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Optimal formulation of an angiogenesis inhibitor, TNP-470, for arterial injection determined by in vitro drug release and stability, and in vivo antitumor activity

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Abstract

TNP-470 (6-*O*-(*N*-chloroacetylcarbamoyl)fumagillol, AGM-1470) is an angiogenesis inhibitor, a new type of anticancer drug which prevents tumor neovascularization, thereby blocking the nutrient supply to tumors. In this study, we sought the optimal formulation of TNP-470 for arterial injection in order to achieve strong anticancer activity due to the tumor-selective targeting of the drug, by investigating in vitro release and stability and in vivo rabbit VX-2 antitumor activity. We found that a medium-chain triglyceride (MCT) solution containing TNP-470 facilitated the 2-week sustained release of TNP-470 in vitro and fairly good long-term stability of the agent, although it was very labile in aqueous solution. In a rabbit VX-2 tumor model, 3 weeks after inoculation on the inner side of the leg, the antitumor activities of various formulations of TNP-470 were evaluated by administration into the femoral artery feeding the tumor. Compared with Lipiodol solution or PLGA microspheres containing TNP-470, the MCT solution containing TNP-470 exerted stronger and more persistent antitumor activity accompanied by tumor regression for 3 weeks subsequently. The release sustainability of TNP-470 in the in vitro release test was suggested to be an important factor in the antitumor activity of each formulation. From these results, we conclude that the MCT solution is the most promising formulation of TNP-470 as an arterial injection for treatment of cancers.

Keywords: TNP-470; Angiogenesis inhibitor; Arterial injection formulation; Medium-chain triglyceride; Sustained release; Rabbit VX-2 carcinoma

1. Introduction

The therapeutic benefits of intra-arterial (i.a.) injection of anticancer drugs into the artery supplying the tumor result from regional elevation of the drug concentration in the tumor site due to the first pass of blood containing high concentration of drugs. Concurrently, systemic side-effects may be reduced (Taguchi, 1984). In order to obtain more enhanced antitumor effects, ideally,

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drugs would be selectively retained in the tumor site following i.a. administration. This has been achieved by chemoembolization in which drugs stay together with embolizing materials and are released in vessels adjacent to the tumor site, or by oily injection in which the drug is specifically retained in the tumor vasculature due to physiological and functional differences in the vasculature of normal and tumor tissue. Chemoembolization combines chemotherapy and tumor devascularization by intra-arterial administration of chemotherapeutic agents together with an embolizing material such as degradable starch (Gyves et al., 1983), albumin (Fujimoto et al., 1985) or poly(lactic acid) (Ichihara et al., 1989) microspheres (msp). This treatment can achieve regional delivery and prolonged retention of drugs at the tumor site, but the development of collateral circulation bypassing the embolized region sometimes becomes problem at the treatment of hepatic cancer (Stridbeck et al., 1984; Tsuji et al., 1987). Drugs dissolved in Lipiodol (LPD), lymphographic oil, show selective accumulation and retention at the tumor site after i.a. administration, due to the difference in time required for the oil to be removed from normal capillaries and the tumor vasculature (Maeda et al., 1979; Konno et al., 1983). Clinical results of this treatment have suggested reinforced antitumor activity with less severe systemic side effects.

TNP-470 (6-*O*-(*N*-chloroacetylcarbamoyl)fumagillol, AGM-1470; Fig. 1), synthesized at Takeda Chemical Ind., Ltd (Osaka, Japan) (Marui et al., 1992) is a new type of anticancer drug that inhibits tumor neovascularization and blocks the supply of nutrients to tumors (Ingber et al., 1990; Kusaka et al., 1991). In a previous study, we found that chemoembolization using TNP-470 msp prepared with a biodegradable polymer, poly(lactic/glycolic) acid (PLGA), caused tumor regression in a rabbit VX-2 tumor model after a single injection into the femoral artery which was feeding the tumor. However, the period of regression of the tumors was poor. An LPD solution of TNP-470 also demonstrated strong antitumor activity, and this was more persistent than that achieved by the PLGA msp (Okada et al., 1992; Kamei et al., 1993).

