Journal of Controlled Release, 28 (1994) 121–129

COREL 00906

SSDI 0168-3659(93)E0090-3

Drug delivery using biodegradable microspheres

Hiroaki Okada, Masaki Yamamoto, Toshiro Heya, Yayoi Inoue, Shigeru Kamei, Yasuaki Ogawa and Hajime Toguchi

DDS Research Laboratories, Takeda Chemical Industries, Ltd., Yodogawa, Osaka, Japan

Received 17 March 1993; accepted in revised form 9 July 1993

Rational delivery systems for leuprorelin acetate, a potent LHRH agonist, have been achieved by developing a microsphere system using biodegradable polymers, poly(lactic/glycolic acid) (PLGA) and polylactic acid, which sustainedly release the drug depending on the biodegradation of polymer used and persistently suppress steroidogenesis for over one and 3 months, respectively, following a single injection. To produce these systems we established a novel microencapsulation technique, the in-water drying method, and microspheres with a high trap ratio and small initial burst were obtained. A microsphere system of TRH prepared using PLGA could also continuously release the drug for 2 or 4 weeks. Using these systems effectively reduced the required dose compared with that needed with daily injection due to more continuous receptor hits on the target organs and could improve patient compliance. Chemoembolization using PLGA microspheres containing an angiogenesis inhibitor, TNP-470, resulted in dramatic regression of VX-2 carcinoma in rabbits. The microsphere system using biodegradable polymers is very useful in designing controlled release delivery and targeted delivery to attain potent and rational therapy.

Key words: Biodegradable microsphere; Long-term release; Leuprorelin acetate; TRH; TNP-470

Introduction

Drug delivery systems using various kinds of biodegradable polymers have been extensively studied [1–4]. Successful commercialization can be seen in the development of depot-forms of LHRH agonists using biodegradable copoly(lactic/glycolic acid) (PLGA) for the treatment of advanced prostatic cancer and endometriosis. Exacerbation in these diseases is highly dependent on the blood levels of testosterone or estradiol, and the suppression of these sex hormone levels by constant blockage of LHRH receptors with an LHRH agonist depot has been proved effective.

One such depot is Lupron Depot, once-monthly injectable microspheres containing leuprorelin acetate (leuprolide acetate), the first highly potent LHRH agonist which was synthesized by Dr Fujino and his colleagues in 1973 [5]. Poly(lactic/glycolic acid) is a biocompatible and biodegradable polymer which has been used for some years as surgical sutures. We synthesized various kinds of PLGA and polylactic acid (PLA) and determined the degradation rate in the rat subcutis to select an appropriate polymer for a 1-month depot formulation [6]. A

Correspondence to: Dr Hajime Toguchi, DDS Research Laboratories, Pharmaceutical Research Division, Takeda Chemical Industries, Ltd.,2-17-85 Jusohonmachi, Yodogawa, Osaka 532, Japan, Tel. (81-6)300-6221.

method for preparation of microspheres of a highly water soluble peptide with a high trap ratio and small initial burst was investigated, and we attained a novel preparation method using in-water drying through a water/oil/water emulsion [7,8]. A single injection of this depot formulation satisfactorily inhibits steroidogenesis and genital organ growth and causes regression of experimental endometriosis in rats as a result of sustained serum drug levels [9-14].

In this paper, formulation studies for the 1 and 3 months of leuprorelin acetate depots and further applications of the PLGA microsphere technology with TRH and TNP-470, a potent antiangiogenic substance, are reviewed.

Experimental methods

Materials and animals

Leuprorelin acetate, D-Leu^6-(des-Gly^{10}-NH_2)-LHRH ethylamide acetate, TRH and TNP-470, 6-*O*-(*N*-chloroacetylcarbamoyl)-fumagillol, were synthesized at Takeda Chemical Ind., Ltd. PLGA and PLA were synthesized in our laboratories or purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). Sprague-Dawley rats (8 and 10 weeks of age) were purchased from Clea Japan, Inc. (Tokyo, Japan). A female rabbit bearing VX-2 carcinoma and male rabbits (Kbl, JW) weighing around 2–2.5 kg were purchased from Funabashi Farm (Chiba, Japan) and Kitayama LABES (Kyoto, Japan), respectively.

Ovulation-inducing activity

The absorption of leuprorelin through various administration routes was estimated by ovulation-inducing activity in rats [15]. Ova in the ampulla of the isolated oviduct were examined microscopically in the morning between 9:30 and 11:30 the day after drug was administered at 14:30 on the day of diestrus. A saline solution of leuprorelin containing 0.1% bovine serum albumin and 20 U/ml of aprotinin was used for intravenous, subcutaneous, nasal and oral administration. For rectal and vaginal administration, drug was dispersed in an oleaginous base (WITEPSOL) without and with 10% citric acid, an absorption enhancer.

