ELSEVIER

International Journal of Pharmaceutics 117 (1995) 151–158

international journal of pharmaceutics

Controlled release of 1-β-D-arabinofuranosylcytosine (ara-C) from hydrophilic gelatin microspheres: in vitro studies

E. Esposito [a], C. Pastesini [a], R. Cortesi [a], R. Gambari [b], E. Menegatti [a], C. Nastruzzi [a,*]

[a] *Department of Pharmaceutical Sciences, University of Ferrara, I-44100 Ferrara, Italy*
[b] *Interdepartmental Biotechnology Center, University of Ferrara, I-44100 Ferrara, Italy*

Received 15 June 1994; accepted 5 September 1994

Abstract

The preparation and characterization of gelatin microspheres containing the antitumor drug 1-β-D-arabinofuranosylcytosine (ara-C) and its release kinetics from microspheres are reported. Spherical gelatin microspheres, showing high encapsulation efficiency (58%) and an average diameter of 18 μm, were obtained by a coacervation method. In vitro release experiments demonstrated that ara-C is released from gelatin microspheres in a controlled and reproducible fashion. The biological effect of ara-C released from gelatin microspheres was determined on in vitro cultured tumor cell lines, showing that after encapsulation ara-C still retains differentiating and antiproliferative activities comparable with those exhibited by the free drug. Taken together, the results obtained suggest that gelatin based microspheres offer excellent potential as carriers for the controlled release of ara-C after in vivo administration.

Keywords: 1-β-D-Arabinofuranosylcytosine; Gelatin; Microsphere; Controlled delivery; Antiproliferative activity; Erythroid differentiation

1. Introduction

The pyrimidine nucleoside analogue cytosine arabinoside (ara-C) is a potent antitumor drug (Luisi de Luca et al., 1984; Fram et al., 1987). It is clinically employed in combination with other antineoplastic agents for the chemotherapeutic treatment of different human neoplasia, and most successfully for acute leukemia. In spite of this interesting pharmacological profile, after administration, ara-C displays a very short plasma half-life (Kufe et al., 1985). In order to obtain an efficient therapeutic response, it is therefore necessary to administer the drug either by continuous infusion or by multiple time-spaced injections, both of which cause low patient comfort and compliance. Because of these adverse phar-

* Corresponding author. Dipartimento di Scienze Farmaceutiche, via Fossato di Mortara 19, 44100 Ferrara, Italy. Tel. +39/532/291259; Fax +39/532/291296.

SSDI 0378-5173(94)00313-0

macokinetic properties, therefore, significant advantages could be gained by the development of controlled delivery devices able to reduce the rapid inactivation of ara-C, increasing the intracellular concentration of the drug possibly with a decrease in unwanted systemic side effects.

An interesting approach that could lead to these results is offered by encapsulating drugs into microparticulate carriers. Liposomes (Janoff, 1992) and microspheres (Langer, 1990) are indeed known to enhance the drug cellular internalization and to preserve the entrapped compound from the activity of degrading enzymes. In addition, microcarriers have been proposed for passive or active targeting strategies aimed at increasing the drug therapeutic index. This opportunity can be related to the altered pharmacokinetics and bioavailability shown by the encapsulated drug (passive targeting) (Kim et al., 1993) or to the specific uptake/absorption of surface modified microparticles (active targeting) (Davis et al., 1993).

With the aim of proposing alternative ways to administer ara-C, the use of gelatin microspheres as carrier system is described here. Gelatin, due to its biocompatibility and biodegradability, is in fact an optimal candidate for producing controlled release systems for a wide variety of drugs (Weltz and Ofner, 1992; Narayani and Panduranga, 1993; Nastruzzi et al., 1994a). Biocompatibility and biodegradability are required characteristics for polymeric materials employed in parenteral formulations, especially if we consider the toxic and/or allergic problems associated with the use of synthetic polymers (i.e., local reactions, poor in vivo metabolization, formation of fibrotic capsules). In this respect, gelatin microspheres are valuable as drug carriers, since after administration, either by subcutaneous injection or via a chemoembolization procedure, they are gradually degraded and can release the incorporated drugs in a controlled fashion (Yoshioka et al., 1981).

