THIONO COMPOUNDS. 4. *IN VITRO* MUTAGENIC AND ANTINEOPLASTIC ACTIVITY OF TEPA AND THIO-TEPA*

ALAN P. BREAU,[1] LAMAR FIELD,[2] AND WILLIAM M. MITCHELL[3]

Departments of Pharmacology,[1] Chemistry,[2] and Pathology[3]
Vanderbilt University, Nashville, TN

Tris (1-aziridinyl) phosphine oxide (TEPA) and tris (1-aziridinyl) phosphine sulfide (thio-TEPA) induced base pair mutations in the Ames mutagenic assay. Thio-TEPA required metabolic activation while TEPA was active without metabolic activation. Growth of a human vaginal carcinoma (A431), a human breast carcinoma (MDA-MB-231), and a human cervical carcinoma (HeLa) were inhibited in soft agar in vitro *at concentrations which induced mutagenesis in the Ames Assay. A fourth line, JEG choriocarcinoma, was sensitive to the antigrowth properties of both drugs at concentrations below that which induced mutagenesis. These data suggest that as more antineoplastic agents become available, and as mean survival times increase, knowledge of the relative* in vitro *sensitivity of a patient's neoplasm to a specific antineoplastic drug (i.e., dose required for growth inhibition) as a function of its mutagenic index might be useful for prediction of clinical remission, as well as the risk of secondary neoplasm induction.*

Tris (1-aziridinyl) phosphine sulfide (thio-TEPA) and tris (1-aziridinyl) phosphine oxide (TEPA), the oxo analog, are antineoplastic drugs which act as polyfunctional alkylating agents (Calabresi and Parks, 1980). In early clinical trials, thio-TEPA replaced TEPA owing to its greater stability (Farber et al., 1956). Thio-TEPA is currently used in the treatment of breast and ovarian adenocarcinoma, as well as intracavitary malignant effusions from serosal surfaces (Physicians Desk Reference, 1983). Thio-TEPA is an *in vivo* mutagen in Drosophila (Epstein and Shafner, 1968), rats (Jackson, Fox and Craig, 1959), rabbits (Nuzhdin and Nizhnik, 1968), and mice (Machemer and Hess, 1971). TEPA is an *in vivo* mutagen in Drosophila (Epstein and Shafner, 1968), Swiss mice (Epstein et al., 1970), C5f BL/GS mice (Sram, Zudova and Benes, 1970; Sram et al., 1970) and causes chromatid damage in rat bone marrow cells (Adler et al., 1971).

*This study was supported by PHS grant No. CA 30321 awarded by the National Cancer Institute (DHHS).

1. Address correspondence to: W.M. Mitchell, Dept. of Pathology, Vanderbilt University, Nashville, TN 37232.

2. Key words: tris (1-aziridinyl) phosphate oxide, tris (1-aziridinyl) phosphate sulfide, base pair mutations, Ames mutagenic assay (*Salmonella* assay), human breast carcinoma, human cervical carcinoma, human vaginal carcinoma.

3. Abbreviations: TEPA, tris (1-aziridinyl) phosphine oxide; thio-TEPA, tris (1-aziridinyl) phosphine sulfide; MEM, Minimal Essential Media.

Cell Biology and Toxicology, 1:21-30, 1984.

TEPA also shows *in vitro* mutagenicity in an auxotrophic strain of *Schizosaccharomyces pombe* (Zetterberg, 1971). In this paper we show that TEPA at dosages required for antineoplastic activity induces back mutation of the histidine operon in strain TA100 of *Salmonella typhimurium* without metabolic activation. The sulfur analog, thio-TEPA, requires metabolic activation with an Aroclor 1254 induced rat liver homogenate preparation (S-9) for TA100 mutagenic activity. These results are of clinical interest since thio-TEPA has been implicated in the induction of acute leukemia in patients treated with the drug for primary malignant neoplasms (Allan, 1970; Garfield, 1970; Greenspan and Tung, 1974; Kaslow et al., 1972; Perlman and Walker, 1973; Ruffner, 1974; Solomon and Firat, 1971; Sypkens-Smit and Meyler, 1970).

