ELSEVIER

International Journal of Pharmaceutics 142 (1996) 25–32

international journal of pharmaceutics

Solid tumor chemotherapy using injectable gelatin microspheres containing free methotrexate and conjugated methotrexate

R. Narayani, K. Panduranga Rao*

Biomaterials Laboratory, Central Leather Research Institute, Adyar, Madras 600 020, India

Received 2 January 1996; revised 5 June 1996; accepted 7 June 1996

Abstract

Biodegradable gelatin microspheres containing free methotrexate (MTX) (GMFM) and gelatin microspheres containing MTX covalently conjugated to gelatin using carbodiimide (GMCM-I) and two types of azide coupling–grafting methods (GMCM-II and GMCM-III) have been prepared and evaluated for their efficacy towards the solid tumor fibrosarcoma in Wistar rats. The tumor was induced by inoculation of a 10% tumor cell suspension in the anterior aspect of the hind limb. Seven groups were studied—untreated control, intratumoral (i.t.) injection of free MTX, i.t. injection of placebo microspheres, i.t. injection of GMFM, i.t. injection of GMCM-I, i.t. injection of GMCM-II and i.t. injection of GMCM-III of mean particle size 10–20 μm. The gelatin-microspheres-entrapped MTX showed an improved antitumor activity over free MTX as evidenced by the gross tumor weight assessments and [^{3}H]thymidine incorporation studies in vitro. This was attributed to the slow and controlled release of MTX from gelatin microspheres, which was more effective compared with free MTX given over the same period of treatment. The drug release from microspheres is due to gradual degradation of the spheres and concomitant diffusion of drug into the external medium. GMCM showed higher antitumor activity than GMFM. The antitumor activity of the various GMCM preparations was of the order: GMCM-I > GMCM-II > GMCM-III. The injectable MTX-containing gelatin microspheres can thus be a potential alternative to conventional therapy using free MTX in the treatment of solid tumors such as fibrosarcoma.

Keywords: Gelatin microspheres; Tumor; Chemotherapy; Free methotrexate; Conjugated methotrexate

1. Introduction

A widespread occurrence of malignant tumors and their poor response to the current methods of treatment call for novel and new strategies for delivering anticancer drugs (Gallo et al., 1989;

* Corresponding author.

PII S0378-5173(96)04650-9

Ghorab et al., 1990; Ike et al., 1992; Hassan and Gallo, 1993; Arshady, 1993). One of the major limitations of cancer treatment is the indiscriminate toxicity of anticancer drugs towards cancer cells and normal proliferating cells. Hence, a high local concentration of the drug at the tumor site with minimal systemic spread is required to obtain maximum antineoplastic activity. The present challenge in cancer chemotherapy is to develop drug delivery systems which will deliver selectively to the neoplastic cells and release the drug in a controlled manner.

