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International Journal of Pharmaceutics 251 (2003) 13–21
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

# Pharmacokinetics and antitumor effects of vincristine carried by microemulsions composed of PEG-lipid, oleic acid, vitamin E and cholesterol

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Received 29 May 2002; received in revised form 15 September 2002; accepted 4 October 2002

**Abstract**

In this study, injectable microemulsions of vincristine (M-VCR) were prepared and its pharmacokinetics, acute toxicity and antitumor effects were evaluated. In M-VCR, the surfactants were PEG-lipid and cholesterol, the oil phase was a vitamin E solution of oleic acid and VCR. The particle size distribution and zeta potential of M-VCR were measured by the laser light dynamic scattering method. The VCR-loading efficiency was measured by Sephadex G50 column chromatography. The stability of M-VCR was monitored by particle size, VCR-loading efficiency and VCR content changes of M-VCR stored at 7 °C. The pharmacokinetics, acute toxicity and antitumor effects of M-VCR were studied in C57BL/6 mice bearing mouse murine histocytoma M5076 tumors. When stored at 7 °C in the dark for 1 year, the average diameter and VCR-loading efficiency of M-VCR changed from $138.1 \pm 1.2$ nm and $94.6 \pm 4.7\%$ to $127.1 \pm 2.4$ nm and $91.3 \pm 4.8\%$ ($n = 3$), respectively, while $7.4 \pm 0.3\%$ VCR decomposition was observed ($n = 3$). The plasma AUC of M-VCR was significantly greater than that of free VCR (F-VCR). The heart, spleen and liver $AUC_{0.08-12\ h}$ of M-VCR were significantly smaller than those of F-VCR while the kidney $AUC_{0.08-12\ h}$ of M-VCR was significantly greater than that of F-VCR. The tumor $AUC_{0.08-12\ h}$ of M-VCR was significantly greater than that of F-VCR. M-VCR had lower acute toxicity and greater potential antitumor effects than F-VCR in M5076 tumor-bearing C57BL/6 mice. M-VCR is a useful tumor-targeting microemulsion drug delivery system.


*Keywords:* Micromulsions; Vincristine; Pharmacokinetics; Stability; Acute toxicity; Antitumor effects

## 1. Introduction

Vincristine (VCR) is a vinca alkaloid antitumor agent isolated from Vinicarosea Linn (Kaplan et al., 1986). The drug exerts its antitumor activity by binding to the tubulins of cancer cells (Rowinsky and Donhower, 1996). The major problems asso-

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0378-5173/02/$ - see front matter 
PII: S0378-5173(02)00580-X

ciated with VCR cancer chemotherapy are its toxic actions, among which loss of reflexes, paraesthesia and muscle weakness are the most serious (Rowinsky and Donhower, 1996). To reduce the toxic responses and increase the antitumor effects of VCR, lipiodol (Miya et al., 1997), microspheres (Marinina et al., 2000), conventional liposomes (Sarris et al., 2000) and stealth liposomes (George et al., 1996) have been used as carriers of VCR. After i.v. injection, the majority of the conventional liposomes of VCR are taken up by the mononuclear phagocytic system (MPS), which means they are MPS-targeting drug delivery system (DDS) instead of tumor-targeting DDS. Microsphere and lipiodol of VCR are administered by local injection to target the tumor tissue or hepatic artery catheter perfusion for treatment of liver cancer (Marinina et al., 2000; Miya et al., 1997). These methods have been confirmed to be effective but are not applicable for the systemic treatment of cancer. To overcome these problems, it is necessary to develop an injectable tumor-targeting DDS as a carrier of VCR.

Over the past 10 years, significant advances have been made in injectable tumor-targeting DDS, of which sterically stabilized liposomes (stealth liposomes) and long-circulating emulsions are the most potentially useful. Stealth liposomes containing a polyethyleneglycol-derivatized phospholipid (PEG-lipid) have been reported to circulate in the bloodstream of mice for several days. Finally, the stealth liposomes accumulate in the tumor tissue because of the higher permeability of the blood vessels of the tumor tissue.

