	.000	.811	53	(15,18)	2.42	0.00 (-2.72)
M2EHP	1.08	2.48	27.4	249	(18)	13.3***	+ 9.16
M2EHP	.718	74.4	95.5	110	(18)	5.24	+ 0.14
M2EHP	.359	113.	95.8	49	(18)	2.42	0.00 (-4.33)
NC	Control	100.	100.	430	(72)	5.12	Control
							Mean t = 3.05
Trial 2 [Exp. #84]							
B(a)P	.000791	.000	75.7	122	(18)	5.92***	+ 6.26
B(a)P	.000250	17.4	114.	81	(18)	3.88***	+ 4.08
M2EHP	1.44	1.0	23.6	30	(18)	1.11	0.00 (-1.07)
M2EHP	1.08	54.4	64.6	43	(18)	1.72	+ 0.44
M2EHP	.718	85.1	76.2	52	(18)	2.24	+ 1.48
M2EHP	.359	126.	89.7	76	(18)	3.35**	+ 3.31
NC	Control	100.	100.	150	(71)	1.52	Control
							Mean t = 1.31
Riddelliine [RIDL, M.W. = 349.]							
Trial 1 [Exp. #48]							
B(a)P	.00791	2.28	47.9	148	(20)	7.06***	+ 16.5
B(a)P	.00250	10.1	90.8	63	(20)	2.68***	+ 6.53

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Table A1. *Continued.*

Treatment Condition[a]		Cytotoxic Activity[b] RCE (%)		Transforming Activity[c] Focus Data Type III	Vessels (N)	Transformation Response[d] Foci/Vessel Focus Type III	Significance[e] *t*-statistic
Drug	Conc. mM	S.A.	CC.A.				
RIDL	2.579	9.77	62.0	79	(20)	3.19***	+ 7.36
RIDL	1.289	39.7	83.0	41	(20)	1.78***	+ 4.58
RIDL	.645	71.0	95.3	34	(19)	1.22*	+ 2.40
RIDL	.322	89.3	88.3	29	(20)	.948	+ 1.77
NC	Control	100.	100.	29	(40)	.537	Control
							Mean t = 4.03
Trial 2 [Exp. #66]							
B(a)P	.000791	2.33	52.0	90	(20)	3.92***	+ 14.1
B(a)P	.000250	6.08	98.4	24	(20)	.795**	+ 3.69
RIDL	3.00	1.01	77.0	21	(20)	.824**	+ 4.68
RIDL	1.50	7.09	93.7	4	(20)	.132	+ 0.91
RIDL	.750	34.1	100.	2	(18)	.080	+ 0.39
RIDL	.375	51.0	103.	7	(18)	.227	+ 1.39
NC	Control	100.	100.	3	(38)	.056	Control
							Mean t = 1.84
Sodium Fluoride [also 300509-S, M.W. = 40.]							
Trial 1 [Exp. #70]							
B(a)P	.000791	1.24	48.9	11	(3)	3.38***	+ 4.23
B(a)P	.000250	11.8	81.0	19	(5)	3.62***	+ 5.73
300509-S	4.50	2.48	1.93	7	(19)	.272	0.00 (-1.67)
300509-S	3.38	3.11	9.32	9	(18)	.339	0.00 (-1.12)
300509-S	2.25	3.11	53.4	9	(20)	.320	0.00 (-1.33)
300509-S	1.13	35.4	96.1	9	(16)	.274	0.00 (-1.400
NC	Control	100.	100.	36	(54)	.526	Control
							Mean t = .000
Trial 2 [Exp. #101]							
B(a)P	.000791	ND	64.8	108	(20)	4.63***	+ 12.7
B(a)P	.000250	ND	83.6	48	(20)	2.11***	+ 9.73
300509-S	4.00	ND	21.8	23	(17)	1.06**	+ 3.51
300509-S	3.00	ND	50.3	12	(13)	.682	+ 1.87
300509-S	2.00	ND	87.7	7	(16)	.330	+ 0.56
300509-S	1.00	ND	104.	9	(20)	.347	+ 0.75
NC	Control	100.	100.	27	(78)	.260	Control
							Mean t = 1.67
12-*O*-Tetradecanoylphorbol-13-Acetate [TPA, M.W. = 616]							
Trial 1 [Exp. #70]							
B(a)P	.000791	1.24	48.9	11	(3)	3.38***	+ 4.23
B(a)P	.000250	11.8	81.0	19	(5)	3.62***	+ 5.73
TPA	.0227	.000	.000	0	(0,13)	.000	NA
TPA	.0170	.000	2.57	0	(2,14)	.000	0.00 (-7.39)
TPA	.0114	57.8	83.6	8	(16)	.389	0.00 (-0.80)
TPA	.0057	87.0	86.5	5	(15)	.260	0.00 (-1.62)
NC	Control	100.	100.	36	(54)	.526	Control
							Mean t = .000
Trial 2 [Exp. #105]							
B(a)P	.000791	5.67	63.1	59	(20)	2.43***	+ 6.70
B(a)P	.000250	18.2	86.5	40	(19)	1.93***	+ 5.54
TPA	.0195	.000	.000	0	(0,20)	.000	NA
TPA	.0146	2.83	28.0	7	(17)	.256	0.00 (-1.55)
TPA	.00974	63.6	90.8	7	(18)	.289	0.00 (-1.77)
TPA	.00487	79.4	93.7	10	(18)	.392	0.00 (-1.07)
NC	Control	100.	100.	58	(77)	.581	Control
							Mean t = .000

