Eur J Cancer Clin Oncol, Vol. 23, No. 1, pp. 55–60, 1987.
Printed in Great Britain

0277-5379/87$3.00+0.00

In Vitro Chemosensitivity of Human Lung Cancer for Vindesine

ELISABETH G.E. DE VRIES,* COBY MEIJER,* NANNO H. MULDER* and PIETER E. POSTMUS†

**Division of Medical Oncology and †Division of Pulmonary Medicine, Department of Internal Medicine, University Hospital, Groningen, The Netherlands*

Abstract—*Firstly, the effect of Vindesine was studied on four different human lung carcinoma cell lines (two small cell, one adeno and one squamous cell) with the Fast Green dye exclusion assay (FGA) and the clonogenic assay. Both methods demonstrate a clear dose response relationship and the estimated drug efficacy is similar for both assays. In the cell lines with the longest doubling time a plateau was reached in the FGA, most probably due to the short culture time in this assay. Secondly, the effect of Vindesine on human lung carcinoma specimens* (n = 64), *mainly bronchoscopy biopsies* (n = 48), *was evaluated with the FGA. The FGA has merits as predictive test in the clinic in the situation that only a small number of cells can be obtained. In this study, due to the high number of bronchoscopy biopsies only in a minority of cases a conclusion could be obtained* (37.5%).

INTRODUCTION

CHEMOTHERAPEUTIC therapy for metastatic non-small cell lung cancer (NSCLC) results in objective response in approx. 15–25% of NSCLC patients with single agent therapy [1]. The results of first line treatment in small cell lung carcinoma (SCLC) are better, but there is a high relapse rate and treatment after first relapse also has a high failure rate [2]. This means that a predictive test for tumor response in patients with inoperable lung cancer would be of great value. In case of metastatic disease often bronchoscopy is sufficient for diagnosis and this way operation can be avoided. Von Hoff *et al.* were able to grow lung cancer colonies from bronchial washings. However, the low number of tumor colonies grown precluded the performance of chemosensitivity studies. Main cause for the limited amount of colonies, was the low yield of cells obtained by bronchial washing [3].

As less cells are needed with the recently developed Fast Green dye exclusion assay (FGA) [4–6], we have evaluated this assay in lung carcinoma. Firstly, we studied the effect of Vindesine, a drug with activity in lung carcinoma [7–10], on four different human lung tumor cell lines with the FGA and clonogenic assay. From one of these cell lines (GLC-4) a cell line with acquired Adriamycin resistance and cross-resistance for Vindesine was derived and tested the same way [11]. Secondly, the effect of Vindesine on human lung tumor specimens, mainly biopsies obtained with rigid bronchoscopy, was evaluated with the FGA. We also attempted to determine if *in vitro* cytotoxicity data were comparable with clinical response patterns.

Accepted 1 July 1986.
Reprint requests to: E.G.E. de Vries, Division of Medical Oncology, Department of Internal Medicine, University Hospital, P.O. Box 30.001, 9700 RB Groningen, The Netherlands.

MATERIALS AND METHODS

Cell lines

Two small cell carcinoma lines (GLC-2, GLC-4), one adenocarcinoma cell line (GLC-A1) and one squamous cell line (N17-4) were maintained in continuous culture. The GLC-2 line, (gift of Dr. L. de Ley, Department of Clinical Immunology, University of Groningen) and the GLC-A1 grow attached, the GLC-4 line grows partly in suspension and partly attached, in Roswell Park Memorial Institute Tissue Culture medium 1640 (RPMI 1640) with 10% heat-inactivated fetal calf serum (FCS). The N17-4 (gift of Dr. J. Klein, TNO Rijswijk, The Netherlands) was maintained in continuous monolayer culture in RPMI 1640 with 10% FCS and 50% conditioned medium.

From the GLC-4 cell line, a subline with acquired Adriamycin resistance and cross-resistance for Vindesine was derived by stepwise incubation with Adriamycin. This resistant subline grows under identical conditions as GLC-4 and has the same growth characteristics.

Doubling time for GLC-2, GLC-4, GLC_4/ADR,

GLC-A1, and N17-4 is respectively 21, 24, 26, 39, 278 hr.

Tumor cell preparation

Fresh human (solid) tumor specimens were placed in RPMI 1640 and 10% FCS with penicillin (125 U/ml), streptomycin (125 μg/ml) and amphotericin B (1 μg/ml). Tissue was kept at 4° C until it was convenient to process, generally 1–2 hr or overnight for late afternoon specimens.

