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# Cell killing action of cell cycle phase-non-specific antitumor agents is dependent on concentration–time product*

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**Summary.** Based on a pharmacokinetic model proposed by Jusko, which assumes that the cell killing action of cell cycle phase-non-specific agents occurs as a bimolecular reaction depending on drug concentration and cell density, we derived a cell kill kinetic equation for these drugs, including the decomposition constant in culture medium. This equation revealed that the cell killing activity of these drugs depends on the value of concentration × exposure time or the area under the drug concentration – time curve (AUC). It was also clarified that the curves for concentration – exposure time necessary for 90% cell kill on a log scale simulated on the basis of the equation differ according as whether drugs are stable or unstable in the culture medium, being expected to be linear with a slope of −1 in the former case, and to take the form of an asymptotic curve in the latter. For three cell cycle phase-non-specific agents, mitomycin C (MMC), 1-(4-amino-2-methylpyrimidine-5-yl)-methyl-3-(2-chloroethyl)3-nitrosourea hydrochloride (ACNU), and nitrogen mustard ($HN_2$), we assessed the concentrations necessary for 90% cell kill ($IC_{90}$) with various exposure times and the degradation rate constants under the culture conditions used. MMC was quite stable during the incubation, while ACNU and $HN_2$ were unstable. When $IC_{90}$'s and exposure times were plotted on the above-mentioned graph, a linear relationship with a slope of −1 was seen for MMC, while for ACNU and $HN_2$ the anticipated asymptotic curves resulted. We also ascertained that the decomposition constants for ACNU and $HN_2$ expected on the basis of these curves showed a good agreement with the corresponding experimentally observed values. These results indicate that the cell killing action of cell cycle phase-non-specific drugs can be well described by a pharmacodynamic model and equation employing their decomposition constants and are dependent on the concentration–time product.

## Introduction

In order to correlate in vitro cytotoxicity quantitatively with in vivo antitumor activity and predict therapeutic effectiveness of a drug from data on its in vitro cytotoxicity, it is quite important to establish a pharmacodynamic model which makes it possible to analyze the relationship between the drug concentration an the exposure time necessary for a definite cell killing effect.

Shimoyama et al. [8, 9] classified cell cycle phase-non-specific agents [10] as concentration-dependent (type I) drugs, and cell cycle phase-specific agents [10] as time-dependent (type II) drugs according to their extensive in vitro colony-forming inhibition studies. They divided type I drugs further into two subtypes, namely, type Ia, with a principally concentration-dependent action, and type Ib, their action dependent on both concentration and time. Drugs belonging to type Ia are nitrogen mustard ($HN_2$), nitrogen mustard *N*-oxide, 4-hydroperoxy cyclophosphamide, BCNU, etc., while type Ib drugs are alkylating agents with the ethyleneimine group, such as thiotriethylene phosphamide, triaziquone, and carbazilquinone, and antitumor antibiotics such as MMC, daunorubicin, adriamycin, bleomycin, actinomycin D, and chromomycin A-3.

Although such a classification has been helpful in enhancing our understanding of the cytotoxic actions of various antitumor agents, in the above-mentioned studies little attention was paid to changes in drug concentration during incubation. This is of particular concern with respect to type Ia drugs, because some of them are known to undergo a fast decomposition in culture medium. In the present study, we assumed that cell killing actions of both type Ia and type Ib would be essentially identical if kinetic analyses were performed with due consideration for the decay of drugs in the culture medium during the incubation period.

Therefore, based on a pharmacodynamic model established by Jusko [2] to describe the kinetics of cell killing effects of cell cycle phase-non-specific agents, we attempted a kinetic analysis of the cell killing effects of three type Ia and Ib antitumor drugs, MMC, ACNU, and $HN_2$, in order to develop a common pharmacodynamic model for these drugs.

## Materials and methods

*Chemicals.* MMC was purchased from Kyowa Hakko Co. Ltd., Tokyo, Japan; $HN_2$ was kindly provided by Yoshitomi Pharmaceutical Industries, Ltd., Osaka, Japan; and 4-(4-nitrobenzyl)-pyridine (NBP) was purchased from

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Aldrich Chemical Company, Inc., Milwaukee, Wis, USA. All other chemicals were of analytical grade.

*Cell culture.* Chinese hamster V79 cells were kindly donated by Dr. S. Okada, Faculty of Medicine, Tokyo University.

V79 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 μg/ml kanamycin at 37° C in a humidified atmosphere of 5% $CO_2$ and 95% air.

