Eur J Cancer Clin Oncol, Vol. 24, No. 8, pp. 1365–1371, 1988.
Printed in Great Britain

0277-5379/88$3.00+0.00

In Vitro and *In Vivo* Responses of a Panel of Murine Colon Tumours to TCNU: a Positive Correlation

R.M. PHILLIPS, M.C. BIBBY and J.A. DOUBLE
Clinical Oncology Unit, University of Bradford, Bradford, West Yorkshire BD7 1DP, U.K.

Abstract—*TCNU is highly active against a panel of three histologically distinct transplantable murine adenocarcinomas of the colon (MAC tumours). Significant reductions in colony formation* (>70%) *were observed* in vitro *in all three cell lines following a* 1 h *exposure to TCNU at experimentally achievable drug* C × t *values. A good correlation exists between* in vivo *tumour responses and* in vitro *cell responses in all cases. Dose–response curves generated at increasing exposure times suggest that no active or long lived products of TCNU are formed as a result of the drug's spontaneous breakdown* in vitro (*rate of breakdown was* 0.078 μg min^{-1} *at* 37°C). *Preliminary studies with the HT-29 human colon cell line have demonstrated that multi-cellular spheroids are more responsive to TCNU* (2 ×) *than the same cells cultured as monolayers.*

INTRODUCTION

1-(2-CHLOROETHYL)3-[2-(dimethylaminosulphonyl)ethyl]-1-nitrosourea (TCNU) is a novel anti-cancer agent. In Phase I clinical trials, objective responses were noted in squamous cell adenocarcinoma and large cell carcinoma of the lung as well as mesothelioma and breast cancer. TCNU was well tolerated with thrombocytopenia being the dose limiting toxicity [1]. Phase II evaluation in non-small cell lung cancer, melanoma, breast cancer and colorectal carcinoma are now in progress.

Studies in this laboratory have shown that TCNU is highly active against a panel of transplantable murine adenocarcinomas of the colon (MAC tumours). This model has been extensively characterized and is similar in terms of cell kinetics, histology and chemosensitivity to tumours of the human colon [2]. The panel of tumours studied included two solid tumours grown intraperitoneally (MAC 26 and MAC 13) and one ascitic tumour grown intraperitoneally (MAC 15A) and systemically (MAC 15A i.v.—induced by the intravenous inoculation of MAC 15A cells via the tail vein). *In vivo* responses to TCNU at the maximum tolerated dose of 30 mg kg^{-1} administered intraperitoneally are described in detail elsewhere [3] and are summarized in Table 1. Briefly, all three MAC tumours are sensitive to TCNU with MAC 15A i.v. tumours being more responsive (9/10 cures) than the same cells grown intraperitoneally. This spectrum of activity represents an improvement over standard nitrosoureas, particularly in the case of MAC 26 which is generally unresponsive to nitrosoureas.

Predictive chemosensitivity testing on human tumours has demonstrated that the tumour colony forming assay described by Hamburger and Salmon [4] predicts for tumour resistance with a greater degree of accuracy (97%) than tumour sensitivity (64%) [5]. In the clinic, however, pharmacokinetic variations in drug exposure parameters between individual patients with different physical and physiological characteristics are known to be extreme [6] and may account for the inconsistent response of tumours designated as sensitive *in vitro*. A more accurate test of a clonogenic assay's ability to predict tumour responses may be obtained in an experimental model where both pharmacokinetic variations in drug exposures are reduced and where a more objective assessment of tumour responses in previously untreated tumours is possible. The extraction, detection and quantification of TCNU in biological fluids together with *in vivo* plasma and peritoneal clearance curves for TCNU following the administration of therapeutic doses of TCNU to non-tumour bearing mice has been described elsewhere [7]. No metabolites of TCNU were detected and the areas under the plasma and peritoneal curves (drug $C \times t$ values) were 6.6 and 17.2 μg h ml^{-1} respectively. As TCNU is reported to be

Accepted 7 April 1988.
This work was supported by the Whyte Watson/Turner Cancer Research Trust, Bradford, West Yorkshire, U.K.