This study compares the *in vitro* growth inhibition of neoplastic cells (by these thiono compounds) to their mutagenicity.

MATERIALS AND METHODS

Ames Mutagenic Assay

The procedures of the Ames assay were performed essentially as published (McCann et al., 1975; Ames, McCann, and Yamasaki, 1975). TEPA (Polysciences, Inc., USA) and thio-TEPA (Lederle Laboratories, USA) were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mg/ml and serially titered in DMSO over a 5-log concentration. In triplicate, 0.1 ml of each dilution was combined with 0.5 ml of a mixture that was 0.1 M Na phosphate (Fisher, USA), pH 7.4, 8 mM $MgCl_2$ (Fisher), 4 mM NADP (Sigma, USA), 5 mM D-Glucose-6-Phosphate (Sigma) and 20% Aroclor 1254 induced male Sprague-Dawley liver homogenate (or sterile water in the non-enzyme incubations). Lastly, 0.1 ml of *Salmonella typhimurium,* strain TA98 or TA100, obtained after 14 hr incubation in Oxoid media (K.C. Biological, USA) at 37°C, was added and the mixture was incubated for 1 hr at 37°C in a shaking water bath. The incubates were combined with 2 ml of molten Vogel's top agar (50°C) and plated immediately onto Vogel-Bonner minimal agar plates. The plates were incubated for 3 days at 37°C, then scored for revertants using a bacterial colony scorer. The spontaneous reversion rate ranged from 100-120 for strain TA100 and from 30-50 for strain TA98. Results are reported with the spontaneous rate subtracted from the total. 2-Aminoanthracene was used routinely as a positive control (i.e., $>$ 2000 revertants per plate for each strain with S9 activation at a concentration of 2 μg per plate). In order to detect acute toxicity of the test materials, plates were examined microscopically for presence of a background bacterial lawn.

In Vitro Cytotoxicity Assay

A modification of the neutral red vital dye uptake assay of Finter (1969) was used to assess *in vitro* cytotoxicity to normal human diploid fibroblasts. Normal human diploid fibroblasts were detached with trypsin (K.C. Biological, USA), counted manually with a hemacytometer, and diluted with Minimal Essential Media (MEM) ob-

tained from GIBCO to a suspension of 300,000 cells/ml. Then, 30,000 cells (0.1 ml) were plated into each well of a 96-well sterile tissue culture plate (Costar), and allowed to attach overnight at 37°C, under 5% CO_2 in a humidified incubator. On the second day weighed amounts of TEPA and thio-TEPA were placed in sterile vials, and 1 ml of DMSO solutions were diluted with MEM to a concentration of 2 mg/ml. Serial dilutions in MEM over a 5-log concentration were made and 0.1 ml of each was added to the wells containing attached fibroblasts. After 24 hr exposure to test chemicals, the chemical containing media was removed, the cells washed with sterile phosphate buffered saline (PBS), and 0.1 ml of Neutral Red in MEM (1:150) was added. The Neutral Red was incubated for 24 hrs at 37°C in a humidified CO_2 incubator. After 2 hrs the dye was removed by aspiration, excess dye washed with PBS, aspirated, and the internalized dye extracted with acidified ethanol (1% acetic acid—50% ethanol solution). The absorbance of the vital dye was measured at 540 nm on a Flow Micro Titertek colorimeter. One hundred percent viability is that color yield of dye obtained from control fibroblasts.

In Vitro Antineoplastic Assay

A modification of the method of Tanigawa et al. (1982) was used, with 4 tumor lines: JEG, human a choriocarcinoma; MDA-MB-231, a human breast carcinoma; A431, a human vaginal carcinoma and HeLa, a human cervical carcinoma. JEG, MDA-MB-231, and HeLa can be obtained from the American Type Culture Collection. A431 was obtained from Dr. Stanley Cohen of Vanderbilt University. The Tanigawa method couples the soft agar growth advantages of the Salmon clonogenic assay (Hamburger and Salmon, 1977) with the speed of ^{3}H-thymidine incorporation as an index of cell division. The technique was modified by using Sea Plaque agar (FMC Corp., USA) which allows avoidance of thermal shock on plating the cells in agar. Normal human fibroblasts do not proliferate in the 0.6% Sea Plaque used in this study (data not shown).

