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Pharmacokinetic Approach to Rational Therapeutic Doses for Human Tumor-bearing Nude Mice

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To improve clinical predictability from therapeutic results of various antitumor agents in human tumor/nude mouse models it seems to be important to use a dose pharmacokinetically equivalent to the clinical dose. Thus, we attempted to find the dose of a given drug that can reproduce in the nude mouse a plasma level similar to that seen in human patients treated with an effective dose of the drug based on comparative pharmacokinetic studies between man and nude mouse. As a result, those of 3 alkylating agents, mitomycin C, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea (ACNU) and cyclophosphamide, and those of 2 antimitotic agents, vincristine and vinblastine, were estimated to be one-fourth or one-fifth of their maximum tolerated doses (MTD's). On the other hand, in the case of adriamycin, its MTD was approximately equivalent to its clinical dose pharmacokinetically. In contrast, clinically equivalent doses of 2 antimetabolites tested, 5-fluorouracil and methotrexate, were significantly greater than their MTD's; i.e., their plasma levels did not reach the effective clinical ones even when their MTD's were administered to the nude mice. These results suggest that the antitumor effects of most antitumor agents are over- or underestimated in this model when MTD's are used as a therapeutic dose, and indicate that the use of clinically equivalent doses determined pharmacokinetically is desirable.

Key words: Antitumor agents — Human tumor — Nude mouse — Plasma level — Clinically equivalent dose

In our previous studies,[1] we attempted experimental chemotherapy of a panel of human gastric tumors implanted in nude mice according to the same regimen, including the use of the MTD*3 for nude mice as a therapeutic dose, and evaluated the effectiveness of various antitumor agents in terms of response rate. We found that such experimental response rates showed good agreement with clinical ones for some drugs, but those of other drugs were much higher than their respective clinical ones. This disagreement was often observed with drugs whose dose per body weight was quite different between man and nude mouse. These results suggested the importance of using appropriate dose levels in the nude mouse to reproduce the clinically equivalent effect in this model. Thus, the purpose of the present study was to find experimentally a reasonable dose of each drug for human tumor-bearing nude mice based on a comparative pharmacokinetic study between man and nude mouse.

Theoretically, it seems most reasonable to use a dose that will produce a drug concentration in the extracellular space of tumor tissue in mice similar to that in human patients given a clinical dose. For most drugs which can easily penetrate the capillary vessel and rapidly reach an equilibrium on both sides, this dose approximates pharmacokinetically to the unbound free drug concentration in the blood.

Thus, in the present study, we administered various doses of selected antitumor agents to

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*3 Abbreviations used: VCR, vincristine; VLB, vinblastine; CPM, cyclophosphamide; 5FU, 5-fluorouracil; MMC, mitomycin C; ADR, adriamycin; MTX, methotrexate; HPLC, high-performance liquid chromatography, MTD, maximum tolerated dose; RD, rational dose; AUC, area under the curve.

nude mice and measured their plasma levels by the same method as used in the clinical studies. Clinical data on the plasma levels of all antitumor agents were taken from papers reported by other investigators. For comparison of plasma levels of both human and nude mouse, we attempted to determine the mouse dose that is needed to achieve the same plasma level as seen in human plasma when the patient is treated effectively.

Materials and Methods

Chemicals VCR and VLB for clinical use were purchased from Shionogi & Co., Osaka. 5FU, MMC and ADR were kindly supplied by Kyowa Hakko Kogyo Co., Tokyo, as pure crystals for experimental use. CPM, ACNU and MTX were provided by Shionogi & Co., Sankyo Co., Ltd., Tokyo, and Lederle Japan Ltd., Tokyo, respectively. [^{3}H]VCR and [^{3}H]VLB were purchased from Amersham International plc, Amersham, England.

