

# Prediction of Chemical Carcinogenicity in Rodents from in Vitro Genetic Toxicity Assays

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Four widely used in vitro assays for genetic toxicity were evaluated for their ability to predict the carcinogenicity of selected chemicals in rodents. These assays were mutagenesis in *Salmonella* and mouse lymphoma cells and chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells. Seventy-three chemicals recently tested in 2-year carcinogenicity studies conducted by the National Cancer Institute and the National Toxicology Program were used in this evaluation. Test results from the four in vitro assays did not show significant differences in individual concordance with the rodent carcinogenicity results; the concordance of each assay was approximately 60 percent. Within the limits of this study there was no evidence of complementarity among the four assays, and no battery of tests constructed from these assays improved substantially on the overall performance of the *Salmonella* assay. The in vitro assays which represented a range of three cell types and four end points did show substantial agreement among themselves, indicating that chemicals positive in one in vitro assay tended to be positive in the other in vitro assays.

**S**HORT-TERM TESTS (STTs) FOR GENOTOXIC CHEMICALS were originally developed to study mechanisms of chemically induced DNA damage and to assess the potential genetic hazard of chemicals to humans. The role of these tests has increased, however, because of accumulating evidence in support of the somatic mutation theory of carcinogenesis (1, 2) and because of reports that many rodent carcinogens are genotoxic in in vitro STTs (3). The in vitro STTs have the advantages that they can be conducted relatively quickly and inexpensively compared to long-term carcinogenicity assays with rodents and do not involve testing in animals. Early studies of concordance between results from in vitro STTs and rodent carcinogenicity tests were highly encouraging (4-7); sensitivities (percentages of carcinogens identified as mutagens) and specificities (percentages of noncarcinogens identified as nonmutagens) of 90% or better were reported, especially for the Ames *Salmonella* mutagenesis assay (5, 6).

As a consequence of these reported concordances and because of concern for heritable damage to future generations, many countries drew up regulatory guidelines requiring submission of STT data for the registration of new chemicals (8). On the basis of a literature-derived study of the performance of STTs conducted by the Gene-Tox Program of the Environmental Protection Agency (9), it became apparent that there were two major impediments to a

thorough evaluation of the ability of these tests to predict rodent carcinogenicity: for most STTs there was a dearth of results for documented noncarcinogens (10), and too few chemicals had been tested in multiple STTs to permit meaningful comparisons of the ability of different STTs and STT combinations to predict carcinogens.

In the early 1970s, the National Cancer Institute (NCI) developed a protocol for rodent carcinogenicity studies that specified long-term exposure of both sexes of two species of rodents, generally F344 rats and B6C3F<sub>1</sub> mice, to high doses of chemicals in 2-year studies, with the use of 50 animals per treatment group (11). This protocol, adopted and refined by the National Toxicology Program (NTP), has been used during the last 12 years to study over 300 chemicals (12). Results from these studies constitute the primary database available today for the evaluation of mammalian carcinogenesis.

In 1984, the NTP initiated a project to develop a database that would permit evaluation of the ability of four of the most commonly used in vitro STTs to predict rodent carcinogenicity: the Ames *Salmonella*/microsome (SAL) mutagenesis assay (13), the assays for chromosome aberration (ABS) and sister chromatid exchange (SCE) induction in Chinese hamster ovary cells (14), and the mouse lymphoma L5178Y (MOLY) cell mutagenesis assay (15). In this article, we present results and conclusions derived from this study.

## Study Design

A number of characteristics of the design of this study distinguish it from previous attempts to evaluate STTs as predictors of rodent carcinogenicity, especially those based on results compiled from the scientific literature. First, standard protocols for the four STTs were developed by the NTP and shown to yield reproducible results in interlaboratory trials with coded chemicals (13, 14, 16). The literature results, by contrast, arise from a highly diverse set of protocols. Second, because literature-based evaluations often reflect the bias of the publication of results on strongly positive mutagens and mammalian carcinogens, a major design concern was selection of test chemicals by a procedure that would minimally bias the evaluation. The 83 chemicals initially selected for this project were those tested for rodent carcinogenicity by NCI and NTP with studies ending

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December 1976 or later, and final NTP peer review approval dates of 1 January 1985 or earlier. Ten chemicals (hexachlorodibenzo-*p*-dioxin mixture, 1,3-butadiene, agar, gilsonite, gum arabic, guar gum, locust bean gum, propylene, tara gum, vinylidene chloride) were excluded from further consideration because the first was not available in the same mixture tested for carcinogenicity, and the physical properties of the remaining nine indicated that they could not be adequately tested with STT protocols used herein. Only the first two of the ten are rodent carcinogens. The remaining 73 chemicals, well-characterized for carcinogenicity in both sexes of two rodent species, were then tested under code with each of the four STTs. Where possible, we used the same chemical lot as was used in the rodent carcinogenicity study. Finally, the STT experiments generally were replicated within each laboratory and evaluated with the aid of statistical analyses (17–19).

## Carcinogenicity Results

Forty-four chemicals (60%) were tumorigenic in at least one site in one of the four combinations of sex and species, the NTP criterion for a determination of a chemical carcinogen. Twenty chemicals (27%) showed no evidence of carcinogenicity, and nine (12%) were reported as “equivocal,” neither clearly positive nor clearly negative. These nine chemicals are treated as noncarcinogens in the statistical analysis. Typically, an equivocal carcinogenic response was described in the NTP reports as follows: “[Chemical name] was not carcinogenic, . . . but [certain tumors] may have been related to chemical administration.” Such an outcome was considered substantially closer to negative than positive. To evaluate comprehensively the performance of STTs, we included all available carcinogenicity data, even those studies that were difficult to interpret; however, the omission of the equivocal studies would not have markedly affected our qualitative conclusions.