Preparation of microspheres

Leuprorelin and TRH

Microspheres containing leuprorelin or TRH were prepared by the in-water drying method as described previously [8,13,16,17]. In brief, an aqueous solution of leuprorelin or TRH was mixed with a dichloromethane solution of PLGA or PLA and agitated vigorously with a homogenizer. The resulting W/O emulsion was then dispersed in a 0.25% polyvinyl alcohol aqueous solution to form a W/O/W emulsion. The dichloromethane was subsequently evaporated off under stirring with a turbine-shaped mixer, leading to drug encapsulation. The resulting microspheres were sieved to remove large particles and lyophilized.

TNP-470

TNP-470 was dissolved in a 50% dichloromethane solution of PLGA (lactic/glycolic molar ratio, 3/1; M_r 6800) containing 0.25% dipalmitoyl phosphatidylcholine [18,19]. The solution was poured into a 0.25% aqueous solution of polyvinyl alcohol to form a O/W emulsion. The subsequent procedures were the same as those for leuprorelin and TRH.

In vitro drug release

Drug (leuprorelin and TRH) released from microspheres was determined by the rotating bottle procedure using a RT-5 rotator (Taiyo Scientific Industrial Co., Tokyo, Japan) at 37 ± 1 °C. The microspheres (50 mg) were suspended in 10 ml of a release medium consisting of pH 7.0, 1/30 M phosphate buffer containing Tween 80 (leuprorelin, 0.05%; TRH, 0.02%). The residual drug in the microspheres was periodically determined after filtering the microspheres through a Millipore filter (1.2 μm).

Leuprorelin content in microspheres

Microspheres (50 mg or less) were dissolved in 10 ml of dichloromethane and extracted with 20 ml of pH 6.0, 1/30 M phosphate buffer. The amount of leuprorelin in the buffer layer was assayed using HPLC (Shimazu LC-3A, Kyoto, Japan). HPLC conditions were: column, Lichrosorb RP-18 (250 mm in length, 4 mm i.d.); column temperature, 30°C; mobile phase, 0.25 M ammonium acetate/methanol (1/1.5); flow rate, 0.8 ml/min; and wavelength, 280 nm.

TRH content in microspheres

The extraction procedure was the same as that used for leuprorelin. HPLC conditions (Shimazu LC-5A) were: column, Zolbax ODS (250 mm in length, 4.6 mm i.d.); column temperature, room temperature; mobile phase, a mixture of 20 ml of acetonitrile and 300 ml of 1/30 M phosphate buffer, pH 6.7; flow rate, 0.8 ml; and wavelength, 215 nm.

In vivo drug release

To estimate the in vivo drug release, the amount of drug remaining at the injection site after microspheres were administered subcutaneously (into the dorsal cervical site) or intramuscularly (into the femoral muscle) to male rats was determined. The excised microspheres, surrounded by a thin layer of connective tissue, were homogenized in 10 ml of pH 6.0, 1/30 M phosphate buffered saline (PBS) containing 0.02% Tween 80 with a Polytrone (Kinematica GmbH, Luzern, Switzerland) and extracted by adding an additional 10 ml of the same PBS and 10 ml of dichloromethane. After the homogenate was centrifuged at 3000 rpm for 15 min, the drug content in the aqueous layer was analysed by HPLC using the same conditions as those for the in vitro drug release.

RIA of serum leuprorelin and testosterone

Serum levels of leuprorelin were determined in duplicate by the double antibody radioimmunoassay (RIA) method as previously described [9]. Serum testosterone was measured in duplicate using commercially available RIA kits (Green Cross Co., Osaka, Japan) utilizing the single antibody and dextran-charcoal method after ether extraction. The sensitivity limit of the assay was 5 pg of leuprorelin acetate and 6.25 pg of testosterone.

Anticancer effects of TNP-470 microspheres

VX-2 carcinoma cell suspension (10%, 0.5 ml) was injected subcutaneously at a position on the inside of the right leg just below the knee of a male rabbit (Kbl, JW) [18,19]. Two weeks after inoculation, the tumor had grown to 10–20 mm in length. To cause the embolization of arteries running to the tumor, TNP470 microspheres were injected into the femoral artery through polyethylene tubing under pentobarbital anesthesia. After administration the blood flow in the femoral artery was reopened by inserting the tubing upward into the artery. The tumor size, length, width and height, was periodically measured through the skin with calipers for 14 days.