The tumor lines were grown to confluence in a 75 cm^2 flask and removed from the plastic surface with 2 ml of 0.19% trypsin (K.C. Biologicals) and suspended in 10 ml of MEM to yield approximately 1-2 $\times$ 10^6 cells/ml. One ml of the cell suspension was combined with 1 ml of TEPA or thio-TEPA to yield a 5-log exposure ranging from 10 μg/ml to 0.001 μg/ml. The cells were exposed to chemicals for 1 hr at 37°C in a CO_2 humidified incubator. Then, 50 μl of 0.9% Sea Plaque agar (FMC Corp.) containing 10% fetal calf serum (K.C. Biological) and 10% tryptose phosphate broth (Difco) was pipetted onto the bottom of a 96-well sterile tissue culture plate and chilled at 6°C to constitute the bottom agar layer. After 1 hr incubation, the 1 ml cell $-$1 ml drug mixture was combined with 4 ml of the 0.9% Sea Plaque agar mixture to yield a cell suspension in 0.6% Sea Plaque agar. Fifty μl of the cell-agar suspension was pipetted onto the chilled bottom layer which hardens on contact. The soft agar plates were incubated for 48 hr at 37°C in a CO_2 humidified incubator. After 48 hr, 1 mCi of H^3-thymidine (16.7 Ci/mmole) in 25 μl MEM, was layered on the top of each well. After a period of 24 hr of thymidine incorporation, the plates were placed

in a −20°C freezer. The frozen plates were heated in an 80°C water bath to melt the agar in each well which is pipetted into test tubes containing 3 ml of 0.86 N KOH. The tubes were heated at 80°C for 1 hr, cooled, then 20 μg of bovine serum albumin was added and the mixture precipitated by adding 2.5 ml of ice-cold 30% trichloroacetic acid. The precipitations are maximized by overnight incubation at 4°C. The next day, the contents of the tubes were poured over glass fiber filter papers and washed 2 times with 5-ml aliquots of ice-cold 30% trichloroacetic acid and two 10-ml aliquots of deionized water. The filters were placed into 20 ml liquid scintillation vials and the precipitates resolubilized in 1 ml of Protosol (New England Nuclear, USA) at 55°C for 1 hr. The Protosol was neutralized with 100 μl of glacial acetic acid, and counted in 10 ml of scintillation cocktail consisting of 29.5% Triton X-100, 2.4% Spectrafluor, and 68.1% toluene (v/v) following dark adaptation to minimize light-induced chemiluminescence.

RESULTS

In vitro dose responses of TEPA and thio-TEPA on general cytotoxicity and antigrowth activities were determined in order to directly compare their mutagenic activity at equivalent concentrations relevant to their clinical usage. Both TEPA and thio-TEPA showed no general cytotoxicity against human foreskin fibroblasts at levels as high as 100 μg/ml (Fig. 1). JEG choriocarcinoma cells were the most sensitive to these antineoplastic agents with significant growth inhibition occurring at 0.01 μg/ml for TEPA and 1 μg/ml for thio-TEPA (Fig. 2A). The other cell lines, MDA-MB-231 breast carcinoma, A431 vaginal carcinoma, and HeLa cervical carcinoma, were approximately equally sensitive to both TEPA and thio-TEPA, with significant antineoplastic activity occurring only at the highest concentration (10 μg/ml) tested (Fig. 2B, C, D).

At concentrations effective for significant antigrowth activity against JEG cells, neither compound was mutagenic for either strain TA98 (data not shown) or TA100 (Fig. 3). However, levels of TEPA and thio-TEPA required for inhibition of growth of HeLa, MDA or A431 cells in soft agar were mutagenic against strain TA100 (Fig. 3). No mutagenic activity was observed for either compound against strain TA98 (data not shown). TEPA does not require metabolic activation for mutagenic activity although S-9 increases mutagenicity at higher concentrations. Thio-TEPA required metabolic activation for mutagenic activity against strain TA100 (Fig. 3).

