Cancer Chemother Pharmacol (1985) 14: 104–107

Cancer Chemotherapy and Pharmacology

Original articles

Time-schedule dependency of the inhibiting activity of various anticancer drugs in the clonogenic assay

Yuka Matsushima[1], Fumihiko Kanzawa[1], Akio Hoshi[1], Eiji Shimizu[2], Hiroaki Nomori[2], Yasutsuna Sasaki[2], and Nagahiro Saijo[2]

[1] Pharmacology Division, National Cancer Center Research Institute, and
[2] Department of Internal Medicine, National Cancer Center Hospital, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan

Summary. *To analyze the discrepancy between the in vitro response in the clonogenic assay and the clinical response, the time-schedule dependencies of various anticancer drugs were determined by comparing the inhibiting effect against colony formation by PC-7 cells treated with the drugs for 1 h with that of those treated for 24 h. According to their schedule dependency the drugs can be divided into a schedule-dependent drug group (5-fluorouracil, methotrexate, bleomycin, pepleomycin, etoposide, cisplatin, teniposide, vindesine, and vinblastine) and a non-schedule-dependent drug group (adriamycin, actinomycin D, ranomustine, mitomycin C, aclacinomycin, daunomycin, nimustine, melphalan, and KW 2083). In the clonogenic assay, the 1-h exposure schedule is appropriate for predicting clinical response for the non-schedule-dependent drugs. However, the effect of the schedule-dependent drugs was underestimated in the same conditions. Therefore, it is necessary to test these drugs in the assay by 24-h exposure for a more accurate assessment of their antitumor activity.*

Introduction

The soft-agar culture method recently developed by Hamburger and Salmon allows growth of human tumor cells while preventing the growth of normal cells such as fibroblasts [5]. With this method anticancer drug sensitivity testing has been able to predict the clinical response to therapy in individual patients with great accuracy [7, 17, 18]. In retrospective and prospective studies, the clonogenic assay had a 60%–92% true-positive rate and an 83%–100% true-negative rate in prediction of the drug sensitivity and resistance, respectively, of cancer patients to specific chemotherapeutic drugs [14]. However, with some kinds of anticancer drugs there were many instances in which the clonogenic assay showed that the patient's tumor would respond to the drug(s) but it did not (false positive) [3, 10, 12]. There were also instances in which the tumor was resistant to drugs in vitro but the patients responded (false negative) [3, 10]. Numerous reasons for these false-positive and false-negative responses could be proposed, including insufficiently strict criteria for evidence of drug activity in vitro. Usually the antitumor effect was tested at a single drug concentration that allowed in vitro testing of a large number of drugs. However, the use of a single drug concentration chosen by taking one-tenth of the peak plasma concentration in man involves several theoretical problems. First, achievable levels of anticancer drugs vary from patient to patient. Second, the drug concentration entering the tumor cell in single-cell suspensions may differ from that in vivo. Moreover, the most important problem in the assay is the arbitrary 1-h incubation with some of the cell-cycle-specific drugs. The studies reported here were, therefore, undertaken to determine the effect of duration of exposure on the inhibition of colony formation by PC-7 cells in soft-agar medium, and to determine the causes of false-negative and false-positive responses in the clonogenic assay system.

Offprint requests to: Y. Matsushima

Materials and methods

Cell line. The human tumor cell line used in this study was PC-7, derived from an adenocarcinoma of the lung (kindly provided by Prof. H. Hayata, Tokyo Medical College). The cells were propagated in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 μg streptomycin per ml, and 100 U penicillin per ml in an incubator under a humidified atmosphere of 5% CO_2 and 95% air, as described by Fischer and Sartorelli [2].

