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Research paper

Preparation of poly-lactic acid microspheres containing the angiogenesis inhibitor TNP-470 with medium-chain triglyceride and the in vitro evaluation of release profiles

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Abstract

TNP-470 (AGM-1470, 6-0-(N-chloroacetylcarbamoyl)-fumagillol), a derivative of fumagillin, is a promising angiogenesis inhibitor. However, as TNP-470 is very unstable in in vitro and in vivo, it has been difficult to verify its pharmacological efficacy in the clinical medicine. The preparation of a drug delivery system (DDS) in a microsphere form was studied for the stable inclusion and controlled release of TNP-470. Medium-chain triglyceride (MCTG) as an effective stabilizer and poly-lactic acid (PLA) as a biodegradable carrier were used for this purpose. The release of TNP-470 from the MCTG containing DDS continued for approximately 2 weeks, while the release of TNP-470 from the one without MCTG stopped after only 5 days. It was proved that TNP-470 could be released much more stable for much longer period from the MCTG containing DDS compared to the one without DDS.

Keywords: TNP-470; Polylactic acid; Microsphere; Medium chain triglyceride; Drug delivery system; Angiogenesis inhibitor

1. Introduction

It is known that angiogenesis is the essential process in the growth, metastasis and formulation of tumors. It is therefore possible to produce an antitumor effect and suppress metastasis by inhibiting angiogenesis. The concept of an angiogenesis inhibitor was initially reported by Folkman et al. [1], and various angiogenesis inhibitors such as TNP-470 [2–6], interferon-α [7], thrombospondin [8], thalidomide [9] and angiostatin [10] have been reported. As TNP-470 had no serious side effects in comparison with the antineoplastic drugs, it was regarded as a very safe antitumor agent. Although the mechanism of the angiogenesis inhibition by TNP-470 is still unclear, its binding to the matrix metalloproteinases such as methionine aminopeptidase 2 (MetAP-2) [11] and the arrest cell cycle at G1 phase in vascular endothelial cells [12] have been reported. These effects can suppress angiogenesis. However, TNP-470 has been difficult to use clinically, because of its instability in aqueous solution and rapid hydrolysis in vivo. Therefore, the development of a new effective dosage form of TNP-470 such as the drug delivery system (DDS) for solving these problems is necessary.

Poly-D,L-lactic acid (PLA) has been used generally as a biodegradable polymeric carrier for DDS [13,14], but it has been difficult to prepare the DDS including an unstable drug. Because it absorbs water and a drug is quickly degraded. On the other hand, TNP-470 is more stable in fat and oil. Research into oleaginous formulations containing TNP-470 has been studied [15]. However, this system has not been proved the long-term release. The PLA microsphere including fatty acid esters to release drugs such as antineoplastic agents has been reported [16]. However, the preparation of PLA microsphere for very unstable drugs such as TNP-470 has not been reported. In this research work, microsphere DDS incorporating TNP-470 (TNP-DDS) was developed. For this purpose, medium-chain triglyceride (MCTG) was used to improve the stability of TNP-470 before and after administration, and the microspheres were prepared successfully.

This study aims to improve the stability and the ability to provide a sustained release of the preparation of micro-

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spheres which permit a greater release duration of the active drug.

2. Materials and methods

2.1. Materials

TNP-470 (Takeda Chemical Industries Ltd., Osaka, Japan); poly-D,L-lactic acid (PLA) (Taki Chemical Co. Ltd., Hyogo, Japan) of a mean molecular weight of 11 000 was used as a carrier.

A medium-chain triglyceride (MCTG; caprylic acid: 58%, capric acid: 40%) (Mitsuba Trading Co. Ltd.) was used as an additive. Poly-vinyl alcohol (PVA) of about 2200 degrees of polymerization (Wako Chemical Industries Ltd., Osaka, Japan) was used as a prime class solvent. Dichloromethane (DCM) and the other reagents were of high purity grade.

