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Assay of anticancer drugs in tissue culture: comparison of a tetrazolium-based assay and a protein binding dye assay in short-term cultures derived from human malignant glioma

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Because of the methodological difficulties associated with the MTT assay in screening short-term cultures derived from human malignant glioma, a chemosensitivity assay based on the protein staining using sulforhodamine B (SRB) has been optimized for use with these cells. SRB at a fixed dye concentration achieved maximal staining density at 20 min for most cell lines and this intensity was not further increased by using dye concentrations above 0.2%. A delay in staining after fixation did not significantly decrease staining intensity, but delay in dye extraction after fixation and staining did. There was an excellent quantitative and qualitative linear relationship between cell number determined by either the SRB assay or by cell counting, but not with the MTT assay which consistently underestimated the number of cells in assay plates. The MTT assay appeared to be incapable of detecting less than about 150 cells/well, while these small numbers of cell were readily detectable by either cell counting or SRB staining. There was a close correlation between chemosensitivity values derived from the MTT and SRB assays for procarbazine, CCNU and vincristine when the endpoint is taken as either the ID_{25}, ID_{50} or ID_{75}. The results indicate that the SRB is capable of producing broadly similar results to the MTT assay, but is more sensitive in the detection of small numbers of cells with a linear relationship between cell number and SRB staining intensity over a wide range of cell numbers. It is capable of producing data from short-term cultures from malignant glioma and offers technical advantages over the MTT assay in that plates may safely be stored at certain points during the assay without the need for immediate processing. The SRB assay provides a useful alternative to the MTT assay for determining the sensitivity of short-term cultures of human glioma to cytotoxic drugs.

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Introduction

Current therapy for human malignant glioma includes surgery, radiotherapy and chemotherapy, and while chemotherapy provides a worthwhile increase in both relapse-free interval and survival, its effect is relatively modest and there is a pressing need to identify new drugs with greater efficacy against these tumors. Organ-specific *in vitro* assays have proved to be of importance in large-scale screening of agents with useful clinical activity. Malignant glioma, unlike many other types of malignant neoplasm, is characterized by a remarkable readiness to produce representative cell cultures *in vitro*,[1] which provides an ideal system for both individualized chemosensitivity testing[2] and for screening agents for potential clinical activity.

One assay which is widely used for these types of study relies on the ability of viable cells to reduce the yellow dye, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), to a purple formazan product which can then be quantified densitometrically.[3,4] This assay can be performed reliably using established cell lines of both human and animal origin. The exact chemical mechanism of this conversion in living cells is not known and appears to be subject to interference from environmental conditions, components of the cell culture medium, the cytotoxic agents being tested or the solvent used to dissolve the MTT-formazan product, at least at low pH.[5] A further major problem is that there are considerable differences between the ability of short-term cultures derived from malignant

glioma to convert MTT to the formazan product. This appears not to be due to differences in simple kinetic characteristics between cultures, like growth rate, but some biochemical characteristic of individual cell lines (Darling *et al.*, unpublished observations). Assays based on tetrazolium dye conversion also include a timed stop, which when large numbers of microplates are being processed, often makes scheduling of experiments difficult. There is also no point at which the assay can be temporarily halted. In order to address these problems, an alternative assay offering considerable technical advantages over the MTT assay has been investigated. This employs a protein binding aminoxanthene dye, sulforhodamine B (SRB) to provide a quantitative analysis of viable cells in culture.[6] The present study describes the optimization the performance of the SRB assay and assesses its suitability for the determination of *in vitro* chemosensitivity of short-term cultures derived from malignant glioma.

Materials and methods

Cells and media

Ten short-term cultures derived from human malignant glioma (Kernohan grades III or IV) were used at passage levels between 5 and 12 (Table 1). Cultures were initiated as previously described[7,8] from biopsies taken during the routine neurosurgical management of these tumors. The cultures were routinely fed with Ham's F-10 medium buffered with 20 mM HEPES and supplemented with 10% selected fetal calf serum (complete growth medium). Antibiotics were not used during routine cell culture or in chemosensitivity assays. All cultures were routinely screened (and found negative) for mycoplasma infection using Hoechst 33258 staining as previously described.[8] Cell counts were routinely carried out using a ZM Coulter Counter calibrated for use with human glioma cells.

