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Rapid chemosensitivity testing of human lung tumor cells using the MTT assay*

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Summary. Numerous procedures have been described which test the chemosensitivity of tumor cell lines. A major disadvantage of most of these assays is that practical limitations prevent the testing of more than a few variables. We have adapted a rapid and efficient colorimetric assay for testing the chemosensitivity of human lung tumor cells. In this assay, a tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, MTT) is converted to a colored formazan product by enzymes active only in living cells. The MTT assay may be carried out entirely in 96-well microtiter plates, so that large experiments examining a number of variables can be readily performed. Thus, drug concentration, time of exposure to drug, length of assay, and cell density can be varied and tested. Moreover, the simplicity of this assay allows simultaneous testing of multiple drugs on multiple cell lines. Finally, the MTT assay is useful for monitoring the development of multidrug-resistant cells in culture.

Introduction

The need for rapid efficient in vitro assays for assessing drug cytotoxicity has accompanied the ever-expanding repertoire of antineoplastic agents. Assays currently in use include those which measure clonogenic cell survival [21, 22] and biochemical assays which measure radiolabelled precursor incorporation into nucleic acids or protein [4, 19]. The effect of drugs on cell proliferation may also be tested by directly counting cells maintained in liquid culture. Large-scale manual counting by trypan blue exclusion is extremely laborious and time-consuming. Therefore, alternative rapid assays to measure cell survival and thus indicate the relative sensitivity of tumor cells to antineoplastic agents are desirable. Landegran [12] has described a method by which cell numbers can be estimated by measuring the activity of the ubiquitous lysosomal enzyme, hexosaminidase. However, since serum contains significant levels of this enzyme it is necessary to remove the tissue culture medium before assaying the enzyme activity. A simpler enzyme activity-based assay is the MTT assay described by Mossman [18]. In this assay, a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, MTT] is reduced to a colored formazan product by reducing enzymes present only in living metabolically active cells [24]. Previous investigators have shown that dead cells are unable to reduce MTT within 30 min of lysis [18]. Thus, the color reaction is a measure of cell viability. Tetrazolium salts have also been used to improve detection of drug cytotoxicity in soft agar colony formation assays [1] and have the advantage over many other stains [10] of being nonlethal [11], allowing cells to be recovered if desired. In the present study, the adaptation of the MTT assay to measure the effect of antineoplastic agents on human lung tumor cells is described.

Materials and methods

Cell lines. Human lung tumor cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (4 m*M*), sodium pyruvate (1 m*M*) and 2-mercaptoethanol (50 μ*M*). Cells were cultured in the absence of antibiotics at all times. Small cell lung cancer (SCLC) cell line NCI-H69 was kindly provided by J. Minna (NIH, Bethesda). Squamous cell line BEN [7] was provided by Prof. A. M. Neville (Ludwig Institute for Cancer Research, London, UK). Large cell lung tumor cell line SK-LC-6 [3] and lung adenocarcinoma A549 were obtained from the Human Tumor Cell Laboratory of the Sloan-Kettering Institute for Cancer Research (Rye, NY). Large cell line QU-DB was established in this laboratory and has been characterized elsewhere [6]. A multidrug-resistant variant of NCI-H69 was established in our laboratory by culturing these cells in increasing concentrations of adriamycin and is designated H69-AR7 (Cole S. and Mirski S., manuscript in preparation).

The Chinese hamster ovary (CHO) cell line AUX B1 and its multidrug-resistant variant CH^RC5 [14] were kindly provided by Dr V. Ling (Ontario Cancer Institute, Toronto). These cell lines were maintained in DMEM medium supplemented with 10% FBS.

All cell lines were tested for mycoplasma contamination using the DAPI (4′-6-diamidino-2-phenylindole) DNA-binding assay [20] and found to be negative.

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MTT assay. Drugs were provided by the Ontario Cancer Treatment and Research Foundation, Kingston Regional Centre. Drugs were dissolved in physiological saline and filtered through a 0.22-μm filter. Drugs were further diluted to 1 m*M* with sterile phosphate-buffered saline (PBS) and stored frozen at −20 °C or used immediately. All subsequent dilutions were made with tissue culture medium.