DISCUSSION

Both TEPA and thio-TEPA induce base pair mutations in the Ames mutagenic assay. Three of the four cell lines tested required mutagenic concentrations of TEPA and thio-TEPA in order to have significant antineoplastic response in a modified Salmon stem cell assay. The fourth line, JEG choriocarcinoma, was sensitive at concentrations of both agents too low for mutagenicity. TEPA is a direct acting base pair

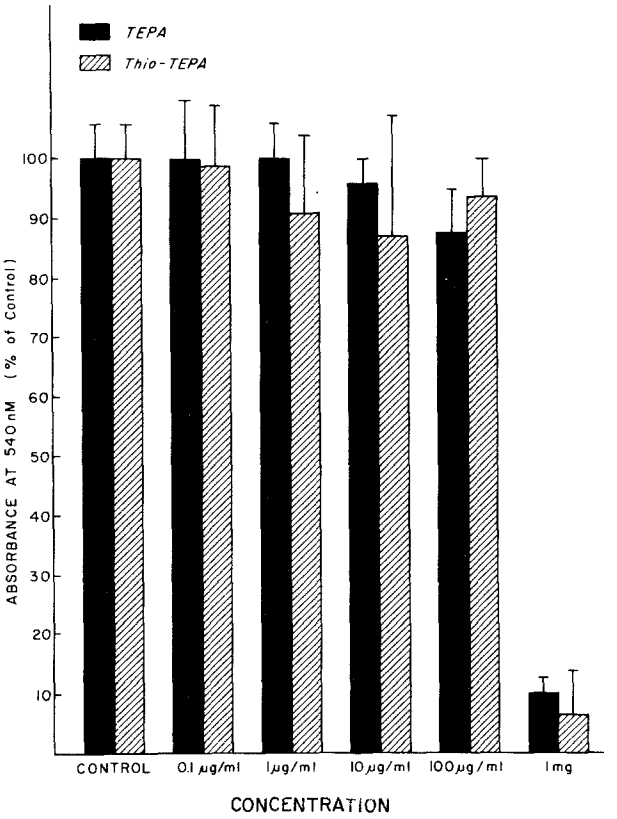
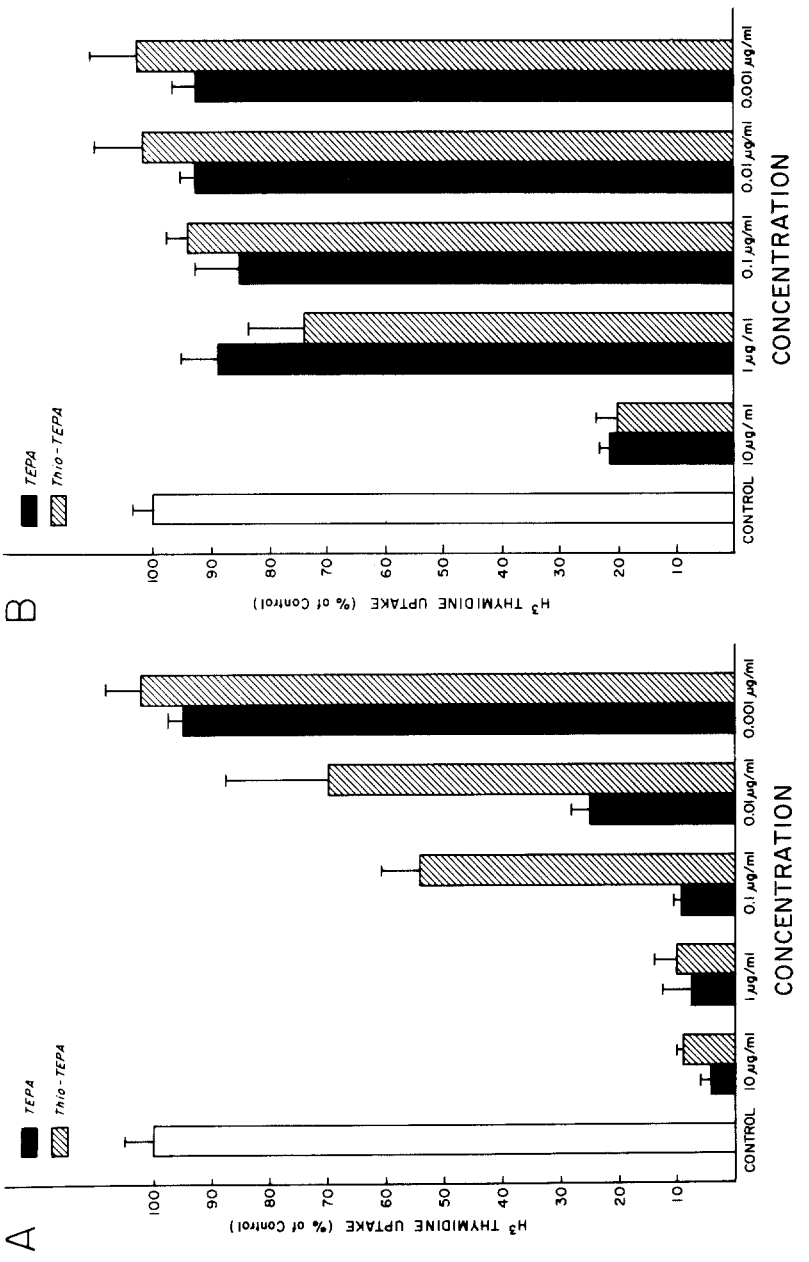


