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Comparison of *in Vitro* Methods for Assessing Cytotoxic Activity against Two Pancreatic Adenocarcinoma Cell Lines[1]

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ABSTRACT

Several models of pancreatic adenocarcinoma are now available for experimental evaluation of newer chemotherapeutic agents. The present study represents an attempt to develop a rapid *in vitro* screening technique that would allow prediction of cytotoxic activity (or lack thereof) as reliably as the clonogenic or colony formation assay. To this end, seven drugs (cisplatin, dactinomycin, doxorubicin, 5-fluorouracil, menogarol, mitoxantrone, and streptozocin) were tested against two pancreatic adenocarcinoma cell lines using a standard colony formation assay and a 24-hr microcytotoxicity assay. The cell lines tested were PANC-1, of human poorly differentiated pancreatic adenocarcinoma origin, and WD PaCa, of hamster well-differentiated pancreatic adenocarcinoma origin. The dose-survival curves and resulting determinations of drug dose (μg/ml/1-hr exposure) at which there is a 50% inhibition of survival as compared to controls were compared for the two cell lines by each assay system. Lack of correlation of the two assays and considerable interdrug and inter-cell line variation were found. In addition, the microcytotoxicity assay was felt to underestimate the *in vitro* drug sensitivity of PANC-1 to three drugs (dactinomycin, doxorubicin, and mitoxantrone) and of WD PaCa to two drugs (5-fluorouracil and mitoxantrone). Despite the possible utility of the microcytotoxicity assay with other experimental models, the colony formation assay technique appears to provide the most reliable *in vitro* assessment of antineoplastic activity for pancreatic adenocarcinoma cell lines and should continue to be the standard to which other assay systems are compared.

INTRODUCTION

The need for experimental evaluation of newer agents and modalities of therapy of pancreatic cancer is emphasized by its increasing incidence (9) and the inability of available treatment to offer meaningful extension of survival to most patients (5). Assays of cultured malignant cells and cell lines permit relatively efficient and economical screening for chemotherapeutic activity when compared to *in vivo* assay systems.

The present study represents an attempt to develop an *in vitro* assay for pancreatic adenocarcinoma cell lines that would provide more rapid screening than the CF[3] assay permits. Roper and Drewinko (11) have carried out a systematic investigation of various methods evaluating drug-induced cellular damage and concluded that the CF assay provides "the most reliable, dose-dependent index of cell lethality." However, they did not compare CF to the MC assay, as developed by Kornblith *et al.* (6, 7). The MC assay has the advantage of using a 24-hr culture of adherent cells in Terasaki plates with counting of individual cells, whereas the standard CF assay requires 5 to 14 days of culture in semisolid medium to allow the formation of identifiable colonies and considerably greater technician time for processing, incubating, and reading cultures.

Seven drugs were tested against 2 adherent pancreatic adenocarcinoma cell lines (one of human and one of hamster origin) by both MC and CF assays. We have reported previously that the drug sensitivities as demonstrated by *in vivo* testing and CF assay for 2 hamster pancreatic cancer models show a close correspondence (2). It was anticipated that the MC assay would be less sensitive than the CF assay, because only short-term cytotoxic effects would be detected by the MC assay. While a dose dependence could be demonstrated by the MC assay with most drugs, the degree of discordance between the 2 assay systems was considerable, especially when interdrug and inter-cell line comparisons are made, and correlation with CF was poor.

MATERIALS AND METHODS

Cell Lines. PANC-1 is a well-established and well-characterized human pancreatic carcinoma cell line derived from a poorly differentiated ductal origin by Lieber *et al.* (8). Our original samples of PANC-1 were obtained from the American Type Culture Collection and have been maintained in continuous culture in our laboratory for about 2 years. *In vitro* doubling time under conditions of routine passage is about 24 to 36 hr.