However, the procedure for preparation of stealth liposomes is very complicated (Moreira et al., 2002; George et al., 1996). In addition, VCR is a lipophilic agent and is not stable in water (Marinina et al., 2000; Beijnen et al., 1986). Therefore, long circulating microemulsions may be more suitable for delivery of VCR. Microemulsions are thermodynamically stable and are formed spontaneously by simple mixing of the various components (Brime et al., 2002). In recent years, considerable emphasis has been given to the development of injectable microemulsions (o/w) used as drug carriers (Kim et al., 1997; Von et al., 1998; Park and Kim, 1999; Brime et al., 2002; Wang et al., 2002). Microemulsions are excellent candidates as potential drug delivery systems because of their improved drug solubilization, long shelf life, and ease of preparation. In this study, we made use of stealth liposome technology and designed a novel kind of injectable microemulsion as a carrier of VCR (M-VCR) prepared by a much easier procedure. The oil phase was composed of oleic acid and vitamin E. VCR was carried by the oil phase. The surfactant and cosurfactant were PEG-lipid and cholesterol, respectively.

The aim of this study was to characterize and evaluate its acute toxicity, antitumor effects, and pharmacokinetics.

## 2. Materials

### 2.1. Chemicals

VCR sulfate was kindly supplied by Eli Lilly Japan K. K. (Japan). PEG-lipid (polyethyleneglycol derivative of distearoylphosphatidyl ethanolamine, PEG-DSPE, mean molecular weight of PEG: 2000) was kindly supplied by NOF Co. Ltd. (Tokyo, Japan). (±)-α-Tocopherol (vitamin E) was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Cholesterol and oleic acid were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Chemicals for HPLC were of HPLC grade and all other chemicals were of analytical grade.

### 2.2. Animal and tumor cells

The animals used were male C57BL/6 mice, weighing 18–20 g, supplied by Tokyo Animal Experiment Center (Tokyo, Japan). All mice used in the study were raised in the SPF animal laboratory. Mouse murine histiocytoma M5076 tumor cells were supplied by the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo, Japan). The tumor cells were used in this study after four transplant generations.

## 3. Methods

### 3.1. Preparation and characterization of M-VCR

The preparation of M-VCR was performed on a clean bench and all materials were pyrogen-free and sterilized. VCR, oleic acid, vitamin E, cholesterol and PEG-DSPE (1/5/5/5/5, w/w) were dissolved in an appropriate volume of ethanol. The ethanol was removed using an evaporator under $N_2$ gas and finally a semi-solid solution was formed. An appropriate volume of 0.9% NaCl aqueous solution containing VCR was added into the semi-solid solution and microemulsions formed instantly. The pH value of the microemulsion suspension was adjusted to 7.4 using 0.1N NaOH. The microemulsion suspension was sterilized by filtration three times through a sterile syringe-driven filter unit with a pore size of 100 nm (Millipore Co., Bedford, MA, USA). Finally, the microemulsions were injected into ampoules (10 ml per ampoule), which were sealed after oxygen was driven out with sterile $N_2$ gas. Each ampoule contained 1 mg of VCR sulfate, 5 mg of vitamin E, 5 mg of PEG-DSPE, 5 mg of cholesterol and 5 mg oleic acid in 10 ml of saline. To study the function of pH adjustment, oleic acid and vitamin E, three other kinds of microemulsions were prepared using the preparation method above with following changes; (1) without pH adjustment, (2) without oleic acid and pH adjustment and (3) without vitamin E, respectively.

The particle size of microemulsions was determined using a laser light scattering instrument (ELS800, Otsuka Electronics, Japan) by the dynamic light scattering method. The VCR-loading efficiency of microemulsions was taken as the percentage of VCR carried by the emulsions and was determined by Sephadex G50 column chromatography. Microemulsions were separated from free VCR (F-VCR) in the Sephadex G50 column using a mobile phase of 0.9% NaCl aqueous solution. The amounts of VCR in the free fraction and the microemulsion fraction were determined by HPLC as described in Section 3.3. The VCR-loading efficiency was calculated according to the following equation:

$$\text{VCR-loading efficiency } (\%) = \left(\frac{1 - A_f}{A_t}\right) \times 100$$

where $A_f$ is the amount of F-VCR and $A_t$ is the total amount of VCR.

The microemulsions without VCR were used as vehicle controls in the subsequent experiments to determine antitumor effects. F-VCR was VCR sulfate saline solution (0.1 mg/ml).

### 3.2. Stability of M-VCR

After preparation, three lots of M-VCR were stored at 7, 40 and 60 °C in the dark for 12 months, 32 days and 32 days, respectively. The stability was monitored by the changes in particle size, VCR-loading efficiency and VCR content of M-VCR during the storage period.

