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Comparison of tetrazolium colorimetric and [^{3}H]-uridine assays for in vitro chemosensitivity testing*

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Summary. We have routinely used a [^{3}H]-uridine microplate assay for assessing chemosensitivity. A colorimetric assay with the advantages of safety, cost and simplicity has previously been described and relies on the ability of living cells to reduce a soluble tetrazolium salt, 3-4,5-dimethylthiazol-2,5-diphenyl-tetrazolium bromide (MMT), into an insoluble formazan precipitate. We compared the chemosensitivity of 14 human tumour cell lines of colonic, lung and cervical carcinoma origin to doxorubicin, vindesine or vindesine immunoconjugates in both the [^{3}H]-uridine assay and a modified MTT assay to evaluate whether we could change to the non-radiolabelled method. Correlation between the concentration of drug causing 50% inhibition of cell growth (IC_{50}) for these agents between the two assays was very poor. However, taking account of recent reports in the literature, we modified the MTT assay by removing serum-containing medium and using dimethyl sulphoxide to solubilise the formazan precipitate. This considerably improved the correlation between the assays for doxorubicin ($r = 0.871$; $P = 0.001$) and vindesine ($r = 0.981$; $P < 0.001$). Our data indicates that the MTT assay can be used to replace the [^{3}H]-uridine assay for chemosensitivity screening, but further modifications are necessary to improve the sensitivity and decrease the problem of cell loss after washing, which was noted with some adherent cell lines.

Introduction

Since the report of a rapid colorimetric assay that uses the ability of viable cells to reduce a soluble tetrazolium salt, 3-4,5-dimethythiazol-2,5-diphenyl-tetrazolium bromide (MTT), into an insoluble formazan precipitate [6], a number of laboratories have investigated its potential for in vitro drug screening with panels of human tumour cell lines [1–3, 7, 10]. In an attempt to improve its sensitivity and reproducibility, factors affecting the technical performance of the MTT assay have also been examined [1, 4, 11]. The MTT assay has been compared with dye exclusion, clonogenic and cellular protein assays, and although there have been comparisons with [^{3}H]-thymidine uptake for the assessment of cell viability, these have involved relatively few cell lines of non-human origin [4, 6]. We have used [^{3}H]-leucine incorporation for the assessment of human tumour chemosensitivity in vitro [12] or [^{3}H]-uridine incorporation for the in vitro evaluation of monoclonal antibody-drug immunoconjugates with the vinca alkaloids [5] or doxorubicin [8]. In this report we describe the results of our direct comparison of the MTT and [^{3}H]-uridine assays for the assessment of the in vitro sensitivity of a panel of human tumour cell lines to vindesine, vindesine immunoconjugates and doxorubicin.

Materials and methods

Tumour cell lines

A total of 14 human tumour cell lines were studied: colon carcinomas COLO320DM, COLO201, SW1116, SW837, WIDR, HT29, LOVO, LS174T and SKCO1; lung carcinomas BENN, CALU-3 and CALU-6; and cervical carcinomas C33A and MS751. BENN was obtained from Dr. M. Ellison at the Ludwig Institute for Cancer Research (Sutton, U. K.); all other cell lines were obtained from the American Type Culture Collection (Rockville, Md, USA). Cells were grown as monolayers in medium containing 10%–16% foetal calf serum (FCS). Media used included Eagle's MEM (SKCO1, LS174T, WIDR, CALU3, C33A and MS751), Dulbecco's Modified Eagle Medium (CALU6), RPMI 1640 (COLO320DM and COLO201), L15 (SW1116 and SW837), Medium 199 (BENN), McCoy's (HT29) and Hamm's F10 (LOVO).