WD PaCa is a cell line adapted in our laboratory from a transplantable solid tumor model of a well-differentiated pancreatic ductal adenocarcinoma originally induced in inbred Syrian hamsters by the carcinogen, *N*-nitrobis(2-oxopropyl)amine. The solid tumor model was developed by Dr. Scarpelli and Dr. Rao, who kindly supplied samples of it to us and who have described it in detail elsewhere (12). In tissue culture, WD PaCa grows as an adherent monolayer with an approximate doubling time of 36 hr. WD PaCa has been grown by us for over 2 years in culture, where it retains mucin positivity and typical epithelial morphology with occasional signet ring cells.

Culture Conditions. Cell lines are maintained in Roswell Park Memorial Institute Tissue Culture Medium 1640, supplemented with 10% heat-inactivated fetal bovine serum, glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Grand Island Biological Co., Grand Island, N. Y.). For both assays, the concentration of fetal bovine serum is raised to 15%; for the CF assay, a final concentration of 0.9% methylcellulose is used for plating. Both cell lines form colonies in the methylcellulose medium. Cell lines are monitored periodically by transmission electron microscopy for mycoplasmal and viral contamination and by karyotyping (to rule out cross-contamination with other cell lines). Cell cultures are also *Mycoplasma* free by the Hoechst staining method (3).

Dose-Survival Assays. For dose-survival determinations, cells are

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[3] The abbreviations used are: CF, colony formation; MC, microcytotoxicity; CCED, cell culture-equivalent dose.

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harvested in late-log or early-stationary phase growth by short exposure to 0.25% trypsin. Cells (1 to 2 × 10^6) were incubated at 37°, 5% CO_2, for 1 hr with drug concentrations of 0, 0.01, 0.1, 1.0, 10, and 100 μg/ml. Drug dilutions were freshly prepared on the day of assay by dissolving the drug being tested (with the exception of menogarol) in Hanks' balanced salt solution to a concentration of 1.0 mg/ml, followed by filter sterilization, and serial dilution to concentrations of 200, 20, 2, 0.2, and 0.02 μg/ml. Mixtures (1:1) of drug solutions and cell suspensions were used to give the desired final drug concentrations. The initial solution of menogarol was prepared in 0.02 M lactic acid, with further dilution as described above. After incubation, cells were washed free of drug and resuspended in assay medium. CF assays were carried out in quadruplicate, using cell concentrations of 1 to 2 × 10^3 cells/ml in methylcellulose medium in 35-mm culture dishes (Lux, Naperville, Ill.) with enumeration of colonies (>50 cells) using an inverted microscope after 5 to 10 days of incubation at 37°, 5% CO_2. MC assays were performed by plating 100 cells/well (16 replicates/drug concentration) in Terasaki microtiter plates (Falcon Plastics, Oxnard, Calif.) and incubating in a 37°, 5% CO_2, humidified atmosphere for 24 hr. MC scoring was accomplished by removing dead cells by washing, staining residual adherent cells with Giemsa stain, and counting (by inverted microscope) the number of cells per well. Results for each assay are expressed as the surviving fraction or *T:C* ratio ($\bar{X}$ treated:$\bar{X}$ control) for each drug concentration. Drug doses (μg/ml/1-hr exposure) at which there is a 50% inhibition of survival as compared to controls were determined from the log dose-survival curves. The following agents were tested and compared: cisplatin [*cis*-diamminedichloroplatinum(II)]; dactinomycin; doxorubicin; 5-fluorouracil; menogarol (7-*O*-methylnogarol, NSC 269148); mitoxantrone (dihydroxyanthracenedione, NSC 301739); and streptozocin (NSC 85998). (The last 3 named agents were obtained courtesy of the Natural Products Branch, Developmental Therapeutics Program, National Cancer Institute. The other agents were obtained commercially.)