In this paper, we report (a) the preparation and characterization of gelatin microspheres containing ara-C, (b) the kinetics of ara-C release from microspheres and (c) a comparative analysis of in vitro antiproliferative and differentiating activity of the free and microencapsulated drug.

2. Materials and methods

2.1. Chemicals

Ara-C and [5-^{3}H]ara-C (50 Ci/mmol) were obtained from Sigma Chemical Co. and Amersham, respectively. The gelatin utilized for the microsphere preparation (Fluka, Switzerland) had the following specifications, as provided by the manufacturer: bloom, 250; pH (1% in water), 3.5–5.0. Isostearyl isostearate was obtained from Gattefossé (Saint-Priest, France).

2.2. Microsphere production

5 ml of a gelatin solution (20% w/v in water) pre-heated to 80°C, containing 10 mg of ara-C plus 50 ml of tracer ([5-^{3}H]ara-C, 50 μCi) and 230 mg of Tween 85 were added to 40 ml of isostearyl isostearate warmed to the same temperature. The biphasic system was stirred under turbulent flow conditions to form a w/o emulsion, using a mechanical stirrer (model IKA RW 20 DZM). After 5 min of continuous stirring, the emulsion was rapidly cooled to 5°C and then 50 ml of acetone were added in order to dehydrate and flocculate the coacervated particles. Gelatin microspheres containing ara-C [GMs(ara-C)] were then isolated by filtering the suspension through a sintered glass filter. Removal of residual oil traces was performed by washing the microspheres with three 80-ml aliquots of acetone.

The choice and adjustment of the manufacturing parameters for the production of microspheres of defined size were performed in accordance with the following equation (Arshady, 1990):

$$d \propto K \frac{D_v R \nu_a \gamma}{D_s N \nu_o C_s} \quad (1)$$

where d is the average particle size, K denotes a variable depending on the geometry of the apparatus (e.g., type and dimension of stirrer), D_v is the diameter of the vessel, D_s represents the diameter of the stirrer, R is the volume ratio between aqueous and oil phases and ν_a and ν_o their respective viscosities, N denotes the stirring speed, γ is the surface tension between the two

immiscible phases and C_s corresponds to the stabilizer concentration.

2.3. Drug content of microspheres

The amount of encapsulated ara-C per mg of dried microsphere was determined by evaluating the unentrapped drug concentration in the filtrate, obtained after microsphere isolation. The determination was performed as follows: the solvent mixture was removed under vacuum in a rotatory evaporator (Buchi, Germany) until complete evaporation of acetone had occurred. 50 ml of ethyl acetate were added to the remaining two-phase system composed of water and isostearyl isostearate. The resulting organic layer was extracted twice with 20 ml of distilled water. The concentration of ara-C in the aqueous phase was then determined by both reverse phase HPLC and quantitating the [5-^{3}H]ara-C with a liquid scintillation counter.

2.4. Microsphere morphological analysis

The morphology of GMs(ara-C) was evaluated by observation on optical and scanning electron microscopy (SEM). Microsphere size and size and volume distributions were determined with a laser granulometer.

2.5. In vitro ara-C release

In vitro ara-C release profiles were obtained by a dialysis method (Nastruzzi et al., 1994b). Briefly, 80 mg of microspheres were poured into a dialysis tube (Mol. Wt cut-off 10000–12000; Medi Cell International, UK), then placed into 30–50 ml of phosphate-buffered saline, pH 7.4, under magnetic stirring at 37°C. After different lengths of time (from 1 to 96 h), 200 μl of receiving buffer (dialyzate) were analysed for ara-C content by both liquid scintillation counting or reverse-phase HPLC.