Table 1. Cell lines used in study

Cell line	Diagnosis	Passage levels tested
478	II–III astrocytoma	7
859	IV astrocytoma	5–9
1265	IV astrocytoma	10
1461	IV astrocytoma	7
1472	IV astrocytoma	6
1528	astrocytoma	11–12
1612	IV astrocytoma	9
1675	IV astrocytoma	11
1719	IV astrocytoma	7–9
1925	IV astrocytoma	4–6

Optimization of the SRB assay

The SRB assay was optimized with regard to a number of parameters using four short-term glioma cell lines. These parameters included determining the optimal time for protein staining of cells and the optimal concentration of dye. Cultures were plated at a density of 2000 cells/well, incubated for 48 h and then processed and stained for 10, 20, 30 or 45 min with SRB solution at a concentration of 0.5 mg/ml, exactly as described below. The same cultures were also stained with SRB solutions at concentrations of 0.5, 1, 2 and 4 mg/ml for a fixed period of 20 min. In routine screening applications it is often convenient to be able to store plates at some stage before the endpoint has been read, and so the effect of delaying the time between fixation and staining and between staining and extraction was determined in these cultures. Cells from two cultures were plated out and fixed with TCA when confluent. Fixation and staining were carried out as described below. Staining was performed immediately and with delays of 48, 96 and 168 h after fixation using a dye concentration of 0.5 mg/ml. Duplicate plates which were stained immediately were left for 168 h before the step of dye extraction with Tris base.

The linearity of the relationship between cell number determined by conventional cell counting or indirectly by either the SRB or MTT assay was examined using IN1925 glioma cells which were plated in a 2-fold dilution series from 8000 to 125 cells/well and incubated for 24 h to allow cell adhesion. Plates were then processed using either MTT or SRB as described below, and further plates were trypsinized and the cell number in each well determined.

Comparison of chemosensitivity determinations using MTT or SRB assays

Chemosensitivity experiments were performed in 96-well microtiter plates. Cells were plated at a density of 1500–2000 cells/well in 100 μl of complete growth medium. Following incubation time for 48 h at 37°C, to allow the cells to reach exponential growth, the medium was replaced with 100 μl of

drug solution. Drug solutions were renewed 24 and 48 h later to give a total exposure time of 72 h. Each cell line was tested against three drugs used in the clinical treatment of malignant glioma: procarbazine (PCB; Natulan, Roche), CCNU (Lundbeck) and vincristine (VCR; Oncovin, Eli Lilly). Stock drug solutions were prepared as follows: PCB at a concentration of 10 mg/ml, VCR at a concentration of 1 μg/ml, both in Ham's F-10 medium, and CCNU at a concentration of 2 mg/ml in ethanol. All stock solutions were subsequently stored at −20°C. For use in individual experiments, drug solutions were diluted in Ham's F-10 supplemented with 10% fetal calf serum. Drug dilutions were prepared freshly for each experiment. The highest concentration to which cells were exposed was 4 mg/ml for PCB, 20 μg/ml for CCNU and 0.02 μg/ml for VCR. Each drug was tested at six different concentration levels. Each drug concentration was tested in 12 wells per assay and 24 wells were used as controls on each plate. The total number of microassays employed for the comparison of MTT and SRB assays amounted to 5760 wells or 60 plates. To ensure that the cells remained in exponential growth phase throughout the assay, a separate plate was prepared for each cell line. This was refed each day with fresh medium and six wells were trypsinized each day, the contents pooled and counted.

After a total drug exposure time of 72 h, wells were rinsed with Hank's balanced salts solution and the cells were allowed a recovery period of 72 h in 100 μl of fresh growth medium per well. The microtitration plates were then processed using either the MTT or SRB assay. MTT (Sigma, St Louis, MO) was used at a concentration of 1 mg/ml in Ham's F-10 and SRB (Sigma) at a concentration of 0.5 mg/ml in 1% v/v acetic acid.

In the MTT assay, cells were incubated with 10 μl of MTT solution at 37°C and after 4 h, the supernatant was gently removed and 100 μl of dimethyl sulfoxide (DMSO, Sigma) added to each of the wells. Plates were the gently agitated on a gyratory shaker to solubilize the formazan crystals and the optical density (OD) was determined *in situ* using a Dynatech MR600 microtitration plate reader at a wavelength of 570 nm.

