Rapid Microtiter Method for Cytotoxicity Screening

ENG-SHANG HUANG*, KUO-HSIUNG LEE†, CLAUDE PIANTADOSI†, T. A. GEISSMAN‡, and JOSEPH S. PAGANO*▲

Abstract □ A simple, rapid, and inexpensive microplate tissue culture method suitable for large-scale cytotoxicity screening is described. The test involves the use of several cell lines, normal human fibroblasts, laryngeal carcinoma, and virus-transformed human cells for the evaluation of the potency of cytotoxicity as well as the selectivity for cancerous tissue of a variety of sesquiterpene lactones, plant products, and synthetic compounds.

Keyphrases □ Cytotoxicity screening, large scale—simple, rapid microplate tissue culture method □ Antitumor agents, screening—simple, rapid microplate tissue culture method □ Microplate tissue culture method—large-scale cytotoxicity screening □ Sesquiterpene lactones—cytotoxicity, microplate tissue culture method

Recently, tissue culture techniques for cytotoxicity tests have been widely used as supplemental tools for screening and detecting antitumor agents (1-7). The reliability and sensitivity of tissue culture cytotoxicity tests were evaluated by Eagle and Foley (4), Toplin (7), and others (2, 5, 6).

Since most investigators have used conventional methods for cytotoxicity tests (4-6), large quantities of the test compounds were necessary. Demands of space, materials, and time and the probability of contamination were often complicating factors. Since microplate techniques are widely used for cell cloning and virus titration, we investigated this reliable technique for routine large-scale cytotoxicity screening and detection of antitumor compounds.

This report describes a cytotoxicity test performed in a microtest plate in which different concentrations of sample compounds were simultaneously tested against different cell lines originating from normal human fibroblasts, human laryngeal carcinoma, and human cells transformed with the tumor virus Simian Virus 40 (SV40). The results obtained by this technique for a group of plant products, *i.e.*, sesquiterpene lactones, and synthetic compounds were presented in separate papers (8-11).

EXPERIMENTAL

Cells and Media—The stock cultures of HEp-2 human carcinoma of larynx[1], WI-38 normal human fibroblast[2], and W18Va2 SV40-transformed human cells (12) were grown as monolayers in 75-cm.^{2} disposable plastic bottles (Falcon) in a growth medium[3]. The cultures were subcultured once every 2 weeks and refed with fresh maintenance medium containing 2% fetal bovine serum twice a week. The stock cells were subcultured 5 days before use in the cytotoxicity tests and also fed with a growth medium[3] before use in the test.

Preparation of Cell Suspensions and Solutions of Test Samples—Cell culture sheets were washed with Hanks' solution twice and removed from the bottles by treatment with 2 ml. of 0.25% trypsin in Hanks' solution without magnesium and calcium. The trypsinized cells were well dispersed by gentle pipeting in the proper amount of growth medium and were then diluted to a final concentration of about 10^5 cells/ml. The test samples of naturally occurring sesquiterpene lactones or plant extracts, dissolved in dimethyl sulfoxide or proper solvents at a concentration of 10 mg./ml., were diluted with the growth medium to the desired drug concentration: 100 mcg./0.9 ml., 20 mcg./0.9 ml., 10 mcg./0.9 ml., 4 mcg./0.9 ml., *etc.*

The concentration should not exceed 0.5% in the final test solution; dimethyl sulfoxide is free of obvious toxicity to the test cell lines at concentrations below 0.5%. Other nontoxic solvents can be used.

For rapid screening of crude plant extracts, final concentrations of 100, 20, and 4 mcg./ml. were employed.

Cytotoxicity Assay with Microtiter Plates—Microtest plates with 60 wells, which were nontoxic for tissue cultures[4], were used for the assays. Each flat-bottom well held about 0.02 ml. The cell suspension (0.1 ml., 1×10^5 cells/ml.) and diluted sample (0.9 ml.) were mixed and inoculated into the culture wells with a disposable tuberculin syringe fitted with a 22-gauge needle. One drop of the mixture (about 0.02 ml.) was placed in the test well by gently rotating the barrel of the syringe. To keep the inoculated volume constant, the syringe should be held at a 30° angle with the beveled edge of the needle facing down. The number of cells in each well was about 200. For a more critical inoculum size, a 20-µl. glass disposable micropipet[5] was used.

For replicate results with the same cells, a series of six wells of cell cultures was used for every diluted sample. The control cell growth of each cell line without drug treatment was done at the same time with the same cell concentration and the same number of wells. All culture plates were incubated in a water-jacketed carbon dioxide incubator with 5% carbon dioxide at 37° without changes of the media.

