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Original Article

Comparison of Chemosensitivity Tests: Clonogenic Assay versus MTT Assay

Kazuhiko Kawada[a], Toshiro Yonei[b], Hiroshi Ueoka[a]*, Katsuyuki Kiura[a], Masahiro Tabata[a], Nagio Takigawa[a], Mine Harada[c], and Mitsune Tanimoto[a]

[a]Department of Internal Medicine II, Okayama University Medical School, Okayama 700–8558, Japan,
[b]Department of Respiratory Medicine, National Okayama Medical Center, Okayama 701–1192, Japan, and
[c]Department of Internal Medicine, Kyushu University School of Medicine, Fukuoka 812–8582, Japan

When the development of chemotherapeutic agents reaches the clinical trial stage, it is necessary to perform drug sensitivity tests quickly in order to select the most promising agents for the treatment of cancer. In order to assess the possibility of using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as a substitute for the human tumor clonogenic assay (HTCA), we evaluated the correlation between the results obtained by these 2 assays in 5 human lung cancer cell lines. The correlation coefficient between the results of the HTCA and the MTT assay was 0.673, indicating a relatively good correlation. The correlation was most prominent in platinum analogues ($r = 0.939$) and good in anthracyclines/anthracenedione ($r = 0.611$). However, no significant correlation was observed in vinca alkaloids, etoposide, irinotecan, SN-38 (an active metabolite of irinotecan), and rhizoxin. The results of the MTT assay showed a high degree of correlation with those of the HTCA in predicting the sensitivity of cancer cell lines to platinum analogues, and anthracyclines/anthracenedione. These results suggest that the MTT assay may be more convenient and quickly performed than the HTCA and can replace HTCA in evaluating the effects of anticancer agents, especially the platinum analogues and anthracyclines/anthracenedione.

Key words: chemosensitivity test, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, clonogenic assay

R ecently, there has been remarkable progress in the development of chemotherapeutic agents. However, an enormous amount of time and money must be invested before an agent is approved for clinical use. Therefore, when the development of chemotherapeutic agents reaches the clinical trial stage, rapid tests of drug sensitivity may be useful for selection of the most promising agents in the treatment of cancer. Although various drug sensitivity tests have been introduced for this purpose, they all possess certain disadvantages [1, 2]. The human tumor clonogenic assay (HTCA) has been widely employed for the evaluation of drug sensitivity in tumor cell lines and tumor tissues obtained by biopsy or surgery [3, 4]. Since HTCA has been proven to have certain degree of correlation with clinical response, it has been routinely used in our laboratory. However, the assay also has a number of disadvantages, including low efficiency and slow turn around time before the results are obtained [5, 6]. Various methods have therefore been developed to allow for more rapid evaluation of drug sensitivity. The

American National Institute of Health (NIH) employed a sulforhodamine B (SRB) assay to screen for effective chemotherapeutic agents using human tumor cell lines in a disease-oriented approach [7, 8]. The MTT assay has also been widely used in many laboratories [9]. We employed the MTT assay and established a panel of human lung cancer cell lines to screen for effective agents against lung cancer [10]. The MTT assay is based on the ability of viable tumor cells to reduce a tetrazolium base compound to a blue formazan product [11]. The MTT assay is generally carried out in a 96-well plate format, and the MTT formazan product is analyzed with a scanning multiple spectrophotometer such that a number of samples can be analyzed quickly and simply [13–15]. However, it is necessary to compare the MTT assay with other assay methods already confirmed to correlate with clinical activity, because the MTT assay does not directly evaluate the cytocidal activity of cancer cells.

The aim of the present study is to assess the usefulness of the MTT assay in comparison with the HTCA in the screening various anticancer drugs of lung cancer cell lines.

Materials and Methods

Human lung cancer cell lines.   Five human lung cancer cell lines, SBC-1 (JCRB 0816), SBC-2 (JCRB 0817), SBC-3 (JCRB 0818), ABC-1 (JCRB 0815), and EBC-1 (JCRB 0820), which have been established and maintained in our laboratory, were employed in the present study [5, 6, 10, 12]. SBC-1, SBC-2, and SBC-3 are human small-cell lung cancer (SCLC) cell lines, ABC-1 is an adenocarcinoma cell line, and EBC-1 is a squamous cell carcinoma cell line. SBC-3 was established from a previously untreated patient with SCLC, while SBC-1 and SBC-2 were established from patients with SCLC who had been clinically resistant to chemotherapy. ABC-1 and EBC-1 were also established from patients receiving anticancer agents. The cells were cultured in RPMI-1640 (GIBCO) supplemented with 15% fetal bovine serum (FBS) at 37 °C with 5% CO_2 in humidified air. Cell concentrations in the culture were adjusted to allow for exponential growth.