Results and discussion

Leuprorelin microspheres

Chronic application of a potent LHRH agonist like leuprorelin acetate (Fig. 1) paradoxically down-regulates the pituitary, decreasing the secretion of LH and FSH and eventually suppressing steroidogenesis of sex-hormones like testosterone and estradiol, resulting in so called 'chemical castration'. This chemical castration has been proved effective in treating advanced hormone-dependent cancers, prostate and breast cancer, and other sex-hormone dependent disorders, endometriosis and uterine fibroids.

To develop a convenient dosage form of leuprorelin for patients we started work on new delivery systems. First we checked the relationship between the ovulation-inducing activity of leuprorelin in rats and various administration routes, namely, oral, nasal, rectal, vaginal, subcutaneous and intravenous administration [15].

5-oxo-Pro-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
1 2 3 4 5 6 7 8 9 10

LHRH

5-oxo-Pro-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NH-C_2H_5
1 2 3 4 5 6 7 8 9

Leuprorelin / Leuprolide
(Leuprorelin acetate: m.w.= 1269.5)

5-oxo-Pro-His-Pro-NH_2
1 2 3

TRH
(m.w.= 364.2)

Fig. 1. Amino acid sequences of LHRH, leuprorelin acetate and TRH.

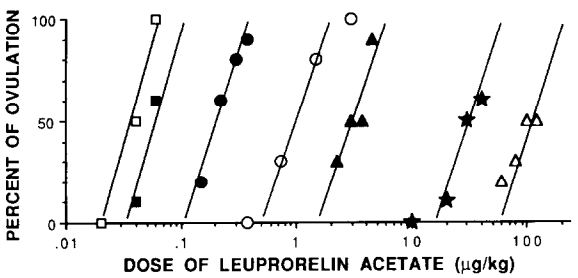


Fig. 2. Ovulation-inducing activity of leuprorelin acetate after intravenous (□), subcutaneous (■), oral (△), rectal (▲), nasal (★) and vaginal (○, without; ●, with 10% citric acid) administration to diestrous female rats.

The ovulation-inducing activity is a good index for bioavailability. As all routes other than intravenous or subcutaneous routes showed poor bioavailability (Fig. 2), we focused on injections and decided to develop a microsphere type depot that would provide constant release of the drug over one month using a biodegradable polymer, PLGA, as the release controlling material. In the body PLGA decomposes slowly to yield lactic acid and glycolic acid.

To obtain microspheres with the desired properties, we adopted the in-water drying method. Very strict harmonization of the formulation, manufacturing devices and manufacturing conditions was required to avoid obtaining deformed or empty microspheres [8]. After in vitro and in vivo polymer screening tests, we decided to use a PLGA with a molecular weight of 14 000 and a lactic/glycolic acid ratio of 3/1 (Fig. 3) [6].

Measurement of distribution of leuprorelin in the microspheres using a field emission SEM revealed that tritium-labelled leuprorelin localized on the surface of the inner pores which were supposedly formed by the evaporation of water from the inner water phase. NMR spectroscopy showed the existence of an ionic interaction between carboxylic acids at the end of PLGA and arginyl and histidyl residues of leuprorelin. Furthermore, viscosity of the W/O emulsion and glass transition temperature of the microspheres were elevated with an increase in peptide loading [20]. Judging from these results, we assume that leuprorelin drug cores in the microspheres should be surrounded by the hydrophobic barriers of the polymer carbohydrate chains, a micelle-like conformation as shown in Fig. 4 [20]. This structure may explain part of the reason that

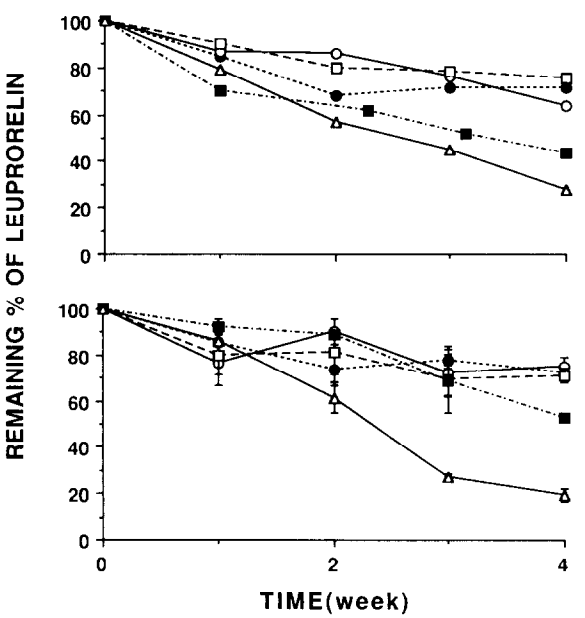


Fig. 3. In vitro (A) and in vivo (B) release of leuprorelin from PLA and PLGA microspheres ●, PLA-22000; ○, PLA-12000; □, PLGA(90/10)-21000; ■, PLA-6000; △, PLGA(75/25)-14500.