In vitro exposure of tumor cells to the drugs. Stock solutions of the anticancer drugs were prepared in sterile buffered 0.9% NaCl solution and stored at −15° C in amounts sufficient for individual assay. Subsequent dilutions for incubation were made with 0.9% NaCl solution. The tumor cells were exposed to standard anticancer drugs at the following final concentrations (μg/ml): 5-fluorouracil (FU), 1.0; methotrexate (MTX), 0.03; bleomycin (BLM), 0.1; pepleomycin (PLM), 0.4; etoposide (VP-16), 0.1; teniposide (VM-26), 3.0; cisplatin (CDDP), 0.2; vindesine (VDS), 0.005; vinblastine (VLB), 0.01; 7-*N*-(*p*-hydroxyphenyl)-mitomycin C (KW 2083), 0.1; mitomycin C (MMC), 0.1; melphalan (1-PAM), 0.4; nimustine (ACNU), 3.0; ranomustine (MCNU), 40; daunomycin (DM), 0.05; aclacinomycin (ACM), 0.06; actinomycin D (Act-D), 0.005; adriamycin (ADM), 0.04. These concentrations of each drug corresponded to approximately one-tenth of the peak plasma concentrations for each drug in humans. The alkylating agent cyclophosphamide is inactive in vitro, therefore, 1-PAM was substituted for cyclophosphamide in vitro.

Effects of the drugs on the colony formation by PC-7 cells were evaluated as follows. Three-milliliter cultures, initially containing 10^5 cells/ml, were incubated in medium containing a drug in capped 15-ml tubes for 1 h or 24 h at 37° C. After drug treatment, the cells were collected by centrifugation (500 *g*, 3 min), washed once, and resuspended in double-enriched CMRL 1066 medium for assay by the in vitro clonogenic assay.

In vitro clonogenic assay. The culture system used in this study was devised by Hamburger and Salmon [6]. Briefly, the cells to be tested were suspended in an overlayer of 0.3% agar in enriched CMRL 1066 medium supplemented with 15% horse serum, penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 m*M*), $CaCl_2$ (4 m*M*), and insulin (3 U/ml). Prior to plating, asparagine (0.6 mg/ml), DEAE-dextran (0.5 mg/ml), and freshly prepared 2-mercaptoethanol (final concentration, 50 m*M*) were added to the medium. Then 1 ml of the resultant mixture was pipetted onto 1-ml underlayers in 35-mm plastic petri dishes (Falcon Plastics). The final concentration of cells in each culture was 10^5 cells in 1 ml agar medium. The underlayers consisted of McCoy's 5A medium plus 10% heat-inactivated fetal calf serum and a variety of nutrients described previously [8]. Immediately before use, 10 ml 3% tryptic soy broth, 0.6 ml asparagine, and 0.3 ml DEAE-dextran were added to 40 ml enriched underlayer medium. Agar (final concentration, 0.5%) was added to the enriched medium, and underlayers were poured into 35-mm petri dishes. After preparation of both bottom and top layers, the plates were incubated at 37° C in 7.5% carbon dioxide and 100% humidity.

Colonies (50 cells) usually appeared by day 14 of culture, and the colonies on control and drug-treated plates were counted by means of an inverted-stage microscope at 40-fold magnification or of a computerized image analyzer (colony analyzer system CA-7A; Oriental Instrument Ltd., Tokyo).

Results

To find the relationship between the number of PC-7 cells plated and the number of colonies formed, the cells were plated at different concentrations (10^4 to 10^6 cells/dish) and the numbers of colonies formed were determined on day 14 of incubation. As shown in Fig. 1, the number of colonies increased linearly with increasing numbers of cells plated up to 3×10^5 cells/dish. Beyond this level there was a decrease in number of colonies. The optimum cell concentration in this system was 10^5 cells/dish, and the plating efficiency was 1.2%.

PC-7 cells were exposed to 18 standard anticancer drugs for 1 h before plating, and inhibition of the colony formation was measured on day 14 of incubation. As shown in Table 1, they exhibited resistance to all the drugs except teniposide up to dose levels of one-tenth peak plasma concentrations, which were used as a standard dose in the clonogenic assay. However, when the drug concentration was increased to the peak plasma concentration some drugs, such as 1-PAM, ADM, and MMC, showed 50% or more reduction in colony formation. On the other hand, FU, MTX, and CDDP did not show any inhibiting activity against colony formation by PC-7 cells even at the high concentration.