2.2. Method

2.2.1. Preparation of TNP-DDS

TNP-DDS was prepared by a solvent evaporation method (water/oil (W/O) emulsion method). The composition ratio is shown in Table 1. TNP-470 was dissolved in MCTG and PLA was added to this solution. DCM was subsequently added, solubilizing this mixture. This DCM solution was added to 0.5% v/v PVA aqueous solution at 15 °C and stirred by a mixer (7000 rpm unloaded; As One Co., Osaka, Japan) to produce a W/O emulsion. The emulsion was stirred for 2 h to evaporate DCM and caking of TNP-DDS. The TNP-DDS was recovered by centrifugal separation, filtered and dried in a vacuum. The control microspheres were produced by the same method but with the exclusion of MCTG.

2.2.2. Shape and particle diameter of TNP-DDS

Formulations were prepared with different composition ratios as given in Table 1. The particle shape was observed under a scanning electron microscope (SEM) (S-900, Hitachi Co., Japan). The particle diameter was measured with image analysis equipment (Luxex-FS, Nireco Co., Japan), and the distribution of particle diameter and the average particle diameter were obtained by those results. Cross-sections of preparations E and G were observed under the SEM.

2.2.3. Measuring the quantity of TNP-470 in TNP-DDS

Ten milligrams of the TNP-DDS was dissolved in 1 ml of acetone and stirred after the addition of 10 ml of physiological saline. The precipitate was removed with a membrane filter (MILLEX-GV 0.22 μm, Millipore). The same volume of acetonitrile was added to give the solution and then stirred. The concentration of TNP-470 in the solution was measured by high-performance liquid chromatography (HPLC), which consisted of a 490E program multi-wavelength detector and a 510-type pump (Waters Co., USA). The column was a Nucleosil 5 C18 4.6×250 mm^2. The measurement was performed using a mobile phase of 50% v/v acetonitrile solution. The flow rate was 1.0 ml/min and the detection wavelength was 217 nm.

2.2.4. Decomposition behavior of TNP-470 in physiological saline

One milligram of TNP-470 was dissolved in 5 ml of physiological saline at 37 °C. The physiological saline was periodically sampled. Each time, acetonitrile of the same amount was added and the TNP-470 concentration in the solution was measured by HPLC. The half-life of TNP-470 was calculated and the decay constant (λ) calculated from these results.

2.2.5. Measuring the in vitro release of TNP-DDS

Samples containing 5 mg of TNP-470 (TNP-DDS (E): 7.22 mg; control (G): 5.69 mg) were dispersed in 1 ml physiological saline at 37 °C. TNP-DDS was periodically recovered by centrifugation at 5000 rpm for 5 min. The quantity of TNP-470 in the TNP-DDS and the solution was measured.

Table 1
Preparation of formulations

Preparation	Material used in preparation				Particle size ($n = 200$) (Mean ± SD, μm)	Encapsulation efficiency (%)	TNP-470 content (mg/MC, MS 100 mg)
	PLA (mg)	Miglyol (mg)	Dichloromethane (ml)	TNP-470 (mg)			
A	400	100	2	50	24.47 ± 21.85	15.01 ± 0.74	2.44 ± 0.12
B	400	100	4	50	17.08 ± 7.96	15.14 ± 1.51	1.88 ± 0.19
C	400	100	10	50	10.69 ± 3.67	12.04 ± 0.92	1.17 ± 0.09
D	400	200	2	50	21.83 ± 15.89	18.04 ± 0.94	2.13 ± 0.11
E	400	100	2	100	20.99 ± 16.91	18.46 ± 1.65	5.54 ± 0.5
F	400	200	2	100	30.66 ± 21.23	16.96 ± 3.28	4.64 ± 0.9
G	400	–	2	100	23.08 ± 14.90	27.01 ± 1.37	8.50 ± 0.43