Microcytostasis assays

[^{3}H]-uridine assay. A terminal uridine assay was used to determine the sensitivity of the cell lines to drug, conjugate or antibody [5]. Cell lines were grown until they were subconfluent, then trypsinised, washed in PBS and resuspended in fresh media. A total of 100 µl medium containing 10^4 viable cells were plated per well into 96-well microtitre plates. Cells were allowed to attach to the plates for 24 h at 37° C in a 5% CO_2 atmosphere. Doxorubicin (DOX; Adria Laboratories Inc., Columbus, Ohio) or vindesine (VDS) (Eli Lilly & Company Inc., Toronto, Ontario) dissolved in sterile saline at 1 mg/ml or vindesine anti-carcinoemb-

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ryonic antigen conjugate were used after sterile filtration through 0.22-μm Millipore filters. Because DOX binds to the sterilizing filters, a small volume was removed aseptically, diluted in PBS (pH 7.4) and the drug concentration was determined from the absorbance at 495 nm. Drug or conjugate dilutions were prepared in culture medium (range, 0.001–100,000 n*M*). Medium was removed from the cells and test dilutions were added in 100 μl fresh medium. Tests were carried out with ten control wells (cells, no drug treatment) and five wells for each test dilution. Cells were exposed to the drug for 24 h at 37° C in 5% CO_2. Medium was removed and the cells were washed three times with PBS. Next, 100 μl fresh medium was added to each well, followed by a 24-h recovery period. Cells were then incubated for 3 h with 1 μCi [^{3}H]-uridine (New England Nuclear, Boston, Mass) in 50 μl medium and cell survival was determined from measurements of uridine uptake. The concentration of drug causing 50% inhibition of cell growth (IC_{50}) was determined for each line. A direct comparison was made with the MTT assay by processing duplicate plates as follows.

MTT assay. The performance of this assay was identical to that of the [^{3}H]-uridine assay up to the end of the 24-h recovery period after test reagent exposure. MTT (M-2128; Sigma Chemical Company, St. Louis, Mo) was dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg/ml, sterile filtered and stored in a dark environment at 4° C for up to a maximum of 3 weeks. In initial experiments, the following procedure was used: 10 μl was added/well, followed by incubation at 37° C for 4 h. Next, 100 μl 0.04 *N* HCl in 10% dimethylsulphoxide (DMSO) (BDH Chemicals, Toronto, Ontario) isopropanol (Sigma Chemical Company) was added to each well with vigorous mixing. Plates were incubated at room temperature (RT) for 20 min, then read spectrophotometrically using dual wavelength filters (570 and 630 nm) with a Bio-Tek EL310 E1A reader interfaced with a Tandy 1200 microcomputer. Absorbance at 630 nm was used as the reference wavelength for detecting artefacts in the plastic plates and was subtracted from the 570-nm values. An additional MTT/medium blank control was used. Wells with medium only (no cells) were processed in exactly the same manner as the rest of the plate, and the mean absorbance for these wells was subtracted from the absorbance values in the other wells. Data analysis was carried out using Bio-Tek Reader and Curve Fitter Software (Mandel Scientific, Rockwood, Ontario). It was often difficult to get complete dissolution of crystals with this procedure. IC_{50} values were determined as described for the [^{3}H]-uridine assay.

Subsequently we modified the procedure as follows. After the 24-h recovery period following drug exposure, medium was aspirated from each well and 100 μl 1:10 dilution of MTT stock (5 mg/ml) was added to each well, followed by a 4-h incubation at 37° C. MTT/medium blank controls with no cells also received 100 μl MTT/well. After incubation, medium was aspirated from each well, 100 μl DMSO was added/well and the plates were agitated on a plate shaker for 20 min at RT. Plates were then processed as above.

VDS conjugates

The preparation of covalent conjugates of vinca alkaloids to IgG has previously been described [5, 9]. Briefly, VDS hemisuccinate was activated as the *N*-hydroxysuccinimide ester and reacted at pH 8.6 for 4 h with monoclonal anti-carcinoembryonic antigen antibody 11-285-14. The background to the use of this antibody has previously been reported [5]. Conjugates were purified by gel filtration on Biogel P6 in PBS and characterised for drug and Ig content by spectrophotometry at 270 and 280 nm, using extinction coefficients previously determined at these wavelengths for the individual components. Two different batches of VDS-11-285-14 conjugate (prepared by Hilary Marsden, Lilly Research Centre, Windlesham, U. K.) were used in this study. Batch 1 contained 5.654 mg/ml immunoglobulin (Ig) and 162 μg/ml conjugated VDS; batch 2 contained 4.088 mg/ml Ig and 168 μg/ml conjugated VDS. Conjugates were stored in a dark environment at 4° C after filtration.