To evaluate the time dependency of the inhibiting activity of the 18 anticancer drugs against the colony formation, the cells were exposed to the drugs at different concentrations for 1 h or 24 h before plating, and the inhibiting activity was determined on day 14. The results were plotted as percent survival versus drug concentration (μg/ml) for both the 1-h and the 24-h exposure experiments shown in Fig. 2. A common phenomenon observed with all drugs tested is that survival curves for tumor cells exposed to the drugs show progressively increasing lethality with increasing drug concentration and that 100% reductions were attained with the high concentration. However, such high concentrations would not be achievable in vivo without excessive toxicity. There were differences among drugs in the distance between the two dose-survival curves recorded in 1-h and 24-h exposure experiments. With cell-cycle-specific drugs the two curves obtained were widely separated. On the other hand, with cell-cycle-nonspecific drugs the two curves were quite near to each other. To estimate the relative dependency on exposure time among the drugs, concentrations of the drugs producing 50% inhibition of colony formation (IC_{50}) were determined from Fig. 2, and are shown in Table 2. The time-schedule dependency index (TDI) was calculated by dividing the IC_{50} with 1-h exposure by that with a 24-h exposure and is given in the last column in Table 2. Changes in the activity sequences with different exposure times were recorded as follows. In the 1-h exposure experiment VLB was the most active drug, followed by DM, Act-D, VDS, ADM, ACM, VM-26, MMC, KW 2083, 1-PAM, PLM, VP-16,

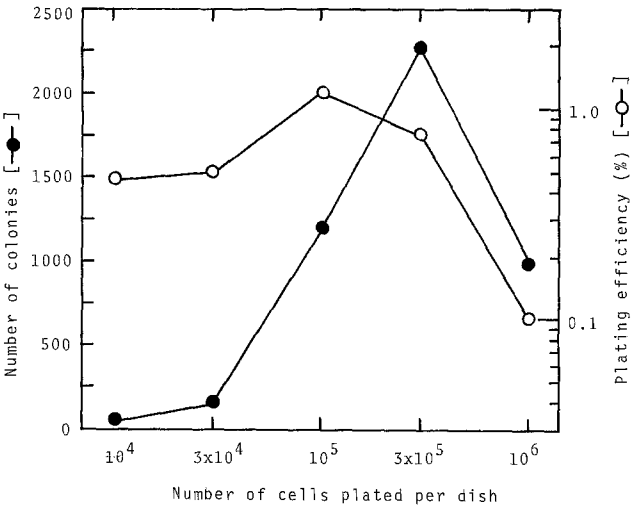

Fig. 1. Relationship between number of colonies formed and PC-7 cells plated in double soft-agar medium

Table 1. Inhibiting activity of various antitumor drugs against colony-forming activity of human lung cancer PC-7 cells

Drug	Tumor colony-forming units (%)	
	Standard dose	Ten-fold dose
5-Fluorouracil	109	98
Methotrexate	108	108
Bleomycin	108	72
Pepleomycin	76	40
Etoposide	111	85
Teniposide	25	–
Cisplatin	111	103
Vindesine	105	63
Vinblastine	114	14
KW 2083	90	32
Mitomycin C	90	30
Melphalan	91	10
Nimustine	89	32
Ranomustine	99	62
Daunomycin	53	13
Aclacinomycin	77	14
Dactinomycin	85	53
Adriamycin	91	13

