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# Antitumor activities and schedule dependence of orally administered MST-16, a novel derivative of bis(2,6-dioxopiperazine)

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**Summary.** We studied bioavailability, treatment schedule dependence, and therapeutic efficacy of orally administered MST-16, a novel derivative of bis(2,6-dioxopiperazine), against murine tumors and human tumor xenografts. The rate of its intestinal absorption was about 50%, and it was immediately metabolized to its parent compound, ICRF-154. Therapeutic efficacy of MST-16 was heavily dependent on the treatment schedule: 9 daily oral administrations and treatment every 4 h on day 1 only were much more effective against s.c.-implanted L1210 leukemia than a single dose or five daily administrations giving the same total dose. Orally administered MST-16 showed potent lifeprolonging effects (196%, 219% and 148%) in mice inoculated i.p. with P388, L1210 leukemia, and C-26 colon adenocarcinoma, respectively, but had no effect on B16 melanoma inoculated in the same way. MST-16 inhibited more than 80% growth of Lewis lung carcinoma, B16 melanoma, and C-38 colon adenocarcinoma implanted s.c., but had only a minor effect on M5076 fibrosarcoma. Lung metastasis of Lewis lung carcinoma was also effectively suppressed. Furthermore, MST-16 significantly inhibited growth of human colon, lung and breast cancers implanted s.c. in nude mice. We also made a kinetic analysis of the in vitro cell-killing effect by ICRF-154, the active form of MST-16 in vivo. It demonstrated a cell cycle phase-specific and time-dependent action, providing a reasonable explanation for the schedule-dependent therapeutic effect of MST-16.

## Introduction

ICRF-159, one of the bis(2,6-dioxopiperazine) compounds, has been reported to possess significant antitumor and antimetastatic activities in several murine tumor models [3, 5, 7, 14, 15], but its inadequate bioavailability seems to have limited its clinical therapeutic efficacy [2, 6]. Accordingly, with the purpose of obtaining a new derivative with improved bioavailability and therapeutic activity, in an earlier study we investigated the antitumor activities of a number of synthetic derivatives of bis(2,6-dioxopiperazine) and selected MST-16, 4,4′-(1,2-ethanediyl)-bis(1-isobutoxycarbonyloxymethyl-2, 6-piperazine dione) as the most promising compound for use as an antitumor agent [1, 10].

MST-16 administered i.p. exhibited potent antitumor activity against various experimental tumors, and its therapeutic ratio was better than that of ICRF-159 [10]. However, since MST-16 is limited in its water solubility, oral administration would presumably be the route of choice for clinical trials. Therefore, in the present study, we examined bioavailability, schedule dependence, and therapeutic efficacy of orally administered MST-16, using murine and xenografted human tumors as models.

## Materials and methods

*Chemicals.* MST-16 and ICRF-154 were synthesized according to the method previously reported [1]. MST-16 was suspended in 1% hydroxypropylcellulose solution for therapeutic use. [3, 5, 3′,5′-$^{14}$C] MST-16 was synthesized by Daiichi Pure Chemicals Co. (Tokyo, Japan). Its specific activity was 9.6 μCi/mg.

*Animals.* Male DBA/2, C57BL/6, BALB/c, BALB/c × DBA/2 ($CDF_1$), and C57BL/6 × DBA/2 ($BDF_1$) mice, 5 weeks of age, were purchased from Charles River Japan (Kanagawa, Japan). Male BALB/c-nu/nu athymic nude mice were purchased from Nihon Clea (Tokyo, Japan). They were maintained under specific pathogen-free conditions at a temperature of 23 ± 1° C and a humidity of 50 ± 10%.

*Murine tumors.* L1210 leukemia (L1210) and P388 leukemia (P388) were maintained by i.p. serial passage in male DBA/2 mice. B16 melanoma (B16), Lewis lung carcinoma (LL), Colon 38 adenocarcinoma (C-38), and M5076 fibrosarcoma (M5076) were serially passaged by s.c. injection in male $C_{57}$BL/6 mice. Colon 26 adenocarcinoma (C-26) was maintained by s.c. passage in male BALB/c mice. All tumor lines were supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo.