3. Results

3.1. Effect of composition ratio on the TNP-DDS

Table 1 summarizes the properties of TNP-DDSs prepared with various compositions of PLA, MCTG, TNP-470 and DCM. The particle size and the TNP-470 content of preparation A was greater than those of preparations B and C. There was no significant difference in particle size among preparations A, D and E, but the TNP-470 content of preparation E was largest compare to those of preparations A and D. The TNP-470 content of preparation E was largest compare to those of preparations A and D. As the TNP-470 content of preparation E was the highest of all preparations, preparation E was chosen for further comparison with preparation G as the control, in the in vitro release test. The particle diameter distribution of preparation C was very narrow (Fig. 1). The average particle diameter increased and the distribution of particle diameters became broader with the increasing ratio of PLA to DCM. The recovery rate and amount of TNP-470 also increased with the increasing ratio of PLA to DCM. No great change in average particle diameters was observed with the change of either the MCTG or TNP-470 amount in system. However, those were increased with the increase of both the MCTG and TNP-470 amount in the system.

Examination of cross-sections revealed that preparation E had a more porous structure than preparation G (Fig. 2).

3.2. Decomposition of TNP-470 in physiological saline

The half-life of TNP-470 was approximately 19.16 h in physiological saline at 37 °C, and after 7 days detection was impossible (Fig. 3). The decay constant (λ) was 0.052. The following equation was obtained from the half-life:

$$D = D_0 e^{-0.052t}$$

(D_0, initial concentration; D, concentration after t hours; t, time).