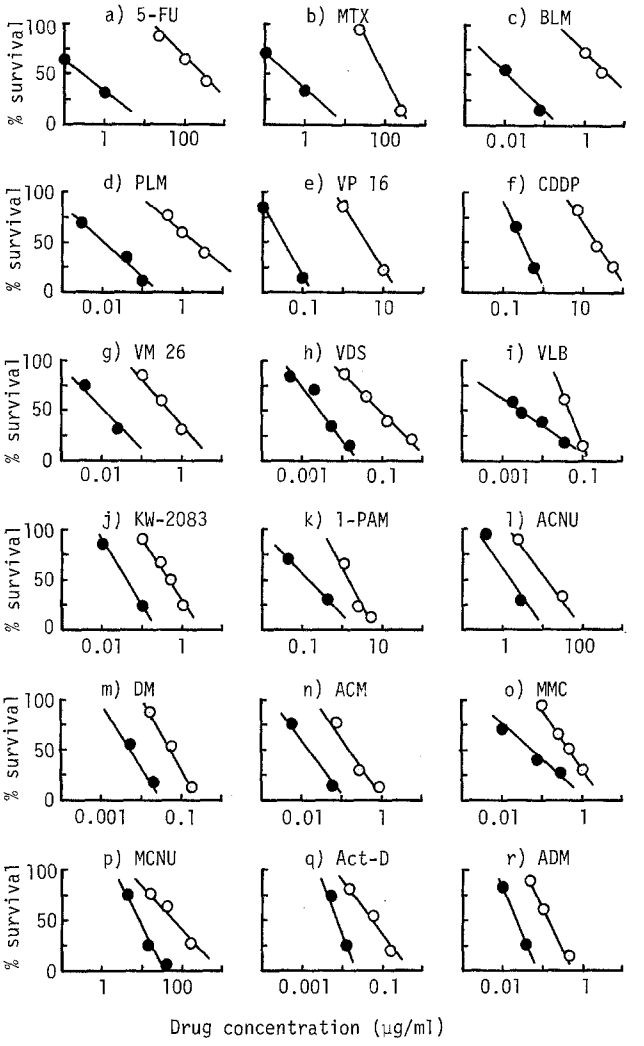

Fig. 2. Cell survival curves for PC-7 cells exposed to anticancer drugs at various concentrations for 1 h (−○−) and 24 h (−●−)

Table 2. Time-schedule dependency of activity of various antitumor drugs

Drug	IC_{50} (μg/ml)		TDI[a]
	1 h	24 h	
5-Fluorouracil	220	0.23	957
Methotrexate	97	0.32	303
Bleomycin	3.5	0.012	292
Pepleomycin	2.2	0.010	220
Etoposide	3.3	0.032	103
Cisplatin	19	0.30	63
Teniposide	0.45	0.012	38
Vindesine	0.096	0.0027	36
Vinblastine	0.044	0.0024	18
KW 2083	0.49	0.040	12
Melphalan	1.3	0.13	10
Nimustine	14	1.5	9.3
Daunomycin	0.050	0.0055	9.1
Aclacinomycin	0.14	0.016	8.8
Mitomycin C	0.48	0.060	8.0
Ranomustine	60	9.0	6.7
Dactinomycin	0.052	0.0081	6.4
Adriamycin	0.13	0.022	5.9

[a] TDI = IC_{50} (1 h)/IC_{50} (24 h)

BLM, ACNU, CDDP, MCNU, MTX, FU in declining order. In the 24-h exposure experiment, however, the corresponding sequence was, VLB, VDS, DM, Act-D, PLM, BLM, VM-26, ACM, ADM, VP-16, KW 2083, MMC, 1-PAM, FU, CDDP, MTX, ACNU, MCNU.

In the 24-h exposure experiment, FU markedly inhibited colony formation and the IC_{50} was much lower than that in the 1-h exposure experiment. Accordingly, the TDI for FU was 957, which is the highest for any of the drugs tested. TDI values for the cell-cycle-specific drugs ranged from 18 (for VLB) to 957 (for FU). On the other hand, the TDI values for the cell-cycle-nonspecific drugs, except for CDDP (TDI 63), ranged from 5.9 to 12.