Table 1. In vitro *and* in vivo *responses of MAC tumours to TCNU*

Cell line	Tumour description	Percentage reduction in colony formation *in vitro* at achievable drug $C \times t$ values	Percentage tumour inhibition
MAC 13	Solid, poorly differentiated, anaplastic adenocarcinoma grown subcutaneously	99%	94%
MAC 26	Solid, well differentiated, cystic adenocarcinoma grown subcutaneously	72%	67%
MAC 15A i.v.	Small, spheroid-like nodules deposited in the lungs following the i.v. inoculation of MAC 15A cells	96%	100% (9/10 cures)
MAC 15A	Ascitic tumour grown intraperitoneally	99%	99%

unstable in aqueous solutions [8], the pharmacokinetics of TCNU *in vitro* are described.

The aim of this study was to assess whether or not a clonogenic assay, in conjunction with pharmacokinetic studies, could retrospectively predict the response of MAC tumours to TCNU.

Finally, a preliminary investigation into the relative cytotoxic effects of TCNU on HT-29 cells (an established human cell line derived from a primary adenocarcinoma of the colon [9]) grown as monolayers or spheroids is described with the aim of explaining the site dependent responses of MAC 15A tumours. HT-29 cells were employed as they readily formed spheroids unlike the MAC cell lines.

MATERIALS AND METHODS

Cell culture

Cell lines were derived by mechanical disaggregation of the solid tumours and were routinely maintained as monolayer cultures in RPMI 1640 tissue culture medium supplemented with foetal calf serum (10%), sodium pyruvate (1 mM), penicillin/streptomycin (50 IU ml^{-1}/50 $\mu g\ ml^{-1}$) and buffered with hepes (25 mM). HT-29 cells were maintained as monolayers in RPMI 1640 as above.

Spheroid formation of HT-29 cells was initiated by seeding approx. 10^5 cells into 75 cm^2 tissue culture flasks that had previously been base coated with 1% agar.

Chemosensitivity studies

TCNU was a gift from Leo Laboratories, Helsingborg, Sweden.

The colony forming ability of tumour cells surviving drug treatment was assessed using a slightly modified version of the Hamburger and Salmon clonogenic assay [4]. In this assay, no soft agar was used as fibroblastic contamination was minimal. Single cell suspensions, derived from monolayer cultures (Trypsin 0.25%) were exposed to a range of experimentally achievable drug concentrations (1.25–10 $\mu g\ ml^{-1}$) in complete RPMI 1640 and incubated at 37°C for various time intervals (1 and 3 h). Following drug exposure, the cells were washed twice in Hank's balanced salt solution and between 2–5 $\times 10^4$ viable cells (Trypan blue exclusion) were plated into 25 cm^2 plastic flasks containing 10 ml of complete RPMI 1640. After 5–7 days incubation at 37°C colonies of $\geq$50 cells were counted using an inverted microscope and plating efficiencies calculated for each drug exposure. Cytotoxic effects of drug treatment were expressed in terms of percentage survival taking control plating efficiencies to represent 100% survival. Triplicate samples for each assay were performed.

In vitro chemosensitivity studies were restricted to cultures of <10 passages in age (except HT-29 cells) and cells in the exponential phase of growth were used throughout.

In vitro sensitivity was defined as a 70% or greater reduction in colony formation following a 1 h exposure to *in vivo* drug $C \times t$ equivalents.

Spheroids: Multicellular spheroids of approx. 250 μm in diameter were exposed to various concentrations of TCNU. Initial concentration ranged from 10 to 1.25 $\mu g\ ml^{-1}$. Following drug exposure, spheroids were washed in Hank's balanced salt solution before being dissociated into a single cell suspension by Trypsin (2.5%). The resulting cell suspensions were washed in Hank's balanced salt solution and assessed for clonogenic properties as described above.

Comparative studies between the effects of TCNU on spheroids and monolayers were run simultaneously using the same stock solution of TCNU.

