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A microtitre cytotoxicity assay useful for the discovery of fermentation-derived antitumor agents

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Summary. A microtiter cytoxicity assay using mammalian cell lines was developed to detect fermentation-derived antitumor agents. Two murine (AKR, B16) and four human (HTB-31, KB, MOSER, RCA) cell lines were used to evaluate 2000 fermentation broth supernatants. The mammalian cell lines tested showed different spectra for fermentation broth activity, as predicted by responses to known antitumor agents. Data were compared with standard antimicrobial assays historically used to screen for antitumor activity for this set of 2000 broths. There was an overlap of approximately 30% of broths identified as containing in vitro bioactivity by the two assay systems. Sixty-three antimicrobially active broths were tested for activity in P388 in vivo; the cytotoxicity assay predictive rate (40%) was twice that of the antimicrobial assays.

Introduction

A variety of in vitro test systems have been used to detect fermentation broths with potential antitumor activities. Screening systems have included enzymic, antimicrobial, and tissue culture approaches [7, 12, 15]. Recent reviews cite the essential features of an effective prescreen system: the ability to select potentially in vivo active samples, to handle large numbers of samples and to be sensitive and cost effective [1, 2, 5, 6, 9–11, 16].

Recent advances in microtiter technology for tissue culture systems led us to a re-evaluation of cytotoxicity-based prescreens for the detection of antitumor activities in fermentation broths. The progress that has been made in establishing a variety of human tumors in continuous culture also adds a new dimension to cytotoxic compound evaluation. Previous studies have examined the use of KB cells [10, 12–14] and the murine leukemia cells, L1210 and P388 [3–5, 8, 14].

This study utilized four human and two murine cell lines for the evaluation of 2000 fermentation broth supernatants. Results were compared with those obtained with standard antimicrobial assays for the same set of broths, and in vivo P388 murine activities were determined for a subset of broth samples as part of an ongoing fermentation screening process. The development of a microtiter-based cytotoxicity assay provides a rapid, sensitive, cost-effective prescreen system that is reliably predictive for in vivo activity.

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Materials and methods

Cell culture. AKR (murine fibroblast), MOSER (human colorectal carcinoma), and RCA (human colorectal carcinoma) cell lines were obtained from Dr Michael Brattain, Bristol-Baylor Laboratory, Houston, Tex. HTB-31 (human cervical carcinoma) cells were obtained from ATCC, Rockville, Md. KB cells (human nasopharengeal) were obtained from Dr Paul Siminoff, Bristol-Myers, Syracuse, NY. B16-F10 (murine melanoma) were kindly provided by Dr Isaiah Fidler, Frederick Cancer Research Center.

The AKR, B16-F10, MOSER, RCA and KB cell lines were maintained in continuous logarithmic culture in minimum essential medium (Eagle) with Earle's salts (Gibco #320-1090) containing 2 m*M* L-glutamine, penicillin/streptomycin (10 units/ml and 10 μg/ml, respectively), 3 m*M* MEM nonessential amino acids (100X, Gibco 320-1140), and 10% fetal bovine serum (enriched MEM). HTB-31 cells were grown in the enriched MEM containing 0.022% sodium pyruvate (Sigma Chemical, St. Louis, Mo). All cells were incubated at 37 °C in a 5% CO_2, balance air, high-humidity incubator. Cells were harvested by mild trypsinization for passage and experiments.

For the cytotoxicity assay, logarithmically growing cells were harvested by mild trypsinization and suspended in enriched MEM at 2.7×10^4 cells/ml. Each well of a 96-well microtiter plate (Costar #3598) received 150 μl of this cell suspension through a Dynatech Auto III dispenser/dilutor (#002-971-0900, Dynatech Laboratories, Inc., Alexandria, Va).

Plates containing 4×10^3 cells/well were incubated at 37 °C, 5% CO_2 overnight to permit cell attachment prior to broth addition.

Fermentation broth assay. Fermentation broths were centrifuged to remove particulate material and 50 μl broth supernatant was transferred to well A of a microtiter plate. Ten broths were added to each plate and two rows served as controls, each receiving 50 μl medium. Broths were seri-

ally diluted four-fold from row A to row H by serial transfer of 50 µl with the Dynatech Auto III dilutor. Broths were assayed, therefore, at dilutions of 1:4, 1:16, 1:64, 1:256, 1:1024, 1:4096, 1:16 384 and 1:65 536. Diluted plates were incubated at 37 °C, 5% CO_2, high humidity for 48 h. All assays were performed in duplicate.