Discussion

In vitro treatment of cells with drugs entails serious problems, because the environment of the cells in vitro is different from that in vivo and may alter their chemosensitivity [11, 15] and because the optimum concentration and exposure time for the drug treatment of patients are not clear. Most investigators have tried to relate drug concentration to achievable plasma levels, but the concentration within a solid tumor may vary quite widely and be impossible to simulate precisely in vivo. However, a single drug concentration (standard dose: one-tenth of the peak plasma concentration) has been reported to be sufficient for detecting in vivo sensitivity of individual tumors to drugs, because the drug effects at standard doses and ten-fold greater doses in corresponding specimens were not very different from each other [9]. Another important characteristic of a schedule-dependent drug is the time-dependent reduction in survival. The standard 1-h incubation was selected because of its similarity to retention duration in plasma after IV injection of a typical short-lived anticancer drug, but may be viewed as arbitrary [11]. Our present study has focused on the difference in the time-schedule dependency of inhibition between the cell-cycle-specific drugs and cell-cycle-nonspecific drugs. The drugs were divided into two separate groups based on their cell-cycle specificities [11] as follows: cell-cycle-specific drugs: FU, MTX, PLM, VP-16, VM-26, VLB, and VDS; and cell-cycle-nonspecific drugs: ADM, DM, ACM, Act-D, MMC, KW 2083, 1-PAM, ACNU, MCNU, and CDDP. Accurate interpretation of the time dependency necessitates determination of the chemical and biological stability of these anticancer drugs in 1-h and 24-h incubation; nevertheless, we were able to evaluate and separate schedule-dependent and non-schedule-dependent drugs by comparing the inhibiting effect on colony growth of 1-h and 24-h drug exposures. With cell-cycle-nonspecific drugs, greater sensitivity is observed in the 24-h exposure experiment than in the 1-h exposure experiment, but the difference is generally less than ten-fold. Examples of this pattern are seen in alkylating agents and antitumor antibiotics, such as MMC and Act-D. CDDP is classified as a cell-cycle-nonspecific drug, but its TDI was high, indicating that CDDP is a schedule-dependent drug. Moreover, CDDP shows a better antitumor effects with less toxicity when given by prolonged infusion at low doses [1]. In contrast, with cell-cycle-specific drugs such as the antimetabolites BLM, PLM, and VP-16, the degree of inhibition with 24-h exposure was more than 100-fold that observed with 1-h exposure. Vinka alkaloids have a mode of action similar to that of colchicine, that is, disordered metaphases are produced and the usual spindle structure is absent. Their effects are defined as completely specific in the

late S-G_2 phase [4]; however, these drugs did not appear to be typically schedule-dependent, as can be seen in Table 2.

In this study, the drugs could be divided into two types as follows: (I) a time-schedule-dependent drug group, to which antimetabolite anticancer antibiotics such as BLM and PLM belong, and (II) a time-schedule-independent drug group, to which alkylating agent anticancer antibiotics such as tetracyclines, MMC, and Act-D belong. It is of interest that the above classification does not always agree with the data on the cell-cycle dependency in studies carried out in the form of continuous drug exposure experiments [13, 16]. This suggests that the assay used in this study with brief drug exposure (i.e., 1 h or 24 h) may help to reflect true drug-related lethality, in that the observed growth inhibition persists in the absence of the drug. On the other hand, the inhibition caused by continuous drug exposure does not allow the distinction between cytocidal and cytostatic drug effects [8]. Also, the schedule dependency [IC_{50} (1-h)/IC_{50} (24-h)] is not always reflected in the time dependency; a net duration time of drug exposure was reduced less than a scheduled time in case of drug such as etoposide [11], which is unstable in cell culture media.

In the clonogenic assay, the use of a standard 1-h incubation is appropriate for the cell-cycle-nonspecific drugs such as alkylating drugs. However, there is a problem in the evaluation of the activity of schedule-dependent drugs that are specific for a particular cell cycle phase. Therefore, it is necessary to test these schedule-dependent drugs in the clonogenic assay by 24-h exposure for a more accurate assessment of their antitumor activity.

Acknowledgements. This work was supported in part by Grants-in-Aid for Cancer Research (56-S-1, 58-12) from the Ministry of Health and Welfare. The authors are indebted to Mitsui Pharmaceuticals Inc., Tokyo, Japan (FU), Nippon Kayaku Co., Ltd., Tokyo, Japan (VP-16 and CDDP), Bristol-Myers K. K., Tokyo, Japan (VM-26), Shionogi & Co., Ltd., Ohsaka, Japan (VDS), Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan (KW 2083), and Tokyo Tanabe Co., Ltd., Tokyo, Japan (MCNU) for kindly supplying the test drugs.