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*Evaluation of antitumor activity against murine tumors.* This study was carried out according to the standard protocols of the Drug Research and Development Program, National Cancer Institute (USA) [4]. A small volume (0.1 ml) of cell suspension in Hanks' balanced salt solution (HBSS) containing $1 \times 10^6$ P388 or $1 \times 10^5$ L1210 cells was inoculated i.p. into male $CDF_1$ mice, and 0.2 ml HBSS with $1 \times 10^6$ L1210 cells was implanted s.c. into $CDF_1$ mice on day 0. Cell suspensions of LL, B16, C-26, and M5076 in HBSS were prepared from surgically removed tumors. The cells were passed through 40-mesh sieves and counted for their viability by trypan blue dye exclusion. LL ($1 \times 10^6$ cells), B16 ($1 \times 10^6$ cells), or M5076 ($2 \times 10^6$ cells) tumor was implanted s.c. into the flank of $BDF_1$ male mice. C-26 ($3 \times 10^5$ cells) was inoculated i.p. into $CDF_1$ mice. Nonnecrotic tissue fragments (50 mg) of C-38 were implanted s.c. with a trocar into $BDF_1$ mice.

MST-16 was administered p.o. into tumor-bearing mice (7 mice/treatment group, 10 mice/control group) in a volume of 0.1 ml/10 g body weight by various schedules. Survival effects were evaluated for i.p.-implanted tumors, and tumor-growth-inhibitory effects were assessed for s.c.-implanted tumors. In the latter (B16, LL, C-38), tumors were excised from each mouse on day 21 and weighed. In these experiments metastatic colonies in the lung were also counted.

*Human tumor xenograft model and experimental design.* Human tumor xenografts used in this study were small-cell lung carcinoma (LX-1), mammary adenocarcinoma (MX-1), and colon adenocarcinoma (Co-4). These tumors were maintained in male BALB/c-nu/nu athymic nude mice. Chemotherapeutic experiments were performed as reported by Inaba et al. [8]. Tumor fragments were implanted s.c. into the right subaxillary region of athymic nude mice. When the tumors had grown to a palpable size (100–300 $mm^3$), the mice were randomly allocated to several experimental groups consisting of six animals each; and MST-16 was given p.o. daily for 5 days. The tumor weight (W) was calculated as follows: $W = 1/2\ ab^2$, where a and b are long and short diameters of the tumor mass in mm. Each tumor weight was calculated twice a week by the above formula and expressed as relative tumor weight (RW), $RW = W_n/W_o$, where $W_n$ is the tumor weight on day n and $W_o$ is initial tumor weight at the time when the treatment was started (day 0).

The effectiveness of each drug was evaluated on day 14 by

$$T/C\ (\%) = \frac{\text{mean RW in the treated group}}{\text{mean RW in the control group}} \times 100$$

Evaluation as "effective" was based on a T/C (%) of 50% or less.

*Estimation of cell-killing effect of drugs.* Chinese hamster V79 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and kanamycin (100 μg/ml) at 37° C in a humidified atmosphere of 5% $CO_2$ and 95% air. Cells were seeded at a cell density of 100, 200, 300, 400, or 500 cells into 60-mm dishes containing 3.0 ml culture medium. The dishes containing 100 or 200 cells were used for controls or for 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 for various lengths of time. At the end of the drug exposure period, the plates were washed twice with 3 ml HBSS; 3 ml culture medium was then added to each dish, followed by further incubation. On the 5th day after seeding, cells were washed once with phosphate-buffered saline and fixed with 10% formalin. The colonies were counted after staining with 0.05% crystal violet. All assays were done in triplicate or quadruplicate.

*Measurement of blood level and urine excretion of MST-16.* MST-16 was administered p.o. to $CDF_1$ male mice (5 mice/group), and blood samples were taken from a tail vein. For analysis of ICRF-154, the 20 μl blood samples were shaken with 40 μl methanol for 10 min, 340 μl 2% methanol solution was added, and the mixtures were centrifuged at 3000 rpm. The supernatant obtained was then injected into a high-performance liquid chromatograph (HPLC) with a column (6.0 ID × 150 mm) of YMC-Pack, AM312, ODS 120A (Yamamura Chemical Lab., Japan). The elution was done under the following chromatographic conditions: monitoring wavelength, 210 nm; flow rate, 1 ml/min; mobile phase, 2% methanol solution. For analysis of MST-16, the 20 μl blood samples were shaken with 40 μl acetonitrile for 10 min, 340 μl 50% acetonitrile solution added and analyzed under the same conditons as ICRF-154, except that the mobile phase was changed to 50% acetonitrile solution. Cumulative excretion of MST-16 in the urine was assessed by measurement of the radioactivity of urine samples.

