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A MAMMALIAN CELL AGAR-DIFFUSION ASSAY FOR THE DETECTION OF TOXIC COMPOUNDS

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ABSTRACT.—A method using murine P-388 leukemia or human HT-29 colon carcinoma cells was developed for the bioautography of potential antitumor agents. Of 18 cancer chemotherapeutic drugs and natural products tested, all were detected by toxicity at 0.01 or 1.0 μg with P-388 cells, and 11 of the 18 were detected at 10 μg or less with HT-29 cells. Bioautography of a crude extract of *Pseudoplexaura wagenaari* and subsequent purification yielded the known compound crassin acetate. With modification, the assay detected specifically toxic DNA-binding agents.

In our search for potential anticancer agents from marine sources, extracts of organisms are screened for toxic activity against cultured tumor cells. Essential for the discovery of antitumor agents present in trace amounts in marine organisms are highly sensitive screening assays for the detection of activity. Bioautographic methods for the detection of antimicrobial agents have proved useful in the isolation of many antibiotics (1–6); however, similar methods for detection of antitumor agents with mammalian cells are not in widespread use. A method to localize toxic activity against cultured tumor cells on analytical tlc plates would be useful in developing solvent systems for subsequent preparative chromatography and identification of common metabolites present in extracts of different organisms (dereplication).

RESULTS AND DISCUSSION

To identify potential antitumor agents in crude extracts of marine organisms, compounds were separated by analytical Si gel tlc and toxicity determined with cells derived from a murine leukemia or human colon carcinoma. Although direct bioautographic tlc assays have distinct technical advantages (6), it was found with murine P-388 cells that a two-layer agar-diffusion method yielded the most reliable results. Before experiments were undertaken to characterize compounds in extracts of marine organisms, the sensitivity of P-388 and HT-29 cells to the toxic effects of known antineoplastic agents was assessed. Because the cell density in the agar-diffusion cultures was greater than the plateau density of HT-29 or P-388 cultures, it was possible that the method would not be suitable for the detection of antiproliferative or cell-cycle-specific agents.

The sensitivity of cultured HT-29 cells to anticancer drugs is shown in Table 1. The concentration of agent that resulted in 50% inhibition of HT-29 cell proliferation ranged from 4 nM (vinblastine) to 4 μM (methotrexate). The plant alkaloids were the most potent antiproliferative agents in this study. IC_{50}'s for these compounds were in the low nM range, and cytotoxic zones were detectable with the agar method at 0.01 or 0.1 μg. *cis*-Platinum(II)diamine dichloride, as an example of an alkylating agent, was also detected as an antiproliferative agent but was not detected with the agar method. Exposure of cells to the majority of DNA-active agents resulted in cytotoxic zones, but these compounds were at least an order of magnitude less active than the plant alkaloids. The least potent class of compounds in the HT-29 agar diffusion assay was antimetabolites. Among those tested, only 5-azacytidine was active.

All of the compounds tested in this study resulted in toxic zones with murine leukemia P-388 cells when 0.01 to 1.0 μg was spotted on Si gel tlc plates (Table 2). In general, the agents were also more potent inhibitors of P-388 cell proliferation in comparison to HT-29 cells. However, adriamycin, mitomycin C, fluorouracil, and cytosine

TABLE 1. Agar-diffusion Detection of Cytotoxic Agents with HT-29 Human Colon Carcinoma.

Compound	IC_{50}		Zone of inhibition (mm)			
	μg/ml	nM	10 μg	1 μg	0.1 μg	0.01 μg
DNA-active agents						
Actinomycin D	0.28 ± 0.04	(223)	ND[a]	ND	ND	ND
Adriamycin	0.02 ± 0.004	(37)		5	ND	ND
m-AMSA	0.22 ± 0.02	(559)	ND	ND	ND	ND
Daunomycin	0.07 ± 0.01	(133)		5	ND	ND
Mithramycin	0.036 ± 0.02	(33)		25	15	ND
Mitomycin C	0.033 ± 0.01	(99)		10	ND	ND
Plant alkaloids						
Colchicine	0.006 ± 0.001	(18)		>40	40	25
Emetine	0.006 ± 0.002	(33)		35	25	ND
Vinblastine	0.003 ± 0.001	(4)		40	25	10
Vincristine	0.006 ± 0.002	(7)		30	15	ND
Podophyllotoxin	0.005 ± 0.001	(11)		>40	>40	25
Antimetabolites						
Cytosine arabinoside	0.016 ± 0.007	(66)	ND	ND	ND	ND
5-Azacytidine	0.93 ± 0.21	(3800)		20	ND	ND
Fluorouracil	0.17 ± 0.05	(690)	ND	ND	ND	ND
Methotrexate	2.0 ± 0.01	(4400)	ND	ND	ND	ND
Thioguanine	0.16 ± 0.006	(960)	ND	ND	ND	ND
Alkylating agent						
cis-Platinum	3.7 ± 0.63	(1240)	ND	ND	ND	ND