Results

The sensitivity of 12 cell lines to DOX, as measured by their IC_{50} values in simultaneous MTT and [^{3}H]-uridine assays, is shown in Table 1. The correlation coefficient was poor (0.587) and not significant ($P > 0.05$) for those lines for which an IC_{50} value was obtained. Of these cell lines, 10 (plus BENN) were evaluated with VDS in both assays; these results are presented in Table 2. In this case the correlation coefficient of 0.819 did indicate a significant relationship between the data obtained with both assays ($P < 0.01$).

Six cell lines were tested with VDS-anti-CEA conjugates in both assays, and these results are shown in Table 3. There was good agreement between the IC_{50} values for SKC01 and WIDR, but it was very poor for the other cell lines. When the two different batches were tested with the same cell line, the results also indicated that batch 2 was less efficacious than batch 1.

The considerable differences in IC_{50} values obtained with DOX, VDS or VDS monoclonal anti-CEA conjugates

Table 1. Sensitivity of cell lines to DOX

Cell line	IC_{50} (n*M*)[a]	
	MTT[b]	[^{3}H]-uridine
SW1116	>18,399[c]	1,472
SW837	9,200	2,208
MS5751	6,440	644
SKC01	4,784	162
COLO320DM	2,392	2,944
HT29	1,693	331
COLO201	920	129
LS174T	764	75
C33A	552	110
LOVO	552	111
WIDR	405	368
CALU3	140	184

[a] Correlation coefficient between IC_{50} values in the MTT and [^{3}H]-uridine assays = 0.587; $P > 0.05$

[b] After 24 h recovery following drug exposure, 10 μl MTT stock (5 mg/ml) was added to each well, followed by incubation at 37° C for 4 h. With vigorous mixing, 100 μl 0.04 *N* HCl in 10%/DMSO/isopropanol was then added to each well. Plates were incubated for 20 min at RT prior to reading

[c] IC_{50} not achieved within the range of concentrations tested

Table 2. Sensitivity of cell lines to VDS

Cell line	IC_{50} (n*M*)[a]	
	MTT[b]	[^{3}H]-uridine
SW1116	>37,603[c]	>37,603[c]
SW837	>37,603[c]	9,400
BENN	7,411	216
LS174T	45	18
COLO201	6	6
HT29	3	9
SKC01	2	2
LOVO	2	2
MS751	0.35	6
WIDR	0.3	3
C33A	20	2

[a] Correlation coefficient between IC_{50} values in the MTT and [^{3}H]-uridine assays = 0.819; $P < 0.01$
[b] Same as in Table 1
[c] IC_{50} not achieved within the range of concentrations tested

between the MTT and [^{3}H]-uridine assays clearly indicated that we could not reliably use the MTT assay (carried out essentially as previously described [6]) as a substitute for the [^{3}H]-uridine assay. In an attempt to improve the performance of the MTT assay, we incorporated some of the modifications reported by Twentyman and Luscombe [11]. Firstly, we evaluated the effect of cell number on both assays for four cell lines (Fig. 1). There was a linear relationship between plated cell number and radioisotope incorporation for all of the lines, even at the lowest cell densities used; however, this was not the case for absorbance. There was a relationship between absorbance and cell number, but it was not linear. LS174T and SKCO1 were very similar, but C33A and CALU3 displayed different curves and showed less sensitivity.

We then re-evaluated sensitivity to DOX in both assays with seven of the cell lines used in the original assay plus three additional ones; these results are presented in Table 4. In comparison with Table 1, a considerable improvement can be seen in the correlation between the two assays ($r = 0.871$; $P = 0.001$). The survival curves were very similar for all cell lines in both assays; representative curves as a percentage of control values are illustrated in Fig. 2 for four of the lines (LS174T, HT29, C33A and SW1116).