2.6. HPLC determination of ara-C

The HPLC analysis of ara-C was conducted by using an HPLC system consisting of a Bruker LC21-C chromatographic pump (Bruker, Germany), a Rheodyne 7125 sample injection valve (Rheodyne, USA) (equipped with a 100 μl loop), and a Chrom-A-Scope rapid scan UV detector (Carlo Erba Strumentazione, Italy) able to measure and store 10 spectra/min. Samples were chromatographed on a 150×4.6 mm reverse-phase stainless-steel column packed with 5-mm particles (Model LC-18-DB, Supelco, USA), and eluted isocratically at room temperature with a mobile phase constituted of 100 mM sodium acetate (pH 5.5) plus 1% acetonitrile, at a flow rate of 1 ml/min. Ara-C detection was monitored at 272 nm and the entire wavelength region between 220 and 320 nm was scanned by mean of the rapid scan detector.

2.7. Assays for cell proliferation and differentiation

Human leukemic K562 cells were grown as suspension cultures in α-medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories, Inc., McLean, VA), 0.1 mg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified atmosphere with 5% CO_2. Cells were plated at an initial density of 5×10^4 cells per 15 mm tissue culture dish. Growth inhibition was assessed in log-phase cells that were counted using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) after 5 days in the presence of free or microencapsulated drug. The assays were carried out in triplicate. Control cultures exposed only to empty microspheres were included with each test. Erythroid induction of K562 cells was achieved in the presence of different concentrations of free or microencapsulated ara-C (1–10 μM). Under these experimental conditions, full activation of erythroid differentiation functions occurs after 5 days of induction. The increase of hemoglobin-containing cells was determined by the benzidine test as described elsewhere (Gambari et al., 1984).

3. Results and discussion

3.1. Microsphere preparation

The use of gelatin for controlled release system production is particularly appealing because

of its biocompatibility, biodegradability and low cost. Moreover, gelatin possesses bioadhesive properties. This further characteristic allows the design and production of drug delivery forms specifically studied for targeting to mucosal epithelial tissues such as eye, mouth, nasal, gastrointestinal and urinary tract. For the preparation of gelatin microspheres we employed a coacervation technique. Since the direct correlation between average size, shape of microspheres and the in vivo activity of the entrapped drug has largely been recognized, our purpose was to investigate the experimental parameters controlling the microsphere dimensions.

In agreement with the equation reported by Arshady (1990) (see section 2), correlating the average particle size with different experimental variables such as apparatus design, speed of mixing and viscosity of the two immiscible phases, we obtained optimal results by using the following parameters: 50 mm diameter vessel, 35 mm diameter rotor, volume ratio of the gelatin solution to isostearyl isostearate of 0.130, stirring speed of 1000 rpm, oil phase constituted by isostearyl isostearate (viscosity at 20°C, 30–60 mPa s) and 0.5% (w/w with respect to the weight of the two phases) of Tween 85.