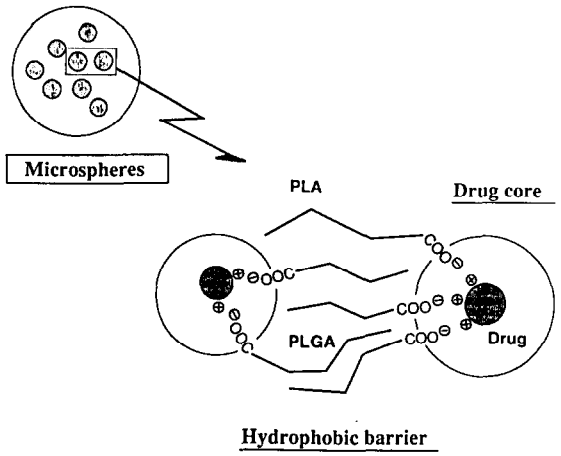


Fig. 4. Hydrophobic diffusion barrier in the PLGA and PLA microspheres of peptide drugs.

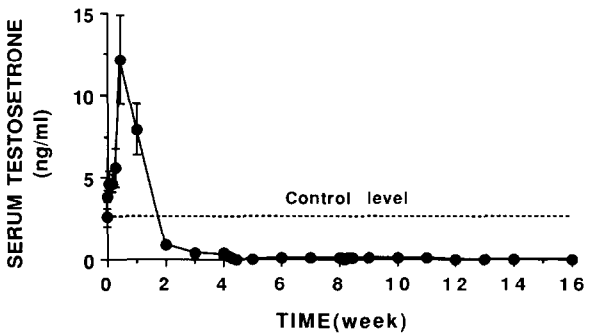


Fig. 5. Serum testosterone in dogs after repeated subcutaneous injection of leuprorelin PLGA microspheres (one-month depot, 50 g/kg/day) (mean ± S.E., $n=5$).

a very water soluble substance like leuprorelin is effectively entrapped during the in-water drying process and that the release of leuprorelin from the microspheres could be controlled dominantly by degradation of the polymer for long periods avoiding a large initial burst.

When the microspheres were administered to beagle dogs at 4-week intervals for a total of three injections, serum testosterone levels were completely suppressed after a 2-week initial rise and no escape was seen (Fig. 5) [11]. A single injection of the microspheres (100 μ g/kg/day) sufficiently inhibited steroidogenesis and consequently suppressed genital organ growth in male rats for over one month [10]. In the female rat study, we revealed that an injection of this depot suppressed serum levels of LH, FSH and estradiol for more than 4 weeks and caused a dramatic regression of growth of experimental endometriosis [9]. These results encouraged our belief that this one-month depot formulation would be potentially useful in the therapy of prostate cancer and endometriosis in human beings. Following these satisfactory results in animal preclinical studies, we proceeded to clinical studies. In the studies carried out in the United States, sustained serum levels of leuprorelin for over one month were obtained after a single injection (Fig. 6) [21]. The serum testosterone suppression pattern with the 1 mg daily injection and that with the 7.5 mg depot overlapped almost perfectly. This means using the 7.5 mg depot can effectively reduce the dose to 1/4 of that required with daily injection.

We are now developing a depot preparation which will release leuprorelin for a longer dura-