Table 3. Sensitivity of cell lines to VDS conjugates

Cell line	Batch number	IC_{50} (n*M*)	
		MTT[b]	[^{3}H]-uridine
LS174T	1	5,453	2,900
	2	18,877	10,427
SKC01	1	317	546
	2	964	1,116
WIDR	1	232	232
	2	567	614
LOVO	2	176	11,516
C33A	1	1,645	87
COLO201	1	>28,202	92

[b] Same as in Table 1

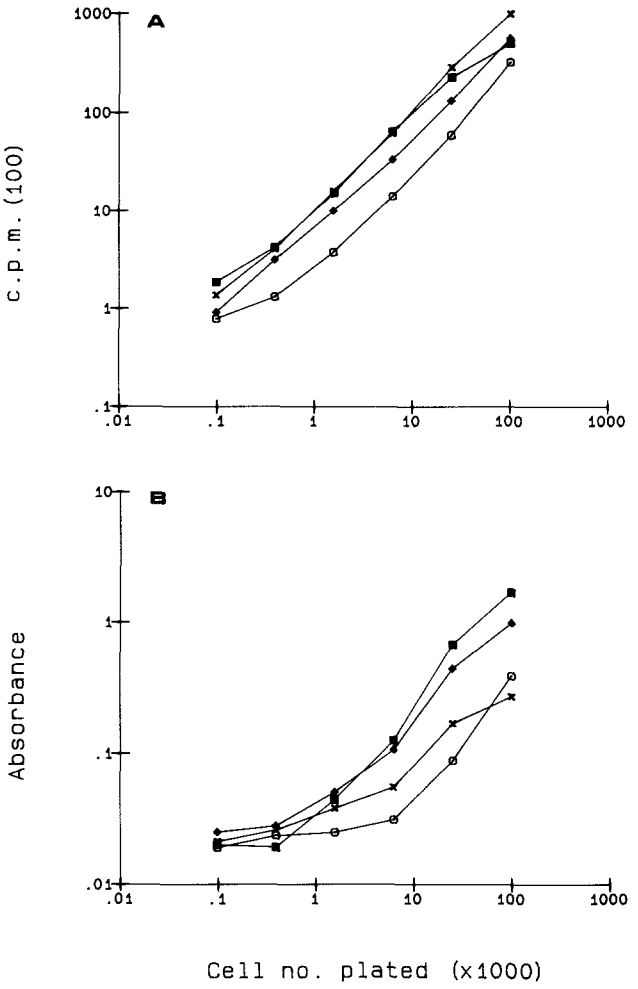

Fig. 1. Relationship between plated cell number and **(A)** cpm in the [^{3}H]-uridine assay and **(B)** absorbance in the modified MTT assay for four cell lines. -■-, LSI74T; -◆-, SKCO1; -O-, C33A; —><—, CALU3. The initial number of cells plated/well were: 98, 391, 1.56×10^3; 6.25×10^3; 2.5×10^4; and 10^5, respectively. Assays were identical in timing and procedure to those described in *Materials and methods*, except that there was no drug incubation and, consequently, no washing on day 2

The IC_{50} values for VDS were also re-examined with the same ten cell lines used for DOX, and the results are presented in Table 5. As with doxorubicin, there was a considerable improvement in the correlation between the two assays ($r = 0.981$, $P < 0.001$), and the VDS survival curves for cell lines in both assays were very comparable. Representative survival curves from both MTT and [^{3}H]-uridine assays are shown in Fig. 3 for the same four cell lines illustrated in Fig. 2. We also noticed a considerable improvement in the intra-assay variation in the modified MTT assay. This is illustrated in Table 6 for both a relatively sensitive (HT29) and a resistant cell line (SW1116) with both DOX and VDS.

Discussion

The reasons for our interest in the MTT assay were its potential advantages in terms of safety, simplicity and cost over radioisotopic methods for the in vitro assessment of human tumour cell sensitivity to chemotherapeutic drugs