The patterns of tumorigenicity exhibited by the 44 carcinogens were highly varied. The most frequent site of tumor induction was the liver (26 out of 44), and this was the only site of activity for 12 of the 44. The concordance, or observed agreement, between rat and mouse carcinogenicity determinations was 67% (49 out of 73) (that is, 12 chemicals were positive only in mice and 12 were positive only in rats, whereas 20 were positive and 29 were negative in both species). This agreement is significantly lower ( $P < 0.01$ ) than the concordance of 85% (211 out of 249) for these species, reported by Purchase (20). Historically, the interspecies concordance for the NTP rodent carcinogenicity assay has been approximately 74% (198 out of 266) (12). Elimination from the 83 chemicals of the 10 not tested in STTs served to depress the interspecies concordance because 9 of those eliminated were concordant and the tenth (1,3-butadiene) was tested only in mice. Nine of the 24 interspecies disagreements are attributable solely to tumors of the liver. For prediction of mouse carcinogenicity, the rat carcinogenicity assay has an estimated sensitivity of 63% (20 out of 32) and specificity of 71% (29 out of 41); when the roles of the rat and mouse in prediction are interchanged, these estimates remain unchanged. These values may represent an approximate upper bound on the concordance that can be achieved between rodent tumorigenicity and STT results.

## STTs as Predictors of Carcinogenicity

The data in Table 1 provide answers to three questions regarding the relation between rodent carcinogenicity and in vitro tests for genotoxicity.

1) What, if any, are the important differences in performance among the four STTs, and is one test clearly better than the others?

2) If SAL is central to in vitro testing schemes, as has often been proposed, which, if any, STT best complements this assay in the sense of raising sensitivity without significant loss in specificity?

3) Can these four STTs in any combination form a battery (multiple concurrent tests) that outperforms SAL in discriminating between carcinogens and noncarcinogens?

The frequencies of positive responses for the four STTs were as follows: SAL, 33% (24 of 73); ABS, 45% (33 of 73); SCE, 66% (48 of 73); and MOLY, 64% (47 of 73). Estimates (and definitions) of sensitivity, specificity, positive and negative predictivity, and concordance, with respect to rodent carcinogenicity for the four STTs, are presented in Table 2. It is apparent that SAL and ABS performed similarly overall, as did SCE and MOLY. These two pairs, however, did differ; SAL and ABS were reasonably specific but relatively insensitive regarding rodent carcinogenicity, while the reverse was true for SCE and MOLY. One should note that the 0.45 sensitivity for SAL is significantly ( $P < 0.001$ ) below the 0.90 reported earlier (5, 6). The negative predictivity of each of the four STTs is essentially the same, approximately 0.50, which should be judged against a prevalence of noncarcinogens in this database of 0.40. The positive predictivities of the STTs range from a high of 0.83 for SAL to a low of 0.66 for MOLY; this needs to be judged against a 0.60 prevalence of carcinogens among the 73 chemicals. As an illustration of the value of a positive STT result, the a priori odds for carcinogenicity among the 73 compounds are 3:2, but a positive SAL result shifts these odds to nearly 5:1. In terms of concordance or percent agreement with the rodent carcinogenicity results, all four STTs had scores of approximately 60% (21, 22). Increasing the stringency of the evaluation criteria for positive STT results produced the classic trade-off between sensitivity and specificity, with little if any consequent overall gain in concordance. On the basis of the current evaluation, there is no single test that is clearly superior to any of the other three STTs studied.

The SAL assay does enjoy advantages when compared to the other three STTs: technical ease of conduct, wide availability, a sizable literature, and low cost. For all these reasons, the SAL test is generally central to any scheme that is intended to screen for carcinogens (23). It has long been recognized, however, that this test does not detect all carcinogens (7, 24); in the current study it missed over one-half (24 of 44). Thus, a pressing question is whether any of the other in vitro tests can serve as a complementary assay to SAL (25); that is, can any assay detect the SAL-negative carcinogens without also detecting as positive an unacceptable number of noncarcinogens?

A way to approach the question of complementarity is to stratify the 73 chemicals by the qualitative (+ or –) results obtained with SAL. The data in Table 3 indicate that when one considers only the 49 SAL-negative chemicals, rodent carcinogenicity results show no association with the results obtained with MOLY, ABS, or SCE. For example, consider the use of ABS to complement SAL, with a positive result in either assay predicting a carcinogen. When the combined results are compared to predictions with SAL alone, an additional eight carcinogens are correctly identified but an additional six noncarcinogens are incorrectly predicted to be carcinogenic. Thus, sensitivity is improved somewhat, but at the expense of specificity, while the overall concordance is barely altered. The data in Table 4 show that ABS, SCE, and MOLY, however, do confirm positive SAL results very effectively; that is, the large majority of the 24 SAL-positive chemicals are also positive in ABS (79%), SCE (88%), and MOLY (96%). From a statistical viewpoint, the results with ABS, SCE, and MOLY lack association with rodent carcinogenicity when they are stratified by the SAL outcome, a feature labeled

**Table 1.** Tumorigenicity and genetic toxicity results for 73 chemicals. This table presents the qualitative STT results, together with the lowest positive or highest negative dose tested for each of the chemicals. Conclusions regarding sites of induced tumors, taken directly from the NTP Technical Reports, are presented separated by sex and species. Detailed STT results for each of the chemicals and evaluation criteria for each of the in vitro assays can be found in one of the following references: SAL (13, 37), ABS and SCE (14, 38), and MOLY (15, 39), or can be obtained on request from the senior author. A negative response is indicated by (-), an equivocal response by "E," and an inadequate study by "T"; all other responses are positive.