For the SRB assay, cultures were fixed in situ on the plates by layering 25 μl of cold (9°C) 50% w/v TCA onto the medium in each well to achieve a final TCA concentration of 10%. After incubation at 4°C for 1 h, microtiter plates were washed five times with tap water and air dried. Staining was performed with 100 μl of SRB solution per well with a staining time of 30 min. Unbound dye was removed by five washes with 7% v/v acetic acid and the plates were air dried. Protein-bound dye was extracted with 100 μl of 10 mM Tris base [tris (hydroxymethyl) aminomethane] with gentle agitation on a gyratory shaker and OD measured as described above.

Results

Optimization of the SRB assay

With a fixed dye concentration maximum staining density was achieved at 10 min with one culture (IN1925) and at 30 min with the remaining two (Figure 1). With a fixed staining time of 20 min staining intensity was not increased with dye concentration above 0.2% indicating maximal uptake of dye by cell protein (Figure 2). Compared with immediate staining, delay in staining after fixation did not significantly decrease staining intensity. With delay in dye extraction after immediate fixing and staining the decrease in intensity was more marked (Figure 3). There was an excellent quantitative and qualitative relationship between cell number determined by either conventional cell counting or by using SRB staining at both high cell densities (Figure 4) or at low cell densities (Figure 5). The relationship between cell number determined by cell counting or SRB staining was linear over the range of 0–8000 cells/well and the line passed through the origin (Figure 5). The MTT assay, however, appears to consistently underestimate the number of cells and this is particularly marked at lower cell densities (Figure 5). MTT seems to be incapable of detecting less than about 150 cells/well, while these small numbers of cell were readily detectable by either cell counting or SRB staining.

Comparison of chemosensitivities determined by the two assay methods

Representative dose–response curves for cell line IN 859 treated with CCNU and the endpoint measured by both the MTT assay and the SRB assay are shown in Figure 6. Survival is expressed as a percentage of survival in control wells. The doses of drug which inhibited protein staining (SRB assay) or MTT-formazan production (MTT assay) by 25% (ID_{25}), 50% (ID_{50}) and 75% (ID_{75}) are indicated. A panel of 10 (nine for CCNU) short-term glioma cell lines were used to assess the agreement between the MTT and the SRB assay. With an overall concentration range of six 2-fold dilution steps tes-

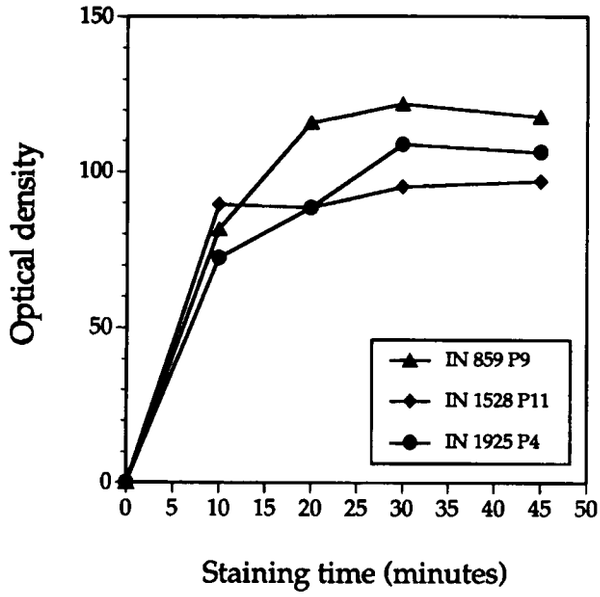

Figure 1. Effect of varying the staining time on the absorbance of cells stained with 0.5 mg/ml SRB.

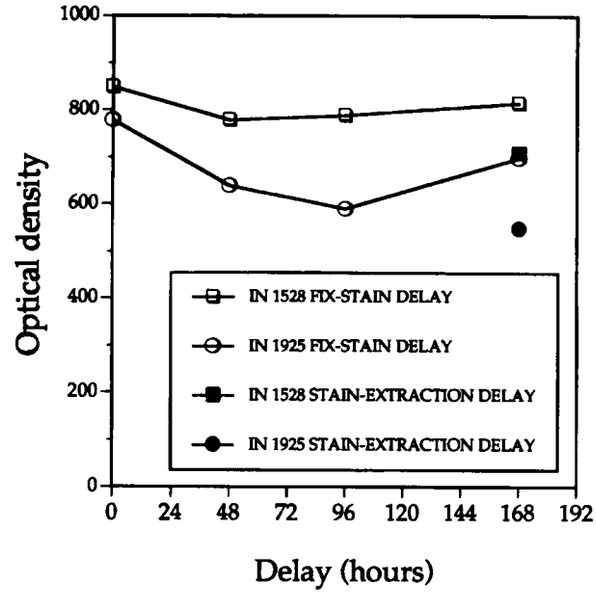

Figure 3. The effect of introducing a delay between fixation and staining or between staining and extraction on the optical density measured.

