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Therapeutic efficacy study of novel 5-FU-loaded PMM 2.1.2-based microspheres on C6 glioma

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Abstract

The aim of this study was to evaluate the potential of poly(methylidene malonate 2.1.2) as a new drug delivery system to the central nervous system. 5-Fluorouracil microspheres were formulated by an emulsion–extraction method, and evaluated on a C6 glioma model. Twenty-seven Sprague–Dawley female rats underwent implantation of various C6 cell concentrations. Magnetic resonance imaging was performed at day 10 to control the setting of the tumor, by using a T2-weighted sequence. At day 12, 18 animals received blank or 5-FU-loaded microspheres, while 9 animals were not implanted and constituted the controls. Thereafter, MRI was performed twice a week to follow the tumor growth. In 12 animals, an alloimmune rejection of the tumor was observed, showing the limitations of the C6 glioma model. When tumor developed, no relationship was observed between the number of C6 cells injected and the tumor volume. 5-FU microsphere efficacy could statistically be demonstrated by significantly improving the median survival of C6 glioma-bearing animals and also by decreasing tumor burden.
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Keywords: PMM 2.1.2; Microspheres; 5-Fluorouracil; C6 glioma; MRI

Therapeutic efficacy of 5-fluorouracil-loaded poly(D,L-lactide-co-glycolide) microspheres (MS) was previously observed on C6 glioma-bearing rats (Menei et al., 1996; Lemaire et al., 2001). The objective of the present study was to assess the therapeutic efficacy of novel 5-fluorouracil-loaded poly(methylidene malonate 2.1.2) microspheres on the same glioma model. Poly(methylidene malonate 2.1.2) (PMM 2.1.2) is a novel material that was described a few years ago (Bru-Magniez et al., 1990). In a biocompatibility study performed in our group, PMM 2.1.2 was observed to degrade more slowly in vivo, compared with the other polymers classically found in the literature (data not shown). Thus, we wanted to develop an efficient system that was capable of delivering a drug over long periods, which could increase the therapeutic efficacy of the drug delivery system.

The C6 glioma cell line was initially established by Benda et al. (1971) by weekly injections of *N*-methylnitrosourea to an outbred rat. The C6 glioma cell line

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used in this study was kindly provided by P. Brachet (Inserm U298, Angers, France). The cells were grown in Ham's F12 medium with L-glutamine containing 10% fetal bovine serum (FBS) and 1% antibiotic and antimycotic solution (penicillin 10000 units/ml, streptomycin 10 mg/ml and amphotericin B 25 μg/ml, Sigma, France) in humidified incubators gassed with 5% carbon dioxide (37 °C).

Sprague–Dawley female rats weighting 250–300 g were obtained from the hospital animal house of Université d'Angers. The animals were kept in standard animal facilities.

The microspheres were prepared using an emulsion–extraction method. This technique is based on the formation of an oil-in-water emulsion obtained under controlled stirring. 5-Fluorouracil (5-FU) crystals (Roche laboratories, Neuilly sur Seine, France) were ground and 300 mg were suspended in 6 ml of methylene chloride (Prolabo, Strasbourg, France) using an UltraTurrax® homogenizer for 2 min. The polymer (300 mg) was then dissolved in the organic solvent under magnetic stirring, which contributed to maintain the 5-FU in suspension. The suspension was driven to a temperature lower than 5 °C. An emulsion was obtained by pouring the organic phase into a polyvinyl alcohol (PVA, 88% hydrolyzed, Rhodoviol® 4/125, Prolabo, Strasbourg, France) aqueous phase (200 ml, 2% w/v, 2 °C) under mechanical stirring (600 rpm, 2 min). Thirteen hundred milliliters of deionized water (4 °C) were then added to the emulsion, allowing the formation of microspheres, which resulted from the solvent extraction (500 rpm, 2 min).

The emulsion and the extraction steps took place in a reactor in which temperature was under control. Thereafter, the particles were collected by filtration under nitrogen pressure, washed with deionized water, resuspended in a minimum of deionized water, frozen in liquid nitrogen and freeze-dried (freeze-drier RP2V, Sérail, Argenteuil, France). Finally, the particles were sterilized by gamma-irradiation (25 kGy).

The drug loading was comprised between 17 and 25% and the average microsphere diameter between 40 and 50 μm. The in vitro release profile of 5-FU from sterilized MS (in PBS pH 7.4, 37 °C) is shown in Fig. 1.

