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EVALUATION OF THE V79 CELL METABOLIC CO-OPERATION ASSAY AS A SCREEN *IN VITRO* FOR DEVELOPMENTAL TOXICANTS*

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Abstract—Inhibition of intercellular communication is proposed to be one of several possible mechanisms of teratogenesis. 38 coded compounds were tested for their effect on intercellular communication in the V79 cell metabolic co-operation assay. Test chemicals were selected from a list of 47 agents recommended for the evaluation of assays *in vitro* for developmental toxicants. In addition to testing the effects of chemicals on intercellular communication, a separate cytotoxicity assay determined the concentration of each chemical that inhibited clonal expansion of V79 cells. Seven of the 29 designated teratogens were positive for inhibition of intercellular communication in the V79 assay. Additionally, four teratogens and one non-teratogen inhibited intercellular communication at only a single concentration or at cytotoxic concentrations and were scored as equivocal. Therefore, the sensitivity of the V79 assay for teratogens was 24% (seven of 29 teratogens tested positive), or 38% if the four equivocal chemicals are considered positive. None of the nine non-teratogens unequivocally inhibited intercellular communication, resulting in a specificity of 100%, which decreased to 89% when the single equivocal score was considered positive. The overall accuracy for correctly identifying teratogens and non-teratogens was 42% when equivocal chemicals were considered negative, and 50% if they were considered positive in the V79 assay. The results demonstrate that despite relatively low accuracy regarding a diverse group of developmental toxicants, chemicals that did inhibit intercellular communication under the present conditions had a high probability of being a teratogen. The low accuracy reported here contrasts with earlier reports on the assay and possible reasons for this are discussed.

INTRODUCTION

Gap junctions are one of several specialized connections that form among adjacent cells (Larsen and Wert, 1988). The individual connexons that comprise the gap junctions lie within the cell membrane and allow electrical coupling and transfer of small molecules between cells (Spray and Bennett, 1985; Zampighi, 1987). The transfer of chemical signals by way of gap junctions has led to the concept that these structures provide for direct intercellular communication (Loewenstein, 1966 and 1979). Loewenstein (1968) first proposed that intercellular communication among contiguous cells is essential for proper orchestration of morphogenesis. Since then, absence of communication, selective communication, and free communication among cells have been observed in various organisms at various stages of development (Cardellini *et al.*, 1988; Fraser *et al.*, 1987; Guthrie, 1987; Warner *et al.*, 1984). The apparent essential role of intercellular communication in normal development has fostered the hypothesis that inhibition of intercellular communication is a mechanism of abnormal development or teratogenesis (Trosko *et al.*, 1982). Although some evidence and considerable discussion have been put forward to support this hypothesis (Elmore *et al.*, 1987; Loch-Caruso, 1990; Loch-Caruso and Trosko, 1985; Trosko and Chang, 1984; Welsch, 1987; Welsch and Stedman, 1984; Welsch *et al.*, 1987), it has yet to be established if inhibition of intercellular communication could serve as the basis of an assay *in vitro* for developmental toxicants.

Elmore *et al.* (1987) compiled a list of 103 'teratogenic and reproductive toxicants' that have been

*Mention of products or company names does not constitute endorsement by the National Institute for Occupational Safety and Health.

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Abbreviations: DMSO = dimethylsulphoxide; HEPM = human embryonic palatal mesenchyme; MOT = mouse ovarian tumour; TPA = 12-*O*-tetradecanoylphorbol-13-acetate.

tested for their effect on intercellular communication in the V79 cell metabolic co-operation assay. On the basis of a non-standardized scoring procedure dependent on outcomes from various laboratories, Elmore *et al.* (1987) reported that the V79 assay had a sensitivity for teratogens of 80%, a specificity for correctly identifying non-teratogens of 61%, and an overall accuracy of 76%. This level of accuracy suggests that the V79 assay has the potential to serve as a screen *in vitro* for developmental toxicants or teratogens. However, it has been noted that, despite an apparent requirement of intercellular communication for normal development, the V79 assay may have limited application as a screen for developmental toxicants (Trosko *et al.*, 1982). Possible limitations stem from the species-, organ- and cell-specificity of inhibitors of intercellular communication (Kihara *et al.*, 1990; Klaunig and Ruch, 1987; Saez *et al.*, 1987), the lack of metabolic activation systems in V79 cells (Trosko *et al.*, 1982) and the use of a single endpoint to predict developmental toxicity. Despite these limitations, the reported high sensitivity of the V79 assay for teratogenic compounds (Elmore *et al.*, 1987) warrants further assessment of the assay as a screen or pre-screen for developmental toxicants that could be used alone or as part of a test battery.

The purpose of the present study was to conduct a preliminary evaluation of the V79 cell metabolic co-operation assay as a possible screen for developmental toxicants. The present study is described as preliminary because, owing to financial limitations, each coded chemical was tested only once. The 38 chemicals tested were selected from a list of 47 agents recommended for development of an assay *in vitro* (Smith *et al.*, 1983).