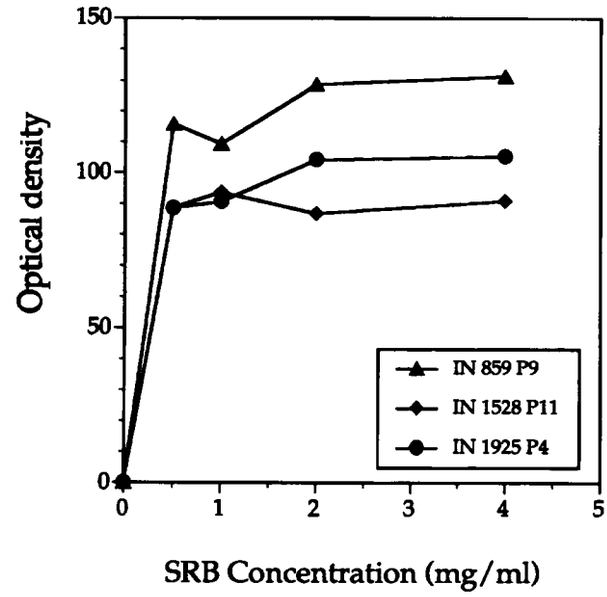

Figure 2. Effect of varying SRB concentration on the absorbance of cells stained for a fixed time of 20 min.

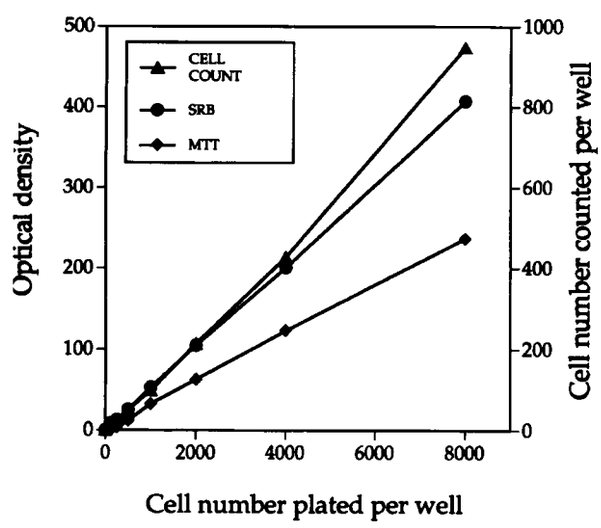

Figure 4. Relationship between cell number assessed by cell counting, the SRB and the MTT assays at high cell densities.

ted for each drug, the ID_{25} and ID_{50} values could be determined in all cell lines, while the ID_{75} value could be determined in only 70% of the lines because some of the lines were markedly resistant to the drugs used. For each cell line a Bland–Altman plot[9] was constructed by plotting the difference between the ID value determined by each of the assay methods against the mean of the two determinations. In this type of plot, if there is complete agreement between the two variables, all the points should lie along the zero line on the ordinate. If the points were to line consistently above or below this line this would be an indication of a systematic difference between the two assay methods, e.g. assay A always produces a result which is consistently

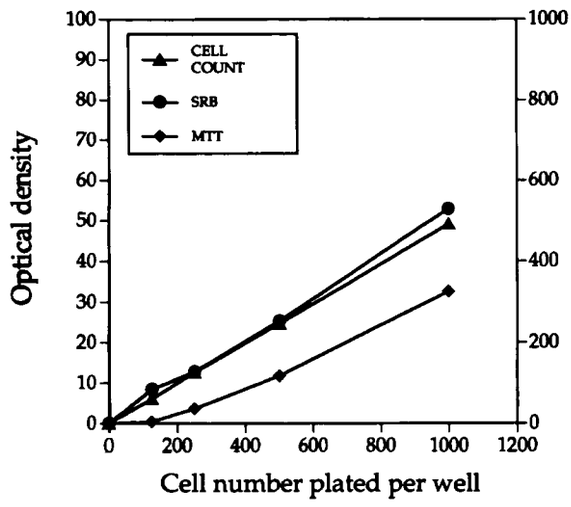

Figure 5. Relationship between cell number assessed by cell counting, the SRB and the MTT assays at low cell densities.