Collection of Plasma Samples Each antitumor agent was dissolved in or diluted with sterile 0.85% NaCl solution just before use. Drug solution was injected into the tail vein of athymic Balb/c-*nu/nu* mice (Clea Japan, Inc., Tokyo). Three female mice weighing an average of 24 g each were used in each group. At specified times after injection, groups of mice were killed by decapitation and the trunk blood was collected from the neck into a heparinized tube. The plasma was obtained by centrifugation at 10,000*g* for 2 min in a centrifuge (Beckman microfuge B) and stored at $-20°$ until the estimation of drug concentration could be performed.

Measurement of Drug Concentration in Plasma Measurement of drug concentrations in plasma of mice was carried out by the same method as used in each clinical study quoted.

MMC was analyzed by a biological assay method with *E. coli* B grown on agar containing essential medium, an assay originally developed by Miyamura *et al.*[2] The lower limit of sensitivity of this method was 0.003 μg/ml.

ADR in plasma was assayed by HPLC according to the method reported by Masuike *et al.*[3] A plasma sample was applied to an HPLC apparatus equipped with a protein-coated column and eluted stepwise with phosphate buffer (PB), that is, 0.005 *M* KH_2PO_4 - 1*M* H_3PO_4 (pH 4.5), PB - CH_3CN (75:25), and PB. The drug was detected fluorometrically. Excitation and emission wavelengths were 470 and 585 nm, respectively.

VCR concentration was measured as total radioactivity in accordance with the method used for clinical study.[4] [^{3}H]VCR solution was injected iv into the nude mice, and the total radioactivity of the plasma (0.1 ml), without isolation of VCR, was measured by liquid scintillation counting.

ACNU concentration in plasma was measured by HPLC according to the method of Nakamura *et al.*[5] Briefly, after extraction with 1,2-dichloroethane, a 50% methanol solution of the extract was assayed. A Waters μ-Bondapack C_{18} reverse-phase column was used and ACNU was eluted from it with a solvent system consisting of PIC-B_7 plus an aqueous solution of 50% methanol at flow rate of 1 ml/min. The lower limit of detection by this method was 0.03–0.04 μg/ml.

Regarding CPM, the concentrations of its activated metabolites, 4-hydroxy CPM and aldophosphamide, were measured according to the method of Wagner *et al.*[6] In this case, a blood sample instead of plasma was used. In brief, the active metabolites were extracted into dichloromethane from the blood sample and the extract was concentrated in an evaporator. A mixture of the filtered extract and dilute HCl solution containing 3-aminophenol and hydroxyammonium chloride was heated at 95° for 20 min, and the fluorescence emission was measured at room temperature in a Hitachi fluorometer, model MPF-4. Excitation and emission wavelengths were 350 and 515 nm, respectively. The fluorescence originating from acrolein, which was liberated from the active metabolites, was carefully measured by using blank tests. The minimum measurable concentration was 0.5 nmol/ml of blood.

Plasma concentrations of unbound VLB were determined according to Lu *et al.*[7] Plasma samples collected from mice injected iv with [^{3}H]VLB were deproteinized by sulfosalicylic acid, neutralized and filtered through an Ekicrodisc 3 (0.45 μm, Gelman Sciences Japan, Ltd.). This preparation was applied to an HPLC apparatus equipped with μ-Bondapack C_{18} column and eluted with a solvent system consisting of acetonitrile and phosphate buffer at a flow rate of 2 ml/min. The eluate was fractionated at intervals of 30 sec, and the radioactivity of VLB fractions (retention time: 11–12 min) was measured.

The concentration of 5FU in plasma was determined by the microbiological assay method originally reported by Fujita,[8] using *Staphylococcus aureus* 209p grown on Muller-Hinton medium without peptone and bouillon. The detection limit was 0.01 μg/ml.

MTX plasma concentrations were measured by dihydrofolate reductase inhibition assay according to the method of Bertino and Fischer.[9] Measurements were taken in a 1.5-ml quartz glass cuvette at 30° and 340 nm wavelength, and the measurement time was 2 min. The lower limit of sensitivity was 0.002 μg/ml.