Chemical	Chemical Abstract Services number	Report number*	Route†	Rodent dose‡	Rat activity§	Mouse activity§	SAL		MOLY		ABS		SCE	
							Dose	Activity¶	Dose#	Activity¶	Dose**	Activity¶	Dose††	Activity¶
Allyl isothiocyanate	57-06-7	234	GA	17.7	M: UB F: E	M: - F: -	1,000	E	0.4	N	5.0	N,A	0.2	A
Allyl isovalerate	2835-39-4	253	GA	43.9	M: HS F: -	M: - F: HS	10,000	-	100.0	N	300.0	A	500.0	N,A
11-Aminoundecanoic acid	2432-99-7	216	FE	300.0	M: L,UB F: -	M: E F: -	10,000	-	320.0	-	1,000.0	-	500.0	N
L-Ascorbic acid	50-81-7	247	FE	6437.5	M: - F: -	M: - F: -	10,000	E	2,000.0	E	3,000.0	-	500.0	N
Benzene	71-43-2	289	GA	17.7	M: ZG,OC,S F: ZG,OC	M: ZG,HS,LU, HG,PG F: ZG,HS,LU, O, MG	1,000	-	905.0	-	5,000.0	-	750.0	N
Benzoin	119-53-9	204	FE	650.0	M: - F: -	M: - F: -	10,000	E	10.0	A	2,000.0	-	2,000.0	E
Benzyl acetate	140-11-4	250	GA	353.7	M: E F: -	M: L,FS F: L,FS	10,000	-	700.0	N	5,000.0	-	5,000.0	-
2-Biphenylamine HCl	2185-92-4	233	FE	386.3	M: - F: -	M: - F: CS	10	A,3	110.0	A	200.0	N	200.0	-
bis(2-Chloro-1-methylethyl) ether	108-60-1	191	GA	70.7	M: - F: -	M: LU,L F: LU	33	A,2	250.0	N	124.0	A	375.0	N,A
Bisphenol A	80-05-7	215	FE	1287.5	M: E F: -	M: - F: -	333	-	50.0	-	50.0	-	50.0	E
Butyl benzyl phthalate	85-68-7	213	FE	594.2	M: I F: HS	M: - F: -	11,550	-	67.2	-	1,250.0	-	1,250.0	-
Caprolactam	105-60-2	214	FE	1931.3	M: - F: -	M: - F: -	10,000	-	5,000.0	-	5,000.0	-	5,000.0	-
Chlorobenzene	108-90-7	261	GA	84.9	M: E F: -	M: - F: -	3,333	-	100.0	N	510.0	-	300.0	N
Chlorodibromomethane	124-48-1	282	GA	71.4	M: - F: -	M: E F: L	10,000	-	100.0	N	2,540.0	-	740.0	A
2-Chloroethanol	107-07-3	275	SP	70.7	M: - F: -	M: - F: -	3,333	A,2	80.0	A	10,100.0	N,A	1,200.0	N,A
3-Chloro-2-methylpropene	563-47-3	300	GA	70.7	M: FS F: FS	M: FS F: FS	10,000	-	23.2	N	120.0	N,A	16.0	N,A
C.I. Acid Orange 10	1936-15-8	211	FE	772.5	M: - F: -	M: - F: -	10,000	-	5,000.0	-	1,250.0	A	5,000.0	-
C.I. Acid Red 14	3567-69-9	220	FE	1238.0	M: - F: -	M: - F: -	5,000	-	5,000.0	-	5,000.0	-	5,000.0	E
C.I. Acid Yellow 73	518-47-8	265	WA	1634.2	M: E F: -	M: - F: -	10,000	-	200.0	N	5,000.0	-	50.1	N,A
C.I. Disperse Yellow 3	2832-40-8	222	FE	198.1	M: L F: -	M: - F: L	10	N,A,3	10.0	A	1,500.0	-	5.0	N
C.I. Solvent Yellow 14	842-07-9	226	FE	19.8	M: L F: L	M: - F: -	0.3	A,2	4.0	A	250.0	-	8.2	N,A
Cinnamyl anthranilate	87-29-6	196	FE	1188.0	M: P,K F: -	M: L F: L	3,333	-	10.0	A	40.0	-	30.0	-
Cytembena	21739-91-3	207	IP	3.0	M: MS F: MG	M: - F: -	100	N,A,3	25.0	N	25.3	N,A	1.0	N,A

