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Polymer-coated albumin microspheres as carriers for intravascular tumour targeting of cisplatin*

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Summary. We used a poly-lactide-co-glycolide polymer (PLAGA 50:50) to formulate cisplatin (cDDP) into microspheres designed for intravascular administration. Two systems were developed, PLAGA-coated albumin microspheres and microspheres consisting of PLAGA only. PLAGA-coated microspheres displayed a mean diameter of 31.8 ± 0.9 μm and a payload of 7.5% cDDP (w/w). Solid PLAGA microspheres exhibited a mean diameter of 19.4 ± 0.6 μm and a payload of 20% cDDP. Release characteristics and in vitro effects on L1210 leukemia and B16 melanoma cell lines were investigated. Both types of microsphere overcame the initial rapid release of cDDP (burst effect), and PLAGA-coated albumin microspheres also showed a lag phase of approximately 30 min before cDDP release began. PLAGA-coated albumin microspheres released most of their payload through diffusion, and the coating eventually cracked after 7 days' incubation in saline supplemented with 0.1% Tween at 37° C, enabling the release of any cDDP remaining. Effects of platinum, pre-released from PLAGA-coated albumin microspheres on the in vitro growth of L1210 cells were comparable with those of standard formulations (dissolved) of cDDP. Material released from non-drug-loaded PLAGA microspheres had no effect on L1210 cell growth, suggesting the absence of cytotoxic compounds in the matrix. The colony-forming ability of B16 cells was also equally inhibited by standard cDDP and pre-released drug. These studies show that formulation of cDDP in PLAGA-based microspheres prevents the rapid burst effect of cDDP seen in previous preparations and offers an improved system of administration for hepatic artery infusion or adjuvant therapy, enabling better clinical handling and the promise of a higher ratio of tumour tissue to normal tissue.

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Introduction

The first-line objective of cisplatin (cDDP) therapy is the treatment of testicular and ovarian cancer. Recently, more attention has been concentrated on the use of cDDP for the treatment of primary or metastatic malignancies of the liver [1, 2, 12]. Since cDDP produces severe side effects, among which nephrotoxicity is often the dose-limiting factor when the drug is given systemically, attempts have been made to confine cytotoxic exposure to the tumour site. Studies in patients presenting with bilobar hepatic metastases and dual arterial hepatic blood supply have established the arterial predominance of the nutritional blood flow to hepatic solid tumours, which is the rationale for hepatic artery infusion of anticancer agents [8]. Hepatoarterial cDDP infusion regimens and targeting of cDDP-loaded controlled-release devices to the liver have been used for this purpose. Among others, liposomes [13, 18], microspheres [9, 17], fibres [6] and microcapsules [9, 11] containing cDDP have been formulated and tested in vitro, in vivo and, in some cases, in the clinical setting. Our laboratory has been concentrating on microspheres for targeting of cisplatin.

Spherical, chemically stabilised albumin microspheres (10–25 μm in diameter) have previously been synthesised in our laboratory and were loaded with 50% cDDP (w/w) [3, 4]. Free platinum levels were detectable in patients for up to 3 days after hepatic arterial administration, which is a strong indication that prolonged cDDP release from this formulation is achievable in humans [10]. In this preparation, however, most of the cDDP payload is depleted in the first few minutes during solubilisation prior to administration (burst effect), which considerably reduces the efficiency of the controlled-release preparation and may fail to reduce systemic toxicity in vivo.

We prepared cDDP-loaded, chemically cross-linked albumin microspheres of appropriate size and coated them with poly-lactide-co-glycolide (PLAGA) using a solvent evaporation technique in an attempt to prevent this initial burst effect and to provide a lag time before drug release. In addition, solid PLAGA microspheres were synthesised

and tested for cDDP release. PLAGA was chosen for these studies because copolymers of lactic and glycolic acid have regularly been used for various medical purposes and have been shown to be biocompatible and biodegradable [5, 7, 14, 15].

In the present report, the formulation, physical characteristics and in vitro release properties of these microspheres are described. The effects of the polymer matrix on cell growth and on cDDP-induced cytotoxicity were also investigated in in vitro cell tests to confirm retention of the antitumour activity of the released drug. For this purpose, material released from microspheres containing PLAGA only or from coated albumin microspheres was tested in an in vitro L1210 leukemia cell-growth assay. Released cDDP was also evaluated for cytotoxicity in an in vitro colony-forming assay using B16 melanoma cells.