### 3.3. Pharmacokinetics

C57BL/6 mice (female, 18–20 g) were selected to evaluate the pharmacokinetics of M-VCR and were inoculated subcutaneously with M5076 tumor cells. Two weeks later, the mice were fasted for 12 h and given only water. Three mice were used at each point. Both M-VCR and F-VCR were administered by i.v. injection at a single dose of 2 mg VCR per kg. The samples (blood, tumor, heart, liver, spleen, lung and kidney) were obtained at 1/12, 1/6, 1/3, 2/3, 1, 2, 4, 6 and 12 h following i.v. injection. The blood was immediately centrifuged at 3000 rpm for 10 min. The plasma was separated and kept at −20 °C with the other samples until analysis. VCR was extracted from the biological samples and determined as described previously (Van et al., 1992) with modification. The HPLC system was composed of an LC-10AS pump (Shimadzu Co. Ltd., Japan), a SIL-10A autoinjector (Shimadzu Co., Ltd., Japan), a SPD-10A UV detector (Shimadzu Co., Ltd., Japan) and a $C_{18}$ 3.9 × 150 mm reverse phase column (Waters Nova-Pak). The mobile phase was 0.01M $NaH_2PO_4$: $CH_3OH$ = 40:60 (V/V, pH 7.0, adjusted with 0.2 N NaOH) and the flow rate was 1.0 ml/min. The concentration of VCR in each sample was determined with a constructed calibration curve. For UV detection, the wavelength was set

to 298 nm. Data were analyzed using the nonlinear least squares fitting program, in which Akaike's information criterion (AIC) was used to determine an appropriate model to fit the plasma VCR concentration data (Yamaoka et al., 1981). The pharmacokinetic parameters were calculated according to the plasma drug concentration-time curves of M-VCR and F-VCR. The areas under the biodistribution curves (from 0.08 to 12 h; $AUC_{0.08-12\ h}$) of M-VCR and F-VCR were calculated using the trapezoid method. The results were analyzed statistically using the Welch $t$-test and expressed as a one-way $P$ value. When comparisons between groups yielded a value for $P < 0.05$, the difference between those groups was considered significant.

### 3.4. Acute toxicity and antitumor effects

To evaluate the acute toxicity of M-VCR, the mice were inoculated subcutaneously with $1 \times 10^6$ M5076 tumor cells per mouse and divided into two groups of 12 animals per group. The tumor cells were removed from normal donor mice under anesthesia with methoxyflurane inhalation. On day 5 after inoculation, the two groups of mice were administered M-VCR and F-VCR i.v. at a dose of 6.0 mg VCR per kg animal. The acute toxicities of M-VCR and F-VCR were reflected in the survival rates of the mice in each group. In the solid tumor model, each mouse was inoculated subcutaneously with $1 \times 10^6$ M5076 tumor cells and divided into four groups of 12 animals per group (day 0). Treatment started on day 5 after inoculation. Animals were treated with the emulsions without VCR (vehicle control), F-VCR or M-VCR by i.v. injection four times with an interval of 5 days at 1.25 mg/kg after inoculation of tumor cells. Mice in the blank control group (Blank) were not treated. The mice in the vehicle control group (Vehicle) were treated with the microemulsions without VCR. The mice were killed on day 21 and the antitumor effect was reflected in the tumor growth suppression rate ($T/C$). $T/C$ was calculated by the following equation: $T/C = (W_c - W_t)/Wc \times 100\%$, where $W_c$ and $W_t$ are the mean tumor weight (gram) of the blank control and the treated mice, respectively. In the ascitic tumor model, each mouse was inoculated intraperitoneally with $1 \times 10^6$ M5076 tumor cells and divided into three groups of 12 animals per group (day 0). Animals were treated with F-VCR or M-VCR i.v. injection three times at 1.25 mg VCR per kg with an interval of 5 days starting on day 5. The results were analyzed statistically using the Welch $t$-test and expressed as a one-way $P$ value. When comparisons between groups yielded a value for $P < 0.05$, the difference between those groups was considered significant.