Chemical	Chemical Abstract Services number	Re-port number*	Route†	Ro-dent dose‡	Rat activity\$	Mouse activity\$	SAL		MOLY		ABS		SCE	
							Dose	Activity¶	Dose#	Activity¶	Dose**	Activity¶	Dose††	Activity¶¶
D & C Red 9	5160-02-1	225	FE	39.6	M: SP, L F: E	M: — F: —	1,000	N, 2	8.0	—	250.0	—	500.0	—
Diallyl phthalate	131-17-9	242 284	GA	212.2	M: — F: E	M: E F: E	10,000	—	67.2	N, A	200.0	A	160.0	A
1,2-Dibromo-3-chloro-propane	96-12-8	206	IN	0.3	M: N, T F: N, T, AG	M: N, LU F: N, LU	0.3	A, 4	83.7	N	50.0	N, A	10.0	N, A
1,2-Dibromoethane	106-93-4	210	IN	4.0	M: N, CS, TV F: N, CS, LU, MG	M: LU F: N, CS, LU, SC, MG	100	A, 3	68.1	N, A	250.6	N, A	5.0	N, A
1,2-Dichlorobenzene	95-50-1	255	GA	84.9	M: — F: —	M: — F: —	333	—	6.5	A	202.0	—	59.0	A
2,6-Dichloro- <i>p</i> -phenylenediamine	609-20-1	219	FE	356.5	M: — F: —	M: L F: L	33	A, 4	25.0	N	250.0	N, A	16.0	N, A
1,2-Dichloropropane	78-87-5	263	GA	88.4	M: — F: E	M: L F: L	1,667	N, 1	11.6	A	1,370.0	N, A	112.7	N, A
1,3-Dichloropropene	542-75-6	269	GA	21.4	M: FS, L F: FS	M: I F: UB, LU, FS	10	N, A, 2	3.0	N	100.0	—	29.9	N, A
di(2-Ethylhexyl) adipate	103-23-1	212	FE	1609.0	M: — F: —	M: L F: L	10,000	—	5,000.0	—	400.0	N	400.0	E
di(2-Ethylhexyl) phthalate	117-81-7	217	FE	297.1	M: L F: L	M: L F: L	10,000	—	122.6	—	5,000.0	—	25.0	N
Diglycidyl resorcinol ether	101-90-6	257	GA	8.5	M: FS F: FS	M: FS F: FS	3.3	N, A, 2	0.1	N	1.6	N, A	0.05	N, A
Dimethyl hydrogen phosphite	868-85-9	287	GA	141.5	M: LU, FS F: E	M: — F: —	10,000	A, 1	1,700.0	A	1,600.0	N, A	250.0	N, A
Dimethyl morpholino-phosphoramidate	597-25-1	298	GA	424.5	M: HS F: HS	M: — F: —	10,000	—	1,845.0	N	5,000.0	N	1,000.0	N
Dimethyl terephthalate	120-61-6	121	FE	638.0	M: — F: —	M: E F: —	333	—	75.0	—	10.0	—	10.0	—
Ethoxylated dodecyl alcohol	9002-92-0	264	FE	1545.0	M: — F: —	M: — F: —	1,000	—	40.0	—	50.0	—	30.6	—
Ethyl acrylate	140-88-5	259	GA	70.7	M: FS F: FS	M: FS F: FS	10,000	—	20.0	N	299.0	A	150.0	A
Eugenol	97-53-0	223	FE	772.5	M: — F: —	M: E F: E	333	—	21.3	N	300.0	A	75.0	N, A
FD & C Yellow No. 6	2783-94-0	208	FE	3218.8	M: — F: —	M: — F: —	10,000	—	1,000.0	A	5,000.0	—	5,000.0	E
Geranyl acetate	105-87-3	252	GA	1414.8	M: — F: —	M: — F: —	3,333	—	18.3	A	150.0	—	70.0	N, A
<i>Hamamelis</i> water (witch hazel)	68916-39-2	286	SP	5536.0	M: — F: —	M: — F: —	10,000	—	5,000.0	—	5,000.0	—	5,000.0	—
HC Blue 1	2784-94-3	271	FE	148.6	M: E F: LU	M: L, TG F: L	100	N, A, 3	20.0	N	960.0	A	119.0	N, A
HC Blue 2	33229-34-4	293	FE	2600.0	M: — F: —	M: — F: —	3,333	N, A, 3	30.0	N	2,500.0	—	240.0	N, A
8-Hydroxyquinoline	148-24-3	276	FE	386.3	M: — F: —	M: — F: —	1	A, 2	0.4	N	7.5	A	2.6	N, A
Isophorone	78-59-1	291	GA	176.8	M: K F: —	M: E F: —	10,000	—	400.0	N	1,600.0	—	500.0	N
Malaonon	1634-78-2	135	FE	130.0	M: — F: —	M: — F: —	10,000	—	123.5	N	3,500.0	—	50.0	N, A
D-Mannitol	69-65-8	236	FE	6437.5	M: — F: —	M: — F: —	10,000	—	5,000.0	—	5,000.0	—	5,000.0	—