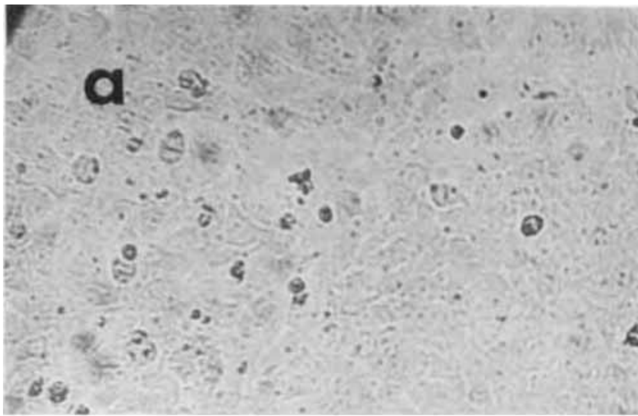
Table I—*In Vitro* Cytotoxicity Tests of Sesquiterpene Lactones for Diploid and Heteroploid Cells

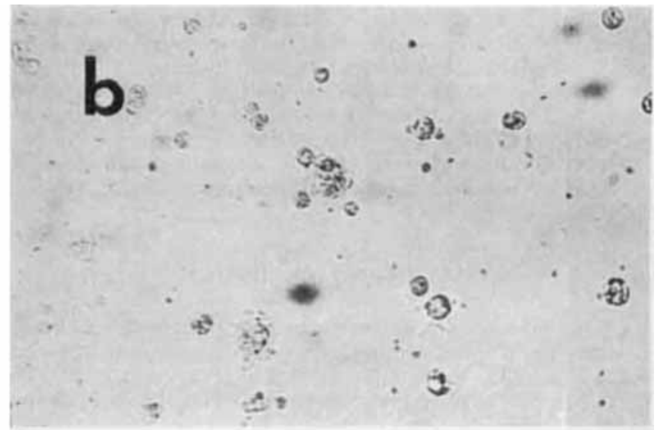
Compound and Reference	ED_{50}, mcg./ml.: Microtiter Method[a] WI-38[c]	HEp-2[d]	W18Va2[e]	CCNSC[b] Screening Data, KB Cell[f]
Vulgarin (8)	19.23	51.55	16.04	
Ludovicin A (8)	5.70	6.32	3.89	
Ludovicin B (8)	7.36	7.52	5.68	
Encelin (8)	0.25	0.92	0.56	
Farinosin (8)	0.23	0.60	0.74	
Xanthinin (8)	0.10	0.62	0.14	
Helenalin (8, 18)[g]	—	0.08	—	0.22
Damsin (15, 18)		0.61	—	0.58
α-Santonin (8, 18)		164.30	—	140.00
Elephantopin (16, 18)	—	0.48	—	0.32
Epitulipinolide (17)	—	1.67	—	2.10

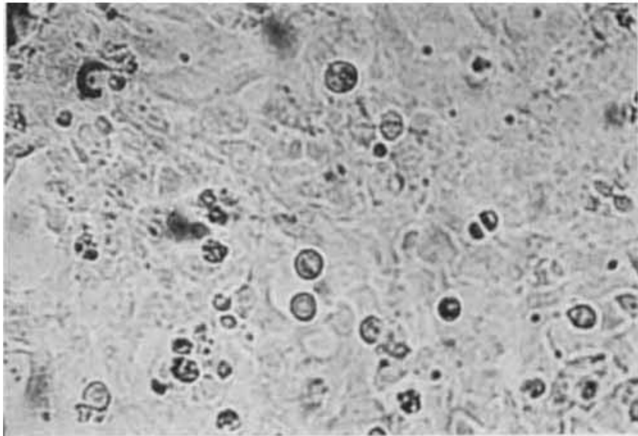
[a] The ED_{50} values were determined based upon the rapid microtiter method described in this article. [b] The ED_{50} values of this column were based upon the screening data of the Cancer Chemotherapy National Service Center (15-18). [c] WI-38 refers to normal human diploid fibroblasts. [d] HEp-2 refers to human epidermoid carcinoma of larynx. [e] W18Va2 refers to Simian Virus 40-transformed cells of human origin. [f] KB refers to human carcinoma of nasopharynx. [g] Further purified from a compound previously tested (8).