## 4. Results

### 4.1. Preparation and characterization of M-VCR

The particle size distribution of M-VCR was 90–150 nm and the average diameters was $138.1 \pm 1.2$ nm ($n = 3$). The VCR-loading efficiency of three batches of M-VCR was $94.3 \pm 0.3\%$ ($n = 3$). Without pH adjustment, the VCR-loading efficiency was decreased to $68.9 \pm 6.7\%$ ($n = 3$). Without pH adjustment and oleic acid, the VCR-loading efficiency was only $30.3 \pm 5.7\%$ ($n = 3$). Stored at 7 °C in the dark for 1 week, the microemulsions without vitamin E was unstable and changed into white crystals. These white crystals were probably cholesterol and oleic acid.

### 4.2. Stability of M-VCR

Stored at 7 °C in the dark for 1 year, the average diameter and VCR-loading efficiency of M-VCR were changed from $138.1 \pm 1.2$ nm and $94.6 \pm 4.7\%$ to $127.1 \pm 2.4$ nm and $91.3 \pm 4.8\%$, respectively, while, $7.4 \pm 0.3\%$ VCR decomposition was observed ($n = 3$). Stored at 40 °C in the dark for 32 days, 68.8% of VCR in M-VCR remained while only 0.7% of VCR in F-VCR remained. Stored at 60 °C in the dark, 32 days, 53.8% of VCR in M-VCR remained while almost 100% of VCR in F-VCR decomposed within 14 days.

### 4.3. Pharmacokinetics of M-VCR

Plasma clearance and biodistribution curves of F-VCR and M-VCR are shown in Fig. 1. The