Melamine	108-78-1	245	FE	178.3	M: UB	M: -	1,111	-	160.0	-	300.0	-	300.0	E
dL-Menthof	15356-70-4	98	FE	515.0	F: -	F: -	666	-	200.0	-	250.0	-	167.0	-
4,4'-Methylene- dianiline 2HCl	13552-44-8	248	WA	8.5	M: TG,L	M: TG,L,AG	1	A,4	250.0	N	800.0	N,A	16.0	N,A
Monuron	150-68-5	266	FE	59.4	F: TG	F: TG,L,HS	5,000	-	1,100.0	E	1,300.0	A	100.0	N,A
4,4'-Oxydianiline	101-80-4	205	FE	7.9	M: L,TG	F: -	1,000	N,A,5	50.0	N	160.0	N,A	50.0	N,A
Pentachloroethane	76-01-7	232	GA	176.8	F: L,TG	F: L,TG,HG	333	-	70.0	N	206.0	-	100.0	N
Phenol	108-95-2	203	WA	990.4	M: E	M: L	3,333	-	300.0	A	2,000.0	A	300.0	N,A
Polybrominated biphenyl mixture	67774-32-7	244	GA	0.2	F: -	F: -	10,000	-	20.0	-	500.0	-	500.0	-
Propyl gallate	121-79-9	240	FE	1545.0	F: L	F: L	1,000	-	50.0	N	5.0	N	5.0	N,A
Propylene oxide	75-56-9	267	IN	48.4	M: E	M: E	333	N,A,3	0.4	N	160.0	N,A	5.0	N,A
Reserpine	50-55-5	193	FE	0.2	M: N	M: N	10,000	-	10.0	-	200.0	-	200.0	-
Selenium sulfide	7446-34-6	194	GA	14.9	F: N	F: N	1,000	N,A,2	0.1	N	5.0	N,A	1.0	N
Sodium (2-ethylhexyl)- alcohol sulfate	126-92-1	256	FE	2575.0	M: AG	M: SV	10,000	-	4,200.0	-	5,010.0	-	4,980.0	-
Stannous chloride	7772-99-8	231	FE	262.5	F: L	F: E	333	-	80.0	-	25.0	N,A	25.0	N,A
Sulfisoxazole	127-69-5	138	GA	1980.8	M: E	F: -	100	-	750.0	N	5,000.0	-	100.0	N,A
2,3,7,8-Tetrachloro- dibenzo- <i>p</i> -dioxin	1746-01-6	209	GA	0.00001	M: -	F: -	1,000	-	1.0	-	0.8	-	0.8	-
1,1,1,2-Tetrachloro- ethane	630-20-6	237	GA	176.8	M: TG	F: L,TG	1,000	-	200.0	A	506.0	-	158.0	N
Titanium dioxide	13463-67-7	97	FE	6440.0	M: E	M: L	10,000	-	1.6	-	25.0	-	25.0	-
2,4- and 2,6-Toluene diisocyanate	26471-62-5	251	GA	43.3	M: -	M: -	33	A,2	50.0	N,A	800.0	N	50.0	N
2,6-Toluenediamine	15481-70-6	200	FE	24.8	F: SC,P, L, MG	F: L,CS	10	A,4	200.0	N	250.0	N	50.0	N,A
Trichloroethylene	79-01-6	243	GA	707.4	M: -	F: -	1,000	-	146.2	A	14,900.0	-	700.0	N,A
tris(2-Ethylhexyl) phosphate	78-42-2	274	GA	707.4	M: I	M: L	10,000	-	74.1	-	1,670.0	-	839.0	-
Zearalenone	17924-92-4	235	FE	12.1	F: -	F: L	1,000	-	65.0	-	15.0	N	12.5	N
Ziram	137-30-4	238	FE	23.8	M: -	M: PI	10	N,A,3	0.063	N	0.025	N,A	1.8	-

\*National Toxicology Program or National Cancer Institute Technical Report number (available from U.S. Government Printing Office). †Route of administration; FE, dosed feed; GA, gavage; IN, inhalation; IP, intraperitoneal injection; SP, skin painting; WA, dosed water. ‡Rodent dose shows the lowest positive dose or highest negative dose tested, expressed as milligrams per kilogram per day for 2 years. For gavage, inhalation, and intraperitoneal injections, doses were taken as reported in the NCI and NTP Technical Reports (and scaled to reflect average daily dose). For dosed feed and water studies, doses expressed as parts per million were converted to milligrams per kilogram per day on the basis of food consumption, water consumption, and body weight data as reported by Gold *et al.* (40). §Activity shown as M, male; F, female; and tumor sites: AG, adrenal gland; CS, circulatory system; ES, forestomach; HG, Harderian gland; HS, hematopoietic system; K, kidney; L, liver; LU, lung; MG, mammary gland; MS, multiple organ sites (mesotheloma); N, nose; O, ovary; OC, oral cavity; P, pancreas; PG, preputial gland; PI, pituitary gland; S, skin; SC, subcutaneous tissue; SP, spleen; SV, seminal vesicle; T, tongue; TG, thyroid gland; TV, tunica vaginalis; UB, urinary bladder; ZG, Zymbal gland. ||*Salmonella* mutagenesis (SAL) dose shown as lowest positive dose or highest negative dose tested (micrograms per plate). ¶Activity: N, positive with no exogenous metabolic activation; A, positive with exogenous metabolic activation; number, number of *Salmonella* strains positive. #Mouse lymphoma cell (MOLY) dose (mutagenesis in L5178Y cells) shown as lowest positive or highest negative dose tested (micrograms per milliliter). If a chemical induced a positive response without exogenous metabolic activation, it was not always tested with the added metabolism. \*\*ABS dose [chromosome aberrations in Chinese hamster ovary (CHO) cells] shown as lowest positive or highest negative dose tested (micrograms per milliliter). ††SCE dose (sister chromatid exchanges in CHO cells) shown as lowest positive or highest negative dose tested (micrograms per milliliter).

**Table 2.** Operational characteristics of each of the four STTs for predicting carcinogenicity. Carcinogenicity of chemical substances as tested in rodents (+, carcinogenic; –, not carcinogenic) may be compared with the results from four STTs; for example, 20 carcinogenic substances tested positive by SAL test, 24 did not.

Measure	Carcino- genicity	SAL		ABS		SCE		MOLY	
		+	–	+	–	+	–	+	–
	+	20	24	24	20	32	12	31	13
	–	4	25	9	20	16	13	16	13
Significance of association (Fisher's Exact Test)		0.004		0.041		0.098		0.139	
Sensitivity* (%)		45 (30–61)†		55 (39–70)		73 (57–85)		70 (55–83)	
Specificity‡ (%)		86 (68–96)		69 (49–85)		45 (26–64)		45 (26–64)	
Positive predictivity§ (%)		83 (63–95)		73 (54–87)		67 (52–80)		66 (51–79)	
Negative predictivity   (%)		51 (36–66)		50 (34–66)		52 (31–72)		50 (30–70)	
Concordance¶ (%)		62 (50–73)		60 (48–72)		62 (50–73)		60 (48–72)	

\*Percentage of carcinogens yielding a positive STT result.  
 ‡Percentage of STT positives that are carcinogens.  
 and rodent carcinogenicity test results.