Employing these manufacturing parameters we

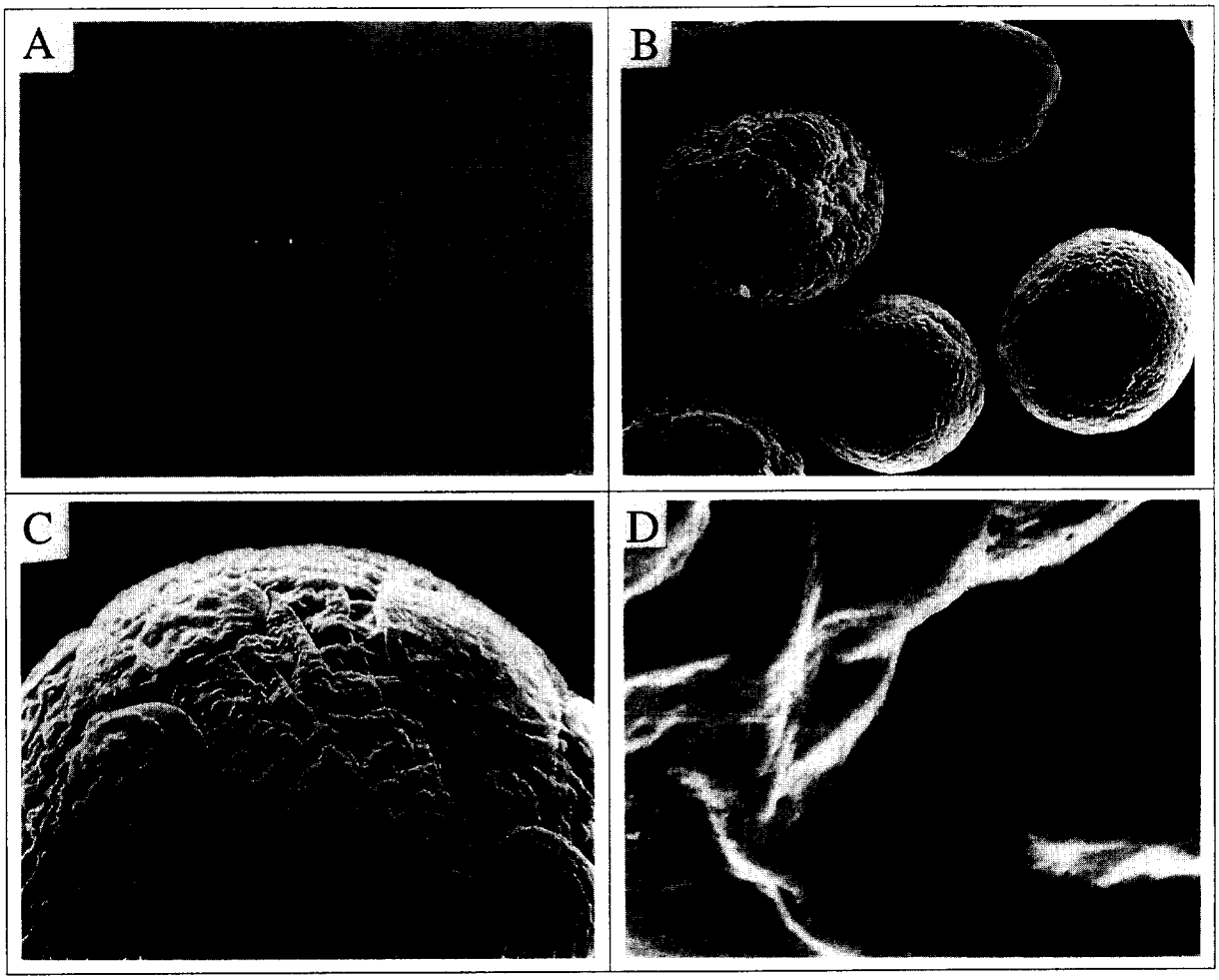

Fig. 1. Optical (A) and scanning electron (B–D) micrographs of ara-C containing gelatin microspheres. Magnifications: 220 × (A), 1800 × (B), 6000 × (C) and 20 000 × (D).

obtained microspheres with an average diameter of 18 μm and a relatively narrow size distribution. In addition, our preparation procedure allows one to obtain a high microsphere recovery (over 85%) with respect to the starting amount of gelatin.

3.2. Ara-C content in microspheres

Samples from three different microsphere batches were analysed for ara-C content. The data obtained indicate that ara-C is encapsulated in microspheres with an efficiency of 58.5 ± 8%.

3.3. Microsphere morphology

The prepared ara-C containing microspheres show good spherical geometry as demonstrated by optical microscopy and SEM analysis (see Fig. 1). Our data establish that the use of isostearyl isostearate permits production of spherical microspheres without aggregation phenomena. Microspheres have a wavy surface with the presence of small plaques, tentatively attributed to the fusion of small droplets to the surface of larger ones. At the highest magnification (panel D) few small pores are detectable on the microsphere surface.

Fig. 2 and Table 1 summarize the geometrical parameters of ara-C containing microspheres, showing that these particles are optimal candidates for subcutaneous or intramuscular administrations.