greater than assay B at a given drug dose. There is no indication that this was the case in this situation, as the points tended to be distributed randomly either side of the zero line. Likewise, there is no evidence of a consistent difference between the assays if the endpoint is taken as either the ID_{25}, ID_{50} and ID_{75}. There was a close correlation between chemosensitivity values derived from the MTT and SRB assays for PCB (Figure 7), CCNU (Figure 8) and VCR (Figure 9). However, the greatest variation between the two assays was most evident when the ID_{75}'s were being compared for PCB (Figure 7) and CCNU (Figure 8).

To quantitate these differences, it is possible to calculate the limits of agreement by adding or subtracting twice the value of the SD from the mean difference between the endpoints measured by each assay.[9] The limits of agreement for each of the drugs tested are given in Table 2. In practice this means, for example, that the SRB assay may produce an ID_{50} value for procarbazine which is 0.85 mg/ml below or 1.39 mg/ml above the ID_{50} value produced by the MTT assay. The limits of agreement shown in Table 2 indicate that the differences are distributed randomly about the mean and that the adoption of one assay over the other is unlikely to affect the ranking of cultures in terms of chemosensitivity either in individualized chemosensitivity tests or in mass drug screening programs. Again, the widest variation between the assays is seen when the ID_{75}'s are compared.

Discussion

Chemosensitivity assays are important tools for a variety of purposes in experimental cancer biology. They can be used to assess individual patient response to cytotoxic drugs and to allow the development of individualized chemotherapy.[2,10,11] They can also be used to screen agents for activity against panels of cell lines derived from defined histological groups of tumor and for biological studies aimed at the elucidation of the mechanisms of drug resistance and attempts to circumvent it development. The characteristics of such assays have been discussed[12] and it is clear that the only worthwhile parameter that needs to be recorded is the number of cells remaining at the end of the assay. Human malignant glioma, perhaps almost uniquely amongst human solid tumors, produces short-term cell lines which appear to be composed of replicating malignant glial cells.[7,13,14,15] Perhaps 90% of surgical biopsies of grade III or IV malignant glioma give rise to short-term cell lines and about half of these will go on to establish in culture. Under these circumstances and in view of the poor clonogenic efficiencies displayed by these short-term lines we have concentrated on the use of microtitration plate-based assays which are capable of producing data rapidly from many cell lines over a wide range of drug concentrations in a semi-automated manner. The chemosensitivity assay we have used, originally described by Freshney *et al.*,[16] has used a variety of methods to determine the endpoint, i.e. [^{3}H]leucine,[16] [^{35}S]methionine,[17] MTT[11] and, in the present paper, SRB staining. The basic design of the assay before the measurement of endpoint has remained consistent, i.e. long drug exposure followed by an extended recovery period. This design has ensured that there is excellent correction between the results obtained with this intermediate duration microtitration assay and clonogenic assays[17] and that the results obtained with any of the non-clonogenic measures of endpoints are extremely consistent. This being the case, it seems reasonable to ignore the biological basis of the measurement of endpoint and to concentrate on the design of the assay. Two main considerations then apply: (i) can the assay be applied to all the cultures which are required to be assayed and (ii) is the assay technically convenient to carry out? These criteria are of particular importance if an assay is to be used in a clinical setting to predict chemosensitivity on an individual basis or for mass screening of drugs.

The present study examined the variables which influence cell staining by SRB. This assay is based

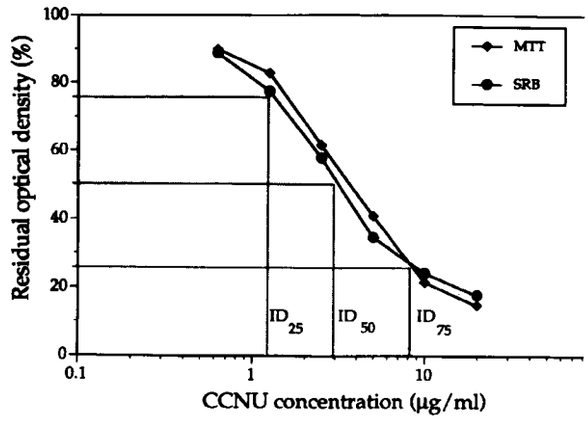

Figure 6. Representative dose-response curves produced by CCNU using the IN 859 cell line and measured using the MTT and the SRB assays showing the derivation of the ID_{25}, ID_{50} and ID_{75} values.