If necessary the specimens were grossly minced with scalpels and scissors. Excessive trauma was avoided, since it was not necessary to obtain a single-cell suspension for the purpose of the dye exclusion assay. Thereafter the suspension was exposed to 0.1% collagenase and 0.003% DNase for at least 1 hr at 37° C. Cells from malignant effusions were collected by means of centrifugation. If necessary red blood cells were lysed with a buffer (8.29 g NH_4Cl, 1.0 g $KHCO_3$, 0.0372 g Na_2-EDTA/l distilled water). Cells from bone marrow aspirates were collected with Ficoll-Hypaque (1.077 g/ml) in all cases the number of viable nucleated cells was quantified using 0.4% trypan blue in 0.15 M NaCl and a hemocytometer counting chamber.

Drug

Purified Vindesine sulfate (gift Eli Lilly, Indianapolis) was dissolved in phosphate-buffered saline (PBS) and frozen at −20° C at 10 times the desired final concentration.

Drug sensitivity assays

FGA. The FGA as described by Weisenthal *et al.* [5, 6] was used with slight modifications. The cells were suspended in fresh Dulbecco's Modified Eagle's Medium (DME)/F_{12} (1 : 1) and 20% FCS with a minimum concentration of 10^5 cells/ml with 10^5 fixed chicken red blood cells as internal standard and incubated for 1 hr at 37° C with Vindesine concentrations of 0.1–10μg/ml and PBS as a control. Tumor specimens were always at least cultured with 10 μg/ml as this is one-tenth of the peak plasma concentration in humans [12, 13].

After the incubation 3 ml PBS was added and the cells were centrifuged and washed twice with PBS and resuspended in 2.5 ml of fresh medium (DME/F_{12}, 20% FCS) in polypropylene tubes for a short-term culture period of 4 days under a humidified atmosphere of 95% air, 5% CO_2 at 37° C. At day 4, cells were stained for 10 min with 1% Fast Green and sedimented onto microscope slides using a Cytospin centrifuge (550 rpm, 5 min) and counterstained with a modified hematoxylin–eosin (H & E) technique. Under the microscope the living cells have their characteristic appearance with H & E (living tumor or non-tumor cells), the dead cells stain green and the fixed chicken red blood cells which are oval and nucleated stain predominantly green. Some cells did phagocytize the fixed chicken red blood cells which resulted in well to distinguish pink stained fixed chicken red blood cells inside living cells. The ratio of living tumor cells over fixed chicken red blood cells was determined for each triplicate of centrifuge slides at each drug concentration. The result was expressed as a percentage of control. The fixed red blood cells did not influence the survival of the tumor cells of the cell lines, but influence of these fixed cells on the culture of tumor specimens can not be excluded.

The fixation of the internal standard of chicken red blood cells as described by Weisenthal [5] was performed with slight modifications. After 10 days fixation with acetaldehyde buffer the cells were washed 3 times with sterile NaCl with streptomycin (100 μg/ml), penicillin (100 U/ml), amphotericin B (5 μg/ml). Thereafter the cells were dissolved in this NaCl solution and this concentrated (± 100 × 10^6/ml) stock solution can be stored indefinitely at 4° C. To make a working solution, a small amount of the stock solution is taken and rediluted with sterile NaCl to a desired final concentration of fixed red blood cells. Before use the working suspension should be vortexed.

Soft agar clonogenic assay (SAA). The agar cloning assay was utilized for GLC-4, GLC_4/ADR and GLC-A1. Cells were plated at 5000 cells/ml in an upper layer of 0.3% agarose in DME/F_{12}, 20% FCS over an underlayer of 0.5% agar in DME/F_{12}, 20% FCS in a 35 mm Petridish. Colonies were counted at day 7 (GLC-4) or at day 14 (GLC-A1). Cloning efficiencies were 20.2% for GLC-4, 10.1% for GLC_4/ADR and 3.3% for GLC-A1. The GLC-2 and N17-4 cell lines were cloned in liquid culture. Cells were plated at 3000 cells/ml in 60 mm tissue culture Petri dishes and grown in 3 ml DME/F_{12}, 20% FCS and respectively 30% and 50% conditioned medium. Adherent colonies were counted day 10 after initiation of the culture. The cells were fixed for 15 min with ethanol 96% followed by a methylene blue staining. Plating efficiency was 8.8% for GLC-2 and 14% for N17-4. The incubation and wash procedure were the same as described for the FGA. Results were expressed as a percentage of control.