†Numbers in parentheses, 95% confidence intervals.  
 ||Percentages of STT negatives that are noncarcinogens.

‡Percentage of noncarcinogens yielding a negative STT  
 ¶Percentage of qualitative agreements between STTs

**Table 3.** Association of rodent carcinogenicity results with ABS, SCE, and MOLY for 49 chemicals that are SAL negative.

Carcino- genicity	ABS		SCE		MOLY	
	+	–	+	–	+	–
+	8	16	15	9	12	12
–	6	19	12	13	12	13

**Table 4.** Association of rodent carcinogenicity results with ABS, SCE, and MOLY for 24 chemicals that are SAL positive.

Carcino- genicity	ABS		SCE		MOLY	
	+	–	+	–	+	–
+	16	4	17	3	19	1
–	3	1	4	0	4	0

conditional independence (26) ( $P = 0.75, 0.42$ , and  $0.98$  for ABS, SCE, and MOLY, respectively). In summary, within the limits of this study, none of the other three in vitro STTs studied is a satisfactory complement to SAL in predicting rodent carcinogenicity.

Because no single STT is adequate to detect all carcinogens, a battery approach to screening for carcinogens has been frequently proposed as an improvement over any single STT. If there is no complementary in vitro assay among these four assays, it is not surprising that batteries of two or more STTs do not appreciably improve the overall predictive performance of SAL alone. In fact, for the carcinogenicity data in Table 5, it can be shown that the maximum concordance for any prediction based on the 16 possible STT outcomes is 0.67 (49 of 73), while SAL alone has a concordance of 0.62 (45 of 73). This incremental gain is not significant. Table 6 summarizes the performance of the four-test battery for predicting carcinogenicity. Concordances range from 0.55 to 0.66, depending on the criteria used in defining a “positive”; similar results hold for two- and three-test batteries.

To summarize the evidence for carcinogenesis into a positive or negative result is to grossly simplify a complex process manifest in a fairly long-term experiment. To determine whether performance by the STTs would improve if the target of prediction were refocused on some specific aspect of carcinogenesis, we considered five aspects. The STTs were then evaluated for their ability to distinguish: (i) the 21 chemicals judged to be “high potency” carcinogens, as determined by the lowest dose (milligrams per kilogram per day) producing statistically significant ( $P < 0.05$ ) increases in tumor incidence; (ii) the 32 chemicals showing evidence of carcinogenesis in more than one sex or species group; (iii) the 20 chemicals exhibiting carcinogenic effects at more than one organ site; (iv) the 32 chemicals judged carcinogenic when liver tumors are excluded; and (v) the 26 chemicals showing increased incidences of malignant neoplasms (all sites combined).

Table 5 summarizes the qualitative data for these responses. These five aspects of carcinogenicity yield prevalence rates ranging from

27% (20 of 73) to 44% (32 of 73), which are close to the frequency of positive SAL responses (24 of 73, 33%). If these aspects are considered to be the targeted response, then the overall performance of SAL improves. For example, concordance for SAL increases from 62% to 67–74%, depending on which aspect of carcinogenicity is considered. Conversely, the performance of the other three STTs tends to diminish. For example, from the data on malignancy in Table 5, it can be shown that the concordance observed for SAL (53 of 73, 73%) is significantly greater than that observed for ABS (55%), SCE (53%), or MOLY (52%). Similar results were found for the other four aspects of carcinogenicity. Further, for the five aspects above, no battery constructed from the STTs exhibited improvement in predictive performance over that of SAL alone. Indeed, in many cases the concordance of the battery strategy was actually lower than that of SAL. Thus, regardless of whether the targeted response is “carcinogenicity” or some aspect of the carcinogenic response, there is little evidence that the four STTs have any enhanced ability to predict carcinogenicity beyond that of SAL.

## Implications for Testing Strategies

For more than a decade, the dominant paradigm motivating the use of STTs to predict chemical carcinogenicity has been that carcinogens are mutagens and, by implication, that mutagens are carcinogens (4). On the basis of the results presented here, it is clear that strong qualifications to these associations are needed. No single in vitro STT adequately anticipates the diverse mechanisms of carcinogenesis; and, more important, the advantage of a battery of in vitro STTs is not supported by results of the present study. These conclusions have major implications for carcinogen screening and regulation based on STT results. They also call into question proposed testing strategies (27) based on results from earlier attempts to evaluate STTs. Before implementing any proposed battery, substantial empirical evidence must be available to document the battery's claimed performance.

The crux of the difficulty encountered by any battery drawn from SAL, ABS, SCE, and MOLY is best illustrated by Table 7. This contains the 12 most potent carcinogens in Table 1 as judged by the criterion of lowest effective dose. There is strong consistency among the carcinogenicity determinations for the four sex and species combinations, and equally strong consistency among the results of the four STTs. Nevertheless, the three most potent carcinogens produced no genetic toxicity in any of the four STTs studied. One may speculate that these three carcinogens do not operate primarily by direct interaction with DNA, as suggested by the tumor-promoting capability of two of the three carcinogens in model systems for two-stage liver carcinogenesis (28, 29). From Table 5 it is apparent that three other weaker carcinogens produced no positive response in any STT, and that three noncarcinogens were positive in all four STTs. Possible explanations for the mutagenic noncarcinogens include low sensitivity of the carcinogenicity assay, in vivo detoxification into innocuous metabolites, or rapid excretion. In vivo STT and pharmacokinetic studies might clarify this point, although they could not "prove" that any carcinogenicity assay result is in error; at present, only a larger and more definitive carcinogenicity assay, or carcinogenicity studies in other species, could do that. Without more extensive carcinogenicity studies or

demonstration of the reproducibility of the rodent studies, the assumption that the carcinogenicity findings are correct remains necessary for the purpose of determining the predictivity of STTs. However, because of health concerns apart from cancer, it seems prudent not to dismiss as insignificant the in vitro mutagenicity of the noncarcinogens (30, 31).