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Table A1. *Continued.*

Treatment Condition[a]		Cytotoxic Activity[b] RCE (%)		Transforming Activity[c]		Transformation Response[d]	
Drug	Conc. mM	S.A.	CC.A.	Focus Data Type III	Vessels (N)	Foci/Vessel Focus Type III	Significance[e] *t*-statistic
2,6-Xylidine [26XY, M.W. = 121.18, Density = 0.98]							
Trial 1 [Exp. #84]							
B(a)P	.000791	49.8	57.9	71	(18)	3.27***	+ 6.82
B(a)P	.000250	ND	71.7	57	(18)	2.44***	+ 6.14
26XY	8.09	.000	.000	1	(6,18)	.122	.00 (-1.52)
26XY	6.07	20.5	50.9	25	(18)	1.20**	+ 3.09
26XY	4.04	43.2	80.0	19	(18)	.823	+ 1.52
26XY	2.06	63.7	92.8	18	(18)	.805	+ 1.44
NC	Control	100.	100.	50	(72)	.511	Control
						Mean t = 2.02	
Trial 2 [Exp. #100]							
B(a)P	.000791	89.7	77.9	65	(18)	3.30***	+ 11.7
B(a)P	.000250	81.0	93.8	62	(18)	2.85***	+ 7.75
26XY	8.09	.000	9.55	2	(6,18)	.260	0.00 (-0.040
26XY	6.07	.000	48.6	18	(18)	.661	+ 1.82
26XY	4.04	.920	76.4	21	(18)	.901***	+ 3.59
26XY	2.06	23.4	98.9	16	(18)	.687**	+ 2.62
NC	Control	100.	100.	28	(72)	.268	Control
						Mean t = 2.68	

Abbreviations: B(a)P, benzo(a)pyrene; CC.A., co-culture clonal survival assay; Conc., concentration; mM, millimole; M.W., molecular weight; N, number of culture vessels; NC, negative control; %RCE, percent relative cloning efficiency; S.A., standard clonal survival assay; ND, not determined.

[a]Treatment Condition: The experimental design for the transformation assay is described in detail in the Materials and Methods. The concentration of the positive control and test chemical treatment are presented in mM, but they can be converted to μg/mL using the molecular weight that is provided with each chemical. The solvent vehicles used for the individual test chemicals were listed in Table 1, and the concentrations of the solvent vehicles are presented in the Materials and Methods.