Materials and methods

Preparation of cDDP albumin microspheres. cDDP-loaded cross-linked albumin microspheres were prepared according to a modification of the method used by Burger et al. [3]. For this purpose, 125 ml highly refined olive oil (Sigma, St. Louis, Mo., USA) was stirred for 30 min at 4500 rpm in a flat-bottomed glass beaker equipped with four baffles (4 mm). Then, 150 mg human serum albumin (HSA; Sigma, St. Louis, Mo., USA) and 150 mg micronised cDDP (Ventron, Karlsruhe, FRG) were solubilised in 0.8 ml phosphate-buffered saline (pH 7.4) and added dropwise to the olive oil. At 15 min thereafter, chemical cross-linking was initiated by the additon of 0.1 ml glutaraldehyde (25% stock solution; Merck, Darmstadt, FRG), giving a final concentration of 0.6% (w/v) in the aqueous phase, and stirring was continued for 1 h. The microspheres were collected by 15 min centrifugation at 3000 g and were resuspended, washed two times with diethyl ether, sieved through stainless steel sieves (Stork Veco, Eerbeek, the Netherlands) to obtain microspheres exhibiting a diameter of between 15 and 40 µm, and dried by lyophilisation prior to further use or storage at 4° C.

Coating of cDDP albumin microspheres. A solution of 2% (w/v) methyl cellulose (15 cps/2% solution; Aldrich, Milwaukee, Wis., USA), 4% (w/v) polyvinyl alcohol (average mol. wt., 10,000 Da; Sigma, St. Louis, Mo., USA) and 0.1% (v/v) Tween-80 (Sigma, St. Louis, Mo., USA) in saline was adjusted to pH 2 with 0.5 M HCl. The solution was stirred in a baffled beaker (as above) at 5500 rpm for 30 min. Then, 64 mg cDDP-loaded HSA microspheres suspended in a solution of 80 mg PLAGA (Medisorb, 50:50 DL-form, M_w, 91,000; M_n, 44,000; M_m/M_n, 2.06; 0.4% H_2O; Dupont de Nemours, USA) in 0.7 ml dichloromethane was added and stirring was continued for 3 h at 22° C. The microspheres were collected by centrifugation at 1500 *g*, washed two times with cold (0° – 4° C) water, and sieved through stainless steel sieves to obtain microspheres displaying a diameter of between 15 and 40 µm.

Preparation of solid PLAGA microspheres. PLAGA microspheres were prepared according to a method described by Spenlehauer et al. [17]. A solution of 3% methyl cellulose, 4% polyvinyl alcohol, and 0.1% Tween-80 in saline was adjusted to pH 2 with 0.5 M HCl and stirred in a baffled beaker at 6000 rpm for 30 min. Then, 80 mg PLAGA and 64 mg micronised cDDP crystals in 0.7 ml dichloromethane was added and the solution was stirred for 3 h at 22° C to obtain microspheres exhibiting a median diameter of 25 µm. After collection by centrifugation and washing with cold double-distilled water, the microspheres were sieved through stainless steel sieves (15 – 40 µm). Microspheres were dried by lyophilisation and stored dry at 4° C.

Characterisation of microspheres. Microspheres were sized using a light microscope equipped with a micrometer calibrated with latex particles of known diameter. Platinum payloads were determined using flameless atomic absorption spectrometric (FAAS) detection (model AA40 AAS equipped with a GTA 96 graphite tube atomiser with Zeeman background correction; Varian, Victoria, Australia) after the digestion of microspheres in 65% HNO_3 at 170° C in Parr Teflon-lined acid-digestion bombs for 2 h. After the mixture had cooled, 5 mg NaCl was added and the liquid was evaporated under a stream of air at 95° C. Residues were dissolved in 0.2 M HCl and 0.15 M NaCl. All standards were treated in the same way as the samples, being either diluted or digested in the appropriate matrix. A 4-stage heating program was used that consisted of drying at 110° C for 65 s, ashing at 1400° C for 75 s, atomizing at 2650° C for 3 s and conditioning at 2550° C for 5 s. Argon was used as the inert gas.