MATERIALS AND METHODS

Chemicals. Test chemicals listed in Table 1, supplied through the Radian Corporation (Austin, TX, USA), were coded and tested blind. Chemicals were dissolved in ethanol, acetone, dimethylsulphoxide (DMSO) or serum-free culture medium (modified Eagle's minimal essential medium; Gibco, Grand Island, NY, USA) as noted in Table 1. The volume of solvent added to each culture dish did not exceed 1% of the medium volume with the exception of culture medium, which was permitted to increase to 10% of the medium in the dish. Volume of medium in culture dishes was adjusted accordingly to maintain a total volume of 5 ml.

V79 cells. The $6TG^s$ cells were derived from Chinese hamster lung fibroblasts (Ford and Yerganian, 1958), and the $6TG^r$ cells were derived from X-ray-irradiated wild-type $6TG^s$ cells (Yotti *et al.*, 1979). Both lines were kindly provided by Dr J. E. Trosko of Michigan State University, East Lansing, MI, USA. Cells were maintained in culture medium containing 3% foetal bovine serum (Hyclone, Logan, UT, USA). The culture medium was modified Eagle's minimal essential medium (MEM) with Earle's salts containing a 50% increase in all vitamins and essential amino acids, and 1 mM-pyruvate. Cultures were incubated at 37°C in a 5% CO_2/air atmosphere. The cells were passaged by trypsinization two to three times a week, and used within 2 months of thawing. Stock cultures were cryopreserved in Eagle's MEM containing 5% serum and 5% DMSO.

Cytotoxicity assays. Test chemicals were initially evaluated in a preliminary cytotoxicity assay to determine the concentration ranges that produced little or no cytotoxicity. In this procedure, cells were plated at 4×10^5 $6TG^r$ cells per 60-mm plastic culture dish. After a 4-hr attachment period, test chemicals were added to dishes at approximately one-half log dilutions ranging from 0.001 to 10,000 μg/ml, depending on estimated toxicity of the chemicals (Steele *et al.*, 1988). Following a 3-day growth period, cells were evaluated microscopically to determine cytostasis or cytotoxicity. From this evaluation, five concentrations ranging from non-toxic to cytostatic were selected and evaluated in a cloning efficiency assay.

Cloning efficiency assay. Two to three independent cloning efficiency assays were performed to establish a concentration range for testing chemicals in the metabolic co-operation assay. The preliminary assays were performed to define a concentration range of approximately one order of magnitude that extended from non-toxic concentrations to slightly toxic concentrations. Ideally, the three lowest concentrations would result in 100% cloning efficiency, and the highest concentration would cause a significant reduction in cloning efficiency. The purpose of identifying a concentration that was cytotoxic was to determine the maximum non-toxic concentration for final testing in the metabolic co-operation assay. In the preliminary cloning assays, 100 $6TG^r$ cells were seeded in 60-mm dishes (four dishes per assay point). After a 4-hr attachment period, dishes were treated with the appropriate concentration of test chemical or vehicle alone. Following a 3-day growth period, culture medium containing test chemical was replaced with fresh culture medium, and the incubation continued. After a 6- to 7-day growth period, cultures were rinsed with phosphate buffered saline and stained and fixed with 1% crystal violet (1 g dissolved in 10 ml ethanol and diluted to 100 ml with water). Colonies were counted and compared with untreated cultures.

V79 metabolic co-operation assay. The V79 metabolic co-operation assay, as modified from Yotti *et al.* (1979) by Bohrman *et al.* (1988), is dependent on the transfer, by way of gap junctions, of the toxic phosphorylated metabolite of 6TG from wild-type $6TG^s$ cells to $6TG^r$ cells. The $6TG^r$ cells are unable to metabolize 6TG to a toxic substrate, and survive to form colonies when intercellular communication is inhibited and the transfer of phosphorylated 6TG from adjacent $6TG^s$ cells is blocked. The metabolic co-operation between the sensitive and resistant V79

cells was assessed using the methodology described in detail by Bohrman *et al.* (1988). In brief, 100 $6TG^r$ cells were co-cultured with 4×10^5 $6TG^s$ cells in 5 ml of medium in 60-mm dishes. Five concentrations of each test chemical were selected on the basis of the outcome in the preliminary cytotoxicity assays. Test chemicals were added 4 hr after plating $6TG^r$ and $6TG^s$ cells, and 6TG was added 15 min later to a final concentration of 10 μg/ml. After 3 days, medium was replaced with fresh medium containing only 6TG but without the test chemical; 3–4 days later, cells were washed with phosphate buffered saline and fixed and stained with 1% crystal violet.

A final cloning efficiency assay was performed concomitantly with the metabolic co-operation assay. Each cloning assay and metabolic co-operation assay included a solvent control and 4 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a positive control. 12 dishes per concentration were tested in each assay.

Selection of chemicals. 38 of the 47 agents listed by Smith *et al.* (1983) were tested. Ethyl nitrosourea, vinyl chloride and hypothermia were not tested for the reasons noted by Steel *et al.* (1988). Carbon tetrachloride and trichloroethylene are too volatile to be tested properly in unsealed culture dishes requiring a 3-day exposure period. Testing of hexahydrophthalamide was not completed because of technical problems. Saccharin, sodium cyclamate and ethanol were not tested because we had previously tested them and found them not to be cytotoxic nor to be effective inhibitors of metabolic co-operation at concentrations of less than 1 mg/ml (Bohrman *et al.*, 1988). One mg/ml was the maximum test concentration, except for ethylene thiourea, which was inadvertently tested at 1.5 mg/ml. A concentration limitation is essential to avoid non-specific effects due to changes in osmolarity or extracellular pH, as well as to prevent exposures to concentrations that would not be achievable *in vivo*. Theoretically, an upper limit of 1 mM may be appropriate (Faustman, 1988; Pratt and Willis, 1985); however, in practice such a limit may be difficult to adhere to. For example, in the present investigation compounds were tested blind without previous knowledge of molecular weights; a similar situation would arise in the testing of unknown compounds. As an alternative to 1 mM, we limited test concentrations to 1 mg/ml. For presentation, concentration–response data are expressed as mg/ml (Table 1) and summary data as μM (Table 2).