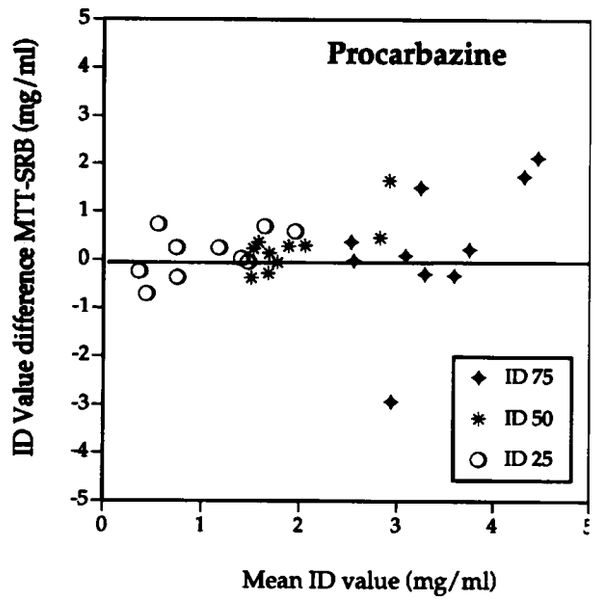

Figure 7. Bland–Altman plot showing the comparison of ID_{25}, ID_{50} and ID_{75} produced from a panel of glioma cell lines tested against PCB.

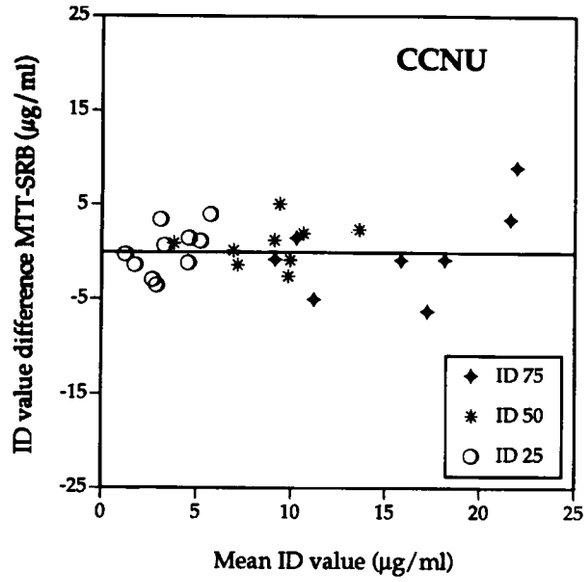

Figure 8. Bland–Altman plot showing the comparison of ID_{25}, ID_{50} and ID_{75} produced from a panel of glioma cell lines tested against CCNU.

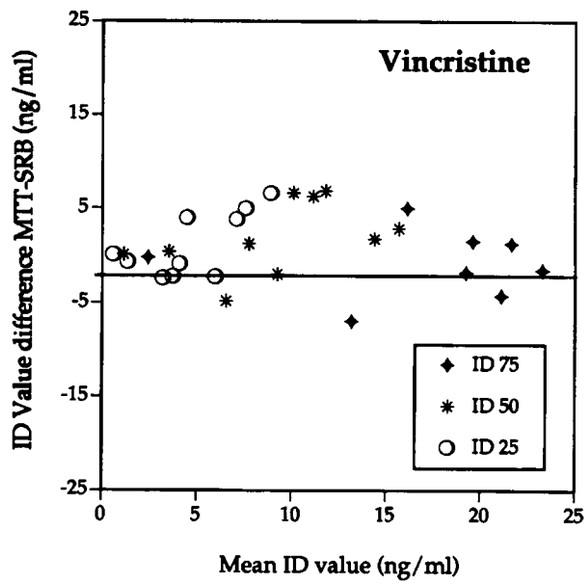

Figure 9. Bland–Altman plot showing the comparison of ID_{25}, ID_{50} and ID_{75} produced from a panel of glioma cell lines tested against VCL.

on a total protein stain and does not rely on the ability of cells to reduce MTT to its formazan product. It is clear that the time taken to stain the cells and SRB concentration are important variable in this assay. Sufficient time (more than 20 min) is required to ensure maximal staining of cells and that reducing the staining time below this could result in 25–30% lower OD values. The relationship between dye concentration and OD is more clear cut. Dye concentrations as low as 0.05% produce near plateau levels of cell staining and that while there is only a small increase in staining intensity by increasing the concentration to 0.2%, no further effect is seen by increasing this concentration further. Con-

