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Interlaboratory Studies With the Chinese Hamster V79 Cell Metabolic Cooperation Assay to Detect Tumor-Promoting Agents

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Three laboratories participated in an interlaboratory study to evaluate the usefulness of the Chinese hamster V79 cell metabolic cooperation assay to predict the tumor-promoting activity of selected chemicals. Twenty-three chemicals of different chemical structures (phorbol esters, barbiturates, phenols, artificial sweeteners, alkanes, and peroxides) were chosen for testing based on in vivo promotion activities, as reported in the literature. Assay protocols and materials were standardized, and the chemicals were coded to facilitate unbiased evaluation. A chemical was tested only once in each laboratory, with one of the three laboratories testing only 15 out of 23 chemicals. Dunnett's test was used for statistical analysis, and differences between treated- and control-cell responses were analyzed at $P \leq .01$. Chemicals were scored as positive (at least two concentration levels statistically different than control), equivocal (only one concentration statistically different), or negative. For 15 chemicals tested in all three laboratories, there was complete agreement among the laboratories for nine chemicals. For the 23 chemicals tested in only two laboratories, there was agreement on 16 chemicals. With the exception of the peroxides and alkanes, the metabolic cooperation data were in general agreement with in vivo data. However, an overall evaluation of the V79 cell system for predicting in vivo promotion activity was difficult because of the organ specificity of certain chemicals and/or the limited number of adequately tested nonpromoting chemicals.

Key words: metabolic cooperation inhibition, tumor promoters, interlaboratory comparison

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INTRODUCTION

In recent years there has been great interest in the development of short-term in vitro assay systems for the identification of carcinogenic chemicals. However, most if not all of these test systems require the chemical or its metabolite to interact with the test organism's DNA to manifest a response. Carcinogenesis is a multistep, multicausal process, and not all chemicals that contribute to the process interact directly with DNA. Thus, most currently employed short-term in vitro assays are not capable of detecting all the chemicals that cause cancer [Tennant et al., 1987]. The chemical structure and mechanisms of these non-DNA reactive (epigenetic) carcinogens are varied [Weisburger and Williams, 1981]. Included within this class of nongenotoxic carcinogens are tumor promoters, which were first defined from initiation-promotion studies on mouse skin, but more recent studies have extended this phenomenon to include other organs [Hecker et al., 1982; Slaga, 1983]. Therefore, tumor promoters represent one of the major classes of carcinogens which are not detected in short-term assays. If short-term assays are to be more useful in identifying potential carcinogens, systems that detect tumor promoters and other non-DNA interacting carcinogens must be developed.

The Chinese hamster V79 cell metabolic cooperation assay has been reported to be useful for detecting tumor promoters [Enomoto et al., 1981; Fitzgerald and Murray, 1980; Jone et al., 1985; Murray and Fitzgerald, 1979; Trosko et al., 1980; 1981; 1982; Trosko and Chang, 1984; Umeda et al., 1980; Yancey et al., 1982; Yotti, 1979]. The biological basis for this assay is the inhibition of gap-junctional communication by test chemicals [Enomoto et al., 1981; Yancy et al., 1982]. Inhibition of metabolic cooperation can be measured in this system by impeded transfer of the toxic, phosphorylated metabolite of 6-thioguanine (6TG) from wild type V79 6TG-sensitive ($6TG^s$) cells to cocultured 6TG-resistant ($6TG^r$) V79 cells. This phenomenon is not limited to V79 cells; it has been observed with other cell types and with other methods of endpoint detection [Fitzgerald and Murray, 1980; Kavanaugh et al., 1986; Madhukar et al., 1983; Murray and Fitzgerald, 1979; Stedman and Welsch, 1985; Zeilmaker and Yamasaki, 1986]. Many chemicals have been studied for their ability to inhibit V79 cell metabolic cooperation [Elmore et al., 1985; Fitzgerald and Murray, 1980; Malcolm, et al., 1983, 1985a; Malcolm and Mills, 1985; Murray and Fitzgerald, 1979; Scott et al., 1985; Slaga et al., 1982; Trosko et al., 1980, 1981, 1982; Trosko and Chang, 1984; Umeda et al., 1980; Welsch and Stedman, 1984; Yotti, 1979; Zeilmaker and Yamasaki, 1986], but the usefulness of the approach as an assay system for detecting tumor promoters is still uncertain. In the present study, three separate laboratories conducted the metabolic cooperation assay with V79 cells, using standardized protocols and chemicals of known tumor-promoting activity. The results from the laboratories were compared both with each other and with previous in vitro and in vivo results.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from Radian Corporation (National Toxicology Program Chemical Repository) San Antonio, TX: 12-O-tetradecanoyl-phorbol-13-acetate (TPA), phorbol, 4-O-methyl-TPA, phorbol-12,13-dibutyrate, me-

zerein, sodium saccharin, sodium cyclamate, d(+)-tryptophan, sucrose, phenobarbital, amobarbital, butylated hydroxyanisole, butylated hydroxytoluene, benzoyl peroxide, t-butyl hydroperoxide, hydrogen peroxide, n-dodecane, 1-phenyldodecane, phenol, catechol, pyrogallol, 2,4-dinitrofluorobenzene, ethyl phenyl propriolate, and 6-thioguanine (6-TG). Modified Eagle's minimum essential medium with Earle's salts, containing a 50% increase in all vitamins and essential amino acids except glutamine, 100% increase in the nonessential amino acids, and 1 mM pyruvate, was obtained from GIBCO (Grand Island, NY) [Yotti et al., 1979]. Fetal bovine serum (FBS) (Flow Laboratories, McLean, VA) was added to a concentration of 3% to make a complete culture medium (CM). A 0.01% trypsin (Worthington Biochemicals, Freehold, NJ) solution was prepared in calcium- and magnesium-free phosphate-buffered saline (PBS). The laboratories received the same lot of each test chemical, solvent, medium, and serum.

The chemicals were dissolved in one of the following solvents: absolute ethanol (Veterans Administration Supply Depot, Hines, IL), acetone (Fischer Scientific), distilled/deionized water, or serum-free culture medium. The volume of the solvent added to each dish was the same for all test-chemical dilutions. The highest concentration of the solvents added did not exceed 2% of the medium volume in the dish, with the exception of serum-free culture medium or distilled/deionized water, whose concentration was permitted to increase to 20% of the media per dish. When the medium or water was employed, the volume of medium containing the required number of cells was reduced per dish so that the total volume per dish was no greater than 5 ml.