In vitro release of platinum from microspheres in a medium consisting of phosphate-buffered saline (0.01 M pH 7.4, supplemented with 0.1% Tween-80) was studied at 37° C under sink conditions (40 ml medium, sufficient to dissolve 10 times more cDDP than could maximally be released) in flat-bottomed vials on a shaker set at 2 Hz. At different intervals, samples were taken, examined under a light microscope and centrifuged at 1000 *g*. The supernatant was subsequently analysed for platinum by FAAS analysis as described above.

Effects on in vitro cell growth and clonogenicity. L1210 cell lines were grown in RPMI 1640 (Gibco, Paisley, Scotland) containing 10% fetal calf serum (Flow Laboratories) and 60 µM 2-mercaptoethanol (Merck, Darmstadt, FRG) in an incubator containing 5% CO_2 at 37° C. In all, 2 million L1210 cells were incubated with PLAGA-coated cDDP albumin microspheres at different concentrations in 8 ml medium and were grown in F25 Falcon tissue-culture flasks for 4 days under the same conditions. Samples of 0.5 ml were taken at regular intervals and counted in a Sysmex CC 108 cell counter. In each sample, the total platinum content in supernatant after centrifugation was measured as described above. The average platinum concentration was calculated and used in control experiments with unformulated (standard) cDDP. L1210 growth assays were carried out in duplicate and cells were counted in triplicate. Microspheres or standard cDDP were added to the cell culture when a new passage was started, and cell growth was followed for 4 days. Cell counts were corrected for microsphere counts, which were expected to amount to <1% of the cell counts in any sample.

Single-cell survival was assessed by a colony-forming assay using a B16 melanoma cell line. B16 cells were grown in DMEM (Dulbecco's modified Eagle's medium; Gibco, Paisley, Scotland) supplemented with 10% NCS (newborn calf serum; Flow Laboratories) in an incubator containing 5% CO_2 at 37° C. Cells were harvested by trypsinisation with 0.05% (w/v) trypsin in phosphate-buffered saline (0.01 M, pH 7.4) and transferred to 6-well tissue-culture plates (Costar, UK) in 3 ml DMEM plus 10% NCS at a density of 100 cells/well. Beginning at 24 h thereafter, the cells were continuously exposed for a further 6 days to several concentrations of standard cDDP or cDDP in the form of drug that had been released over a period of 72 h from solid PLAGA microspheres. Released material was standardised for cDDP by platinum FAAS analysis. To prevent bacterial contamination, the material released was filtered through a 0.22-µm Millipore filter. Colony formation was quantitated by counting of colonies that contained >50 cells as determined under a dissection microscope. Six replicate dishes were counted per point. Surviving fractions were calculated as the ratio of treated to control plating efficiencies. The plating efficiency for untreated cells (colonies per plated cell) was between 60% and 70%.

Statistical analysis. Results were expressed as mean values (± SEM) unless otherwise stated. Estimation of release parameters was carried out by nonlinear regression using the simplex optimisation method. Fitted parameters (k and n) were expressed as the results (± SD) of the fit.

Results

Both microsphere preparations were easily resuspendable in saline supplemented with 0.1% Tween-80, which is a prerequisite for clinical handling. Diameters of PLAGA-

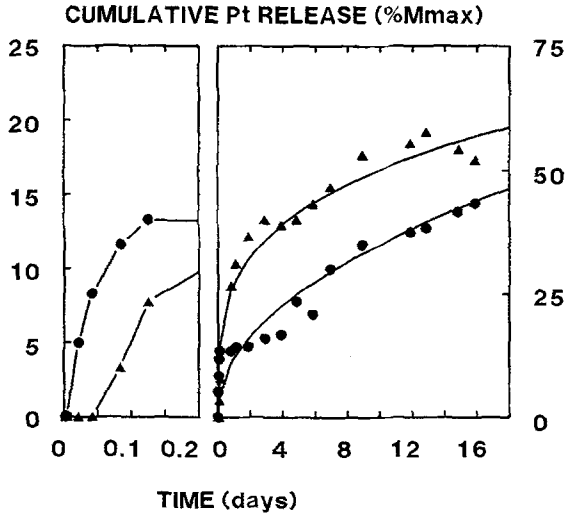

Fig. 1. In vitro cumulative release profiles of cDDP-loaded PLAGA-coated albumin microspheres (▲) and cDDP-loaded solid PLAGA microspheres (●). *Smooth lines* depict release characteristics calculated after nonlinear regression. Data represent mean values for 6 and 2 experiments, respectively. The *left panel* is a close-up of the first period of platinum release. *Mmax*, theoretically maximally releasable amount of platinum from the preparation