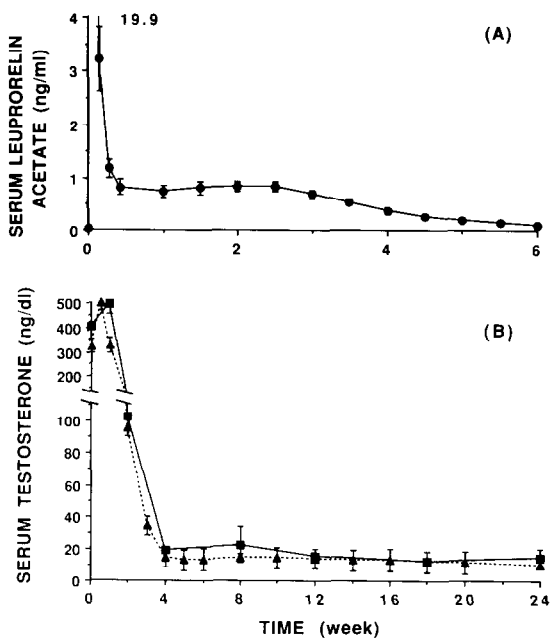


Fig. 6. Plasma leuprorelin acetate (A) and serum testosterone (B) in humans after intramuscular injection of the PLGA microspheres (mean ± S.E., $n=53$) ●, microspheres (7.5 mg); ■, solution (daily subcutaneously, 30 mg/month); ▲, microspheres (every 4 weeks, 7.5 mg).

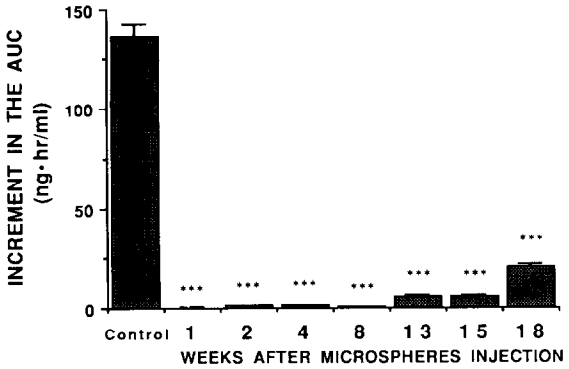


Fig. 7. Increment in the AUC of serum testosterone for 8 h in rats following challenge with leuprorelin solution (100 μg/kg) at different weeks after intramuscular injection of leuprorelin PLA microspheres (3-month depot, 100 μg/kg/day) (mean ± S.E., $n=5$); *** $P<0.001$.

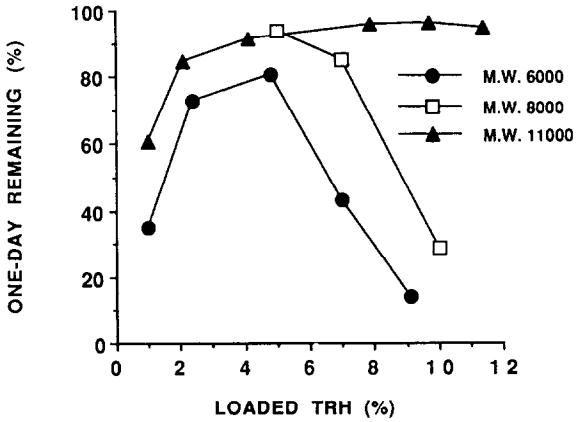


Fig. 8. Effect of molecular weight of PLGA on initial release (in vitro) from TRH PLGA microspheres with different levels of drug loading.

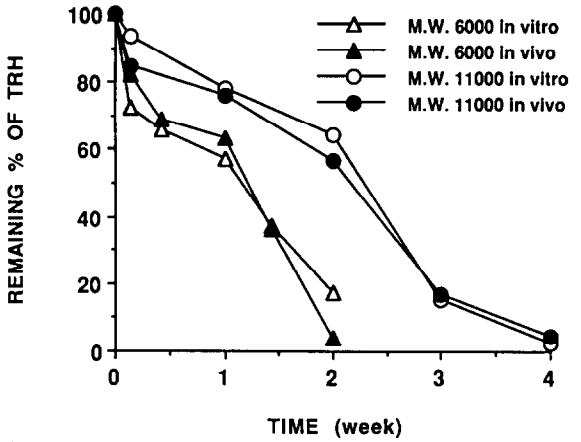
tion. Figure 7 shows the increment in the AUC of serum testosterone for 8 h in rats following challenge with leuprorelin aqueous solution at different weeks after intramuscular injection of the new 3-month depot [16]. Testosterone secretion following challenge was sufficiently suppressed for longer than 13 weeks.

TRH Microspheres

TRH (Fig. 1) enhances physiological activities of the brain besides stimulating the release of TSH and daily intravenous injection of TRH aqueous solution is used to treat disturbances of consciousness caused by head injury and hemorrhage. The efficacy of TRH for the treatment of spinocerebellar degeneration, an incurable disease, has also been investigated. The response to bolus intravenous injection, however, was not satisfactory. Therefore, to enhance the activity of TRH we investigated the development of a depot formulation using the PLGA microsphere technique.