In vitro *pharmacokinetics*

The stability of TCNU in complete RPMI 1640 tissue culture medium at 37°C in the dark was determined by the analytical methods described previously [7]. Areas under the TCNU stability curve were calculated by the trapezoid rule.

RESULTS

TCNU breaks down at a rate of 0.078 μg min^{-1} in 'complete' RPMI 1640 at 37°C in the dark (Fig. 1). No TCNU could be detected after 3 h and no breakdown products were detected. The unstable nature of TCNU is reflected in a series of dose–response curves generated at various exposure times (Fig. 2). Increasing the duration of exposure beyond 3 h resulted in no further increase in cytotoxicity. The correction of *in vitro* drug concentrations for the breakdown of TCNU shifts the dose–response curve to the left (Fig. 3). In the case of MAC 26 (Fig. 3) the differences in the reduction in colony formation at *in vivo* drug $C \times t$ equivalents as a result of this shift (34% cell kill compared to 72% cell kill for incorrected and corrected *in vitro* exposures, respectively) has a significant influence on the predictive value of this assay.

There were marked differences in the inherent sensitivity of MAC cell lines following a 1 h exposure to TCNU with MAC 26 cells being more resistant than MAC 13 cells (Fig. 4). Reductions in colony formation *in vitro* following a 1 h exposure to

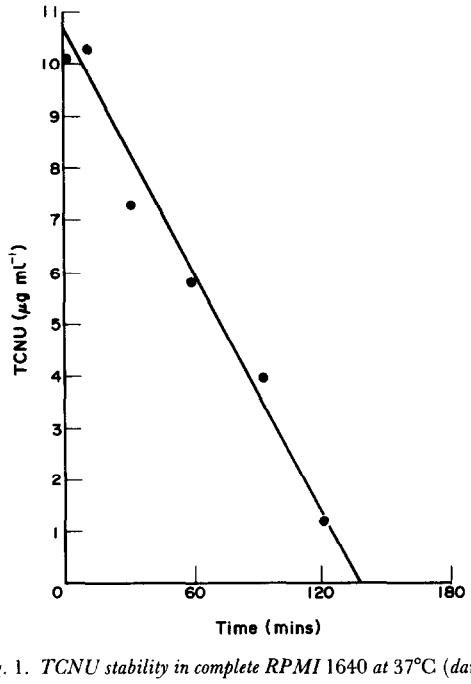

Fig. 1. TCNU stability in complete RPMI 1640 at 37°C (dark).

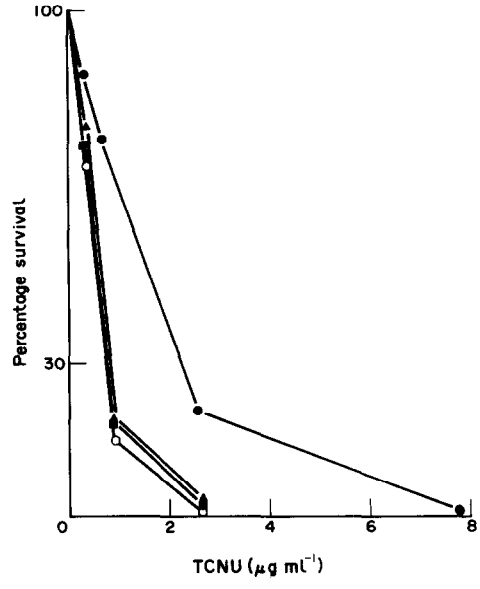

Fig. 2. In vitro chemosensitivity of MAC 15A cells exposed to TCNU for 1 h (●—●), 3 h (▲—▲), 6 h (■—■) and 24 h (○—○).