RESULTS

Comparison of FGA and SAA in lung tumor cell lines

Both methods demonstrate a clear dose response relationship and the estimated drug efficacy is similar for both assays.

For the small cell lines the FGA gave a somewhat lower estimate for cell survival than did the

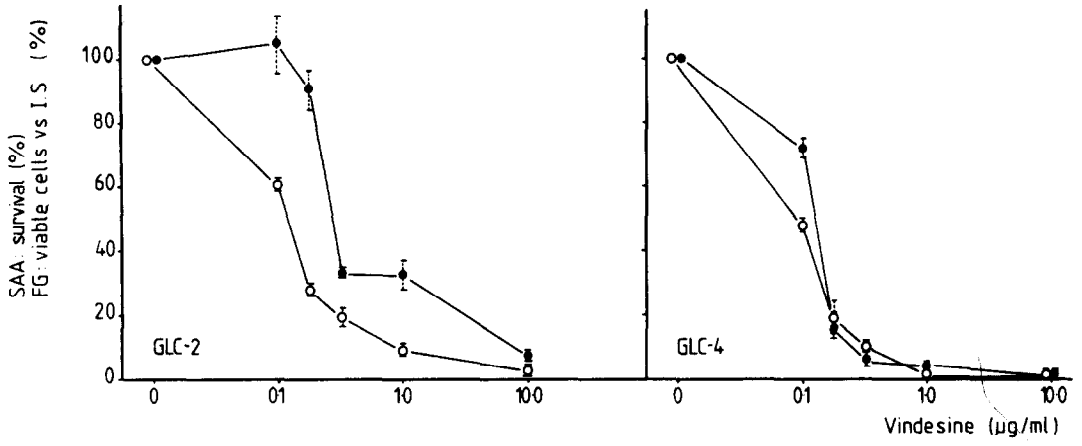

Fig. 1. Dose response curves of the two small cell tumor cell lines, GLC-2, GLC-4, to Vindesine (1 hr exposure). Soft agar cloning assay (●——●) and Fast Green assay after a 4 days culture period (○——○).

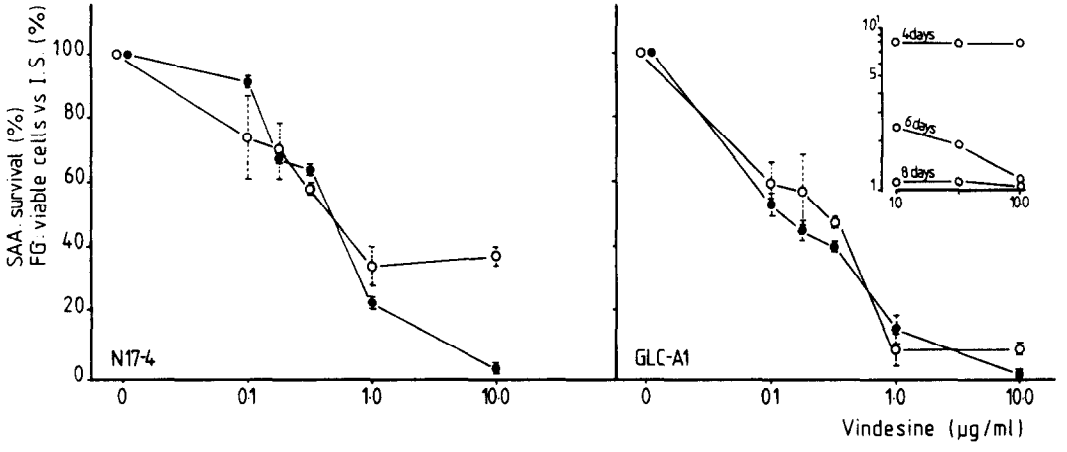

Fig. 2. Dose response curves of N17-4 and GLC-A1 to Vindesine (1 hr exposure). Soft agar cloning assay (●——●) and Fast Green assay after a 4 days culture period (○——○). The inset shows the results for the Fast Green assay after various culture periods (y- axis logarithmic scale).