Results

We expected it to be difficult to find the dose of a given drug which would accurately reproduce in the nude mouse the clinically observed "concentration-time curve" in man, since the slope of the plasma clearance curve of most drugs is usually quite different between man and nude mouse. As a preliminary experiment, three graded doses of each drug, for example, the MTD and one-half and one-fourth of it were injected into nude mice to measure their plasma levels at a few time points, and rough plasma clearance curves were obtained. To determine a dose for nude mouse pharmacokinetically equivalent to the clinically effective dose, comparison of plasma levels between man and nude mouse was made with an emphasis on drug concentration in plasma at a relatively early time after injection. This is very important, particularly for antitumor drugs with a small difference in the slope of their clearance curve between the two species, because relatively higher drug concentrations at early time make a greater contribution to the antitumor effect.

The MMC, ADR, and VCR plasma clearance curves demonstrated in Fig. 1 seem to belong to the above category. In the case of MMC, used as a reference, data for both the MTD (6.7 mg/kg) and 1/4 MTD (1.7 mg/kg) in the nude mouse are shown in comparison with those of patients given the clinical dose[10] (Fig. 1-A). The plasma level of MMC at its MTD was obviously higher than that in the case of the clinical dose. However, when MMC at 1.7 mg/kg was administered to nude mice, its plasma clearance curve was found to be similar to that of human patients. In the case of MMC, therefore, 1.7 mg/kg or thereabouts might be regarded as the clinically equivalent dose for nude mice on a pharmacokinetic basis.

In contrast to MMC, a plasma level of ADR similar to that in patients given a clinical therapeutic dose of 60 mg/m^2 [11] was observed in nude mice when the MTD was administered (Fig. 1-B). With VCR, compari-

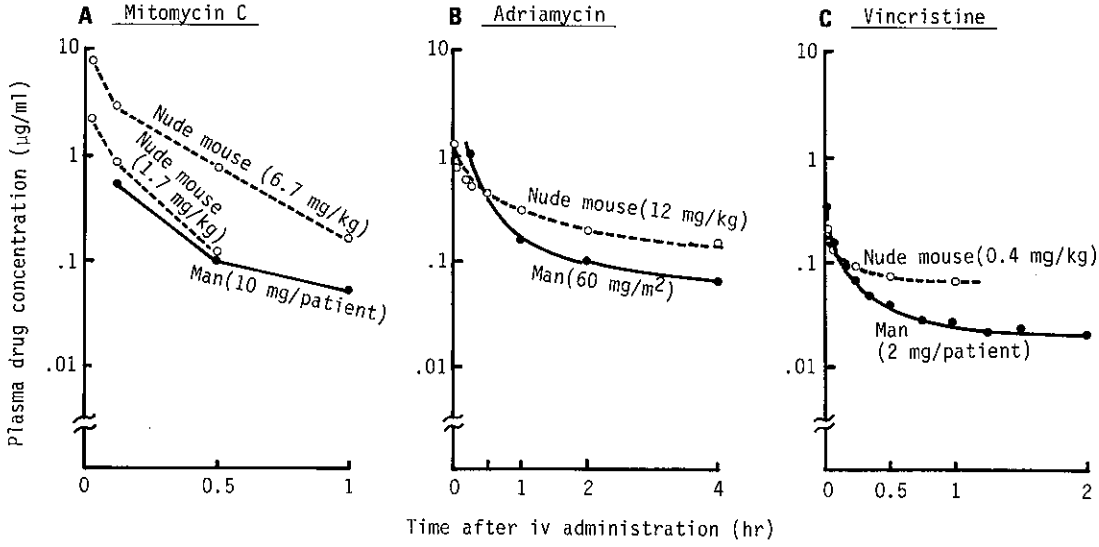

Fig. 1. Comparison of plasma levels of mitomycin C, adriamycin, and vincristine between man and the nude mouse. Plasma concentrations of antitumor agents in nude mice given a single dose of each agent (2 doses for MMC) were measured and compared with those of human patients administered a clinical dose of the same agent. Determination of plasma levels of each drug was made by the same methods as used in clinical studies. Each value was the mean of 3 to 4 determinations with less than 20% standard deviation. Clinical data of each drug are quoted from the literature.[2-4] Clinical doses of MMC, ADR and VCR can be converted into 0.17, 1.6 and 0.033 mg/kg, respectively, assuming that mean body weight is 60 kg or 1 m^2 of body surface area corresponds to 37.5 kg of body weight.

