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Assessment of antineoplastic agents by MTT assay: partial underestimation of antiproliferative properties

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Summary. In the present study a slightly modified MTT assay was used in conjunction with cell counting to determine the antiproliferative efficacy of *N*-(2-chloroethyl)-*N*-nitroso-*N'*-2-hydroxyethylurea (HECNU), vinblastine, and hexadecylphosphocholine (HPC) in a panel of six tumor cell lines. This panel consisted of two human (MDA-MB231, MCF-7) and two rodent (1/C2, 1/C32) mammary-carcinoma cell lines as well as of two tumor cell lines of gastrointestinal origin (HT-29, KB). It was shown that the use of acid isopropanol as a solvent of the formazan crystals produced correlations between cell number and absorption that were as good as, if not better than, those seen after dimethylsulfoxide (DMSO) application. The optimal period of incubation with the MTT dye was 2 h. A comparison of the antiproliferative activity of HECNU revealed that the HT-29 cell line was most resistant [50% inhibition concentration (IC_{50}), 138.7 μmol/l], followed by MCF-7 cells (IC_{50}, 127.7 μmol/l), whereas MDA-MB231 cells showed the highest sensitivity (IC_{50}, 6 μmol/l). Vinblastine induced the highest (MCF-7 cells; IC_{50}, 0.68 nmol/l) and the lowest (1/C2 cells; IC_{50}, 7.69 nmol/l) degrees of growth inhibition in cell lines derived from mammary carcinoma. This contrasted with the activity of HPC, which was considerably less effective in the four mammary-carcinoma cell lines (IC_{50} from 29.4 to 69.9 μmol/l) than in the two cell lines of gastrointestinal origin (IC_{50}, 1.9 and 3.1 μmol/l). Interestingly, treatment with HPC stimulated the growth of 1/C32 cells in the lower dose range. After treatment with HECNU, the average IC_{50} value determined in the MTT assay was 2.4-fold that disclosed by cell counting, whereas the average values found for HPC and vinblastine by both methods corresponded fairly well, with the respective values obtained using the MTT assay being only 26% and 14% higher than those measured by cell counting. A dose-dependent increase in the mean size of MCF-7 cells was observed after exposure to HECNU, which – if taken into account – considerably reduced underestimation of this parameter by the MTT assay. No variation in cell size was noted following treatment with HPC and vinblastine. Thus, depending on the antitumor agent used, the MTT assay can result in slight or even considerable underestimation of the antitumor efficacy of certain compounds and may need correction by consideration of the effect of the drugs on cell size.

Introduction

Experimental chemotherapy assesses the antiproliferative activity of potential antineoplastic compounds in tumor systems. These include in vivo models such as transplanted tumors [12, 41] and chemically induced malignancies [35, 46] or in vitro screening assays. A disease-oriented anticancer drug-screening panel using established tumor cell lines of many types of malignancies has recently been employed for large-scale drug-sensitivity testing with the aim of assaying compounds for selective growth inhibition [1, 6]. Among various in vitro assays, the MTT assay first described by Mosmann [26] as a rapid colorimetric assay was chosen as a valid, simple, and semiautomated method of assessing selective growth inhibition in established cell lines [1, 6, 37]. In this assay, a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, MTT] is reduced to a blue formazan product by reducing enzymes present only in living, metabolically active cells. Since 1983, the MTT assay has been adopted worldwide in many cell-culture laboratories. Besides its use in drug screening, it has been applied in various other fields, e.g., for measuring radiosensitivity [15, 32, 42], cell-mediated cytotoxicity using human immunocompetent cells [10, 14,

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HECNU, *N*-(2-chloroethyl)-*N*-nitroso-*N'*-2-hydroxyethylurea; HPC, hexadecylphosphocholine; MNU, methylnitrosourea; DMSO, dimethylsulfoxide; IC_{50}, 50% inhibitory concentration; APC, alkylphosphocholines; PBS, phosphate-buffered saline; fl, femto litre

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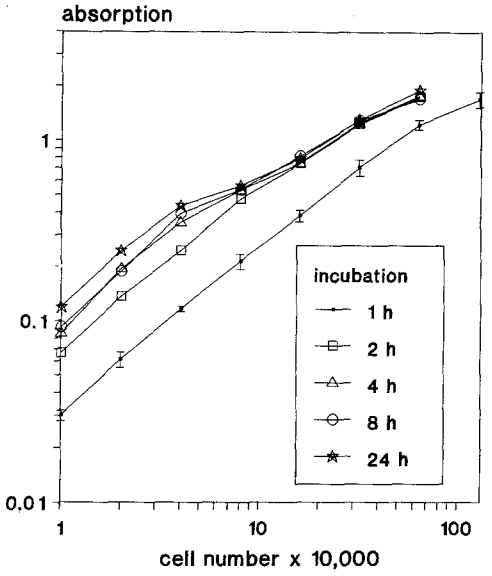