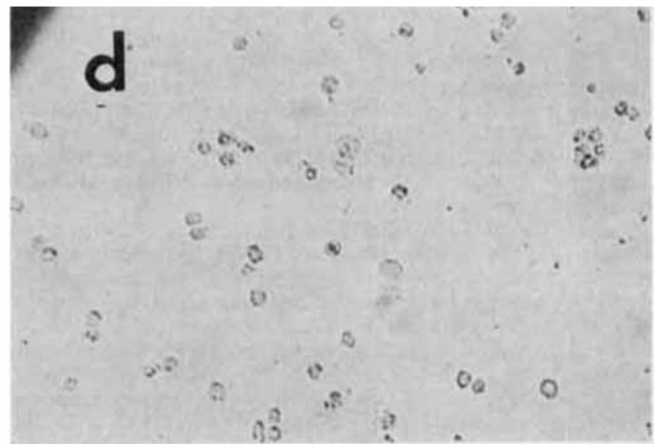
[1] Moore *et al.* Obtained from Grand Island Biological Co.
[2] Hayflick and Moorhead. Obtained from Microbiological Associates.
[3] Eagle's Minimal Essential Medium, Grand Island Biological. Co., with 10% fetal bovine serum and 100 mcg./ml. neomycin.

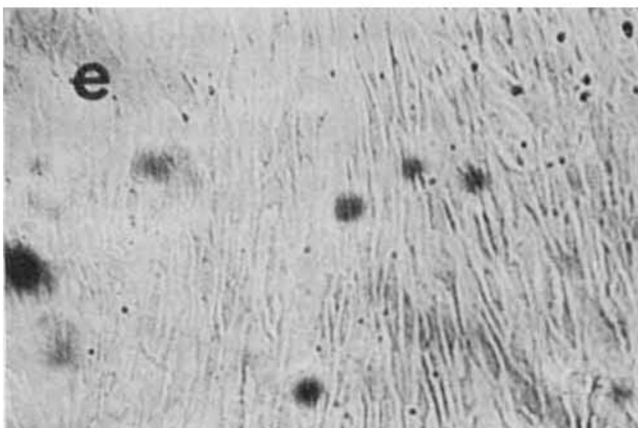
[4] Falcon No. 3034.
[5] Corning 7099-5.











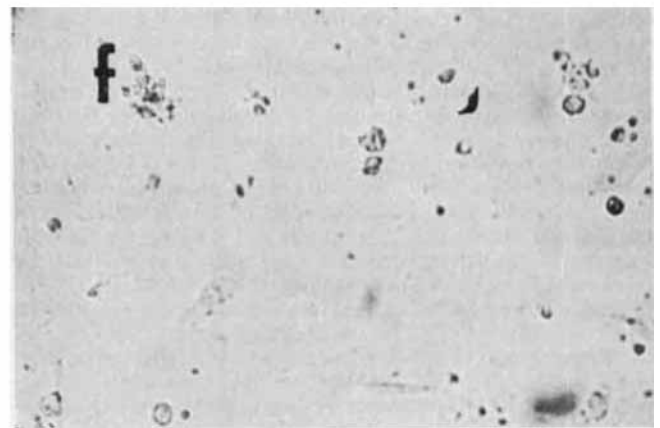


Figure 1—*Microscopic appearance of control and assay cells 4–6 days after inoculation. Key:* (*a*) *HEp-2, control* (*4 days*); (*b*) *HEp-2, helenalin, 1 mcg./ml.* (*4 days*); (*c*) *W18Va2, control* (*4 days*); (*d*) *W18Va2, helenalin, 1 mcg./ml.* (*4 days*); (*e*) *WI-38, control* (*6 days*); *and* (*f*) *WI-38, helenalin, 0.1 mcg./ml.* (*6 days*).

For a laboratory without a water-jacketed carbon dioxide incubator, a Lucite box with a sealable air inlet and outlet can be used in a 37° incubator. The test plates are placed horizontally in the upper portion of the box and a pan of water is put on the bottom to prevent evaporation of the test samples. Carbon dioxide in a volume of about 5% of the total box volume is blown into the chamber by a balloon. To keep the media close to neutral pH, the chamber should be completely sealed and refilled with carbon dioxide after each observation.

Scale System of Cytotoxicity—All assays were examined daily for 1 week with a low power, inverted binocular microscope[6]. Usually at 12 hr. after inoculation a rough estimate of the degree of cytotoxicity can be obtained by observing the percentage of cells adhering to the plastic plate; however, a precise value is determined by a final examination. The cytopathic cells always appear round, shrunk, or cytolytic. The surviving cells usually become sheets stretching and adhering to the well bottom (Fig. 1).

Between 2 and 7 days after inoculation, the percentage of the area of the bottom surface occupied by adherent surviving cells can be roughly estimated. On the day (3–4 days for HEp-2 and W18Va2 and 6–7 days for WI-38) when the control cells reached 100% confluence, the average percent of the confluence areas of six assay cultures was designated as the degree of assay cell growth. The ED_{50} values are used for expressing the cytotoxic potency, which is the calculated effective dose that inhibits the net cell growth to 50% of control growth.