RESULTS

The results of the dose-survival determinations for a number of standard and experimental agents are summarized in Table 1. The response curves of doxorubicin and mitoxantrone are shown in Charts 1 and 2. Using both the CF and MC assays, the dose-survival curves for cisplatin and 5-fluorouracil (not shown) are fairly flat until the dose of 100 μg/ml is reached; only then is 70 to 90% inhibition seen for these agents. Streptozocin does not inhibit either cell line at any dose level, and it can best

Table 1
Comparison of CF and MC assays

Drug	Cell line	ID_{50}[a] CF	ID_{50}[a] MC	ID_{50} ratio (MC:CF)
Cisplatin	PANC-1	13.0	62	4.8:1
	WD PaCa	2.8	>100	>35:1
Dactinomycin	PANC-1	0.16	23	144:1
	WD PaCa	1.1	7.6	6.9:1
Doxorubicin	PANC-1	0.025	11.0	440:1
	WD PaCa	4.0	100	25:1
5-Fluorouracil	PANC-1	27	>100	>3.7:1
	WD PaCa	12	>100	>8.3:1
Menogarol	PANC-1	0.7	5.0	7.2:1
	WD PaCa	1.5	3.7	2.5:1
Mitoxantrone	PANC-1	0.013	>100	>7692:1
	WD PaCa	0.93	>100	>107:1

[a] ID_{50}, drug dose (μg/ml/1-hr exposure) at which there is a 50% inhibition of survival as compared to controls.

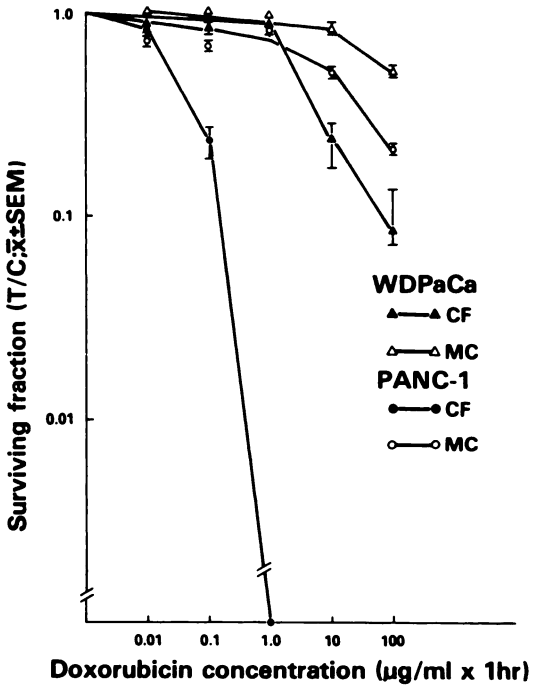

Chart 1. Dose-survival curves of doxorubicin against WD PaCa and PANC-1 cell lines comparing MC and CF assays.

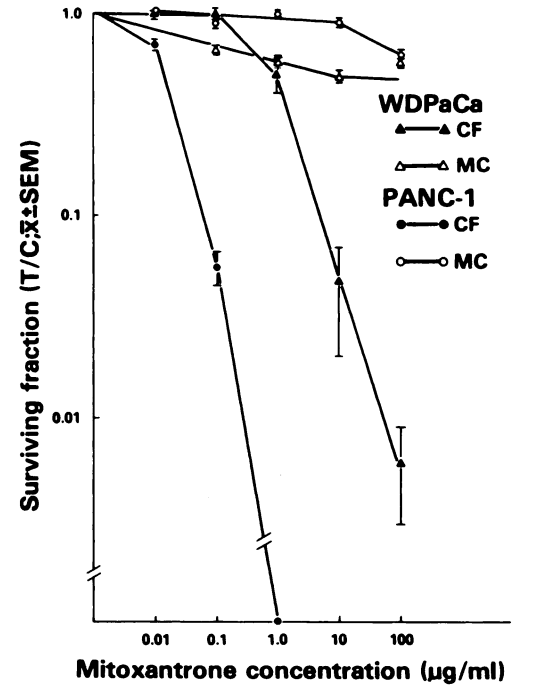

Chart 2. Dose-survival curves of mitoxantrone against WD PaCa and PANC-1 cell lines comparing MC and CF assays.

be characterized as "ineffectual," as described by Drewinko *et al.* (4).