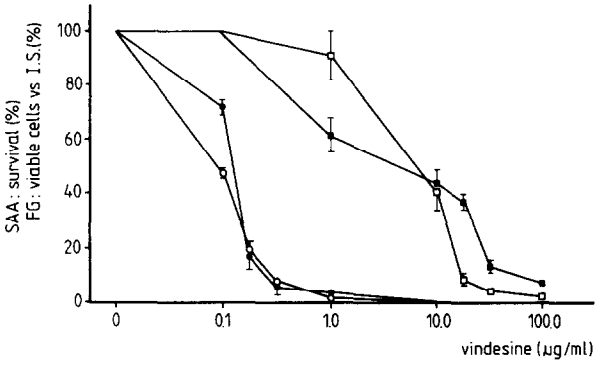

Fig. 3. Dose response curves of the sensitive GLC-4 and resistant GLC_4/ADR cell line, to Vindesine (1 hr exposure). Soft agar cloning assay (■——■ GLC_4/ADR, ●——● GLC-4) and Fast Green assay (□——□ GLC_4/ADR, ○——○ GLC-4), after a 4 days culture period.

SAA (Fig. 1). This is contrary to the GLC-A1 and N17-4 (Fig.2). Here the FGA shows a plateau at 5–10 µg/ml Vindesine for 1 hr after 4 days culture.

The primary mode of action of Vindesine is mitotic accumulation [14] and the doubling times of the adeno and squamous cell lines are longer than for the small cell lines. For this reason the effect of culture time after drug exposure was evaluated for the adenocarcinoma, GLC-A1 cell line. Figure 2 shows that with increasing culture time there is an increase in cell kill as measured with the FGA for the GLC-A1 cell line. This suggests that differences in doubling time between the cell lines may explain the different results of the FGA after 4 days culture. Figure 3 shows that the results for both assays are identical for the Vindesine sensitive GLC-4 cell line and Vindesine resistant GLC_4/ADR cell line.

Patient specimens evaluated with FGA

Sixty-four tumor specimens were received. Table 1 shows the origin and histology of the specimens. Most of the specimens were obtained by rigid bronchoscopy ($n = 48$). Rigid bronchoscopy is performed routinely in patients suspected of centrally

Table 1. Origin and histology of the tumor specimens

	Number of specimens	Small cell	Adeno	Large cell	Squamous cell
Bronchoscopic biopsies	48	20	2	1	25
Thoracotomy material	6	1	1	–	4
Lymph node specimen	5	3	1	–	1
Subcutaneous metastasis	1	–	1	–	–
Malignant pleural effusion	3	3	–	–	–
Bone marrow aspiration	1	1	–	–	–
Total	64	28	5	1	30

located lung cancer, or in patients suspected of tumor relapse after chemo- and/or radiotherapy. The major advantage of rigid bronchoscopy is the possibility to obtain larger biopsies. In all patients at least two biopsies were taken: one for light microscopy and one for the presented study. If possible, dependent on bleeding tendency at the biopsy site, one or two additional biopsies were taken for this purpose. Only biopsies were used for the FGA. Ten of the bronchoscopy specimens (six small cell carcinoma, four squamous carcinoma) and two of two other specimens (two small cell carcinoma) were taken from previously treated patients. Table 2 shows the results of the FGA. Finally 24 specimens (37.5%) could be evaluated (25.0% of bronchoscopy specimens, 75% of the other specimens). Of the rigid bronchoscopy specimens 48% of the failures was due to an initially insufficient number of viable cells. Mean number of cells obtained after bronchoscopic biopsy was 43.5×10^4 (range 10^4–390×10^4) with a mean viability (trypan blue exclusion) of 10%. We found that for the FGA with bronchoscopic biopsies a minimum of 2×10^5 viable cells are necessary to perform a control and drug treated culture. This high number of cells compared to other publications [4, 6] is necessary due to the fact that there is a high percentage of cell death in the control culture after 4 days. In 21% of the bronchoscopic biopsies all cells were dead in the control culture, which made drug evaluation impossible. The assay on bronchoscopy specimens was only successful in small cell (20%) and squamous cell (32%) carcinoma.

Mean number of cells obtained from the other specimens was 7.8×10^6 (range 0.8×10^6–20.4×10^6) with a viability (trypan blue) above 10% (range 5–75%). In this group there was no failure due to an initially insufficient number of viable cells and the percentage in which all cells were dead in the control culture was much lower (caused by a higher cell concentration and a higher viability at the start of the incubation).

The histology of the specimens that could be evaluated was small cell carcinoma 12, adenocarcinoma 3, large cell 0, squamous carcinoma 8. Figure 4 shows the results of the FGA if evaluable. If enough cells were available apart from 10 μg Vindesine/ml, extra drug concentrations were evaluated.

Only 20.8% (5/24) had over 70% reduction of viable cells versus internal standard in the FGA with a Vindesine concentration of 10 μg/ml.