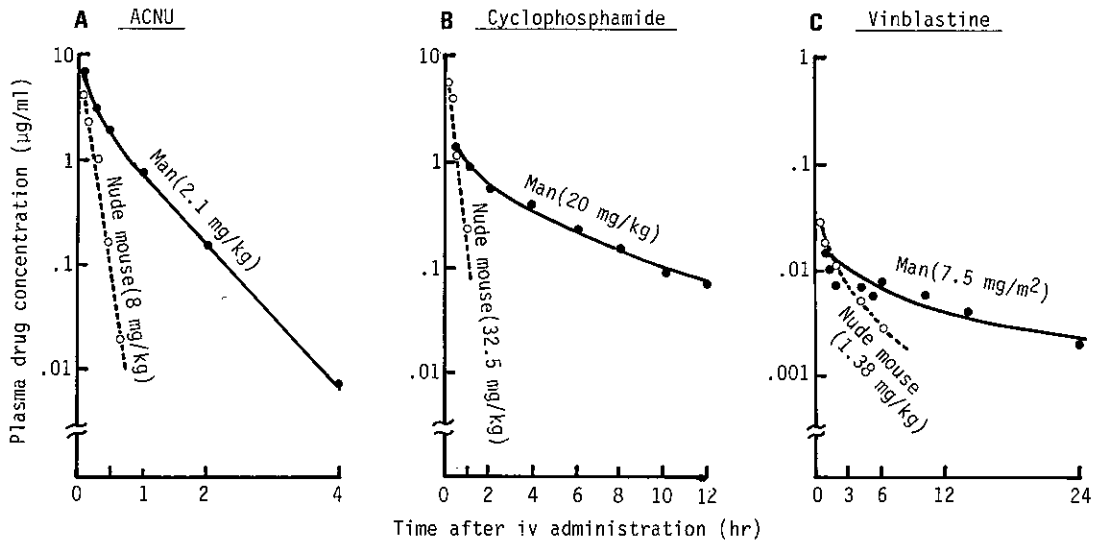

Fig. 2. Comparison of plasma levels of ACNU, cyclophosphamide and vinblastine between man and the nude mouse. Plasma concentrations of antitumor agents in nude mice given a single dose of each agent were measured and compared with those of human patients administered a clinical dose of the same agent. Determination of plasma levels of each drug was made by the same methods as used in clinical studies. Each value was the mean of 3 to 4 determinations with less than 20% standard deviation. Clinical data of each drug are quoted from the literature.[5-7] Clinical dose of VLB can be converted into 0.2 mg/kg assuming that 1 m^2 of body surface area corresponds to 37.5 kg of body weight.

son of the plasma level in nude mice treated with 0.4 mg/kg with that[4] of human patients given an effective dose, as shown in Fig. 1-C, reveals similar VCR levels between the two species at the early stage, i,e., up to 15 min after injection. On the other hand, in the second phase of clearance, the plasma level of VCR was significantly higher in nude mice than in man. If coincidence of VCR plasma level at the late phase between man and nude mouse is required, some dose less than 0.4 mg/kg may correspond to the clinically equivalent dose.

As illustrated in Fig. 2, the drugs ACNU, CPM, and VLB exhibited markedly different clearance rates between man and nude mouse. In the case of ACNU, its half-lives in the plasma of man[5] and nude mouse were 25 and 4.6 min, respectively (Fig. 2-A). Thus with this kind of drug it is very difficult to reproduce the clinical clearance curve in nude mice by a single injection. As an approach to get a clearance curve of ACNU in the nude mouse similar to the clinical one, intermittent injection of three graded doses (8, 2, and 0.8 mg/kg at 0, 25, and 70 min, respectively) was attempted, as shown in Fig. 3-A. Rather better tracing of the clinical clearance curve was thus obtained, although coincidence was still incomplete.

