APPLICATION OF A TISSUE CULTURE MICROTITER TEST FOR THE DETECTION OF CYTOTOXIC AGENTS FROM NATURAL PRODUCTS

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A method is described by which the growth inhibitory effects of cytotoxic compounds and fermentation broth cultures on adherent tumor cell lines can be quantitated. Cells are seeded into 96-well microtiter plates and 16 hours later the test compounds or broths are added to the wells. Cell growth is measured after three days (B16 mouse melanoma cells) or six days (HT-29, human colon carcinoma cells) by first fixing adherent cells, staining with Giemsa stain, washing away excess stain, then solubilizing stained cells with HCl. Absorbance is determined using a microELISA spectrophotometer and the data are transferred to and analyzed by a computer. The assay is rapid and reproducible and can be used to identify fermentation broths with cytotoxic components. Addition of DNA into the assay mixture (cells plus compound) inhibits the cytotoxic activities of certain DNA-reactive agents. The results of this study demonstrate the application of this assay system for primary and secondary evaluation of fermentation broths for *in vitro* antitumor activity.

A number of natural products have been shown to have significant activities against human malignancies[1]. These drugs have been discovered as a result of a variety of screening protocols. A critical factor, therefore, in the discovery of novel natural products with antineoplastic activity is the quality of the screening techniques employed. Screening methods should be rapid, reproducible, inexpensive and help select compounds for advanced testing, *i.e.* predict for a higher probability of clinically useful anticancer activity[2].

Although a variety of *in vitro* and *in vivo* screening systems have been reported, among the most useful are *in vitro* cytotoxicity assays employing malignant cell lines[2,3]. In these protocols, natural products are screened for potential activity by determining the cytotoxicity of various dilutions of broths or extracts against tumor cell such as P388 mouse leukemia or B16 mouse melanoma cells. The principle advantage of these methods is that they directly assay cytotoxicity against cancerous cell lines. They are also reasonably rapid and inexpensive.

Nevertheless, current *in vitro* cytotoxicity methods employ relatively large numbers of cells, substantial quantities of media and serum, excessive technical manipulation and the labor-intensive evaluation of data is time consuming. They are also relatively difficult to automate. To improve these assays, we have developed a microtiter technique. This allows for more rapid, less expensive, high throughput, automatable and the computer assisted cytotoxicity screening of natural products.

In this report we describe the approach, results with standard agents and results from the random screening of natural products of unknown character. We also describe a rapid procedure of identifying agents that are cytotoxic and bind to DNA in this assay.

Materials and Methods

Cell Lines

B16 (F10) mouse melanoma[4] and HT-29 human colon carcinoma cells[5] were used in this study and maintained as monolayer cultures in Minimal Essential Media (MEM) (Grand Island Biological Co., Grand Island, NY) supplemented in 10% calf serum, 1% penicillin-streptomycin in a 5% CO_2 humidified incubator at 37°C. They were passaged in 75 cm^2 tissue culture flasks and were subcultured weekly using Versene to harvest the cells. Cells used in these studies were shown to be free of mycoplasma by the fluorescent Hoechst 33258 staining method of CHEN[6].

Drugs and Fermentation Broths and Their Preparation

Daunorubicin, mitomycin C, cycloheximide, vincristine, vinblastine, 5-fluorouracil, rifamycin were purchased from Sigma Chemical Co., St. Louis, MO. Actinomycin D was purchased from Calbiochem-Behring, La Jolla, CA. Bleomycin, phleomycin and talisomycin were generously provided by Bristol Laboratories, Syracuse, NY. All other compounds were generously provided by the National Cancer Institute, Bethesda, MD.

All compounds were first solubilized in DMSO at a concentration of 1 mg/ml and were then diluted to the appropriate concentrations in MEM.

Samples of 200 randomly selected crude broths were obtained from fermentation of various soil microorganisms. Crude culture broths were centrifuged at 10,000 × g for 30 minutes to separate mycelia. The resulting clarified broths were stored at 4°C and assayed within 48 hours. Dilutions of the fermentation broths were made in MEM. Partial characterization of a selected group of broths was achieved by their antimicrobial profile and physico-chemical characteristics.[7,8,9] This system has been developed for the identification of known antibiotics within fermentation broths evaluated in our natural products screening program.