Table 2. Results Fast Green Assay of tumor specimens

	Bronchoscopy biopsies	Other specimens
Number	48	16
Evaluable with Fast Green Assay	12	12
Failure Fast Green Assay due to:		
—initial insufficient number of cells	23	0
—contamination	3	2
—after 4 days all cells dead in control culture	10	2

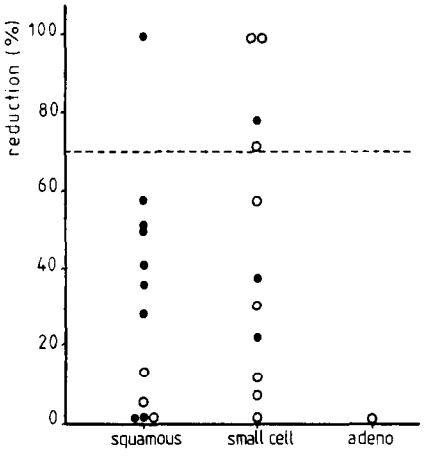

Fig. 4. Chemosensitivity for Vindesine (10 μg/ml for 1 hr) measured with FGA in human tumor specimens. Bronchoscopy biopsies (●), other specimens (○).

DISCUSSION

The ability to predict the sensitivity of an individual human tumor to the effects of cytostatic agents in advance of the actual use *in vivo* would have a major impact on the practice of clinical oncology. This is even more important if for a certain tumor type effect of the drug can be expected in only a limited number of patients as is the case in initial treatment of NSCLC and treatment of SCLC relapse.

Recently Weisenthal *et al.* developed a new dye exclusion assay [4–6], which circumvents a number of the disadvantages of the older dye exclusion assays [15, 16].

The advantages of this assay are that the slides are counterstained after Fast Green staining with H & E staining, which makes it possible to distinguish dead and viable, tumor and non-tumor cells. Furthermore, the counting of living tumor cells over chicken red blood cells as internal standard gives the possibility to correct for disintegration of killed cells and to correct for continued proliferation of living cells, and there is no perfect single cell suspension necessary. This assay is easier to perform and faster than the clonogenic assay and requires a smaller number of cells.

In the study here described we evaluated the effect of Vindesine on human lung carcinoma cell lines and patient tumor specimens. Vindesine was studied because of the known effect in NSCLC and in SCLC [1, 7–10, 17]. A phase 2 study with a new dose schedule [18] was started and monotherapy makes correlation of *in vitro* data with the clinical response in the future possible.

As there are conceptual advantages of clonogenic assays as chemosensitivity test [19] a comparison was made for four different lung tumor cell lines and Vindesine sensitivity with the SAA and FGA. There was an excellent correlation between the two assays if culture time was corrected for doubling time of the cell line. There was also a good correlation for the Vindesine resistant small cell lung carcinoma cell line.

With the FGA the effect of Vindesine on various tumor specimens from lung tumor patients was evaluated. Major emphasis was paid to make the test applicable to rigid bronchoscopy biopsies. In case of metastatic lung disease bronchoscopy is often the easiest procedure to obtain tumor material. In bladder cancer with biopsies obtained after cytoscopy only 5×10^4 cells were required per assay tube for FGA [4]. But in the case of bronchoscopy material and also in the case of lung tumor specimens obtained by thoracotomy we needed a larger number of cells, namely 10^5 due to the low initial cell viability and the low viability after 4 days culture of the initial viable cells in the control tube. Von Hoff *et al.* report in a large study on clonogenic assay, that the viability derived from solid tumor ranged from 0 to 100% (median 26%) while viability of cells from effusions and bone marrow ranged from 0 to 100% (median 90%) [13]. The low viability after 4 days and the low initial yield of cells made this assay applicable to only 25.0% of the bronchoscopy biopsies. On the other hand a clonogenic assay would not be possible on this material for at least 5×10^5 cells per dish are needed to give enough colonies for one drug concentration. Furthermore, in the clonogenic assay only about 50% of the human tumors grow with a sufficient number of colonies for evaluation. In other than bronchoscopy specimens the success rate of the FGA was higher. The reason for the low percentage of samples with 70% reduction in viability may be the fact that there is an increase in sensitivity if the exposure time to Vindesine is increased [20, 21], and in this study only 1 hr drug exposure was performed. On the other hand, the results for the tumor specimens (20.8% sensitivity) for Vindesine, are compatible with response rates in NSCLC and recurrent SCLC [10].