*Estimation of cell killing effects of drugs.* V79 cells were harvested by trypsinization and enumerated in a model ZBI Coulter counter. The cells were diluted and seeded at a cell density of 100, 200, 400, 800 or 1600 cells per dish into 60-mm dishes each containing 3.0 ml culture medium. The dishes containing 100 or 200 cells were used for controls and relatively low drug concentrations, and those containing more cells were used for higher drug concentrations. On the day after seeding 30 μl drug solution was added to each dish, and the cultures were then incubated further for various lengths of time in the presence of the drug. At the end of the incubation with drug, the plate was washed twice with 3 ml Hanks' balanced salt solution, and 3 ml culture medium was added to each dish.

On the 5th or 6th day after seeding, all dishes were washed once with PBS, fixed with 10% formalin, and stained with 0.05% crystal violet. Colonies were enumerated by means of a Colony Analyzer CA-7 (Oriental Instruments Ltd., Tokyo, Japan). The surviving fraction was calculated by dividing the colony number of the cells exposed to drug by that of the control.

The $IC_{90}$ value of each drug, i.e. the concentration reducing the surviving fraction to 10% of the control, was determined from the dose-response curve for each exposure period.

*Determination of decay during the incubation in the culture medium.* To determine the decomposition of drugs in the culture medium during the incubation period, the concentrations of MMC and ACNU were measured by HPLC and that of $HN_2$, by colorimetric assay using NBP [1, 3, 12].

The drugs were incubated in the culture medium at 37° C for appropriate periods of time. At the end of each incubation period the free drug was ultrafiltered by centrifugation using a CENTRIFREE ultrafiltration unit (Amicon). The concentrations of MMC and ACNU in the filtrate were determined by HPLC. The HPLC conditions for ACNU were: column, μBondapak $C_{18}$ (Waters); mobile phase, 50%-MeOH, 0.1% PIC-$B_7$ (Waters); for MMC: column, Nucleosil 100–5; mobile phase, $CHCl_3$:MeOH; $H_2O$ (90:10:0.15).

The $HN_2$ concentration was determined by alkylation of NBP. The filtrate containing free $HN_2$ was incubated with 0.1% NBP at 37° C for 2 h. The reaction mixture was subsequently made alkaline with sodium hydroxide, and the colored material was extracted with ethyl acetate. The absorbance of the ethyl acetate layer was then determined at 540 nm.

## Results

### *Model analysis*

The basic pharmacodynamic model proposed by Jusko [2] for the characterization of the effects of chemotherapeutic agents is shown in the scheme described below.

Drug administration

$$\downarrow \qquad \uparrow k_r \quad k$$
$$C_m \rightarrow C_e + C_s \rightarrow \text{cell death},$$
$$k_s$$

where $C_m$ and $C_e$ are drug concentrations in medium and at the site of action, respectively, and $k_s$, $k_r$, and k are cell proliferation rate constant, physiologic degradation rate constant, and drug-induced irreversible cell death rate constant, respectively. If it is assumed that both concentrations rapidly reach equilibrium, such a relationship can be expressed by the followings equation:

$$C_e = KC_m, \tag{1}$$

where K is an equilibrium constant. Considering the bimolecular reaction of drug and cell, the rate of change in cell density ($C_s$) can be expressed as follows:

$$\frac{dC_s}{dt} = -kC_e \cdot C_s + k_sC_s - k_rC_s. \tag{2}$$

$C_m$ and $C_e$ are constant for drugs whose degradations are negligible. Substitution of Eq. (1) into Eq. (2) yields

$$\frac{dC_s}{dt} = -kKC_m \cdot C_s + (k_s - k_r) \cdot C_s. \tag{3}$$

Considering cell density at 0 time of drug exposure (i.e., $t = 0$, $C_s = C_s^0$), Eq. (3) is converted upon integration to:

$$l_n \frac{C_s}{C_s^0} = (k_s - k_r - kKC_m) \cdot t. \tag{4}$$

If $C_s'$ is the cell density of control ($C_m = 0$), Eq. (4) becomes

$$l_n \frac{C_s'}{C_s^0} = (k_s - k_r) \cdot t. \tag{5}$$

If we subtract Eq. (5) from Eq. (4), surviving fraction ($C_s/C_s'$) can be written as

$$l_n \frac{C_s}{C_s'} = -kKC_m \cdot t. \tag{6}$$

If the surviving fraction is reduced to 0.1 ($C_s/C_s' = 0.1$) by exposing cells to drug for exposure time t at a concentration of $C_{m,90}$, Eq. (6) becomes

$$AUC = C_{m,90} \times t = \frac{2.3}{kK} = \text{constant}. \tag{7}$$

According to this equation, a log $C_{m,90}$ – log t curve is expected to be linear with a slope of $-1$, as shown in Fig. 1 A.