Among the colloidal particulate delivery systems, microspheres and liposomes have featured in many investigations concerning controlled delivery of anticancer drugs. However, the stability of liposomes in the blood stream and their rapid clearance pose major problems. Nontheless, the more recently designed 'stealth' liposomes are proving useful because of their reduced recognition and uptakes by the immune system. Reports that sterically stabilised liposomes-entrapped doxorubican was more effective in curing mice with implanted tumors when compared with free drug or conventional liposomes have appeared (Kim et al., 1994; Lilley et al., 1994). Another powerful approach for effective systemic chemotherapy is to use antibodies to target the delivery systems to the tumor site (Rowland et al., 1994; Balthasar and Fung, 1995). Although antibody-linked drug carriers have ability to target tumor sites, the number of drug molecules that can be linked to the antibodies is limited and also such linking may inactivate the drug or the antibody. In conventional therapy, the injected drug diffuses even to normal tissues and therefore only a small proportion of the drug reaches the malignant tissues. This necessitates injections of high doses of the drug repeatedly. Moreover, the exposure of normal cells to the anticancer drug will result in several toxic side-effects in the body. By contrast, the application of microspheres-entrapped drug will circumvent some of these problems of repeated systemic therapy by releasing therapeutically effective concentrations of the drug at regular time intervals for a prolonged time period. Exposure of healthy cells to the drug, wastage of drug and the need for repeated injections are thereby mitigated. The process is cost effective and also improves patient compliance. Therefore, the utilisation of polymeric microspheres as an injectable controlled-release system for anticancer drugs has been extensively studied (Kim and Oh, 1988; Jones et al., 1989; Goldberg, 1992; Cremers et al., 1995). In the last few years, we have directed considerable efforts towards the development of controlled-release systems for anticancer agents, namely mitomycin, bleomycin and 5-fluorouracil (Jeyanthi and Panduranga Rao, 1987, 1989). Methotrexate (MTX) is one of the most widely used drugs for the treatment of neoplastic diseases in humans. Recently, we have studied the preparation, characterisation and in vitro evaluation of gelatin microspheres containing free MTX (GMFM) (Narayani and Panduranga Rao, 1994). Many researchers have reported that macromolecular drug conjugates exhibited lesser toxicity and greater antitumor activity compared with free drug. Greater uptake of drug by macrophages was observed when presented as conjugates of macromolecules, and tumor cells exhibited a higher uptake of macromolecules by endocytosis than normal cells (Jeong and Kim, 1986). Therefore, conjugation of drugs to macromolecules provides a method for introducing substantial amounts of drugs to tumor cells and minimise systemic toxicity which would not be possible with the use of pure drug only. Many macromolecular carriers have been investigated as drug delivery systems for methotrexate (Chu and Howell, 1981; Ghosh et al., 1988; Kim and Oh, 1988; Naoji, 1992). The preparation, characterisation and in vitro stability of hydrophilic gelatin microspheres, using gelatin MTX conjugate with the carbodiimide coupling method and two types of azide coupling method were recently reported by us (Narayani and Panduranga Rao, 1992, 1996). In this paper, the antitumor activity of gelatin microspheres containing free MTX (GMFM) and gelatin microspheres containing conjugated MTX (GMCM) for the chemotherapy of a solid tumor fibrosarcoma in rats is discussed.

2. Materials and methods

2.1. Materials

MTX was a gift sample from Tamil, Nadu Dadha Pharmaceuticals, Madras, India. Gelatin (Oxoid, UK), glutaraldehyde (25%) (Fluka, Germany) potassium persulfate and sodium bisulphate (Loba, India) were used as obtained. Methylmethacrylate (Siso, India) was purified by distillation under reduced pressure. [^{3}H]-Thymidine (*methyl*-T) (high specific activity) was obtained from the Isotope Group, Bhaba Atomic Research Institute, Bombay, India. Scintillation grade naphthalene, dioxane, 2,5-diphenyl oxazole (PPO), 1-4-bis-(5-phenyloxazol-2-yl) benzene (POPOP) were obtained from Sisco Research Laboratory, India. All other chemicals used were of analytical grade.

2.2. Animals

Female albino Wistar rats weighing about 150 g were used in all the animal experiments. The rats were maintained on a standard Hindustan Lever Rat Feed pellet diet and water ad libitum.

2.3. Tumor cell line

Methylcholanthrene-induced rat fibrosarcoma tumor cell line was obtained from the Cancer Institute, Madras. The solid tumor cells could be propagated by inoculating the tumor cells subcutaneously once a week.

2.4. Preparation of gelatin microspheres containing free and conjugated MTX

Gelatin microspheres were prepared as reported earlier by us. Briefly the process is as follows: polymethylmethacrylate (PMMA) was prepared by polymerising distilled MMA using $K_2S_2O_8$–$NaHSO_3$ redox initiation technique in aqueous medium. Gelatin dissolved in phosphate buffer (pH 7.4) was dispersed using concentrated PMMA solution in organic medium. The microspheres were crosslinked with glutaraldehyde-saturated toluene solution. GMFM were prepared in the same manner, except that the drug in phosphate buffer pH 7.4 was added to gelatin solution before dispersing in PMMA. Gelatin was conjugated to MTX using 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) as coupling agent to link the amino group of gelatin to the carboxyl group of the drug in GMC-I. Similarly in GMCM-II, the carboxyl group of MTX was conjugated to the amino group of gelatin by the MTX azide coupling method and in GMCM-III, the carboxyl group of gelatin was conjugated to the amino group of the drug by the gelatin azide coupling method. GMCM-I were also prepared in a similar manner, by dispersing a solution of GMC-I/GMC-II/GMC-III in phosphate buffer (pH 7.4) using PMMA in organic medium. During addition of PMMA and the crosslinking agent, the drug-containing gelatin solution was stirred using a vortex mixer (Remi, India). By using appropriate concentration of PMMA, placebo microspheres GMFM and GMCM in the mean particle size range of 10–20 μm were obtained. The antitumor activity of GMFM and GMCM was tested in vivo for intratumoral chemotherapy of rat fibrosarcoma.