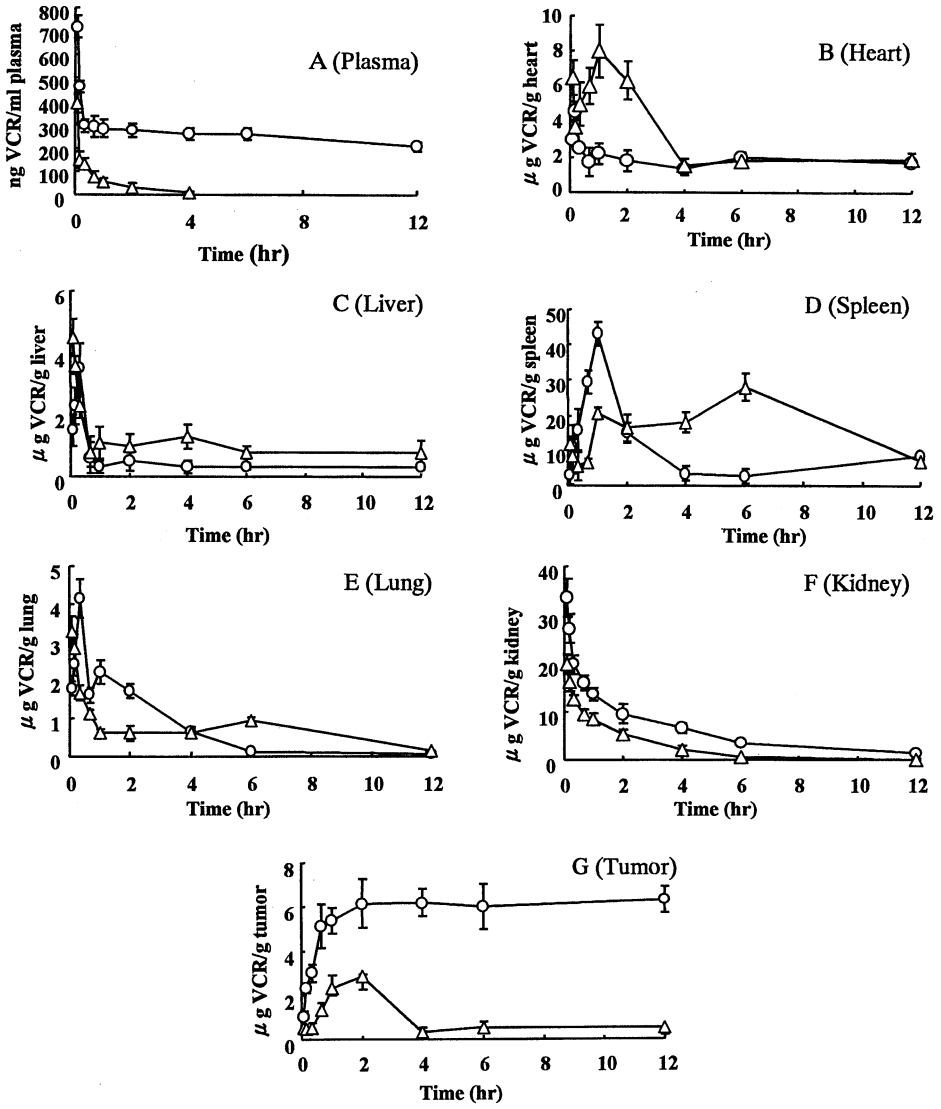


Fig. 1. Plasma concentration–time curves and biodistribution comparisons of microemulsions of vincristine (M-VCR) and free vincristine (F-VCR) following i.v. injection at a dose of 2 mg VCR per kg in C57BL/6 mice bearing solid M5076 tumors. (A) Plasma; (B) heart; (C) liver; (D) spleen; (E) lung; (F) kidney; (G) tumor. Sampling times were 1/12, 1/6, 1/3, 2/3, 1, 2, 4, 6 and 12 h. ○, M-VCR; Δ, F-VCR. Results are given as mean ± S.D. of three mice.

plasma concentration-time curves of M-VCR and F-VCR were best fitted to two biexponential decay curves, respectively, and the pharmacokinetic parameters are presented in Table 1. The $T_{1/2\alpha}$ and $T_{1/2\beta}$ of M-VCR were significantly longer than those of F-VCR. The AUC $_{0.08-12\ h}$ values of M-VCR and F-VCR are shown in Table 2. The plasma AUC of M-VCR was significantly greater than that of F-VCR (Table 2). After i.v. injection of F-VCR, VCR left the blood circulation system immediately and was taken up mainly by the spleen tissues (Fig. 1 and Table 2). However, after i.v. injection of M-VCR, VCR taken up by the tumor and kidney was significantly increased while

Table 1
Comparisons of pharmacokinetic parameters of M-VCR and F-VCR (VCR dose: 2 mg/kg, iv administrated in C57BL/6 mice)

| Agents | F-VCR* | M-VCR* |
|---|---|---|
| $C_0$ (μg/ml) | 1.11±0.16 | 0.85±0.12 |
| $K_{12}$ (1/h) | 31.74±4.24 | 0.30±0.01 |
| $K_{21}$ (1/h) | 6.78±1.52 | 0.21±0.08 |
| $K_e$ (1/h) | 1.37±0.28 | 0.07±0.01 |
| $T_{1/2\ \alpha}$ (h) | 0.02±0.01 | 1.25±0.31** |
| $T_{1/2\ \beta}$ (h) | 2.96±0.43 | 25.76±3.88** |
| $V_1$ (l/kg) | 1.82±0.37 | 2.35±0.41 |
| $V_2$ (l/kg) | 8.51±2.32 | 3.52±0.59 |
| $V_{ss}$ (l/kg) | 10.33±3.45 | 5.88±1.21 |
| Cl (l/h per kg) | 3.28±0.25 | 0.17±0.09** |

* Results were given as mean±S.D., $n=3$.
** $P<0.01$, compared with those of F-VCR.

Table 2
AUC 0.08–12 h comparisons of biodistribution curves of F-VCR and M-VCR

| Tissues | $AUC_{0.08-12}$ (μg/ml per h)[a] | |
|---|---|---|
| | F-VCR | M-VCR |
| Heart | 41.7±5.9 | 23.7±3.5[b] |
| Liver | 12.9±2.8 | 5.7±1.2[b] |
| Spleen | 228.5±34.6 | 143.1±24.1[b] |
| Lung | 8.9±2.6 | 9.9±2.8 |
| Kidney | 36.7±5.3 | 82.6±11.5[b] |
| Tumor | 12.9±3.5 | 76.5±15.7[b] |
| Plasma | 0.2±0.1 | 3.3±0.3[b] |

[a] Results were given as means±S.D., $n=3$.
[b] $P<0.01$, comparisons between M-VCR and F-VCR.

VCR taken up by the spleen, heart and liver was significantly decreased as compared with those of F-VCR (Table 2).

### 4.4. Acute toxicity of M-VCR

The death rates of each group after i.v. injection of F-VCR and M-VCR at a dose of 6 mg/kg starting on day 5 after tumor inoculation are shown in Fig. 2. Fig. 2 indicates that within 9 days, 80% of the mice in the F-VCR group died, while no mice in the M-VCR group died during the same period. In the M-VCR group, 50% of the mice survived for over 20 days. The longest survival of the mice of M-VCR group was 36 days, while that of the F-VCR group was only 29 days.

