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# A metronomic schedule of cyclophosphamide combined with PEGylated liposomal doxorubicin has a highly antitumor effect in an experimental pulmonary metastatic mouse model

Emi Shiraga [a], Jose Mario Barichello [a,b], Tatsuhiro Ishida [a,*], Hiroshi Kiwada [a]

[a] *Department of Pharmacokinetics and Biopharmaceutics, Subdivision of Biopharmaceutical Science, Institute of Health Biosciences, The University of Tokushima, 1-78-1 Sho-machi, Tokushima 770-8505, Japan*
[b] *Japan Association for the Advancement of Medical Equipment, Tokyo 113-0033, Japan*

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**Abstract**

Metronomic chemotherapy is a novel approach to the control of advanced cancer, as it appears to preferentially inhibit endothelial cell activity in the growing vasculature of tumors. Doxorubicin-containing sterically stabilized liposomes (DXR-SL) accumulate in large amounts in tumor tissue, resulting in enhanced antitumor effects of the encapsulated DXR. In the present study, it was hypothesized that metronomic chemotherapy may further augment the accumulation of DXR-SL, improving its therapeutic efficacy. This study tests the antitumor efficacy for the combination of a metronomic cyclophosphamide (CPA)-dosing schedule with sequential intravenous injections of DXR-SL in the treatment of lung metastatic B16BL6 melanoma-bearing mice. Three dosing schedules for the combination of metronomic CPA injections (s.c. 170 mg/kg every 6 days) plus either a low or a high dose of DXR-SL (i.v. 1 or 5 mg/kg every 6 days) were set: Schedule I, DXR-SL was given 3 days before the first CPA treatment; Schedule II, DXR-SL and CPA were given simultaneously; and, Schedule III, DXR-SL was given 3 days after the first CPA treatment. Lung weight and median survival time (MST) were evaluated. As expected, both the dosing schedule as well as the dose of DXR-SL improved therapeutic efficacy. Schedule I with the low DXR dose and Schedule II with the low or high DXR dose significantly increased MST, compared with regular metronomic CPA therapy. Under the dosing schedules (Schedule I with the low DXR dose and Schedule II with the high DXR), there was a strong relationship between increased MST and decreased lung weight. However, Schedule I with high DXR dose resulted in significantly lower lung weights, but did not increase MST, suggesting that chemotherapy may result in increased toxicity in some conditions. Although treatment regimens require optimization, the results of the present study may prove useful in further explorations of combining metronomic chemotherapy with liposomal anticancer drugs in the treatment of solid tumors.


*Keywords:* Combination therapy; Metronomic chemotherapy; Liposomal anticancer drug; PEGylated liposome; Cyclophosphamide; Doxorubicin

## 1. Introduction

Traditionally, systemic anti-cancer therapy has been dominated by the use of cytotoxic chemotherapeutics, which often are administered as a single dose or in short courses of therapy using the maximum tolerated dose (MTD) (conventional chemotherapy). MTD chemotherapy requires prolonged breaks between successive cycles of therapy due to toxicity (Kerbel and Kamen, 2004). On the other hand, a novel chemotherapeutic regimen, metronomic chemotherapy, recently has been advocated (Kerbel and Kamen, 2004; Gille et al., 2005; Munoz et al., 2005; Laquente et al., 2007; Tonini et al., 2007). Metronomic chemotherapy refers to the frequent administration of

*Abbreviations:* CHOL, cholesterol; CPA, cyclophosphamide; DXR, doxorubicin; DXR-SL, DXR-containing PEGylated liposomes; EPR, enhanced permeability and retention; HEPC, hydrogenated egg phosphatidylcholine; MPS, mononuclear phagocyte system; $mPEG_{2000}$-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000]; MST, median survival time; MTD, maximum tolerated dose; PEG, polyethylene glycol; TβR-I, transforming growth factor type I receptor; TSP-1, thrombospondin-1; WBC, white blood cells.

* Corresponding author. Tel.: +81 88 633 7260; fax: +81 88 633 7260.
*E-mail address:* ishida@ph.tokushima-u.ac.jp (T. Ishida).

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chemotherapeutics at doses significantly below the MTD without prolonged drug-free breaks. The therapy shows lower toxicity, allowing prolonged treatment.

The target of metronomic chemotherapy is believed to be the genetically stable endothelial cells within the vascular bed of the tumor, rather than tumor cells with a high rate of mutations (Browder et al., 2000). Thus, this strategy is categorized as an anti-angiogenic chemotherapy (Browder et al., 2000; Kerbel and Kamen, 2004; Munoz et al., 2005; Laquente et al., 2007; Tonini et al., 2007). Various chemotherapeutics, such as cyclophosphamide (CPA), vinblastine, methotrexate, etoposide and tegafur, are used for metronomic chemotherapy (Klement et al., 2000, 2002; Bello et al., 2001; Shaked et al., 2005; Klink et al., 2006; Munoz et al., 2006); among these, CPA is most frequently used. CPA is traditionally used for chemotherapy as an alkylating agent, which kills the tumor cells directly. However, it is reported that at lower doses, CPA enhances expression of thrombospondin-1 (TSP-1) in stromal and tumor cells (Hamano et al., 2004). TSP-1, a component of the extracellular matrix, which is secreted and found in circulation, is a well-known endogenous inhibitor of angiogenesis (de Fraipont et al., 2001; Lawler, 2002). The molecule primarily binds to CD36 receptors, which are expressed by endothelial cells of tumors (Dawson et al., 1997). It is thought that this interaction blocks proliferation and induces apoptosis in tumor endothelial cells, thereby inducing collapse of angiogenic vessels (Dawson et al., 1997; Guo et al., 1997; Jimenez et al., 2000). Consequently, metronomic CPA-dosing reduces tumor neovascularization, thus suppressing tumor growths (Browder et al., 2000; Kerbel and Kamen, 2004).