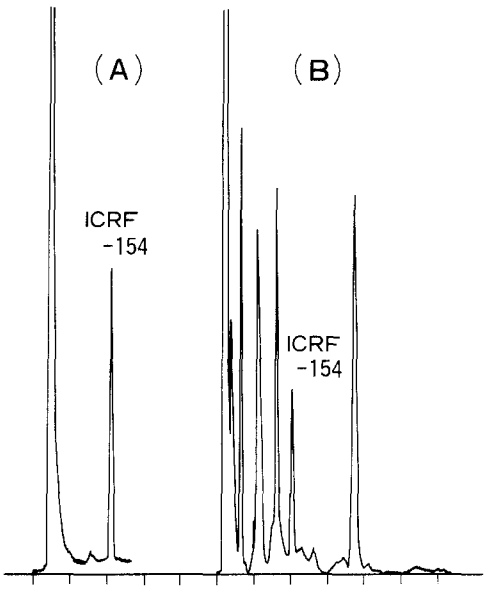


**Fig. 1.** High-performance liquid chromatograms of ICRF-154 in standard (*A*) and blood samples (*B*). Column: (6.0 ID × 150 mm) YMC Pack, AM312, ODS 120A; wave length: 210 nm, flow rate; 1 ml/min; mobile phase: 2% methanol solution

## Results

### *Bioavailability of orally administered MST-16*

MST-16 was administered p.o. to $CDF_1$ mice, and its blood levels were measured. ICRF-154 in the blood was detected by HPLC as shown in Fig. 1. MST-16 was also identified by HPLC under different conditions (data not shown). Analysis of the blood sample by HPLC revealed no MST-16 even only 30 min after administration, but the parent compound of MST-16, that is, ICRF-154, was observed. This result suggests that 1-isobutoxycarbonyloxymethyl groups are rapidly removed from MST-16 and that ICRF-154 is an active metabolite of MST-16 in vivo. Chemical structures of MST-16 and ICRF-154, together with the structure of ICRF-159, are shown in Fig. 2. The peak blood concentration, approximately 10 μg/ml, of ICRF-154 appeared 1 h after administration of 200 mg/kg MST-16 (Fig. 3). Cumulative radioactivity in the urine and expired gases up to 120 h after administration of 50 mg/kg of [$^{14}$C]MST-16 was 46.4% and 0%, respectively. Since biliary excretion was not detected up to 48 h after its administration, the rate of intestinal absorption of MST-16 was estimated to be about 50% (data not shown).

### *Schedule dependence of MST-16 given p.o.*

We examined how much the therapeutic efficacy of MST-16 depends on the treatment schedule in the s.c.-p.o. mod-