Cell Cultures

The wild type 6-TG^s cells were derived from Chinese hamster V79 lung fibroblasts [Ford and Yerganian, 1958], and 6-TG^r V79 cells were derived from x-ray-irradiated wild-type cells [Trosko et al., 1981; Yotti et al., 1979]. Both cell lines were kindly provided by Dr. J.E. Trosko, Michigan State University, East Lansing, MI. The cells were maintained in CM, passaged by trypsinization two to three times per week, and used within 2 months from thawing. All cultures were incubated at 37°C in a humidified 5% CO_2 air atmosphere. The cultures were routinely checked and found to be free of mycoplasma contamination (Flow Laboratories, McLean, VA). Stock cultures were cryopreserved in CM containing 5% FBS and 5% dimethysulfoxide. To eliminate cell clumping, all cultures were passaged on the day before use and then were reseeded at the appropriate density on the day the experiment was initiated.

Preliminary Cytotoxicity Assays

All test chemicals were initially evaluated to determine the concentration ranges required to produce little or no cytotoxicity, as modified from the method of Tsushimoto et al. [1982]. The first assay used a high cell density (4×10^5 6-TG^r cells/dish) in each of ten 60-mm dishes (LUX®, Miles Laboratories). After a 4-hour cell-attachment period, the test chemical was added to each dish approximately in one-half log dilutions ranging from 0.001 to 10,000 ug/ml, depending on the estimated solubility and/or toxicity of the chemical, as reported in the literature. Following a 3-day growth period, the cells in each dish were evaluated microscopically to determine the cytostasis or degree of cytotoxicity.

Five concentrations, ranging from nontoxic to marginally cytostatic, were selected for the second toxicity determination. In this clonal assay, 100 6-TG^r cells were seeded in each 60-mm dish (four dishes per assay point). After a 4-hour attachment period, the appropriate concentration of test chemical was added to the medium in each dish. Simultaneous solvent controls consisted of adding an amount of solvent to each dish identical to the volume of test chemical solution added. Following a 3-day growth period, the CM was replaced with fresh CM without test chemical, and the incubation continued. After a 6- to 7-day growth period, the cultures were rinsed with PBS and stained and fixed with 1% crystal violet (1 g dissolved in 10 ml ethanol and diluted to 100 ml with deionized water), and the colonies were counted. The relative cytotoxicity was estimated by comparison with the solvent control cloning efficiency.

For the third cytotoxicity determination, five or six chemical concentrations were selected based on the concentrations that produced 70–100% cell survival in the second assay. The assay procedure was identical to that of the second assay.

Inhibition of Metabolic Cooperation

The assay was adapted from that described by Yotti et al. [1979] and Tsushimoto et al. [1982]. Five or six chemical concentrations were selected, based on the concentrations that produced 70% to 100% cell survival in the third cytotoxicity assay. CM containing 100 6-TG^r and 4×10^5 6-TG^s cells were dispensed into each 60-mm dish. After an attachment period of 4 hours, the appropriate concentration of test chemical or solvent was added to each dish (20 dishes per concentration). After 15 minutes, 6-TG was added to each dish to a final concentration of 10 ug/ml in 5 ml total CM/dish. After a 3-day incubation period, the treated CM was removed and replaced with 5 ml fresh CM containing 6-TG but without test chemical. On the sixth or seventh day, the culture dishes were rinsed with PBS and stained and fixed with 1% crystal violet. The colonies present on each dish were counted.

For the parallel cytotoxicity assays, all procedures were similar to the one in the metabolic cooperation assay, except that no 6-TG^s cells were seeded wth 100 6-TG^r cells. Each cytotoxicity and metabolic cooperation assay included a solvent control and a positive control (TPA, 4 ng/ml). In addition, each cytotoxicity assay included a medium control without solvent.

Statistical Analysis

Means and standard deviations of colonies/dish were calculated for each experimental group. Values from cells treated with test chemical in the metabolic cooperation assay were compared with values from cells treated only with solvent with Dunnett's test [Kirk, 1982]. Data were analyzed relative to controls at the ≤ 0.01 confidence level. A chemical was classified as positive if it inhibited metabolic cooperation at two sequential concentration levels and if the cloning efficiencies in the concurrent cytotoxicity assay was at least 70% of the solvent control. A chemical that did not statistically inhibit metabolic cooperation at any concentration was scored as negative. Chemicals were scored as equivocal if they statistically inhibited metabolic cooperation only at a single concentration level and/or if greater than 30% cytotoxicity relative to controls occurred at the statistically positive concentration levels.

RESULTS AND DISCUSSION

In the present study, three laboratories investigated the V79 metabolic cooperation system as a short-term assay for tumor promoters. To minimize interlaboratory variables, the laboratories all used identical lots of test chemicals, solvents, serum, medium, and trypsin and used a common supplier of culture dishes. Furthermore, before the start of the study, the laboratories accepted a standard protocol, including numbers of dishes and the number of 6-TG^s and/ or 6-TG^r cells needed. Concentration, sequence, and the time of addition of test substance and 6-TG were also standardized. The chemicals were tested coded, although each laboratory was given an initial concentration range for cytotoxic determination based on literature values. The final concentration selections for the metabolic cooperation assay were chosen by agreement among the laboratories. The criteria for scoring of the colonies (time of staining, required number of cells per colony) were also common and the statistical approach was uniform among the laboratories. In the present work, Dunnett's test was employed, and the responses are scored as positive at the $P \leq .01$ significance level of treated samples relative to controls. A $P \leq .01$ was used because in studies where two control groups were included within the same assay, these groups sometimes differed from each other at the $P \leq .05$ level but not at the $P \leq .01$ level.