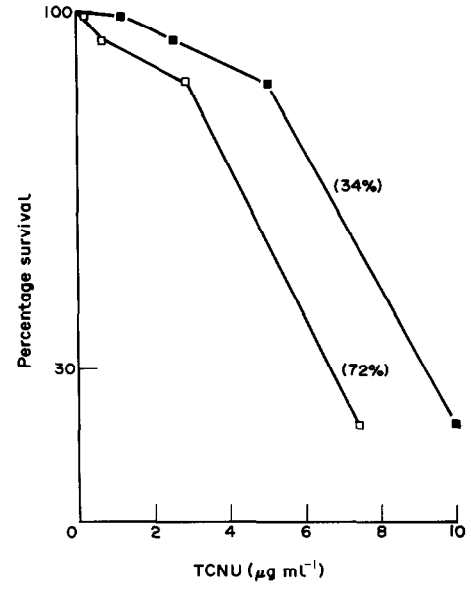

Fig. 3. MAC 26 dose–response curve corrected (□—□) and uncorrected (■—■) for TCNU breakdown in vitro. (Values in parentheses represent the percentage survival of colony forming units following a 1 h exposure to experimentally achievable plasma drug C × t values.)

TCNU at experimentally achievable drug $C \times t$ values were, nevertheless, greater than 70% in all the cell lines tested (Table 1), which under the criteria stated above is sufficient to allow sensitivity to be predicted. As all the MAC tumour lines are sensitive to TCNU *in vivo* (sensitivity *in vivo* was defined as a 70% or greater tumour inhibition) a good correlation exists between the *in vivo* and *in vitro* responses of MAC tumours to TCNU.

HT-29 spheroids were more responsive to TCNU than the same cells cultured as monolayers (Fig. 5). MAC 15A (i.v.) lung nodules resemble HT-29

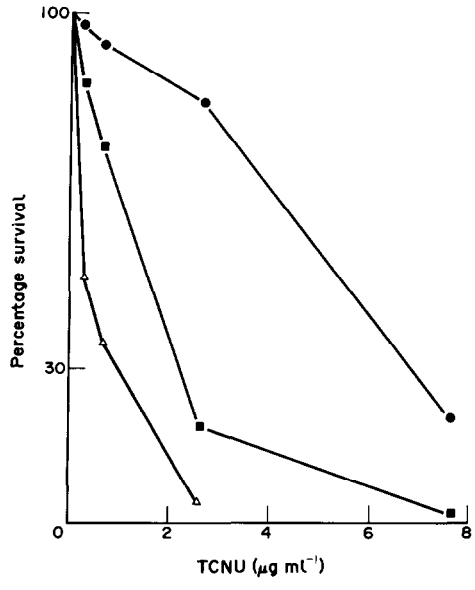

Fig. 4. In vitro *chemosensitivity of MAC cell lines following a* 1 h *exposure to TCNU. MAC* 26 (●—●), *MAC* 15*A* (■—■), *MAC* 13 (△—△).

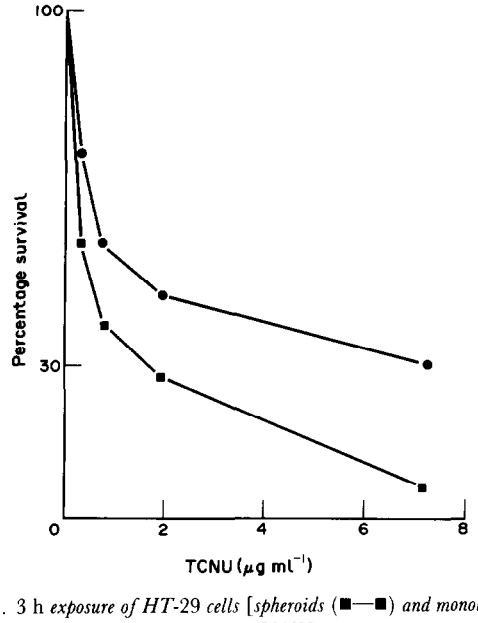

Fig. 5. 3 h *exposure of HT*-29 *cells* [*spheroids* (■—■) *and monolayers* (●—●)] *to TCNU.*

spheroids histologically (Plate 1). Both appear to have peripheral proliferating cells with deeper lying viable but non-proliferating cells and necrotic centres.