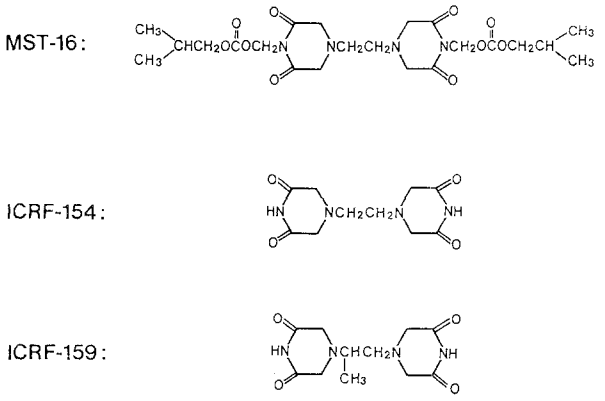


**Fig. 2.** Chemical structures of MST-16, ICRF-154, and ICRF-159

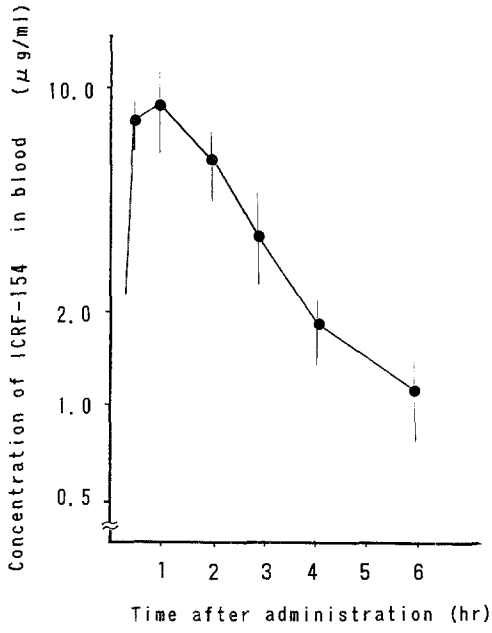


**Fig. 3.** Blood levels of MST-16 following administration p.o. of 200 mg/kg to male $CDF_1$ mice. Blood levels were measured by HPLC at various time points. MST-16 itself was not detected at all. Accordingly, the major metabolite, ICRF-154, was measured

el of L1210 leukemia. MST-16 was administered according to the following four different schedules, each of which involved the same total dosage: single dose on day 1 only; 6 doses at 4-h intervals on day 1 only; 1 dose daily on each of 5 consecutive days; and 1 dose daily on each of 9 consecutive days. As clearly demonstrated in Fig. 4, 9 daily administrations yielded the best results. Life-prolonging effects were clearly dose-dependent with this schedule, and the maximum effect (T/C) was 223% at a total dose of 1800 mg/kg. In contrast, with the single treatment on day 1 no significant survival effect was seen at any dose examined. However, even with the treatment on day 1 only, remarkable survival effects were observed when the drug was administered as divided doses: the survival effect was only 112% when 2500 mg/kg MST-16 was given as a single dose, but it increased to as much as 200% when the same total amount of drug was divided into doses given every 4 h on day 1. All these results obviously indicate that

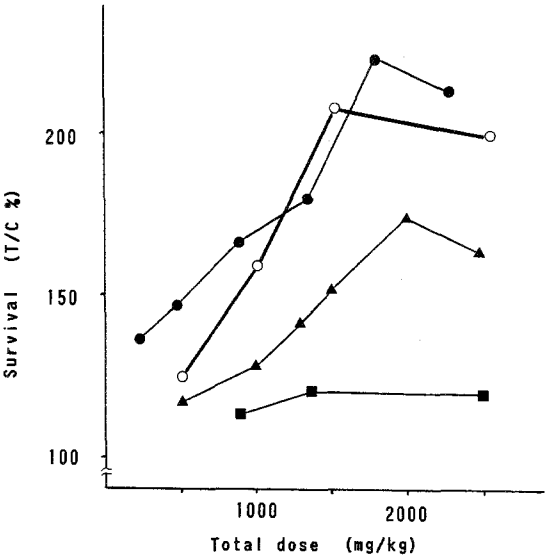


**Fig. 4.** Antitumor effects of MST-16 given with different treatment schedules in L1210 leukemia. MST-16 was administered p.o. by three different schedules to mice with s.c.-implanted L1210 leukemia: single dose on day 1 (■); daily dose for 5 consecutive days (▲); daily dose for 9 consecutive days (●); divided doses every 4 h on day 1 only (○)

the therapeutic efficacy of MST-16 given p.o. is heavily dependent on the treatment schedule.

*Antitumor effects on murine tumors*

With P388, L1210, C-26, and B16 tumors, survival effects of MST-16 were studied in the i.p.-p.o. model, in which tumor cells were implanted i.p. and drug was administered