Animals were anesthetized with an intraperitoneal injection of 0.75–1.5 ml/kg of a solution containing 2 parts ketamine (100 mg/ml) (Clorketam®, Bayer, France) and 1 part xylazine (20 mg/ml) (Rompun®, Vétoquinol, France).

The anesthetized animals were immobilized in a stereotaxic head frame (Lab Standard Stereotaxic, Stoelting, Chicago, IL, USA) and a burr hole was drilled at the following coordinates: 0.2 mm posterior from bregma, 3.5 mm lateral from the sagittal suture, and 5.0 mm below dura (with the incisor bar set at −3.0 mm).

A cultured tumor monolayer was trypsinized briefly, washed twice with Ham's F12 medium without FBS or antibiotics, counted and resuspended to the final concentration desired. Ten microliters of cell suspension were injected via a cannula at a flow rate of 1 μl/min at the previously mentioned coordinates. The cannula was left in place for four additional minutes to avoid

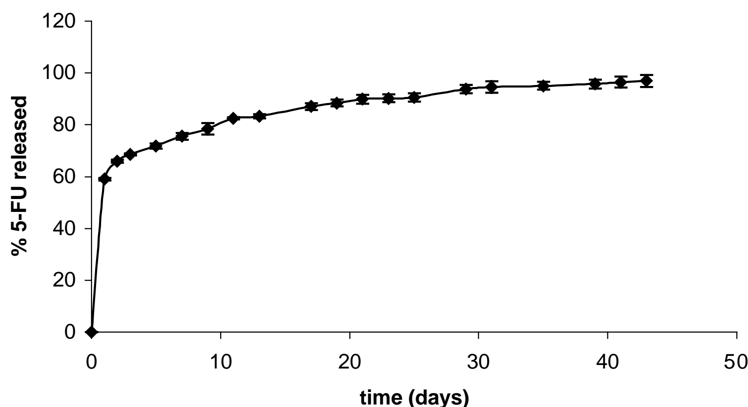


Fig. 1. Release profile of 5-FU from gamma-sterilized microparticles.

the suspension to be expelled from the brain during the withdrawal of the syringe, which was done very slowly (0.5 mm/min).

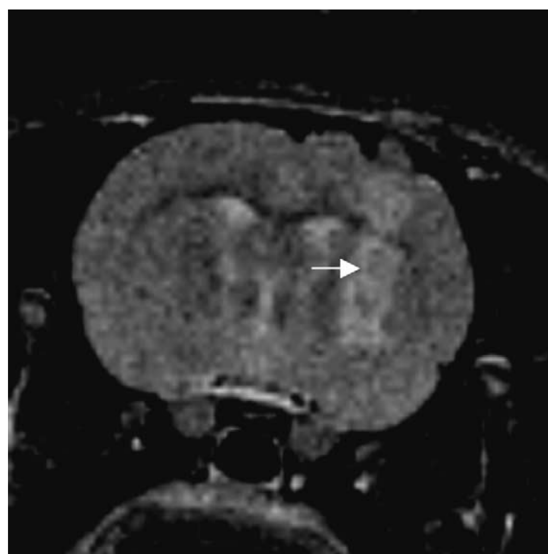
Freeze-dried blank and 5-FU-loaded MS were sieved and resuspended in a sterile aqueous medium consisting of 1.25% (w/v) carboxymethylcellulose (low viscosity) (Cooper Pharmaceutique, France), 1% (w/v) polysorbate 80 (Prolabo, Paris, France) and 4% (w/v) mannitol (Cooper Pharmaceutique, France). Ten microliters of MS suspension were injected at the same coordinates as the tumor cells (dose of 5-FU/animal: 10 mg/kg).

MRI experiments were performed on a Bruker Avance DRX 300 (Bruker, Germany) equipped with a vertical superwide-bore magnet operating at 7 T and shielded gradient insert (maximum gradient strength 144 mT/m, rising time $<300\,\mu s$). The resonant circuit of the NMR probe was a 64 mm diameter birdcage. T2-weighted images were obtained in less than 10 min using a multi-spin multi-echo sequence (TR = 2500 ms; TE = 15, 30, 45, 60, 75, 90, 105, 120 ms; FOV = 3 cm × 3 cm; slice thickness 1 mm; 128 × 128 matrix) (Lemaire et al., 2000). This sequence was shown to be specially adapted to detect and follow C6 glioma growth, and to evaluate therapeutics (Lemaire et al., 2001).