First we studied the relationships among the loading amount of TRH, the entrapment ratio of the drug into the microspheres and the one-day release of the drug from the microspheres in the in vitro release test [17,22]. In Fig. 8, the percentage remaining after one day is plotted vs TRH loading using PLGA of different molecular weights. At a low level of drug loading (1%) a large initial burst was observed at all molecular weights of the polymer and the loading range of 5–7% was found to produce a minimal initial burst. When TRH tartrate salt was used, the amount remaining after one day was zero regardless of the loading amount. From these results, we assumed that ionic interaction between the polymer and the drug, as seen in the leuprorelin microspheres (Fig. 4), plays an important role both in efficient drug entrapment and decreasing the initial burst and that a certain amount of the peptide is necessary for the formation of the rigid structure of the microspheres. Tartaric acid possibly competes with the carboxylic acid of the polymer as the counter ion for the basic moiety of TRH, increasing the amount not bound to PLGA. Choosing an appropriate PLGA, we can control the release period of TRH for 2 and 4 weeks (Fig. 9).

Items where amelioration or enhancement was seen in the pharmacological responses in rats and mice were (1) antagonism of barbitone-induced sleeping in rats, (2) amelioration of memory and learning impairment and abnormal behavior such as low food neophobia and (3) enhancement of the metabolism of acetylcholine and monoamines in the brain and glucose utilization in the brain [23,24]. For these pharmacological responses, the efficacy of TRH was increased 10-



1
Fig. 9. In vitro and in vivo release profiles of TRH PLGA microspheres.

fold or more compared with that after repeated injection of TRH. Clinical studies for the use of the TRH depot formulation in the treatment of senile dementia are under way.

Chemoembolization with microspheres containing TNP-470

Chemoembolization is a potential cancer treatment that combines chemotherapy and embolization of the blood vessels that supply nutrients to the tumor. The advantage of this treatment is high efficacy with less systemic side effects due to the regional elevation of drug concentration and the selective blockage of nutrient supply to the tumor. For the rapid growth of a tumor, angiogenesis, the formation of new capillaries, is essential. Chemoembolization using conventional chemotherapeutic agents cannot inhibit the formation of the development of collateral circulation which could bypass the arteries occluded by embolization. Theoretically, it is obvious that if we can embolize the blood vessels of the tumor with microspheres which sustainedly release an anti-angiogenic substance, the formation of bypassing circulation would be inhibited, resulting in strong anti-tumor activity.

TNP-470, a fumagillin derivative (Fig. 10), has strong antiangiogenic activity [25]. We prepared PLGA microspheres containing TNP-470

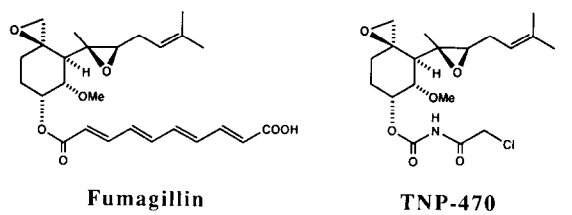


Fig. 10. Chemical structures of fumagillin and TNP-470

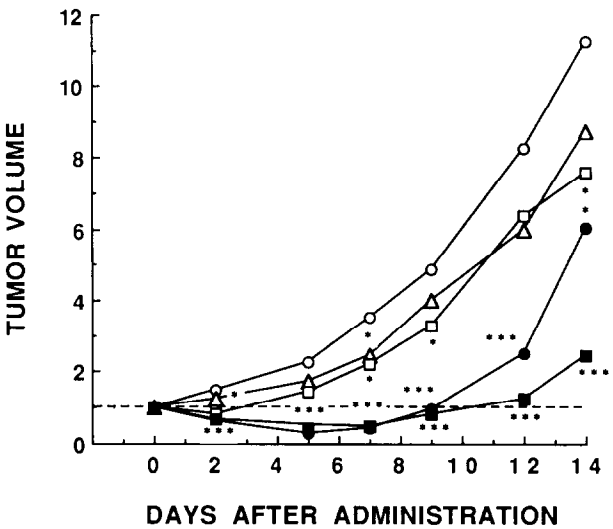


Fig. 11. Anticancer activity after a single intra-arterial injection of TNP-470 microspheres in rabbits bearing VX-2 carcinoma ○, untreated control ($n=16$); △, TNP-470 aqueous solution ($n=3$); □, placebo microspheres ($n=4$); ●, 0.95 mg TNP-470 microspheres ($n=5$); ■, 3.8 mg TNP-470 microspheres ($n=4$).

and determined the efficacy in rabbits bearing VX-2 squamous cell carcinoma. Compared with the untreated control, placebo microspheres and TNP-470 solution caused only slight inhibition of the tumor growth, whereas TNP-470 microspheres caused striking inhibition (Fig. 11) [18,19]. Synergistic effects were obtained when an aqueous solution of doxorubicin was co-administratered with TNP-470 microspheres.