We conclude that the FGA assay has definite merits as predictive test in the clinic in the situation that a small number of cells is obtained. However, in our study, due to the high number of bronchoscopy biopsies, only in a minority of cases (37.5%) could a conclusion be obtained.

REFERENCES

1. Hansen HH, Rorth M. Lung Cancer. In: Pinedo HM, Chabner BA, eds. *Cancer Chemotherapy Annual* 6. Amsterdam, New York, London, Elsevier, 1984, 299–321.
2. Ihde DC. Current status of therapy for small cell carcinoma of the lung. *Cancer* 1984, **54,** 2722–2728.
3. Von Hoff DD, Weisenthal LM, Ihde DC, Mathews MJ, Layard M, Makuch R. Growth of lung cancer colonies from bronchoscopy washings. *Cancer* 1981, **48,** 400–403.
4. Weisenthal LM, O'Tayo Lalude A, Miller JB. *In vitro* chemosensitivity of human bladder cancer. *Cancer* 1983, **51,** 1490–1496.
5. Weisenthal LM, Dill PL, Kurnick NB, Lippman ME. Comparison of dye exclusion assays with a clonogenic assay in the determination of drug-induced cytotoxicity. *Cancer Res* 1983, **43,** 258–264.
6. Weisenthal LM, Marsden JA, Dill PL, Macaluso CK. A novel dye exclusion method for testing *in vitro* chemosensitivity of human tumors. *Cancer Res* 1983, **43,** 749–757.
7. Natale RB, Gralla RJ, Wittes RE, Golbey RB, Young CW. Vindesine chemotherapy in lung cancer. *Cancer Treat Rev* 1980, **7,** 59–63.

8. Maltson K, Helshi LR, Salmo M, Saastamoinen M, Ahlstedt S, Hetsli P. Vindesine in the treatment of small cell and non-small cell bronchogenic carcinoma: preliminary results. *Cancer Treat Rev* 1980, **7,** 65–70.
9. Gralla RJ, Raphael BG, Golbey RB, Young CW. Phase II evaluation of vindesine in patients with non-small cell carcinoma of the lung. *Cancer Treat Rep* 1979, **63,** 1343–1346.
10. Jewkes J, Harper PG, Tobias JS, Geddes DM, Souhari RL, Spiro SG. Comparison of vincristine and vindesine in the treatment of inoperable non-small cell bronchial carcinoma. *Cancer Treat Rep* 1983, **67,** 1119–1121.
11. Zijlstra JG, de Vries EGE, Mulder NH, de Ley L, de Jong B. Adriamycin (ADR) resistance in a human small cell lung cancer (SCLC) cell line. *Am Assoc Cancer Res* 1986, abstract 1083.
12. Hande K, Gay J, Gober J, Greco FA. Toxicity and pharmacology of bolus vindesine injection and prolonged vindesine infusion. *Cancer Treat Rev* 1980, **7,** 25–30.
13. Von Hoff DD, Clark GM, Stogdill BJ *et al.* Prospective clinical trial of a human tumor cloning system. *Cancer Res* 1983, **43,** 1926–1931.
14. Sweeney MJ, Boder GB, Cullinan GJ *et al.* Antitumor activity of deacetyl vinblastine amide sulfate (Vindesine) in rodents and mitotic accumulation studies in culture. *Cancer Res* 1978, **38,** 2886–2891.
15. Roper PR, Drewinko B. Comparison of *in vitro* methods to determine drug induced cell lethality. *Cancer Res* 1976, **36,** 2182–2188.
16. Roper PR, Drewinko B. Cell survival following treatment with antitumor drugs. *Cancer Res* 1979, **39,** 1428–1430.
17. Young CW. Vindesine trials at Memorial Sloan Kettering Center. *Cancer Treat Rev* 1980, **7,** 53–57.
18. Mulder NH, Postmus PE, Sleijfer DTh, de Vries EGE, Willemse PHB. Phase one study of twice weekly vindesine. *Eur J Cancer Clin Oncol* 1985, **21,** 1555–1556.
19. Buick RN, Selby P, Tannoch IF. *In vitro* tests for predicting response to chemotherapy. *The Cancer Bulletin* 1984, **36,** 179–184.
20. Hill BT, Whelan RDH. Comparative cell killing and kinetic effects of vincristine or vindesine in mammalian cell line. *JNCI* 1981, **67,** 437–443.
21. Hill BT, Whelan RDH. Comparative effects of vincristine and vindesine on cell cycle kinetics *in vitro*. *Cancer Treat Rev* 1980, **7,** 5–15.