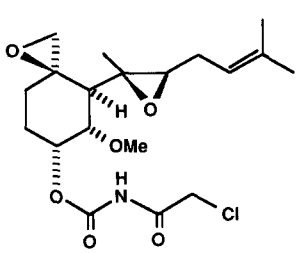

Fig. 1. Chemical structure of TNP-470.

In the present study, we have investigated the optimal formulation feasible as a commercial product with sufficient antitumor activity after i.a. administration by evaluating its in vitro release, stability and in vivo antitumor activity in a rabbit VX-2 tumor model.

2. Materials and methods

2.1. Materials

TNP-470 was synthesized at Takeda Chemical Ind. (Osaka, Japan). PLGA (molecular ratio of lactic and glycolic acid, 75:25; weight-average molecular weight, around 6800) was obtained from Wako Pure Chemical Ind. (Osaka, Japan), Miglyol 812 (caprylic and capric acid triglyceride, MCT) and Miglyol 829 (caprylic, capric and succinic acid triglyceride) were from Huls A.G. (Marl, Germany), LPD was from Gurbert Lab. (Aulnay-Sous-Bois, France), and sesame oil was from Takemoto Yushi (Aichi, Japan). Decaglyceryl decaoleate (Decaglyn 10-O) and polyoxyethylene hydrogenated castor oil (HCO-10) were obtained from Nikko Chemicals (Tokyo, Japan). Other oils and reagents were commercially available. [^{3}H]TNP-470 (spec. act. 241 MBq/mg) was obtained from Amersham (Bucks, UK) and [^{3}H]oleic acid (spec. act. 74–370 MBq/μmol) was from NEN Research Products (Boston, MA, USA).

2.2. Animals

Male Sprague-Dawley rats weighing 280–300 g (8 weeks old) were obtained from Clea Japan Inc.

(Tokyo, Japan). Female rabbits bearing the VX-2 carcinoma and male rabbits (Kbl, JW) weighing 2.5–3.0 kg were purchased from Funabashi Farm (Chiba, Japan) and Kitayama Labs (Kyoto, Japan), respectively. Food and water were available ad libitum.

2.3. Methods

2.3.1. Preparation of dosage forms

TNP-470 is soluble in various oily bases and especially in MCT with a solubility of approx. 100 mg/ml at 25° C. TNP-470 was dissolved in oily bases with mild shaking at room temperature and the solution was sterilized by filtration (0.22 μm, Dimex filter, Millipore Japan, Tokyo, Japan). TNP-470 with or without a tracer amount of labeled TNP-470 was microencapsulated in PLGA by an in-water drying method. Briefly, TNP-470 was dissolved in 50% PLGA-methylene chloride containing 0.25% dipalmitoylphosphatidylcholine. The solution was poured into a 0.15% aqueous solution of polyvinyl alcohol under stirring with a turbine-shaped mixer. The oil/water emulsion was continuously stirred for 2 h to evaporate the methylene chloride. The hardened msp were sized using sieves with apertures of 53–125 and 125–250 μm and lyophilized. The resulting msp (10 mg) contained 0.91–1.0 mg of TNP-470. Msp were administered with vehicle (0.1% Tween 80, 0.5% carboxymethylcellulose, 5% D-mannitol) or saline in animal experiments. An aqueous solution of TNP-470 was prepared by dissolving TNP-470 with maltosyl-β-cyclodextrin.

2.3.2. Drug release and long-term stability in vitro

PLGA msp (125–250 μm) containing 1 mg of TNP-470, or oils (1 ml) containing 10 mg of TNP-470 were dispersed in pH 7.0 phosphate buffer (5 ml) containing 0.02%(w/v) Tween 80 (Kao, Tokyo, Japan) using a rotator (10 rpm) at 37° C for designated times. After centrifugation at 3000 rpm, msp or oils were collected, and the remaining TNP-470 was quantified by HPLC.

1 ml of MCT or Lipiodol solution containing 10 mg of TNP-470 was stored in a vial filled with nitrogen at 25, 40 and 60° C for 1–6 months, and the remaining TNP-470 was quantified by HPLC. The degradation rate constant was calculated by fitting data at each temperature to the equation describing first-order degradation kinetics.