Staining and evaluation. Following incubation, medium and detached cells were removed by inverting and shaking the plates. Formalin (10%) in PBS was added to the plates with the Dynatech Dynawasher II (≠011-950-0900), and cells were fixed for 10 min. The fixative was removed by aspiration and plates were air dried prior to staining with 0.09% crystal violet. After a 15-min staining period plates were washed with saline twice and air dried. The stain pattern for each broth was examined visually and a maximum dilution for an approximate IC-50 was determined relative to control well stain density.

Results

Mammalian cell lines were selected for inclusion in this study on the basis of their response to known antitumor agents. Table 1 summarizes the IC-50 values of six clinically effective antitumor agents to the six cell lines that were eventually used for evaluation of the 2000 fermentation broth supernatants. These six cell lines demonstrated spectrums of drug sensitivity different from one another, thus allowing a broad-based analysis of antitumor activity in the fermentation-derived material.

The assay system historically used in our fermentation screening operation is an agar plate zone of inhibition of microbial growth. Four organisms, *Bacillus subtilis* M45 (recombinant deficient), *Bacillus subtilis* H17, *Saccharomyces cerevisia* Sc-1-109, and *Saccharomyces cerevisia* ATCC 9763, were used to evaluate this series of 2000 fermentation broths. Summary data were provided by Dr Kawaguchi, of the Bristol-Myers Research Institute, Tokyo, as part of the normal screening operation. Of the 2000 fermentation broths that were analyzed in the antimicrobial prescreen, 152 were confirmed to be active giving a pass rate of 7.6% (152/2000).

Table 2 indicates the percentage of broths that were active to given dilutions for each cell line. Cytotoxicity at a dilution of 1/1024 or greater was arbitrarily considered active in this study, to best approximate the pass rate obtained in the antimicrobial prescreen.

All cell lines were compared in a pairwise fashion to determine whether similar activity profiles were being obtained by the cell lines. The chi-square analysis indicated

Table 1. IC-50 (µg/ml) of standard antitumor agents

	AKR	B16-F10	HTB-31	KB	MOSER	RCA
Adriamycin	4	0.1	0.02	0.02	16	0.02
Bleomycin	250	250	40	250	250	50
Platinol	16	12	4	5	14	7
Mitomycin C	6	5	4	4	8	1
Vincristine	90	90	0.02	0.6	0.3	0.2
VP-16	35	20	12	20	35	5

Table 2. Percentage of fermentation broths with bioactivity at various dilutions

Dilution	AKR	B16-F10	HTB-31	KB	MOSER	RCA
1/4	80.7	87.7	76.5	86.9	69.0	88.8
1/16	44.8	50.7	47.8	52.2	34.1	42.2
1/64	24.1	26.5	28.8	20.4	14.9	17.1
1/256	11.9	12.8	14.3	7.7	7.0	8.6
1/1024	4.2	6.2	7.9	3.8	3.5	4.1
1/4096	1.8	3.4	5.4	2.4	2.0	2.2
1/16384	1.2	2.4	3.6	1.8	1.3	1.5
1/65536	0.6	1.8	2.4	1.4	0.9	1.1

Table 3. Comparison of antimicrobial and cytotoxicity assays

	Antimicrobial	AKR	B16-F10	HTB-31	KB	MOSER	RCA
No. of broths active[a]	162	82	122	154	75	68	80
No. of broths in common with antimicrobial assay	–	30	44	33	28	15	22

[a] A zone of inhibition ≥ 15 mm was required for antimicrobial activity. Cytotoxic activity at a dilution of 1/1024 or greater was required for cell line activity

Table 4. Bioactivity profiles of prescreen-selected fermentation broths[a, b]

Broth no.	AKR	B16-F10	HTB-31	KB	MOSER	RCA	P388 in vivo	%T/C in vivo	
								Test	Confirmation
138							+	135	130
182		+	+	+	+		+	140	140
495	+	+		+	+	+	+	135	150
543	+	+							
599	+	+	+	+		+	+	150	140
603						+			
618		+	+						
621			+			+			
633	+	+	+	+			+	140	140
665	+	+	+	+		+			
782			+						
819	+	+	+			+	+	170	160
832	+	+	+	+	+	+			
841	+	+							
845	+	+	+		+		+	125	135
871	+	+	+		+			Toxic	–
887								125	–
934			+						
980		+							
993	+	+	+	+	+				
1017		+					+	140	140
1166							+	140	130
1287			+						
1343		+				+			
1371		+				+			
1375				+					
1377				+					
1383		+							
1553	+	+					+	125	125
1591								125	–
1596	+	+				+			
1626	+	+							
1731		+		+		+	+	130	130