Table 2. Comparison of the MTT and SRB assay

Drug	ID	Mean difference	Standard deviation	Limits of agreement	
		(x)	(s)	x−2s	x+2s
Procarbazine	25	0.11	0.48	−0.85	1.07
	50	0.27	0.56	−0.85	1.39
	75	0.24	1.42	−2.6	3.08
CCNU	25	0.097	2.49	−4.9	5.1
	50	0.75	2.28	−3.81	5.31
	75	1.25	5.9	−10.6	13.1
Vincristine	25	1.05	3.38	−5.7	7.8
	50	1.87	3.85	−5.8	9.6
	75	−0.95	3.68	−8.3	6.4

centration effects appear to be particularly important in the MTT assay and a number of workers have emphasized the need for increased concentrations of MTT to be used at very high cell densities.[5] It is also clear that there is considerable variability in the concentration of MTT required to give optimal results between established cell lines and that this may require optimization on an individual basis.

Once the plates have been fixed there appears to be little diminution in the eventual OD values produced if the plates are stored unstained for at least a week. However, if the plates are stained, but left unextracted, for this period of time, there is a marked effect on the OD values subsequently achieved of around 15–20%. This presumably indicates that cells are not lost from fixed plates in significant numbers over this period of time, but that significant amounts of dye are lost or degraded over this period.

The linearity of the relationship between cell number and OD is one of considerable importance in the MTT assay. Clearly, any assay which is required to determine the number of residual cells must have a linear relationship between cell number and OD. However, the MTT assay has rarely been shown to have such a relationship.[18,19] In these experiments, we have chosen to use cell densities we have previously encountered typically when assaying drugs against short-term cultures of malignant glioma. Other workers, using established cell lines, have reported very considerable deviation from the linear relationship which is particularly marked at very high cell densities.[18,19] Our data certainly gives the impression that a similar deviation is occurring in our MTT assays, but not when cell numbers are estimated using SRB staining or by Coulter counting. At low cell densities, it is clear that short-term glioma cells are incapable of reducing MTT to the formazan product in sufficient amounts to be detectable, although sufficient cellular protein is present to be stained with SRB. It is possible that small numbers of scattered cells which may not be actively dividing may not metabolize MTT efficiently. It should be remembered that these cells were not drug treated and it is possible that larger numbers of drug-treated cells may also not be capable of detection by MTT assay should their metabolism be affected.

It is clear that a significant minority of short-term cell lines derived from malignant glioma are incapable of carrying out this conversion efficiently, resulting in a small difference between the absorbencies in the control and test wells (data not shown). This does not occur in the SRB assay; it is therefore applicable to all adherent short-term cultures including those which are 'poor converters' of MTT.

When suggesting the adoption of one assay method over another, it is self evident that there must be a strong relationship between the results of one assay and another. This may not in itself be sufficient, there must also be good *agreement* between the assay results if they are to be used interchangeably. Certainly, when the endpoints of two assays are compared using a scatter plot and the relationship between them examined by regression analysis there will always be an apparently good relationship between the two assays, but this may disguise rather poor agreement. The use of Bland–Altman plots to examine the degree of agreement has demonstrated that when comparing ID_{25}'s and ID_{50}'s, the MTT and SRB assays are comparable. However, for two drugs, PCB and CCNU, the limits of agreement are largest for the ID_{75} and this may be a reflection of the inability of the MTT assay to detect small numbers of residual cells in the culture plates. In practical terms, this type of microtitration-based assay is not designed to measure very high cell kills (i.e. detect very small numbers of residual cells) and the ID_{50} value is very often chosen as the index of chemosensitivity. This is because the dose–response curve at this point is rather stable (see Figure 6) and small changes in drug dose do not bring about large changes in cell viability. However, it is clear that portions of the curve which are rapidly changing, e.g. the ID_{75}, may be a more unreliable endpoint resulting in larger apparent differences between the assays.

The SRB assay is simple to perform and as demonstrated results which correlate well with the established MTT assay when applied to *in vitro* chemosensitivity testing of short-term cultures

derived from malignant glioma. The advantages of the SRB assay recommend its wider application in drug sensitivity studies in neuro-oncology.

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