**Table 1.** Antitumor activity of MST-16[a] against i.p.-inoculated P388, L1210 leukemias, and C-26 adenocarcinoma

| Tumor[b] | Dose (mg/kg) | Median survival (days) | T/C (%) |
|---|---|---|---|
| P388 | 250 | 12.1 | 118.6 |
| | 200 | 20.0 | 196.1 |
| | 175 | 18.3 | 179.4 |
| | 150 | 15.5 | 152.0 |
| | 125 | 14.4 | 141.2 |
| | 100 | 13.8 | 135.3 |
| | 0 | 10.2 | – |
| L1210 | 300 | 17.1 | 192.1 |
| | 250 | 19.1 | 214.6 |
| | 200 | 19.5 | 219.1 |
| | 150 | 15.0 | 168.5 |
| | 100 | 15.0 | 168.5 |
| | 50 | 12.1 | 136.0 |
| | 0 | 8.9 | – |
| C-26 | 200 | 17.5 | 100.0 |
| | 150 | 26.0 | 148.6 |
| | 100 | 24.0 | 137.1 |
| | 50 | 21.0 | 120.0 |
| | 25 | 21.1 | 120.5 |
| | 0 | 17.5 | – |

[a] MST-16 was given p.o. on days 1–9

[b] P388, L1210 and C-26 cells were inoculated i.p. into $CDF_1$ mice on day 0

**Table 2.** Antitumor activity of MST-16 against s.c.-implanted Lewis lung carcinoma

| Dose (mg/kg) | Tumor implanted s.c. | | Metastasis | |
|---|---|---|---|---|
| | Tumor wt (g) | Inhibition of tumor growth (%) | Number of colonies[a] | Inhibition (%) |
| 250 | 0.66±0.20 | Toxic | 0.14±0.10 | 99.6 |
| 200 | 1.79±0.23 | 82.4 | 0.57±0.27 | 98.3 |
| 150 | 3.79±0.31 | 62.8 | 1.14±0.29 | 96.6 |
| 100 | 3.48±0.19 | 65.8 | 0.28±0.28 | 99.2 |
| 50 | 5.58±0.56 | 45.2 | 6.16±0.62 | 81.6 |
| 25 | 5.74±0.43 | 43.7 | 7.43±0.66 | 77.8 |
| 0 | 10.19±0.29 | | 33.50±1.60 | |

LL cells were implanted s.c. into $BDF_1$ mice on day 0. MST-16 was given p.o. on days 1–8

[a] Number of metastasic colonies in the lung on day 21 (mean ± SE)

**Table 3.** Antitumor activity of MST-16 against s.c.-implanted murine tumors

| Tumor | Schedule | Dose (mg/kg) | Tumor wt (g) | Inhibition of tumor growth (%) |
|---|---|---|---|---|
| B16 | Days 1–8 | 250 | 1.312 | toxic |
| | | 200 | 1.286 | 86.6 |
| | | 150 | 1.915 | 80.1 |
| | | 100 | 3.303 | 65.7 |
| | | 50 | 5.436 | 43.5 |
| | | 25 | 8.572 | 10.9 |
| | | 0 | 9.622 | |
| C-38 | Days 1–8 | 250 | 0.251 | toxic |
| | | 200 | 0.263 | 81.9 |
| | | 150 | 0.391 | 72.5 |
| | | 100 | 0.516 | 63.7 |
| | | 50 | 0.807 | 43.4 |
| | | 25 | 1.061 | 25.5 |
| | | 0 | 1.425 | |
| M5076 | Days 1–5 | 300 | 1.766 | 49.7 |
| | | 200 | 2.552 | 27.3 |
| | | 0 | 3.511 | |

B16, M5076 and C-38 fragments were implanted s.c. into $BDF_1$ mice on day 0. Tumor sizes were measured on day 21

**Table 4.** Antitumor activity of MST-16 against s.c.-implanted L1210 leukemia

| Schedule | Dose (mg/kg) | Median survival(days) | T/C (%) |
|---|---|---|---|
| Days 1– 9 | 250 | 17.3 | 213.6 |
| | 150 | 15.1 | 186.4 |
| Days 5–13 | 250 | 17.5 | 216.0 |
| | 150 | 14.5 | 179.0 |
| | 0 | 8.1 | |

L1210 leukemia cells were implanted s.c. into CDF1 mice on day 0. MST-16 was given p.o. in each schedule

**Table 5.** Antitumor activity of MST-16 against human xenograft tumors implanted in nude mice

| Tumor xenograft | Dose (mg/kg) | Relative tumor weight | T/C (%) |
|---|---|---|---|
| Co4 | 200 | 2.50 | 30.1 |
| | 0 | 8.32 | |
| LX-1 | 200 | 5.21 | 45.7 |
| | 0 | 11.41 | |
| MX-1 | 200 | 6.91 | 35.9 |
| | 0 | 19.26 | |