The concept of the CCED as developed by Wilkoff *et al.* (14) is used to deal with the problem of defining *in vitro* drug sensitivity. While a culture dose producing a 90% cell kill of less than or equal to the CCED, were it precisely known, would provide a more accurate prediction of antitumor activity *in vivo*, we have chosen a culture dose producing a 50% cell kill ≤ the CCED as

Table 2
Estimated CCEDs

Drug	Estimated CCED (μg/ml)	Ref.
Cisplatin	1.94	1
Dactinomycin	0.182	1
Doxorubicin	1.56–3.84	1
5-Fluorouracil	16.33	1
Menogarol	32.5	10
Mitoxantrone	52.0	*[a]

[a] Estimated from data supplied by the Developmental Therapeutics Program, National Cancer Institute.

suggestive of drug sensitivity. Some overestimation of drug sensitivity is not considered a problem, since confirmatory *in vivo* testing will have to be performed later; rather, our concern is that too narrow of a definition of sensitivity may exclude potentially active agents.

CCEDs, as outlined in Table 2, were estimated from published pharmacokinetic data of the concentration × time (or areas under the plasma drug elimination curves) in humans for cisplatin, dactinomycin, doxorubicin, and 5-fluorouracil (1), and from the 10% lethal doses in animal studies for menogarol, mitoxantrone, and steptozocin using the method of Wilkoff *et al.* (14), wherein the CCED is defined as 1.3 times the 10% lethal dose in mg/kg, converted to μg/ml. (This method assumes complete and instantaneous equilibration of a drug in total body water, which is estimated to be 75% of body weight.)

Using the criteria outlined above, the potential sensitivity of PANC-1 to dactinomycin, doxorubicin, and mitoxantrone and of WD PaCa to 5-fluorouracil and mitoxantrone would have been missed by the MC assay. Of the agents tested, the only one for which activity was detected by the MC assay (as well as the CF assay) was menogarol.

DISCUSSION

Two *in vitro* assay systems, CF and MC, have been compared in 2 cell lines of pancreatic adenocarcinoma. While dose-response relationships could be demonstrated with the MC assay for all except the most ineffectual agent (*i.e.*, streptozocin), this assay generally underestimated responsiveness in a manner that was unpredictable and showed poor correspondence to the CF assay. The most serious problem with the MC as compared to the CF assay was that the magnitude of the discordance was quite variable both between cell lines, even when the same drug was studied, and between drugs, which is perhaps less surprising considering that the drugs studied here represent several different mechanisms of action. The reason for the discrepancy undoubtedly has to do with the different end points each assay assesses; *i.e.*, CF measures the reproductive capacity of clonogenic cells, and MC measures short-term survival of adherent cells. It has been argued that in fact clonogenic renewal is the only relevant end point for *in vitro* testing of antitumor agents (13). Nevertheless, if there had been a constant predictable ratio of activity shown in the MC as compared to the CF assay, then the MC assay might have had some utility in drug testing with pancreatic adenocarcinoma cell lines. Longer incubations (*e.g.*, 2 to 3 days) prior to enumeration of MC assays were not tested but might improve the sensitivity of the MC assay. However, due to our disappointing experience with the 24-hr MC assay, we are reluctant to undertake the detailed testing that would be required to explore further refinements of the MC assay.

It should be noted that Kornblith and Szypko (7) have found the 24-hr MC assay to have predictive value for the clinical response (which is the ultimate test of any assay system) of human malignant gliomas to 1,3-bis(2-chloroethyl)-1-nitrosourea. However, it is quite possible that the usefulness of the MC assay is limited to certain drugs against certain types of tumors. Thus, the relative merits of the MC assay even with brain tumors may have to await ascertainment by comparison with CF assays and by testing with other cytotoxic agents.

On the basis of the present study, we feel that the CF assay still provides the most reliable assay system for pancreatic adenocarcinoma cell lines and probably for other cell lines as well. The CF assay should continue to be the standard to which other, especially short-term, assays should be compared.

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