3.4. In vitro ara-C release from microsphere

Considering the assumption that the pharmacological activity of any biological response modi-

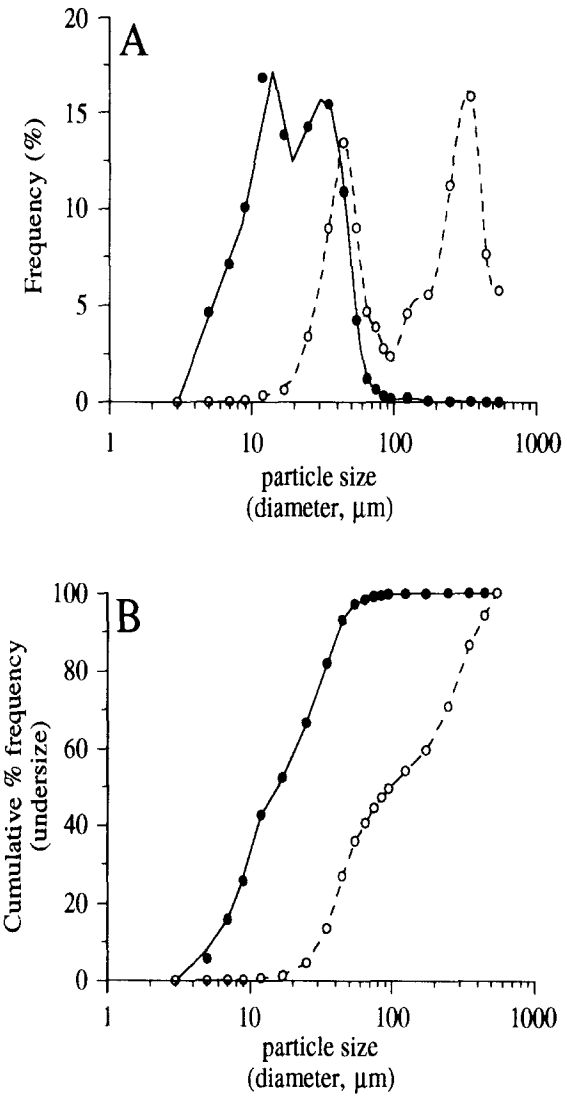

Fig. 2. Size distribution (A) and cumulative frequency plot (B) of ara-C gelatin microspheres. (●) Particle number; (○) particle volume. The data represent the average of three independent determinations on different microsphere batches.

Table 1
Geometrical parameters of ara-C containing gelatin microspheres

Coacervate yield [a]	Incorporation efficiency (%)	Microsphere size [b]			Surface aspect
		Number	Area	Volume	
87.7	58.5	18.1 (35.34)	45.9 (48.6)	104.1 (330.1)	wavy, porous

[a] Theoretical solids (expressed as % of gelatin weight). [b] The values represent the median diameter and the mode (in parentheses) expressed in μm.

fier is directly related to its concentration at the site of action, the determination of both the amount and the mechanism(s) of drug release from microspheres represents a key step in the rationale design of microparticulate delivery systems.

In order to achieve reproducible and reliable quantitations of ara-C during release experiments, two different analytical approaches were employed, namely, liquid scintillation counting and an HPLC method (see section 2). Both approaches gave similar results, as demonstrated by the low variation (< 3%). Fig. 3 shows a typical chromatogram of ara-C containing solution from release experiments (panel A) and the complete release kinetics (panel B). As is clearly appreciable, the ara-C release profile from gelatin microspheres is typically constituted of two different phases: an initial period, characterized by relatively fast release, followed by a second stage, distinguished by slower release of the drug.