Statistical analysis. For both the metabolic co-operation assay and the final cloning efficiency assay, Dunnett's test ($P < 0.01$) was used to compare the number of colonies in cultures treated with the test compounds with control cultures treated only with solvent. The level of $P < 0.01$ was considered statistically significant because it had previously been determined that groups of identically treated cultures were too frequently found to be significantly different at the $P < 0.05$ level (Bohrman *et al.*, 1988).

Criteria for summary scores. The results of each chemical assay were reduced to a single summary score for comparison with developmental toxicity of the chemicals *in vivo*. The summary scores separated the chemicals into three categories: chemicals were given a positive (+) score if they significantly inhibited metabolic co-operation at at least two non-cytotoxic concentrations; chemicals were given a negative (−) score if they did not significantly inhibit metabolic co-operation at any concentration; chemicals were given an equivocal (±) score if they inhibited intercellular communication at less than two non-cytotoxic concentrations.

RESULTS

Inhibition of intercellular communication

Of the 38 compounds tested in the V79 assay, only seven inhibited intercellular communication at two non-cytotoxic concentrations (Tables 1 and 2). Of these seven, five have previously been tested in the V79 assay. Four of the five were previously reported to inhibit intercellular communication: these were cyclophosphamide (Elmore *et al.*, 1987), mirex (Tsushimoto *et al.*, 1982), all-*trans*-retinoic acid (Davidson *et al.*, 1985) and trichlorophenoxy-acetate (Rubinstein *et al.*, 1984). Cytochalasin D was a potent inhibitor of intercellular communication in the present study, but was previously reported to be negative in the V79 assay (Elmore *et al.*, 1987). The two positive chemicals not previously tested were 13-*cis*-retinoic acid, and sodium arsenate. However, 13-*cis*-retinoic acid has been shown to inhibit dye-coupling in limb bud mesenchyme cells (Welsch *et al.*, 1987) and in $10T\frac{1}{2}$ cells (Mehta *et al.*, 1989).

Testosterone propionate, coumarin, diazepam, dilantin and doxylamine succinate were found to inhibit intercellular communication, but only at a single concentration or concentrations that were also cytotoxic. Testosterone propionate inhibited intercellular communication at double the concentration that was cytotoxic, and gave no indication of blocking intercellular communication at non-toxic concentrations. Testosterone has been reported to inhibit dye coupling in JTC-30 and JTC-23 carcinoma cell lines but not in human fibroblasts (Kihara *et al.*, 1990). Diazepam and coumarin both caused a non-significant increase in $6TG^r$ cells at non-cytotoxic concentrations, and a significant increase in $6TG^r$ cells at the lowest significantly cytotoxic concentration. Dilantin and doxylamine succinate both inhibited metabolic co-operation at a single non-cytotoxic concentration. Diazepam (Trosko *et al.*, 1982) and dilantin (Welsch and Stedman, 1984) have previously been reported to inhibit metabolic co-operation in V79 cells. Although a number of compounds required concentrations greater than 1 mM to demonstrate toxicity, coumarin was the only compound for which more than 1 mM (1.026 mM) was needed to inhibit intercellular communication. This