With CPM, a steeper plasma clearance was also observed with the nude mouse as compared with the clearance in man.[6] For this drug, plasma levels of 4-hydroxy CPM instead of CPM itself were compared between man and nude mouse, since changes in the concentrations of activated CPM are thought to be more important as far as antitumor activity is concerned. However, it is hard in practice to mimic the clinical clearance curve in the nude mouse by intermittent injections, because the clearance rate of 4-hydroxy CPM in man is extremely slow. The AUC values of 4-hydroxy CPM in the plasma of both man and nude mouse were calculated from concentration-time curves (Fig. 2-B) by computer anal-

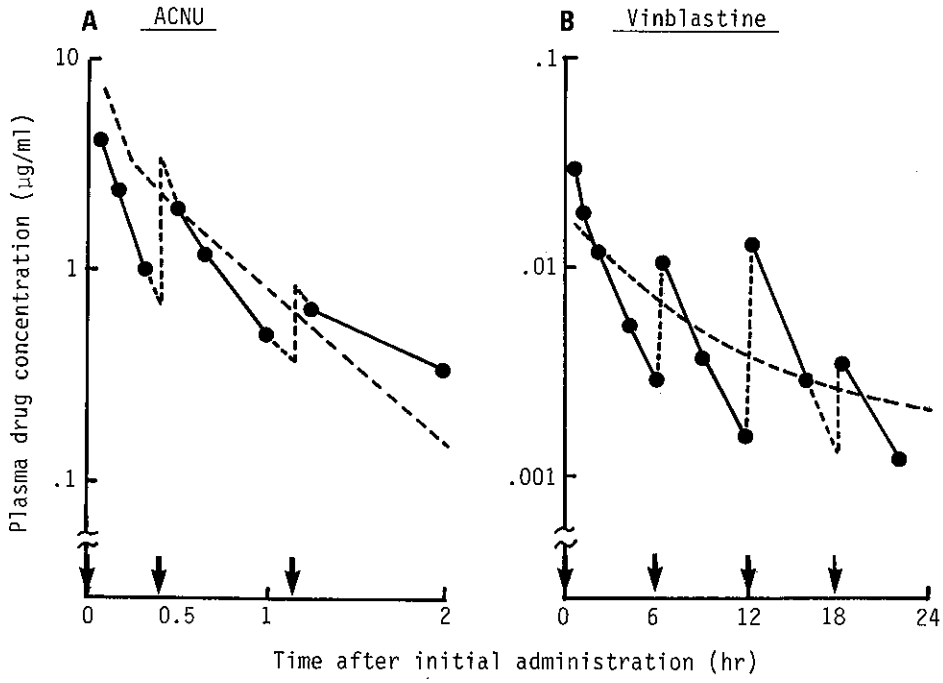

Fig. 3. Comparison of clinical plasma clearance curves of ACNU and vinblastine with the respective curves obtained by intermittent injection of the drugs into nude mice. ACNU was intermittently injected into nude mice at 3 doses of 8 (0 min), 2 (25 min), and 0.8 mg/kg (70 min). Similarly, VLB was injected at 4 doses of 1.38 (0 hr), 0.6 (6 hr), 0.3 (12 hr) and 0.1 mg/kg (24 hr). At appropriate intervals, the plasma concentrations were measured and compared with those of human patients administered a single clinical dose[5,7] (----). Each value was the mean of 3 to 4 determinations with less than 20% standard deviation.

ysis and found to be 16.4 and 8.37 nmol·hr/ml, respectively. Therefore, if the cell-killing activity of CPM depends on "concentration × exposure time" i.e., AUC in the case of plasma, a doubling of the 32.5 mg/kg dose of CPM should correspond to the clinical dose.