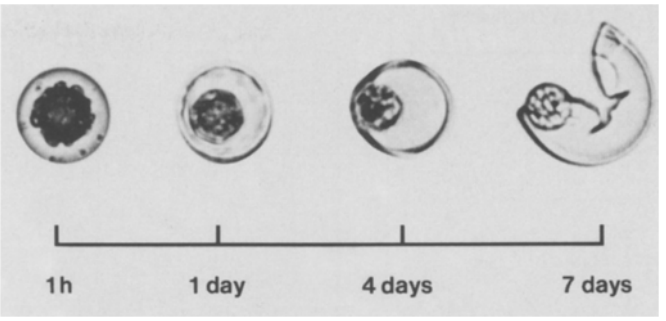

Fig. 2. Transmission micrographs of PLAGA-coated cDDP-loaded albumin microspheres suspended in saline plus 0.1% Tween-80 for the indicated times

coated cDDP-loaded albumin microspheres were suitable for intravascular application (31.8 ± 0.9 μm; payload, 7.5% cDDP). The in vitro cumulative platinum release was followed for 16 days and is displayed in Fig. 1 (mean of six experiments). After a lag phase of 33 ± 13 min during which no detectable platinum was released, the cDDP release rate was initially rapid and decreased after 2 days. At that time, >30% of the incorporated cDDP had been released.

cDDP-loaded solid PLAGA microspheres [diameter, 19.4 ± 0.6 μm; payload, 20% cDDP (w/w)] exhibited neither a lag phase nor a burst effect (Fig. 1, mean of two experiments). Platinum release started almost immediately and levelled off at 12 h, with 85% of the encapsulated cDDP remaining in the microspheres. However, release continued, albeit at a much lower rate. Release profiles of both microsphere formulations were analysed by a general equation for drug release that was proposed by Sinclair and Peppas [16]:

$$\frac{M_t}{M\infty} = kt^n,$$

where $M_t/M\infty$ is the fraction percentage of compound released at time t, k is the rate constant expressed in percent per hour^n. The exponent n is an indication for the drug-release mechanism and correlates with the release order. In general, these assumptions are valid for a release rate of <60%. In spherical delivery systems, values for n are 0.43 and 0.85 for Fickian diffusion and Case-II transport, respectively. At values for n that lie between 0.43 and 0.85, the transport is considered to be non-Fickian. The values for k and n were estimated by nonlinear regression. For release of cDDP from polymer-coated albumin microspheres, k and n values were 11.42 ± 1.81 (SD of the fit) and 0.27 ± 0.03, respectively. cDDP release from solid PLAGA microspheres was characterised by values of 2.58 ± 0.82 and 0.48 ± 0.06 for k and n, respectively.

At regular intervals, samples from in vitro release experiments were examined using a light microscope. The PLAGA coating around the albumin microspheres tended to soften, and a swelling of the microspheres occurred that increased with incubation time (Fig. 2). Most of these microspheres displayed a ruptured PLAGA coating after 7 days. However, by that time, most of the incorporated cDDP had been released by diffusion (as measured by FAAS) as reflected by a reduction in the light-density of the albumin cores of the microspheres during that period. Some of the microspheres aggregated into a cluster exhibiting an apparent reduction in matrix viscosity, causing a further packing of the microspheres. This phenomenon was even more obvious in the solid PLAGA microspheres; within 1 day, most of the cDDP-containing solid PLAGA microspheres had aggregated into several larger conglomerations.

Control cell growth was neither inhibited nor stimulated by empty, solid PLAGA microspheres (0.2 mg microspheres/ml cell-culture medium; data not shown), suggesting that the procedures used for the formulation of PLAGA-coated albumin microspheres and solid PLAGA microspheres were satisfactory and did not result in detectable incorporation of growth-stimulating or toxic compounds.