The chemicals selected for testing were those of known tumor-promoting activity and/or related chemical structure. Although most chemicals selected were positive in in vivo promotion assays, some were selected as negative controls (i.e., phorbol, amobarbital, pyrogallol, and sucrose). However, whereas phorbol is a negative control for skin [Boutwell, 1974; Slaga et al., 1978], it has been reported as a positive promoter for liver and lung in mice as well as mammary tissue [Armuth and Berenblum, 1972; Hecker et al., 1982; Shellabarger et al., 1979] and for leukemia in rats [Armuth and Berenblum, 1974]. Furthermore, during the course of this study, sucrose was reported as positive for mammary tumor promotion and enhancement of liver foci [Hei and Sudilovsky, 1985; Klurfield et al., 1984]. Amobarbital, a structural analogue of phenobarbital, did not promote hepatoma induction in rats [Diwan et al., 1985; Peraino et al., 1975], although during the course of this study it was reported positive for rat liver foci induction [Shinozuka et al., 1982]. Pyrogallol has been reported negative in mouse skin tumorigenesis [Boutwell and Bosch, 1959]. Therefore, the shortage of adequately tested nonpromoting chemicals makes it difficult to validate this assay for distinguishing between promoting and nonpromoting chemicals.

Table I shows the cloning efficiencies and the mean number of colonies ± standard deviation for the metabolic cooperation studies for each chemical tested. A summary of each chemical's response at the $P \leq .01$ significance level and an overall call for the chemical are shown in Table II. The concentration range tested for each chemical, as well as the minimally effective concentration for the positive chemical, is also given. For the results in Table II, the chemicals were scored as positive (+), negative (−), or equivocal (±), as described in Materials and Methods. Previous in vitro metabolic cooperation and in vivo promotion results are also shown in Table II.

Phorbol Esters. The data in Tables I and II demonstrate that phorbol esters are potent inhibitors of metabolic cooperation and that there was agreement in response among the laboratories. The results are also in agreement with documented studies using this system [Malcolm et al., 1983; Scott et al., 1985; Trosko et al.,

TABLE I. Effect of Test Chemical on Metabolic Cooperation

Compound	Concentration	Cytotoxicity[a] (laboratory) 1	2	3	Metabolic cooperation[b] (laboratory) 1	2	3
12-0-tetradecanoyl-phorbol-13-acetate (TPA)							
	Ethanol	91±7	81±10	76±12	38±5	14±4	6±3
	Positive (TPA)	84±8	83±7	72±10	100±9[d]	66±9[d]	72±8[d]
	0.05 μg/ml	ND[c]	ND	67±10	ND	ND	49±8[d]
	0.1	90±7	86±7	76±9	70±9[d]	43±6[d]	44±5[d]
	0.2	ND	83±9	ND	ND	41±6[d]	ND
	0.25	87±8	ND	73±9	69±10[d]	ND	41±6[d]
	0.4	ND	83±10	ND	ND	46±10[d]	ND
	0.5	90±7	ND	73±10	83±11[d]	ND	46±10[d]
	0.75	87±9	ND	ND	93±9[d]	ND	ND
	0.8	ND	80±12	ND	ND	51±6[d]	ND
	1.0	73±7	81±10	72±8	80±9[d]	51±7[d]	56±7[d]
4-0-Methyl TPA							
	Ethanol	47±6	68±12	76±8	18±4	10±3	15±4
	Positive (TPA)	49±6	65±9	72±11	52±9[d]	54±7[d]	65±9[d]
	0.1 μg/ml	47±6	70±8	76±7	20±5	10±2	39±7[d]
	0.5	48±6	64±9	69±6	64±6[d]	55±7[d]	78±11[d]
	1.0	45±6	66±10	70±13	67±8[d]	68±9[d]	78±10[d]
	1.5	45±6	69±8	64±7	57±6[d]	72±10[d]	76±8[d]
	2.0	37±7	70±10	62±14	46±7[d]	80±11[d]	68±16[d]
Phorbol-12,13-dibutyrate							
	Ethanol	125±9	58±8	70±14	14±3	14±5	21±4
	Positive (TPA)	131±13	54±7	71±13	117±10[d]	46±7[d]	102±14[d]
	1.0 μg/ml	133±12	55±7	70±10	70±10[d]	27±6[d]	77±9[d]
	5.0	130±14	55±10	66±7	76±8[d]	32±5[d]	68±11[d]
	10.0	128±19	56±9	65±13	95±8[d]	30±7[d]	73±9[d]
	20.0	6±9(T)[c]	55±8	41±15(T)	107±12[d]	34±5[d]	85±8[d]
	30.0	0±0(T)	53±7	1±2(T)	85±16[d]	40±7[d]	93±11[d]
Phorbol							
	Ethanol	63±10	49±9	74±15	11±3	9±4	23±6
	Positive (TPA)	63±9	50±9	74±12	62±9[d]	40±5[d]	86±13[d]
	1.0 μg/ml	65±7	51±7	73±11	12±5	8±3	26±5
	5.0	60±9	49±10	72±9	11±3	9±3	23±5
	10.0	61±8	50±9	75±12	14±4	8±3	24±5
	20.0	59±8	54±7	73±13	14±4	10±4	26±5
	30.0	59±8	60±11	74±10	12±4	10±3	27±8
Mezerein							
	Ethanol	72±7	59±9	94±12	30±5	17±5	12±4
	Positive (TPA)	72±7	66±7	91±9	61±6[d]	64±7[d]	92±11[d]
	0.1 μg/ml	73±7	63±6	91±9	53±5	55±9[d]	82±13[d]
	0.5	79±11	69±10	87±8	80±7[d]	48±9[d]	79±10[d]
	1.0	56±11	68±8	91±7	79±9[d]	51±8[d]	73±6[d]
	1.5	76±7	65±7	94±11	77±6[d]	57±8[d]	77±8[d]
	2.0	72±8	63±7	76±11	70±8[d]	60±7[d]	76±9[d]
Phenobarbital							
	Ethanol	86±4	76±9	66±15	9±3	12±3	10±4
	Positive (TPA)	86±5	75±9	72±12	85±8[d]	58±8[d]	56±9[d]
	50.0 μg/ml	89±6	ND	69±13	12±3	ND	8±3
	100	88±6	76±8	64±10	18±5[d]	13±4	9±2
	150	87±8	74±9	66±9	19±3[d]	16±4	12±3
	200	82±8	80±10	64±11	22±4[d]	19±5[d]	10±3
	250	83±7	77±8	81±8	22±5[d]	21±6[d]	10±3
	300	73±8	76±8	67±12	26±5[d]	23±6[d]	9±2

(continued)

TABLE I. Effect of Test Chemical on Metabolic Cooperation (Continued)