In the case of VLB, a difference in the slope of the clearance curve was also seen between man and nude mouse. Its plasma level in nude mice administered 1.38 mg/kg of VLB was initially somewhat higher than that in human patients given 7.5 mg/m^2 of VLB,[7] but became lower later due to its relatively rapid clearance in the nude mouse. VLB has been classified by cell-killing kinetic analysis as a time-dependent drug,[12] suggesting that its cytotoxicity is not dependent on the AUC. Then, intermittent injections (1.38, 0.6, 0.3, and 0.1 mg/kg at 0, 6, 12 and 24 hr, respectively) were attempted, and they were found to reproduce the clinical clearance curve roughly, as shown in Fig. 3-B. However, such treatment is impractical in therapeutic experiments. Therefore, with this drug, we tried to find a dose for a single injection equivalent to the above four intermittent dosings in terms of antitumor activity. Although the data are not shown, a single injection of 2.6 mg/kg of VLB inhibited *in vivo* tumor growth to approximately the same extent as the intermittent administration in 2 different xenograft models.

With the 2 antimetabolites 5FU and MTX, no marked difference in plasma clearance rate was observed between man and nude mouse, at least over the period of time examined. Different from the above-mentioned drugs, the plasma levels of 5FU and MTX in nude mice did not reach those in human patients

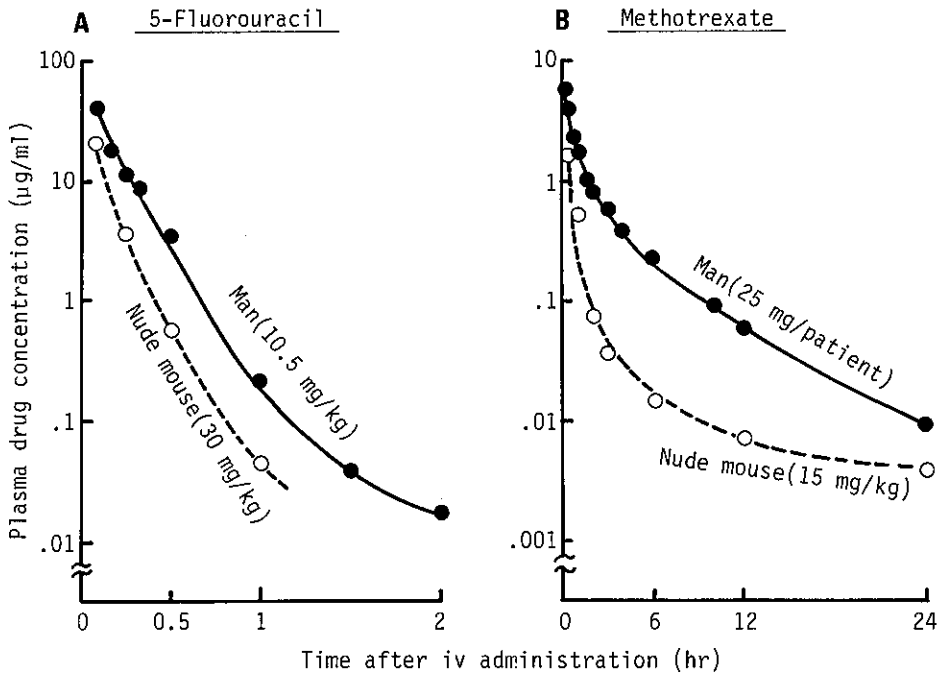

Fig. 4. Comparison of plasma levels of 5-fluorouracil and methotrexate between man and the nude mouse. The plasma concentration of each antitumor agent in nude mice given a single dose of agent was measured and compared with that of human patients administered a clinical dose of the same agent. Determination of plasma levels of each drug were made by the same method as used in clinical studies. Each value was the mean of 3 to 4 determinations with less than 20% standard deviation. Clinical data of drugs are quoted from the literature.[8,9] The clinical dose of MTX can be converted into 0.42 mg/kg assuming that the mean body weight is 60 kg.

treated with the therapeutic dose,[13,14] even when the MTD of MTX or greater than the MTD of 5FU was injected (Fig. 4).