[a] Thirty antimicrobial active broths were inactive in the cytotoxicity assay and the P388 in vivo assay, and are not listed in this table

[b] Bioactivity at a dilution of 1/1024 or greater is considered active in the cytotoxicity assay. Confirmed 125% treated/control increase in life-span is required for P388 in vivo activity

Table 5. Predictive rates for in vivo P388 activity

Test system	No. active in vitro	No. tested in vivo	No. actives in vivo	No. of true-positives[a]
Antimicrobial	162	63	11	17.5
AKR	82	14	6	42.9
B16-F10	122	22	9	40.9
HTB-31	154	14	5	35.7
KB	75	10	5	50.0
MOSER	68	6	3	50.0
RCA	80	11	4	36.4
≥ 2 Cell lines	116	20	8	40.0
≥ 3 Cell lines	68	12	7	58.3
≥ 4 Cell lines	51	10	6	60.0
≥ 5 Cell lines	36	5	2	40.0

[a] % True positive = no. active in vivo/no. prescreen positive tested in vivo X 100

that among the 15 possible cell line pairs, 10 comparisons revealed significant differences ($P<0.003$). The cell line pairs that were not statistically different from one another were AKR/B16-F10, AKR/RCA, B16-F10/RCA, KB/MOSER, and KB/RCA. Further analysis indicates, however, that even for the most closely similar cell lines, AKR and RCA, the activity profiles are actually quite different. Of the 124 fermentation broth supernatants which demonstrated cytotoxic activity in either cell line, only 38 (30.6%) were common to both cell lines, 42 were active on AKR cells only, and 44 were active on RCA cells only (data not shown).

Cell line data were also analyzed for correlation to antimicrobial data in a pairwise fashion by chi-square analysis. McNemar's two-sided test of concordance of the chi-square analysis indicated that there was no correlation of any cell line to the antimicrobial activity profile. Table 3 shows the actual number of broths for a given cell line which were also identified by the antimicrobial assay; it also gives the total number of broths considered active in the assay system.

A group of 63 broths continued beyond the in vitro prescreens to an in vivo P388 evaluation. Dereplication to known activities accounted for most broths not tested in vivo. All fermentation broths tested in vivo were active in the antimicrobial prescreen, as this was part of the normal screening operation. Table 4 presents the cell line activity profiles of this set of 63 broths in the microtiter cytotoxicity assay as well as the P388 in vivo activities. Of the 63 broths, 30 demonstrated no cytotoxic activity and no P388 in vivo activity, and are not detailed in Table 4. Although there are examples of P388 in vivo active broths which were not active in the cytotoxicity assay (broths 138, 1166) the vast majority (9/11) of broths confirmed to be active in vivo were identified by the microtiter prescreen. Predictive rates of in vitro/in vivo activity can be determined from this set of 63 broths. Table 5 demonstrates that activity in any given cell line, as well as activity in multiple cell lines, predicted reasonably well for in vivo P388 activity.

Discussion

This study was undertaken to evaluate the usefulness of a mammalian-cell-based cytotoxicity assay for the detection of fermentation-derived antitumor activities. Microtiter technology offers a system which is rapid and easily semiautomated; if an assay system is sensitive and predictive for in vivo activity, then an efficient, cost-effective prescreen could easily be developed for large screening operations. This report describes the evaluation of 2000 fermentation broths in such a microtiter cytotoxicity assay and compares the results with the antimicrobial profile traditionally used to screen fermentation broths for antitumor activity.

Twenty-eight cell lines were analyzed for response to known antitumor compounds (data not shown), and six were chosen for the prescreen panel on the basis of their differing chemosensitivity profiles. Incubation of adherent cells with various broth dilutions for 48 h proved to be a sensitive assay system for cytotoxic evaluation. Cytotoxic activity was observed for approximately 5% of fermentation broths at a dilution of 1/1024 or greater. Thus it is possible to adjust the pass rate of the prescreen by defining minimal dilution activity levels or by requiring activity in two or more cell lines of a given panel.