2.5. Induction of fibrosarcoma and evaluation of antitumor activity

Prior to treatment, rats were inoculated subcutaneously with 0.5 ml of 10% tumor cell suspension in sterile 0.9% NaCl (saline) in the anterior aspect of the hind limb. Treatment commenced after 8 days of inoculation when the tumor was palpable. In conventional therapy, the required dose of free anticancer drug has to be periodically injected. The superiority of controlled-delivery technology lies in the fact that the entire dosage can be injected as a single injection of microspheres containing the drug. Therefore in our treatment protocol, different schedules of administration for free MTX and microspheres-entrapped MTX were selected. The free MTX was administered repeatedly (i.e. three times) on days 8, 10 and 12 post inoculation (p.i.), whereas GMFM and GMCM containing MTX (equal to

the amount of free MTX given as three doses) was administered as a single injection on day 8 p.i. In each group, four animals were studied. The treatment protocol was as follows: (i) untreated control by intratumoral (i.t.) injection of saline on days 8, 10 and 12 p.i; (ii) i.t. injection of placebo microspheres on day 8 p.i; (iii) free drug (30 mg/kg) injected i.t. as three equally divided doses on days 8, 10 and 12 p.i; (iv) gelatin microspheres with free MTX (30 mg/kg) injected i.t. on day 8 p.i (single dose); (v) gelatin microspheres with conjugated MTX (GMC-I) (30 mg/kg) injected i.t. on day 8 (single dose); (vi) gelatin microspheres with conjugated MTX (GMC-II) (30 mg/kg) injected i.t. on day 8 (single dose); (vii) gelatin microspheres with conjugated MTX (GMC-III) (30 mg/kg) injected i.t. on day 8 (single dose). In all the seven groups studied, animals were assessed for weight change and tumor size.

2.6. Tumor size measurements

Tumor measurements were made using vernier callipers and two diameters at right angles to each other were recorded every day from the time of treatment (day 8) up to day 15 of tumor growth. Tumor weights (W) (g) were calculated using the formula (Geran et al., 1972).

$$W = a \times \frac{b^2}{2}$$

where a is the longest diameter (cm) and b is the shortest diameter (cm).

2.7. Viability studies

On day 15 p.i., animals were killed and the tumors were excised and weighed. Treated tumors were tested (i) for their ability to grow after transplantation in vivo and (ii) for their inhibition of DNA synthesis which was assessed by their uptake of [^{3}H]thymidine in vitro.

2.7.1. Transplantation studies

The tumors excised 15 days p.i. were transplanted subcutaneously by injecting 0.5 ml of 10% tumor cell suspension in sterile saline in the anterior aspect of the hind limb of syngeneic strains of rats (recipient rats). The weight change and tumor size were studied for these rats at regular time intervals. Tumor size was measured on days 8, 11 and 15 post transplantation and tumor weights were calculated as described earlier.

2.7.2. Study of the inhibition of [^{3}H]thymidine incorporation in vitro

Another part of the tumor cell suspension prepared in phosphate buffered saline (PBS) containing 5% rat serum was used in the [^{3}H]thymidine uptake studies. 200 μl of this suspension was incubated in triplicates with 2 μCi of [^{3}H]thymidine at 37°C for 3 h. The uptake of [^{3}H]thymidine was arrested after 3 h by the addition of cold PBS. The suspension was then vacuum filtered through 0.45-μm millipore filter and the proteins precipitated with cold 5% trichloroacetic acid. The filter circles were dried at 60°C in the oven, placed in 10 ml scintillation cocktail and counted in the scintillation counter (LKB Rackbeta 1211 Liquid Scintillation Counter). Zero time samples were taken into account for non-specific adsorption.

2.8. Statistical analysis

Statistical evaluation of the experimental results was performed by analysis of variance (ANOVA) by one-way classification to test the significance of the different treatments given to rats induced with the solid tumor. The tumor growth of the rats in the untreated group was compared with that of groups receiving free MTX, GMPM and GMCM treatments, to test the significance of these treatments compared with untreated controls. Moreover, to study the efficacy of microspheres-entrapped MTX compared with free MTX, the tumor growth of rats in the group given free MTX treatment was compared with that of the groups given GMFM and GMCM treatments, respectively.