Table 1. Cytotoxicity and metabolic co-operation in V79 cells

Compound* concentration	Number of colonies: Cytotoxicity† assay	Number of colonies: Metabolic co-operation† assay
Acetazolamide 59–66–5		
DMSO‡	61 ± 9	12 ± 2
TPA§	69 ± 9	72 ± 8*
250¶	64 ± 7	17 ± 3
400	57 ± 9	16 ± 4
550	53 ± 5	13 ± 4
700	47 ± 10*	14 ± 3
850	45 ± 7*	9 ± 4
Acetylsalicylic acid 50–78–2		
ETOH	123 ± 6	18 ± 6
TPA	122 ± 7	114 ± 9*
50	119 ± 10	18 ± 3
100	110 ± 13	19 ± 4
150	113 ± 10	16 ± 4
200	101 ± 8*	19 ± 7
250	80 ± 12*	19 ± 3
Amaranth 915–67–3		
Medium	104 ± 10	5 ± 2
TPA	98 ± 9	86 ± 10*
25	104 ± 10	6 ± 2
50	99 ± 7	6 ± 3
100	84 ± 7*	4 ± 3
150	76 ± 9*	5 ± 3
200	64 ± 7*	4 ± 2
6-Amino nicotinamide 329–89–5		
DMSO	96 ± 9	22 ± 8
TPA	91 ± 10	73 ± 9*
0.1	93 ± 12	18 ± 4
0.2	95 ± 9	14 ± 3
0.4	90 ± 13	15 ± 3
0.6	81 ± 14	13 ± 2
0.8	75 ± 9*	10 ± 4
Caffeine 58–08–2		
Medium	106 ± 13	7 ± 3
TPA	92 ± 11	76 ± 5*
50	106 ± 8	8 ± 3
100	99 ± 12	10 ± 4
200	97 ± 13	11 ± 4
300	80 ± 9*	9 ± 2
400	64 ± 13*	7 ± 3
Chlorambucil 305–03–3		
ETOH	90 ± 11	5 ± 2
TPA	78 ± 10	68 ± 5*
0.125	87 ± 13	5 ± 2
0.250	86 ± 10	7 ± 3
0.500	75 ± 9	6 ± 2
1.000	75 ± 11	6 ± 3
1.500	65 ± 7*	7 ± 3
Coumarin 91–64–5		
ETOH	121 ± 10	16 ± 5
TPA	122 ± 8	108 ± 11*
25	124 ± 9	17 ± 5
50	116 ± 10	20 ± 5
100	108 ± 13	23 ± 3
150	93 ± 10*	28 ± 8*
200	74 ± 13*	28 ± 10*
Cyclophosphamide 50–18–0		
ETOH	81 ± 7	26 ± 3
TPA	74 ± 6	95 ± 10*
50	81 ± 9	32 ± 5
100	80 ± 10	33 ± 4
150	83 ± 7	42 ± 7*
200	75 ± 9	46 ± 6*
250	78 ± 8	51 ± 6*
Cytochalasin D 22144–77–0		
DMSO	86 ± 11	27 ± 4
TPA	86 ± 8	84 ± 8*
0.01	90 ± 13	35 ± 6
0.025	88 ± 10	48 ± 8*
0.05	85 ± 7	68 ± 8*
0.1	75 ± 12	105 ± 10*
0.15	64 ± 7*	115 ± 10*
Dexamethasone 50–02–2		
DMSO	68 ± 6	14 ± 4
TPA	65 ± 7	76 ± 9*
12.5	67 ± 7	10 ± 4
25.0	65 ± 5	9 ± 4
50.0	55 ± 9*	8 ± 2
100.0	41 ± 6*	4 ± 2
150.0	31 ± 5*	4 ± 3
Diazepam 439–14–5		
DMSO	79 ± 15	14 ± 4
TPA	80 ± 12	61 ± 6*
2.5	74 ± 10	16 ± 4
5	70 ± 12	17 ± 4
10	69 ± 8	17 ± 5
20	64 ± 11*	24 ± 6*
30	55 ± 8*	18 ± 3
Diethylstilboestrol 56–53–1		
ETOH	83 ± 9	17 ± 5
TPA	88 ± 7	83 ± 7*
0.1	83 ± 9	17 ± 4
0.2	87 ± 4	17 ± 5
0.4	86 ± 9	17 ± 3
0.6	81 ± 8	21 ± 6
0.8	77 ± 11	20 ± 4
Dilantin 57–41–0		
DMSO	77 ± 9	18 ± 3
TPA	76 ± 8	87 ± 9*
25	71 ± 8	23 ± 4*
50	55 ± 5*	15 ± 3
75	36 ± 10*	15 ± 4
100	19 ± 6*	15 ± 3
150	20 ± 6*	15 ± 4
Diphenhydramine HCl 147–24–0		
Medium	105 ± 8	5 ± 2
TPA	100 ± 6	81 ± 13*
8	98 ± 6	8 ± 3
12	97 ± 7	10 ± 3
16	91 ± 11*	11 ± 3
20	86 ± 10*	8 ± 3
24	62 ± 9*	6 ± 3
Doxylamine succinate 562–10–7		
Medium	80 ± 12	6 ± 2
TPA	71 ± 5	66 ± 5*
20	80 ± 9	7 ± 3
40	80 ± 6	8 ± 3
80	75 ± 9	12 ± 4*
120	66 ± 8*	13 ± 3*
160	42 ± 9*	16 ± 5*
EM-12 26581–81–7		
DMSO	106 ± 9	18 ± 6
TPA	112 ± 7	120 ± 6*
100	103 ± 11	18 ± 5
200	105 ± 15	16 ± 4
300	103 ± 10	16 ± 6
400	104 ± 12	18 ± 4
500	92 ± 14	20 ± 4
Ethylene thiourea 96–45–7		
DMSO	72 ± 9	13 ± 3
TPA	79 ± 11	66 ± 7*
100	80 ± 8	12 ± 4
250	79 ± 9	12 ± 4
500	67 ± 5	15 ± 4
1000	63 ± 8	15 ± 4
1500	52 ± 3*	18 ± 5
5-Fluorouracil 51–21–8		
ETOH	86 ± 6	25 ± 5
TPA	81 ± 9	95 ± 8*
0.1	87 ± 7	27 ± 6
0.2	88 ± 6	21 ± 4
0.3	72 ± 4*	14 ± 3
0.4	62 ± 8*	11 ± 5
0.5	38 ± 11*	1 ± 1
Formaldehyde 50–00–0		
Medium	94 ± 12	6 ± 2
TPA	84 ± 9	80 ± 7*
0.5	90 ± 12	7 ± 2
1.0	85 ± 7	4 ± 2
2.0	80 ± 7	7 ± 2
3.0	65 ± 5*	8 ± 4
4.0	34 ± 10*	8 ± 3
Hydroxyurea 127–07–1		
Medium	84 ± 12	5 ± 2
TPA	79 ± 7	78 ± 6*
2	69 ± 9*	7 ± 3
4	54 ± 12*	8 ± 3
6	33 ± 6*	7 ± 2
8	26 ± 7*	4 ± 3
10	6 ± 5*	7 ± 3