Discussion

In the present study, we attempted to find the dose of various antitumor agents for nude mice that could reproduce the clinical plasma clearance curve observed with patients treated effectively, because we considered from a pharmacokinetic point of view such a dose to be a reasonable therapeutic one for human tumor-bearing nude mice. Practically speaking, we found it difficult to determine such a dose in a precise manner, since the plasma clearance rate of most drugs in nude mice was significantly greater than that found clinically in man. However, to observe the clinically equivalent antitumor effect in this human tumor/nude mouse model, determination of such a pharmacokinetically appropriate dose instead of the MTD is necessary even if it is not so accurate. Therefore, we have designated such a dose the "rational dose (RD)" and tentatively estimated such doses based on the present comparative study on plasma levels of each drug in man and nude mouse.

With MMC and ADR, 1.7 and 12 mg/kg, respectively, seem to be reasonable as such a dose. In the case of VCR, its plasma level in nude mice was clearly higher than its clinical one in the late phase of clearance. Since clearance rates in this phase are so slow that such levels might predominantly represent a bound form of VCR, especially in the present assay method, we regard 0.4 mg/kg of VCR as the RD.

Of the 3 drugs with relatively rapid clearance in nude mice, ACNU seemed to give reasonable results when administered as 3 in-

termittent injections to the animals. However, such intermittent treatment seemed impractical in the case of CPM, since the clinically observed plasma clearance was too slow to be reproduced in nude mice by intermittent administration. Very recently, we found from experiments involving *in vitro* colony forming that the cell-killing activity of alkylating agents such as nitrogen mustard depends on "concentration × exposure time" or AUC value. This finding was made by subjecting their decomposition rate in the culture medium during incubation to a cell-kill kinetic analysis.[15] Therefore, it is probably possible to regard the dose of CPM in nude mice equivalent to the clinically effective dose in terms of plasma AUC as its RD. This line of reasoning, however, could not be applied in the case of VLB, for this drug does not demonstrate the same pharmacokinetics as CPM, i.e., the cell-killing activity of this drug has been proved to be independent of the AUC. Accordingly, in this case, a single injection of 2.6 mg/kg was found equivalent to intermittent administrations with respect to antitumor effect and was regarded as its RD.

The estimated RD's of 8 kinds of antitumor agents are listed with their MTD's[1] in Table I. The RD's of MMC, VCR, ACNU, CPM, and VLB corresponded to 1/5–1/4 of their respective MTD's. These results can reasonably explain the higher experimental response rates observed with these antitumor agents, except for VCR, as compared with the respective clinical ones when human gastric tumors in nude mice were treated with the MTD.[1] On the other hand, the RD of ADR was very close to its MTD. In the case of 5FU and MTX, their plasma levels in nude mice did not reach those in human patients given the effective doses even if their MTD's were injected into the nude mice. These results suggest that the clinical antitumor effects of these agents might be underestimated if treatment in nude mice was done even at the MTD, although the degree of underestimation is not clear. In this respect, some examples of a lack of therapeutic efficacy of 5FU[16] and MTX[17] against human tumor xenografts at doses that are effective in an *in vitro* clonogenic assay or in clinical treatment have been reported by a group in the Netherlands.

In the succeeding paper, the results of experimental chemotherapy using these clinically equivalent doses against a panel of human gastric tumors implanted in nude mice will be reported in comparison with those obtained by using the MTD's.

Table I. Maximum Tolerated and "Rational" Doses of Various Antitumor Agents for Nude Mice

Drug	MTD (mg/kg)	RD (mg/kg)	RD/MTD
MMC	6.7	1.7	0.25
ADR	12	12	1
VCR	1.6	0.4	0.25
ACNU	48	8 (0 min) 2 (25 min) 0.8 (70 min)	ca. 0.2
CPM	260	65	0.25
VLB	11	2.6	0.24
5FU	19 (×5)	>19 (×5)	>1
MTX	15 (×5)	>15 (×5)	>1

MTD values, which were determined as maximum nonlethal doses, are quoted from our previous paper.[1]

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