Human tumor clonogenic assay (HTCA). HTCA was performed according to the Salmon-Hamburger method [3, 4]. For the suspension culture, cells were separated by pipetting. For the monolayer culture in flasks, a single cell suspension was prepared at a density of 5×10^4 cells/ml by treatment with 0.25% trypsin + 0.05% EDTA. After several concentrations of drugs prepared by serial dilution, were added to the cultures, the cells were incubated for 1 h at 37 °C. After they were washed in order to remove the drug, the cells were suspended in 15% FBS + RPMI-1640 + 0.3% agarose (Takara Shuzo Co., Ltd. Kyoto, Japan) and plated onto a feeder layer (15% FBS + RPMI-1640 + 0.5% agarose), followed by culture with 5% CO2 in air (NAPCO) at 37 °C for 2 weeks. The number of colonies at each drug concentration was counted using a particle counter (Shiraimatsu Instrument Company, CP-3000).

MTT assay.   The MTT assay was performed according to the method of Mosmann [11]. Serial dilutions of chemotherapeutic agents prepared by the multiplication method were placed in a 96-well microplate. Since the cell lines employed in the present study had different proliferation rates, the number of cultured cells was adjusted to a density that allowed the untreated control cells to grow exponentially. That is, SBC-3 was inoculated into the microplate wells at a density of 2,000 cells/well and the other cell lines were inoculated at a density of 5,000 cells/well. After exposure of the cells to the test drug for 96 h at 37 °C, 10 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) solution in PBS (5 mg/ml) was added, and the cells were incubated for another 4 h. Then 125 ml of isopropanol + 0.04 N HCl was added, and the absorbance was determined at a wavelength of 560 nm using an ELISA reader.

Chemotherapeutic agents.   The agents used in the present study were as follows: Anthracyclines: doxorubicin (ADM), daunorubicin (DNR), epirubicin (EPI), pirarubicin (THP), aclarubicin (ACR), amrubicin (SM-5887), ME2303, and KRN8602 (MX-2); anthracenedione: mitoxantrone (MXT); platinum analogues: cisplatin (CDDP), carboplatin (CBDCA), nedaplatin (254-S), NK121, and DWA2114R; vinca alkaloids: vincristine (VCR), vindesine (VDS), vinblastine (VLB), and vinorelbine (VNR); a podophyllotoxin; etoposide (VP-16); topoisomerase I inhibitors: irinotecan (CPT-11) and its active metabolite (SN-38); and a new macrolide: rhizoxin (RZX). ADM, EPI, and VNR were provided by Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan, CDDP, CBDCA, and VP-16 by Bristol-Myers Squibb K.K., Tokyo, Japan, NK121 by Nippon Kayaku Co., Ltd., Tokyo, Japan, CPT-11 and SN-38 by Yakult Honsha Co., Ltd., Tokyo, Japan, SM-5887 by

Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan, DWA2114R by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan, DNR, THP and ME2303 by Meiji Seika kaisha, Ltd., Tokyo, Japan, ACR by Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan, KRN8602 (MX-2) by Kirin Brewery Co., Ltd., Tokyo, Japan, MXT by Lederle Japan, Ltd., Tokyo, Japan, VCR and VLB by Eli Lilly Japan K.K., Kobe, Japan, 254-S and VDS by Shionogi & Co., Ltd., Osaka, Japan, and RZX by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. The drugs were diluted with physiological saline, mannitol, or dimethyl sulfoxide (DMSO). Each drug was then examined at various dose levels, including at a dose of 1/10 peak plasma concentration, as reported in the clinical phase I study.

Study Design.   Using duplicate cultures of each cell line, the HTCA was performed 3 times. Considering the colony count of untreated tumor cells as a control, the percentage of viable cells was plotted against the logarithm of the drug concentration. From the regression line thus obtained, the 70% lethal drug concentration (LD70) was determined as an index of the cytocidal effect of the drug. The MTT assay was performed at least 3 times for each cell line in quadruplicate cultures. The ratio of absorbance of treated cultures to that of untreated control cultures was obtained for all concentrations of every drug. From the dose-response curve thus obtained, a 50% inhibitory concentration (IC50) was determined as an index of antitumor activity. The degree of correlation of the data obtained with these 2 assay methods was evaluated using Pearson's test.