Chemotherapy delivered in nanocarriers has been developed to improve the clinical treatment of solid tumors by achieving high accumulation of chemotherapeutic agents in tumor tissues but limited accumulation in healthy organs. Doxil, doxorubicin-containing sterically stabilized (PEGylated) liposomes, is one such drug that has already been used clinically (Grunaug et al., 1998; Safra et al., 2000; Krown et al., 2004). It is well-known that sterically stabilized (PEGylated) liposomes (SL) show prolonged circulating times as a result of reduced opsonization by serum proteins and lowered recognition by cells of the mononuclear phagocyte system (MPS) (Lasic et al., 1991; Torchilin et al., 1994). Consequently, SL accumulate in solid tumors via angiogenic blood vessels that have increased permeability (Jain, 1987; Papahadjopoulos et al., 1991; Wu et al., 1993; Yuan et al., 1995; Forssen et al., 1996; Vaage et al., 1997), due to the so-called "enhanced permeability and retention (EPR) effect" (Maeda et al., 2000). As described above, metronomic injection of low-dose CPA induces the collapse of tumor angiogenic vessels. This may enhance the EPR effect for SL, resulting in increased nanocarrier accumulation in tumor tissue. The use of metronomic CPA chemotherapy combined with DXR-SL may thus have clinical significance and practical importance in treating solid tumors.

Therefore, the objective of the present study was to determine whether the combination of metronomic cyclophosphamide (CPA)-administration and sequential intravenous injections of DXR-SL improves antitumor efficacy in lung metastatic B16BL6 melanoma-bearing mice. The results indicated that a combination approach may improve therapeutic efficacy under some dosage regimens, although this approach is accompanied by an increase in toxicity.

## 2. Materials and methods

### 2.1. Materials

Hydrogenated egg phosphatidylcholine (HEPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000] (mPEG$_{2000}$-DSPE) were generously donated by Nippon Oil and Fat (Tokyo, Japan). Doxorubicin (DXR) was generously donated by Daiichi Pharmaceutical. (Tokyo, Japan). Cholesterol (CHOL) and cyclophosphamide (CPA) were purchased from Wako Pure Chemical (Osaka, Japan). FITC-labeled rabbit anti-rat IgG heavy and light chain polyclonal antibody was purchased from Abcam (Cambridge, UK). Rat monoclonal anti-mouse CD45 antibody was purchased from R&D systems (CA, USA). All other reagents were of analytical grade.

### 2.2. Animal and tumor cell line

Male C57BL/6 mice, 5 weeks old, were purchased from Japan SLC (Shizuoka, Japan). The experimental animals were allowed free access to water and mouse chow, and were housed under controlled environmental conditions (constant temperature, humidity, and 12 h dark–light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

The pulmonary metastatic mouse melanoma cell line, B16BL6, was maintained in DMEM (Wako Pure Chemical) supplemented with 10% heat-inactivated FBS (Japan Bioserum, Hiroshima, Japan), 10 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin in a 5% $CO_2$ air incubator at 37 °C.

### 2.3. Preparation of liposomes

PEGylated liposomes (sterically stabilized liposomes, SL) were composed of HEPC/CHOL/mPEG$_{2000}$-DSPE (2/1/0.1 molar ratio). Liposomes were prepared using the thin-film hydration technique (Ishida et al., 2003). Briefly, the lipids were dissolved in chloroform and, after evaporation of the organic solvent, the resulting lipid film was hydrated with 250 mM ammonium sulfate solution (pH 5.5). The liposomes were sized by subsequent extrusion through polycarbonate membrane filters (Nuclepore, CA, USA) with pore sizes of 400, 200, and 100 nm. The mean diameter of the liposomes was approximately 100 nm, as determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The phospholipid concentration was determined by colorimetric assay (Bartlett, 1959). DXR was encapsulated into the liposomes by remote loading using an ammonium sulfate gradient, as previously described (Bolotin et al., 1994). Following extrusion, the external buffer was exchanged by eluting through a Sephadex G-50 column equilibrated with 10% sucrose. DXR was dissolved in a sucrose solution at a concentration of 10 mg/ml.

The DXR solution then was added to the liposome solution at a concentration of 0.2 mg DXR/1 mg phospholipid. The mixture was incubated in a 65 °C water bath for 1 h with slow agitation. After loading DXR into the liposomes, unencapsulated DXR was removed using a Sephadex G-50 column in HEPES buffered saline (25 mM HEPES, 140 mM NaCl, pH 7.4). DXR-loading efficiency was >90%.

### 2.4. In vivo assessment of tumor growth

B16BL6 cells were grown to 80–90% confluence in a 10 cm culture dish, harvested, and resuspended in cold PBS (51 mM $Na_2HPO_4$, 12 mM $NaH_2PO_4$, 77 mM NaCl, pH 7.4). The cells ($5 \times 10^4$) in 0.2 ml PBS were inoculated into the tail vein of C57BL/6 mice.