The HPLC equipment consisted of a Waters Model 700 autoinjector, a model 600E solvent delivery pump (all from Millipore Japan) and an L-4000 UV detector (Hitachi, Tokyo, Japan). The detector was operated at 210 nm. The detector signal was recorded using a D-2500 integrator (Hitachi). An Asahi-Pack ODP-50 (150 × 6.0 mm internal diameter, Asahi Chemical Ind., Tokyo, Japan) was used as an analytical column. The eluent comprised 50% (v/v) acetonitrile in distilled water and the flow rate was 0.7 ml/min.

The rheological properties of oils at 25 and 37° C were measured using a cone-and-plate viscometer (Visconic ELD, Tokyo Keiki, Tokyo, Japan) with the range of rate of shear being 0–38.3 s^{-1}. The apparent viscosities of oils were determined as follows: The shearing stress on the various rates of shear was determined and the apparent viscosity (centipoise, cP) was calculated as the slope of the regression line.

2.3.3. Retention of oily drug carrier in the liver and distribution of TNP-470 encapsulated in PLGA msp, after administration via the hepatic artery in rats

Rats (9 weeks old) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Laparotomies were carried out to allow administration of test solutions via the hepatic artery and subsequent evaluation according to previously reported methods (Yanai et al., 1994). The midline of the rat abdomen was opened and the celiac, hepatic and gastroduodenal arteries were exposed. The gastroduodenal artery was temporarily ligated so that the test solution would not flow into it. The oil solution (25 μl/rat) containing a tracer amount of [^{3}H]oleic acid (37 kBq), or PLGA msp (53–125 μm, 8.7 mg of msp/kg) encapsulating a tracer amount of [^{3}H]TNP-470 and unlabeled TNP-470 was then injected into the hepatic artery using a 23-gauge needle, immediately followed by closing the needle hole with an adhesive agent (Aron Alpha, Sankyo, Tokyo, Japan) and removing the ligature in order to restore circulation. At the designated times after administration, rats were

killed by collection of blood from the inferior vena cava. Specimens of various organs were removed, weighed, minced and dried. Samples were then oxidized with a sample oxidizer (Model 307, Packard Japan, Tokyo, Japan), and the radioactivity recovered in scintillation fluid (Monophase S, Packard Japan) was determined using a liquid scintillation counter (LSC-903, Aloka, Tokyo, Japan).

2.3.4. Antitumor effects in rabbits bearing the VX-2 carcinoma

Experiments were carried out according to a modification of the method of Kamei et al. (1993) (Yanai et al., 1995). The transplantable anaplastic VX-2 carcinoma, originating from a spontaneously transformed Shope papilloma, was used. A VX-2 carcinoma was minced with scissors and sieved through wire mesh (60 mesh). Tumor cells were suspended in Hank's solution containing 10% rabbit serum, 120 μg/ml penicillin and 100 μg/ml streptomycin. Male rabbits were inoculated subcutaneously with a VX-2 carcinoma cell suspension (10% (w/v), 0.5 ml) at a position on the inside of the right leg just below the knee. The VX-2 carcinoma cell line was maintained by successive inoculation of untreated rabbits. 3 weeks after inoculation, i.a. administration was carried out. Oil solutions (0.5 ml) or PLGA msp containing 5 mg of TNP-470 was injected into the femoral artery through polyethylene tubing (PE-50, Clay Adams, NJ, USA) under pentobarbital anesthesia. After administration, the blood flow to the femoral artery was reopened by inserting the tubing upward into the artery after being shortened to a length of about 4 cm. Total ischemia occurs for less than 3 min during the administration procedure, but should have no effect on tumor growth. Antitumor activities were evaluated in comparison with control rabbits that were untreated. The tumor volume was taken to be the product of the length, width and height as measured with calipers through the skin and is expressed as a ratio to the volume just before treatment (tumor volume ratio). The T/C value was calculated as tumor volume ratio$_{(\text{treated})}$/tumor volume ratio$_{(\text{untreated control})}$.

3. Results and discussion

Although TNP-470 possesses specific antitumor activity since angiogenesis is highly active in tumor tissue compared to normal tissues (Folkman, 1985; Kusaka et al., 1994), it was considered to be a rational strategy to deliver this novel drug topically at the tumor site in order to obtain more enhanced selective antitumor activity. In the present study, therefore, we tried to formulate TNP-470 for arterial injection to treat cancers such as unresectable hepatic cancer. In formulating TNP-470 for arterial injection, the efficiency of selective delivery to the tumor region, drug release, and drug stability must be taken into consideration.