Tumor fragments were inoculated s.c. into the flank of BALB/c-nu/nu athymic nude mice. When the tumor volume had reached 100–300 $mm^3$, MST-16 was given p.o. daily for 5 days (days 0–4). Evaluation was made on day 14

p.o. (Table 1). With nine daily treatments, MST-16 exhibited marked survival effects against both leukemias, the maxima being 196% for P388 and 219% for L1210 at 200 mg $kg^{-1}$ $day^{-1}$. On the other hand, MST-16 showed a maximum of 149% with C-26, and was ineffective against B16 (data not shown). Growth-inhibitory effects of MST-16 on solid tumors were also investigated in the s.c.-p.o. model of murine tumors. Drug treatments were given on days 1–8, and growth inhibition was determined on day 21. MST-16 exhibited dose-dependent effects, and greater than 80% growth inhibition against LL, B16, and C-38 was noted at a daily dose of 150 or 200 mg/kg (Tables 2, 3). However, its effect against M5076 was weak in this model. The activity of MST-16 against s.c.-inoculated L1210, already shown in Fig. 4, was also assessed as a survival effect, and T/C values of approximately 200% were observed (Table 4). In addition, the survival effect of the treatment started in the advanced stage, i.e. days 5–13, was found to be 216% at 250 mg $kg^{-1}$ $day^{-1}$, which is almost the same as that when the drug was given on days 1–9.

In the case of LL, the inhibitory effect of MST-16 on lung metastasis was studied. As shown in Table 2, formation of metastatic colonies in the lung was markedly inhibited over a relatively wide dose range.

### *Therapeutic effects on human tumor xenografts*

Growth-inhibitory effects of MST-16 on human tumors grown in nude mice were investigated. In this experiment, MST-16 was administered p.o. daily for 5 consecutive days after the tumors had reached a predetermined size. It was found that 200 mg/kg MST-16 significantly suppressed the growth of Co-4 colon adenocarcinoma, LX-1 lung carcinoma, and MX-1 mammary adenocarcinoma with T/C (%) of 30, 46, and 36, respectively (Table 5).

### *Kinetic analysis of cell-killing action*

To confirm the schedule-dependent therapeutic effect of MST-16, we attempted kinetic analysis of its cell-killing action by an in vitro colony assay [13, 14]. In this experiment, ICRF-154 was used instead of MST-16, because the former is the active form of MST-16 in vivo, as previously stated. V-79 cells were exposed to various concentrations

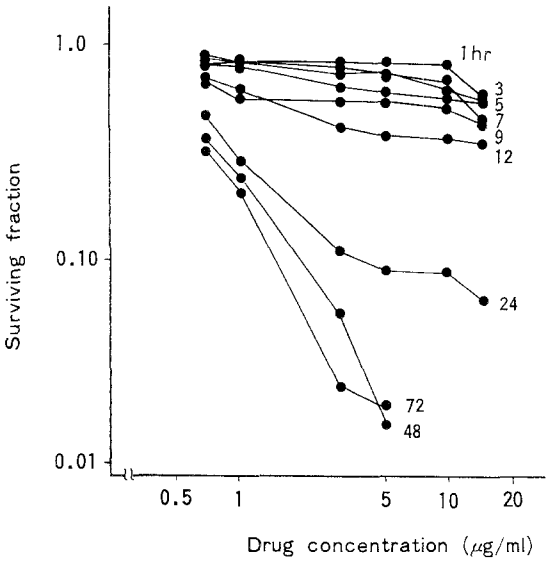


**Fig. 5.** Relationship between concentration of ICRF-154 and surviving fraction with different exposure times. V79 cells were exposed to various concentrations of ICRF-154 for each period of incubation, and fractions surviving were determined from the colony assay

of ICRF-154 for each period of incubation, and the surviving fraction was determined by the colony assay. Concentration-surviving fraction curves for various exposure times are shown in Fig. 5. With relatively short exposure times of 1–12 h, its cell-killing action was not correlated with drug concentration. However, when cells were exposed to the drug for 24 h or longer, the data clearly showed that its cell-killing activity became greater with increasing drug concentration. This kinetic mode of cell-killing by ICRF-154 is quite similar to that by cell cycle phase-specific agents such as vincristine and cytosine arabinoside [11, 12].