Fig. 1. 1/C2 cells. Correlation of optic density and cell number after different periods of incubation with the MTT dye. The correlation coefficients for the linear regression equation y = a+bx were 0.999 (1 h), 0.990 (2 h), 0.987 (4 h), 0.983 (8 h), and 0.983 (24 h)

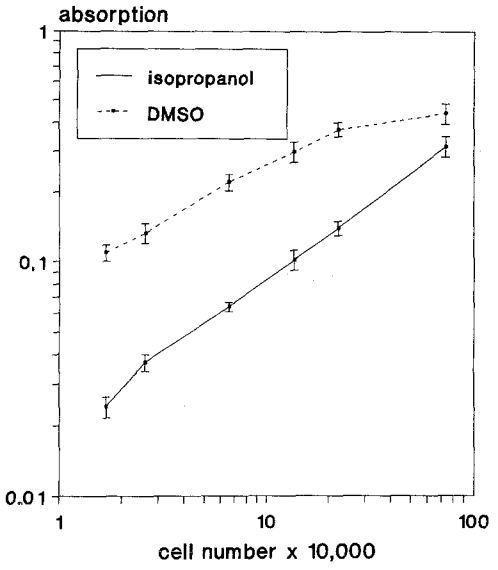

Fig. 2. HT-29 cells. Correlation of optic density and cell number using isopropanol and DMSO as MTT formazan solvents. The correlation coefficients for the linear regression equation y = a+bx were 0.992 (isopropanol) and 0.979 (DMSO; cell number, $<2 \times 10^5$ cells/well) or 0.836 (DMSO; cell number, up to 7.5×10^5 cells/well)

40], and sensitivity of malignancies to standard cytostatics. Recent results indicate that the MTT assay is useful in predicting the clinical effectiveness of various chemotherapeutic agents against human malignancies [5, 17, 21, 29, 30, 34, 45]. Apart from established human-tumor cell lines and human ex vivo tumor cells, the assay has been performed using normal cells [11, 20, 24, 25], viral plaques [22, 44], bacteria [28], fungi [23], and filariae [8]. Its overall performance was quite good.

To establish the validity, predictability, and limitations of a slightly modified MTT assay for drug-sensitivity testing in our laboratory and to delineate the limitations with respect to its use in clinical drug-sensitivity assessment, we investigated vinblastine (mitosis inhibitor), *N*-(2-chloroethyl)-*N*-nitroso-*N'*-2-hydroxyethylurea (HECNU; alkylating agent), and hexadecylphosphocholine (HPC; alkylphosphocholine, a membrane-directed antiproliferative agent) as examples of different groups of antitumor agents. Their antiproliferative activity was compared in human and methylnitrosourea (MNU)-induced rat cell lines by cell counting and MTT assay. The chosen panel of mammary and gastrointestinal cell lines represents two of the most frequent sites of human malignancy.

Materials and methods

Cell lines and culture

Rat-cell clones. The cell clones 1/C2 and 1/C32, derived from an MNU-induced mammary carcinoma growing in a female BD-VI rat during the stage of invasive growth, were kindly provided by Dr. H. R. Scherf [2]. The 1/C2 clone ist characterized by a mean cell volume of 800 μm^3, 44 chromosomes, and a doubling time in culture of 14.7 h, whereas the 1/C32 clone exhibits a mean cell volume of 3,000 μm^3, 88 chromosomes, and a doubling time of 26.3 h. Both cell clones induced tumor growth after transplantation.

Human-tumor cell lines. Two human mammary-carcinoma cell lines were used: MDA-MB231, a high-grade anaplastic carcinoma cell line that displays a doubling time of 26.2 h, and MCF-7, a well-differentiated cell line containing estrogen, androgen, progesterone, and glucocorticoid receptors that exhibits a doubling time of 31.7 h. The MDA-MB231 cell line was obtained from the American Type Culture Collection (Rockville, Md., USA), and the MCF-7 cell line was supplied by the Tumor Bank of the German Cancer Research Center (Heidelberg, FRG). In addition, a human colon-carcinoma cell line (HT-29) was provided by the Tumor Bank of the German Cancer Research Center, and a human oropharyngeal-carcinoma cell line (KB) was obtained from the American Type Culture Collection. HT-29 cells and KB cells showed doubling times of 23.9 and 26 h, respectively.

Cell culture. All cell lines were adapted to RPMI 1640 medium containing sodium bicarbonate (2.0 g/l) supplemented with 10% (v/v) heat-inactivated (57° C; 40 min) fetal calf serum (FCS), streptomycin (100 µg/ml), penicillin (100 IU/ml), and L-glutamine (4 µmol/ml); all of these substances were obtained from Serva (Heidelberg, FRG). All cell lines were free of pathogenic contamination during the experiments and were grown as monolayers in a humidified atmosphere (5% CO_2/95% air) at 37° C.