DISCUSSION

The main conclusion of this study is that the clonogenic assay is capable of predicting the response of a panel of murine colon tumours to TCNU when *in vitro* and *in vivo* drug $C \times t$ parameters are considered. Other studies using human tumour xenografts have also demonstrated that a good correlation exists between *in vitro* and *in vivo* responses to cytotoxic drugs when plasma drug $C \times t$ and peak drug concentrations are utilized [10–12]. These results emphasize the importance of including drug exposure parameters in the analysis of a 'predictive' chemosensitivity test and suggest that large variations in drug $C \times t$ values between individual patients may account for the poor prediction of tumour sensitivity witnessed in the clinic. Similar studies in this laboratory, however, have demonstrated poor *in vitro/in vivo* correlations despite the inclusion of drug exposure parameters and have suggested that other factors have a significant influence on the final outcome of chemotherapy [13].

One potential source of error in chemosensitivity testing is the failure to acknowledge the anti-tumour effects of metabolites of cytotoxic drugs. For a number of anti-cancer drugs, e.g. cyclophosphamide, metabolites make a significant contribution to anti-tumour effects and their omission from chemosensitivity tests *in vitro* will introduce inaccuracies in the prediction of tumour responses. There is, however, no evidence for the presence of long lived active metabolites of TCNU in this or previous studies [7] although recent results have indicated the presence of two TCNU metabolites *in vivo* [Hartley-Asp, personal communication]. *In vitro* studies have shown that the cytotoxic effects of TCNU do not increase when exposure times are extended beyond 3 h. This result first of all reflects the unstable nature of TCNU *in vitro* and secondly suggests that no active products of TCNU are formed as a result of the drug's spontaneous breakdown *in vitro.*

The three-dimensional structure of solid tumours also introduces additional factors which have a significant bearing on the final outcome of chemotherapy such as problems with drug penetration, proliferation gradients and differences in the microenvironment (i.e. pH, pO_2, nutrients etc.) as a function of distance from a supporting blood vessel. These properties can to some extent be imitated *in vitro* by the use of multicellular spheroids [14] and several studies have documented significant drug resistance in spheroids (10–100 × more resistant [15]) compared to monolayer cultures of the same cell type [15–17]. As these factors are not included in the design of a clonogenic assay, the influence of the three-dimensional structure of solid tumours on the cytotoxic potency of certain anti-cancer drugs will make the prediction of tumour responses uncertain. In this study HT-29 cells cultured as spheroids were more responsive to TCNU (approx. 2 ×) than monolayer cultures of HT-29 cells. The cytotoxic potency of TCNU is therefore not adversely influenced by environmental conditions within the spheroid. These results are consistent with those of Deen *et al.* [18] where BCNU was found to be more potent (approx. 2 ×) against 9L rat brain tumour cell spheroids than the same cells cultured as monolay-