[a]ND = no zone detected.

arabinoside (ara-C), were equipotent against cultured P-388 and HT-29 cells, but were at least tenfold less active in the HT-29 agar method. Although many of the agents tested are cell-cycle-specific inhibitors of cell division (7), all were detected with plateau density cultures using the P-388 bioautography method. These results indicate that the P-388 agar-diffusion assay should be a reliable method for detecting potential antitumor agents in crude extracts. In addition, the HT-29 and P-388 cells were exposed to the agents in parallel, so it was unlikely that differences in cytotoxic potency between the two cell types were related to binding to Si gel, diffusibility, or other physical characteristics of the drug. If differential toxicity reflects biochemical or molecular differences between the tumor types, then comparison of cytotoxic activity, similar to the two-tumor cell cloning assay (8,9), may lead to the discovery of agents specific for solid tumors. However, the low sensitivity of HT-29 cells would make them of minimal use for many extracts.

Crude extracts of *Pseudoplexaura wagenaari* Stiasny (Plexauridae) were chromatographed and cytotoxic compounds identified with the P-388 and HT-29 methods. Analytical tlc plates were spotted with 1–10 μg of a crude EtOAc extract of *P. wagenaari*. After elution with EtOAc-heptane (1:1), 8–10 metabolites were visualized by uv absorbance (λ = 254 nm) and/or charring with 2% vanillin in H_2SO_4. A single cytotoxic zone was identified at an R_f of 0.4 (Table 3). This toxic zone corresponded to a uv-absorbing compound which was present in comparatively large amounts in the extract. Subsequent cc of the crude extract was followed by both conventional microtiter assay with P-388 cells and Si gel tlc. The known compound crassin acetate (10,11) was found to correspond with the toxic zone. Crassin acetate inhibited the proliferation of both HT-29 and P-388 cells in culture (IC_{50}'s, mean ± SD, $n = 3$ duplicate determinations, of 0.26 ± 0.10, and 1.0 ± 0.16 μg/ml, respectively).

TABLE 2. Agar-diffusion Detection of Cytotoxic Agents with P-388 Murine Leukemia.

Compound	IC_{50}		Zone of inhibition (mm)		
	μg/ml	nM	1 μg	0.1 μg	0.01 μg
DNA-active agents					
Actinomycin D	0.0002 ± 0.0005	(0.2)	5	ND[a]	ND
Adriamycin	0.04 ± 0.01	(74)	25	17.5	5
m-AMSA	0.09 ± 0.03	(229)	20	10	ND
Daunomycin	0.015 ± 0.004	(28)	20	12.5	10
Mithramycin	0.12 ± 0.006	(111)	30	15	ND
Mitomycin C	1.0 ± 0.01	(3000)	25	5	ND
Plant alkaloids					
Colchicine	0.008 ± 0.001	(24)	>40	40	17.5
Emetine	0.004 ± 0.005	(7)	>40	40	20
Vinblastine	0.006 ± 0.0001	(0.7)	>40	32.5	12.5
Vincristine	0.01 ± 0.0002	(1.2)	>40	32.5	12.5
Podophyllotoxin	0.002 ± 0.0002	(5.9)	40	32.5	12.5
Antimetabolites					
Cytosine arabinoside	0.03 ± 0.01	(1233)	>40	20	10
5-Azacytidine	1.3 ± 0.09	(5000)	30	12.5	ND
Fluorouracil	0.15 ± 0.02	(609)	>40	15	ND
Methotrexate	0.03 ± 0.009	(66)	40	27.5	ND
Thioguanine	0.05 ± 0.001	(300)	>40	>40	>40
Alkylating agent					
cis-Platinum	1.7 ± 0.6	(560)	10	ND	ND

[a]ND = no zone detected.