Modified MTT assay

Cell monolayers in the exponential growth phase were trypsinized, and single-cell suspensions were obtained by repeated pipetting. The percentage of viability was determined by the trypan blue exclusion test. To determine the relationship of cell number to extinction in the MTT assay, increasing numbers of cells were plated in wells of 24- and 96-well culture plates. Alter 24 h incubation, the cells were counted and the MTT assay was performed concomitantly.

For testing of different concentrations of the formazan dye, MTT was dissolved in phosphate-buffered saline (PBS) at concentrations of 2 and 5 mg/ml. The solutions were filtered through a 0.22-µm filter. Following appropriate incubation of cells, 50 µl of one of the MTT stock solutions

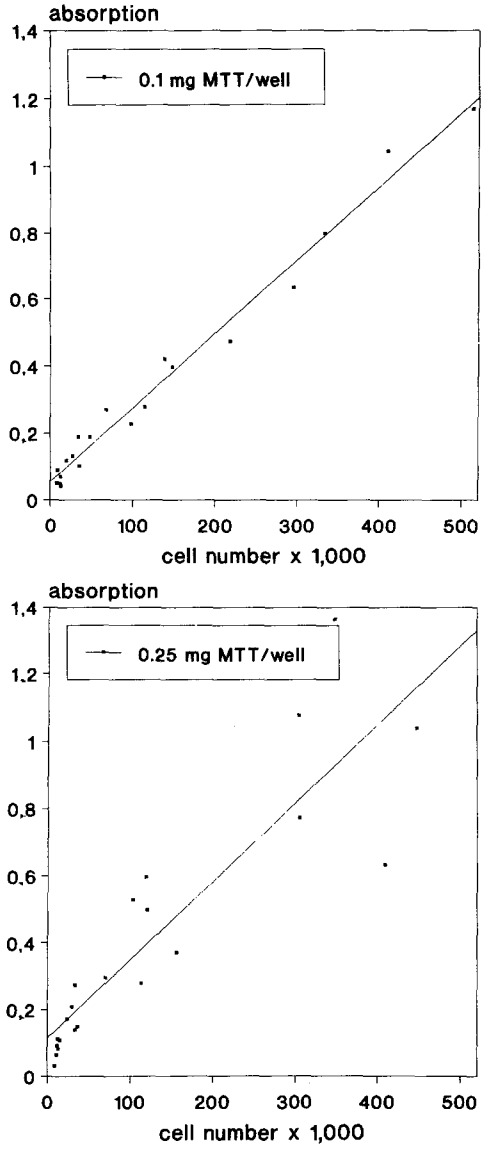

Fig. 3. 1/C2 cells. Correlation of optic density and cell number using different amounts of the MTT dye. The correlation coefficients for the linear regression equation y = a+bx were 0.983 (0.1 mg/well) and 0.792 (0.25 mg/well)

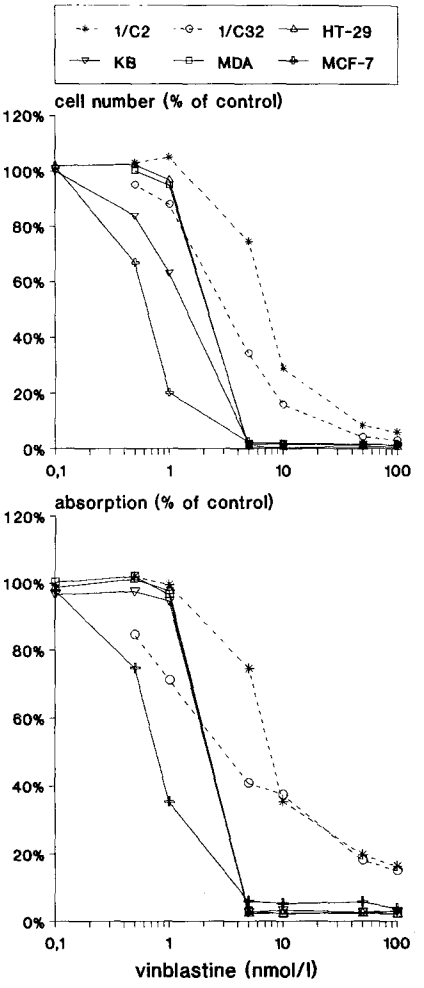

Fig. 4. Dose-dependent growth-inhibitory effect of vinblastine following continuous 72-h exposure of cells to drug as estimated by cell counting and MTT assay. Each point represents the mean value for 3 (cell count) and 8 (MTT assay) independent determinations performed in 1 experiment. Error bars have been omitted for clarity (SD of all values, <10%)

was added to each well of 96-well culture plates. After incubation for 2 h at 37° C, the medium was discarded using a suction pump and a drying cabinet. Then, 100 µl acid-isopropanol (0.1 ml 0.04 *N* HCl in isopropanol) or dimethylsulfoxide (DMSO) was added to each well to dissolve the MTT formazan crystals; the use of a culture-plate shaker accelerated the dissolution of formazan. Within 1 h after addition of the solvent, the absorbance at 540 nm was measured by a Titertek Multiskan (MCC) reader using 96-well microtiter plates. For determination of the optimal MTT incubation period, increasing numbers of 1/C2 cells were incubated with MTT for 1, 2, 4, 8, and 24 h; the best results were obtained following 1- or 2-h incubation intervals.