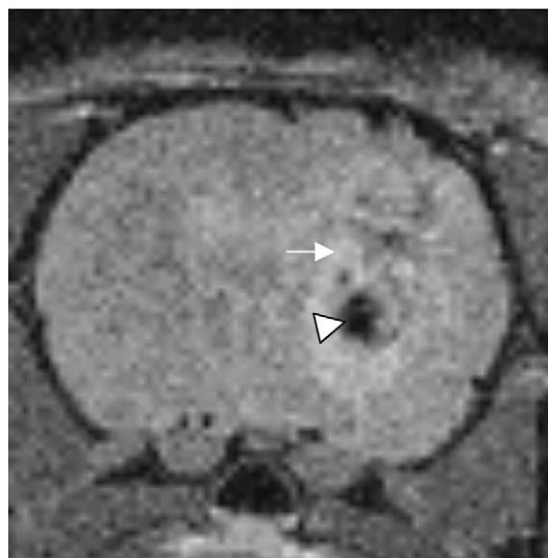
Three groups of nine rats underwent implantation of 4×10^4, 6×10^4 and 2×10^5 tumor cells, respectively. After 10 days, MRI was performed to control the setting of the tumor. At day 12, three rats/group received blank MS, three received 5-FU MS (10 mg/kg 5-FU) and three were not implanted and constituted the controls. Thereafter MRI was performed twice a week to follow the tumor growth (Fig. 2). The survival times of the implanted and control groups were recorded. Rats too weak to feed or stand were sacrificed.

Comparison of survival between groups was made using the nonparametric Mann–Whitney *U*-test.

Among the 27 animals of this study, 5 treated, 4 controls and 3 unloaded MS implanted rats developed a tumor that regressed and disappeared between days 25 and 35. At least for the controls or the unloaded MS-treated rats, this phenomenon evoked an alloimmune-like rejection of the tumor. Indeed, the origin of the cell line remains in question even though the randomly bred Wistar character of this tumor type is now established (Peterson et al., 1994). Moreover, a previous study performed in our laboratory has shown that the tumor take was better in Sprague–Dawley rats than in Wistar rats (Menei et al., 1996). Taken all together, all these points drove us to withdraw from the statistics all the cured animals.



(a)



(b)

Fig. 2. T2-weighted image of a C6 glioma-bearing rat implanted with 4×10^4 cells, before (D10) (a) and after (D14), (b) implantation of the 5-FU microspheres. The hyposignal corresponds to the MS (white arrowhead), the hypersignal to the lesion (white arrows).

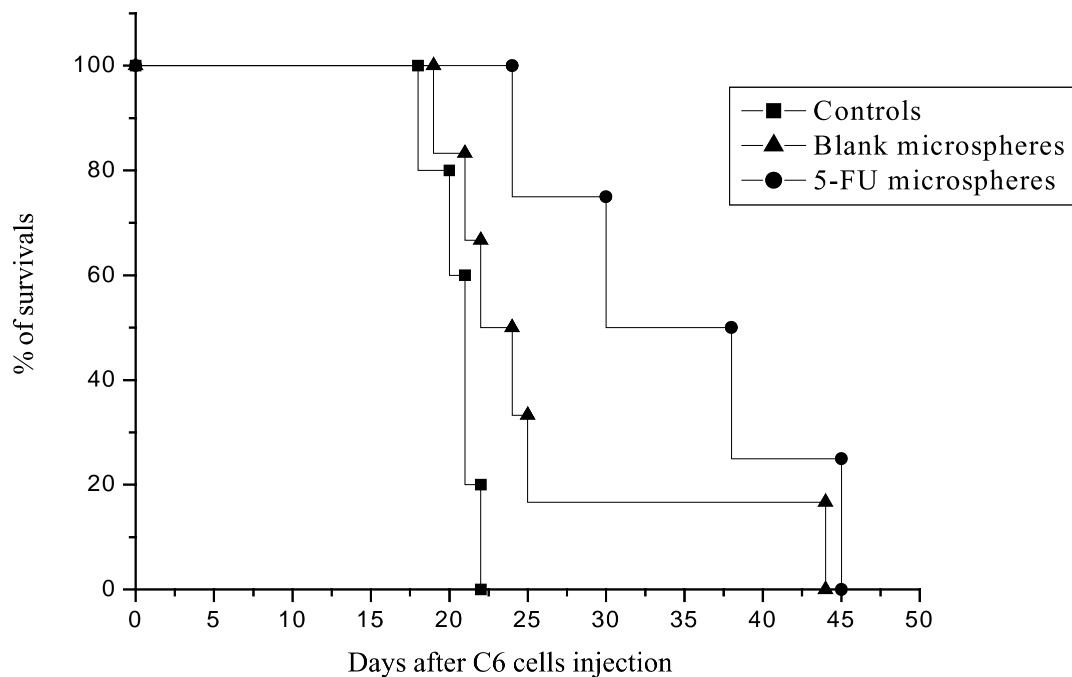


Fig. 3. Kaplan–Meier survival curves.