Cytotoxicity Assays in 96-Well Microtiter Plates

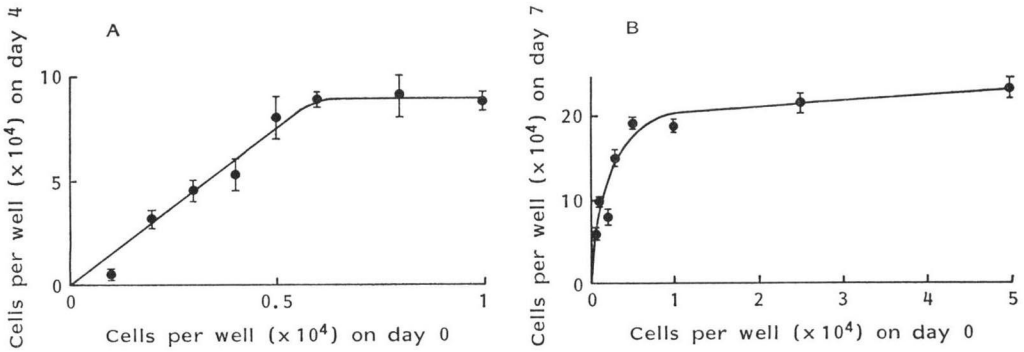
Cells were harvested from monolayers and centrifuged at 1,000 × g for 5 minutes and resuspended in MEM. The cell concentration was determined by hemocytometer counting and the cells were then diluted in MEM to the appropriate cell concentration. Three thousand (B16) and four thousand (HT-29) cells in 0.1 ml were pipetted into the wells of 96-well microtiter plates (Linbro) using a 5-ml repetitive pipette (Tridak, Brookfield Centrer, CT). Medium without cells was added to one well per plate to serve as spectrophotometric blanks. Plates containing cells were incubated for 16 hours before the addition of drugs or fermentation broths. Drug or fermentation broth at twice the required final concentration was added (0.1 ml) to three wells. Sequential two-fold dilutions were then performed by progressively transferring 0.1 ml of media (in triplicate) using a 40-channel micropipet ("Titertek", Flow Laboratories). Controls (no drug) included appropriate dilutions of media containing the ethanol and DMSO solvent solutions. Cultures were then continued for a further 3 (B16) or 6 (HT-29) days.

In experiments in which calf thymus DNA was incubated with the cells in the presence of drugs or fermentation broth, the following modifications were made. The cells were added to the wells in a volume of 75 μl. Before the addition of the test compound, 25 μl of calf thymus DNA (2.6 mg/ml) in MEM was added to each well. The final concentration of the DNA in each well during the assay was 325 μg/ml. The calf thymus DNA (type I; highly polymerized, Sigma Chemical Co., St. Louis, MO) was prepared as follows. Five mg/ml of DNA was first dissolved in sterile H_2O and solubilized overnight at 4°C. In order to reduce the potential for nuclease and bacterial contamination, the DNA solution was boiled for 20 minutes and then cooled at 22°C for 18 hours. Aliquots were stored at −70°C. Immediately before use in an assay, the DNA solution was thawed and diluted to 2.6 mg/ml in MEM.

Following the appropriate incubation period, culture supernatants were aspirated and the cells were washed one time with 0.1 ml of phosphate buffered saline (PBS) and aspirated. One-tenth ml of

Fig. 1. Growth of B16 and HT-29 cells in the microtiter plate assay.

B16 cells (A) and HT-29 cells (B) were seeded into microtiter plate wells at various cell densities. Following the appropriate growth period (See Materials and Methods), the cells were harvested and counted by hemocytometer.



absolute methanol was added to each well for 10 minutes at room temp to fix the cells. The methanol was then removed and 0.1 ml of Giemsa stain (lot 720559, Fisher Scientific Co., Fairlawn, NJ) (1 stain: 10 saline) was added for 5 minutes to stain the cells and was then aspirated. Cells were washed with 0.2 ml of H_2O to remove unbound stain. Stained cells were solubilized with 0.2 ml of 0.1 N HCl. Plates were placed in a microtiter plate shaker apparatus for 10~20 minutes to allow for thorough mixing of the stain into the HCl.

Absorbances were read by a microELISA (MR580, Dynatech, Alexandria, Va) at a sample wavelength of 600 nm and a reference wavelength of 405 nm. The data were transferred directly into an Apple II Plus computer for analysis. Data were displayed as total absorbance and percent of the control absorbances (relative to untreated cells).