To confirm that crassin acetate was the active component initially identified by bioautography, the pure compound was chromatographed by thin layer under the same conditions, and the cytotoxicity was determined with both the HT-29 and P-388 agar-diffusion methods. As shown in Table 3, a single cytotoxic zone with an R_f virtually identical to that found with the crude extract was observed.

To determine if compounds that bind to nucleic acids could be selectively detected, exogenous DNA was included in the assay. As shown in Table 4, the addition of DNA to the cell layer blocked or lowered the toxicity of the known DNA-active agents adriamycin, mithramycin, and actinomycin D. Because toxicity of most agents is mediated by cellular uptake, decreased toxicity is probably related to binding to extracellular DNA and decreased effective concentrations of the drug. Thus, it appears possible to detect DNA-active agents in a mixture of toxic compounds present in a

TABLE 3. Bioautography of Crude Extracts of *Pseudoplexaura wagenaari* and Crassin Acetate.

Compound	Amount	Zone (mm)	R_f of zone	R_f of compound
P-388 bioautography				
P. wagenaari	5 μg	14	0.41	—
	1 μg	5	0.41	—
Crassin acetate	5 μg	20	0.41	0.41
	1 μg	9	0.42	0.41
HT-29 bioautography				
P. wagenaari	10 μg	11	0.41	—
Crassin acetate	5 μg	17	0.42	0.42

TABLE 4. Effects of DNA on toxicity of DNA-active Drugs

Compound	Amount	Zone of inhibition (mm)	
		(−)DNA	(+)DNA
Adriamycin	0.5 μg	12	ND[a]
	0.1 μg	8	ND
Mithramycin	1.0 μg	17	8
	0.5 μg	13	6
Actinomycin-D	10.0 μg	13	ND

[a]ND = no zone detected.

crude extract by bioautography in the presence and absence of DNA. It is likely that incorporation of other appropriate salvageable metabolites, as has been previously investigated with microbial and mammalian agar-diffusion assays (12–14), would allow specific detection of an antimetabolite of interest.

The P-388 agar-diffusion method, which detected the toxic activity of all 18 agents tested, was more sensitive than the HT-29 method (11 of 18 detected). Although limits of detection were specific to each compound, results with the P-388 agar-diffusion assay indicate that cytotoxic compounds comprising 0.01–0.1% of the total material in an extract could be detected. Preliminary results with extracts in addition to *P. wagenaari* suggests that cytotoxic compounds comprising less than 0.001% can be detected with the P-388 bioautography method. However, crude extracts of certain organisms that strongly inhibited the proliferation of cultured P-388 cells did not yield detectable zones of inhibition with the P-388 agar-diffusion assay. Thus, the method described offers to advantages over conventional screening techniques, but it has utility in the localization of toxic zones on analytical tlc plates for bioautographic identification of compounds. Although less toxic but potentially interesting compounds may be masked by the toxicity of the major compound, determining the R_f of the predominant toxic compound in several solvent systems is useful in the development of solvent systems for preparative chromatography and dereplication of compounds. In our experience, less active compounds become apparent during subsequent cc followed by conventional microtiter cytotoxicity assays. In conclusion, the current study indicates that this mammalian cell bioautography has utility in the early chemical characterization and dereplication of potential anticancer agents in crude extracts of marine organisms and can be modified with the addition of DNA to detect DNA binding agents.

EXPERIMENTAL

CELL CULTURE.—Cultured cells were maintained at 37° in a humidified atmosphere of 5% CO_2 in air. Uncloned P-388 (15) cells, obtained from Dr. J. Mayo, National Cancer Institute, Bethesda, Maryland, and strain ATCC HTB 38 HT-29 cells (16) purchased from American Type Culture Collection, Rockville, Maryland, were grown in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% horse serum or 10% fetal bovine serum, respectively. Cultures of both cell lines were passaged in antibiotic-free growth medium and were reinitiated at 3-month intervals from frozen stocks (cells suspended in growth medium supplemented with 20% serum and 10% DMSO were frozen at a rate of 1°/min with a programed freezer and stored in liquid N_2) that were free of *Mycoplasma* as determined with Hoechst 33258 stain (17). Gentamicin sulfate (50 μg/ml; Schering Corporation, Kenilworth, New Jersey) was added to growth media used in experiments to determine toxicity of agents in suspension and agar cultures. RPMI 1640 was purchased from Biologos Inc., Naperville, Illinois, and heat-inactivated fetal bovine and donor horse sera were obtained from GIBCO Laboratories, Grand Island, New York.