Compound	Concentration	Cytotoxicity[a] (laboratory) 1	2	3	Metabolic cooperation[b] (laboratory) 1	2	3
Amobarbital							
	Ethanol	76±4	60±8	69±18	18±5	7±2	13±3
	Positive (TPA)	79±5	65±10	61±12	79±3[d]	55±8[d]	68±12[d]
	50 μg/ml	78±6	67±9	62±12	22±3	8±2	11±4
	75	75±6	69±7	64±12	25±4[d]	8±4	14±3
	100	73±4	71±6	61±8	29±5[d]	10±4[d]	13±4
	125	72±4	66±9	57±9	28±4[d]	13±4[d]	13±4
	150	74±5	66±8	55±9	31±6[d]	14±3[d]	16±5[d]
Butylated hydroxytoluene							
	Ethanol	84±5	86±11	63±12	14±4	5±3	19±4
	Positive (TPA)	83±7	83±11	69±14	87±7[d]	32±6[d]	66±9[d]
	0.25 μg/ml	ND	89±11	ND	ND	5±2	ND
	0.5	89±10	82±10	72±15	11±5	7±2	16±3
	1.0	84±10	85±8	67±10	12±4	7±3	17±4
	2.0	84±5	ND	70±14	11±5	ND	22±6
	2.5	ND	83±11	ND	ND	8±3[d]	ND
	3.0	86±9	ND	69±10	22±7[d]	ND	23±5
	5.0	85±11	83±9	68±10	22±7[d]	8±3[d]	20±4
Butylated hydroxyanisole							
	Ethanol	89±10	84±13	63±10	18±8	23±5	16±5
	Positive (TPA)	93±7	82±11	61±8	97±7[d]	62±8[d]	56±9[d]
	2.5 μg/ml	ND	85±6	ND	ND	27±6[d]	ND
	5.0	91±7	86±10	57±9	29±8[d]	26±5	15±2
	7.5	90±6	80±9	56±12	31±6[d]	31±5[d]	16±3
	10.0	86±8	73±11	55±13	36±7[d]	37±6[d]	19±5
	12.5	89±7	ND	57±10	39±9[d]	ND	17±3
	15.0	88±8	66±8	52±11	45±11[d]	44±6[d]	18±5
Benzoyl peroxide							
	Acetone	61±8	78±9	63±12	20±5	24±7	19±6
	Positive (TPA)	58±7	73±9	61±11	84±11[d]	56±7[d]	90±12[d]
	0.5 μg/ml	56±10	77±8	63±9	21±6	10±3	16±4
	1.0	58±6	76±10	65±10	20±4	10±3	16±6
	2.0	43±18(T)	75±9	61±11	20±6	11±4	19±4
	3.0	34±9(T)	75±13	64±11	20±4	14±5	18±5
	4.0	28±11(T)	72±12	54±11	19±4	15±4	16±5
Hydrogen peroxide							
	Ethanol	88±8	86±8	52±10	11±4	5±2	20±4
	Positive (TPA)	77±14	74±8	48±7	93±10[d]	57±10[d]	59±9[d]
	0.5 μg/ml	82±10	82±9	43±8	12±3	6±2	24±6
	1.0	86±8	89±9	49±8	11±6	6±2	22±6
	2.5	74±10	80±9	40±8	15±4	5±2	22±5
	5.0	29±7(T)	89±11	25±7(T)	14±4	7±4	18±5
	7.5	31±16(T)	80±10	15±4(T)	11±3	6±3	15±4
t-Butyl hydroperoxide							
	Ethanol	74±10	95±14	74±13	19±6	11±3	14±5
	Positive (TPA)	80±12	94±9	76±16	83±10[d]	67±9[d]	77±13[d]
	0.5 μg/ml	78±11	93±13	67±9	14±3	12±4	11±5
	1.0	72±12	81±9	68±9	14±3	15±5	14±3
	2.0	60±13	80±14	46±7(T)	17±5	15±4	18±5[d]
	2.5	46±8(T)	71±11	41±10(T)	16±5	14±3	19±4[d]
	3.0	37±10(T)	59±10(T)	39±12(T)	19±6	17±5[d]	19±5[d]

(*continued*)

TABLE I. Effect of Test Chemical on Metabolic Cooperation (Continued)

Compound	Concentration	Cytotoxicity[a] (laboratory) 1	2	3	Metabolic cooperation[b] (laboratory) 1	2	3
D(+)-tryptophan							
	Water	78±5	66±9	67±13	12±2	3±2	4±2
	Positive (TPA)	76±4	71±6	59±8	84±7[d]	55±10[d]	60±9[d]
	100 μg/ml	77±5	65±7	66±10	14±3	4±2	4±2
	250	78±5	63±9	64±9	18±4[d]	3±1	6±2
	500	78±5	65±10	63±10	19±5[d]	8±3[d]	11±3[d]
	750	77±4	62±7	63±8	26±4[d]	13±3[d]	10±3[d]
	1000	78±5	63±9	61±7	26±5[d]	19±5[d]	14±7[d]
Sodium saccharin							
	Serum-free culture media	70±12	73±10	ND	9±3	4±2	ND
	Positive (TPA)	72±10	68±9	ND	83±9[d]	63±9[d]	ND
	1.0 mg/ml	74±12	75±11	ND	8±3	5±2	ND
	2.0	70±10	70±9	ND	11±4	7±2[d]	ND
	3.0	71±15	70±8	ND	14±4	8±4[d]	ND
	4.0	63±11	61±9	ND	12±3	11±4[d]	ND
	5.0	57±11	57±8	ND	12±5	13±4[d]	ND
Sodium cyclamate							
	Water	78±6	59±7	82±11	20±4	11±3	15±4
	Positive (TPA)	71±12	57±9	79±7	82±10[d]	50±5[d]	72±8[d]
	1.0 mg/ml	73±7	59±10	82±13	17±5	11±4	24±5[d]
	1.5	77±9	55±9	82±11	20±8	12±4	24±5[d]
	2.0	71±11	58±6	80±9	18±3	13±3	24±6[d]
	2.5	73±8	58±5	70±14	20±5	14±3	22±4[d]
	3.0	63±12	58±6	60±15	20±5	14±4	22±4[d]
	5.0	ND	57±7	ND	ND	16±3[d]	ND
Sucrose							
	Serum-free culture media	111±10	79±10	ND	11±3	7±3	ND
	Positive (TPA)	105±14	80±9	ND	89±13[d]	55±6[d]	ND
	5.0 mg/ml	122±12	74±8	ND	13±3	10±3	ND
	10.0	115±10	69±8	ND	19±4[d]	12±3[d]	ND
	15.0	115±11	77±8	ND	17±4[d]	10±3	ND
	20.0	105±20	74±8	ND	22±5[d]	12±3[d]	ND
	25.0	101±18	71±6	ND	22±7[d]	14±3[d]	ND
n-Dodecane							
	Ethanol	69±7	61±8	ND	18±4	11±4	ND
	Positive (TPA)	66±11	58±10	ND	71±11[d]	63±8[d]	ND
	0.1 μg/ml	69±9	61±8	ND	12±5	10±3	ND
	0.5	72±9	64±8	ND	14±6	10±2	ND
	1.0	66±6	62±9	ND	19±5	9±4	ND
	2.5	65±10	58±9	ND	19±5	11±3	ND
	5.0	56±9	60±7	ND	17±5	12±3	ND
1-Phenyldodecane							
	Ethanol	70±10	83±9	ND	10±3	16±6	ND
	Positive (TPA)	69±7	86±11	ND	68±8[d]	85±7[d]	ND
	0.5 μg/ml	69±9	86±9	ND	9±3	12±4	ND
	1.0	69±11	92±8	ND	10±4	16±4	ND
	2.5	74±13	90±9	ND	11±3	16±4	ND
	5.0	72±9	82±9	ND	11±4	17±5	ND
	7.5	57±10	47±13(T)	ND	16±6[d]	17±4	ND