For cell-growth studies, serially increasing numbers of cells were plated in different columns on 24- and 96-well microtiter plates, and cell counting and the MTT assay were performed daily. No major variation in cell growth was found between the growth rates obtained in 24- vs 96-well cell-culture plates. Growth rates varied among the cell lines as well as between treated and untreated cells. The number of cells plated were chosen such that untreated cells were in the exponential phase of growth throughout the experiments.

Drug-sensitivity assessment was carried out using continuous exposure of cells to drugs. Equal numbers of cells dispersed in 500 µl (100 µl) medium were inoculated into 24-well (96-well) microtiter plates. At day 1 after plating, the double-concentrated drugs were dissolved in 500 µl (100 µl) medium and added to the plates. The microculture tetrazolium assay was performed on each of the subsequent 5 days to control for balanced cell growth during the experiments. On the 3rd day of drug incubation, cells from parallel treated wells were also counted by Coulter counter. For comparisons of the MTT assay with cell counting, the IC_{50} values for the 3rd day were calculated for both methods. An incubation period of 3 days was selected for comparisons of the two methods to allow sufficient time for drug-induced cell death to occur within the exponential growth phase of the control cells.

Cell counting

In a parallel experiment, cells dispersed in 1 ml medium in the presence and absence of drugs were grown in 24-well microtiter plates. The medium was discarded, and the monolayers were trypsinized with 500 µl trypsin-EDTA solution by incubation at 37° C for 15 min. Single-cell suspensions were produced by repeated pipetting and were checked microscopically prior to counting by Coulter counter (model ZF, Coulter Electronics Ltd., Krefeld, FRG). In addition, the percentage of viable cells was determined by the trypan blue exclusion test. The viability always exceeded 93%.

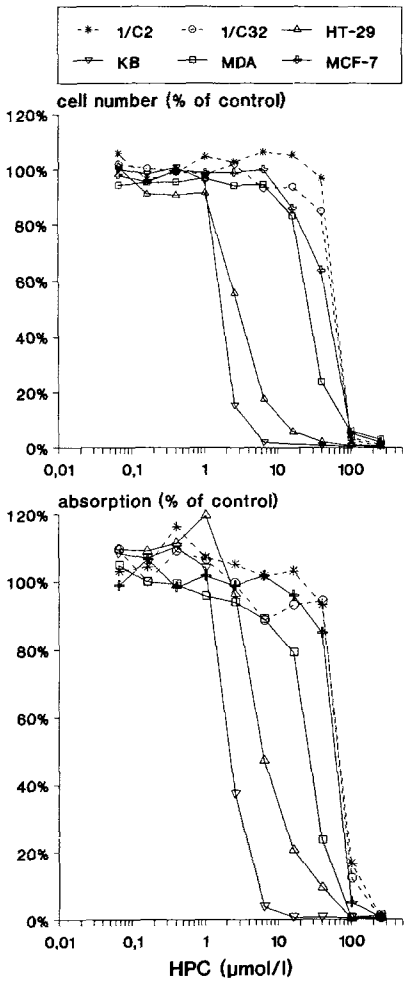

Fig. 5. Dose-dependent growth-inhibitory effect of HPC following continuous 72-h exposure of cells to drug as estimated by cell counting and MTT assay. Each point represents the mean value for 3 (cell count) and 8 (MTT assay) independent determinations performed in 1 experiment. Error bars have been omitted for clarity (SD of all values, <10%)

Cell volume

Changes in cell volume were checked microscopically and were exemplarily quantified in MCF-7 cells grown in 24-well microtiter plates. The volume of control cells and cells exposed to drugs for 24, 48 and 72 h was determined in single-cell suspensions using a Coulter Channelyzer 256 (calibration was carried out using 67.8-fl latex particles; Coulter Electronics Ltd., Krefeld, FRG).

Drugs

Vinblastine was provided by Rhone-Poulenc Pharma GmbH (Norderstedt, FRG), HPC was obtained from Asta Pharma AG (Frankfurt, FRG), and HECNU was synthesized by Prof. G. Eisenbrand. The crystalline stock materials were stored at –20° C. Test agents were dissolved by sonication in medium containing no FCS, and drug solutions were freshly prepared for each experiment to account for drug instability.

Results

The correlation of optic density (determined by MTT assay) and cell number (determined by Coulter counter) is exemplarily given in Fig. 1 for the 1/C2 clone after different periods of incubation with the MTT dye. Linear regression analysis revealed high correlation at between 10,000 and 500,000 cells/well (24-well culture plate) for all incubation periods ($r > 0.98$). By 2 h incubation, the maximal absorption had nearly been reached. Thus, a 2-h incubation period was considered to be optimal. Table 1 shows the respective linear regressions obtained for all cell lines, with all correlation coefficients exceeding 0.98.