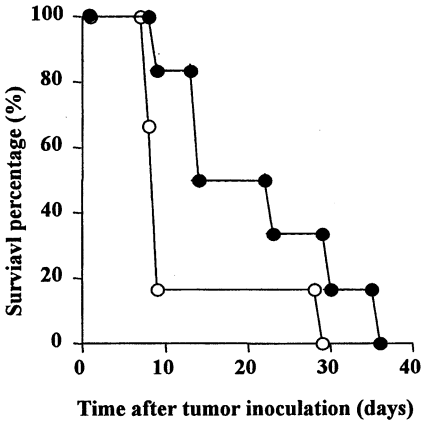


Fig. 2. Acute toxicity comparisons of microemulsions of vincristine (M-VCR) and free vincristine (F-VCR) in C57BL/6 mice bearing solid M5076 tumors. On day 5 after tumor inoculation, both agents were administered by single i.v. injection at a dose of 6 mg VCR per kg ($n=12$). ●, M-VCR; ○, F-VCR.

### 4.5. Antitumor effects of M-VCR

The anticancer effects of M-VCR and F-VCR are compared in Table 3 and Fig. 3. Table 3 shows that in the solid M5076 tumor model, the tumor weight inhibition rate of M-VCR was 67%, significantly higher than that of F-VCR (42%). Fig. 3 shows that in the ascitic M5076 tumor

Table 3
Comparisons of antitumor effects of F-VCR and M-VCR in M5076 solid tumor model in C57BL/6 mice

| Agents | Dose[a] (mg VCR per kg) | MTW[b] (g, ± S.D.) | TWR[c] (%) |
|---|---|---|---|
| Blank | Control | 1.2±0.2 | – |
| Vehicle | Control | 1.2±0.3 | 0 |
| F-VCR | 1.25 | 0.7±0.2 | 42* |
| M-VCR | 1.25 | 0.4±0.2 | 67* |

* $P<0.01$, comparison between F-VCR and M-VCR.
[a] iv × 4 with interval of 5 days starting on day 5 after tumor inoculation.
[b] Mean tumor weight.
[c] Tumor weight reduction.

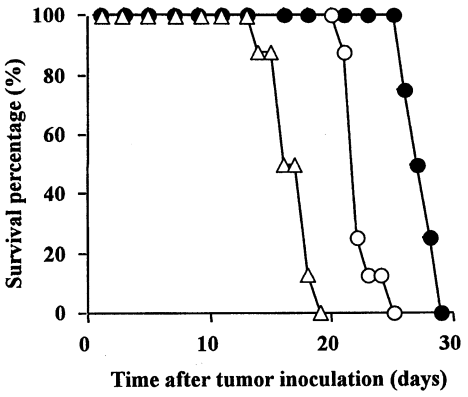


Fig. 3. Comparisons of the antitumor effects of M-VCR and F-VCR administered by i.v. injection three times with an interval of 5 days at a dose of 1.25 mgVCR per kg in the ascitic M5076 tumor model in C57BL/6 mice ($n=12$). ●, M-VCR; ○, F-VCR; Δ, Control (untreated).

model, the anitumor effect of M-VCR was significantly greater than that of F-VCR.

## 5. Discussion

In M-VCR, the oil phase was composed of oleic acid and vitamin E, while the surfactant and co-surfactant were PEG-DSPE and cholesterol, respectively. Oleic acid and cholesterol are in their crystal states at 7 °C and, therefore, without the vitamin E, M-VCR was not stable and changed in white crystals at 7 °C. However, vitamin E solution of oleic acid and cholesterol is in its liquid state even at −10 °C. Therefore, when vitamin E was added into the oil phase of M-VCR, M-VCR kept stable for 1 year at 7 °C.

VCR is an alkaloid and exhibits lower solubility in aqueous solutions and higher lipid solubility when pH is increased. Without pH adjustment, the pH of M-VCR was 5.6. Therefore, when the pH of M-VCR was adjusted to 7.4, the VCR-loading efficiency of M-VCR was increased from 68.9±6.7 to 94.6±4.7%. When oleic acid was used in the oil phase of the microemulsions, the carboxyl groups on the surface of the microemulsions might be combined with the amino groups of VCR and increase the VCR-loading efficiency of M-VCR. Therefore, without oleic acid and pH adjustment, the VCR-loading efficiency of M-VCR was only 30.3±5.7%. The preparation procedure of M-VCR was much easier than the ammonium sulfate gradient method and the polyanionic-compound gradient method used in the preparation of VCR-liposomes reported previously (George et al., 1996; Maurer et al., 1999).