Cells to be tested were harvested, in the case of adherent cell lines (non-SCLC and CHO) by incubation in 0.02% EDTA in phosphate-buffered saline for 10 min at 37 °C and in the case of suspension cultures (SCLC), by centrifugation. After resuspension in fresh medium, cells were plated in a volume of 100 μl at 5×10^4 cells/well, unless otherwise specified, in 96-well microtiter plates (Linbro 76-032-05) using a multichannel pipette. The plates were incubated at 37 °C for 2–4 h to allow cells to reattach and reequilibrate. Without removing any medium, drugs were then added at twice the final concentration in a volume of 100 μl. In some experiments, cells were exposed to adriamycin for a time and then the drug was removed using a multichannel pipette followed by addition of drug-free medium. The cells were incubated for selected times at 37 °C. At 3 h before the end of drug exposure, 100 μl medium was removed from each well and 25 μl MTT (Sigma) solution (2 mg/ml in PBS) was added. Then, at the end of drug exposure the enzyme reaction was stopped by the addition of 1 *N* HCl:*i*-propanol (1:24) (100 μl/well) followed by thorough mixing with a multichannel pipette. The plates were read on a Flow Multiskan MC plate reader at 540 nm. All drug concentrations were tested in four to five replicate wells on each plate, and each experiment was repeated at least twice. Controls included wells with cells but no drugs and wells with medium and the highest drug concentration but no cells.

Results

To determine the relationship of cell number to absorbance values, a standard curve was generated for each cell line. The results for SCLC cell line NCI-H69 are shown in Fig. 1. For this cell line, linearity extended from 1×10^5 cells/well to approximately 8×10^2 cells/well. This range of linearity generally applied to the other cell lines (results not shown).

NCI-H69 cells were exposed to various concentrations of adriamycin and assessed for viability using the MTT assay 6, 24, 48 and 72 h later. The apparent sensitivity of these cells increases with time as seen in Table 1. For example, after 6 h toxicity was seen at 6 μ*M* drug, while after 72 h toxicity was observed at 0.2μ*M* adriamycin. Similar results were obtained at 72 h even if the drug was removed after 24 h (Fig. 2).

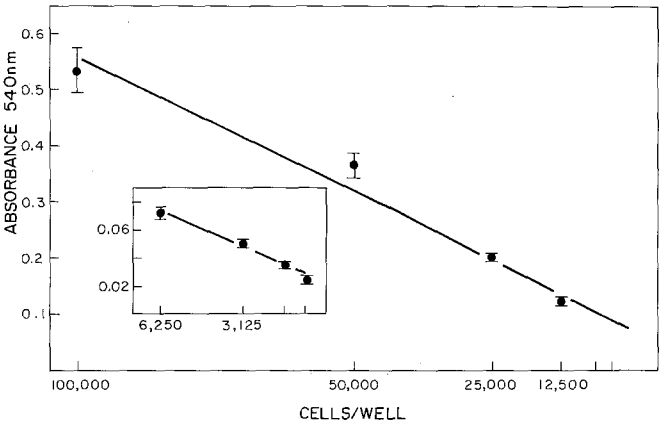

Fig. 1. Linearity of the MTT assay with small cell lung cancer cell line NCI-H69. Each *point* represents the mean of five determinations ±SD

The effect of adriamycin on four human non-small cell lung tumor cell lines is shown in Fig. 3. Lung adenocarcinoma A549 and squamous cell lung carcinoma BEN were quite insensitive to adriamycin, as cytotoxicity was observed only at concentrations greater than 2 μ*M*. Large cell lines SK-LC-6 and QU-DB were much more sensitive, with cytotoxicity seen at concentrations of less than 0.2 and 0.6 μ*M*, respectively.

The effects of mitoxantrone, cyclophosphamide, daunomycin and nitrogen mustard on A549 cells and NCI-H69 cells were tested, and the results are shown in Fig. 4. Cyclophosphamide had no effect on viability of either cell type at the concentrations of drug tested. Nitrogen mustard and mitoxantrone were active on NCI-H69 cells at concentrations greater than 0.2 μ*M*, while cytotoxicity in A549 cells was not observed until concentrations of greater than 6 μ*M* were used. Thus, there is about a 30-fold difference between the sensitivity of these cell lines to these