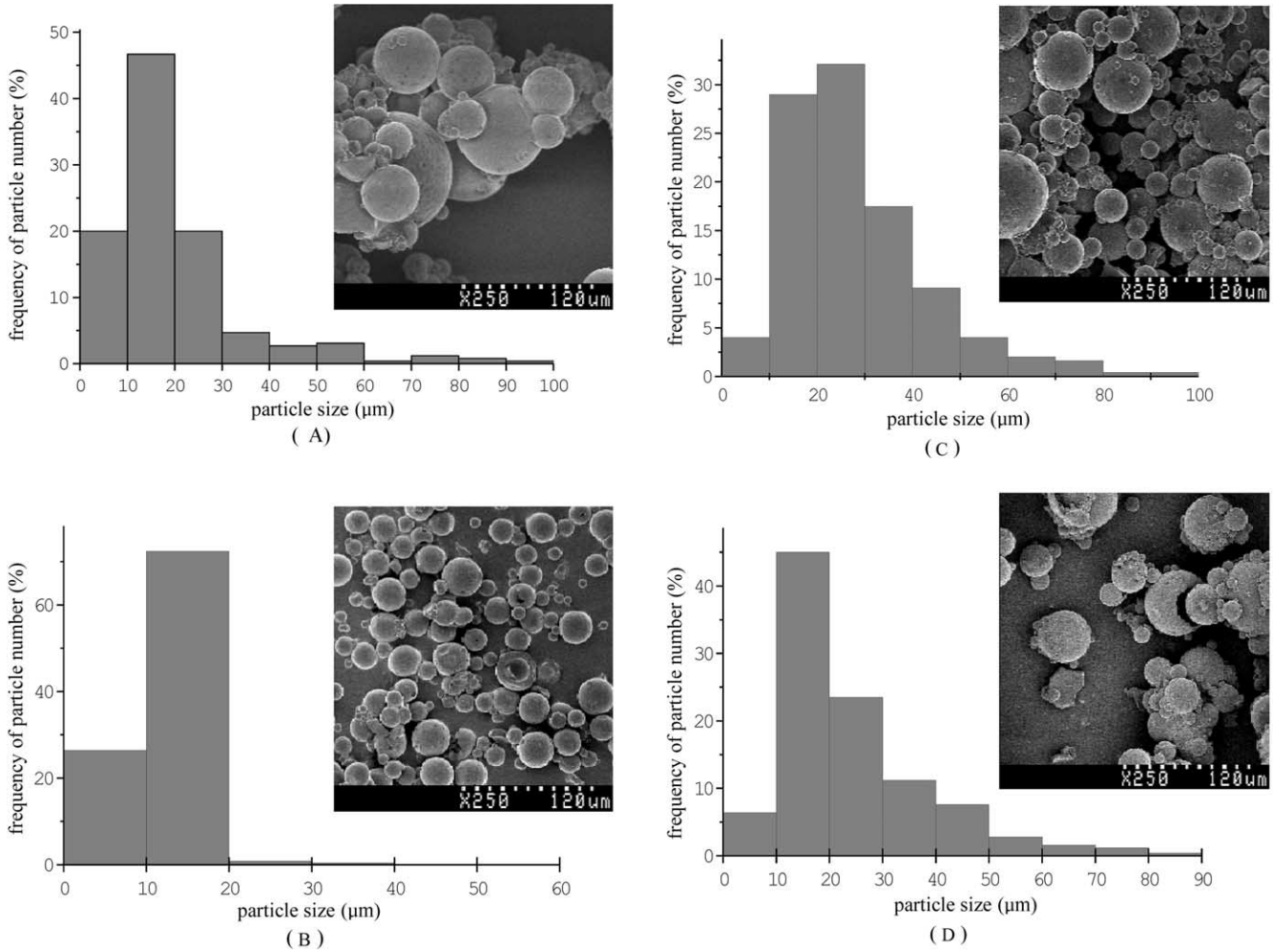
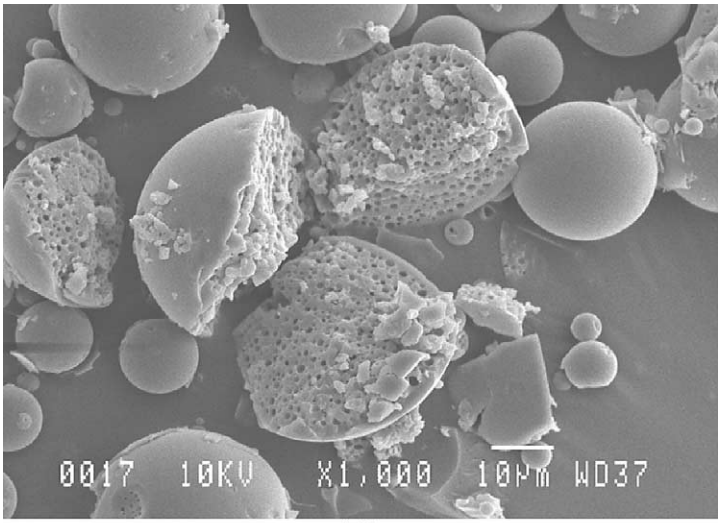
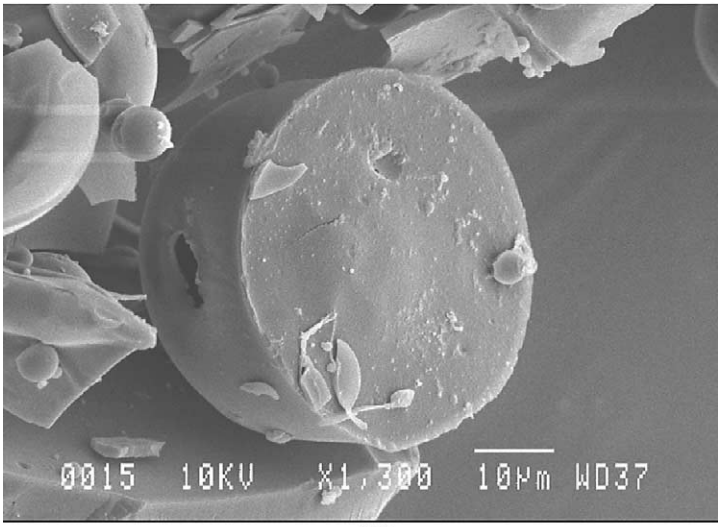


Fig. 1. Particle size distribution and scanning electron photomicrographs of preparations.