For observation of the cytotoxicity for cells with a suspended, nonadherent habit, *e.g.*, human Burkitt lymphoma cells [Raji

[6] 100×, Olympus.

cells (13, 14)], uptake of trypan blue is used for the enumeration of surviving cells. In this case, the final observation can be made 5 days after inoculation. Five microliters of 0.2% trypan blue in phosphate-buffered saline solution (pH 7.4) is added to the culture well with gentle mixing and without bubbling; after 30 min. at 37° the stained cells are counted as surviving cells. The degree of assay cell growth is obtained by calculating the number of surviving cells in the test sample as compared with that of the control wells.

RESULTS AND DISCUSSION

A large number of compounds, including naturally occurring sesquiterpene lactones, synthetic compounds, and plant extracts, were tested by this method. Some data were reported previously (8–11). The results of several representative compounds are summarized in Table I.

Figure 1 shows the morphology of the confluent cell sheet of control cells (Figs. 1*a*, *c*, and *e*) and helenalin-treated cytopathic cultures (Figs. 1*b*, *d*, and *f*) 4–6 days after inoculation. Heteroploid HEp-2 and W18Va2 control cells grow faster than diploid WI-38 fibroblasts. It only takes 3–4 days for HEp-2 and W18Va2 to become confluent; in contrast, it requires 6–7 days for WI-38. The cytopathic cell always appears rounded, with vacuole formation or cytolysis, and is easy to distinguish from cells at the mitotic stage by a followup examination. In this case, the mitotic cell will stretch out and attach to the bottle in several hours.

The value of the degree of cell growth in the assay as compared to cell growth in the controls reflects two factors: the amount of cells surviving and the rate of surviving cell growth. The criteria for ED_{50} of this method are based on the surviving population without marked morphological change. The time course of progressive cellular degeneration is not emphasized because some compounds could not be categorized by the cytotoxicity scale system of Toplin (7). Theoretically, the ED_{50} determined by this method requires a lower drug concentration than the lethal end-point (LE) of Toplin and requires higher doses than the ID_{50} of Eagle and Foley (4).

Assays performed to date indicate that the ED_{50} of all compounds assayed with HEp-2 carcinoma cell lines is higher than that for WI-38 normal fibroblasts. Consequently, higher doses of compounds are necessary for inhibition of cancer cells *in vitro*.

In Table I the data for five selective compounds (helenalin, damsin, α-santonin, elephantopin, and epitulipinolide) obtained with the microtiter method are compared with the screening data of the Cancer Chemotherapy National Science Center (CCNSC). The concentration required for the inhibition of growth of tumor cell line HEp-2 tested by the present method is not greatly different from data for the same agents screened in KB cells by the CCNSC program (15–18). The differences in these results may be due to the different cell lines, technique, and purity of the compounds.

The variations in the ED_{50} from six experiments with the same lot of helenalin are 0.089, 0.083, 0.091, 0.090, 0.089, and 0.078 mcg./ml. The standard error is about 6%. The ED_{50}'s of testosterone from two experiments are 0.58 and 0.44 mcg./ml., respectively. The results obtained by this microtiter method probably will vary somewhat, but these results with helenalin indicate that the ED_{50} determinations should be within $\pm 15\%$ limits of the mean.

Dimethyl sulfoxide appears to be a useful, relatively nontoxic solvent for many of these rather insoluble compounds. All the test samples in this report were readily dissolved in dimethyl sulfoxide.

Several important factors will influence the observations, such as uneven cell distribution, uneven sample size or dilution, and too high or too low a pH of the medium, but these factors can be minimized by careful pipeting and mixing of the cells before distribution. The test cells will sediment upon standing and must be distributed promptly to prevent large variations in the number of cells in each well.

Beside being simple, rapid, and economical, there are other advantages provided by the method. First, it offers a method of direct investigation of the selectivity of antitumor compounds against normal, carcinomatous, and tumor virus-transformed human cells. Also, viruses have become increasingly likely candidates as causal agents of tumors, for example, the Epstein-Barr virus in Burkitt's lymphoma (13, 14, 19, 20), so the potency of a drug to inhibit the growth of virus-transformed cells, such as Raji cells which are derived from Burkitt's lymphoma, becomes an important subject for investigation.

A second advantage is that bacterial and fungal contamination is rarely encountered with the microtiter technique, in part because it is not necessary to change the culture medium to complete the assay and because only a small amount of medium is used for assay.

A third advantage is that the test requires only a minute quantity of test sample and is conducive to large-scale use. Other advantages are that the technique facilitates direct comparison of at least three variables at different concentrations, it allows replicate results, almost any cell type can be used, and controls are strictly comparable.

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▲ To whom inquiries should be directed.