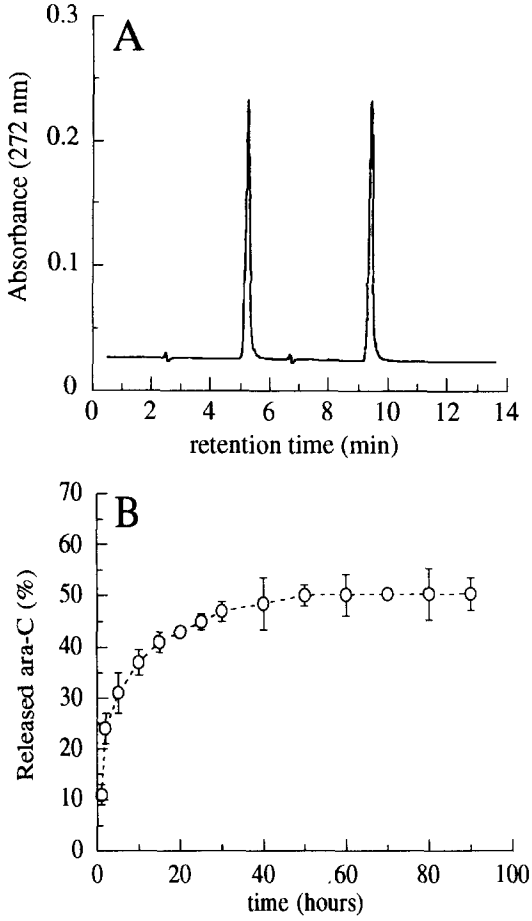

Fig. 3. (A) Typical ara-C chromatogram obtained from two consecutive injections of the same sample of receiving buffer from release experiments. Release experiment and chromatographic conditions are described in section 2. (B) Resulting ara-C release profile; the reported values represent the average of three independent experiments ± SD.

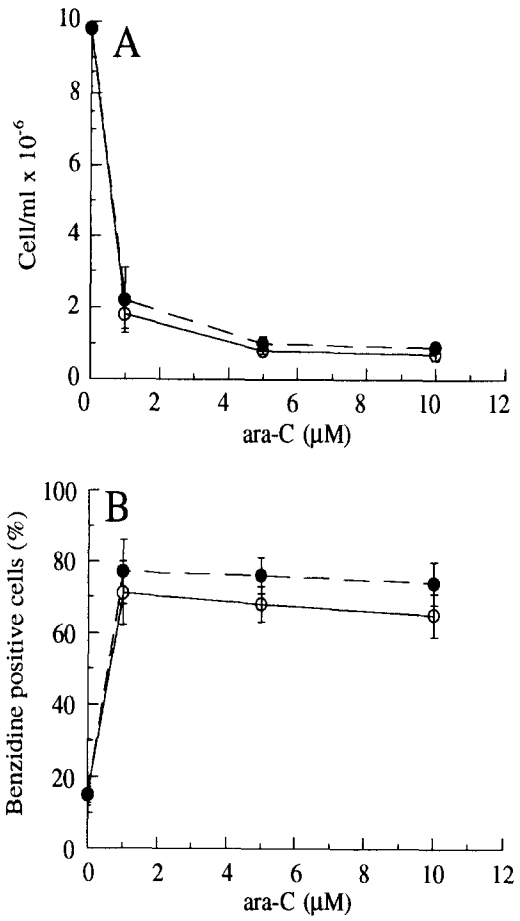

Fig. 4. Comparative analysis of the effect of free (•) and microencapsulated ara-C (○) on proliferation (A) and erythroid differentiation (B) of K562 cells. The empty and ara-C containing microspheres were added at the same indicated concentrations. Determinations were performed after 5 days of cell culture. Data represent the average of three independent experiments ± SD.

The use of HPLC for the determination of release kinetics from pharmaceutical formulations is particularly attractive, since it allows the determination of drug degradation products and/or matrix components, possibly released from microspheres. This consideration is especially important if we consider that released compounds could (a) interfere with the analytical procedure or (b) represent bioactive or toxic agents showing pharmacological activity after in vivo administration. In this respect, it should be stressed that the comparison of the ara-C spectra collected by the rapid scan UV detector with that of pure compound (data not shown) indicates that no interference due to ara-C degradation products or microsphere impurities is present. In conclusion, the use of rapid scan UV detectors to identify and quantitate drugs by both retention time and absorption spectrum gives more reliable results than the use solely of HPLC with conventional single wavelength UV detectors or simple UV determinations.

3.5. In vitro activity of microencapsulated ara-C

In order to determine whether ara-C entrapped in gelatin microspheres maintains its cytotoxic and differentiating activity, human leukemic K562 cells were treated with the same concentrations of free or encapsulated ara-C (in term of ara-C molarity). After 5 days of cell culture, the cells were electronically counted and the erythroid differentiation evaluated by benzidine test. Fig. 4 reports the results of this experiment, showing that GMs(ara-C) maintain antiproliferative (panel A) and differentiating activities (panel B) comparable to those of the free compound. Moreover, we evaluated possible antiproliferative effects of empty microspheres, in the same way, on cultured K562 cells. The data obtained demonstrate that empty microspheres do not cause any inhibition of in vitro cell growth (data not shown). These data, together with the HPLC analysis of the released ara-C (Fig. 3, panel A), clearly indicate that the encapsulation of ara-C into gelatin microspheres, does not lead to any appreciable chemico-physical modification of the molecule.