The two most frequently used formazan crystal solvents isopropanol and DMSO are compared in Fig. 2 according to the growth of HT-29 cells over 5 days. Although DMSO showed considerably higher absorption values than did isopropanol, the correlation found between cell counting and MTT assay for the latter solvent ($r = 0.992$) was as good as that observed for the former ($r = 0.979$) up to a cell number of 200,000 cells/well. At higher concentrations, DMSO seemed to be less advantageous, as it yielded insufficient absorption values ($r = 0.836$). However, the high isopropanol coefficient depended on complete removal of the medium prior to dissolution of the formazan crystals. Figure 3 shows the correlation between cell number and absorption resulting from five and seven growth curves after incubation of cells with MTT dye for 2 h at 0.1 ($r = 0.983$) and 0.25 mg/well ($r = 0.792$), respectively. The corresponding linear regression parameters demonstrate that the lower concentration was associated with a much better correlation than was the higher one.

Table 1. Regression parameters for correlation of optic density and cell number

Cell lines	y = a+bx				
	a±σ	b±σ[a]	*r*	u	*df*
1/C2	0.069 ± 0.053	1.26 ± 0.039	0.993	15	13
1/C32	0.021 ± 0.009	3.24 ± 0.043	0.998	15	13
HT-29	0.052 ± 0.059	0.91 ± 0.038	0.986	18	16
KB	0.056 ± 0.069	2.04 ± 0.088	0.987	16	14
MDA-MB231	0.011 ± 0.012	1.89 ± 0.037	0.998	12	10
MCF-7	0.003 ± 0.004	1.88 ± 0.030	0.998	12	10

[a] values $\times 10^{-6}$

y, absorption (MTT assay); x, cell number (Coulter counter); a, ordinate distance; b, gradient; *r*, correlation coefficient; u, number of experiments; *df*, degrees of freedom; σ, standard deviation

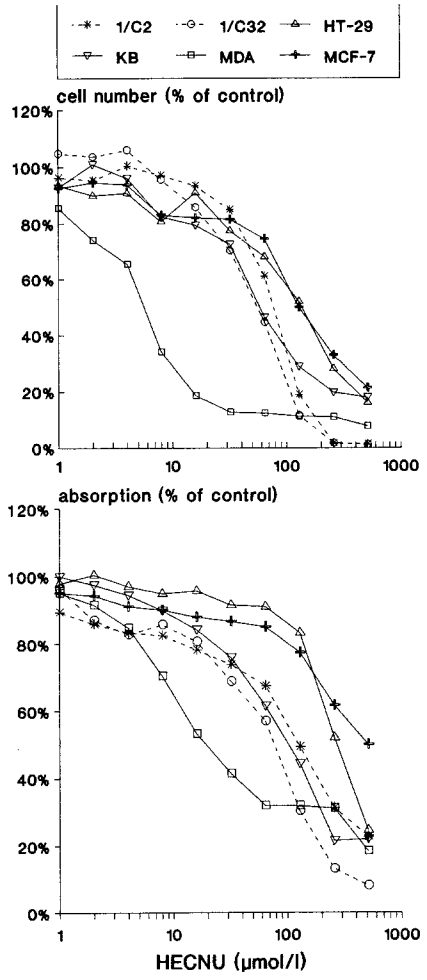

Fig. 6. Dose-dependent growth-inhibitory effect of HECNU following continuous 72-h exposure of cells to drug as estimated by cell counting and MTT assay. Each point represents the mean value for 3 (cell count) and 8 (MTT assay) independent determinations performed in 1 experiment. Error bars have been omitted for clarity (SD of all values, <10%)

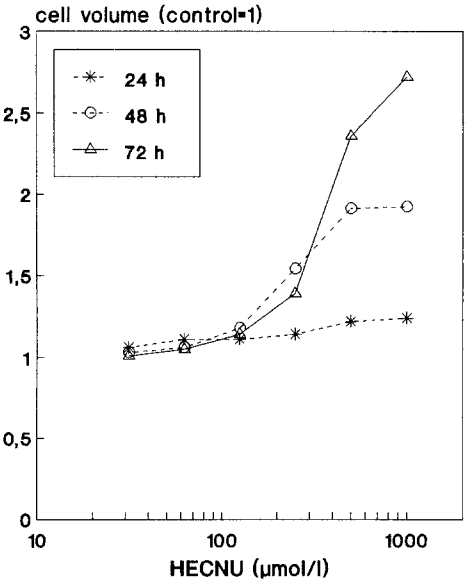

Fig. 7. Dose-dependent increase in the mean cell volume of MCF-7 cells after continuous exposure to HECNU for 24, 48, and 72 h. Each point represents the mean value for 3 independent determinations performed in 1 experiment. Error bars have been omitted for clarity (SD of all values, <7%)