Table 1. Effect of adriamycin on NCI-H69 cell viability

Drug incubation time (h)	μ*M* Adriamycin					
	0	0.2	0.6	2	6	20
6	0.324 (±0.035)	0.289 (±0.031)	0.296 (±0.029)	0.294 (±0.025)	0.257 (±0.020)	0.268 (±0.022)
24	0.325 (±0.027)	0.311 (±0.030)	0.288 (±0.014)	0.249 (±0.024)	0.183 (±0.010)	0.152 (±0.012)
48	0.314 (±0.051)	0.277 (±0.039)	0.250 (±0.024)	0.214 (±0.027)	0.160 (±0.021)	0.093 (±0.018)
72	0.632 (±0.043)	0.491 (±0.013)	0.423 (±0.015)	0.291 (±0.007)	0.163 (±0.010)	0.107 (±0.005)

Cells were plated at 5×10^4 cells/well and reequilibrated for 3 h at 37°C before addition of the drug. At 3 h before the end of the drug incubation period the MTT was added. The enzyme reaction was stopped by addition of *i*-propanol/HCl. Values given are the absorbance values at 540 nm. Each number represents the mean (±SD) of four to five determinations

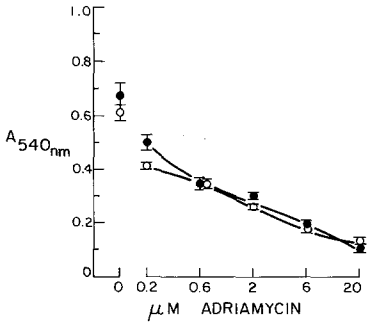

Fig. 2. Effect of adriamycin on small cell lung cancer cell line NCI-H69 after a 72-h incubation period. ●———●, continuous exposure for 72 h; ○———○ drug removed after 24 h. Each *point* represents the mean ±SD of four to five determinations

drugs. NCI-H69 cells were also more sensitive to daunomycin than A549 cells.

The effect of cell density on the sensitivity of NCI-H69 and A549 cells to adriamycin was tested on initial cell densities of 1×10^4 and 5×10^4 cells/well. With NCI-H69 cells (Fig. 5, upper panel), little difference was seen in the relative sensitivity at the two cell densities. With A549 cells (Fig. 5, lower panel), cytotoxicity was observed at adriamycin concentrations greater than 2 μ*M* when cells were plated at 5×10^4 cells/well. By contrast, cytotoxicity was observed at 0.2 μ*M* when cells were initially plated at 1×10^4 cells/well.

To determine whether the MTT assay would be useful for distinguishing drug-resistant variants from their sensitive parent cells, the sensitivity of the well-characterized CHO cell line AUX B1 and its multidrug-resistant variant, CH^RC5, to adriamycin was determined, and the results are shown in Fig. 6a. Cytotoxicity was observed in the drug-

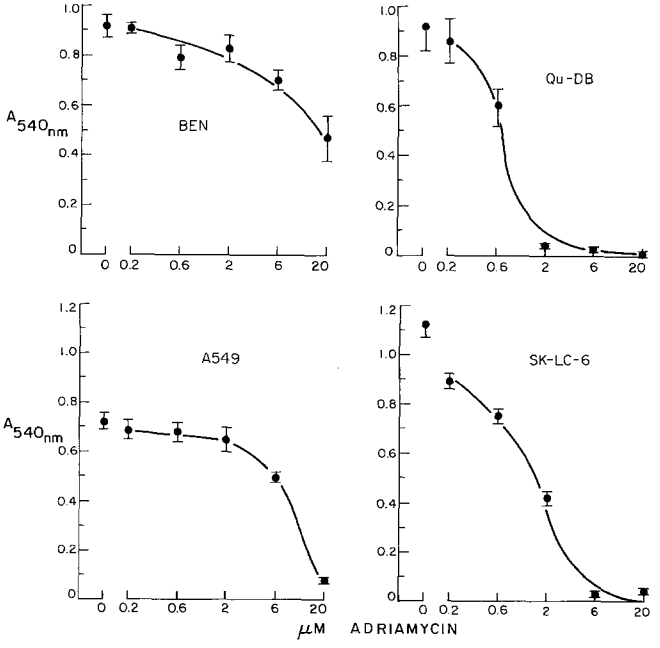

Fig. 3. Effect of adriamycin on the viability of human non-small cell lung tumor cell lines following a 72-h exposure to the drug. Each *point* represents the mean ±SD of four to five determinations