Drug potency is described by the IC_{50}, the concentration of drug required to reduce the absorbance to 50% of that in control cultures (drug-free). Fermentation broth potency is described by the ID_{50}, the dilution of the clarified broth required to reduce the absorbance by 50% of that in control cultures.

Monolayer Clonogenic Assay

Cells were harvested and replated at 5,000 cell/plate in sterile 60 mm×15 mm plates. Plates were incubated for 16 hours to allow attachment of cells to the plate surface. The treatment protocol was as follows. Culture medium was aspirated and 5 ml of culture media containing a drug or fermentation broth was added to cells. Plates were incubated for 3 days at 37°C in a CO_2 incubator; the media was then removed and 3 ml of fresh media was added to the plates which were then incubated for 2 days. Viability was measured by the ability of a cell to form a colony greater than 50 cells. Colonies wer efixed with 0.5% crystal violet in 95% ethanol. Plates were dried and counted with a Biotran III Automatic Count Totalizer (New Brunswick Scientific Co, Edison, NJ). Means and standard deviations of triplicate samples were determined for each drug concentration. The data were analyzed by plotting the log of the survival fraction (number of colonies in drug treated plates/number of colonies in controls) *versus* the drug concentration.

Results

Figs. 1A and 1B show the growth curves of the B16 and HT-29 cells in the microtiter plates are shown as function of the initial concentration of cells plated. As can be seen, there was a linear increase in B16 cell number on day 4 at initial plating densities between 1×10^3 and 6×10^3 cells. Similarly for HT-29 cells, a linear increase in cell number on day 7 was observed for the same range of initial plating densities. Consequently, for subsequent experiments and for incorporation into a standard protocol, B16 and HT-29 cells at an initial density of 3×10^3 and 4×10^3 cells per well, respectively were used. Fur-

Fig. 2. Quantitation of cell density by absorbance measurement in the microtiter plate assay.

B16 cells (A) and HT-29 cells (B) were seeded into 96-well plates at various cell densities. Following the appropriate growth period, cells were fixed and stained and the resulting absorbance reading (at 600 nm) per well was measured. In replicate plate the cell number per well was quantitated by harvesting and counting the cells by hemocytometer counting.

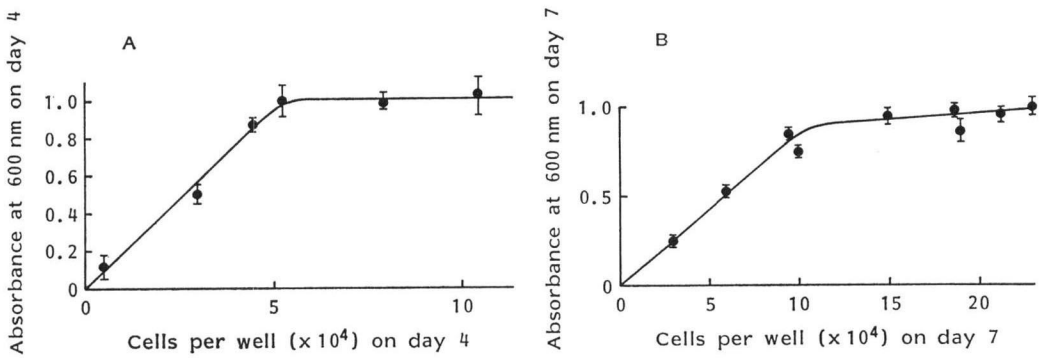
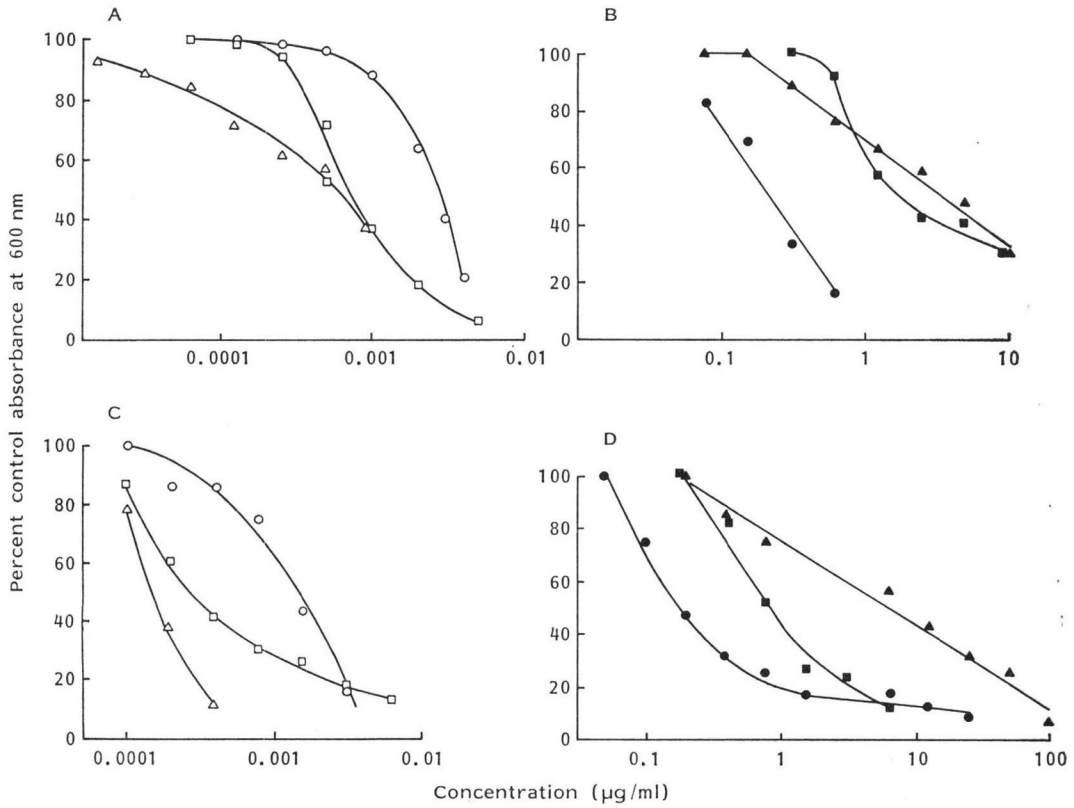