(A)



(B)

Fig. 2. Scanning electron photomicrographs of cross sections of preparations E and G.

3.3. Comparing TNP-470 release from TNP-DDS (E) and the control (G) in vitro

TNP-DDS (E) had a larger particle diameter and a higher content of TNP-470 than the other TNP-DDSs, as shown in Table 1. The remaining amount of TNP-470 in TNP-DDS (E) and the control (G) in the in vitro release test in physiological saline at 37 °C, are shown in Fig. 4. After 2 weeks, the rates of recovery of TNP-470 from TNP-DDS (E) and the control (G) were about 20%.

The released amount of TNP-470 from TNP-DDS (E) and the control (G), was measured in physiological saline at 37 °C (Fig. 5). The release of TNP-470 from both TNP-DDS (E) and the control (G) increased for about 12 h and then decreased. TNP-470 release from TNP-DDS (E) was still detected after about 2 weeks, but very little TNP-470 was detected from the control (G) after 5 days.

4. Discussion

4.1. Effect of the composition ratio on the TNP-DDSs

The different results in TNP-470 amount, the average particle diameter and its distribution, are attributed to the large difference in viscosity of DCM solution with a change of the composition. The recovery ratio and the amount of TNP-470 was greatest in preparation G, because a certain amount of MCTG containing TNP-470 leaked out with the DCM into the aqueous PVA solution from the microspheres. Therefore, the composition ratio has an important effect in controlling the characteristics of microspheres.

Furthermore, the results of the cross-section examination for preparations E and G (Fig. 2) showed that preparation E has a porous structure. As preparation G had no MCTG and no porous structure, it is supposed that the MCTG contain-

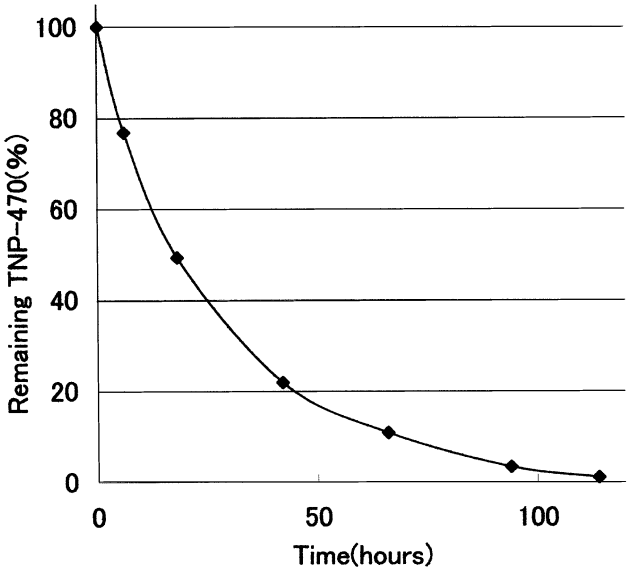


Fig. 3. Decomposition of TNP-470 in physiological saline at 37 °C.

ing TNP-470 was dispersed uniformly inside the microspheres in preparation E.

4.2. Comparison of TNP-470 releases in TNP-DDS (E) and the control (G)

Both TNP-DDS (E) and the control (G) retained TNP-470 for approximately 2 weeks in physiological saline at 37 °C, and there was no significant difference in the retained TNP-470 between those two samples (Fig. 4). It has been reported that TNP-470 in oleaginous formulation is hydrolyzed quickly in the buffer solution. However, the hydrolysis of TNP-470 was retarded by entrapping with PLA in TNP-DDS (E) and the control (G). It is supposed that water could not so easily access the TNP-470 enveloped