Results

The IC50 values of each drug obtained with the MTT assay for the 5 human lung cancer cell lines are shown on the right side of Table 1 and the LD70 values obtained with HTCA are shown on the left side. Fig. 1 shows the

Table 1   The LD70 values obtained by the human tumor clonogenic assay (HTCA) and IC50 values obtained by the MTT assay

	The LD70 values of each drug obtained by the HTCA (nM)					The IC50 values of each drug obtained by the MTT assay (nM)				
	SBC-1	SBC-2	SBC-3	ABC-1	EBC-1	SBC-1	SBC-2	SBC-3	ABC-1	EBC-1
doxorubicin	190	235	60	520	2,800	29	69	22	62	70
daunorubicih	NO	NO	72	NO	NO	NO	NO	35	NO	NO
epirubicin	NO	NO	130	NO	NO	NO	NO	19	NO	NO
pirarubicin	98	92	16	120	320	76	74	7	105	156
aclarubicin	92	60	58	85	360	23	14	5	12	14
SM-5887	NO	NO	23,600	NO	NO	NO	NO	832	NO	NO
ME2303	NO	NO	2,400	NO	NO	NO	NO	14	NO	NO
KRN8602	NO	NO	1,400	NO	NO	NO	NO	45	NO	NO
mitoxantrone	210	270	34	180	860	86	140	15	350	440
cisplatin	3,800	6,600	4,830	9,170	84,100	646	2,188	603	1,622	6,310
carboplatin	62,400	51,900	77,100	134,800	606,800	6,500	5,800	4,571	14,000	39,000
254-S	10,200	13,900	14,100	19,500	108,300	1,150	2,000	891	3,000	8,600
NKl21	32,400	49,200	23,400	158,000	490,400	2,200	4,000	2,000	5,000	15,600
DWA2114R	95,700	91,300	111,300	186,800	685,800	7,600	17,000	9,800	16,000	80,000
vincristine	673	420	12,400	5,500	NO	8	2	2	2	4
viNOesine	500	NO	5,000	3,450	2,400	2	1	4	2	4
vinblastine	750	420	3,200	2,000	420	4	3	2	2	3
vinorelbine	680	260	1,900	3,280	8,900	53	3	5	4	6
etoposide	315,000	438,000	745,000	820,000	245,000	245	1,202	288	1,549	3,802
irinotecan	55,000	32,000	72,300	200,000	500,000	5,754	2,455	316	665	501
SN-38	20	34	61	81	107	5	3	1	2	2
rhizoxin	NO	33	19	92	20	NO	1	1	1	1

NO, not obtained.

correlation of mean IC50 values obtained with the MTT assay and the mean LD70 values obtained with the HTCA. The common logarithms of LD70 values were plotted along the longitudinal axis and the common logarithms of IC50 values were plotted along the horizontal axis. The correlation coefficient was 0.673 ($P < 0.01$), indicating a relatively good correlation between the results of the HTCA assay and those of the MTT assay. The correlation coefficients of the data obtained from different cell lines are as follows: 0.794, 0.861, 0.752, 0.691, and 0.861 in SBC-1, SBC-2, SBC-3, ABC-1, and EBC-1, respectively. Fig. 2 shows the stratification of data according to the class of each drug (platinum analogues, anthracyclines/anthracenedione, and vinca alkaloids). In the platinum analogues, the data obtained with the MTT assay showed the most prominent correlation (r = 0.939, $P < 0.01$) with the data obtained by HTCA. In anthracyclines/anthracenedione, a good correlation (r = 0.611, $P < 0.01$) was also confirmed. However, no significant correlation was observed in the case of the vinca alkaloids. The plots, within a 5% control limit of vinca alkaloids, were distributed on the upper left side, in contrast to those of the platinum analogues and anthracyclines/anthracenedione. Fig. 3 shows the stratification data of etoposide, irinotecan, SN-38, and rhizoxin. No significant correlation was observed with these drugs. The plots within a 5% control limit were similarly distributed at the left, suggesting that the MTT assay

may predict higher activity for these drugs than HTCA.