Fig. 3. The effect of cytotoxic compounds on the growth of tumor cell lines as measured in the microtiter plate assay.

B16 cells (A and B) and HT-29 cells (C and D) were treated with echinomycin (○), actinomycin D (□), hedamycin (△), 5-fluorouracil (●), bleomycin (▲) or cisplatin (■).



thermore, Figs. 2A and 2B show that the absorption at 600 nm corresponded linearly as a function of final cell concentration between the densities of 0.5 and 6×10^4 cells per well for B16 cells and 0.3 and 10×10^4 cells per well for the HT-29 cells. The fact that different cell lines have distinctly different

Table 1. IC_{50} values for standard antitumor agents as measured in the microtiter and clonogenic assays.

Compound	IC_{50} (μg/ml) as determined in		
	B16 cells[a] microtiter	B16 cells clonogenic	HT-29 cells microtiter
Hedamycin	0.0006		0.0002
Actinomycin D	0.0007	0.0005	0.0003
Rhodomycin B	0.0008	0.0006	0.0005
CC-1065	0.001		
Echinomycin	0.003	0.002	0.002
Dnacin B_1	0.008		
Rubeomycin A_3	0.008		
Vincristine	0.008	0.007	0.004
Pyrromycin	0.01		
Sibiromycin	0.01	0.007	
Daunorubicin	0.01	0.008	0.009
Kidamycin	0.02		
Olivomycin	0.1	0.06	
Cycloheximide	0.1		
Geldanamycin	0.1		
Gilvocarcin V	0.1	0.01	0.06
Streptonigrin	0.2		
Anthramycin	0.2		0.09
5-Fluorouracil	0.2		0.2
Mitomycin C	0.4	0.3	
Phleomycin	1.5		
Bleomycin	1.7	3.0	6.0
Cisplatin	3.5	1.0	1.0

[a] Cell type and assay procedure used.

relationships between absorbance and cell number illustrates the importance of developing standard curves for all cell lines used in this assay.

Figs. 3A and 3B show the concentration-response curves for six known antitumor agents against B16 cells. Note that the potency of each agent varied and that different drugs produced different types of concentration-response curves. These curves were highly reproducible and the standard error in the absorbance measured between triplicate wells at each concentration was from 1 to 10%. In Figs. 3C and 3D, similar curves are presented for the HT-29 cells. In general, rank order potencies and the shapes of the curves for the different drugs were similar for the two cell lines. The IC_{50} concentrations determined for a number of natural products and synthetic agents (5-fluorouracil and cisplatin) against B16 cells and HT-29 cells in the microtiter assay are compared in Table 1. The two cell lines display only slight differences in sensitivity towards their diverse antitumor antibiotics and show a rank order potency among compounds that is virtually equivalent. The slight increase in sensitivity of the HT-29 cells may be the result of the longer incubation time with the compounds for the HT-29 cells (6 days) compared to the B16 cells (3 days). Bleomycin was the only compound tested that demonstrated a greater degree of potency against B16 cells relative to HT-29 cells.