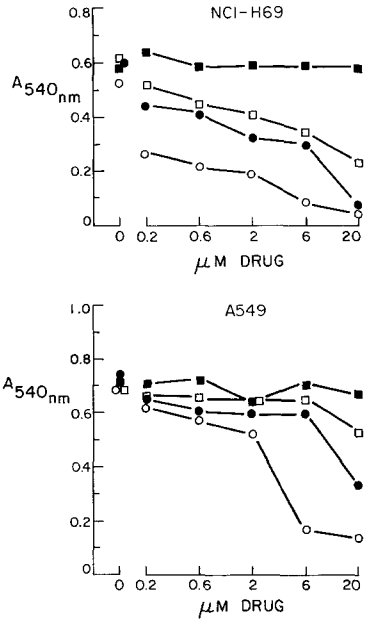

Fig. 4. Effect of antineoplastic agents on NCI-H69 cells *(upper panel)* and A549 cells *(lower panel)* after a 72-h continous exposure to t the drugs. ●, mitoxantrone; ○, daunomycin; □, nitrogen mustard; ■, cyclophosphamide. Each *point* represents the mean of four to five determinations. Error bars have been omitted for the sake of clarity but errors were similar to those in the other figures

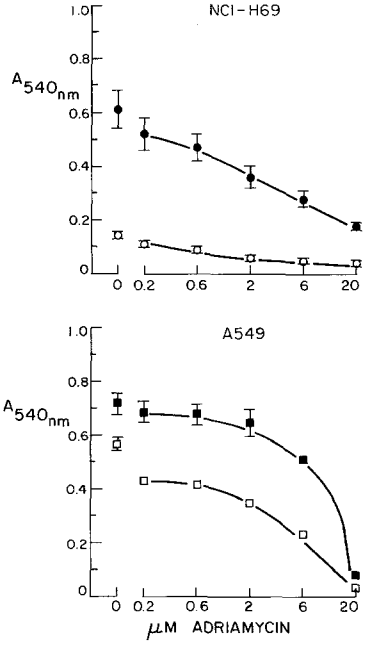

Fig. 5. Effect of adriamycin after 72 h on the viability of NCI-H69 cells *(upper panel)* and A549 cells *(lower panel)* initially plated at different cell densities *(open symbols,* 1×10^4 cells/well; *closed symbols,* 5×10^4 cells/well). Each *point* represents the mean of four to five determinations ±SD

sensitive AUX B1 cells at about 0.6 μ*M* adriamycin, while cytotoxicity was not observed in the resistant cells at the concentrations of drug tested (up to 20 μ*M*). The MTT assay works equally well with human cells. The sensitivity of the NCI-H69 parent line and its multidrug-resistant var-

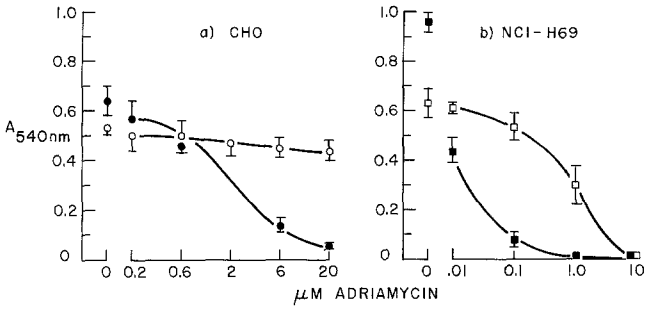

Fig. 6a, b. Effect of adriamycin on **a** Chinese hamster ovary cell line AUX B1 (●———●) and its multidrug-resistant variant, CH^RC5 (○———○) after a 72-h exposure, and **b** small cell lung cancer cell line, NCI-H69 (■———■) and its multidrug-resistant variant, H69AR7 (□———□) after a 7-day exposure. Each *point* represents the mean of four determinations ±SD

iant H69-AR7 to adriamycin after a 7-day exposure was determined, and the results are shown in Fig. 6b. Cytotoxicity was observed in NCI-H69 cells at doses as low as 0.01 μ*M* adriamycin, while cytotoxicity was not observed in resistant H69-R7 cells until concentrations greater than 0.1 μ*M* adriamycin were used.

Discussion

Most new antineoplastic drugs are initially screened on the murine leukemia P388 cell line before further testing on mouse and human tumors [25]. The relevance of this test to the chemotherapy of human tumors may be limited. Shoemaker et al. [23] have reported that a significant proportion (27/105) of P388 prescreen-negative compounds showed activity in a human tumor colony forming assay, suggesting that screening assays using human cells might be more meaningful [9]. Such assays would be useful for testing single drugs and drug combinations already in clinical use as well as for testing experimental drugs.