3. Results and discussion

Gelatin microspheres entrapping free MTX and conjugated MTX were prepared and tested in rats

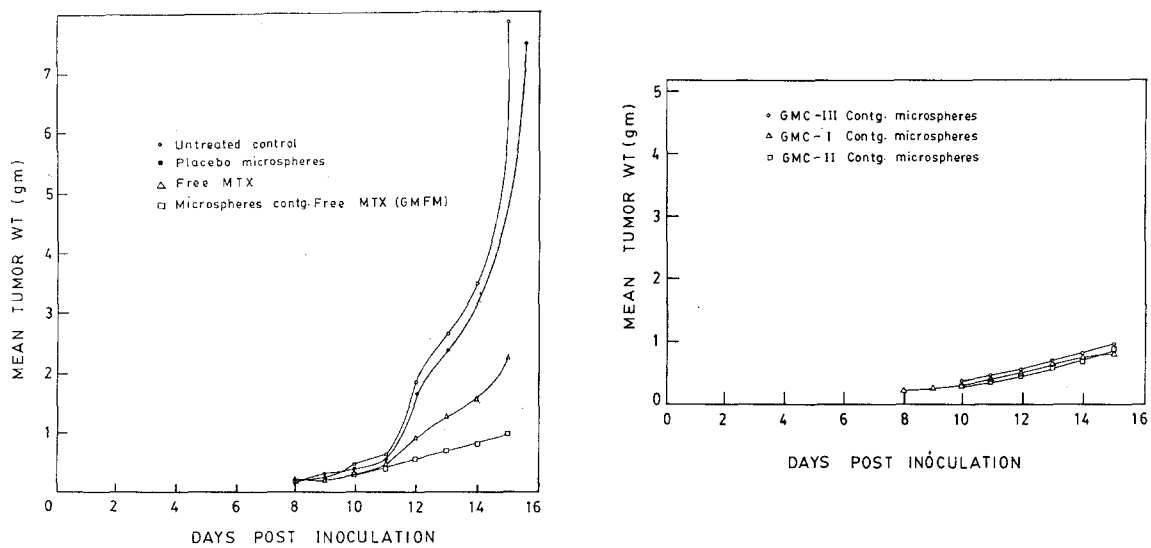

Fig. 1. (a) and (b) Tumor growth curves of different groups of rats (post inoculation).

induced with solid tumor fibrosarcoma for their ability to prevent or delay the growth of tumor by intratumoral therapy. In the present study, microspheres were prepared from gelatin which has a well-known history in parenteral formulations. Gelatin is a natural and biodegradable polymer and hence will be degraded to low molecular weight non-toxic substances and eliminated from the body. Besides, no surfactants were used to disperse the microspheres during preparation, which will adversely influence tissue interactions, drug activity and drug release. The use of glutaraldehyde in small amounts as a crosslinking agent for polymers has appeared in the literature. However, in this study the use of glutaraldehyde directly as a crosslinking agent was not adopted and glutaraldehyde-saturated solution of toluene was used for crosslinking microspheres.

The potential advantage of intratumoral chemotherapy is that it minimises systemic drug absorption and cytotoxic effects on the normal tissue located beyond the injected tumor and maximises tumor cell destruction. Placebo microspheres, GMFM and GMCM whose mean particle size ranged from 10 to 20 μm were selected for the animal studies. The loading of free MTX in GMFM was 53.3 μg/mg microspheres, whereas by covalently coupling MTX to gelatin, a higher drug loading of about 200 μg/mg microspheres was achieved in the various GMCM preparations. We have also reported earlier that GMFM of mean particle size 10–20 μm released about 96% of MTX in 7 days, whereas GMCM-I, GMCM-II and GMCM-III of the same size released about 98% of MTX at a slower rate i.e. for 10–11 days in phosphate buffer pH 7.4. In the present investigation, the inhibitory effect of placebo microspheres, free MTX, GMFM and GMCM on the growth of tumor cells was evaluated by following changes in size and weights of tumors up to 15 days post inoculation. The ability of the treated tumor cells to grow in vivo was tested by transplanting them into their respective syngeneic strains of rats and also by the inhibition of [^{3}H]thymidine incorporation in vitro.