FIGURE 1. Cytotoxicity of TEPA and thio-TEPA on human diploid foreskin fibroblasts (Sg-181). Each bar is the mean ± one standard deviation from 3 determinations.

mutagen while thio-TEPA requires metabolic activation. Since the conversion of P=S to P=0 has been shown to occur via a cytochrome P450 (Neal, 1967), TEPA is probably the active mutagen in S-9 induced mutagenicity by thio-TEPA. Higher doses of thio-TEPA are mutagenic without S-9 activation (data not shown) presumably via non-enzymatic hydrolysis to TEPA.

The observation that thio-TEPA requires metabolic activation for mutagenic activity while TEPA is an apparent direct acting mutagen can be explained on the relative electronegativities (Allred, 1961; Allred and Rochow, 1958; Pritchard and Skinner, 1955) of oxygen (3.17-3.50) and sulfur (2.41-2.58). Transmission of the greater electron withdrawing powers of the oxygen through the intervening phosphorus and nitrogen should render the carbon atoms of the aziridine ring more electronegative, and thus, more susceptible to attack by the biological nucleophiles involved in mutation. In support of the effect of oxygen versus sulfur atoms, a greater susceptibility to nucleophilic attack on the phosphorus of =P(=O)- compounds in comparison to that of =P(=S)- compounds is indicated by the work of Lauwerys and Murphy (1969) and Cohen and Murphy (1972, 1974). Both laboratories have noted that the oxo analogs


A
B
TEPA
Thio-TEPA
H³ THYMIDINE UPTAKE (% of Control)
100
90
80
70
60
50
40
30
20
10
CONTROL
10 µg/ml
1 µg/ml
0.1 µg/ml
0.01 µg/ml
0.001 µg/ml
CONCENTRATION