## Discussion

As previously reported, i.p.-administered MST-16 exhibited potent therapeutic activity against i.p.- or s.c.-implanted murine tumors regardless of the treatment schedule [10]. However, owing to its limited aqueous solubility, MST-16 would presumably be administered p.o. in clinical trials. Thus, we studied the bioavailability, optimal treatment schedule, and therapeutic efficacy of orally administered MST-16.

Pharmacokinetic study revealed that MST-16 is very rapidly changed to its parent compound, ICRF-154. However, when ICRF-154 itself was administered p.o. it was not found in the plasma. Therefore, this change seems to take place after MST-16 is absorbed from the intestine [1]. On the other hand, we found that MST-16 was also rapidly changed to ICRF-154 when it was incubated with serum or small intestinal homogenate [1]. These results suggest that MST-16 is metabolized to ICRF-154, probably by some esterase immediately after its intestinal absorption and that ICRF-154 is the active metabolite of MST-16 in vivo.

In contrast to i.p. administration, following oral administration the effect of MST-16 was strikingly dependent on the treatment schedule. A single administration at a relatively high dose showed neither antitumor effect nor host toxicity. However, when the dose was divided, even when it was all given on day 1, significant antitumor effects were observed. Daily administration for 5 or 9 consecutive days also provided good therapeutic results. Such schedule dependence was confirmed by in vitro kinetic analysis of the cell-killing effect of ICRF-154. With less than 12 h exposure, ICRF-154 showed little cell-killing effect on Chinese hamster V79 cells at as high a concentration as 10 μg/ml. However, when cells were exposed for longer than 24 h, concentration-dependent cell-killing effects were observed. Since the concentration-surviving fraction curves observed were quite similar to those for cytosine arabinoside and vincristine [11, 12], this strongly suggests that the cell-killing action of ICRF-154 is cell cycle phase-specific and time-dependent.

Such cell cycle phase-specific action of ICRF-154, the active metabolite of MST-16, would be expected to correlate closely with the mode of antitumor action of MST-16. From our study using synchronized cells, the ICRF-154-sensitive phase seems to be the $G_2 \cdot M$-phase, and not the S-phase. However, the treated cells did not display a metaphase arrest such as occurs with vinca alkaloids. These results will be presented elsewhere (Ishida R, Narita T, Miki T, Yui R, Utsumi K, Andoh T (1990) Inhibition by bis(2,6-dioxopiperazine) derivatives of topo-isomerase II-mediated DNA strand breaks in a human cell: different mode of cell growth inhibition from other topo-isomerase II Inhibitors (Cancer Res., in press). This observation is in agreement with the cell cycle phase-specific action of ICRF-159 reported on the basis of a flow-cytometric study [13].

Orally administered MST-16 demonstrated significant antitumor effects on both the i.p.- and the s.c.-implanted tumors. It was significantly active against the i.p. model of P388, L1210, and C-26 and against the s.c. model of LL, B16, and C-38. It also showed marked inhibitory effects on lung metastases of LL over a very wide dose range. In their studies on the mechanism of the antimetastatic effect of ICRF-159, Le Serve and Hellman [9] proposed that the compound inhibited metastasis by normalizing tumor blood vessels. This mechanism of MST-16 would be expected to be similar to that of ICRF-159. It should be particularly noted that MST-16 exhibited significant growth-inhibitory effects on human colon, lung, and breast tumors implanted in nude mice. However, on the whole, the therapeutic effects of orally administered MST-16 seem to be somewhat smaller than those obtained with i.p. injection in all tumor models, particularly in i.p.-implanted tumors. The prominent therapeutic effects seen in the i.p.-i.p. model seem to result from very long exposure of tumor cells to MST-16 or ICRF-154 in the peritoneal cavity owing to the gradual dissociation of MST-16 injected i.p. as a suspension. This again indicates that continuous exposure of tumor cells to the drug by frequent administration is essential for the antitumor effect of MST-16.

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