3.1. Post inoculation studies

The tumor growth curve and the mean tumor weights of the different groups of rats studied between 8 and 15 days post inoculation is shown in Fig. 1 and Table 1, respectively. Control rats

Table 1
In vitro [^{3}H]thymidine uptake by treated cells of different groups (post inoculation)

	Untreated control	Placebo microspheres	Free MTX	GMFM	GMCM-I	GMCM-II	GMCM-III
[^{3}H]Thymidine counts	8431	8321	5358	2831	1775	1835	2770

MTX, methotrexate; GMFM, gelatin microspheres containing free MTX; GMCM-I, GMCM-II, GMCM-III, gelatin microspheres containing conjugated MTX.

receiving no treatment, as well as placebo microspheres-, free MTX-, GMFM- and GMCM-treated rats demonstrated an increase in tumor weight initially. Although all rats showed similar rate of tumor growth initially, significant differences were obtained from day 11 post inoculation. Between days 11 and 15, the untreated control tumors and placebo tumors showed rapid growth up to 7.8 g on day 15, whereas the free MTX- and GMFM-treated rats demonstrated a very gradual increase in tumor weight and attained mean tumor weights of 3.5 g and 0.95 g, respectively, on day 15. On the other hand, the GMCM-I-, GMCM-II- and GMCM-III-treated rats demonstrated a more delayed growth rate and reached mean tumor weights of only 0.81, 0.84 and 0.90 g, respectively, at the time of sacrifice on the day 15. Thus, from days 11 to 15 p.i., the mean tumor weights of free MTX-, GMFM- and GMCM-treated rats were significantly lower than those of untreated control and placebo microspheres-treated rats ($P \leq 0.01$). The mean weights of GMFM- and GMCM-treated tumors from 11 to 15 days p.i. were significantly lower than those of free MTX-treated tumors ($P \leq 0.01$). This showed that the free MTX, GMFM and GMCM treatments differed significantly from the untreated controls. The results also showed that the GMFM and GMCM treatments differed significantly from free MTX treatment ($P < 0.01$).

3.2. *In vivo transplantation studies*

Tumor growth curves of treated tumor cells after transplantation in vivo in recipient rats are shown in Fig. 2. The transplanted cells of untreated control rats and placebo microspheres-administered rats showed a rapid growth of tumor and attained mean tumor weight of about 5.6 g and 5.5 g, respectively, after 15 days. The free MTX-treated tumor cells showed very slow rate of growth and reached mean tumor weight of 0.53 g during the same time. The GMFM-treated cells showed a more delayed tumor growth compared with free MTX-treated cells and reached only 0.14 g of mean tumor weight. However, in the case of transplanted GMCM-I-, GMCM-II- and GMCM-III-treated cells, it was interesting to observe that there was no appearance of tumor growth until 15 days post transplantation. These results indicated that the controlled and slow release of small doses of MTX can effect more tumor cell killing than a multiple high dosing schedule of free MTX. Moreover, from these re-

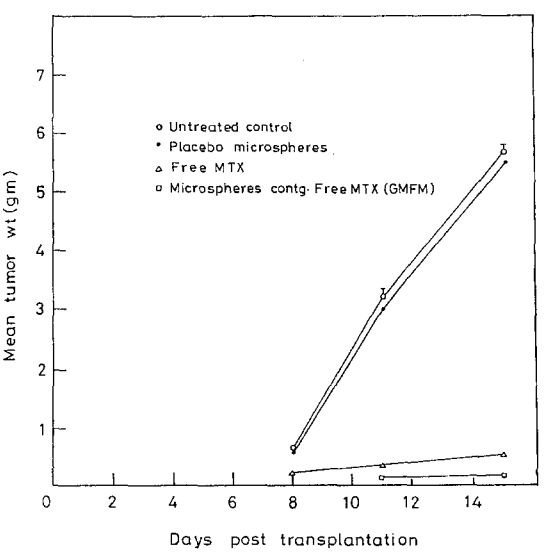

Fig. 2. Tumor growth curves of treated tumor cells of different groups of rats (post transplantation).

sults it is also evident that the conjugated MTX-containing microspheres (GMCM) were more effective than free MTX-containing microspheres (GMFM) in causing tumor cell killing, because no tumor growth was seen in the conjugated MTX-containing microspheres until the end of the experimental period (15 p.i.). This may be attributed to the more delayed release rate of conjugated MTX, compared with free MTX, from microspheres.