Encapsulation of cDDP in a matrix may cause the drug to become less active due to cross-linking with proteins or other compounds during the microsphere preparation process or during cDDP release. Since platinum released from macro-aggregated albumin microspheres has been proven to be active, we focused on the possible effects of PLAGA on cDDP activity [10]. We therefore determined the inhibitory effect of cDDP-loaded PLAGA-coated albumin microspheres on the growth of L1210 leukemia cells over 4 days in vitro. Increasing cDDP concentrations caused progressive growth reduction, with the highest concentration used (14.2 μM Pt) totally abolishing the increase in cell numbers. A comparison of the left and right panels in Fig. 3 reveals that cisplatin's toxicity to L1210 cells was not reduced by the microsphere formulation, demonstrating that incorporation in PLAGA did not lead to the degradation or inactivation of the drug. In contrast, the lowest concentration of Pt (1.4 μM) used in these experiments

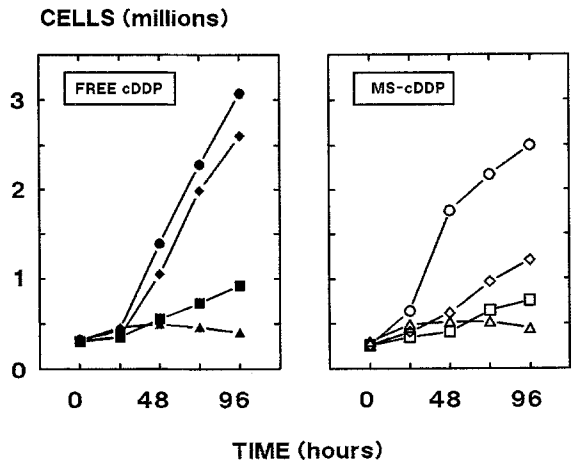

Fig. 3. Effect of free cDDP (*left panel*) and cDDP-loaded PLAGA-coated albumin microspheres (*MS-cDDP, right panel*) on the in vitro growth of L1210 leukemia cells. Concentrations of Pt were: none (●, and ○), 1.4 μM (◆, ◇), 3.4 μM (■, □), and 14.2 μM (▲, △) Data represent mean values for 2 experiments

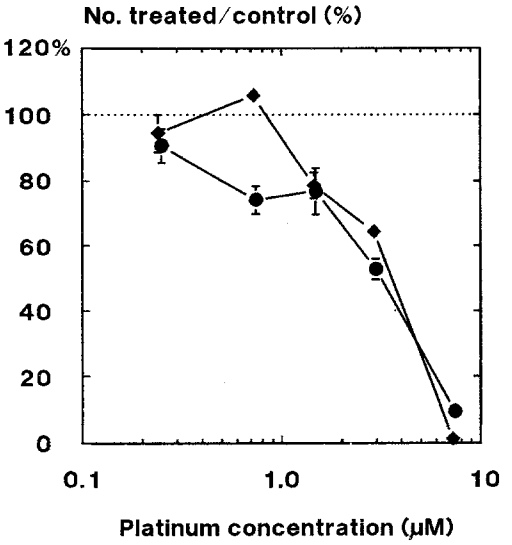

Fig. 4. Colony-forming assay of B16 melanoma cells during continuous cDDP exposure. ◆, Pt from standard (free) cDDP; ●, Pt released from solid PLAGA microspheres

seemed to produce a stronger cytotoxic or cytostatic effect than did 1.4 μM Pt from standard cDDP (Fig. 3, diamonds).

Clonogenic assays were performed using continuous incubation with both standard cDDP and cDDP encapsulated in PLAGA microspheres. At equal concentrations, no differences were seen in the colony-forming ability of B16 cells following treatment with free cDDP vs microsphere-released drug (Fig. 4). These results suggest that all platinum released from the microspheres occurred as cDDP and that its activity was therefore equal to that of platinum from dissolved (standard) cDDP.

Discussion

Encapsulation of cytostatic agents in microspheres is a procedure that is widely used to achieve higher or prolonged tumour exposure and lower systemic exposure as compared with those obtained using conventional parenteral drug formulations. By taking advantage of the preferential arterial blood flow to particular hepatic tumours and retaining nutrient flow to the liver via the portal vein, treatment of hepatic malignancies or palliation of patients exhibiting liver metastases may be accomplished. Metastases from mammary or colon carcinoma are particularly suitable for this type of treatment, since these are usually highly vascularised tumours. In patients undergoing abdominal surgery, adjuvant therapy with micro-encapsulated cytostatic agents that are locally delivered to the liver via the portal vein may result in higher ratios of tumour tissue to normal tissue and thus improve the therapeutic index.