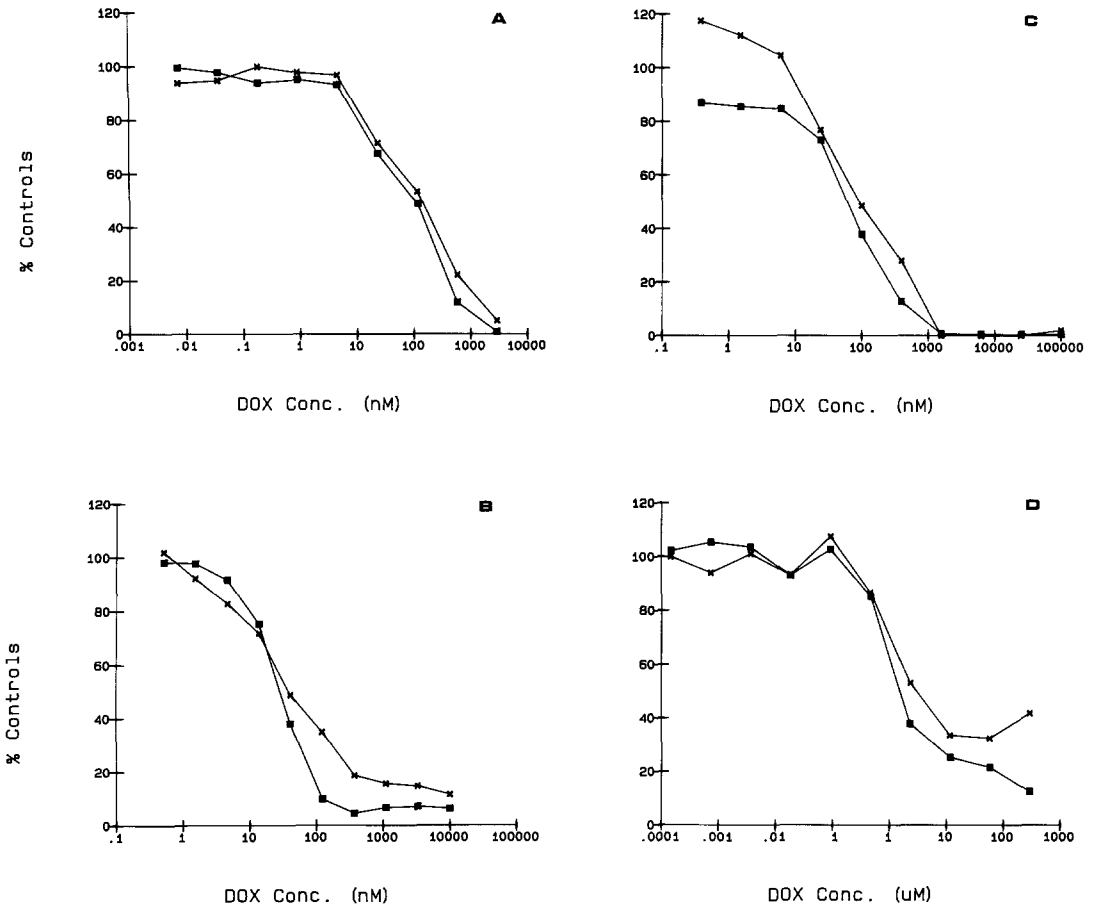

Fig. 2. Comparison of survival curves for four cell lines in either the [^{3}H]-uridine assay (–■–) or the modified MTT assay (———) with doxorubicin. **A** LSI74T; **B** HT29; **C** C33A; **D** SW1116. Each point represents the mean of five wells. Error bars have been omitted for clarity

and immunoconjugates. The initial results of our comparison of the two assays were disappointing and particularly for doxorubicin (Table 1), indicated that we could not use the MTT assay as a substitute for the [^{3}H]-uridine assay. Although there was a better correlation between the [^{3}H]-uridine and MTT assays with VDS (Table 2), the data were not good enough to permit a change from one assay to the other for our in vitro drug screening. VDS-anti-CEA conjugates were not as extensively compared as uncon-

Table 4. Sensitivity of cell lines to DOX with the modified MTT assay

Cell line	IC_{50} (n*M*)[a]	
	MTT[b]	[^{3}H]-uridine
BENN	12,000	4,000
SW1116	2,800	1,600
CALU6	800	600
SKC01	600	21
WIDR	100	48
LS174T	100	100
C33A	90	60
CALU3	55	13
HT29	40	30
A549	25	28

[a] Correlation coefficient between IC_{50} values in the modified MTT assay vs [^{3}H]-uridine assay = 0.871; $P = 0.001$