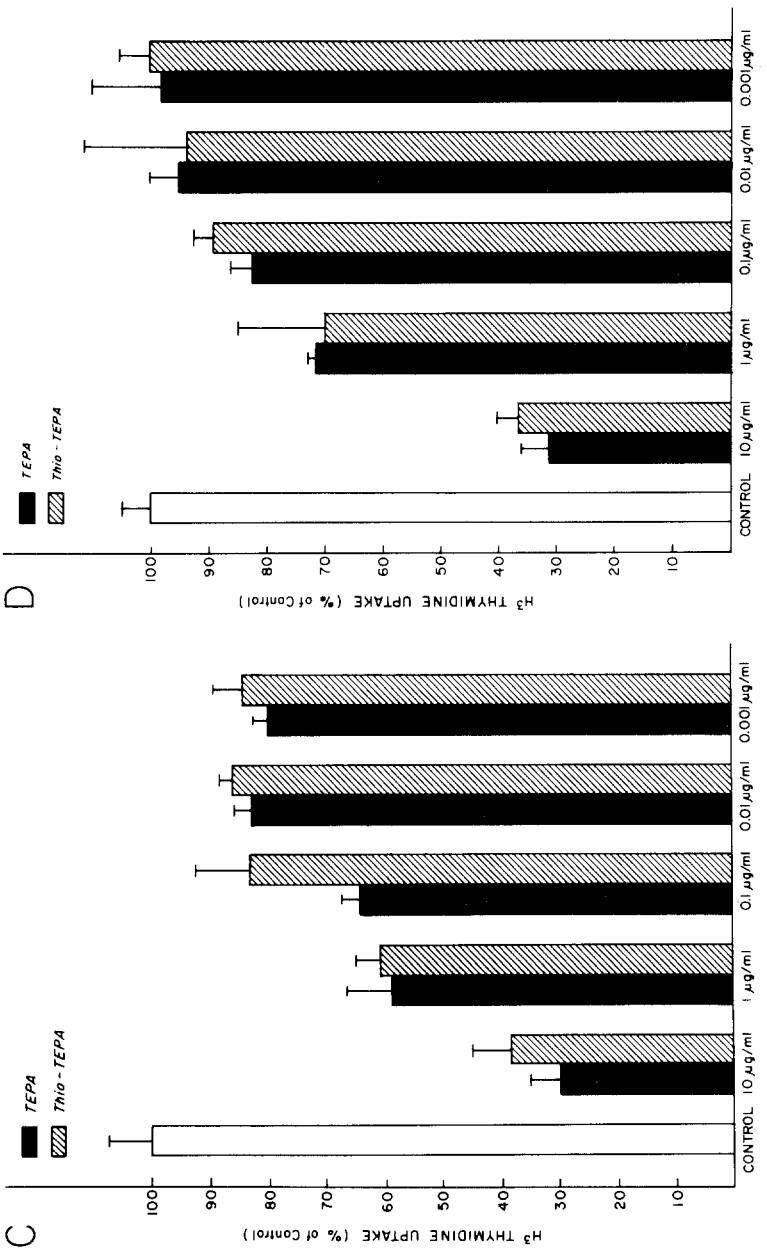


FIGURE 2. Comparative antigrowth activities of TEPA and thio-TEPA against four human *in vitro* cancer lines. (A) JEG, choriocarcinoma; (B) A431, vaginal carcinoma; (C) MDA-MB-231, breast carcinoma; (D) HeLa, cervical carcinoma. Each bar is the mean ± standard deviation from 3 determinations in one experiment. Control values were 44,580 ± 2146 cpm for JEG cells, 56,744 ± 1964 cpm for A431 cells, 12,737 ± 990 cpm for MDA-MB-231 cells and 54,466 ± 6758 cpm for HeLa cells.