4. Conclusions

This investigation shows that ara-C gelatin microspheres possess promising potential as a delivery system for ara-C (possibly reducing the toxicity problems associated with the administration of the free drug). A further advantage of this system is represented by the chance of the concomitant administration of gelatin microspheres containing other anticancer drugs (for instance, polyamidines) (Nastruzzi et al., 1994a). This approach could be applied in combination therapy in order to overcome the resistance problems often encountered in cancer chemotherapy.

Acknowledgements

This work was supported by the AIRC and Regione Emilia-Romagna.

References

Arshady, R., Albumin microspheres and microcapsules: methodology of manufacturing techniques. *J. Controlled Release*, 14 (1990) 111–131.

Davis, S.S., Illum, L., Moghimi, S.M., Davies, M.C., Porter, C.J.H., Muir, I.S., Brindley, A., Christy, N.M., Norman, M.E., Williams, P. and Dunn, S.E., Microspheres for targeting drugs to specific body sites. *J. Controlled Release*, 24 (1993) 157–163.

Fram, R.M., Robichaud, N., Bishow, S.D. and Wilson, J.M., Interactions of *cis*-diamminodichloroplatinum (II) with 1-β-D-arabinofuranosylcytosine in LoVo colon carcinoma cells. *Cancer Res.*, 47 (1987) 3360–3365.

Gambari, R., del Senno, L., Barbieri, R., Viola, L., Tripodi, M., Raschellà, G. and Fantoni, A., Human leukemia K562 cells induction of erythroid differentiation by 5-azacytidine. *Cell Diff.*, 14 (1984) 87–92.

Janoff, A.S., Lipids, liposomes, and rational drug design. *Lab. Invest.*, 66 (1992) 655–658.

Kim, C., Hwang, S. and Lee, M.G., The organ targetability of small and large albumin microspheres containing free and HSA conjugated methotrexate. *Int. J. Pharm.*, 89 (1993) 91–102.

Kufe, D. W., Griffin, J.D. and Spriggs, D., Molecular pharmacology of cytosine arabinoside. In Gale, R.P. and Golde, D.W., (Eds), *Leukemia: Recent Advances in Biology and Treatment*, A.R. Liss, New York, 1985, pp. 547–561.

Langer, R., New methods of drug delivery. *Science*, 249 (1990) 1527–3153.

Luisi de Luca, C., Mitchell, T., Spriggs, D. and Kufe, D.W., Induction of terminal differentiation in human K-562 erythroleukemia cells by arabinofuranosyl cytosine. *J. Clin. Invest.*, 64 (1984) 821–827.

Narayani, R. and Panduranga, R., Preparation, characterization and in vitro stability of hydrophilic gelatin microspheres using a gelatin-methotrexate conjugate. *Int. J. Pharm.*, 95 (1993) 85–91.

Nastruzzi, C., Esposito, E., Cortesi, R., Gambari, R. and Menegatti, E., Kinetics of bromocriptine release from microspheres: comparative analysis between different in vitro models. *J. Microencapsul.*, 11 (1994a) 565–574.

Nastruzzi, C., Pastesini, C., Cortesi, R., Esposito, E., Gambari, R. and Menegatti, E., Production and in vitro evaluation of gelatin microspheres containing an antitumor tetra-amidine. *J. Microencapsul.*, 11 (1994b) 249–260.

Weltz, M.M. and Ofner, C.M., Examination of self-cross-linked gelatin as a hydrogel for controlled release. *J. Pharm. Sci.*, 81 (1992) 85–90.

Yoshioka, T., Hoshia, M., Muranish, S. and Sezaki, H., Specific delivery of mitomycin C to the liver, spleen and lung: nano- and microspherical carriers of gelatin. *Int. J. Pharm.*, 8 (1981) 131–141.