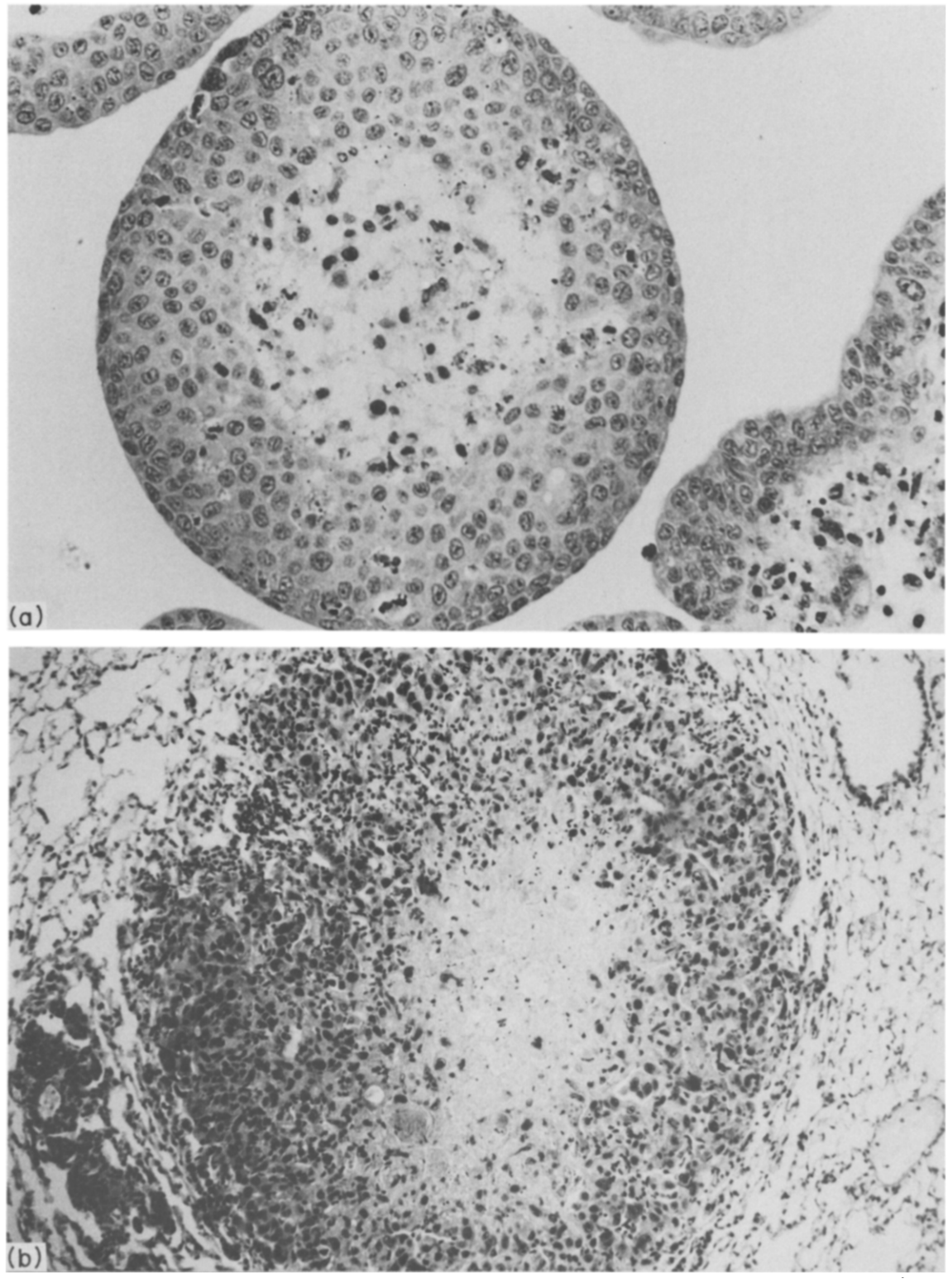

Plate I. (*a*) *Cross-section through an HT-29 multi-cellular spheroid*; (*b*) *Cross-section through MAC* 15.1 *i.v. lung nodule.*

ers. Although an explanation for these observations is not clear, the improved response of spheroids may be the result of greater drug stability within the acidic interior of the spheroid thereby prolonging drug exposures *in vitro*. This result nevertheless suggests that the response of a tumour to TCNU closely reflects the inherent chemosensitivity of tumour cells as assessed by the clonogenic assay.

These properties, in conjunction with the determination of *in vitro* and *in vivo* drug $C \times t$ values, may explain the good correlation between the *in vitro* and *in vivo* responses of MAC tumours to TCNU and suggest that the clonogenic assay may be of value in predicting responses to TCNU in the clinic. On a more cautious note, however, although the clonogenic assay correctly predicts the response of both MAC 15A tumours as sensitive, the site dependent response of MAC 15A tumours could not have been foreseen on the basis of differences in the inherent sensitivity of these cells to TCNU. Although the increased sensitivity of HT-29 spheroids to TCNU provides a possible explanation for the increased response of the histologically similar MAC 15A i.v. nodules, the results of a clonogenic assay on tissue taken from one site may not be a good indicator of the sensitivity of metastases at different sites throughout the body.