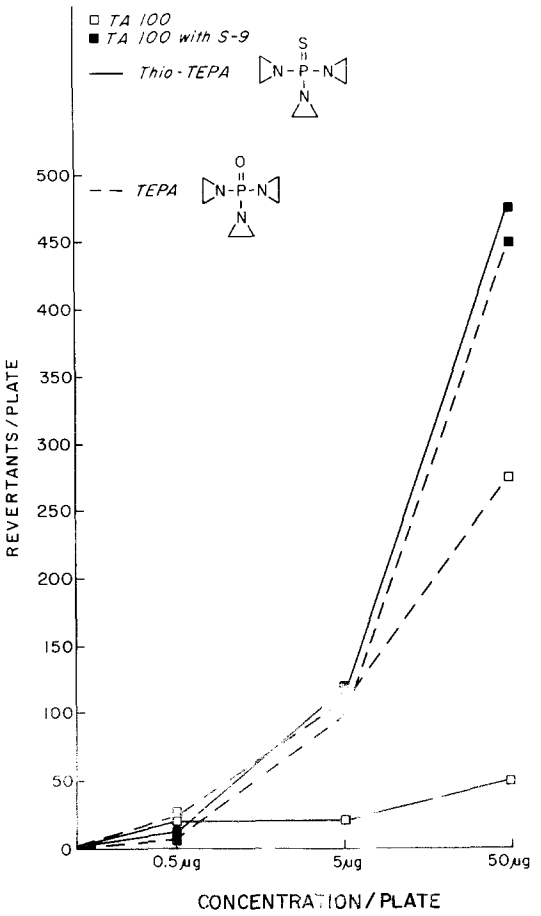


FIGURE 3. Comparative mutagenicity of TEPA (—) and thio-TEPA (---) on TA 100 (□) and TA 100 plus S-9 (■). Data are reported as revertants per plate corrected for spontaneous reversion rate. Each point is the mean from 3 observations of one experiment. The dose of drug per ml of bacteria (1:10 dilution of maximal log growth phase) during the 1 hr incubation period prior to soft agar plating can be obtained by multiplying the values given on the abscissa by 1.4 (see Methods).

of malathion and parathion show increased "binding" inactivation, with respect to anticholinesterase activity in liver and other tissues.

Our data suggest that as more antineoplastic agents become available, and as mean clinical survival times increase, knowledge of the relative *in vitro* sensitivity of a given patient's neoplasm to a specific antineoplastic drug (i.e., dose required for growth inhibition) as a function of its mutagenic index might be useful for prediction of clinical remission as well as the risk of secondary neoplasm induction. The Ames assay using mammalian microsomal activation is the simplest and most widely used *estimate* of relative mutagenicity. However, since the Ames assay uses prokaryotic DNA as a target, similar studies in mammalian cells might be useful. The technique

has been used as a screen for over 5,000 chemicals to date and has successfully identified approximately 83% of known carcinogens for eukaryotic organisms (Maron and Ames, 1983). The reliability of the Salmon clonogenic assay in predicting response of individual tumors to a specific drug has not been definitively demonstrated. Nevertheless, in the hands of experienced investigators the technique is clinically useful (Sikic and Tuker, 1981). With improvements in tumor cell isolation (e.g., enzyme digestion) and culture conditions (e.g., growth factors), it is likely that the clonogenic assay will become an accepted laboratory procedure for the clinical oncologist. Thus, given two or more drugs with equal *in vitro* activity against a specific patient's malignancy (e.g., as determined by the Salmon clonogenic assay), selection of the least mutagenic compound would appear to be clinically prudent. It is clear from our data that antineoplastic activity can be achieved in sensitive tumors *in vitro* at a concentration that has no apparent mutagenic activity.

ACKNOWLEDGMENTS

Dr. Stanley B. Garbus of Lederle Laboratories kindly provided the thio-TEPA used in this study.

REFERENCES

ADLER, I.D., RAMARAO, G. AND EPSTEIN, S.S. (1971). *In vivo* cytogenetic effects of trimethyl phosphate and of TEPA on bone marrow cells of male rats. Mutat. Res. **13**: 263-273.

ALLAN, W.S.A. (1970). Acute myeloid leukemia after treatment with cytostatic agents. Lancet ii: 775.

ALLRED, A.L. (1961). Electronegativity values from thermochemical data. J. Inorg. Nuclear Chem. **17**: 215-221.

ALLRED, A.L. AND ROCHOW, E.G. (1958). A scale of electronegativity based on electrostatic force. J. Inorg. Nuclear Chem. **5**: 264-268.

AMES, B.N., McCANN, J. AND YAMASAKI, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutat. Res. **31**: 347-364.

CALABRESI, P. AND PARKS, R.E. JR. (1980). Antiproliferative agents and drugs. In *Pharmacological Basis of Therapeutics* (A.E. Gilman et al., eds.) 1256-1314. Macmillan, NY.

COHEN, S.D. AND MURPHY, S.D. (1972). Inactivation of malaoxon by mouse liver. Proc. Soc. Exp. Biol. Med. **139**: 1385-1389.

COHEN, S.D. AND MURPHY, S.D. (1974). A simplified bioassay for organophosphate detoxification and interactions. Toxicol. Appl. Pharmacol. **27**: 537-550.

EPSTEIN, S.S., ARNOLD, E., STEINBERG, K., MACKINTOSH, D., SHAFNER, H. AND BISHOP, Y. (1970). Mutagenic and antifertility effects of TEPA and METEPA in mice. Toxicol. Appl. Pharmacol. **17**: 23-40.