We investigated the relationship between the viscosities of oily bases and the degree of retention in microvessels in the liver due to local microembolization after intra-hepatic arterial administration. Fig. 2 shows the retained [^{3}H]oleic acid activity of various oily bases of different viscosities (25–164 cP at 37° C, Table 1), in the

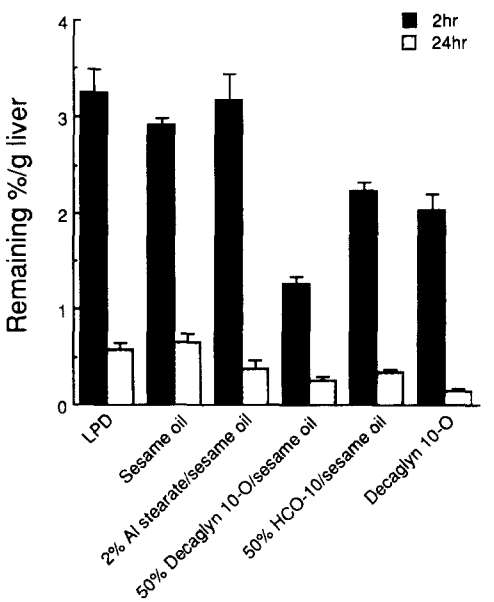

Fig. 2. Radioactivity retained in rat liver after administration via the hepatic artery of various oily bases containing tracer amounts of [^{3}H]oleic acid. Each bar represents the mean ± S.E. of three rats.

Table 1
Viscosities (cP) of various oily bases

	25° C	37° C
MCT	21	15
LPD	37	25
Rosehip oil	41	8
Soybean oil	49	29
Cottonseed oil	53	28
Corngerm oil	57	22
Sesame oil	57	36
Olive oil	58	40
Camellia oil	71	20
2% aluminium stearate [a]	105	61
50% Decaglyn 10-O [a]	122	71
Miglyol 829	147	75
50% HCO-10 [a]	294	149
Decaglyn 10-O	317	164
Castor oil	488	262

[a] Mixed with sesame oil.

liver after administration via the hepatic artery in normal rats. It had been expected that the higher the viscosity of the oily bases, the slower would be the disappearance by the hepatic blood flow. However, this was not borne out by the result. It is possible that much higher viscosity, i.e., solidity, is necessary in order for the formulation base to be retained in the normal microvasculature for a long time. Therefore, oily injection was expected as a system for selective delivery to the tumor site depending on the difference in the vascular structure and function, rather than the embolizing system. As shown in Fig. 3, concentrations of radioactivities of [^{3}H]TNP-470 in tissues were measured in normal rats following intrahepatic arterial administration of msp or an aqueous solution containing [^{3}H]TNP-470. For msp, the liver showed a high level of radioactivity 2–72 h after administration, probably due to the embolization of hepatic microvessels (Cho and Lunderquist, 1983; Kamei et al., 1992). The level in the liver was 7.7-fold higher than that of the aqueous formulation 2 h after administration and a relatively high level was maintained until even 72 h after administration, confirming the topical localization of drugs by PLGA msp.

It is well known that an oil such as LPD remains selectively in tumor tissue because it is not removed as quickly by the tumor neovasculature as by normal vasculature. If TNP-470 was delivered to the tumor site together with an oil base as a drug carrier, it could be more effective that the drug was sustainedly released from the oil solution at the tumor site because continuous i.v. infusion of an aqueous solution of TNP-470 was more effective than i.v. bolus injection, due to continuous angiogenesis inhibition (Yamaoka et al., 1993). Therefore, we then investigated the