The sensitivity of B16 cells to a number of the compounds in the microtiter assay was compared to the sensitivities measured in a clonogenic assay (see Materials and Methods). As shown in Table 1, 10 out 11 compounds had lower IC_{50} values against B16 cells when measured in the clonogenic assay than in the microtiter plate assay. The increase in sensitivity for the clonogenic assay relative to the microtiter plate assay was less than two-fold for 9 out of 10 compounds. The rank order potencies

Fig. 4. The percentage of the total number of fermentation broths which inhibited B16 cell growth in the microtiter plate assay by 50 % (relative to controls) at various dilutions of the broths.

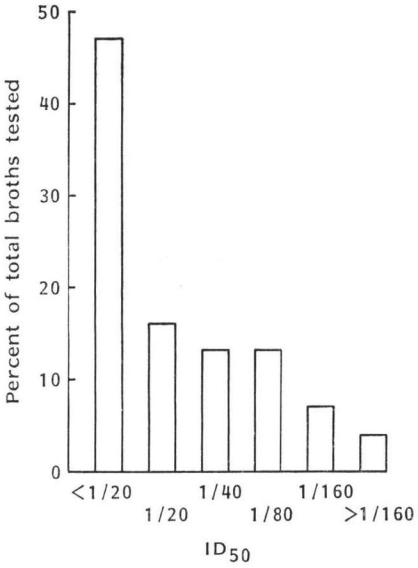
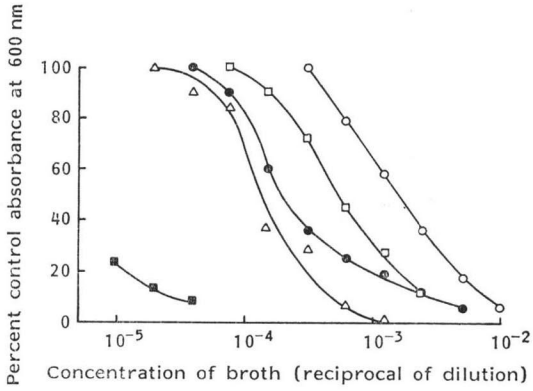


Fig. 5. Effects of selected fermentation broth cultures on the growth of B16 cells.

Broth cultures, AAH188 (○), AAJ004 (□), AAF072 (●), AAJ059 (△), and AAJ046 (■) were incubated with B16 cells as described in Materials and Methods.



measured in each of the respective assays was equivalent for those compounds whose IC_{50}'s were less than 0.01 μg/ml. Of those compounds evaluated in both assay procedures, bleomycin and cisplatin were least active.

To determine the utility of the microtiter assay for screening natural products, 200 fermentation broths were tested for cytotoxicity. Fig. 4 shows the percentage of the total broths displaying activity as a function of the dilution. Approximately 90% of these randomly selected cultures displayed cytotoxic activity at dilutions at and below 1: 80. Clearly, the assay is sensitive and using a cut off ID_{50} dilution at and above 1: 160 to identify cultures with cytotoxic activity probably represents an appropriate compromise between sensitivity and nonspecific cytotoxicity (*i.e.* false positives). Fig. 5 shows the dose-responses obtained when 6 broths were tested in the microtiter assay against B16 cells. The ID_{50} dilutions for these broths ranged from 1: 600 to greater than 1: 100,000.

Another potential advantage of the microtiter assay that derives from the small volumes employed and the sensitivity of the assay is that specific antagonists can be efficiently employed to identify agents with particular biochemical targets. For example, we have defined assay conditions which allow for the identification of cytotoxic compounds with DNA-binding characteristics. Specifically, compounds were incubated with cells in the absence or in the presence of 325 μg/ml of calf thymus DNA. As shown in Fig. 6A, the IC_{50} for rhodomycin B was increased from 0.8 ng/ml to 0.65 μg/ml (an 800-fold difference) when it was assayed in the microtiter cytotoxicity assay in the absence or presence of 325 μg/ml calf thymus DNA, respectively. The addition of DNA into the incubation mixture also decreased the cytotoxic potency of daunorubicin and gilvocarcin V (Figs. 6B and 6C), although the degree of the inhibition of cytotoxicity by DNA differed with each to the three agents (Table 2). The addition of DNA did not however, alter the shapes or slopes of the linear portions of the dose-response curves. Fig. 6D shows that the cytotoxic potency of vincristine was not affected by the addition of DNA into the assay system. Table 2 shows that the addition of DNA dramatically reduced the potency of agents that reportedly bind to DNA such as actinomycin D and echinomycin and had