EPSTEIN, S.S. AND SHAFNER, H. (1968). Chemical Mutagens in the Environment. Nature **219**: 385-386.

FINTER, N.B. (1969). Dye uptake methods for measuring viral cytopathogenicity and their application to interferon assays. J. Gen. Virol. **5**: 419-427.

GARFIELD, D.H. (1970). Acute erythromegakaryocytic leukemia after treatment with cytostatic agents. Lancet ii: 1037.

GREENSPAN, E.M. AND TUNG, B.G. (1974). Acute myeloblastic leukemia after cure of ovarian cancer. JAMA **230**: 418-420.

HAMBURGER, A. AND SALMON, S.E. (1977). Primary Bioassay of Human Myeloma Stem Cells. J. Clin. Invest. **60**: 846-854.

JACKSON, J., FOX, B.W. AND CRAIG, A.W. (1959). The effect of alkylating agents on male fertility. Brit. J. Pharmacol. **14**: 149-157.

KASLOW, R.A., WISCH, N. AND GLASS, J.L. (1972). Acute leukemia following cytostatic therapy. JAMA **219**: 75-76.

LAUWERYS, R.R. AND MURPHY, S.D. (1969). Comparison of assay methods for studying o-odiethyl, o-p-nitrophenyl phosphate (paraoxon) detoxification *in vitro*. Biochem. Pharmacol. **18**: 789-800.

MACHEMER, L. AND HESS, R. (1971). Comparative dominant lethal studies with phenylbutazone, thio-TEPA and MMS in the mouse. Experientia **27**: 1050-1057.

MARON, D.M. AND AMES, B. (1983). Revised methods for the *Salmonella* mutagenicity test. Mutat. Red. **113**: 173-215.

McCANN, J., CHOI, E., YAMASAKI, E. AND AMES, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: Assay of 300 chemicals. Proc. Natl. Acad. Sci. (U.S.A.) **72**: 5135-5139.

NEAL, R.A. (1967). Studies on the Metabolism of Diethyl 4-Nitrophenyl Phosphorothionate (Parathion) *in vitro*. Biochem. J. **1032**: 183.

NUZKDIN, N.I. AND NIZHNIK, G.V. (1968) Fertilization and embryonic development of rabbits after treatment of spermatozoa *in vitro* with chemical mutagens. Dokl. Biol. Sci. **181**: 419-422.

PERLMAN, M. AND WALKER, R. (1973). Acute leukemia following cytotoxic chemotherapy. JAMA **224**: 250.

PRITCHARD, H.O. AND SKINNER, H.A. (1955). The concept of electronegativity. Chem. Rev. **55**: 745-786.

RUFFNER, B.W. (1974). Androgen role in erythroleukemia after treatment with alkylating agents. Ann. Int. Med. **81**: 118-119.

SIKIC, B.I. AND TABER, R.L. (1981). Human tumor clonogenic assays. An overview. Cancer Chemother. Pharmacol. **6**: 201-203.

SOLOMON, R.B. AND FIRAT, D. (1971). Acute leukemia following treatment with irradiation and alkylating agents. N.Y. State J. Med. **71**: 2422-2425.

SRAM, R.J., BENES, Y. AND ZUDOVA, Z. (1970). Induction of dominant lethals in mice by TEPA and HEMPA. Folia Biol. (Praha) **16**: 407-416.

SRAM, R.J., ZUDOVA, Z. AND BENES, Y. (1970). Induction of translocations in mice by TEPA. Folia Biol. (Praha) **16**: 367-368.

SYPKENS-SMIT, G.C. AND MEYLER, L. (1970). Acute myeloid leukemia after treatment with cytostatic agents. Lancet, ii: 58-62.

TANIGAWA, N., KERN, D.H., HIKASA, Y. AND MORTON, D.L. (1982). Rapid assay for evaluating the chemosensitivity of human tumors in soft agar culture. Cancer Res. **42**: 2159-2164.

ZETTERBERG, G. (1971). Bacteriophage development in lysogenic *Escherichia coli* and mutations in fungi induced with analogs of tris (1-aziridinyl)-phosphine oxide, TEPA. Hereditas **68**: 245-254.