If drugs are unstable and undergo first-order decomposition during incubation in the culture medium, the change in drug concentration can be written as

$$\frac{dC_m}{dt} = -\alpha, \tag{8}$$

where α is a first-order degradation rate constant of the drug.

Upon integration, Eq. (8) becomes:

$$C_m = C_m^0 e^{-\alpha t}, \quad (9)$$

where $C_m^0$ is concentration of drug at 0 time. Substitution of Eq. (9) into Eq. (3) followed by solution of the resulting equation yields:

$$\ln \frac{C_s}{C_s^0} = (k_s - k_r)t - \frac{kKC_m^0}{\alpha}(1 - e^{-\alpha t}). \quad (10)$$

In the control.

$$\ln \frac{C_s'}{C_s^0} = (k_s - k_r)t. \quad (5)$$

Subtraction of Eq. (5) from Eq. (10) yields:

$$\ln \frac{C_s}{C_s'} = -\frac{kKC_m^0}{\alpha}(1 - e^{-\alpha t}). \quad (11)$$

From Eq. (11), relation between the initial drug concentration ($C_{m,90}$) necessary for reducing survival fraction ($C_s/C_s'$) to 0.1 and exposure time can be expressed as:

$$C_{m,90} \times \frac{1-e^{-\alpha t}}{\alpha} = \frac{2.3}{kK} \quad (12)$$

Since AUC can be written as $\int_0^t C_{m,90} \times e^{-\alpha t} dt$,

$$AUC = \int_0^t C_{m,90} \times e^{-\alpha t} dt =$$

$$C_{m,90} \times \frac{1-e^{-\alpha t}}{\alpha} = \frac{2.3}{kK} \quad (13)$$

is obtained from Eq. (12).

$C_{m,90}$ – t curves on a log scale for various degradation constants, α 's, can be simulated as asymptotic, as shown in Fig. 1B. If drugs are stable and almost no decomposition occurs during the exposure time ($\alpha \to 0$), Eq. (12) turns out to be the same as Eq. (7) for these.

Furthermore, important relationships of AUC to cell survival can be derived. For example, when cell survival is suppressed to x% ($C_s/C_s' = x/100$) by exposing cells to drug for exposure time t at a concentration of $C_{m,100-x}$, Eq. (6) becomes

$$AUC = C_{m,100-x} \times t = -\frac{1}{kK} \ln \frac{x}{100} \quad (14)$$

Also, for unstable drugs, substitution of $C_s/C_s' = x/100$ and $C_m = C_{m,100-x}$ into Eq. (11) produces:

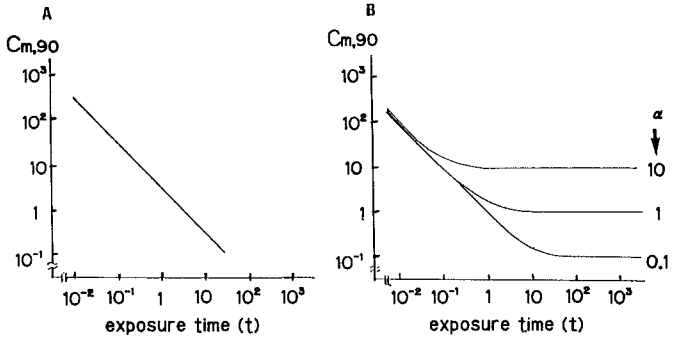


**Fig. 1A, B.** Log-log relationships between $C_{m,90}$ (i.e., $IC_{90}$) and exposure time (t) simulated from a kinetic model for cell killing effect of antitumor agents that are stable (A) and unstable (B) during the incubation

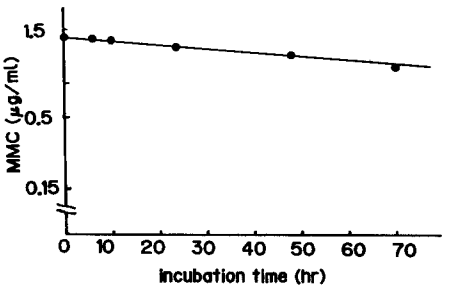


**Fig. 2.** Degradation of MMC in culture medium. MMC was added to the culture medium at concentration of 1.4 μg/ml and incubated at 37° C for various periods of time. MMC concentrations were estimated by HPLC (see *Materials and methods*)