To determine the feasibility of the MTT assay for drug assessment, we compared the antiproliferative activity of vinblastine, HPC, and HECNU used at different concentrations in six cell lines over 3 days. In Figs. 4–6, the concentration-dependent MTT values (absorption expressed as a percentage of control values) obtained after continuous 72-h exposure of cells to drugs are compared with the values resulting from Coulter counting of parallel treated cells. The respective interassay variations were below 10% as determined from control growth values. Concentrations inhibiting cell growth by 50% (IC_{50}) were calculated from the curves (see Table 2). The results indicated that after treatment with the alkylating agent HECNU, average IC_{50} values obtained in the MTT assay were 2.4-fold those resulting from cell counting. Quite a good correspondence between the two methods was found following treatment with HPC and vinblastine, with the average MTT values being only 26% and 14% higher, respectively, than the Coulter-counter findings. An increase in cell size was observed microscopically after exposure to HECNU. This was exemplarily quantified in MCF-7 cells, yielding a dose-dependent increase in the mean cell size (see Fig. 7); at a concentration corresponding to the IC_{50} value for HECNU in the MTT assay (512 µmol/l) a 2.4-fold increase in cell size was found. When this was taken into account, a corrected MTT IC_{50} value of 213 µmol/l was calculated (Table 2). Following treatment of cells with vinblastine

Table 2. IC_{50} values obtained following continuous 72-h exposure of cells to vinblastine, HPC, and HECNU using cell counting and the MTT assay

Cell lines	Vinblastine (nM)		HPC (µM)		HECNU (µM)	
	CC	MTT	CC	MTT	CC	MTT
1/C2	7,69	8,13	69.9	73.8	80.8	125.9
1/C32	3.83	3.78	65.8	72.4	57.3	80.7
HT-29	2.94	3.00	3.1	6.2	138.7	276.4
KB	1.85	2.94	1.9	2.3	59.6	106.9
MDA-MB231	2.92	2.99	29.4	28.4	6.0	20.3
MCF-7	0.68	0.81	54.0	66.3	127.7	512.0[a]

CC, Coulter counter, MTT, MTT assay

[a] 213 µmol/l if corrected for the 2.4-fold increase in cell volume at the respective HECNU concentration (Fig. 7)

Table 3. Comparison of the time-dependent efficacy of HPC as evaluated by cell counting and MTT assay

Cell lines	HPC dose	Survival (% of control)					
		24 h		48 h		72 h	
		CC	MTT	CC	MTT	CC	MTT
1/C2	27 µmol/l	94.3 ± 3.8	92.7 ± 6.6	93.1 ± 3.1	98.1 ± 4.5	93.4 ±42.3	96.2 ± 4.8
1/C2	68 µmol/l	78.3 ± 2.2	91.2 ± 5.1	64.1 ± 2.2	92.5 ± 0.7	60.2 ± 1.5	81.1 ± 2.3
1/C2	170 µmol/l	25.1 ± 1.1	58.4 ± 6.6	6.9 ± 0.8	30.1 ± 5.6	1.1 ± 0.4	6.0 ± 0.4
1/C32	29 µmol/l	110.0 ± 7.9	109.7 ± 9.6	119.2 ± 4.6	111.9 ± 3.2	109.8 ± 2.9	106.6 ± 2.6
1/C32	73 µmol/l	100.8 ± 9.7	112.6 ± 5.8	121.9 ± 3.3	128.4 ± 7.1	104.5 ± 5.8	110.3 ± 9.9
1/C32	183 µmol/l	9.0 ± 1.6	37.2 ± 4.8	4.0 ± 0.6	1.6 ± 0.6	2.9 ± 1.6	2.7 ± 0.3
HT-29	1 µmol/l	101.2 ± 9.3	93.7 ± 2.5	97.1 ± 3.4	94.1 ± 8.9	107.2 ± 2.5	108.4 ± 6.7
HT-29	5 µmol/l	99.3 ± 9.6	86.1 ± 7.2	80.0 ± 0.7	78.1 ± 7.1	76.1 ± 6.0	67.2 ± 6.3
HT-29	25 µmol/l	92.3 ± 9.4	86.1 ± 3.8	40.8 ± 3.2	55.0 ± 0.6	27.4 ± 0.9	45.0 ± 1.7
KB	0.3 µmol/l	97.9 ± 6.7	92.9 ± 3.5	95.2 ± 4.8	94.0 ± 1.2	92.1 ± 4.0	93.7 ± 2.4
KB	1.3 µmol/l	100.5 ± 2.4	91.8 ± 8.2	68.3 ± 3.5	61.1 ± 4.2	29.1 ± 0.8	42.6 ± 2.1
KB	6.5 µmol/l	52.5 ± 1.4	56.5 ± 0.7	15.5 ± 3.3	8.4 ± 2.4	1.7 ± 0.2	4.7 ± 1.3
MDA-MB231	12 µmol/l	85.2 ± 8.8	87.0 ± 1.3	65.2 ± 1.6	72.9 ± 9.8	64.8 ± 3.2	78.0 ± 1.4
MDA-MB231	30 µmol/l	86.7 ± 2.3	89.6 ± 1.3	67.9 ± 1.9	79.0 ±.1.5	59.3 ± 3.5	72.9 ± 2.5
MDA-MB231	75 µmol/l	69.0 ± 1.2	62.3 ± 2.5	19.7 ± 1.6	45.9 ± 2.3	11.5 ± 0.3	26.0 ± 2.5
MCF-7	22 µmol/l	78.9 ± 4.3	83.3 ± 5.6	62.7 ± 2.1	69.6 ± 1.4	63.2 ± 1.1	64.8 ± 1.6
MCF-7	54 µmol/l	77.3 ± 1.9	75.0 ± 5.6	60.4 ± 5.6	60.9 ± 4.3	53.2 ± 4.3	54.9 ± 2.5
MCF-7	135 µmol/l	31.3 ± 0.7	36.1 ± 2.8	16.4 ± 3.6	10.1 ± 1.4	8.1 ± 3.4	4.1 ± 1.6