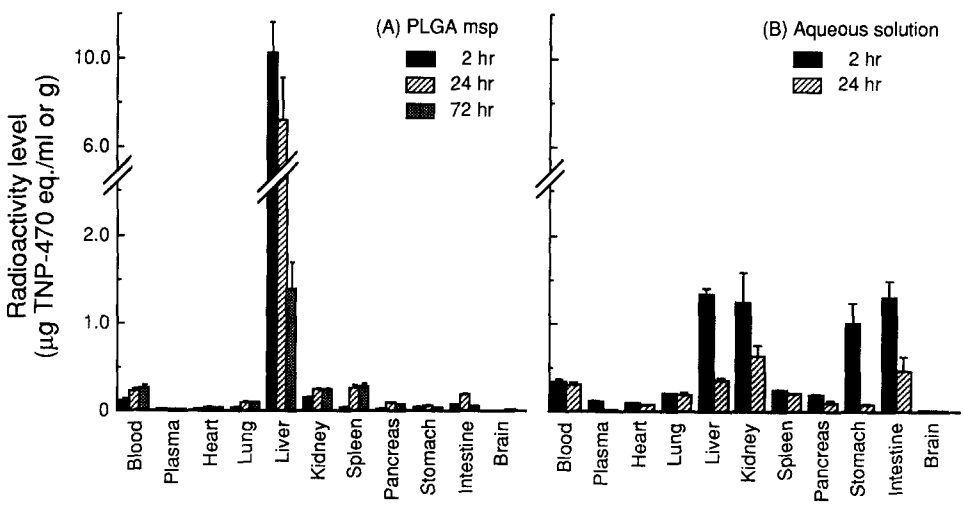

Fig. 3. Concentrations of radioactivity in various tissues in rats after administration of (A) PLGA msp and (B) an aqueous solution containing a tracer amount of [^{3}H]TNP-470 with unlabeled TNP-470. Dose: (A) 0.79 mg of TNP-470/8.7 mg of msp/kg. Data were normalized to 1 mg of TNP-470/kg. (B) 1 mg/100 μl per kg. Each bar represents the mean ± S.E. of four rats.

in vitro release of TNP-470 from several oil bases, the viscosities of which are listed in Table 1. We have studied the retention of TNP-470 in the oil phase after 1-day mixing of several oil bases containing TNP-470 with phosphate buffer, which was considered to reflect the release sustainability of each oil solution. As shown in Fig. 4, the remaining percentage of TNP-470 was relatively high in MCT, LPD, Miglyol 829 and caster oil, indicating that these oily bases could attain superior sustained release of TNP-470. However, administration of highly viscous oil bases such as Miglyol 829 and caster oil through a catheter was difficult. As the viscosity of the oily base in the present study was not related to the ability of embolizing vessels, our investigations into the oil bases were limited to MCT and LPD. MCT is caprylic and capric acid triglyceride which is used clinically as a component in a lipid emulsion formulation for parenteral nutrition, indicating satisfactory biocompatibility. Fig. 5 shows the time courses of the release profile of TNP-470 from MCT, LPD solutions and PLGA msp in the same in vitro test system. Sesame oil solution was also tested for comparison. The MCT, LPD and sesame oil solutions demonstrated that the release of TNP-470 occurred according to first-order release kinetics with half-lives of 2.0, 1.1 and 0.5 day^{-1}, respectively, indicating that MCT is the

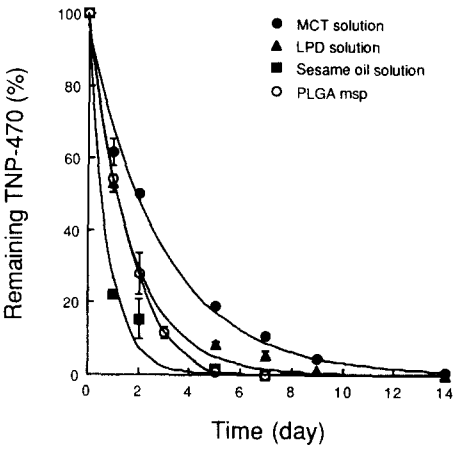

Fig. 5. Time courses of TNP-470 retention in various formulation bases in in vitro release test. The initial concentration of TNP-470 in oily bases was 10 mg/ml and initial content of TNP-470 in msp was 1 mg. Lines for oily formulations were obtained by fitting the data to the equation describing first-order release kinetics. Each point represents the mean ± S.D. of three independent experiments.