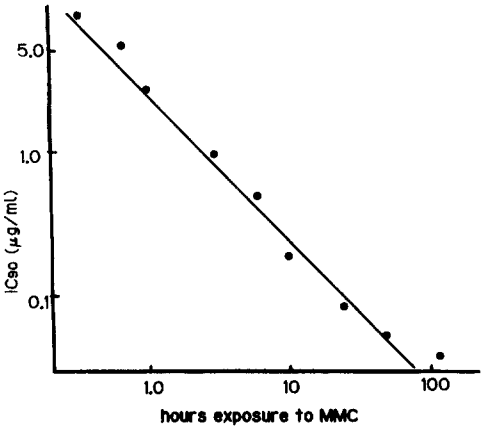


**Fig. 3.** Log-log relationship between $IC_{90}$ value and exposure time for MMC. $IC_{90}$ values obtained from the dose-response curves for MMC were plotted against exposure times on a log scale

$$C_{m,100-x} \times \frac{1-e^{-\alpha t}}{\alpha} = -\frac{1}{kK} \ln \frac{x}{100} \quad (15)$$

Considering Eq. (15), AUC can be expressed as:

$$AUC = \int_0^t C_{m,100-x} \times e^{-\alpha t} dt = C_{m,100-x} \times \frac{1-e^{-\alpha t}}{\alpha} = -\frac{1}{kK} \ln \frac{x}{100}. \quad (16)$$

($C_{m,100-x}$ is the initial drug concentration necessary to reduce cell survival to x%).

These results indicate that the cell killing action of cell cycle phase-non-specific agents can be determined in terms of C × T or AUC whether the drugs are stable or not.

### *Description of cytotoxic action caused by MMC as a stable drug*

Degradation kinetics of MMC in the culture medium are shown in Fig. 2. MMC was relatively stable, and residual MMC amounted to 65% of the initial level even after a 70-h incubation at 37° C. $IC_{90}$ values for various exposure times were obtained from the dose-response curves. The linear relationship of log (exposure time) to log ($IC_{90}$) or log ($C_{m,90}$) with a slope of −1 resembled that in Fig. 1A and showed good agreement with Eq. 7; and kK was calculated as $1.46 \times 10^{-2}$ (ml/μg/min) by linear least-squares regression analysis (Fig. 3). Thus, the AUC for

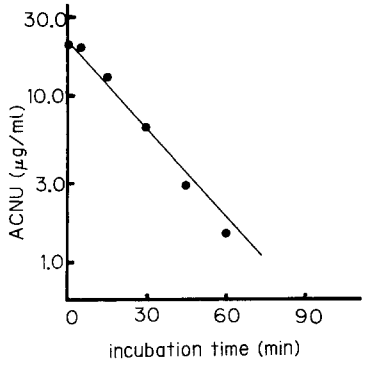


**Fig. 4.** Degradation of ACNU in the culture medium. ACNU was added to the medium at a concentration of 20.3 μg/ml and incubated at 37° C for various periods of time. At the end of the incubation, ACNU concentration was determined by HPLC (see *Materials and methods*)

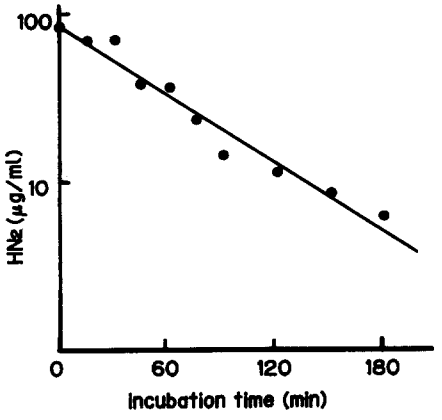


**Fig. 5.** Degradation of $HN_2$ in the culture medium. $HN_2$ was added to the medium at a concentration of 89 μg/ml and incubated at 37° C for various periods of time. $HN_2$ was assayed in terms of its alkylation of NBP (see *Materials and methods*)

90% cell kill for V79 cells is expressed as 2.3/kK = 158 (μg·min/ml), which means that AUC is constant (Eq. 7).