CPA and DXR treatments were started 14 days after inoculating the mice with B16BL6 cells, because our earlier study demonstrated that extensive progression of tumor nodules on the surface of the lungs was evident 14 days post-inoculation (Li et al., 2005). The antitumor effect of the treatments was evaluated by survival time and lung weight at time of death. Body weight also was evaluated as a surrogate marker of toxicity.

### 2.5. CPA and DXR-SL dosing schedules

The CPA and DXR-SL treatments were as follows:

(1) *Conventional dosing of CPA*. Mice ($n = 5$) received two cycles of CPA treatment separated by a 3-week interval. Each cycle consisted of a total of three doses of CPA (150 mg/kg per dose) administered subcutaneously every other day (total dose of 450 mg/kg, i.e., MTD, maximum tolerated dose) (Browder et al., 2000).
(2) *Metronomic dosing of CPA*. Mice received eight doses of CPA (170 mg/kg per dose) administered subcutaneously at 6-day intervals (Browder et al., 2000).
(3) *Conventional dosing of DXR-SL*. Mice received DXR-SL (1 or 5 mg/kg) intravenously at 6-day intervals until the mice died.
(4) *Combination dosing of CPA with DXR-SL*:
    (i) *Schedule I* (*low or high dose of DXR-SL*). CPA (170 mg/kg) was administered subcutaneously at 6-day intervals. Three days *before* CPA injection (11 day after tumor inoculation), high (5 mg/kg) and low (1 mg/kg) dose DXR-SL administered intravenously at 6-day intervals.
    (ii) *Schedule II* (*low or high dose of DXR-SL*). CPA (170 mg/kg) was administered subcutaneously and high (5 mg/kg) or low (1 mg/kg) dose DXR-SL intravenously at 6-day intervals. The treatment was started at day 14 after tumor inoculation.
    (iii) *Schedule III* (*low or high dose of DXR-SL*). CPA (170 mg/kg) was administered subcutaneously at 6-day intervals. Three days *after* the first CPA injection (17 day after tumor inoculation), high (5 mg/kg) or low (1 mg/kg) DXR-SL was administered intravenously and the injections were continued at 6-day intervals.

### 2.6. Toxicity assessment

To avoid the influence of tumorigenesis on toxicity assessment, normal C57BL/6 mice were used. Mice received six cycles of CPA or DXR-SL treatment administered at 6-day intervals. One cycle consisted of either subcutaneous administration of CPA (170 mg/kg) or intravenous injection of DXR-SL (1 or 5 mg/kg). After treatment was initiated, body weight was measured every 3 days. To determine change in the number of circulating white blood cells (WBC), blood was collected via retro-orbital puncture 7, 19 and 31 days after treatment was begun. Blood samples were washed twice with cold PBS and blood cells were collected by centrifugation for 5 min at 2000 rpm and 4 °C. Blood cells were blocked with 1% BSA/PBS for 15 min at room temperature and then were incubated with primary antibody (rat monoclonal anti-mouse CD45 antibody) for 30 min. After washing with cold PBS, samples were incubated for an additional 30 min with secondary antibody (FITC-labeled rabbit anti-rat IgG heavy- and light-chain polyclonal antibody). WBC number was determined using flow cytometry (Guava EasyCyte Mini System, GE Healthcare, CA, USA).

### 2.7. Statistics

All values are expressed as the mean ± S.D. Statistical analysis was performed with a two-tailed unpaired *t*-test using GraphPad InStat software (GraphPad Software, CA, USA). The level of significance was set at $p < 0.05$.

## 3. Results

### 3.1. Antitumor effect of a metronomic CPA-dosing schedule on pulmonary metastatic B16BL6 melanoma-bearing mice

The pulmonary metastatic B16BL6 melanoma bearing mouse model was employed in the present study because the antitumor effects of metronomic CPA chemotherapy have not yet been reported for a plumonary metastatic model. Conventional and metronomic CPA-dosing schedules significantly improved median survival time (MST) of tumor-bearing mice compared with controls (no-treatment). In addition, the metronomic schedule remarkably prolonged MST compared with the conventional schedule (control = 24.0 days, conventional schedule = 36.5 days and metronomic schedule = 50.0 days) (Fig. 1A). In experimental pulmonary metastasis models, lung weight reflects the growth of pulmonary metastases (Fig. 1B). Conventional and metronomic CPA-dosing schedules significantly inhibited the growth of cells that had metastasized to the lungs compared with control (no-treatment). Moreover, the metronomic schedule was significantly more efficient than the conventional schedule (Fig. 1B). Throughout these experiments, animals did not exhibit significant weight loss (data not shown). These results confirmed that the metronomic CPA-dosing schedule is more effective than the conventional dosing schedule with no severe side effects in lung metastatic B16BL6 melanoma-bearing mice. In contrast, when the dosing was started 3 days after B16BL6 cell-inoculation, lung weights