Discussion

We compared the usefulness of the MTT assay with that of the HTCA with respect to drug sensitivity testing. The sensitivity of the human lung cancer cell lines evaluated with these 2 assay methods generally showed relatively
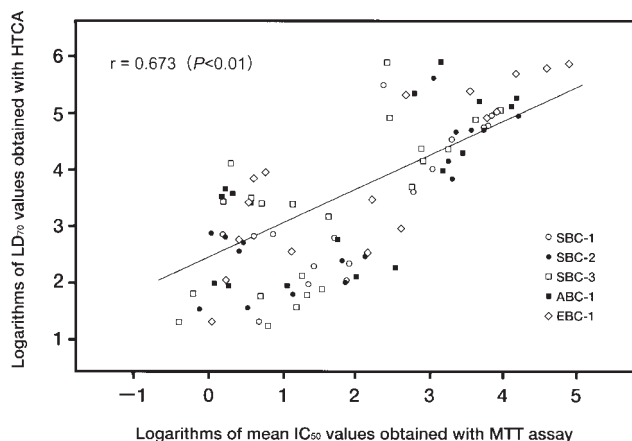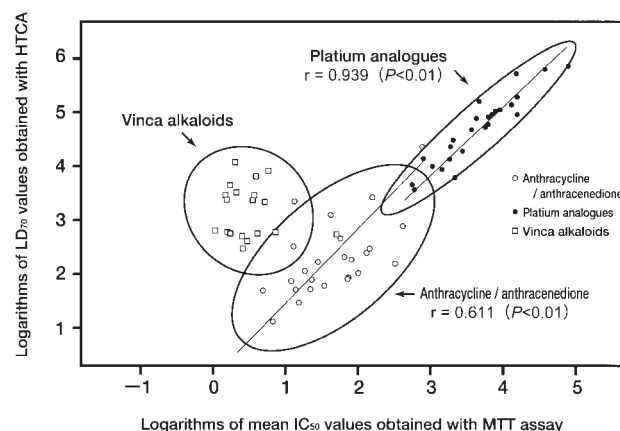

Fig. 2 The stratification of data according to the class of drugs (platinum analogues, anthracyclines/anthracenedione, and vinca alkaloids). The data obtained by the MTT assay showed a significant correlation with the data obtained by the HTCA with respect to platinum analogues and anthracyclines/anthracenedione (r = 0.939, $P < 0.01$; r = 0.611, $P < 0.01$, respectively). No significant correlation was observed in the case of vinca alkaloids. The plots within a 5% control limit are circled.

Fig. 1 Correlation of mean IC50 values obtained by the MTT assay and mean LD70 values obtained by HTCA. The common logarithms of the LD70 values were plotted along the longitudinal axis and the common logarithms of the IC50 values were plotted along the horizontal axis. The correlation coefficient was 0.673 ($P < 0.01$).
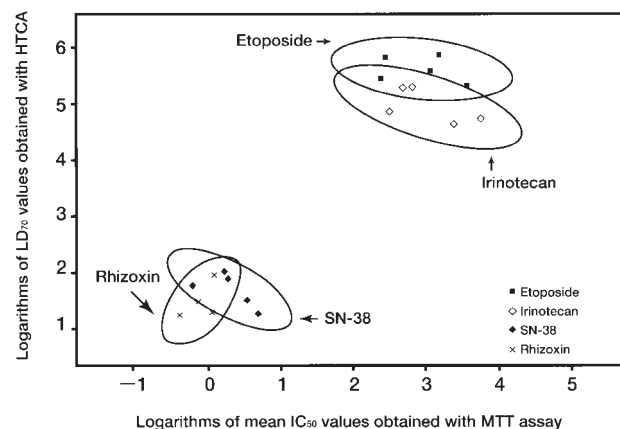

Fig. 3 The stratification data obtained with etoposide, irinotecan, SN-38, and rhizoxin. No significant correlation was observed with these drugs. The plots within a 5% control limit are circled.

good correlation among various types of chemotherapeutic agents. In particular, for the platinum analogues and anthracyclines/anthracenedione, close correlations were confirmed in the results evaluated by HTCA and MTT assay. However, in vinca alkaloids, etoposide, irinotecan, SN-38, and rhizoxin, the sensitivities predicted by the MTT assay were generally higher than those obtained by the HTCA. These results may reflect the time dependency of the anticancer effect in the latter agents.