[*contd*]

Table 1—*contd*

Compound* concentration	Number of colonies: Cytotoxicity† assay	Number of colonies: Metabolic co-operation† assay
Isoniazid 54–85–3		
Medium	81 ± 13	6 ± 3
TPA	76 ± 8	95 ± 13*
25	79 ± 12	11 ± 4
50	77 ± 10	10 ± 3
75	70 ± 9	15 ± 5
100	68 ± 11	15 ± 4
125	57 ± 13*	16 ± 4
Meprobamate 57–53–4		
Medium	83 ± 9	5 ± 2
TPA	81 ± 6	61 ± 2*
200	82 ± 7	5 ± 2
400	81 ± 7	4 ± 2
600	77 ± 7	5 ± 2
800	71 ± 7*	4 ± 2
1000	46 ± 9*	3 ± 2
Methotrexate 59–05–2		
DMSO	80 ± 10	11 ± 4
TPA	85 ± 14	68 ± 5*
0.01	76 ± 8	12 ± 5
0.02	72 ± 10	9 ± 4
0.04	62 ± 5*	4 ± 2
0.06	53 ± 8*	4 ± 3
0.08	36 ± 9*	1 ± 1
Methyl mercury chloride 115–09–3		
Acetone	102 ± 11	7 ± 3
TPA	91 ± 5	69 ± 9*
0.02	98 ± 6	7 ± 4
0.04	90 ± 17	8 ± 2
0.06	80 ± 13*	6 ± 3
0.08	55 ± 10*	6 ± 3
0.10	39 ± 8*	5 ± 2
Mirex 2385–85–5		
DMSO	77 ± 6	12 ± 4
TPA	84 ± 16	64 ± 5*
10	71 ± 8	41 ± 6*
30	70 ± 9	47 ± 9*
50	70 ± 7	45 ± 5*
70	66 ± 8	40 ± 7*
90	58 ± 12*	35 ± 6*
Nitrilotriacetic acid 5064–31–3		
Medium	95 ± 11	6 ± 2
TPA	85 ± 12	60 ± 3*
0.2	99 ± 7	7 ± 2
0.4	92 ± 10	8 ± 2
0.6	91 ± 10	9 ± 3
0.8	86 ± 9	9 ± 6
1	84 ± 9	8 ± 4
Penicillin G 69–57–8		
Medium	72 ± 9	15 ± 3
TPA	65 ± 6	68 ± 9*
100	67 ± 7	18 ± 5
250	61 ± 8*	17 ± 3
500	64 ± 8	19 ± 4
750	59 ± 11*	20 ± 5
1000	64 ± 8	21 ± 4
L-Phenylalanine 63–91–2		
Medium	66 ± 9	17 ± 3
TPA	66 ± 10	69 ± 7*
100	66 ± 9	15 ± 5
250	67 ± 9	16 ± 3
500	67 ± 8	15 ± 3
750	59 ± 6	20 ± 6
1000	61 ± 9	20 ± 4
Phthalimide 85–41–6		
DMSO	100 ± 12	19 ± 5
TPA	102 ± 14	106 ± 11*
25	96 ± 8	22 ± 4
50	104 ± 10	23 ± 5
100	96 ± 8	23 ± 6
150	100 ± 10	24 ± 5
200	99 ± 5	22 ± 6
Procarbazine 671–16–9		
DMSO	87 ± 9	22 ± 5
TPA	85 ± 10	78 ± 9*
25	90 ± 17	19 ± 5
50	89 ± 8	22 ± 7
100	85 ± 12	23 ± 6
200	83 ± 6	22 ± 7
400	79 ± 12	19 ± 5
All-*trans*-retinoic acid 302–79–4		
DMSO	106 ± 9	22 ± 7
TPA	101 ± 12	98 ± 9*
0.5	105 ± 12	48 ± 12*
1.0	103 ± 12	84 ± 17*
2.0	87 ± 14*	89 ± 10*
3.0	53 ± 6*	56 ± 21*
4.0	7 ± 5*	1 ± 2
13-*cis*-retinoic acid 4759–48–2		
Acetone	64 ± 4	19 ± 4
TPA	63 ± 6	71 ± 7*
0.2	58 ± 9	22 ± 6
0.6	61 ± 4	29 ± 6*
1.0	57 ± 10	31 ± 4*
1.4	52 ± 7*	30 ± 5*
1.8	50 ± 7*	31 ± 4*
Sodium arsenate heptahydrate 7631–89–2		
Medium	80 ± 9	10 ± 4
TPA	81 ± 16	74 ± 6*
3	70 ± 8	18 ± 4
4	70 ± 9	28 ± 7*
5	66 ± 10	28 ± 5*
6	56 ± 6*	30 ± 6*
7	55 ± 6*	33 ± 6*
Testosterone propionate 57–85–2		
ETOH	81 ± 9	6 ± 2
TPA	75 ± 8	61 ± 5*
2	79 ± 7	5 ± 3
4	68 ± 6*	6 ± 3
6	68 ± 8*	11 ± 4
8	56 ± 10*	19 ± 10*
10	19 ± 10*	47 ± 7*
Thalidomide 50–35–1		
DMSO	107 ± 7	19 ± 4
TPA	106 ± 11	119 ± 8*
20	108 ± 8	17 ± 4
40	108 ± 11	21 ± 3
60	107 ± 14	26 ± 6
80	107 ± 14	23 ± 5
100	101 ± 11	25 ± 6
Trichlorophenoxy acetate 93–76–5		
ETOH	95 ± 9	22 ± 3
TPA	89 ± 11	73 ± 9*
20	94 ± 9	40 ± 8*
60	93 ± 8	55 ± 5*
100	83 ± 7*	62 ± 10*
140	67 ± 8*	59 ± 6*
180	34 ± 7*	44 ± 9*
Urethane 51–79–6		
DMSO	120 ± 11	5 ± 4
TPA	116 ± 8	90 ± 13*
100	108 ± 14	6 ± 2
200	104 ± 13	7 ± 4
300	114 ± 13	10 ± 4
400	90 ± 9*	11 ± 4
600	87 ± 12*	11 ± 2
Vincristine 2068–78–2		
Medium	89 ± 7	5 ± 2
TPA	81 ± 7	63 ± 5*
0.0002	98 ± 8	7 ± 3
0.0005	94 ± 7	6 ± 1
0.001	92 ± 6	7 ± 3
0.002	89 ± 8	5 ± 2
0.003	66 ± 9*	4 ± 2