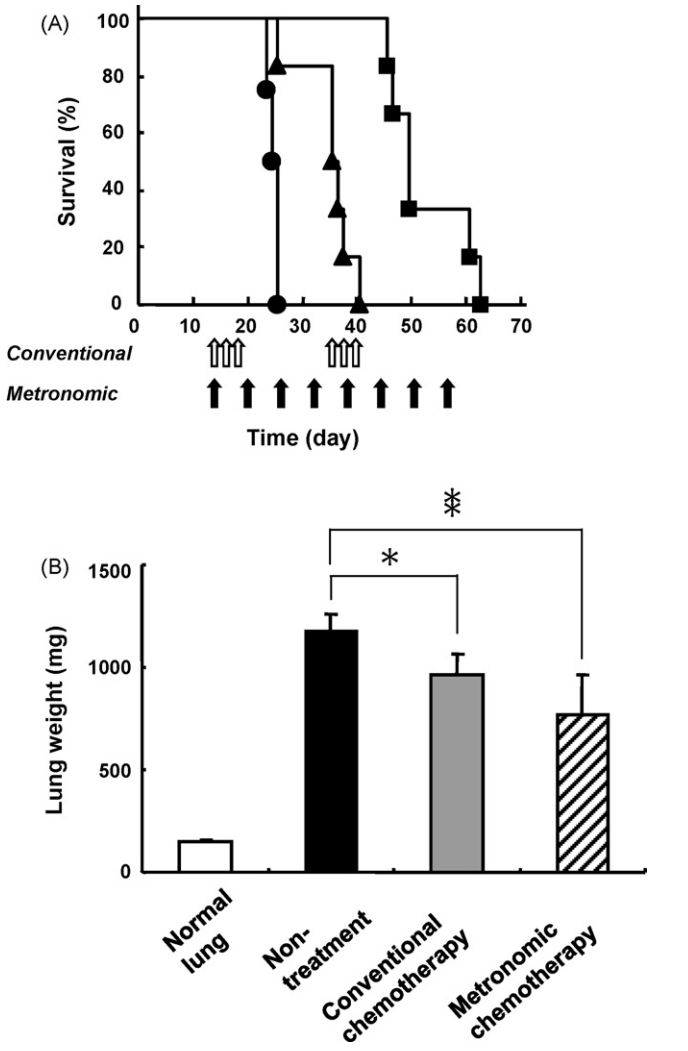


Fig. 1. Effect of either conventional (maximum tolerated dose) or metronomic CPA-dosing on (A) survival of B16BL6-bearing mice and (B) lung weight at time of death. Control (no treatment (●), conventional dosing (▲), 2 cycles consisting of 3 doses (white arrows) of 150 mg/kg administered every other day (total 450 mg/kg) with 3 weeks between cycles. Metronomic dosing (■), 8 doses of 170 mg/kg (black arrows) administered at 6-day intervals. $N = 5$ mice per group. $^{*}p < 0.05$; $^{**}p < 0.005$ vs. control.

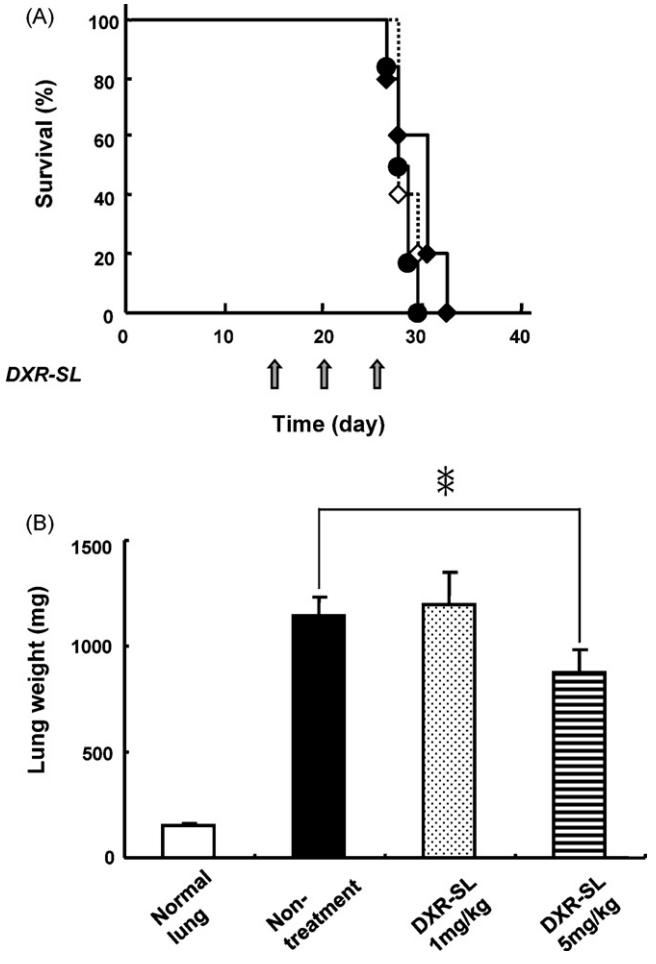


Fig. 2. Effect of DXR-SL (high or low dose) on (A) survival of B16BL6-bearing mice and (B) lung weight at time of death. Control (●). DXR-SL (◇ with dotted line), 3 doses of 1 mg/kg administered at 6-day intervals. DXR-SL (♦), 3 doses of 5 mg/kg administered at 6-day intervals. $N = 5$ mice per group. $^{**}p < 0.005$ vs. control.

did not increase, regardless of which CPA-dosing schedule was used. No tumor nodules were evident on the surface of lungs (data not shown). Thus, when the growth and formation of pulmonary metastases were minimal, because only 3 days had passed since inoculation with melanoma cells, both schedules had remarkable therapeutic efficiency in this animal model.