[b] After 24 h recovery following drug exposure, the medium was aspirated and 100 µl 1 : 10 dilution of MTT stock (5 mg/ml) was added to each well, followed by 4-h incubation at 37° C. The medium was carefully aspirated and 100 µl DMSO was added to each well, followed by incubation at RT for 20 min on a shaker prior to reading

Table 5. Sensitivity of cell lines to VDS with the modified MTT assay

Cell line	IC_{50} (n*M*)[a]	
	MTT[b]	[^{3}H]-uridine
SW1116	77,600	82,300
LS174T	12	11
SKC01	7	3
HT29	6	9
CALU6	41	15
A549	14	5
BENN	6	2
CALU3	14	20
C33A	4	4
WIDR	3	3

[a] Correlation coefficient between IC_{50} values in the modified MTT assay vs [^{3}H]-uridine assay = 0.981; $P < 0.001$

[b] Same as in Table 4

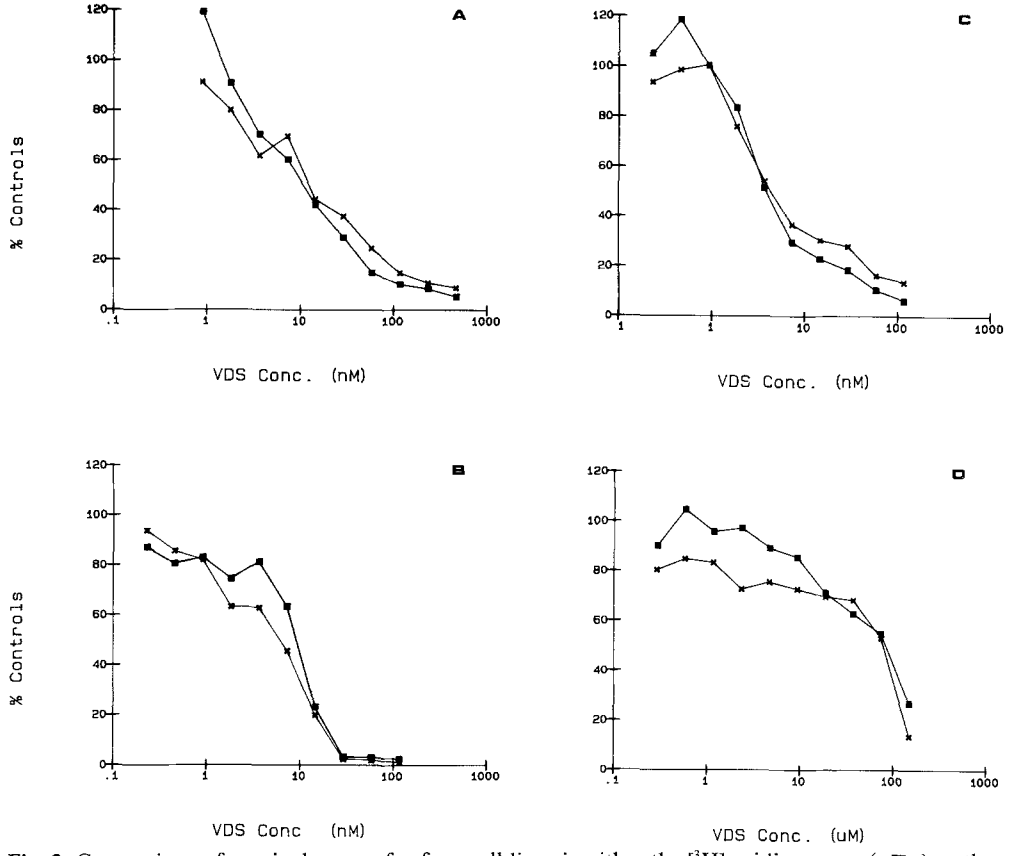

Fig. 3. Comparison of survival curves for four cell lines in either the [^{3}H]-uridine assay (–■–) or the modified MTT assay (—×—) with vindesine. **A** LSI74T; **B** HT29; **C** C33A; **D** SW1116. Each point represents the mean of five wells. Error bars have been omitted for clarity

Table 6. Comparison of intra-assay variation – modified MTT assay and [^{3}H]-uridine assay[a]