Data represent mean values ± SD for 3 (Coulter counter, CC and 8 (MTT assay, MTT) independent determinations in 1 experiment, respectively

and HPC, however, no significant variation in cell volume was noted (data not shown). Parallel to the increases observed in cell size, steadily decreasing IC_{50} values were obtained over time during the experimental period (data not shown), thus excluding the quick regrowth of cells exposed to HECNU.

The cell lines used showed a varying rank order in their sensitivity to treatment with the three drugs (Table 2). The human and MNU-induced rat mammary-carcinoma cell lines, for example, were very resistant to treatment with HPC in vitro, with MDA-MB231 cells being the most sensitive of these four cell lines. The human gastrointestinal-carcinoma cell lines HT-29 and KB, on the other hand, were very sensitive to this agent (Fig. 5). MCF-7 cells reacted 4 times more sensitively to treatment with vinblastine than did MDA-MB231 cells. Following treatment with HPC or HECNU, only slight differences between the IC_{50} values for 1/C2 and 1/C32 cells were found, whereas the former cell line was twice as resistant as the latter to vinblastine treatment (Fig. 4). MCF-7 cells and HT-29 cells were very resistant to treatment with HECNU, whereas MDA-MB231 cells appeared to be extremely sensitive to this alkylating agent (Fig. 6).

Finally, to illustrate the time-dependent efficacy of HPC treatment, three HPC concentrations were tested on 3 days using cell counting and MTT assay. In Table 3, the MTT values (absorption expressed as a percentage of control values) obtained on days 1–3 are compared with the results of cell counting. The findings indicate a fairly good correspondence between the two methods for the investigation of time-dependent drug effects within the exponential growth phase of control cells. Surprisingly, the HPC-treated 1/C32 cells showed stimulation of growth in the lower concentration range, with maximal values of 122% (cell counting) and 128% (MTT assay) being obtained after 48 h incubation at an HPC concentration of 73 µmol/l.

Discussion

In the present study, a slight modification of the MTT assay described by Mosmann in 1983 [26] was used to assess cell proliferation and drug-induced cytotoxicity in established human and rodent cell lines in vitro. To simplify culture conditions, all cell lines were adapted to a single culture medium. Methodological investigations revealed that removal of the medium after MTT incubation distinctly improved the solubility of the nonpolar MTT formazan crystals in isopropanol. The additional time involved in using a suction pump and a drying cabinet was negligible in view of the total assay duration. As a result of the lacking interference by the culture medium, the correlation found between absorption and cell number using isopropanol as a solvent was at least as good as that obtained using DMSO.

A comparison of the two solvents revealed interesting differences. DMSO yielded higher absorption values than did isopropanol [6]. At high cell numbers, however, the solution of MTT formazan in DMSO yielded absorption values that did not form a straight regression line. Instead, the absorption tended to underestimate control-cell growth and, hence, cytostatic efficacy. Furthermore, when the regression lines generated for the respective absorptions were extrapolated back, the deviation from the original value was considerably greater for DMSO (for $x = 0$, $y = 0.109$) than for isopropanol (for $x = 0$, $y = 0.035$), thus indicating greater overestimation of low cell numbers for

the former solvent. Apparent MTT formazan production in the absence of cells has also been observed by other groups using DMSO [31]. Accordingly, the inherent disadvantages of the olfactory irritant DMSO, including the safety hazard of human exposure and the deleterious effects on laboratory equipment, were avoided in our experiments by the use of isopropanol.

Under the described test conditions, incubation of cells for 2 h with 0.1 mg MTT/well produced the best linear correlation between cell number and absorption as well as nearly maximal absorption values. Exact maintenance of a 1-h incubation period, as previously used in other studies [19], yielded a similarly good correlation, but it was associated with low absorption values at small cell numbers and was more dependent on strict adherence to the time protocol.