Although point mutations are phenomenologically different from cytogenetic effects, the four STTs showed good interassay agreement. All four STTs agreed for 33 of the 73 chemicals (45%), whereas three of the four STTs concurred for an additional 26 (36%) chemicals. In fact, the pairwise associations among the STTs were highly significant (all  $P < 0.01$ ) and uniformly greater than the association between any one STT and the carcinogenicity assay. In short, chemicals that were positive in one in vitro STT tended to be positive in other in vitro STTs representing three cell types and four end points.

## Summary

To help put this project into its proper context, we emphasize certain features of the study:

**Table 5.** Patterns of STT and rodent carcinogenesis results. The qualitative results in Table 1 are summarized and, as with the tumorigenicity results, the equivocal STT results were treated as negative.

STT results				Overall carcinogenicity*		Aspects of carcinogenicity†								Malignancy‡	
SAL	ABS	SCE	MOLY			Potency		>1 group		>1 site		Without liver			
				+	-	High	Low	+	-	+	-	+	-	+	-
+	+	+	+	<b>14</b>	<b>3</b>	<b>9</b>	<b>5</b>	<b>13</b>	<b>1</b>	<b>10</b>	<b>4</b>	<b>12</b>	<b>2</b>	<b>11</b>	<b>6</b>
+	+	+	-	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
+	+	-	+	<b>2</b>	<b>0</b>	<b>1</b>	<b>1</b>	<b>0</b>	<b>2</b>	<b>0</b>	<b>2</b>	<b>2</b>	<b>0</b>	<b>0</b>	<b>2</b>
+	+	-	-	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
+	-	+	+	<b>3</b>	<b>1</b>	<b>2</b>	<b>1</b>	<b>3</b>	<b>0</b>	<b>1</b>	<b>2</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>1</b>
+	-	+	-	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
+	-	-	+	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
+	-	-	-	<b>1</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>1</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>1</b>	<b>0</b>
-	+	+	+	<b>5</b>	<b>4</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>1</b>	<b>0</b>	<b>5</b>	<b>5</b>	<b>0</b>	<b>2</b>	<b>7</b>
-	+	+	-	<b>2</b>	<b>1</b>	<b>2</b>	<b>0</b>	<b>1</b>	<b>1</b>	<b>2</b>	<b>0</b>	<b>2</b>	<b>0</b>	<b>0</b>	<b>3</b>
-	+	-	+	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
-	+	-	-	<b>1</b>	<b>1</b>	<b>0</b>	<b>1</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>2</b>
-	-	+	+	<b>5</b>	<b>6</b>	<b>0</b>	<b>5</b>	<b>3</b>	<b>2</b>	<b>0</b>	<b>5</b>	<b>1</b>	<b>4</b>	<b>2</b>	<b>9</b>
-	-	+	-	<b>3</b>	<b>1</b>	<b>1</b>	<b>2</b>	<b>2</b>	<b>1</b>	<b>2</b>	<b>1</b>	<b>2</b>	<b>1</b>	<b>2</b>	<b>2</b>
-	-	-	+	<b>2</b>	<b>2</b>	<b>0</b>	<b>2</b>	<b>2</b>	<b>0</b>	<b>2</b>	<b>0</b>	<b>2</b>	<b>0</b>	<b>1</b>	<b>3</b>
-	-	-	-	<b>6</b>	<b>10</b>	<b>3</b>	<b>3</b>	<b>3</b>	<b>3</b>	<b>2</b>	<b>4</b>	<b>4</b>	<b>2</b>	<b>4</b>	<b>12</b>
Totals				<b>44</b>	<b>29</b>	<b>21</b>	<b>23</b>	<b>32</b>	<b>12</b>	<b>20</b>	<b>24</b>	<b>32</b>	<b>12</b>	<b>26</b>	<b>47</b>

\*Carcinogenicity for each chemical as reported in Table 1. The empirical decision for each STT result that produces maximum concordance with carcinogenicity outcome is indicated in boldface type. This maximum concordance is 0.67 (49 of 73). †The 44 carcinogens are subdivided on the basis of four different criteria: (i) potency (high-potency carcinogens defined as chemicals producing effects at doses <60 mg/kg per day); (ii) chemicals producing carcinogenic effects in more than one sex and species group; (iii) chemicals producing carcinogenic effects at more than one organ site; and (iv) chemicals producing carcinogenic effects when liver tumors are excluded. ‡Results based on increased incidences of malignant tumors (all sites combined); not necessarily a subset of the carcinogens evaluated on the basis of site-specific effects.