This report describes the formulation of two types of microspheric cDDP delivery systems exhibiting characteristics that render them suitable for both intra-arterial and intraportal administration to the liver. Inactivation of cDDP in vivo by Kupffer-cell capture of microspheres is unlikely due to the chosen microsphere diameter range of 15–40 μm. Practical handling has been improved by better resuspendability. In addition, the cDDP-loaded PLAGA-coated albumin microspheres display a lag time of approximately 30 min, thus avoiding the rapid initial release of cDDP (also absent in cDDP-loaded solid PLAGA microspheres) and facilitating the preparation of the formulation for administration. This theoretically reduces the systemic exposure to free cDDP. Sterilisation of the formulation for parenteral use also poses no obstacle, since gamma irradiation of PLAGA-based microspheres is feasible. As long as this is performed after the completion of microsphere preparation, the characteristics of cDDP release from lactic acid-co-glycolic acid polymer matrices will not be altered during storage [17].

Initial in vivo experiments using cDDP-loaded solid PLAGA microspheres showed that payloads were sufficiently high for intravascular liver targeting (>10 μg platinum/g wet liver tissue in healthy rats, unpublished data). The parameters of release obtained by mathematical analysis of release characteristics indicated that none of the release profiles could be described as representing Fickian or Case-II transport. Apparently, the cDDP release from cDDP-loaded solid PLAGA microspheres was limited by the aggregation of softened microspheres in the in vitro release experiments. Following the distribution of microspheres into the microvascular system of the liver or tumour(s) in vivo, aggregation of this kind probably does not occur; this was confirmed by preliminary in vivo rat experiments in which no microspheres or microsphere aggregate could be found in livers at 26 days after venoportal administration (unpublished data). Release in vivo is therefore likely to be faster than that indicated in our in vitro experiments.

To verify that the platinum doses used in (standard cDDP) control experiments and in microsphere experiments were equal, we measured exact Pt concentrations in cell-culture medium from microsphere experiments and calculated control growth curves using the measured Pt

concentrations 1 week thereafter. The L1210 cell line proved to be stable and reliable in vitro and we could establish growth curves reproducibly.

Since the use of PLAGA as a microsphere carrier for cytotoxic agents is quite new, tests were designed to detect the interference of (released) PLAGA material with the toxicity of platinum to tumour cells examined both in suspension (L1210) and as adherent monolayers (B16). The L1210 assay could be performed without any technical problems; released Pt could be immediately taken up by cells. However, the B16 clonogenicity assay, had to be carried out indirectly due to mechanical interference of the microspheres with cell adhesion and, hence, with plating efficiency. As a result, B16 cells were incubated with platinum released from cDDP-loaded solid PLAGA microspheres. To avoid drug inactivation by protein binding during the 72 h release period in the platinum-collecting medium, cDDP encapsulated in coated albumin microspheres was not used.

In vitro cell growth assays showed that encapsulating cDDP in PLAGA had no influence on the inhibition of either in vitro L1210 cell growth or the in vitro clonogenic capacity of B16 cells. This indicates the absence of any interaction between cDDP and the polymer that would cause inactivation of the drug. The PLAGA formulation itself had no effect on cell growth, and we therefore assume that no cytotoxic compounds were present in the formulation. It seemed that the effect of platinum released from PLAGA microspheres at a concentration of 1.4 μM was stronger than that of 1.4 μM standard cDDP. However, this difference may have been caused by small inaccuracies in FAAS platinum measurements that were used to calculate the cDDP concentration for control-cell growth-inhibition experiments. In that concentration range, the steepness of the cDDP concentration-response curve induces a relatively large shift in growth inhibition.

The formulation of cDDP in biodegradable PLAGA-based microspheres exhibiting suitable release properties and lacking the burst effect opens perspectives for further improvement in the treatment of hepatic secondary lesions by hepatic artery infusion and in microsphere-based adjuvant chemotherapy via the portal vein following abdominal resection of colon carcinomas or other selected solid tumours.

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