Due to the validity and the short duration of this modified MTT assay and because it does not involve the use of radioisotopes, it appears to be highly suitable for the performance of proliferation studies in established human and nonhuman cell lines. However, for assessments of drug-induced cytotoxicity, the MTT assay should be used with reservation because of the varying validity observed after the administration of different antineoplastic agents. In our experiments a slight (as seen after treatment with HPC and vinblastine) or even considerable (as measured after treatment with HECNU) underestimation of antiproliferative efficacy was noted. Several factors contributing to this effect might be of relevance. If absorption of the drug or its metabolites can be excluded, another direct influence might result from interaction of the drug with enzymes of the mitochondrial electron-transport system such as succinate dehydrogenase, leading to increased or decreased formazan production. Alternatively, indirect changes in the metabolism of the dye might be related to the induction of cell differentiation, which is partially based on increased metabolic capacity as determined by the conversion of nitrotetrazolium blue [3,7] or on changes in cell volume.

Cell swelling is a phenomenon that has commonly been observed after the exposure of cells to S-phase-active cytotoxic agents [33]. In our experiments, cell swelling was found in MCF-7 cells after exposure to HECNU but not following incubation with HPC or vinblastine. The cell volume at the MTT IC_{50} was 2.4-fold that of control cells, and this increase in cell size might have accounted to a considerable extent for the 4-fold increase in formazan production noted at this concentration. Apart from this possibility, other factors such as increased mitochondrial activity might have played a role, as previously reported by Jabbar et al. [18] for interferons. These authors also described a negative influence of old medium on formazan production in control cells, which can also lead to underestimation of cytotoxic efficacy. The latter influence might be of some relevance, since a decrease of 8% – 40% in glucose content was seen in control cells after 3 days of incubation (data not shown).

A comparison of the antiproliferative activity of HECNU in the six cell lines studied revealed that the MER + cell line HT-29 [9] was most resistant, followed by MCF-7 cells, whereas MDA-MB231 cells showed the highest sensitivity. Other authors [43] have described similar differences in sensitivity to alkylating agents between the latter two cell lines, which might well be ascribed to different alkyltransferase levels. Despite the relatively short half-life of HECNU in aqueous media (<1 h) no decrease in efficacy was noted during the experimental period, as IC_{50} values steadily decreased with increasing incubation periods. Thus, the effect of HECNU obviously lasted longer than would have been expected from its half-life.

Vinblastine showed a profile of sensitivity that differed substantially from that of HECNU. The most sensitive (MCF-7) and the most resistant (1/C2) cell lines differed by only a factor of 11, which is low as compared with ratios resulting from multidrug resistance (MDR), which approach a 750-fold difference [36]. Thus, the altogether low IC_{50} values determined in the present study, which were similar to each other, favor the assumption that the cell lines used were devoid of the MDR phenotype.

HPC belongs to a new group of compounds that are completely different from all known classes of antineoplastic agents. Accordingly, its sensitivity profile differed from that of HECNU and vinblastine. It is noteworthy that the dose-response curves generated for HPC showed growth stimulation at low doses, indicating a bimodal effect on tumor-cell growth. In our experiments, the two cell lines derived from the gastrointestinal tract were most sensitive to this agent, whereas the four mammary-carcinoma cell lines showed low to moderate sensitivity. Similar sensitivity relationships have also been observed for other alkylphosphocholine (APC) derivatives [38], and these contrast – at least for the two rodent cell lines – with the exceptionally high sensitivity to APC of MNU-induced, primary mammary carcinomas growing in Spraque-Dawley rats [4]. Moreover, the sensitivity of the colon-cancer cell line HT-29 differed from the resistance previously observed during treatment of autochthonous, acetoxymethylmethylnitrosamine-induced colorectal cancer in rats with HPC [27]. The question remains open as to whether these differences are ascribable to species differences, to the observation that tumors can be composed of cells with different sensitivities, or to other yet unknown mechanisms. The mechanism of action of HPC as well as the factors contributing to resistance to the drug are unknown. Presumably, the APC interferes with mitogenic signal transduction and alters gene expression [16, 39]. The sensitivity to HPC of the two human gastrointestinal-carcinoma cell lines may have been related to inhibition of protein kinase C, which has been found to be strongly expressed in HT-29 cells as well as in many other gastrointestinal tumors [13].

In summary, the MTT assay is a simple, valid, and semiautomated system for the measurement of cell proliferation. However, it should be used with some reservation in predicting the clinical effectiveness of antineoplastic agents and in identifying new antitumor drugs in vitro, since the antiproliferative efficacy of certain compounds might be underestimated. It can nonetheless be recommended in conjunction with cell counting and determination of cell size, as it is capable of detecting cell-directed drug properties that are related to qualities other than basal cytotoxicity.

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