### *Description of cell killing action caused by ACNU and $HN_2$ as unstable drugs*

ACNU and $HN_2$ were rapidly eliminated during the incubation, with half-lives of 17.7 min and 44 min, respectively. Degradation rate constants were $3.91 \times 10^{-2}$ for ACNU and $1.57 \times 10^{-2}$ ($min^{-1}$) for $HN_2$, determined by HPLC and by alkylation of NBP, respectively, as in Figs. 4 and 5. For $HN_2$, the degradation rate constant in the presence of a considerable number of V79 cells ($3.3 \times 10^4$ cells/ml) was also determined as $1.52 \times 10^{-2}$ ($min^{-1}$), which was similar to that in the absence of the cells.

$IC_{90}$ values for ACNU and $HN_2$ with various exposure times were obtained from the dose-response curves, and relationships between log (exposure time) and log ($IC_{90}$) of ACNU and $HN_2$ are shown in Figs. 6 and 7, respectively; these curves were found to be very similar to the asymptotic curves presented in Fig. 1B. Equation (12) was fitted to the experimental data to obtain the values for kK and α by iterative nonlinear least-squares regression [14]. kK values

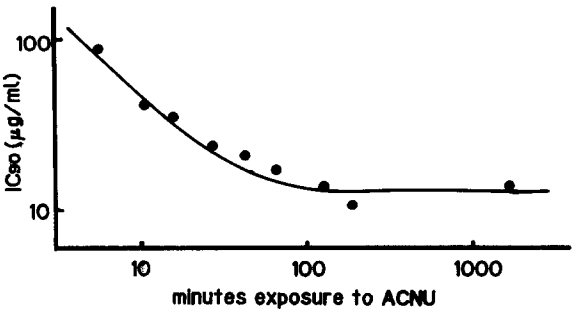


**Fig. 6.** Log-log relationship between $IC_{90}$ value and exposure time for ACNU. $IC_{90}$ values obtained from the dose-response curves for ACNU were plotted against exposure times on a log scale

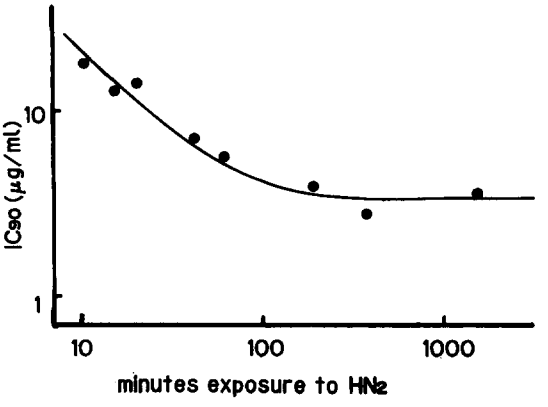


**Fig. 7.** Log-log relationship between $IC_{90}$ value and exposure time for $HN_2$. $IC_{90}$ values obtained from the dose-response curves for $HN_2$ were plotted against exposure times on a log scale

**Table 1.** Calculated and observed decomposition constants and half-lives for ACNU and nitrogen mustard

| Drug | Decomposition rate composition (α)[a] | | Half-life ($t_{1/2}$)[b] | |
|---|---|---|---|---|
| | Calculated | Observed | Calculated | Observed |
| ACNU | 0.0314 | 0.0391 | 22.1 | 17.7 |
| Nitrogen mustard | 0.0175 | 0.0157 | 39.6 | 44.1 |

[a] $min^{-1}$ [b] min

(means ± SD) calculated for ACNU and $HN_2$ were $(5.64 \pm 0.42) \times 10^{-3}$ and $(1.18 \pm 0.11) \times 10^{-2}$ (ml/μg/min), respectively. As in Table 1, α and half-life of the drug ($t_{1/2}$) calculated from the curves in Figs. 6 and 7 were in a good agreement with the values observed by way of chemical assays for ACNU and $HN_2$. These results suggest that the cell killing action of unstable drugs, such as ACNU and $HN_2$, can also be quantitatively analyzed in the same way as that of MMC if drug decay in the medium is taken into account. Therefore, common cell kill kinetics is proposed for cell cycle phase-non-specific agents.

## Discussion

After i.v. administration of antitumor agents in humans, the plasma concentration of the drug generally decreases

rapidly. The drug concentration in tumor tissues may also change rapidly. In contrast, tumor cells are exposed to a drug at a constant concentration in the case of in vitro assays of cytotoxicity. Therefore, to predict the in vivo response to antitumor agents on the basis of cell killing activity determined in vitro, the relationship between drug concentration and exposure time necessary for a certain cytotoxic activity must be determined.