One of our primary interests was to develop an assay system which detected a different spectrum of activities from the traditional antimicrobial prescreen and which had a higher rate of prediction for in vivo activity. This study demonstrated that all the cell lines tested were significantly different from the antimicrobial assay, and furthermore that for this set of fermentation-derived samples the cytotoxicity assay prediction rate for P388 in vivo activity was at least twice that of the antimicrobial assay.

A microtiter cytotoxicity assay has been developed which is rapid, sensitive, and easily semiautomated. The cell lines detect a different spectrum of activities in fermentation broths than traditional antimicrobial assays. Cell lines can be chosen for differing response patterns; the flexibility of using cell lines with specific resistance patterns may avoid commonly encountered antitumor antibiotics. A prospective study is being undertaken to determine the efficiency and accuracy of the microtiter cytotoxicity assay in identifying potential antineoplastic compounds in a large fermentation screening operation.

References

1. Bradner WT, Claridge CA (1984) Screening systems. In: Remers WA (ed), Antineoplastic agents. Wiley, New York, p 41
2. Bunge RH, McCready DE, Balta LA, Graham BD, French JC, Dion HW (1978) Fingerprint methods used to identify known antineoplastic agents in culture filtrates. In: Carter SK, Umezawa H, Douros J, Sakurai Y (eds) Antitumor antibiotics. Springer, Berlin Heidelberg New York, p 77 Recent results in cancer research, vol 63
3. Bunge RH, McCready DE, Balta LA, Graham BD, French JC, Dion HW (1979) Rapid identification of known antitumor antibiotics in culture filtrates. Dev Ind Microbiol 20: 393
4. Buskirk HH (1969) Assay of cytotoxic agents with L1210 cells. Proc Tissue Culture Assoc 20: 23
5. Coetzee ML, Ove P (1979) In vitro assays as a screening test for anti-tumour agents. Process Biochem 14: 26
6. Douros JD (1978) National Cancer Institute's Fermentation Development Program. In: Carter SK, Umezawa H, Douros J, Sakurai Y (eds), Antitumor antibiotics. Springer, Berlin Heidelberg New York, p 33 (Recent results in cancer research, vol 63)
7. Foley GE, McCarthy RE, Binns VM, Snell EE, Guirard BM, Kidder GW, Dewey VC, Thayer PS (1958) A comparative study of the use of microorganisms in the screening of potential antitumor agents. Ann NY, Acad, Sci 76: 413
8. Garretson AL, Elespuru RK, Lufriu I, Warnick D, Wei T, White RJ (1981) In vitro prescreens for the detection of antitumor agents. Dev Ind Microbiol 22: 211
9. Hanka LJ, Bhuyan BK, Martin DG, Neil GL, Douros JD (1978a) A multi-end point in vitro system for detection of new antitumor drugs. Antibiot Chemother 23: 26
10. Hanka LJ, Kuentzel SL, Martin DG, Wiley PF, Neil GL (1978b) Detection and Assay of antitumor antibiotics. In: Carter SK, Umezawa H, Douros J, Sakurai Y (eds) Antitumor antibiotics. Springer, Berlin Heidelberg New York, p 69 (Recent results in cancer research, vol 63)
11. Hanka LJ, Martin DG, Neil GL (1978c) In vitro methods used in detection and quantitation of antitumor drugs produced by microbial fermentations. J Nat Prod 41: 85
12. Smith CG, Lummis WL, Geiersbach HJ (1959) An improved tissue culture assay. I: Methodology and cytotoxicity of antitumor agents. Cancer Res 19: 843
13. Thayer PS, Gordon HL, MacDonald M (1971a) In vitro growth inhibition by 3837 compounds tested for antitumor activity: Comparison of tumor cell culture and microbial assays. Cancer Chemother Rep [2] 2: 27
14. Thayer PS, Himmelfarb P, Watts GL (1971b) Cytotoxicity assays with L1210 cells in vitro: Comparison with L1210 in vivo and KB cells in vitro. Cancer Chemother Rep [2] 2: 1
15. Weber G (1980) Key enzymes and tumor cell heterogeneity. Antibiot Chemother 28: 53
16. White RJ (1981) In vitro prescreens. In: Fidler IS, White RJ (eds) Design of models for testing cancer therapeutic agents. Van Nostrand Reinhold, New York, p 1

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