### *3.2. Antitumor effect of sequential DXR-SL administration on pulmonary metastatic B16BL6 melanoma bearing mice*

Two different doses of DXR-SL (low and high) were administered intravenously every 6 days, 3 cycles, into tumor bearing mice from 14 day after tumor cells-inoculation. No significant increases in the MST were observed for either DXR-SL dose when administered according to a conventional dosing schedule (Fig. 2A): MSTs of control (no-treatment), low and high DXR-SL doses were 28.4, 29.0 and 32.0 days, respectively. However, lung weights of tumor-bearing mice indicated that the higher DXR-SL dose slightly inhibited the growth of metastasized cells (Fig. 2B). These findings demonstrate that sequential administration of DXR-SL had a very mild antitumor effect, although the effect did not appear to be related to extension of survival time in tumor-bearing mice. Throughout this experiment, no significant weight loss was observed (data not shown).

### *3.3. Antitumor effect of the combination of a metronomic CPA-dosing schedule and sequential administration of DXR-SL*

To examine the effect of the combination therapy, CPA was metronomically administered either alone or plus DXR-SL (low or high dose). Sequential administration of DXR-SL (every 6 days) was started either 3 days before (Schedule I), on the same day as (Schedule II), or 3 days after (Schedule III), the first CPA injection because the metronomic CPA-dosing schedule may have affected the accumulation of SL in tumor tissue by increasing the permeability of angiogenic blood vessels. Schedules I, II

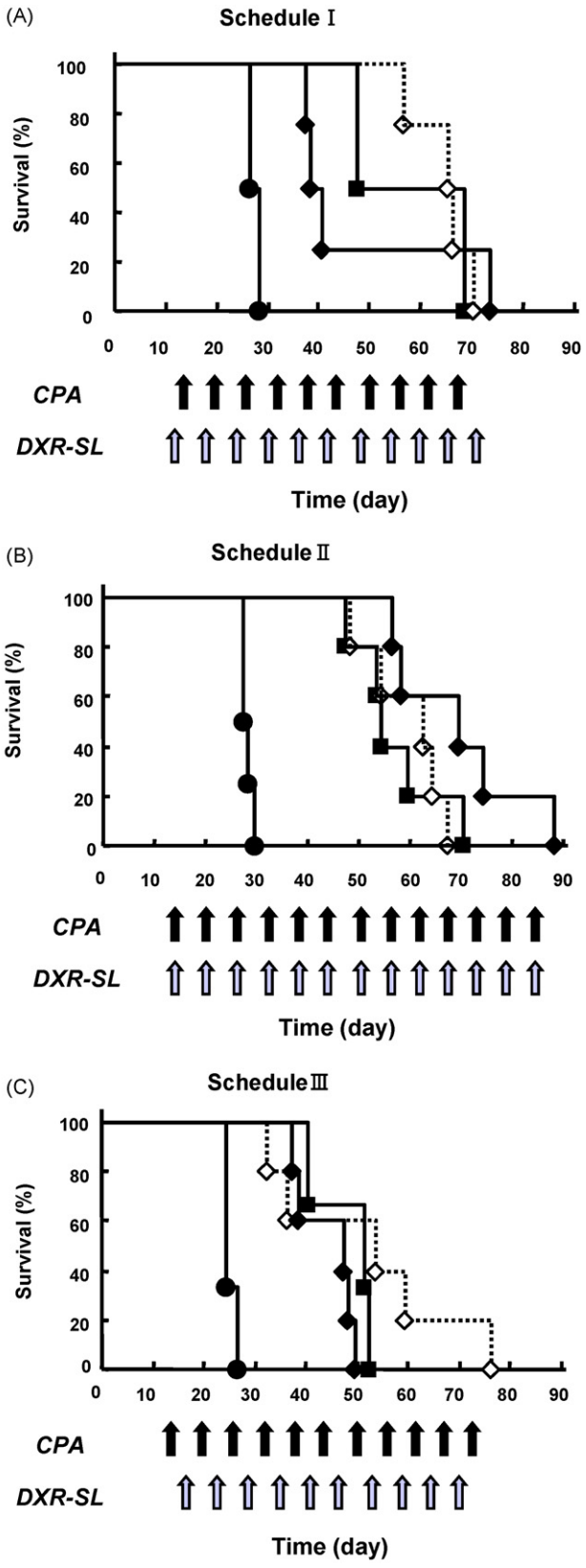


Fig. 3. Effect of the combination of metronomic CPA- and sequential DXR-SL-dosing on survival of B16BL6-bearing mice. (A) Schedule I (low or high dose of DXR-SL): CPA (170 mg/kg) was administered subcutaneously at 6-day intervals. Three days *before* CPA injection (11 days after tumor inoculation), high (5 mg/kg) or low (1 mg/kg) DXR-SL was administered intravenously at 6-day intervals. (B) Schedule II (low or high dose of DXR-SL): CPA (170 mg/kg) was administered subcutaneously and high (5 mg/kg) or low (1 mg/kg) DXR-SL intravenously at 6-day intervals (14 days after tumor inoculation). (C) Schedule III (low or high dose of DXR-SL): CPA (170 mg/kg) was administered subcutaneously at 6-day intervals. Three days *after* the first CPA injection (17 day after tumor inoculation), high (5 mg/kg) or low (1 mg/kg) dose DXR-SL was administered intravenously and the injections were continued at 6-day intervals. Control (●). Metronomic schedule (■), 170 mg/kg administered at 6-day intervals. Combination of metronomic CPA-dosing (170 mg/kg, thin black arrows) and low dose DXR-SL (1 mg/kg, thin gray arrows) (◊ with dotted line). Metronomic CPA-dosing schedule (170 mg/kg, thin black arrows) and high dose DXR-SL (5 mg/kg, thin gray arrows) (♦).