HT29					
	DOX			VDS	
n*M*	MTT	[^{3}H]-uridine	n*M*	MTT	[^{3}H]-uridine
10,000	11.5 (1)	6.2 (1.3)	117.0	1.1 (0.6)	2.4 (0.41)
3,300	14.7 (1.4)	7.0 (0.7)	58.8	1.9 (0.4)	3.0 (0.2)
1,100	15.6 (2.1)	6.6 (0.5)	29.4	2.4 (1.1)	3.3 (0.9)
370	18.7 (0.9)	4.5 (0.9)	14.7	19.9 (0.8)	23.2 (2.1)
123	34.9 (2.9)	9.8 (0.6)	7.5	45.7 (2.1)	63.5 (5.4)
41	48.8 (6.5)	38.0 (5.1)	3.7	62.9 (12)	81.4 (5.8)
14	71.7 (21.1)	75.1 (12.6)	1.8	63.3 (7.9)	74.5 (4.4)
5	82.7 (12)	91.5 (14.2)	0.9	82.0 (10.3)	83.1 (7.4)
1.5	92.1 (11)	97.6 (7.6)	0.45	85.6 (15.5)	80.6 (7.2)
0.5	101.9 (9.2)	98.1 (13.2)	0.2	3.6 (8.2)	87.1 (3.9)
SW1116					
μ*M*			μ*M*		
289.0	41.9 (4.2)	12.6 (2.1)	150.0	13.6 (9.1)	27.0 (8.4)
57.8	32.5 (4.2)	21.7 (1.9)	75.0	53.0 (10.6)	54.7 (5.2)
11.6	33.6 (6.8)	25.4 (2.6)	37.5	68.2 (9.1)	62.8 (4.7)
2.3	53.2 (12.5)	38.0 (4.6)	18.8	69.7 (4.5)	71.2 (6.3)
0.5	86.8 (5.3)	85.5 (4.9)	9.4	72.7 (9.1)	85.6 (4.6)
0.09	107.6 (20.4)	102.8 (10.8)	4.7	75.7 (10.6)	89.4 (12.6)
0.02	93.2 (5.3)	93.2 (6.6)	2.4	72.7 (10.6)	97.3 (7.5)
0.004	101.1 (7.5)	103.6 (9.5)	1.2	83.3 (7.6)	95.8 (3.4)
0.0007	94.0 (8.3)	105.4 (7.6)	0.6	84.8 (4.5)	104.7 (11.1)
0.0001	100.0 (10.2)	102.2 (18.4)	0.3	80.3 (6.1)	90.1 (12.4)

[a] Mean of five test wells as a percentage of control values with standard deviations in parentheses

jugated drugs, but sufficient data were generated to confirm the results obtained with VDS (Table 3), i.e. great variability in the data obtained with most cell lines between the two assays. Another factor (data not shown) was that the intra-assay standard deviations in the MTT assay were often very large (>15%), being considerably greater than those in the [^{3}H]-uridine assay, which were usually <10%.

Three major problems were apparent. The first, the fact that certain cell lines were more prone to becoming detached during the washing procedures, was common to both assays. We therefore concentrated on the second and third, which involved the incomplete dissolution of the crystals in the MTT assay and the variable occurrence of protein precipitation.

These last two problems have been reported by others [4, 11]. Twentyman and Luscombe [11] confirmed the report of Carmichael et al. [2] that DMSO was a suitable solvent for the formazan product, and also demonstrated that residual medium in the wells of the microtitre tray used in the assay could affect the shape of the absorbance spectrum. We modified our procedure to take account of these observations by completely aspirating medium after the 4-h incubation with MTT, adding 100 µl DMSO to each well and agitating the samples at RT for 20 min prior to plate reading. As can be seen from Tables 4 and 5, this considerably improved the correlation between the MTT and [^{3}H]-uridine assays for DOX and VDS. This is more clearly illustrated with representative survival curves for four cell lines with DOX (Fig. 2) and VDS (Fig. 3), which show good agreement between the curves obtained in both assays.

An important observation was that the intra-assay standard deviations for values obtained in the MTT assay had also improved considerably and were approaching those of the [^{3}H]-uridine assay as exemplified by two representative cell lines HT29 and SW1116 (Table 6).