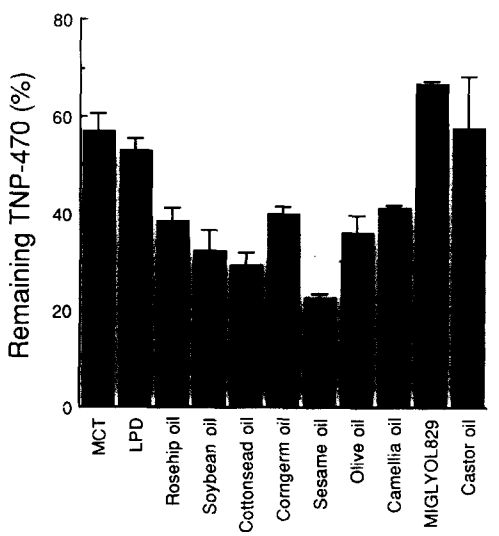

Fig. 4. TNP-470 retained in various oily bases (1 ml) after mixing with phosphate buffer (5 ml) for 1 day at 37° C. The initial concentration of TNP-470 in oil bases was 10 mg/ml. Each bar represents the mean ± S.D. of three independent experiments.

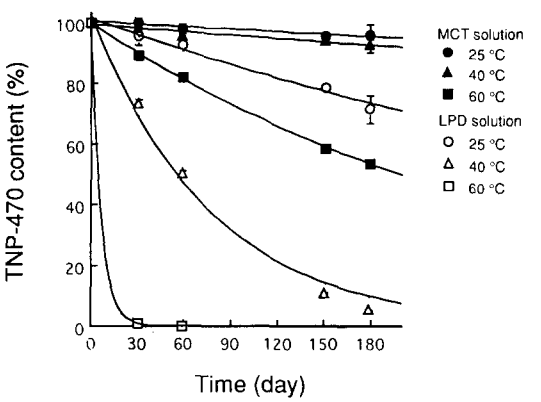

Fig. 6. Long-term stability of TNP-470 in MCT or LPD solution stored at 25, 40 and 60° C. The initial TNP-470 content was 10 mg/ml, and data show the TNP-470 remaining in the solutions. Lines except for LPD solution stored at 60° C were obtained by fitting the data to the equation describing first-order degradation kinetics. Each point represents the mean ± S.D. of three independent experiments.

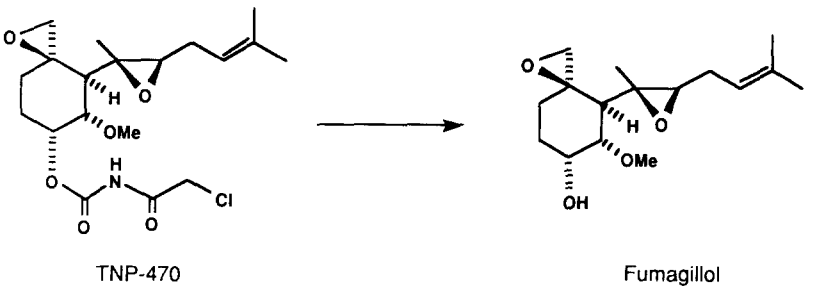

Fig. 7. Degradation scheme of TNP-470 in MCT solution.

most preferable oily base for the sustained release of TNP-470. However, TNP-470 showed relatively rapid disappearance from PLGA msp within 5 days of mixing. It has also been confirmed that TNP-470 was released from oil solution as an active form using a shell-less chorioallantoic membrane assay system (Yanai et al., 1995). It was not clear whether the disappearance of TNP-470 from PLGA msp meant release from or degradation in the msp.

Fig. 6 shows the long-term stability of TNP-470 in MCT and LPD solutions. The MCT was able to retain TNP-470 so stably that 92.6% of the initial TNP-470 content was preserved even when the solution was stored at 40° C for 6 months, while only 5.3% of the initial content remained in the LPD solution. The apparent first-order degradation rate constants at 25° C of TNP-470 in the MCT and LPD solution were calculated as 2.67×10^{-4} and 1.79×10^{-3} day^{-1}, respectively. It was also confirmed that the release profiles of TNP-470 from MCT solution stored at 25 and 40° C for 1 month were identical with that obtained immediately after preparation (data not shown), also indicating the pharmaceutical superiority of the TNP-470 MCT solution. The degradation scheme of TNP-470 in the MCT solution is exhibited in Fig. 7. A major degradation product of TNP-470 during storage at 60° C was detected as a clear single peak in the HPLC chromatogram and identified as fumagillol by liquid chromatography/mass spectrometry. Degradation products of TNP-470 in LPD solution could not be resolved by HPLC. It is interesting that although TNP-470 is readily degraded in aqueous solution, with a degradation half-life of 4–5 h (pH 7.0 at 37° C), fumagillol is scarcely generated in the aqueous medium.