(*continued*)

TABLE I. Effect of Test Chemical on Metabolic Cooperation (Continued)

Compound	Concentration	Cytotoxicity[a] (laboratory) 1	2	3	Metabolic cooperation[b] (laboratory) 1	2	3
Phenol							
	Ethanol	72±10	78±6	ND	13±4	15±4	ND
	Positive (TPA)	65±9	78±8	ND	73±8[d]	68±6[d]	ND
	0.5 μg/ml	64±10	72±7	ND	12±5	16±3	ND
	10.0	64±9	76±9	ND	13±5	12±4	ND
	25.0	67±9	77±9	ND	17±5[d]	15±4[d]	ND
	50.0	71±9	69±8	ND	18±4[d]	16±4	ND
	75.0	64±11	72±8	ND	19±5[d]	17±5	ND
Catechol							
	Ethanol	135±11	115±8	ND	14±4	25±6	ND
	Positive (TPA)	125±15	109±8	ND	142±17[d]	84±5[d]	ND
	0.05 μg/ml	134±10	110±12	ND	15±5	23±5	ND
	0.10	130±14	108±10	ND	19±5	28±5	ND
	0.25	126±13	110±10	ND	29±6[d]	32±5	ND
	0.50	123±9	98±9	ND	37±6[d]	46±8[d]	ND
	0.75	121±12	91±10	ND	43±5[d]	49±5[d]	ND
Pyrogallol							
	Ethanol	61±8	55±5	ND	13±3	9±3	ND
	Positive (TPA)	65±9	58±6	ND	86±10[d]	47±5[d]	ND
	0.25 μg/ml	66±10	59±9	ND	15±3	9±3	ND
	0.5	63±10	57±6	ND	13±5	8±4	ND
	1.0	78±13	53±6	ND	19±4[d]	8±3	ND
	2.0	68±10	54±7	ND	17±3	9±2	ND
	3.0	64±8	56±9	ND	18±5[d]	8±3	ND
2,4-Dinitrofluorobenzene							
	Ethanol	72±7	76±10	49±10	17±5	10±3	13±4
	Positive (TPA)	71±8	68±9	46±7	86±11[d]	60±5[d]	54±7[d]
	0.010 μg/ml	69±9	70±9	44±10	18±4	10±4	12±3
	0.025	64±7	66±7	47±7	16±5	12±3	11±4
	0.050	66±6	61±11	43±8	20±5	10±3	14±4
	0.075	65±9	51±6	36±10	19±2	11±2	12±3
	0.100	62±9	34±6(T)	22±8(T)	21±5	10±3	11±4
Ethyl phenyl propriolate							
	Ethanol	86±11	90±8	ND	10±3	8±3	ND
	Positive (TPA)	79±11	85±11	ND	77±10[d]	70±7[d]	ND
	0.10 μg/ml	82±9	89±9	ND	9±4	10±3	ND
	0.25	84±8	85±9	ND	12±3	14±4[d]	ND
	0.50	84±8	88±6	ND	13±3[d]	12±4[d]	ND
	0.75	72±9	82±6	ND	9±4	15±3[d]	ND
	1.00	80±14	75±8	ND	8±3	15±2[d]	ND

[a]Mean and standard deviation of colonies formed from 100 $6TG^R$ cells plated into each dish for the total of 20 dishes.

[b]Mean and standard deviation of colonies formed from 100 $6TG^R$ cells cocultured with 4×10^{-5} cells per dish for the total of 20 dishes per assay point.

[c]ND = not determined; i.e., the test was not performed for that assay point.

[d]Highly significant difference from solvent control ($P \leq .01$).

[e](T) = Toxic level; i.e., the value is below 70% of the solvent control.

1981, 1982; Welsch and Stedman, 1984; Yotti et al., 1979] as well as with other in vitro studies [Boutwell, 1974; Weinstein et al., 1979; Yuspa et al., 1976]. It has been suggested that phorbol esters act in the V79 system through a receptor mechanism [Hartman and Rosen, 1985], which may account for their activity at low concentra-

tions. The responses with mezerein, which is not a phorbol ester but is a weak complete skin-tumor promoter and a strong second-stage tumor promoter [Slaga et al., 1980], suggest that both first- and second-stage tumor promoters are active in the V79 system. Phorbol was negative in all three laboratories up to 30 ug/ml. Whereas phorbol is routinely used as a negative control in mouse-skin promotion studies, it has been reported to have promotional activity in other organ systems (Table II).