[b]Cytotoxic Activity: The experimental design for the standard clonal survival assay and the co-culture clonal survival assay were described in detail in the Materials and Methods. The test chemical cytotoxic response was expressed as % RCE and was calculated as described in the Materials and Methods.

[c]Transforming Activity: The criteria used to evaluate the transformed foci of BALB/c-3T3 cells is described in the Materials and Methods. The number of type III foci > 2-mm in diameter per culture vessel scored are recorded in this table.

[d]Transformation Response: The transformation responses are expressed as type III foci/vessel and were calculated using a $\log_{10}$ mathematical transformation procedure (refer to Materials and Methods). The arithmetic value or foci/vessel represents the antilog of the $\log_{10}$ mean transformation response minus one.

[e]Significance: The significance of test chemical transformation responses were calculated by a computer using the SAS statistical software (*25*), and the method is described in detail in Materials and Methods. The correct *t*-statistic according to the F-test is presented in this table. The *t*-statistics of each treatment dose of the test chemical in a single experiment were averaged to determine the mean *t*-statistic of the test chemical for the experiment (refer to Table 2). The mean *t*-statistic for two or experiments for each chemical was weighted according to the number of treatment doses evaluated and averaged to determine the rank *t*-statistic which was used to rank-order the test chemical transformation responses in Table 3. Arbitrarily, transformation responses with negative (−) *t*-statistics were given a value of zero (0).

*Significant BaP or test chemical transformation response, $0.01 < p \leq 0.05$.

**Significant BaP or test chemical transformation response, $0.001 < p \leq 0.01$.

***Significant BaP or test chemical transformation response, $p \leq 0.001$.

Appendix B.

Table A1. Comparison of transformation responses of coded chemicals detected in assays conducted using two different assay protocols.[a]

Chemical[b]	Transformation Response[c] I[d]			II[e]		
Chemicals Active in Protocol II						
11-aminoundecanoic acid	-	SN		++	LN,LN,SP,SP	
allyl isothiocyanate	-	SN		++	LA,SP	
barium chloride-2H_2O		-	SN	++	SP,LA	
benzidine-2HCl		-	SN	++	SP,SP	
2-biphenylamine		-	SN	++	SP,SP	
4-biphenylamine		-	SN	++	SP,LA	
4-chloro-o-toluidine-HCl	-	SN		++	LN,SP,SP	
C.I. direct blue 15	-	SN		++	SP,SP	
cinnamaldehyde		-	SN	++	LA,SP	
C.I. solvent yellow 14		-	SN	++	SP,SP,LA,SP	
D&C red 9		-	SN	++	SP,LA,SP	
2,6-dichloro-*p*-phenylenediamine	+	SP		++	SP,SP	
diglycidyl resorcinol ether	-	LA		++	SP,SP	
epichlorohydrin		-	SN	++	LA,SP	
ethylene thiourea	-	SN		+	LN,SP	
FD & C yellow no. 6	-	LA		++	SP,SP,SP	
H.C. red 3		-	SN	++	SP,SP,LA,SP	
manganese sulfate-H_2O		-	SN	++	SP,SP	
melamine	-	SN		++	SP,LA	
melphalan	+	SP		++	LA,SP	
2-mercaptobenzimidazole	-	SN		++	SP,SP	
methyl carbamate	-	SN		++	SP,LA,SP,SP,SP	
4,4-methylenedianiline-2HCl	-	SN		++	SP,SP	
2-naphthylamine	-	SN		++	SP,SP	
diethanolnitrosamine	ND			+	SP	
4,4-oxydianiline		-	LA	++	SP,SP	
polybrominated biphenyl mixture	-	LA		++	SP,SP	
riddelliine	+	SN,SP		++	SP,LA	
2,6-xylidine		-	SN	++	LA,SP	
Chemicals Inactive in Protocol II						
benzoin		-	SN,SN	-	SN,SN	
bisphenol A		-	SN	-	SN,LA,SN,SN	
caprolactam		-	SN,SN	-	SN,SN	
carisoprodol		-	SN	-	SN,SN	
chloramphenicol sodium succinate	-	SN		-	SN,SN	
4-chloro-2-nitroaniline		-	SN	-	SN,SN	
C.I. acid orange 10	-	SN		-	SN,SN	
C.I. acid red 114	-	SN		-	SN,SN	
C.I. basic red 9		-	SN	-	SN,SN,SN,SN,SN	
C.I. direct blue 218	-	SN		E	LA,LA	
cinnamyl anthranilate	-	SN		-	SN,SN,SN	
dichlorvos		NA		-	SN,LA,SN,SN,SP	
2,4-dinitrotoluene	-	SN		-	SN,SN,SN	
diphenylhydantoin	-	SN		-	SN,SN	
geranyl acetate		-	SN	-	SN,SN	
H.C. blue 1			ND	E	SN,SN,SP	
8-hydroxyquinoline	-	SN		E	SP,SN,SN	
isobutyraldehyde	+	SP		-	LA,SN	
methapyrilene-HCl		NA		-	SN,SN,SN	
methdilazine-HCl	-	SN		-	SN,SN	