To assess the antiproliferative effects of agents against P-388 cells, 200-μl cultures (96-well tissue culture plates, Nunc, Denmark) were established at 1×10^5 cells/ml in drug-free medium or medium containing the drug to be tested. After 48-h exposures, P-388 cells were enumerated using 3-(4,5-dimethyl-

thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) (18) as described below. To assess the antiproliferative effects of agents against HT-29 cells, 200-μl cultures (96-well tissue culture plates) were established with 4000 cells/cm^2 in drug-free medium or medium containing the agent to be tested. After 96-h incubations, cells were enumerated with MTT. To quantitate effects of drugs on cell proliferation, 75 μl of warm growth medium containing 5 mg/ml MTT was added to each well, and cultures were returned to the incubator and left undisturbed for 90 min. Formation of reduced formazan was spectrophotometrically quantitated with a microplate reader (570 ± 10 nM band pass filter, Dynatech model MR700, Dynatech Laboratories, Chantilly, Virginia). The absorbance of test wells was divided by the absorbance of untreated wells and normalized as a percent of control. The concentration of agent that resulted in 50% of the absorbance of untreated wells (IC_{50}) was determined by linear regression of logit-transformed data (19). A linear relationship between P-388 or HT-29 cell number and production of formazan was found over the range of cell densities observed in this study.

AGAR-DIFFUSION ASSAY.—To determine the minimum amounts of drug detectable with the agar-diffusion method, 0.01–10 μg of compound was spotted on small pieces of Si gel and air-dried. The pieces were placed with the Si gel face down on a layer of 1.5% Noble agar in PBS-growth medium (20:80 v/v). Material was allowed to diffuse into the agar for 60 min. After the piece of Si gel was removed with sterile tweezers, cells (2×10^6/ml) in 0.3% low-temperature agarose (Type II, mp 37°, Sigma, St. Louis, Missouri) were layered over the surface of the hard agar. The plates were transferred to a cell culture incubator and left undisturbed for 48 h. Areas of cytotoxicity were visualized by gently pipetting 1 ml of a 5 mg/ml MTT solution in growth medium over the surface of the agarose. After 1–2 h at 37°, areas of toxicity were clear, while unaffected areas of the plate were stained a deep uniform purple by the production of reduced formazan.

EXTRACTION AND ISOLATION OF CRASSIN ACETATE.—*P. wagenaari* was collected at a depth of 10 m by SCUBA on 2 July 1987, at Crooked Island, Bahamas. The organism was frozen immediately upon collection for transport to the laboratory. The organism was identified by John Reed and Steve Viada, Division of Biomedical Marine Research, Harbor Branch Oceanographic Institution, and a voucher specimen (IRCZM Catalog Number 012:00403) was deposited in the Indian River Coastal Zone Museum, Ft. Pierce, Florida. The frozen organism (25 g) was repeatedly extracted with EtOAc, the crude extract filtered, and the filtrate concentrated by distillation under reduced pressure to yield 1.56 g of a brown oil. A portion of the crude extract (800 mg) was repeatedly chromatographed under vacuum liquid chromatographic conditions on a Si gel stationary phase (Kieselgel 60H, EM Science) using a step gradient of EtOAc/heptane as eluent. The known compound crassin acetate (310 mg) was eluted with EtOAc-heptane (3:2) and identified by spectroscopic methods.

BIOAUTOGRAPHY.—The extracted material (1–10 μg) was spotted on 5 × 10 cm plastic-backed Si gel plates (DC-Plastikfolien Kieselgel 60 F_{254}, E. Merck, Darmstadt) and developed with EtOAc-heptane (1:1). Lanes of the plate were cut out and overlaid on the 1.5% agar in 90 × 90 mm tissue culture plates. The tlc plate was left on the surface of the agar for 60 min to allow diffusion of material into the agar. After removal of the tlc plate, HT-29 or P-388 cells (2×10^6/ml) suspended on 0.3% low-temperature agarose in growth medium at 37° were overlaid. Cytotoxicity was visualized with MTT staining after plates were incubated 48 h in a tissue culture incubator.

DRUG SOURCES.—4′-(9-Acridinylamino)methane-sulfon-*m*-anisidide (*m*-AMSA) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. All other drugs were obtained from Sigma. *m*-AMSA and other drugs were used as obtained from the supplier without further purification.

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