REFERENCES

1. Vibe-Petersen J, Hansen HH. A phase I clinical evaluation of TCNU: a new water soluble nitrosourea compound, 5th NCI/EORTC Symposium on New Drugs in Cancer Therapy. 22–24 October, Free University, Amsterdam, The Netherlands, 1986.
2. Double JA, Ball CR. Chemotherapy of transplantable adenocarcinomas of the colon in mice. *Cancer Chemother Rep* 1975, **59**, 1083–1089.
3. Bibby MC, Double JA, Morris CM. Anti-tumour activity of TCNU in a panel of transplantable murine colon tumours. *Eur J Cancer Clin Oncol* 1988, **24**, 1361–1364.
4. Hamburger AW, Salmon SE. Primary bioassay of human tumour stem cells. *Science* 1977, **197**, 461–463.
5. Salmon SE, Alberts DS, Meyskens FL Jr *et al.* In: Salmon SE, ed. *Cloning of Human Tumour Stem Cells*. New York, Alan R Liss, 1980, 223–245.
6. Alberts DS, George Chen H-S. Tabular summary of pharmacokinetic parameters relevant to *in vitro* drug assay. In: Salmon SE, ed. *Cloning of Human Tumour Stem Cells*. New York, Alan R Liss, 1980, 351–359.
7. Double JA, Bibby MC, Loadman PM, Bloomer JC. Effects of routes of administration of TCNU on its plasma, tissue and tumour concentrations. *Eur J Cancer Clin Oncol* 1988, **24**, 1355–1360.
8. Phillips RM, Loadman PM, Bibby MC, Double JA. Relationship between pharamcokinetics and stability of TCNU and *in vitro* sensitivity of murine colon tumour cells. *Br J Cancer* 1987, **56**, 199–200.
9. Fogh J. In: Fogh J, ed. *Human Tumour Cells* In Vitro. New York, Plenum Press, 1975, 119.
10. Rice JM, Houchens DP, Sanchez MS, Overtera AA. Correlation of drug sensitivity in human tumour cells grown in soft agar and in nude mice. *Proc Am Assoc Cancer Res*1976, **21**, 274.
11. Bateman AE, Peckham MJ, Steel GG. Assay of drug sensitivity for cells from human tumours: *in vitro* and *in vivo* tests on a xenografted tumour. *Br J Cancer* 1979, **40**, 81.
12. Bateman AE, Selby PJ, Steel GG,Towse GDW. *In vitro* chemosensitivity tests on xenografted human melanomas. *Br J Cancer* 1980, **41**, 189–198.
13. Phillips RM, Bibby MC, Double JA. Experimental correlations of *in vitro* drug sensitivity with *in vivo* responses to ThioTEPA in a panel of murine colon tumours. *Cancer Chemother Pharmacol* 1988, **21**, 168–172.
14. Sutherland RM, Durand RE. Radiation response of multicell spheroids: an *in vitro* tumour model. *Curr Top Radiat Res* 1976, **11**, 87–139.
15. Wibe E, Ofrebro R. A study of factors related to the actions of 1-propargyl-5-chloropyrunidin-2-one (NY3170) and vincristine in human multicellular spheroids. *Eur J Cancer Clin Oncol* 1981, **17**, 1053–1059.
16. West GN, Weichselbaum R, Little JB. Limited penetration of methotrexate into human osteosarcoma spheroids as a proposed model for solid tumour resistance to adjuvant chemotherapy. *Cancer Res* 1980, **40**, 3665–3668.
17. Sutherland RM, Eddy HA, Bareham B, Reich K, Vanantwerp D. Resistance to adriamycin in multicellular spheroids. *Int J Radiat Oncol Biol Phys* 1979, **5**, 1225–1230.
18. Deen DF, Hoshino T, Williams M, Muraoka I, Knebel KD, Barker M. Development of a 9L rat brain tumour cell multicellular spheroid system and its response to 1,3-bis-(2-chlorlethyl)-1-nitrosourea and radiation. *J Natl Cancer Inst* 1980, **64**, 1373.