We finally compared the antitumor activities of several formulations after arterial administration in the rabbit VX-2 tumor model. Although our present tumor model may not be realistic for hepatic cancer which is the ultimate object of our present treatment, it has the great advantage of allowing tumor size to be measured easily and continuously through the skin. Fig. 8 shows the effects of MCT or various dosage forms of TNP-470 on well-developed VX-2 tumors 3 weeks after inoculation. Untreated tumors grew extensively, and their volume increased 7.4-fold during the

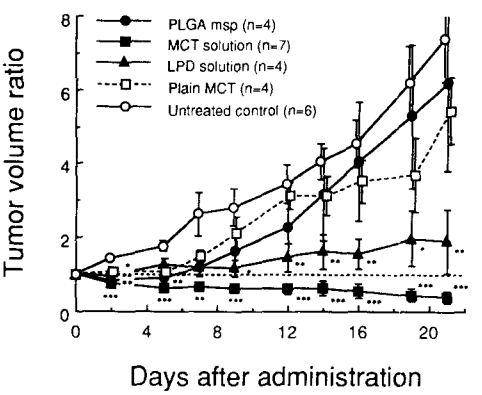

Fig. 8. Antitumor activities of MCT solution, LPD solution, PLGA msp, all containing 5 mg of TNP-470, or plain MCT against VX-2 carcinoma in rabbits following i.a. injection on week 3 after tumor inoculation. Each point represents the mean ± S.E. Significantly different from the corresponding point for the untreated control by Student's t-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

following 3 weeks. The tumors in animals receiving a plain MCT also grew extensively after transient initial suppression probably due to partial embolization of the tumor microvasculature by the oil droplets for a short time following administration, as was observed in a rat hepatic cancer model (Yanai et al., 1994). The tumors in animals receiving 5 mg of TNP-470 dissolved in LPD were persistently suppressed, and the tumor volume ratio and the T/C value at 3 weeks after administration were 1.9 and 0.26, respectively. Moreover, the tumors administered TNP-470 MCT solution were persistently suppressed with regression for the entire 3 weeks, and the tumor volume ratio and the T/C value at 3 weeks after administration decreased to 0.39 and 0.05, respectively. Since plain MCT caused little tumor suppression past that observed initially, retention and release of the angiogenesis inhibitor around the tumor are probably responsible for the distinct antitumor activity. However, for PLGA msp containing TNP-470, tumor growth was completely suppressed with slight regression for an initial 5 days after i.a. administration, followed by vigorous growth to reach the tumor volume ratio and the T/C value of 6.18 and 0.83, respectively, at 3 weeks later. No severe effects on body weight were observed. These results suggest that because TNP-470 disappeared from the PLGA msp within 5 days in in vitro release test as shown in Fig. 5, the PLGA msp containing TNP-470 could not exert sufficient antitumor effects despite achieving topical localization of the drug initially as observed in Fig. 3. Therefore, the release sustainability of TNP-470 from each formulation could be one reason for the differences observed in the antitumor activity. In addition, it was demonstrated that the oil formulation could facilitate highly selective delivery and retention of TNP-470 at the tumor site (Yanai et al., 1994), which is another reason for the strong antitumor activity of the oil formulations. Our present tumor model is thought to be the model for metastatic cancer. As for primary hepatoma, the vascular system is known to be dominated by the hepatic artery and hypervascular. Therefore, we consider that our present therapeutic strategy would also be effective in treating this disease.

In conclusion, the MCT solution containing TNP-470 could achieve the most prolonged, sustained release of TNP-470 in vitro and this formulation showed fairly good long-term stability of TNP-470. Moreover, the TNP-470 MCT solution exerted the strongest and most persistent antitumor activity accompanied by tumor regression in the rabbit VX-2 tumor model. We therefore conclude that the MCT solution is the most promising formulation of TNP-470 as an arterial injection for treatment of cancers.

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