Table 1
Median survival time for each treatment schedule group

| | Median survival time (day) | | | |
|---|---|---|---|---|
| | None | Schedule I | Schedule II | Schedule III |
| Control | | 28.0 | | |
| Metronomic dosing | | 52.0 | | |
| CPA (170 mg/kg) + DXR-SL (high, 5 mg/kg) | – | 40.5 | 69.00 | 51.0 |
| CPA (170 mg/kg) + DXR-SL (low, 1 mg/kg) | – | 66.5 | 63.0 | 54.0 |

The results were calculated on the basis of the data presented in Fig. 3.

and III (described in detail in Section 2) were tested in pulmonary metastatic B16BL6 melanoma bearing mice. All MSTs are summarized in Table 1. The combination of a metronomic schedule of CPA with DXR-SL prolonged MST compared to control (non-treatment) (Fig. 3). For Schedule I, only the combination of CPA with the *high* dose of DXR-SL prolonged MST compared with the metronomic schedule of CPA (Fig. 3A, Table 1). In contrast, for Schedule II, the combination of CPA with either the *low* or *high* dose of DXR-SL prolonged MST compared with the metronomic CPA-dosing schedule (Fig. 3B, Table 1). Interestingly, for Schedule III, the combination of CPA with DXR-SL did not prolong, but rather shortened, MST compared with the metronomic CPA-dosing schedule (Fig. 3C, Table 1). The results shown in Fig. 4 indicate that the combined therapy significantly inhibited the growth cells that had metastasized in the lung compared to control (no-treatment). The Schedule I combined therapy significantly inhibited growth of metastasized cells, compared with the metronomic CPA-dosing schedule, regardless of the dose of DXR-SL (Fig. 4A). The Schedule II combined therapy with the high dose of DXR-SL significantly inhibited growth of metastasized cells, while the low dose of DXR-SL did not (Fig. 4B). The Schedule III combined therapy did not induce any significant inhibition of metastatic cell growth (Fig. 4C).

### *3.4. Toxicity of the combination of the metronomic CPA-dosing schedule and sequential administration of DXR-SL*

Because the presence of a tumor influences bone marrow function and because treatment of tumor-bearing mice with chemotherapeutic agents makes data interpretation extremely