Barbiturates. Two of the three laboratories found both phenobarbital and amobarbital positive, and results from the third laboratory were scored as negative and equivocal, respectively, for phenobarbital and amobarbital (Tables I and II). The overall call was therefore positive for both chemicals. The minimal effective concentration was 100 ug/ml for phenobarbital and 75 ug/ml for amobarbital. Phenobarbital is a liver-tumor promoter [Peraino et al., 1971], but mixed results have been previously reported in the V79 cell system [Elmore et al., 1985; Jone et al., 1985; Trosko et al., 1981; Umeda et al., 1980]. Amobarbital was initially chosen as a nonpromoter based on its failure to induce hepatic carcinogenesis [Diwan et al., 1985; Peraino et al., 1975], although it was found to promote the development of liver foci [Shinozuka et al., 1982]. Amobarbital has not been previously studied in this assay.

Antioxidants. Butylated hydroxyanisole produced a concentration related positive response in two of the three laboratories (Tables I and II). Positive responses were observed at concentrations of 7.5 ug/ml and higher in both laboratories. Butylated hydroxytoluene showed a weak response in laboratories 1 and 2, and it has previously been reported to be positive in V79 cells [Malcolm et al., 1983; Malcolm and Mills, 1985; Trosko et al., 1981, 1982]. Butylated hydroxytoluene, but not butylated hydroxyanisole, has been demonstrated to be a tumor promoter in the lungs [Witschi, 1983; Witschi and Cote, 1976; Witschi and Doherty, 1984]. Butylated hydroxyanisole appears to be an antipromoter in the liver [Imida et al., 1983; Tsuda et al., 1984a, 1984b] although it enhanced forestomach and urinary bladder carcinoma [Tsuda et al., 1984c].

Peroxides. Benzoyl peroxide did not inhibit metabolic cooperation between 0.5 and 4 ug/ml in any of the three laboratories (Tables I and II) although cytotoxicity was apparent in laboratory 1 at 2.0 ug/ml and higher. Hydrogen peroxide was also negative (0.5–7.5 ug/ml) in all three laboratories, with cytotoxicity observed at concentrations of 5.0 and 7.5 ug/ml in laboratories 1 and 3. Statistically significant inhibition of metabolic cooperation by t-butyl hydroperoxide was observed in two laboratories, but only at concentrations (2.0–3.0 ug/ml) that caused greater than 30% toxicity, and therefore, based on the outcome from the third laboratory, the chemical was scored as negative. By contrast to the present results, benzoyl peroxide has been shown to inhibit metabolic cooperation in V79 cells [Slaga et al., 1982] and human epidermal keratinocytes [Lawerence et al., 1984]. All peroxides are tumor promoters in skin [Hoshino et al., Klein-Szanto and Slaga, 1982], and hydrogen peroxide is a promoter in rat duodenum [Ito et al., 1981]. The reason for the discrepancy between our results with benzoyl peroxide and those previously reported [Lawerence et al., 1984; Slaga et al., 1982] is unknown, although the instability of such chemicals under our test conditions may be a contributing factor; t-Butyl hydroperoxide effects in vitro have not been previously reported.

Bladder-tumor promoters. D(+)-tryptophan was positive in all laboratories in which it was tested, with the minimum active concentration 250 ug/ml (Tables

I and II). Sodium cyclamate gave disparate results, with one laboratory finding it negative, one equivocal, and one positive but without a dose response (Tables I and II). Sodium cyclamate was given an overall call of equivocal. Sodium saccharin was only positive in one of two laboratories. In the cases where cyclamate and saccharin were positive, it was at levels of 1 and 2 mg/ml, respectively. These chemicals previously have been reported to inhibit metabolic cooperation in V79 cells [Malcolm et al., 1983; Malcolm and Mills, 1985; Trosko et al., 1980; Umeda et al., 1980]. In in vivo studies, saccharin and cyclamate have been found to promote tumors in the bladders of rats (Table II).

The activity of sucrose in the metabolic cooperation assay was surprising. Sucrose was originally chosen as a nonpromoter for comparison with sodium saccharin and sodium cyclamate; sucrose was similar to sodium saccharin and sodium cyclamate in that it inhibited metabolic cooperation at high concentrations. The need for high concentrations for positive responses raises the question of osmolarity effects and other nonspecific effects of these chemicals under the test conditions. A recent paper has reported that sucrose increased the number of γ-glutamyltranspeptidase-positive foci in rat livers [Hei and Sudilovsky, 1985]; another paper has suggested that sucrose may act as a promoter to mammary carcinogenesis [Klurfield et al., 1984]. However, whether the in vivo responses are a result of caloric imbalances or promotional mechanisms such as gap-junction alteration is not known at present, and the possibility exists that V79 cells detected sucrose as positive but for a reason different from its action in vivo.

Alkanes. In mouse skin, n-dodecane and 1-phenyldodecane are tumor promoters [Bingham and Falk, 1969; Bingham and Nord, 1977; Horton et al., 1976], but they were scored as overall negative from the results obtained by two laboratories (Tables I and II). For n-dodecane, the maximum concentration achievable was limited by solubility, and significant toxicity was not achieved in these studies. 1-Phenyldodecane concentrations were also limited by solubility, but toxicity was apparent at the highest concentration test (Table I). These alkanes have been reported to enhance V79 cell mutagenesis by a comutagenic mechanism [Lankas et al., 1978].

Phenol and metabolites. Phenol was detected as positive at 25 ug/ml in one of the two laboratories, whereas catechol, a metabolite of phenol, was positive in both laboratories, with a lowest effective concentration of 0.25 ug/ml. Pyrogallol, tested at the concentration range of 0.25 to 3 ug/ml, was positive in one laboratory and negative in the other. The overall calls were phenol, equivocal, catechol, positive, and pyrogallol equivocal (Table II). Phenol has been reported as a weak mouse-skin-tumor promoter [Barauskaite, 1983; Boutwell and Bosch, 1959; Van Duuren, 1976], whereas catechol and pyrogallol are cocarcinogenic but not tumor promoting [Boutwell and Bosch, 1959; Van Duuren et al., 1978]. Previous metabolic cooperation studies showed phenol to be negative and catechol to be positive [Chen et al., 1984; Malcolm et al., 1983, 1985a, 1985b; Malcolm and Mills, 1983]. Pyrogallol has not been previously tested in this system.