*Chemical Abstracts numbers are listed adjacent to each chemical.

†A cytotoxicity and metabolic co-operation assay were performed as described in the text. Each chemical assay included a negative and positive control experiment in addition to a concentration response experiment with each test chemical.

‡The negative controls were treated only with solvent–ethanol (ETOH), dimethylsulphoxide (DMSO), culture media or acetone.

§The positive control was 12-*O*-tetradecanoylphorbol-13-acetate (TPA) at 4 ng/ml.

¶All test chemical concentrations are in μg/ml of culture medium. In the cytotoxicity assay, asterisks indicate that the number of colonies was significantly ($P < 0.01$) less than in cultures treated with solvent. In the metabolic co-operation assay, asterisks indicate that the number of colonies was significantly ($P < 0.01$) greater than in cultures treated only with solvent.

Table 2. Comparison of teratogenicity data *in vivo* with results *in vitro*

Chemical	*In vivo* Summary score*	Cytotoxicity† (μM)	Metabolic co-operation‡ (μM)	*In vitro* Summary score§
Acetazolamide	±	3108	3774	−
Acetylsalicylic acid	+	1110	1388	−
Amaranth	−	165	331	−
6-Aminonicotinamide	+	5.83	5.83	−
Caffeine	±	1545	2060	−
Chlorambucil	+	4.93	4.93	−
Coumarin	±	1026	1026	±
Cyclophosphamide	+	(896)	537	+
Cytochalasin D	±	0.295	0.049	±
Dexamethasone	+	127	255	−
Diazepam	±	70.2	70.2	±
Diethylstilboestrol	+	(2.98)	2.98	−
Dilantin	+	199	99	±
Diphenhydramine	−	54.8	82.2	−
Doxylamine succinate	−	309	206	±
EM-12	+	(1233)	1233	−
Ethylene thiourea	+	9000	9000	−
5-Fluorouracil	+	2.31	3.84	−
Formaldehyde	−	99.8	133	−
Hydroxyurea	+	26.3	131	−
Isoniazid	−	911	911	−
Meprobamate	−	3.66	7292	−
Methotrexate	+	0.088	0.176	−
Methyl mercury chloride	+	0.239	0.398	−
Mirex	±	165	18.3	+
Nitrilotriacetic acid	−	3.63	3.63	−
Penicillin G	−	2104	2805	−
L-Phenylalanine	+	(6053)	6053	−
Phthalimide	−	(1359)	1359	−
Procarbazine	+	(1552)	1552	−
All-*trans*-retinoic acid	+	6.66	1.66	+
13-*cis*-retinoic acid	±	4.66	1.99	+
Sodium arsenate	+	19.2	12.8	+
Testosterone propionate	+	11.6	23.2	±
Thalidomide	±	(387)	387	−
Trichlorophenoxy acetate	±	391	78.3	+
Urethane	+	4489	6734	−
Vincristine sulphate	+	0.0033	0.0033	−

*Developmental toxicity scores are from human and animal data summarized by Smith *et al.* (1983): (+) teratogen, (±) weak or variable teratogen, (−) non-teratogen.

†Values are minimum cytotoxic concentrations ($P < 0.01$) or the maximum concentration (in parentheses) that was evaluated.

‡Values are the minimum concentration that inhibited metabolic co-operation, or maximum concentration tested if chemical did not inhibit metabolic co-operation.

§A (+) indicates inhibition of metabolic co-operation at at least two non-cytotoxic concentrations, a (±) indicates inhibition at less than two non-cytotoxic concentrations and (−) indicates no inhibition.

concentration exceeds the upper limit suggested for testing in developmental toxicity assays *in vitro* (Faustman, 1988; Pratt and Willis, 1985).