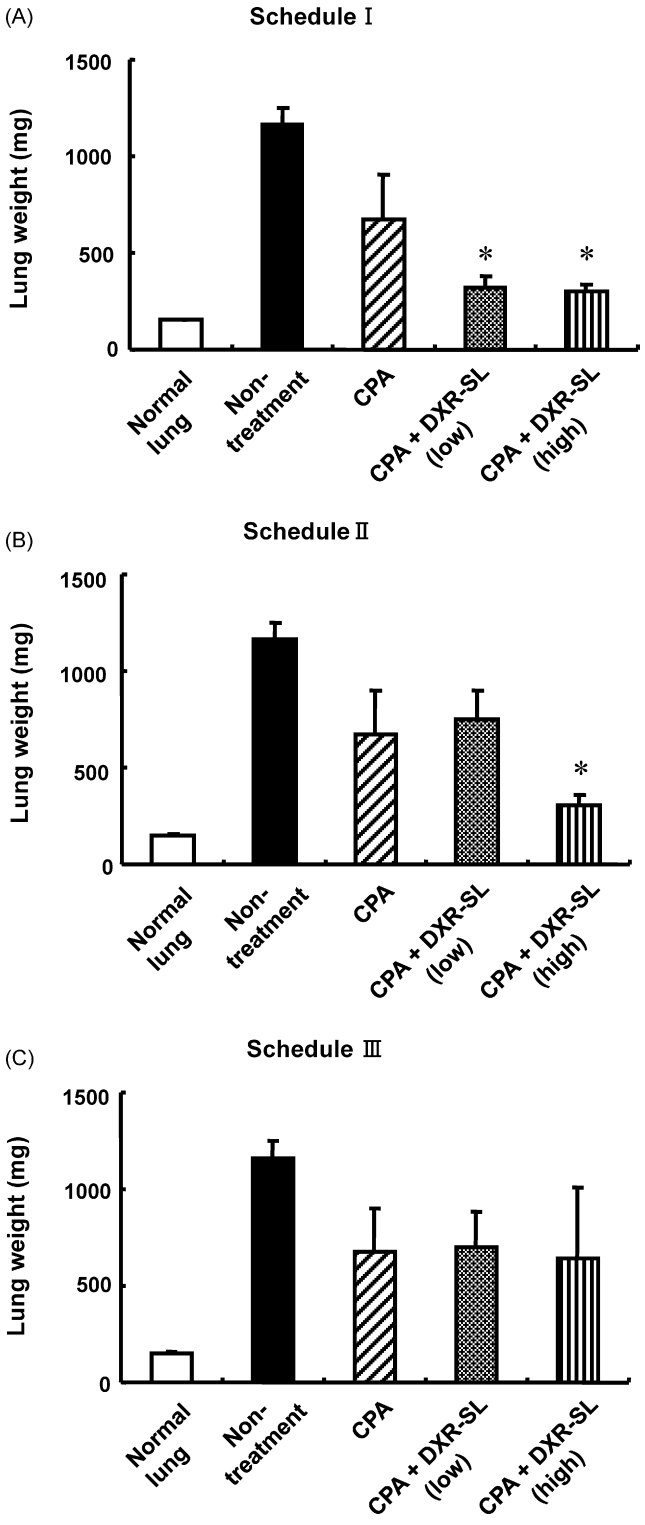


Fig. 4. Effect of the combination of metronomic CPA- and sequential DXR-SL-dosing on lung weight in B16BL6-bearing mice. The dosing schedules used were the same as those described in the legend of Fig. 3: (A) Schedule I (low or high dose of DXR-SL); (B) Schedule II (low or high dose of DXR-SL); (C) Schedule III (low or high dose of DXR-SL). *$p < 0.001$ vs. metronomic CPA-dosing schedule.

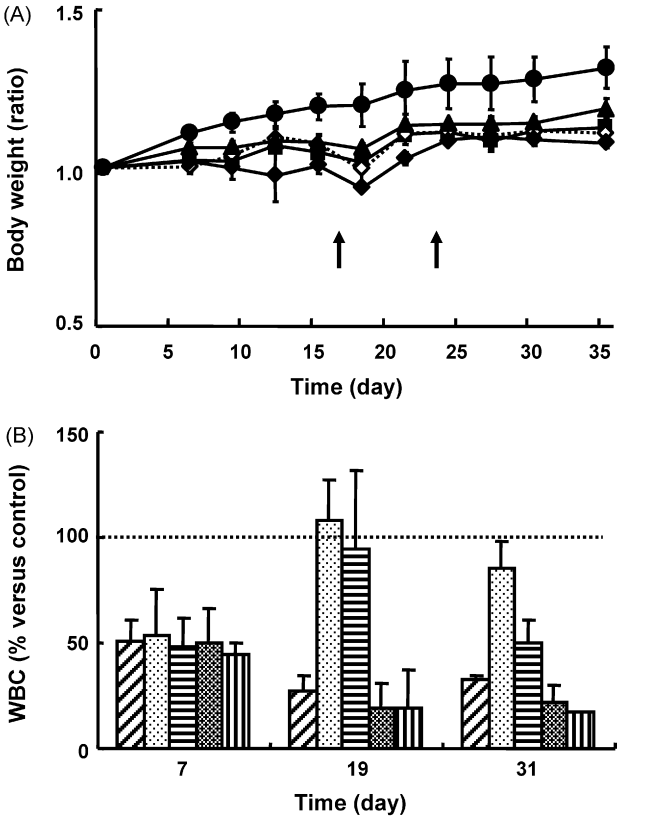


Fig. 5. Toxicity studies for the combination metronomic CPA-dosing plus intravenous administration of DXR-SL. (A) Body weight change. The body weight change expressed as ratio of post-treatment to initial body weight. Control (●), no treatment. CPA (■), 170 mg/kg administered at 6-day intervals. Combination of CPA (170 mg/kg) and low dose DXR-SL (1 mg/kg) administered at 6-day intervals (◊ with dotted line). Combination of CPA (170 mg/kg) and high dose DXR-SL (5 mg/kg) administered at 6-day intervals (♦). DXR-SL (▲), 5 mg/kg administered at 6-day intervals. Arrows indicate time of death for mice treated with combination of CPA (170 mg/kg) and DXR-SL (5 mg/kg). (B) Change in white blood cell number relative to control (no treatment). CPA (▨), 170 mg/kg administered at 6-day intervals. Low dose DXR-SL (▩), 1 mg/kg administered at 6-day intevals. High dose DXR-SL (▤), 5 mg/kg administered at 6-day intervals. Combination of CPA (170 mg/kg) and low dose DXR-SL (1 mg/kg) (▩) administered at 6-day intervals. Combination of CPA (170 mg/kg) and high dose DXR-SL (5 mg/kg) (▥) administered at 6-day intervals.

difficult, the toxicity of the combination therapy of CPA and DXR-SL was examined in not-tumor-bearing mice. In addition, six cycles of the Schedule II combination treatment were employed in the toxicity assessment. At day 35 after initiation of the dosing schedule, the toxicity of the combination therapy was estimated by measuring changes in body weight and WBC number, surrogate markers for treatment-related toxicity.

All combination regimens led to transient weight loss related to growth suppression in treated versus untreated mice (Fig. 5A). Two of the five mice treated with CPA (170 mg/kg) plus the high DXR-SL dose (5 mg/kg) died—one on day 17 and the other on day 24 (Fig. 5A, arrow). This demonstrates that the higher dose of DXR (5 mg/kg) in SL is toxic when combined with CPA. However, tissue weights of treated mice (heart, lung, liver, kidney and spleen) that survived were not different than those of control mice (data not shown).

The metronomic CPA-dosing schedule reduced the number of peripheral WBC (Fig. 5B). DXR-SL also dramatically reduced WBC number, regardless of DXR dose, followed by a rebound in WBC number. The combination regimen (CPA and DXR-SL) reduced the number of WBC, but there was no subsequent rebound in WBC number. The reduction in WBC number with the combination therapy was similar to that observed for the metronomic CPA-dosing schedule. This finding indicates that adding DXR-SL to the metronomic CPA-dosing schedule does not increase the bone marrow toxicity of CPA.