References

1. Drewinko B, Gottlieb JA (1975) Action of *cis*-dichlorodiamine-platinum (II) (NSC-119875) at the cellular level. Cancer Chemother Rep 59: 665
2. Fischer GA, Sartorelli AC (1964) Development, maintenance and assay of drug resistance. Methods Med Res 10: 247
3. Greenberg BR, Salmon SE (1984) Human tumor clonogenic assay in patients with unknown primary carcinomas. J Clin Oncol 2: 46
4. Grieder A, Maurer R, Stahelin H (1974) Effect of an epipodophyllotoxin derivative (VP-16-213) on macromolecular synthesis and mitosis in mastocytoma cells in vitro. Cancer Res 34: 1788
5. Hamburger AW, Salmon SE (1977) Primary bioassay of human tumor stem cells. Science 197: 461
6. Hamburger AW, Salmon SE, Kim MB, Trent JM, Soehnlen BJ, Alberts DS, Schmidt HJ (1978) Direct cloning of human ovarian carcinoma cells in agar. Cancer Res 38: 3438
7. Johnson PA, Rossof AH (1983) The role of the human tumor stem cell assay in medical oncology. Arch Intern Med 143: 111
8. Kanzawa F, Hoshi A, Kuretani K (1980) Mechanism of cytotoxicity of 5-fluorouracil: Distinction between the irreversible cytotoxic effect of 5-fluorouridine and the reversible cytotoxic effect of 5-fluoro-2′-deoxyuridine on murine lymphoma L5178Y cells in culture. J Cancer Res Clin Oncol 98: 85
9. Kanzawa F, Hoshi A, Shimizu E, Saijo N, Miyazawa N, Shimabukuro Z (1984) Clonogenic cell assay for carcinoma of the lung. Gan 75: 81
10. Kitten CM, Von Hoff DD, Bennett EV Jr, Trinkle JK, Grover FL (1983) The human tumor clonogenic assay in the treatment of patients with lung cancer. Ann Thorac Surg 36: 408
11. Ludwig R, Alberts DS, Miller TP, Salmon SE (1984) Evaluation of anticancer drug schedule dependency using an in vitro human tumor clonogenic assay. Cancer Chemother Pharmacol 12: 135
12. Rosenblum ML, Dougherty DV, Deen DF, Wilson CB (1981) Potentials and limitations of a clonogenic cell assay for human brain tumors. Cancer Treat Rep (Suppl) 65: 61
13. Rupniak HT, Whelan RDH, Hill BT (1983) Concentration and time-dependent inter-relationships for antitumor drug cytotoxicities against tumour cells in vitro. Int J Cancer 32: 7
14. Salmon SE (1984) Human tumor colony assay and chemosensitivity testing. Cancer Treat Rep 68: 117
15. Shimoyama M (1975) Cytocidal action of anticancer agents: Evaluation of the sensitivity of cultured animal and human cancer cells. In: Ito Y, Dutcher RM (eds) Comparative leukemia research 1973. Leukemogenesis. University Press, Tokyo; Karger, Basel, p 117
16. Van Putten LM (1974) Are cell kinetic data relevant for the design of tumour chemotherapy schedules? Cell Tissue Kinet 7: 493
17. Von Hoff DD, Casper JT, Bradley E, Sandbach J, Jones D, Makuch R (1981) Association between human tumor colony-forming assay results and response of an individual patient's tumor to chemotherapy. Am J Med 70: 1027
18. Von Hoff DD, Clark GM, Stogdill BJ, Sarosdy MF, O'Brien MT, Casper JT, Mattox DE, Page CP, Cruz AB, Sandbach JF (1983) Prospective clinical trial of a human tumor cloning system. Cancer Res 43: 1926

Received May 7, 1984/Accepted September 19, 1984