Miscellaneous chemicals. The mouse-skin-tumor promoter 2,4-dinitrofluorobenzene [Bock et al., 1969] was found to be negative (0.01–0.10 ug/ml) in all laboratories even at cytotoxic concentrations (0.1 ug/ml) (Table I). This chemical was previously shown to inhibit metabolic cooperation in V79 cells [Warren et al., 1982]. Ethyl phenyl propriolate was chosen for this study because it is a hyperplastic agent with weak promoting potential in mouse skin [Raick, 1974]. This chemical, not

TABLE II. Response of Chemicals in V79 Assay

Compound	Laboratory response 1	2	3	Overall response[a]	Concentration range μg/ml	LEC[b] μg/ml	Previously reported data V79	In vivo
TPA	+[c]	+	+	+	0.05–1.0	(0.05)	+ [Malcolm et al., 1983; Welsch and Stedman, 1984; Yotti et al., 1979]	+ Skin [Boutwell, 1974; Slaga et al., 1978]
4-0-methyl TPA	+	+	+	+	0.1–2.0	(0.1)	+ [Trosko et al., 1982]	+ Skin [Boutwell, 1974; Hecker et al., 1982; Slaga et al., 1978]
Phorbol-12,12-dibutyrate	+	+	+	+	1.0–30.0	(1.0)	+ [Trosko et al., 1982]	+ Skin [Boutwell, 1974; Slaga et al., 1978]
Phorbol	−[d]	−	−	−	1.0–30.0	(−)[e]	− [Scott et al., 1985]	− Skin [Boutwell, 1974; Slaga et al., 1978] ± Mammary [Shellabarger et al., 1979; Armuth and Berenblum, 1974] + Leukemia [Armuth and Berenblum, 1974] + Liver [Armuth and Berenblum, 1972] + Lung [Armuth and Berenblum, 1972]
Mezerein	+	+	+	+	0.1–2.0	(0.1)	+ [Trosko et al., 1982]	+ Skin [Slaga et al., 1978; 1980]
Phenobarbital	+	+	−	+	50–300	(100)	+/− [Jone et al., 1985; Umeda et al., 1980]	+ Liver [Peraino et al., 1971, 1975, 1977] + Liver Foci [Tsuda et al., 1984a, 1984c]
Amobarbital	+	+	±[f]	+	50–150	(75)	NA[g]	− Liver [Diwan; et al., 1985; Peraino et al., 1975] + Liver Foci [Shinozuka et al., 1985]
Butylated hydroxytoluene	+	+	−	+	0.25–5.0	(2.5)	+ [Malcolm and Mills, 1985; Trosko et al., 1982]	+ Lung [Witschi and Doherty, 1985] + Liver [Peraino et al., 1977] − Liver Foci [Imida et al., 1983; Tsuda et al., 1984a] + Bladder [Imida et al., 1983] − Colon [Shirai et al., 1985]
Butylated hydroxyanisole	+	+	−	+	2.5–15.0	(2.5)	NA	− Liver Foci [Imida et al., 1983; Tsuda et al., 1984a, 1984c] + Forestomach [Tsuda et al., 1984c] + Bladder [Imida et al., 1983; Tsuda et al., 1984c] − Lung [Witschi and Doherty, 1985]

								+ Kidney [Tsuda et al., 1984b] − Colon [Shirai et al., 1985]
Benzoyl peroxide	−	−	−	−	0.5–4.0	(–)	+ [Slaga et al., 1982]	+ Skin [Slaga et al.,1982; Klein-Szanto and Slaga, 1982]
Hydrogen peroxide	−	−	−	−	0.5–7.5	(–)	NA	+ Skin [Klein-Szanto and Slaga, 1982] + Duodenum [Ito et al., 1981]
t-Butyl hydroperoxide	−	±	±	−	0.5–3.0	(–)	NA	+ Skin [Hoshino et al., 1970]
D(+)tryptophan	+	+	+	+	100–1,000	(250)	NA	+ Bladder [Cohen et al., 1979; Dunning et al., 1950; Radomski and MacDonald, 1977] + Liver [Dunning et al., 1950]
Sodium saccharin	−	+	ND[h]	E[a]	1,000–5,000	(2,000)	+ [Trosko et al., 1980]	+ Bladder [Hicks et al., 1975; Cohen et al., 1984] − Liver Foci [Tatematsu et al., 1983]
Sodium cyclamate	−	±	+	E	1,000–5,000	(1,000)	+ [Malcolm and Mills, 1983	+ Bladder [Hicks et al., 1975]
Sucrose	+	+	ND	+	5,000–25,000	(10,000)	NA	+ Liver Foci [Hei and Sudilovsky, 1985] + Mammary [Klurfield et al., 1984]
n-Dodecane	−	−	ND	−	0.1–5.0	(–)	NA	+ Skin [Bingham and Falk, 1969; Bingham and Nord, 1977; Lankas et al., 1978]
l-Phenyldodecane	±	−	ND	−	0.5–7.5	(–)	NA	+ Skin [Sice, 1966]
Phenol	+	−	ND	E	5–75	(25)	− [Malcolm and Mills, 1983; Malcolm et al., 1983, 1985b]	+ Skin [Boutwell and Bosch, 1959; Van Duuren et al., 1978]
Catechol	+	+	ND	+	0.05–0.75	(0.25)	+ [Malcolm and Mills, 1983; Malcolm et al., 1985]	− Skin [Boutwell and Bosch, 1959; Van Duuren et al., 1978]
Pyrogallol	+	−	ND	E	0.25–3.0	(1.0)	NA	−Skin [Boutwell and Bosch, 1959; Van Duuren et al., 1978]
2,4-Dinitro-fluorobenzene	−	−	−	−	0.01–0.10	(–)	+ [Trosko et al., 1982; Warren et al., 1982]	+ Skin [Block et al., 1969]
Ethyl phenyl propriolate	±	+	ND	+	0.1–1.0	(0.25)	NA	+ Skin [Raick, 1974]

[a]At least two laboratories must have the same score to receive the rating; if only two laboratories tested the chemical and got conflicting results, the overall score is E, or equivocal.
[b]LEC = lowest effective concentration where statistically significant inhibition of metabolic cooperation took place.
[c]Positive, two concentration levels were significantly ($P \leq .01$) greater than solvent control, with 70% or greater cloning efficiency in the cytotoxicity assay.
[d]Negative; there was no significant difference from the solvent-control value at any concentration tested.
[e]The dash in parenthesis indicates that no LEC was observed.
[f]Positive/negative or equivocal for an individual laboratory because of cytotoxicity at concentrations that inhibited metabolic cooperation or because inhibition was statistically significant at only a single concentration.
[g]NA = no literature available.
[h]ND = not determined.

previously studied in the V79 system, elicited a positive response in laboratory 2 and an equivocal response in laboratory 1.