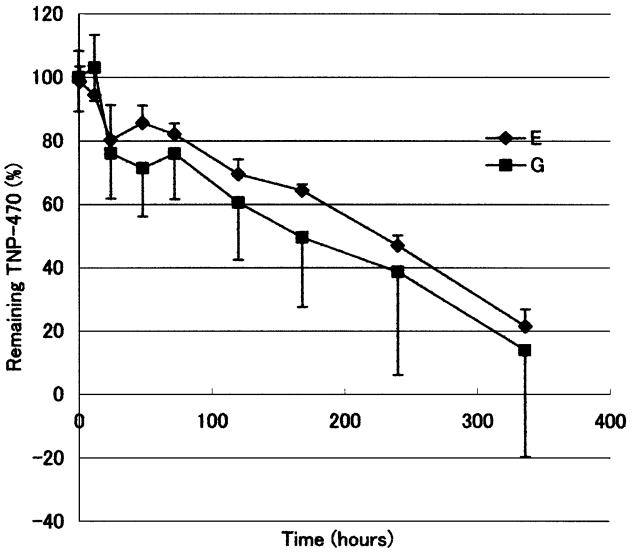


Fig. 4. TNP-470 retention in TNP-DDS (E) and the control (G) in vitro release test ($n = 3$).

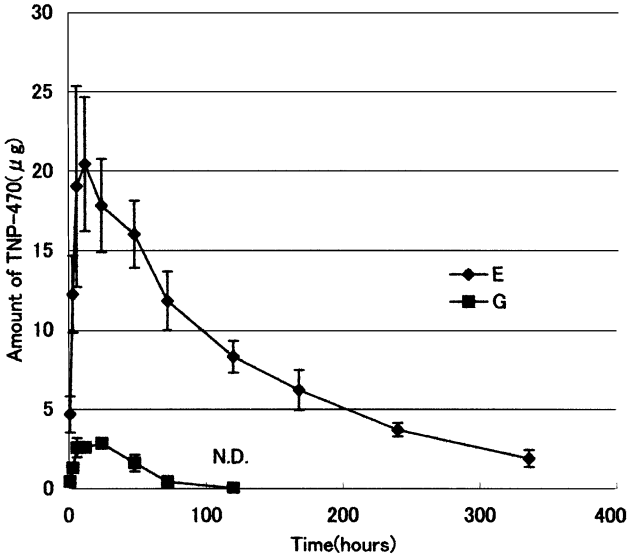


Fig. 5. In vitro release test of TNP-470 from TNP-DDS (E) and the control (G) in physiological saline at 37 °C.

by fat and oil molecules. Furthermore, the released amount of TNP-470 from TNP-DDS (E) was much larger and the release period was much longer than the control (G). TNP-470 was not detected after 120 h in the control (G) (Fig. 5) and the half-life of TNP-470 was very short (Fig. 3). It is probable that TNP-470 release occurred only in the initial stages from the control (G).

Although TNP-DDS (E) retained almost the same amount of TNP-470 as the control (G), the released amount and the release period of TNP-470 were obviously superior in TNP-DDS (E). The lower released amount in the control (G) was attributed to the lack of porous structure, and long-term release was difficult without MCTG. In the control (G), the remaining TNP-470 gradually decreased with the permeation of water into the PLA particles and as the TNP-470 inside was hydrolyzed. On the other hand, in the TNP-DDS (E), TNP-470 remained and was released easily and in a stable fashion as TNP-DDS (E) had a porous structure due to the addition of MCTG. The porous structure of TNP-DDS (E) promoted the release of TNP-470, and TNP-470 was protected from hydrolysis by the presence of MCTG. It is concluded that the present system is very effective and enhanced releasing of unstable drugs such as TNP-470.

5. Conclusion

TNP-470 was stably entrapped and released over a period of 2 weeks in the in vitro test from a new microsphere system using MCTG and PLA. These effects are attributed to the porous structure to promote the release and uniform distribution of MCTG to protect the TNP-470 in the DDS. These results suggest the system has a possibility of applying to the clinic.

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