Currently, *in vitro* drug sensitivity tests include a cell proliferation assay (HTCA), a differential staining cytotoxicity (DiSC) assay, and modified assays such as an adherence matrix assay and a three-dimensional gel assay [1]. HTCA, developed by Salmon and Hamburger, judges cell viability as clearly based on the presence or absence of cell proliferation, *i.e.*, based on colony-forming ability [3, 4]. Roper and Drewinko compared HTCA with a labeling index assay, a dye exclusion assay, a ^{51}Cr release assay, measurement of [^{3}H] thymidine uptake, and measurement of doubling time [16]. They found that HTCA was the most definitive indicator of cell death. In the present experiment, we employed the 2-layer soft agar medium method of HTCA. With this method, it was possible to freely change exposure time and to express cell death quantitatively. Thus, our method appears to be the most useful method for performing HTCA [17]. The drug concentration employed for HTCA was based on the blood drug concentration achieved with clinical doses. The blood drug concentration at the start of the beta-phase of elimination, following intravenous bolus administration at the standard clinical dose, is used as the pivotal concentration for HTCA. The cells are judged to be sensitive to an anticancer agent when at least 70% inhibition of colony-forming activity is attained as a result of a 1-hour exposure of cells to the drug at that concentration. Therefore, the cell death observed as a result of 1-hour exposure to the drug in HTCA is considered to be useful for an assessment of the relationship between HTCA data and the clinical effect. However, disadvantages of the HTCA include the complexity of the procedure, the time required to obtain results, and the limitations on the carcinostatic agents and the number of cell lines that can be tested simultaneously [6].

On the other hand, the MTT assay, which is in widespread clinical use at present, allows for the use of various types of carcinoma cells and thus facilitates more efficient assessment of a chemotherapeutic agent or an agent expected to have carcinostatic activity [13-15]. The MTT assay is also useful for performing small-scale experiments using an expensive drug [7]. However, it has some drawbacks that can lead to misleading results, *e.g.*, evaluation using the MTT assay is based on the existence of a proportional relationship between the absorbance and the number of viable cells in a culture [18]. Therefore we confirmed this proportional relationship between the absorbance and the cell count by preparing a calibration curve prior to the present experiment. Since the size and doubling time of cancer cells vary from cell line to cell line, we also performed a preliminary experiment to find the optimal number of cells to be cultured for each cell line. In addition, cells should be exposed to a test drug for an adequate amount of time in order to cause maximum cell death and loss of dehydrogenase activity. We previously determined the optimal number of cells per well and the optimal duration of culture for each of our cancer cell lines so that the maximum absorbance was obtained while exponential growth was maintained [10, 12].

We performed the present study to evaluate the correlation between tumor sensitivity evaluated by HTCA, which has been traditionally used at our institution, and that by the MTT assay, which is widely used to test the sensitivity of various anticancer agents. There have been only a few reports comparing the MTT assay and the HTCA. Perez *et al.* showed that the MTT assay was comparable to a clonogenic assay as regards cisplatin sensitivity; the correlation coefficient was 0.810 ($P = 0.0074$). However, they did not report the sensitivity of other drugs [19]. We intended to assess the possibility of replacing the HTCA with the MTT assay for drug sensitivity testing in the case of 22 anticancer agents using 5 lung cancer cell lines. Generally, a high degree of correlation was confirmed between the results obtained with these 2 assay methods. However, the MTT assay predicted a higher level of anti-tumor activity of vinca alkaloids than that was predicted with HTCA. Vinca alkaloids are type IIa drugs and they have a time-dependent action, according to Shimoyama's classification of drug mechanisms[20]. Since the cells were exposed to the drug for a longer duration in the MTT assay (96 h) than in the HTCA (1 h), this may have led to differences in the results obtained by these 2 types of assay. Similar results were also obtained for camptothecin analogues, etoposide, and rhizoxin, and the

mechanism of these agents may also be time-dependent.

In conclusion, HTCA and MTT assays showed a high degree of correlation as regards drug sensitivity testing of platinum analogues and anthracyclines/anthracenedione. The present results suggest that the MTT assay can be employed instead of the HTCA in order to estimate the activity of such agents against tumor cells; the advantages of the MTT assay are its simplicity, low cost, and shorter duration. However, when assessing time-dependent agents such as vinca alkaloids, etoposide, camptothecin analogues, and rhizoxin, it should be noted that the MTT assay predicts higher cytotoxic activity than that predicted by HTCA.

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