Dunnett's test was used in the present evaluation to identify cytotoxicity at the $P < 0.01$ level. Frequently, a 30% reduction in colony formation of V79 cells is used as an indication of cytotoxicity (Bohrman *et al.*, 1988; Malcolm *et al.*, 1985; Trosko *et al.*, 1981). One rationale for using 70% as a cut-off is the difference in cell density between the cytotoxicity assay and the metabolic co-operation assay: in the cytotoxicity assay, the plating efficiency of only 100 $6TG^r$ cells is measured, whereas in the metabolic co-operation assay the plating efficiency of 100 $6TG^r$ cells in the presence of 5×10^5 $6TG^s$ cells is determined; this results in a much lower mole-to-cell ratio in the metabolic co-operation assay. Consequently, inhibition of metabolic co-operation may be occurring at less toxic concentrations than the cytotoxicity assay indicates because test compounds are less toxic to the co-cultures, owing to higher cell density. Despite the disparity in cell density between the two assays, Dunnett's test was chosen because it is more sensitive and objective than arbitrarily selecting 70% as the cut-off between toxic and non-toxic concentrations. In the present study, therefore, any concentration that significantly reduced colony number was considered cytotoxic. Although preliminary cytotoxicity assays indicated that the maximum concentrations used in the metabolic co-operation assay were cytotoxic, for seven of the 38 chemicals tested, the maximum concentration did not significantly reduce cloning efficiency in the concurrently run assay. These concentrations are in parentheses in Table 2. It must be realized that although these concentrations were not significantly effective in the final cytotoxicity assay, they had previously exhibited some degree of cytotoxicity in the range-finding assays.

Table 3. Summary comparison of results *in vitro* and evaluations *in vivo*

	Sensitivity*	(%)	Specificity†	(%)	Accuracy‡	(%)
Smith *et al.*, 1983§						
Criterion 1¶	7/29	24	9/9	100	16/38	42
Criterion 2‖	11/29	38	8/9	89	19/38	50
Steele *et al.*, 1988**						
Criterion 1	7/31	26	7/7	100	14/38	36
Criterion 2	11/31	35	6/7	86	17/38	45
Jensen *et al.*, 1989††						
Criterion 1	7/27	30	11/11	100	18/38	47
Criterion 2	11/27	41	10/11	91	21/38	55

*Sensitivity, number of teratogens that tested positive *in vitro*/total number of teratogens tested.
†Specificity, number of non-teratogens that tested negative *in vitro*/total number of non-teratogens tested.
‡Accuracy, total number of correct responses *in vitro*/total number of chemicals tested.
§*In vivo* data from Smith *et al.* (1983) as noted in Table 2.
¶Chemicals with an equivocal score (±) in the metabolic co-operation assay considered negative for this comparison.
‖Chemicals with an equivocal score in the metabolic co-operation assay considered positive for this comparison.
**Smith list as modified by Steele *et al.* (1988) where meprobamate and phthalimide are classified positive (+) *in vivo*.
††Modification to Smith list based on recommendations of Jensen *et al.* (1989) to classify acetazolamide and chlorambucil as negative (−) *in vivo*.

Comparison of data in vitro and in vivo

On the basis of the evaluation of developmental toxicity by Smith *et al.* (1983), compounds were designated (+) if they were defined as strong positive or positive teratogens, (±) if they were considered weak or variable teratogens, and (−) if they were negative or not demonstrated to be teratogens. These scores are listed in Table 2. Table 2 also summarizes data from Table 1 and lists the concentrations that significantly reduced cloning efficiency and metabolic co-operation. A summary score of the outcome in the V79 assay is provided for each chemical. A comparison of data *in vivo* and *in vitro* is shown in Table 3. The most conservative and traditional criterion was that chemicals that inhibited metabolic co-operation at two non-cytotoxic concentrations were considered positive (+). This resulted in low sensitivity (percentage of teratogens that were positive in the V79 assay) and high specificity (percentage of non-teratogens that were negative in the V79 assay) when compared with the original evaluation by Smith *et al.* (1983). Extending the criteria for considering a chemical to be positive, to include those chemicals scored as equivocal, improved sensitivity of the V79 assay and decreased specificity. This inclusion of equivocal results as positive seemed appropriate for the present data, as three of the equivocal chemicals had previously been reported to inhibit intercellular communication. Table 3 also shows that three interpretations of developmental toxicity *in vivo* of the compounds tested had little impact on sensitivity, specificity and accuracy of the V79 assay.

DISCUSSION

Screens *in vitro* for developmental toxicants are especially challenging to develop and validate because of the variety of mechanisms believed to result in growth retardation, impaired function, physical malformation or death. Faustman (1988) identified two general approaches that have been used in the development of assays *in vitro*: one approach relies on the examination of a limited aspect of embryogenesis, such as chondrogenesis in limb bud cultures or early organogenesis of post-implantation cultures; the second approach focuses on a specific event in a model *in vitro* that is similar to a developmental process *in vivo*, and examples include the mouse ovarian tumour (MOT) cell assay (Braun *et al.*, 1982) which examines cell attachment, and the human embryonic palatal mesenchymal (HEPM) cell assay (Pratt *et al.*, 1982; Pratt and Willis, 1985) which assess growth of embryonic cells. The V79 metabolic co-operation assay falls into the latter category, and assesses the potential of a chemical to inhibit intercellular communication.