As a preliminary study of the drug distribution, the concentration of tritium in the tissues was measured in normal rats given an intra-hepatic arterial dose of microspheres containing tritium-labelled TNP-470 or an aqueous solution of tritium-labelled TNP-470. Following in-

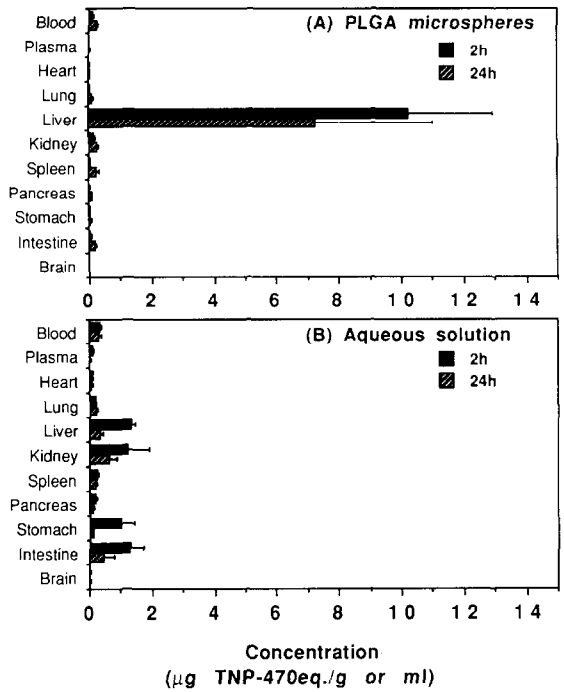


Fig. 12. TNP-470 concentrations in various tissues of rats after a single injection into the hepatic artery (1 mg/kg, mean ± S.D., $n=4$).

jection of the microspheres, the liver showed high radio-activity at 2 and 24 h, suggesting less systemic side effects (Fig. 12).

In summary, we can see that drug delivery using biodegradable microspheres provides potential methods for treating various diseases, and we are looking at additional applications of this microsphere technique with other peptides, proteins and chemical drugs.

References

1 A.D. Schwope, D.L. Wise and J.F. Howes, Lactic/glycolic acid polymers as narcotic antagonist delivery systems, Life Sci., 17 (1975) 1877–1886.

2 D.L. Wise, H. Rosenkrantz, J.B. Gregory and H.J. Esber, Long-term controlled delivery of levonorgestrel in rats by means of small biodegradable cylinders, J. Pharm. Pharmacol., 32 (1980) 399–403.

3 L.M. Sanders, J.S. Kent, G.I. McRae, B.H. Vickery, T.R. Tice and D.H. Lewis, Controlled release of a luteinizing hormone-releasing hormone analogue from poly(D,L-lactide-co-glycolide) microspheres, J. Pharm. Sci., 73 (1984) 1294–1297.

4 T.W. Redding, A.V. Schally, T.R. Tice and W.E. Meyers, Long-acting delivery systems for peptides: inhibition of rat prostate tumors by controlled release of [D-TRP^6]luteinizing hormone-releasing hormone from injectable microcapsules, Proc. Natl. Acad. Sci. USA, 81 (1984) 5845–5848.

5 M. Fujino, T. Fukuda, S. Shinagawa, S. Kobayashi, I.Yamazaki, R. Nakayama, J.H. Seely, W.F. White and R.H.Rippel, Synthetic analogs of luteinizing hormone releasing hormone (LH-RH) substituted in position 6 and 10, Biochem. Biophys. Res. Commun., 60 (1974) 406–413.

6 Y. Ogawa, H. Okada, M. Yamamoto and T. Shimamoto, In vivo release profiles of leuprolide acetate from microcapsules prepared with polylactic acids or copoly(lactic/glycolic) acids and in vivo degradation of these polymers, Chem. Pharm. Bull., 36 (1988) 2576–2581.

7 H. Okada, Y. Ogawa and T. Yashiki, Prolonged release microcapsules and its production, US Patent, No. 4,652,441(1987).

8 Y. Ogawa, M. Yamamoto, H. Okada, T. Yashiki and T. Shimamoto. A new technique to efficiently entrap leuprolide acetate into microcapsules of polylactic acid or copoly(lactic/glycolic) acid, Chem. Pharm. Bull., 36 (1988) 1095–1103.