3.3. Inhibition of [^{3}H]thymidine incorporation in vitro

The reduced viability of the GMFM- and GMCM-treated tumor cells was further confirmed by the data obtained from the in vitro uptake of [^{3}H]thymidine (Table 1). The inhibition of DNA synthesis in GMFM- and GMCM-treated cells was much higher than in free MTX-treated cells as evidenced by the lower uptake of [^{3}H]thymidine in GMFM- and GMCM-treated cells, respectively. However, the uptake of [^{3}H]thymidine in GMCM-treated cells was slightly lower than in GMFM-treated cells, which indicated more inhibition of DNA synthesis in GMCM-treated cells. The placebo-treated cells and untreated controls behaved similarly and showed a higher uptake of labelled thymidine than all the other groups of treated cells, which indicated a higher rate of DNA synthesis. These results indicated that GMFM and GMCM treatments caused more inhibition of DNA synthesis in the tumor cells than free MTX treatment. It was also observed that, among the three types of GMCM treatments, GMCM-I and GMCM-II exhibited higher inhibition of DNA synthesis than GMCM-III. The post inoculation and [^{3}H]thymidine uptake studies revealed that GMCM-I and GMCM-II caused greater tumor suppression and inhibition of DNA synthesis in the treated animals. These results showed that the conjugates formed by coupling the carboxyl group of the anticancer drug methotrexate with the amino group of the polymer gelatin had more efficient antitumor activity.

Although generally applicable dosage principles of 0.4 mg/kg (maximum dose 25 mg) daily for 4–5 days or 0.4 mg/kg i.v. twice weekly (maximum dose = 12.5 mg) have been proposed for MTX, the application of folinic acid rescue has permitted the use of a greater than 100-fold dose of MTX from microspheres. This may be considered as one of the means to mitigate MTX toxicity and make possible the principle of high-dose methotrexate (HDMTX) therapy without calcium folinate protection.

However, to meet this end and make HDMTX clinically feasible, a high incorporation efficiency (%) of MTX in microspheres has to be achieved. Otherwise, a large quantity of microspheres have to be injected to produce the desired therapeutic effect. In the present study, GMFM showed 5.3% MTX loading, whereas a 4-fold higher incorporation rate of 20% MTX loading was obtained in the case of GMCM. When compared with GMFM, GMCM holds more promise in the realisation of microsphere-entrapped HDMTX therapy in the clinic.

In the present tumor studies, all the animals injected with the free drug as well as the entrapped drug behaved in a manner similar to controls and survived until the end of the experimental period. This proved not only the biocompatibility of the gelatin microspheres but also that the animals tolerated the selected dosage of the anticancer drug.

4. Conclusion

The results of the in vivo post inoculation and post transplantation studies, and of the in vitro [^{3}H]thymidine studies, established that MTX-loaded gelatin microspheres had higher antitumor activity than free MTX on the rat fibrosarcoma. Furthermore, of GMFM and GMCM, the latter had higher antitumor activity. The slow and controlled release of free MTX and conjugated MTX from microspheres was more efficient in inhibiting tumor growth, compared with the multiple high-dose regimen of free MTX. The gelatin microspheres developed in the present study offer additional possibilities as an injectable delivery system for the better management of cancer by virtue of their ability to inhibit tumor growth more effectively than conventionally administered free drug.

References

Arshady, R., Microspheres for biomedical applications: preparation of reactive and labelled microspheres. *Biomaterials*, 14(1) (1993) 5–15.

Balthasar, J.P. and Fung, H.L., High affinity rabbit antibody directed against methotrexate production: purification, characterisation and pharmacokinetics in the rat. *J. Pharm. Sci.*, 4(1) (1995) 2–6.

Chu, B.C.F. and Howell, S.B., Pharmacological and therapeutic properties of carrier bound methotrexate against tumor confined to a third space body compartment. *J. Pharmacol. Exp. Ther.*, 219 (1981) 389–393.