Interlaboratory comparisons of solvent and positive controls. A solvent and a positive control (TPA, 4 ng/ml) were included in each assay performed. Table I shows that the solvent-control cloning efficiency mean of 6-TG^r cells alone ranged from 70.7 to 82.2%, whereas the recovery of 6-TG^r mutants in control cocultures with 6-TG^s ranged from 11.3 to 15.4% For the TPA positive controls, the cloning efficiencies of 6-TG^r cells alone and 6-TG^r cocultivated with 6-TG^s were also in close agreement in all laboratories, with the possible exception of the latter in laboratory 2 (Table I). Although interlaboratory agreement appears good when mean values for cloning efficiency for the entire study are examined, the range of individual-experiment means within a laboratory was quite large. For example, the number of background colonies per dish of 6-TG^r cells cocultivated with 6-TG^s in laboratory 1 ranged from a low of 9 ± 3, when phenobarbitol was tested, to a high of 38 ± 5 for TPA. If these control values had been obtained in the same experiment, they would have been significantly different, even though they were both solvent (ethanol) controls. Despite such variation between experiments, considerably smaller differences in cloning-efficiency values within an experiment were considered biologically different, based on statistical analysis. A subsequent study revealed that when two independent sets of solvent controls are run during an experiment, they usually differed by less than 10%, only occasionally significantly differed at the $P < .05$ level, and never significantly differed at the $P < .01$ level, by Dunnett's test (unpublished results). Therefore, significant differences at the $P < 0.01$ value were considered to indicate biological difference. A few TPA positive controls also varied considerably from the study mean, and it is recommended for future studies that upper and lower limits on negative and positive controls be set for the acceptability of an experiment.

Agreement among laboratories for test chemicals and comparison of V79 results to in vivo data. The laboratories agreed completely on 9 of the 15 chemicals tested in all three laboratories (Table II). Of the 23 chemicals tested in laboratories 1 and 2, there was agreement on 16. To facilitate comparisons with in vivo tumor-promoting activities, overall calls were assigned to the V79 results, based on individual laboratory responses (Table II). In cases where all three or both laboratories agreed, the overall call equaled the individual calls. When the three laboratories disagreed, the overall call was based on the majority of individual responses, unless the majority call was equivocal. When only two laboratories tested a chemical and those two disagreed, the overall call was equivocal (E). When an equivocal call (±) was obtained for each individual laboratory, the responses from the other laboratories were used to determine the overall call. In the present study, repeat experiments in an individual laboratory were not built into the protocol because it was believed that individual calls from the separate laboratories would have the same effect. However, it now appears that repeat experiments may have resolved some of the overall equivocal calls. More-recent studies include repeat experiments [Langenbach et al., 1988].

The difficulty in assigning overall calls was not limited to V79 results; discrepancies in the in vivo responses in the various systems were also encountered. The extreme examples of this were phorbol and butylated hydroxyanisole, as discussed above. Such discrepancies in in vivo responses may be due to organ and/or

species differences in responses. Furthermore, it should be noted that certain of these in vivo systems used as standards for comparison with V79 results are also in validation stages, and presently may not be suitable or properly validated to call a chemical a "promoter."

Nevertheless, all chemicals tested in vivo were considered positive except catechol and pyrogallol, which are negative for promotion in vivo but have been tested only in the mouse-skin system. Catechol received an "overall" positive score in the V79 system and was the only false positive. Nine of the 15 positive chemicals tested in all three laboratories were overall positive in the V79 system, which results in 60% agreement with in vivo responses. For the 21 positive chemicals tested in two laboratories, 11 were overall positive in the V79 system for 52% agreement. Three of the in vivo positive chemicals (cyclamate, saccharin, and phenol) received equivocal overall scores in the V79 system and were not considered positive or negative for this comparison.

Without an adequate number of nonpromoters tested in this study, it is impossible to determine the specificity of the V79 assay system for identifying promoters. However, the data do indicate that the system lacks sensitivity for a wide variety of tumor promoters. Although in the present study the metabolic cooperation assay was quite sensitive for detecting the phorbol esters, it was insensitive to the peroxides and alkanes, which are also tumor promoters in vivo. Other known promoters showed varying degrees of responses, which may be due to their differing modes of action.

On evaluating the data, several characteristics of this assay may limit its applicability for screening broad classes of chemicals for potential activity as tumor promoters. Many chemicals show organ-specific effects, and it may be unrealistic to expect any individual cell type (in vivo or in vitro) to detect all such chemicals. Furthermore, the requirements for metabolic activation for tumor promoters are virtually unknown, but it is reasonable to assume that certain classes of chemicals will require metabolism (i.e., phenol and metabolites, Table I). The V79 cells used in this metabolic-cooperation assay have very low intrinsic metabolic activation potential and would not respond to chemicals requiring metabolism. Compounds that alter the pH of the medium or change the osmolarity may also have an effect in the system that would not mimic in vivo exposure. Toxic chemicals may also lead to results in the V79 system that would not be observed in vivo because of pharmacodynamics. In the present study, 70% cell survival in the cytotoxicity assay was required to call a chemical positive; however, although this figure is used by others [Malcolm et al., 1985a; Trosko et al., 1981], it is subjective and does not take into account differences resulting from mass culture effects. Only a 100 6-TG^r are used in the cytotoxicity assay, whereas the metabolic cooperation assay also includes 4×10^5 6-TG^s cells. Ideally, toxicity should be determined in mass culture, and methods employing V79 mutants lacking gap junctions are being considered.

Finally, the proper validation of any in vitro assay for tumor promoters requires the use of adequately tested nonpromoting chemicals. As stated earlier and as shown in Table II for the limited number of chemicals studied the identification of such nonpromoting chemicals is difficult because of organ, species, and test-system differences. To facilitate test-system development, a better mechanistic understanding of tumor promotion in vivo is needed, and the role of gap-junctional communication in this process needs to be elucidated to fully evaluate the usefulness of the V79 assay.

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