The chemicals chosen by Smith *et al.* (1983) still stand as the only published list of agents selected specifically for evaluation of short-term tests for developmental toxicants. In the most comprehensive use of chemicals from this group, Steele *et al.* (1988) tested 44 compounds in the MOT cell assay and in HEPM cell assay. Steele *et al.* (1988) updated the summary scores by reducing the animal and human results to (+) for compounds that caused death, malformation, or growth retardation in the absence of maternal toxicity, and (−) where results were clearly negative or only slight skeletal alterations were noted. More recently, the original summary scores of Smith *et al.* (1983) and the re-evaluation by Steele *et al.* (1988) have been criticized by Jensen *et al.* (1989), who focused on specific problems that they found with both evaluations and recommended a re-evaluation of the list before validation of additional assays *in vitro*. This, of course, would be a major task best undertaken by a consensus workshop. In addition, further evaluation of the compounds by us to serve the purpose of the present study would not be appropriate. However, we did evaluate sensitivity, specificity and overall accuracy of the V79 assay bearing in mind the assessments of the above authors regarding the developmental toxicity *in vivo* of the tested compounds. As shown in Table 3, the minor modifications by Steele *et al.* (1988) or Jensen *et al.* (1989) to the original interpretation

of Smith *et al.* (1983) did not affect the overall accuracy of the V79 assay.

The poor accuracy of the V79 assay for detecting teratogens was the result of low sensitivity to the diverse group of compounds tested. This contrasts with the 80% sensitivity obtained by Elmore *et al.* (1987) in their evaluation of published results. Elmore *et al.* (1987) did not evaluate data or algorithms for reaching a positive score in the V79 assay, but accepted the conclusions reached by the individual authors; there are therefore several factors that may have contributed to the discrepancy between our results and that review. Possible contributors include the testing by us of coded chemicals in a single laboratory using a standardized protocol, the evaluation of compounds selected specifically for validation of assays *in vitro*, and the use of strict criteria for excluding the testing of overtly toxic concentrations. With regard to this last point, several chemicals that are considered to be potent developmental toxicants were found to be negative in the present study: for example, methotrexate, methylmercury, vincristine sulphate and cytochalasin D were all negative in the V79 assay. However, these chemicals were also extremely cytotoxic. Interestingly, the cloning efficiency assay used here to assess cytotoxicity is somewhat analogous to the HEPM assay. The chemicals noted above had EC_{50} concentrations below 1 μM in the HEPM assay (Steele *et al.*, 1988). Furthermore, of 11 compounds tested in the present study that were cytotoxic at less than 10 μM, nine were designated teratogens (Table 2). For this reason, chemicals exhibiting high cytotoxicity at low concentrations in the V79 assay should possibly be considered for further study in other teratogenesis-testing systems.

Of the chemicals tested here, the retinoic acids are the only teratogens for which the effects on development have been investigated in conjunction with their effects on gap junctional communication (Mehta *et al.*, 1989; Welsch, 1987; Welsch *et al.*, 1987). Mehta *et al.* (1989) reported that inhibition of growth of $10T\frac{1}{2}$ cells and 3T3 cells by retinoic acids correlated inversely with inhibition of gap junctional communication: the retinoic acids inhibited intercellular communication at concentrations that stimulated growth, and enhanced intercellular communication at concentrations that inhibited growth. In contrast, Welsch *et al.* (1987) did not observe a consistent pattern between the action of various retinoids on intercellular communication and their effect on chrondrogenic differentiation of cultured limb bud mesenchymal cells. They indicated that a morphometric analysis of gap junction size and number relative to cell surface area is needed to interpret properly the action of these teratogens on gap junctional communication.

Despite low sensitivity, the V79 assay proved to be highly specific for identifying developmental toxicants. With any *in vitro–in vivo* comparison the probability of obtaining a false positive *in vitro* decreases as the ratio of positive/negative toxicants *in vivo* increases. In the present study, approximately three-quarters of the chemicals were categorized as developmental toxicants: a random designation of the chemicals as positive would therefore have resulted in a specificity of 75%. Although this detracts somewhat from the 100% specificity obtained with the V79 assay, the present findings are in agreement with the hypothesis that chemicals that inhibit intercellular communication pose a risk of disruption of normal development.

Recently, more direct methods dependent on the transfer of fluorescent dyes have been developed to measure intercellular communication (Loch-Caruso *et al.*, 1990). Their primary advantage lies in the freedom to perform measurements in other cell lines and primary cells from various species and tissues (Klaunig and Ruch, 1987; Mesnil and Yamasaki, 1988). Improved sensitivity and specificity may be attained with alternative cell models possessing the capability for metabolic activation or detoxification. Especially promising are cells transfected with genes/cDNA for metabolizing enzymes that mimic species- and organ-specific metabolism (Langenbach *et al.*, 1990). Further consideration of inhibition of intercellular communication as a screen for developmental toxicity should be aided by incorporation of these new technologies. This may be especially true for the compounds evaluated here, as the majority are considered to require metabolism to be effective (Smith *et al.*, 1983).

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