**Table 6.** Operational characteristics of batteries of STTs for predicting carcinogenicity.

Measure	Number of positive STTs required for prediction of carcinogenicity*			
	One or more	Two or more	Three or more	All four
Sensitivity† (%)	86 (38 of 44)	70 (31 of 44)	55 (24 of 44)	32 (14 of 44)
Specificity‡ (%)	34 (10 of 29)	48 (14 of 29)	72 (21 of 29)	90 (26 of 29)
Positive predictivity§ (%)	67 (38 of 57)	67 (31 of 46)	75 (24 of 32)	82 (14 of 17)
Negative predictivity   (%)	62 (10 of 16)	52 (14 of 27)	51 (21 of 41)	46 (26 of 56)
Concordance¶ (%)	66 (48 of 73)	62 (45 of 73)	62 (45 of 73)	55 (40 of 73)

\*When the members of a battery of STTs are treated as equals, there are four criteria for determining whether or not the test results from the battery are positive. These range from requiring only one of four tests to be positive to requiring that all four give positive results. None of the possible strategies results in significant improvement beyond that obtained by a single STT (see Table 2). These data also illustrate the "trade-off" in sensitivity and specificity (or equivalently, in positive and negative predictivity) that occurs as the criteria are made progressively more stringent. †Proportion of carcinogens yielding a positive STT result. ‡Proportion of noncarcinogens yielding a negative STT result. §Proportion of STT positives that are carcinogens. ||Proportion of STT negatives that are noncarcinogens. ¶Proportion of qualitative agreements between STT and rodent carcinogenicity test results.

**Table 7.** Carcinogenicity and STT results for the 12 most potent rodent carcinogens as defined by lowest effective dose from Table 1.

Chemical name	Carcinogenicity				Lowest effective rodent dose*	STT			
	Rat		Mouse			SAL	MOLY	ABS	SCE
	M	F	M	F					
2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin	+	+	+	+	$1.0 \times 10^{-5}$	—	—	—	—
Polybrominated biphenyl mixture	+	+	+	+	0.2	—	—	—	—
Reserpine	+	—	+	+	0.2	—	—	—	—
1,2-Dibromo-3-chloropropane	+	+	+	+	0.3	+	+	+	+
Cytembena	+	+	—	—	3.0	+	+	+	+
1,2-Dibromoethane	+	+	+	+	4.0	+	+	+	+
4,4'-Oxydianiline	+	+	+	+	7.9	+	+	+	+
Diglycidyl resorcinol ether	+	+	+	+	8.5	+	+	+	+
4,4'-Methylenedianiline 2HCl	+	+	+	+	8.5	+	+	+	+
Zearalenone	—	—	+	+	12.1	—	—	+	+
Selenium sulfide	+	+	—	+	14.9	+	+	+	+
Allyl isothiocyanate	+	E†	—	—	17.7	E	+	+	+

\*Milligrams per kilogram per day. †E, equivocal.

1) Standard protocols were used to mimic the major use of STTs worldwide—screening for mutagens and carcinogens; no attempt was made to optimize protocols for specific chemicals.

2) The 73 NTP chemicals and their 60% incidence of carcinogenicity are probably not representative of the universe of chemicals but rather reflect the recent chemical selection process for the NTP carcinogenicity assay.

3) The small, diverse group of chemicals precludes a meaningful evaluation of the predictive utility of chemical structure information.

4) The NTP is currently testing these same 73 chemicals in two in vivo STTs for chromosomal effects.

5) Complete data for an additional group of 30 to 40 NTP chemicals will be gathered on carcinogenicity and the four in vitro STTs to attempt to confirm the current findings.

The standard against which the performance of STTs is measured has changed dramatically in the past decade. The high levels of concordance published in the early 1970s were accurate at the time. Nearly all known carcinogens tested were genotoxic, and there was little experimental evidence on which to base a judgment of noncarcinogenicity which, taken together, restricted assessment of test performances with noncarcinogens. With the increasing availability of results from NCI and NTP 2-year carcinogenicity studies in rodents, higher frequencies of nongenotoxic carcinogens and genotoxic noncarcinogens have been observed; this has resulted in the reduced concordance of the STT results with carcinogenicity results. It is clear that even with a battery of assays, not all rodent carcinogens are in vitro mutagens nor are all in vitro mutagens rodent carcinogens. If current in vitro STTs are expected to replace long-term rodent studies for the identification of chemical carcinogens, then that expectation should be abandoned. STTs do, however, continue to offer an economical, rapid, and dependable means to detect genotoxic chemicals. There is a range of applications in which STTs have been used successfully, from the identification of mutagenic fractions in complex mixtures such as cooked meat (32, 33) or air pollutants (34) to the early identification of genetic toxicity in the development of new chemical products (35).

Requirements for the use of STT have not been consistent in both the national and international regulatory agencies. This is evident in the variety of testing requirements (8) and the different impacts that positive test results have on the registration or further testing requirements of chemicals. Consensus on these matters is not likely to occur in the near future, but agreement should be possible in certain areas. For instance, any time a new test or strategy is proposed, it is imperative that there be documentation by a substantial set of systematically acquired test results on well-defined rodent carcinogens

and noncarcinogens (36). The current study represents a prototype of the evaluative effort needed for such documentation.

Results of the current study focus attention on two questions involving discordances between carcinogenicity and genotoxicity test results: (i) Do nongenotoxic rodent carcinogens pose the same carcinogenic risk to humans as those that are genotoxic? (ii) Can the apparent high frequency of in vitro genotoxic rodent noncarcinogens be explained as a combination of artifacts arising from extremely high dosing in in vitro tests or the failure of many bona fide in vitro genotoxins to express their genetic toxicity in whole animals? Until these questions are resolved, chemicals that show mutagenic effects, particularly if such effects are observed in vivo, must be initially considered to pose human health risks as long as the somatic mutation theory of cancer remains a viable explanation for the etiology of some chemically induced cancers.

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- Dunkel *et al.* (21) and 69 of the 73 from the present study. In both studies, a higher sensitivity and lower specificity and positive predictivity than seen here were reported. The values reported by Zeiger were as follows: sensitivity, 0.54; specificity, 0.70; and positive predictivity, 0.69. Differences among the three studies can be attributed to differences in the relative proportions of the different chemical classes represented in the databases. In all three of these studies, however, the concordances were similar.
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