According to the theory of emulsion, macro-emulsion formation generally requires vigorous mixing, while microemulsions tend to form spontaneously and have an average particle size of less than 150 nm. Unlike the long circulating emulsions reported previously (Liu and Liu, 1995), M-VCR formed spontaneously. In addition, M-VCR could be sterilized by simply passing emulsions through a sterile syringe-driven filter unit with a pore size of 100 nm by hand. M-VCR is a thermodynamically stable system stabilized by not only the PEG chains of PEG-lipid but also the charges of PEG-lipid and oleic acid. Therefore, M-VCR seems to be a microemulsion system.

The $T_{1/2\alpha}$ and $T_{1/2\beta}$ of M-VCR were significantly greater than those of F-VCR while the Cl of M-VCR was significantly smaller than that of F-VCR, which indicated that when administered in the M-VCR formulation, the drug circulated in the bloodstream for a longer time as compared with F-VCR. The total plasma and blood volumes of mice are approximately 0.49 and 0.75 l/kg, respectively (Seki et al., 1981). The $V_1$ and $V_2$ of M-VCR were 2.4 and 3.5 l/kg, respectively, which was much greater than the total mouse plasma volume. Therefore, while M-VCR was circulating in the bloodstream, VCR was released into the plasma. When emulsions are administered by i.v. injection, they are rapidly taken up by the MPS in the liver and spleen (Singh and Ravin, 1986; Poste et al., 1982). This is advantageous to target drugs to the MPS, but is an obstacle for delivery of drugs to targets outside the MPS. Unlike the emulsions described above, the spleen and liver $AUC_{0.08-12\ h}$ of M-VCR were significantly decreased compared with those of F-VCR (Table 2), which is maybe due to the steric barrier of the surface-grafted PEG chains of M-VCR. In addition, M-VCR (90–150 nm) dose not efficiently cross the endothelial cell barrier present in most normal tissues because the diameter of the pores of the capillaries in the

normal tissues is generally less than 80 nm (Kodama et al., 1997). Therefore, after i.v. injection of M-VCR, less VCR was taken up by the heart as compared with F-VCR. The lung $AUC_{0.08-12\ h}$ of M-VCR was not significantly different from that of F-VCR (Table 2), which indicated that the steric barrier of the PEG chains of M-VCR could not decrease the uptake of M-VCR by the MPS of the lung. The kidney $AUC_{0.08-12\ h}$ of M-VCR was significantly higher than that of F-VCR, which indicated that M-VCR could not decrease the VCR level of the kidney. Therefore, when administered in the M-VCR formulation, the drug circulated in the bloodstream for a longer time compared with F-VCR, which seemed to be mainly due to the reduced biodistribution of M-VCR to the MPS and some other normal tissues, such as the heart. Unlike the normal tissues in living systems, the tumor vasculature has been shown to be relatively leaky and less permeoselective than normal vessels (Matsumura and Maeda, 1986; Jain, 1994). The vascular permeability in the tumor tissue is probably governed by diffusion across the vessel wall that allows the penetration of particles up to 400 nm in diameter (Fan et al., 1995). The particle size distribution of M-VCR is less than 150 nm, which made it possible for long circulating M-VCR to accumulate in tumor tissues. Therefore, the tumor $AUC_{0.08-12\ h}$ of M-VCR was significantly greater than that of F-VCR (Table 2). In our laborotary, VCR concentrations were determined using HPLC, which was not sensitive enough to detect VCR less than 1 μg/ml. Further work is need to establish a more sensitive analysis method to study the pharmacokinetics of M-VCR and F-VCR.

In conclusion, M-VCR was a microemulsion system and was physically stable during the 1-year storage period at 7 °C in the dark. M-VCR has lower toxicity and higher antitumor effects than F-VCR and is useful tumor-targeting DDS.

## Acknowledgements

This work was supported by grants from Japan Society for Promotion of Science and the Ministry of Education, Science, Sports, and Culture, Japan.

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