Fig. 6. Effect of calf thymus DNA on the cytotoxic potency of known cytotoxic compounds.
Rhodomycin B (A), daunorubicin (B), gilvocarcin V (C) and vincristine (D) were incubated with B16 cells in the absence (○) or presence (●) of 325 μg/ml of calf thymus DNA in the microtiter plate assay.

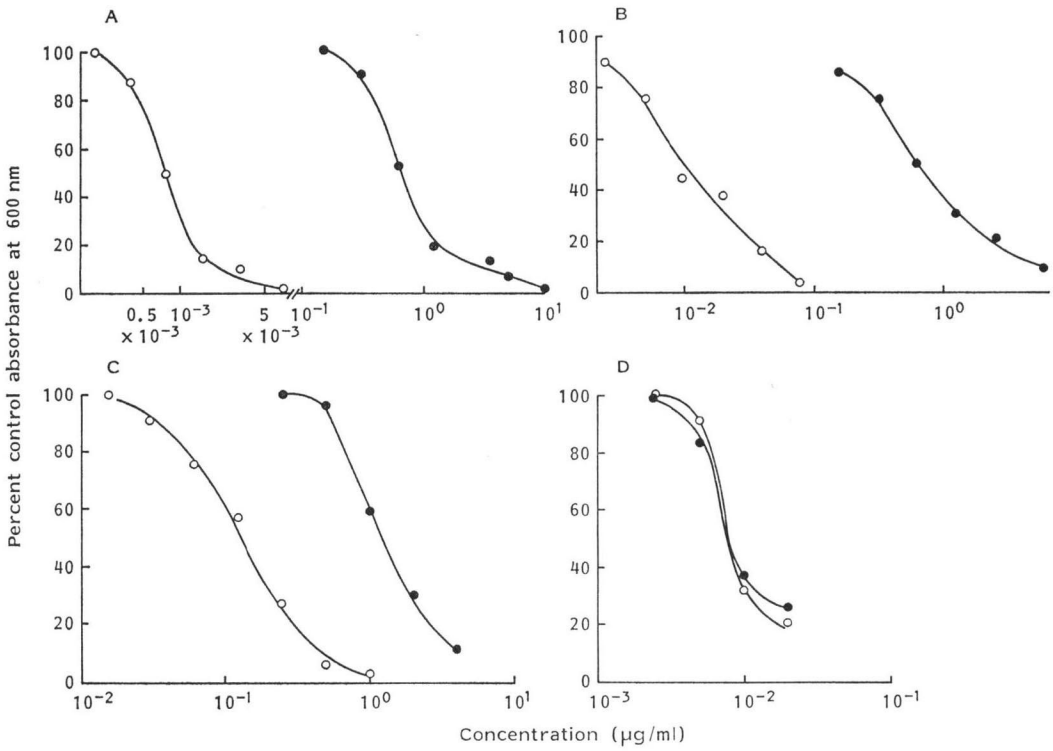


Table 2. Effect of calf thymus DNA on the cytotoxic activity of standard compounds and partially characterized fermentation broths.

Compound	$\frac{IC_{50}(+DNA)^a}{IC_{50}(-DNA)}$	Fermentation broth	$\frac{ID_{50}(+DNA)^b}{ID_{50}(-DNA)}$
Actinomycin	2.8×10^5	AAJ059 (luzopeptin)[c]	$>2\times10^4$
Hedamycin	8.0×10^4	AAJ188 (echinomycin)	$>1.5\times10^3$
Echinomycin	1.0×10^4	AAJ005 (carminomycin)	1×10^3
Rhodomycin B	1.0×10^3	AAH809 (actinomycin)	>700
Daunorubicin	90	AAF046 (actinomycin)	>700
Gilvocarcin V	20	AAH277 (actinomycin)	>200
Olivomycin	15	AAJ072 (anthracycline)	100
Vincristine	1	AAJ004 (virenomycin)	8
Vinblastin	1		
Rifamycin	1		
Cycloheximide	1		