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Table A1. *Continued.*

Chemical[b]	Transformation Response[c] I[d]			II[e]		
methylphenidate-HCl	-	SN		-	SN,SN	
molybdenum trioxide	-	SN		-	LA,SN	
mono(2-ethylhexyl)adipate	-	SN		-	LA,SN	
mono(2-ethylhexyl)phthalate	-	LA		E	LA,LA	
reserpine	-	SN		-	LA,SN,SN	
tetrahydrofuran		-	LA		-	SN,LA

Abbreviations for Chemical Transformation Response in Individual Experiments: SP, sufficient positive; LA, limited activity; SN, sufficient negative; LN, limited negative; ND, not determined.

Abbreviations for Overall Chemical Transformation Response: + +, active; +, weakly active; −, inactive; E, equivocal activity.

[a]Protocols: The information contained in this table have been previously discussed in a poster presentation (*10*). The data summarize the results of testing chemicals in two different BALB/c-3T3 cell transformation assay protocols, including: 1) Protocol I which was used on a interagency contract with the EPA and the NIEHS [NIEHS Contract No. 68-02-3682[, and 2) Protocol II which used in this investigation (*12,16–18*) [NIEHS Contract No. NO1-ES-65150].

[b]Chemical: A total of 59 coded chemicals were furnished to be tested using Protocol I; however, data was obtained for only 55 of these chemicals. All 59 chemicals were retested in the current investigation and these data are reported in detail in either this paper or in part V (*12*).

[c]Transformation Response: This table presents a summary of the transformation responses detected for chemicals tested in one or more experiments using either Protocol I or Protocol II. The method used to call individual experiments, as well as two or experiments, is described in detail in the Materials and Methods.

[d]Protocol I: The major procedural differences between protocols I versus II which enhanced the sensitivity of Protocol II, included: treatment time duration [72 versus 48 hours]; initiation of chemical treatments [day 1-4 versus day 2-4]; seeding density [1×10^4 versus 3.2×10^4 cells/vessel]; number of treated cells/dose [$\sim 3 \times 10^4$/20 vessels versus 10×10^4 cells/20 vessels]; method used to select test chemical treatment doses [standard clonal survival assay (Materials and Methods) versus co-culture clonal survival assay (*17*)]; FBS concentration in maintenance medium [5.0% versus 7.5%]; and solvent vehicles [DMSO and acetone versus organic solvent and pluronic F68 (refer to Materials and Methods)].

Other differences between Protocols I versus II included: experimental trials/chemical [1 versus 2 or more]; treatment doses/chemical [5 versus 4]; number of chemicals/experiment [1 or 2 versus 3 to 6]; positive control [MCA versus B(a)P]; and type of foci scored [type III versus types I, II and III].

[e]Protocol II: The methods used in Protocol II are described in Materials and Methods.