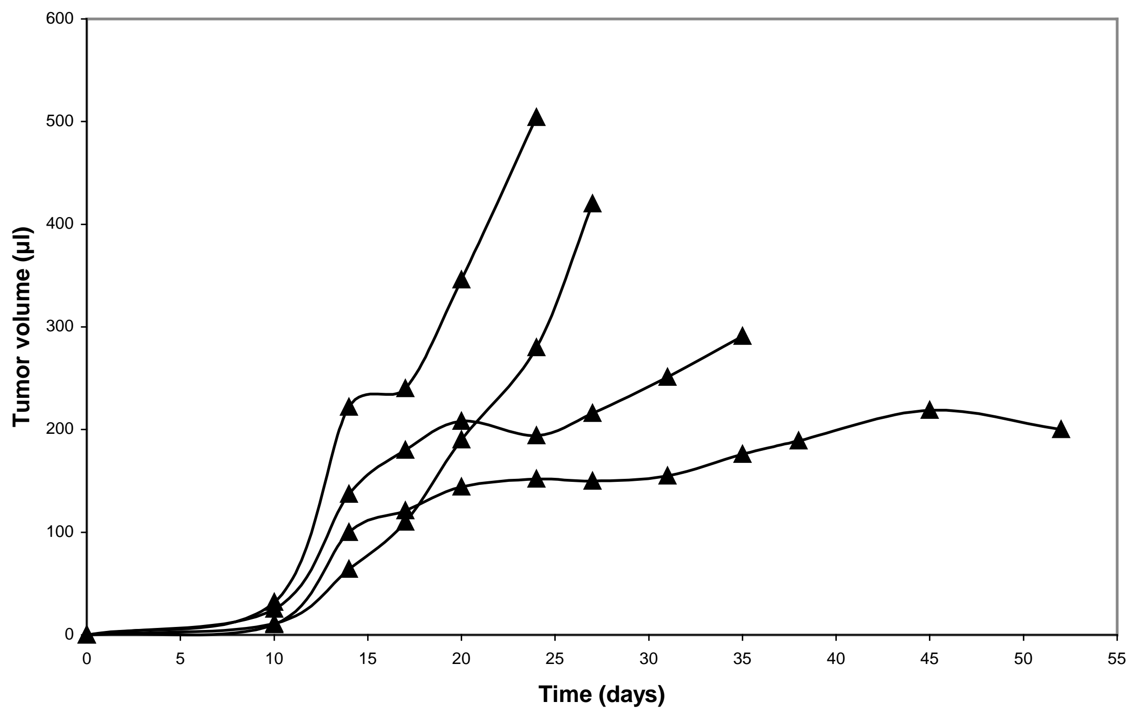


Fig. 4. Tumor volume evolution as determined by T2-weighted MRI in presence of 5-FU microspheres. The curves presented correspond to the rats involved in the statistical analysis.

When tumor developed, no relationship was observed between the number of cells injected and the tumor volume, as previously shown (Raila et al., 1999). After gathering the three groups, a positive effect of the 5-FU PMM 2.1.2 MS on the survival of the animals was observed (Fig. 3). Indeed, the median survival of the treated group was 34.3 ± 9.2 days ($n = 4$) and was significantly longer than the controls (20.6 ± 1.7; $n = 5$; $P = 0.02$). Thus, our system displayed a comparable 50% increase in life span versus 5-FU-loaded PLGA microspheres (Roullin et al., 2003).

The increase in life span observed after 5-FU PMM 2.1.2 MS treatment appeared to be linked to a limitation of C6 glioma cell proliferation as shown by the growth curves measured in vivo by using MRI, where a 4–15-day long lag was observed (Fig. 4).

In conclusion, excluding the long survivors from the analysis confirmed the limitations of the C6 glioma tumor model injected in immunocompetent animals, especially when therapeutic efficacy is addressed (Barth, 1998). Nevertheless, the present study emphasizes the potentialities of 5-FU PMM 2.1.2 MS on gliomas since this therapy significantly increased median survival and decreased tumor burden.

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