9 H. Okada, T. Heya, Y. Ogawa and T. Shimamoto, One-month release injectable microcapsules of a luteinizing hormone-releasing hormone agonist (leuprolide acetate) for treating experimental endometriosis in rats, J. Pharmacol. Exp. Ther.,244 (1988) 744–749.

10 H. Okada, T. Heya, Y. Igari, Y. Ogawa, H. Toguchi and T. Shimamoto, One-month release injectable microspheres of leuprolide acetate inhibit steroidogenesis and genital organ growth in rats, Int. J. Pharm., 54 (1989) 231–239.

11 Y. Ogawa, H. Okada, T. Heya and T. Shimamoto, Controlled release of LHRH agonist, leuprolide acetate, from microcapsules: serum drug level profiles and pharmacological effects in animals. J. Pharm. Pharmacol., 41 (1989) 439–444.

12 H. Toguchi, Pharmaceutical manipulation of leuprorelin acetate to improve clinical performance, J. Int. Med. Res.,18 (1990) 35–41.

13 H. Okada, T. Heya, Y. Ogawa, H. Toguchi and T. Shimamoto, Sustained pharmacological activities in rats following single and repeated administration of once-a-month injectable microspheres of leuprolide acetate, Pharm. Res., 8 (1991) 584–587.

14 H. Okada, Y. Inoue, T. Heya, H. Ueno, Y. Ogawa and H.Toguchi, Pharmacokinetics of once-a-month injectable microspheres of leuprolide acetate, Pharm. Res., 8 (1991) 787–791.

15 H. Okada, I. Yamazaki, Y. Ogawa, S. Hirai, T. Yashiki and H. Mima, Vaginal absorption of a potent luteinizing hormone-releasing hormone analog (leuprolide) in

rats. I. Absorption by various routes and absorption enhancement, J. Pharm. Sci.,71 (1982) 1367–1371.

16 H. Okada, Y. Inoue, Y. Ogawa and H. Toguchi, Three-month release injectable microspheres of leuprorelin acetate, Proc. Int. Symp. Control. Rel. Bioact. Mater., 19 (1992) 52–53.

17 T. Heya, H. Okada, Y. Tanigawara, Y. Ogawa and H. Toguchi, Effects of counteranion of TRH and loading amount on control of TRH release from copoly(DL-lactic/glycolic acid) microspheres prepared by an in-water drying method, Int. J.Pharm., 69 (1991) 69–75.

18 H. Okada, S. Kamei, T. Yoshioka, Y. Inoue, Y. Ogawa and H.Toguchi, Anticancer effects of chemoembolization using microspheres of an angiogenesis inhibitor (TNP-470) in rabbits bearing VX-2 carcinoma, Drug Del. Syst. 7(1992) 97–102.

19 S. Kamei, H. Okada, Y. Inoue, T. Yoshioka, Y. Ogawa and H.Toguchi, Antitumor effects of angiogenesis inhibitor TNP-470 in rabbits bearing VX-2 carcinoma by arterial administration of microspheres and oil solution, J. Pharmacol. Exp. Ther.,264 (1993) 469–474.

20 H. Okada, T. Heya, Y. Inoue, M. Yamamoto, Y. Ogawa and H.Toguchi, Sustained release injection of therapeutic peptides for long periods, Polymer Preprints, Japan, 41 (1992) 3343–3345.

21 R. Sharifi, M. Soloway, the Leuprolide Stydy Group, Clinical study of leuprolide depot formulation in the treatment of advanced prostate cancer, Fertil. Steril., 54 (1990) 68–71.

22 T. Heya, H. Okada, Y. Ogawa and H. Toguchi, Factors influencing the profiles of TRH release from copoly(DL-lactic/glycolic acid) microspheres, Int. J. Pharm., 72 (1991) 199–205.

23 T. Hashimoto, T. Wada, N. Fukuda and A. Nagaoka, Effect of thyrotropin-releasing hormone on pentobarbitone-induced sleep in rats: continuous treatment with a sustained release injectable formulation, J. Pharm. Pharmacol., 45 (1993) 94–97.

24 M. Miyamoto, K. Hirai, H. Takahashi, K. Kato, M. Nishiyama, H. Okada and A. Nagaoka, Effects of sustained release formulation of thyrotropin-releasing hormone on learning impairments caused by scopolamine and AF64A in rodents, Eur. J. Pharmacol., 238 (1993) 181–189.

25 D. Ingber, T. Fujita, S. Kishimoto, K. Sudo, T. Kanamaru, H. Brem and J. Folkman, Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth, Nature, 348(1990) 555–557.