None of the assays currently used to test the chemosensitivity of human tumor cells are ideal [19, 22, 26, 27]. A major drawback of most assays is that only a single drug exposure time, cell density, and even single drug concentration can be tested because of practical limitations. The main advantage of the MTT assay is that the ease with which it can be performed allows a large number of parameters to be examined in a very short time.

In many instances, a cursory inspection of the dose-response curve may be all that is necessary where only a relative estimate of drug sensitivity is required. However, estimates of actual cell numbers can be determined using the MTT assay from a standard curve of absorbance (enzyme activity) versus cell number if desired. For NCI-H69 cells, the relationship between absorbance and cell number is linear over a wide range (Fig. 1); nevertheless, it is essential that the range of linearity be determined for each cell type.

In many cytotoxicity assays, the time at which viability is assessed after exposure to the drug is fixed, and may be somewhat arbitrary. In certain instances, this choice may profoundly affect the results obtained [8]. Thus, it would be preferable to be able to test a number of time points, since some drugs may take longer than others to exert their cytotoxic effects. In some studies tumor cells are continually exposed to drug for the duration of the assay. Frequently, however, drug exposure time may be as little as 1 h. The choice is often one of convention and may not necessarily be appropriate for all drugs or cells being tested [15]. Using the MTT assay, the effect of drug exposure time can be readily determined (Table 1). Moreover, the effect of removing the drug at a certain time can be easily compared with the continuous exposure of the cells to the drug. In our study, no difference was observed when NCI-H69 cells were exposed to adriamycin for only 24 h or continuously over the entire 72-h assay period (Fig. 2).

Because of the rapidity and efficiency of the MTT assay, multiple cell lines can be simultaneously tested with multiple drugs. This may be important if one is interested in determining the variability of chemosensitivity of cell lines within a particular type of tumor [16, 17] or among different tumor types. Our results clearly show that different lung tumor cell lines have different sensitivities to adriamycin (Fig. 3). Furthermore, SCLC cell line NCI-H69 was shown to be consistently more sensitive to three of four drugs tested than adenocarcinoma A549 cells (Fig. 4). The fourth drug tested, cyclophosphamide, had no effect on cell viability at the concentrations of drug tested (Fig. 4). This is probably because this drug must be metabolically activated before it can exert its cytotoxic effect [2]. The necessary biotransformation processes may not be present in these cell lines.

It has been reported that the degree of cytotoxicity observed with adriamycin can depend on cell density [5]. With most in vitro cytotoxicity assays only a single cell density is tested. The effect of cell density on chemosensitivity can be readily tested with the MTT assay. Little difference was seen in the relative sensitivity of NCI-H69 cells at the two cell densities tested (Fig. 5, upper panel), while an approximately 10-fold difference in sensitivity was seen with A549 cells (Fig. 5, lower panel). Thus, cell density is important with some tumor cell lines but not with others.

The increasingly widespread use of multidrug therapy has been accompanied by the emergence of the multidrug resistance phenotype. This phenomenon has been extensively studied in a CHO cell model system using a drug-sensitive cell line, AUX B1, and a number of variants, including CH^RC5, which exhibit multidrug resistance [13]. The difference in drug sensitivity of these two lines is readily apparent using the MTT assay (Fig. 6a). Recently a mutlidrug-resistant variant of the NCI-H69 human SCLC cell line has been isolated in our laboratory by selection in adriamycin (Cole S and Mirski S, manuscript in preparation). The MTT assay has been very useful for monitoring the gradual development of drug resistance in this cell line (Fig. 6b) and for quick determination of its cross-resistance to a wide variety of drugs (results not shown).

In conclusion, the experiments described in this paper establish the usefulness of the MTT assay for measuring in vitro chemosensitivity of human lung tumor cell lines and for monitoring the emergence of drug-resistant variants of such lines.