If IC_{50} values for DOX are compared between different [^{3}H]-uridine assays (Table 4 with Table 1), quite large inter-assay variations can be seen for certain lines. For example, the IC_{50} values for SKCO1, HT29, WIDR and CALU3 are generally 1 log lower for DOX in later assays, whereas for other lines (e.g. SW116 and LS174T) they are consistent. This was more of a problem with DOX than with VDS as, in general, there was excellent agreement in [^{3}H]-uridine IC_{50} values between assays (Table 5 compared with Table 2). The exception to this was BENN. Although such variation is undesirable in an in vitro assay used for drug screening, it is not unusual, which has been shown by Park et al. [7] with the MTT assay. These authors found considerable variation in the inter-assay mean ID_{50} values for colorectal cell lines exposed to certain chemotherapeutic drugs, for example 5-fluorouracil (5FU). However, their reported reproducibility with DOX was good. Some of the variation in the present assays was due to the tendency of certain lines to wash off, particularly in the case of BENN and, to a lesser degree, A549 and CALU3. This variability in DOX IC_{50} values has previously been noted by us [8], and we are undertaking studies to see if the reproducibility can be improved with "problem" lines. These lines have all been shown to be mycoplasma-free; thus, microbial contamination is not the cause.

Carmichael et al. [2] have found the MTT assay to be particularly good for adherent cell lines, where the standard deviation of absorbance readings approximated ±5%, and have found it less good for floating lines, where standard deviations of ±15% were observed. However, these authors used either a 1-h exposure, in which cells were washed after exposure and then dispensed into microtitre plates, or a continuous 4-day drug exposure, after which wells containing the cells were not washed. The loss of cells during the washing procedures may therefore explain the higher variations noted by us with certain adherent lines in comparison with the results obtained by Carmichael et al. [2].

There was a very good linear relationship between the number of plated cells and subsequent radioisotope incorporation in the [^{3}H]-uridine assay (Fig. 1A). Although there was also a clear relationship between cell number and absorbance in the MTT assay (Fig. 1B), the shape of the curve, which was not linear, and the number of cells required to produce an observable effect were greater. For example, with C33A there was little difference between final absorbance, ≤6,250 cells being the initial number of plated cells. Only with 2.5×10^4 and 10^5 cells was there a significant increase in absorbance; this is in contrast to the results obtained with LS174T and SKCO1, where initial plated cells numbering $>10^3$ resulted in significant increases in the subsequent absorbance values. Although we evaluated only four cell lines at different plating densities, our data with the MTT assay are in contrast to those from several groups of authors who have demonstrated that the reduction of MTT correlates linearly with the number of human cancer cells [2, 3, 10]. The most extensive evaluation has been carried out by Alley et al. [1] with 86 human cancer cell lines. These investigators calculated the inoculation densities that resulted in exponential or near-exponential growth and a linear, detectable range of absorbance values (minimum, >0.05; maximum, >0.5 units) following culture of each cell line for 7 days.

Clearly, factors such as the loss of cells during the washing procedures and the fact that the MTT assay depends on both the number of cells present and the mitochondrial activity per cell [4] affect the ability of individual cell lines to reduce MTT. Taken as a whole, the results from several laboratories and the work presented in this paper confirm that whereas there is a relationship between cell number and absorbance, individual cell lines can vary considerably in their ability to reduce MTT.

Our results demonstrate that the MTT assay modified according to Twentyman and Luscombe [11], with its advantages of safety, cost and simplicity, may be used to replace the [^{3}H]-uridine assay for the assessment of in vitro cell line chemosensitivity. The correlation between the [^{3}H]-uridine and MTT assays was excellent for VDS and acceptable for DOX. However, with certain adherent lines there were problems of cell loss during the washing procedure we used; modifications to the assay will have to be considered for these "problem" lines. Other improvements in the MTT assay are also desirable, and one that has been reported to increase sensitivity is the use of a reference wavelength of 690 nm rather than 620 or 630 nm [4]. It remains to be seen whether sensitivity can be further improved with this approach and whether other chemotherapeutic drugs and immunoconjugates will show a good correlation between the assays after these modifications. Once a standardised assay has been established, it will also be possible to evaluate inter-assay variation more thoroughly.

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