Cremers, H.F.M., Verrijk, R., Bayon, L.G., Werseling, M.M., Wondergem, J., Heuff, G., Meijef, S., Kwon, G.S., Bae, Y.H. and Kim, S.W., Improved distribution and reduced toxicity of adriamycin bound to albumin heparin microspheres. *Int. J. Pharm.*, 120(1) (1995) 51–62.

Gallo, J.M., Hung, C.T., Gupta, P.K. and Perrier, D.G., Physiological pharmacokinetic model of adriamycin delivered via magnetic albumin microspheres in the rat. *J. Pharm. Biopharm.*, 17(3) (1989) 305–326.

Geran, R.I., Greenberg, N.H., MacDonald, M.M., Schumacher, A.M. and Abbot, B.J., Protocols for screening chemical agents and natural products against tumors and other biological systems. *Cancer Chemother. Rep.*, 3 (1972) 51–53.

Ghorab, M.M., Zia, H. and Luzzi, L.A., Preparation of controlled release anticancer agents. I: 5-Fluorouracil-ethyl cellulose microspheres. *J. Microencapsulation*, 7(4) (1990) 447–454.

Ghosh, M.K., Dane, K.O. and Mitra, A.K., Preparation and characterisation of methotrexate–immunoglobulin conjugates. *Drug Des. Del.*, 4 (1988) 13–25.

Goldberg, I.A., An in vivo assessment of adriamycin-loaded albumin microspheres. *Cancer*, 65(3) (1992) 393–395.

Hassan, E.M. and Gallo, J.M., Targeting anticancer drugs to the brain: enhanced brain delivery of oxantrazole following administration in magnetic cationic microspheres. *J. Drug Targetting*, 1 (1993) 7–14.

Ike, O., Shimizu, R., Wada, S.H., Hyon, S.H. and Ikada, Y., Controlled cisplatin delivery system poly(D-L-lactic acid). *Biomaterials* 13(40) (1992) 230–234.

Jeong, S.Y. and Kim, S.W., Biodegradable polymeric drug delivery systems. *Arch. Pharm. Res.*, 9(2) (1986) 63–73.

Jeyanthi, R. and Panduranga Rao, K., Preparation of gelatin microspheres of bleomycin. *Int. J. Pharm.*, 35 (1987) 177–179.

Jeyanthi, R. and Panduranga Rao, K., Release characteristics of bleomycin mitocycin C and 5-fluorouracil from gelatin microspheres. *Int. J. Pharm.*, 55 (1989) 31–37.

Jones, C., Burton, M.A. and Gray, B.N., Alubmin microspheres as vehicles for the sustained and controlled release of doxorubicin. *J. Pharm. Pharmacol.*, 41 (1989) 813–816.

Kim, C.K. and Oh, Y.K., Development of hydrophilic human serum albumin microspheres using a drug–albumin conjugate. *Int. J. Pharm. Bull.*, 38 (1988) 2871–2873.

Kim, C.K., Lee, M.K., Han, J.H. and Lee B.J., Pharmacokinetics and tissue distribution of methotrexate after intravenous injection of differently charged lipozome entrapped methotrexate in rats. *Int. J. Pharm.*, 108(1) (1994) 21–30.

Lilley, J.C., Patterson, L.H. and Taylor, M.J., Preparation and stabilization of liposomes encapsulated doxorubicin and mitoxantrone and their internalization by murine peritoneal macrophages. *Int. J. Pharm.*, 107(2) (1994) 149–158.

Naoji, U., Molecular design of MTX antibody conjugate for targeted cancer treatment. *Biocomp. Polym.*, 7(2) (1992) 191–219.

Narayani, R. and Panduranga Rao, K., Preparation, characterisation and in vitro stability of hydrophilic gelatin microspheres using a gelatin methotrexate conjugate. *Int. J. Pharm.*, 95 (1992) 85–91.

Narayani, R. and Panduranga Rao, K., Controlled release of anticancer drug methotrexate from biodegradable gelatin microspheres. *J. Microencapsulation*, 11(1) (1994) 69–77.

Narayani, R. and Panduranga Rao, K., Biodegradable microspheres using two different gelatin drug conjugates for the controlled delivery of methotrexate. *Int. J. Pharm.*, 128 (1996) 261–268.

Rowland, A.J., Mekenzie, I.F.C. and Pietersz, G.A., Enhanced antitumor effects using a combination of two antibodies conjugated to different drugs. *J. Drug Targetting*, 2(2) (1994) 113–122.