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References

1. Alley MC, Uhl CB, Lieber MM (1982) Improved detection of drug cytotoxicity in the soft agar colony formation assay through use of a metabolizable tetrazolium salt. Life Sci 31: 3071
2. Alley MC, Powis G, Appel PL, Kooistra KL, Lieber MM (1984) Activation and inactivation of cancer chemotherapeutic agents by rat hepatocytes cocultured with human tumor cell lines. Cancer Res 44: 549
3. Anger B, Bockman R, Andreeff N, Erlandson R, Jhanwar S, Kameya T, Saigo P, Wright W, Beattie EJ, Oettgen HF, Old LJ (1982) Characterization of two newly established human cell lines from patients with large cell anaplastic lung cancer. Cancer 50: 1518
4. Bech-Hansen NT, Sarangi F, Sutherland DJA, Ling V (1977) Rapid assays for evaluating the drug sensitivity of tumor cells. J Natl Cancer Inst 59: 21
5. Chambers SH, Bleehen NM, Watson JV (1984) Effect of cell density on intracellular adriamycin concentration and cytotoxicity in exponential and plateau phase EMT6 cells. Br J Cancer 49: 301
6. Cole SPC, Campling BG, Dexter DF, Holden JJA, Roder JC (1986) Establishment of a human large cell lung tumor line (QU-DB) with metastatic properties in nude mice. Cancer (in press)
7. Ellison M, Woodhouse D, Hillyard C, Dowsett M, Coombes RC, Gilby ED, Greenberg PB, Neville AM (1975) Immunoreactive calcitonin production by human lung carcinoma cells in culture. Br J Cancer 32: 373
8. Ferguson PJ, Phillips JR, Selner M, Cass CE (1984) Differential activity of vincristine and vinblastine against cultured cells. Cancer Res 44: 3307
9. Finlay GJ, Baguley BC (1984) The use of human cancer cell lines as a primary screening system for antineoplastic compounds. Eur J Cancer Clin Oncol 20: 947
10. Finlay GJ, Baguley BC, Wilson WR (1984) A semiautomated microculture method for investigating growth inhibitory effects of cytotoxic compounds on exponentially growing carcinoma cells. Anal Biochem 139: 272
11. Klebe RJ (1984) Rapid cloning of mammalian cells with honeycomb cloning plates and nonlethal vital stains. In Vitro 20: 127
12. Landegran U (1984) Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Applications to detection of lymphokines and cell surface antigens. J Immunol Methods 67: 379
13. Ling V (1982) Genetic basis of drug resistance in mammalian cells. In: Drug and hormone resistance in neoplasia. CRC Press, Miami, p 1
14. Ling V, Thompson LH (1974) Reduced permeability in CHO cells as a mechanism of resistance to colchicine. J Cell Physiol 83: 103
15. Matsushima Y, Kanzawa F, Hoshi A, Shimizu E, Nomori H, Sasaki Y, Saijo N (1985) Time-schedule dependency of the inhibiting activity of various anticancer drugs in the clonogenic assay. Cancer Chemother Pharmacol 14: 104
16. Merry S, Kaye SB, Freshney RI (1984) Cross-resistance to cytotoxic drugs in human glioma cell lines in culture. Br J Cancer 50: 831
17. Metcalfe SA, Whelan RDH, Masters JRW, Hill BT (1983) In vitro responses of human prostate tumour cell lines to a range of antitumour agents. Int J Cancer 32: 351
18. Mossman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55
19. Roobol C, Sips HCM, Theunissen J, Atassi G, Bernheim JL (1984) In vitro assessment of cytotoxic agents in murine cancers: comparison between antiproliferative and antimetabolic assays. JNCI 72: 661
20. Russell WC, Newman C, Williamson DH (1975) A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature 253: 461
21. Salmon SE, Von Hoff DD (1981) In vitro evaluation of anticancer drugs with the human tumor stem cell assay. Semin Oncol 8: 377
22. Selby P, Buick RN, Tannock I (1983) A critical appraisal of the "human tumor stem-cell assay". N Engl J Med 308: 129
23. Shoemaker R, Wolpert-Defilippes M, Kern D, Lieber M, Melnick N, Miller W, Salmon S, Simon R, Venditti J, Von Hoff D (1984) Antitumor drug screening with a human tumor colony forming assay (HTCFA). Proc Am Assoc Cancer Res 25: 1292 (abstract)
24. Slater TF, Sawyer B, Strauli U (1963) Studies on succinate-tetrazolium reductase systems: III. Points of coupling of four different tetrazolium salts. Biochim Biophys Acta 77: 383
25. Venditti JM (1983) The National Cancer Institute Antitumor Drug Discovery Program, current and future perspectives: a commentary. Cancer Treat Rep 67: 767
26. Weisenthal LM, Lippman ME (1985) Clonogenic and nonclonogenic in vitro chemosensitivity assays. Cancer Treat Rep 69: 615
27. Wilson AP, Ford CHJ, Newman CE, Howell A (1984) A comparison of three assays used for the in vitro chemosensitivity testing of human tumours. Br J Cancer 49: 57

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