[a] IC_{50} (mg/ml) as determined in the microtiter cytotoxicity assay against B16 melanoma cells; in the presence of 325 μg/ml of calf thymus DNA/in the absence of DNA.
[b] ID_{50} values determined by dilution of clarified fermentation broth.
[c] Fermentation broths were partially characterized by antimicrobial and chemical evaluation, as containing members of known classes of antitumor antibiotics (see Materials and Methods).

no effect on drugs that do not bind to DNA significantly, *e.g.* rifamycin. Furthermore, partially characterized culture broths containing components of classes of known DNA binding agents were affected similarly by DNA in the assay. Note that the broth AAJ004 which was found to contain virenomycins V and M, a gilvocarcin congener, was the least affected of the broths tested. This correlates with the data obtained using a purified sample of the related antibiotic, gilvocarcin V.

Discussion

Primary screening of fermentation broths for potential antitumor activity requires assay procedures which allow for the evaluation of large numbers of samples (200~1,000 per week) with a reproducible end-point obtained within relatively short period (3~7 days). The assay described in this paper fits these criteria and in addition, it is highly sensitive, reproducible and automatable. Moreover, as the final determination is simply the absorption at 600 nm with a microELISA spectrophotometer, the assay can easily be incorporated into numerous laboratory automation systems, assuring rapid, efficient data storage, retrieval and comparative data analysis.

FINLAY *et al.*[10] have reported on a similar type of cytotoxicity assay employing adherent cell lines grown in 24- and 96-well plates. Similar to that demonstrated in our report, these authors concluded that the method was reproducible and offered the advantage that any adherent cell line could be used without the need for tedious cell counting procedures. Our studies go beyond these initial findings to further demonstrate that the microtiter plate cytotoxicity assay is nearly equivalent in terms of sensitivity to the clonogenic cell assay system, which is used routinely to assess the cytotoxic potency of antitumor agents.[11] The comparative data for B16 and HT-29 cells also demonstrate no dramatic differences in their respective sensitivities to the 11 agents studied. Therefore, use of B16 melanoma cells in the microtiter plate system would appear to be a primary screening protocol of appropriate sensitivity and assay duration. Use of a slower growing human neoplastic cell line (HT-29) and tumor cells of defined resistance patterns (to known antitumor agents) in the assay, may be useful in secondary evaluation of cultures originally selected in the primary protocol. Our present study extends these findings in an attempt to use the assay procedure as a screening system to 1) detect fermentation broth cultures which contain molecules with cytotoxic activity and 2) to identify the biochemical properties of these cytotoxic components.

While cytotoxicity is an important activity for a presumptive antineoplatic agent, the identification of the mechanism by which it produces its cytotoxic effects would aid in determining its potential for activity against human neoplasias. For example, the vast majority of clinically useful antineoplastic agents produce their cytotoxic effects through interaction with DNA or interference with DNA metabolism.[12] Therefore, screening systems designed to aid in the identification of specific mechanistic classes of agents are valuable when evaluating compound sources such as fermentation broths. In our studies we have identified a system whereby the cytotoxic potencies of known DNA reactive antitumor agents are inhibited in the presence of double-stranded calf thymus DNA. Similar inhibitory effects by calf thymus DNA were observed when broth cultures (partially characterized as containing members of known classes of DNA reactive cytotoxic agents) were evaluated for cytotoxic activity. Although in our studies we employed a heterogeneous preparation of DNA (calf thymus) to inhibit DNA binding agents, other inhibitors could also be used such as homogeneous preparations of DNA (*e.g.* poly dA-dT), specific nucleosides, sugars, amino acids, *etc.* Such screening protocols would be of utility not only in identifying specifically desired mechanistic classes of cytotoxic agents but also in identifying undesired classes of cytotoxic agents; allowing one to disregard such broths rapidly and efficiently.

In conclusion, this study demonstrates the practical application of a mammalian cell cytotoxicity assay which employs microtiter plates, automatic spectrophotometric quantitation and the capacity for complete automation. The system is highly adaptable to the use of multiple cell types and varying assay protocols. This microtiter plate cytotoxicity assay procedure may 1) be useful in primary and secondary screening protocols for fermentation broths programs; 2) serve as a biological assay for following the isolation of cytotoxic agents in broths and 3) be used in mechanism of action studies.

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