## 4. Discussion

Metronomic chemotherapy – the frequent administration of an anticancer agent at relatively low, minimally toxic doses with no prolonged drug-free breaks – is a novel approach to the control of advanced cancer. In this study, it was confirmed that the metronomic CPA-dosing schedule exhibited superior therapeutic efficacy in B16BL6-bearing mice, compared with the conventional dosing schedule using the CPA maximum tolerated dose (Fig. 1). Furthermore, the combination of the metronomic CPA-dosing schedule with sequential injections of DXR-SL increased therapeutic efficacy compared to administration of either treatment alone, although the effect was dependent on the schedule and dose of DXR-SL (Figs. 1–3).

Metronomic chemotherapy using CPA is believed to induce apoptosis in the endothelial cells of the growing tumor vasculature (Browder et al., 2000; Kerbel and Kamen, 2004). Thus, it was hypothesized that the metronomic CPA-dosing schedule would increase the permeability of tumor microvessels to macromolecules, including SL, resulting in enhanced accumulation of SL in tumors. Consequently, the therapeutic efficacy of anticancer agents would be enhanced. As expected, the results of the present study showed that some of the combined CPA and DXR-SL dosing schedule had improved therapeutic efficacy (Fig. 3). It should be noted that injection of DXR-SL alone (every 6 days, three times) showed no improvement in MST, regardless of the dose of DXR. Only the high dose of DXR-SL slightly suppressed the growth of cells that had metastasized to the lung (Fig. 2). This result strongly supports the hypothesis that metronomic CPA therapy changes the tumor neovasculature, resulting in enhanced extravasation and subsequent accumulation of SL in tumors. However, the ability of metronomic chemotherapy to enhance the EPR effect remains to be determined.

Interestingly, the improvement was dependent on the dosing schedule, as well as on the DXR-SL dose. Schedule I (low dose of DXR-SL) and Schedule II (high dose of DXR-SL) significantly increased the MST of tumor-bearing mice and inhibited the growth of cells that had metastasized to the lung, compared with the metronomic CPA-dosing schedule (Figs. 3 and 4). In contrast, Schedule III did not increase MST. It is thought that SL, which has a long half-life in circulation, accumulates in solid tumors due to the EPR effect (Jain, 1987; Papahadjopoulos et al., 1991; Wu et al., 1993; Yuan et al., 1995; Forssen et al., 1996; Vaage et al., 1997; Maeda et al., 2000), if the tumor vessels are still permeable. Preclinical and clinical evidence shows that anti-angiogenic therapies normalize the endothelial cells of tumor vessels and consequently decrease vessel permeability (Huber et al., 2005; Jain et al., 2007). These reports describing the biodistribution of SL led to the hypothesis that the metronomic CPA-dosing schedule (an anti-angiogenic therapy) transiently increases tumor vessel permeability and then decreases it as vessels are "normalized". Therefore. It is possible that for Schedule III, according to which DXR-SL is administered 3 days after the first CPA injection, SL accumulation was inhibited, thereby preventing a therapeutic effect (Figs. 3 and 4).

Schedule I (high dose of DXR-SL) resulted in significantly reduced lung weights without any improvement in MST. This apparent inconsistency may be due to enhanced toxicity of the combination of metronomic CPA therapy and intravenous liposomal DXR treatment. Increased toxicity of the combination treatment was supported by the death of two out of five normal mice during six cycles of the combination of CPA and DXR-SL administered at 6-day intervals (Fig. 5A). Furthermore, the combined therapies suppressed weight gain in normal mice. However, it appears that the increased toxicity in this study was not caused by enhancement of the myelosuppressive effects of CPA (Gale, 1985) by the DXR-SL injections (Fig. 5B). Although SL has a long half-life in circulation because of reduced opsonization by serum proteins and lowered recognition by cells of the MPS (Lasic et al., 1991; Torchilin et al., 1994), a fraction of administered SL is ultimately cleared by hepatic macrophages. Daemen et al. (1995, 1997) reported that injection of DXR-SL has a toxic effect on hepatic macrophages, reducing both specific phagocytic activity and cell numbers. Hence, it may be presumed that the toxic effects of CPA and DXR-SL can be either additive or synergistic, resulting in death in some experimental animals.

Other researchers have already demonstrated that the combination of several drugs administered by metronomic dosing with specific antiangiogenic reagents significantly increased the antitumor effects of metronomic dosing (Browder et al., 2000; Klement et al., 2000, 2002; Takahashi et al., 2001; Bello et al., 2001). Recently, Kano et al. (2007) reported that a low dose of transforming growth factor type I receptor (TβR-I) inhibitor promoted accumulation of macromolecules, including nanocarriers (polymeric micelles incorporating DXR), in tumors. TβR-I inhibitor specifically increases the permeability of the tumor neovasculature by decreasing coverage of the endothelium without decreasing endothelial area. This observation clearly suggests that the low dose of TβR-I inhibitor enhances the EPR effect in solid tumors, thereby increasing the therapeutic efficacy of DXR-containing nanocarrier. To the best of our knowledge, this is the first study to demonstrate that the combination of metronomic chemotherapy and liposomal anticancer drug (DXR) treatment increases antitumor effect, presumably by enhancing accumulation of liposomal anti-cancer drugs within the tumor. Thus, the combination of metronomic chemotherapy and liposomal anticancer agents such as Doxil is an innovative strategy in cancer chemotherapy. However, additional studies are needed to determine optimal dosages and treatment schedules to enhance therapeutic efficacy without any adverse effects.

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