A number of studies performed to this end have been reported. Rupniak et al. [7] found that treatment with cisplatin, adriamycin, or vinblastine generated exponential survival curves, with increasing cell kill resulting either when the drug concentration was increased or when exposure time was extended. On the other hand, Wu et al. [13] presented the following three distinct patterns of drug sensitivity: (a) dose- and time-dependent, (b) time-dependent and (c) time-independent. Weinkam et al. [11] also described a quantitative dose-response relationship for the cytotoxic activity of chloronitrosourea cancer chemotherapeutic agents in cell culture. These studies were very suggestive, but not necessarily satisfactory in terms of quantitative analysis that would lead to general conclusions regarding cell kill kinetics of antitumor agents. From this point of view, Shimoyama's afore-mentioned study was outstanding. It yielded the conclusion that for their cell killing action cell cycle phase-non-specific agents obey the following equation:

$$C^n \times T = K, \tag{17}$$

where C and T are the drug concentration and exposure times, respectively, necessary for a certain degree of cell kill, and n and K are constants. It was also stressed that the cell killing action of this class of antitumor agents was principally concentration-dependent and that its degree of dependence on concentration relies on the value of n. According to their study, a number of drugs denoted as type Ib demonstrated n values of nearly 1, while those of type Ia, such as nitrogen mustard, 4-hydroperoxy-cyclophosphamide, and nitrosoureas, had values larger than 2. This result directly means that the cell killing action of type Ib drugs is dependent on C × T or AUC in view of Eq. (17), although they did not refer to AUC dependence.

Our present pharmacodynamic analysis of cell kill kinetics for cell cycle phase-non-specific agents, which takes account of their decomposition during the incubation, showed that log $IC_{90}$ – log exposure time relationships for type Ia drugs could be expressed by an asymptotic curve and that the cell killing action of type Ia drugs also depends on C × T or AUC. We ascertained that MMC, which has a negligible rate of decomposition, had a n value of 1, indicating its complete dependence on AUC (Fig. 3). Experimentally derived log $IC_{90}$ – log t relationships of ACNU and $HN_2$ were expressed as asymptotic curves, as predicted by our model analysis (Figs. 6 and 7), and their decomposition constants calculated from the log $IC_{90}$ – log t curves turned out to be in good agreement with those determined by chemical assay (Table 1).

These results, in keeping with those of Shimoyama and coworkers, demonstrate that the cell killing action of both type Ia and Ib drugs can be analyzed with reference to a common kinetic if their decomposition is taken into account, and allow the conclusion that the cell killing action of cell cycle phase-non-specific drugs is principally dependent on C × T or AUC. Such (C × T)-dependence of cell killing action seems to be due to (C × T)-dependence of the bimolecular and irreversible reaction of these drugs with cellular macromolecules. Several previous studies have suggested that C × T might be the best pharmacokinetic parameter for predicting response to an antitumor agents [4, 6]. Our present model analysis and experimental results give such suggestions a theoretical basis.

The conclusion obtained in the present study is highly applicable to clinical sensitivity test for individual tumors and prediction of the clinical antitumor activity of new drugs in preclinical testing. In human tumor clonogenic assays, one-tenth of the peak plasma concentration has been used as the standard drug concentration. It has been often pointed out that this setting of drug concentration lacks a theoretical basis and may therefore be one of the major causes for relatively poor predictability of a positive response. Our study makes it possible to determine reasonable concentrations to be employed in this in vitro sensitivity testing of cell cycle phase-non-specific agents, if their clinically achievable plasma AUCs of unbound drug are already estimated. On the other hand, poor clinical predictability of the current screening model for antitumor agents has been one of the most serious problems in cancer chemotherapy. The situation was not significantly improved even by the introduction of human tumor implantation in nude mice. However, if we know the clinically achievable plasma AUC of new drugs belonging to the type I category at the end of clinical phase I testing, we can perform experimental phase II tests on various human tumor lines in vitro or in vivo using reasonable drug concentrations or treatment doses. These tests would be expected to yield important information as to what type of tumors are sensitive to the agent in question. Furthermore, if we can foresee their clinically achievable plasma AUC by means of some kinetic approaches such as animal scale-up procedure [5], it might become possible to predict the clinical effectiveness of such new drugs in the preclinical stage.

Our present efforts are directed at finding a general parameter to determine the cell killing potency of cell cycle phase-specific agents.

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