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Controlled Release of 4-Hydroperoxy-cyclophosphamide from the Fatty Acid Dimer–Sebacic Acid Copolymer

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ABSTRACT

Controlled polymeric release of chemotherapeutic agents has shown promise in the management of malignant gliomas. 4-Hydroperoxycyclophosphamide (4HC), loaded on the fatty acid dimer–sebacic acid copolymer (FAD:SA, 1:1), significantly prolonged survival in rats implanted with F98 and 9L gliomas. Here, we studied the in vitro *and* in vivo *release kinetics in phosphate-buffered saline and rat brain of 20% 4HC/FAD:SA (wt:wt), the optimal dose for treatment of rat gliomas.* In vitro *release under infinite sink conditions was steady over the initial 12 hr to a peak of 20–35% of impregnated drug, consistent with early phase control via surface erosion. Release over the next 3 weeks was minimal, consistent with barrier formation around the polymer by an oily fatty acid dimer degradation product and consequent slowing of release. However, the polymer started to disintegrate by day 4, and there were minimal visible remnants by 3 weeks. Thus, a considerable amount of polymer-carried drug was probably lost in the disintegrating fragments. Also, drug loss is expected from its inherent hydrolytic instability.* In vivo *release into brain revealed two peak levels of drug at 0–1 hr and 5–20 days. With loaded polymer implanted intraperitoneally or cyclophosphamide injected systemically, peak brain drug levels were measured in 2–8 hr, with substantial decrease by 48 hr without a second peak. Brain levels were substantially higher than blood levels at all time periods. We conclude that FAD:SA (1:1) adequately protects the otherwise labile 4HC, allowing effective and sustained drug release* in vivo. *Furthermore, it should be possible to modify the polymer to adjust the time of peak release for more beneficial therapeutic effects.*

KEYWORDS: Controlled release, Polymeric release, Drug delivery, Malignant glioma, Cyclophosphamide

INTRODUCTION

Interstitial chemotherapy with biocompatible controlled release polymers results in sustained, high, local drug concentration without the toxicity associated with systemic therapy [1–4]. Using the biocompatible, nonbiodegradable polymer ethylene–vinyl acetate (EVAc), we demonstrated the sustained local release of carmustine (BCNU) *in vitro* and *in vivo* [5]. These studies revealed steady high concentrations adjacent to the polymer over several days, with minimal spillage into the systemic circulation or contralateral brain. In order to optimize local delivery of the lipid-soluble BCNU, it was incorporated into the poly[bis(*p*-carboxyphenoxy)propane–sebacic acid] copolymer (PCPP:SA, 20:80). PCPP:SA is a biocompatible, biodegradable polymer that provides optimal protection and delivery of lipid-soluble chemotherapeutic agents such as the nitrosoureas [1–4]. Efficacy studies in the 9L gliosarcoma brain tumor model demonstrated significantly increased survival in rats treated with BCNU-loaded PCPP:SA polymer, versus control rats and rats treated systemically with BCNU. These experimental

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studies resulted in the testing of BCNU loaded on PCPP in a randomized, placebo-controlled study in patients with malignant gliomas [4, 6]. We have since extended these studies, seeking the optimal cytotoxic agent–polymer combination.

4-Hydroperoxycyclophosphamide (4HC) is a preactivated, hydrophilic derivative of cyclophosphamide (cytoxan) that spontaneously generates active metabolites. Unlike cyclophosphamide, 4HC does not require the hepatic cytochrome P-450 enzyme system for its activation [7, 8], thus making 4HC ideal for local delivery. Because of its hydrophilic properties, 4HC was incorporated into the fatty acid dimer–sebacic acid copolymer (FAD:SA, 1:1) which was designed to provide optimal release of hydrophilic moieties [6]. Using this delivery system, we found that treatment with 4HC prolonged survival in the 9L gliosarcoma and F98 rat tumor models. In the present study, we report the *in vitro* and *in vivo* release kinetics of 4HC loaded on the FAD:SA copolymer.

Symbols and Abbreviations

4HC, 4-hydroperoxycyclophosphamide; FAD, fatty acid dimer; SA, sebacic acid; CSF, cerebrospinal fluid; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; EVAc, ethylene–vinyl acetate; BCNU, 1,3-bis (2-chloroethyl)-1-nitrosourea; PCPP:SA, poly[bis(*p*-carboxyphenoxy)propane–sebacic acid] copolymer.

EXPERIMENTAL

Preparation of Polymeric Devices

Empty and 20% 4HC-loaded FAD:SA (1:1) polymer disks were fabricated and supplied by the Nova Pharmaceutical Corporation (Baltimore, MD, USA). The devices were fabricated via melt casting [6, 9, 10]. The polymer was first melted on a hot plate at 70°C, removed from the plate, and mixed quickly with 4HC by use of a spatula. The mixture was then pressed between two metal plates to obtain the appropriate thickness, 1 mm in this study. The 2 mm diameter devices were finally punched out with a cork borer. The 20% (wt:wt) 4HC-loaded FAD:SA (1:1 ratio) polymer was used because it has previously been determined experimentally to afford the longest survival in rats with the 9L gliosarcoma [11, 12].

In vitro kinetics

The polymer disk, measuring 2 × 1 mm (diameter × thickness), was placed in a 5 cm^3 solution of Dulbecco's phosphate-buffered saline (PBS) mixed with 20 cm^3 (per liter of PBS) of a solution containing penicillin G (10^4 U/cm^3) and streptomycin (10^4 μgm/cm^3). The polymer was carried at the tip of a 1.5 inch, 22 gauge needle, with the base of the needle affixed by glue to the inside of the cover of a scintillation counter vial. The latter carried the 5 cm^3 buffer aliquots, providing an infinite sink for polymeric drug release. The vial containing the polymer was placed in an incubator, with the temperature set at 37.5°C. For each time point, 1 cm^3 of the solution was withdrawn with a syringe and mixed with the reagent solution (see Assay) for later analysis of the amount of 4HC released. After withdrawal of each 1 cm^3 sample, the disk was transferred to another scintillation vial with a fresh 5 cm^3 of buffer solution. The procedure was repeated at hourly intervals over the first 12 hr. The disk was then incubated in the same 5 cm^3 of solution for another 12 hr, yielding the 24 hr release point. Subsequently, the solution was changed once a day for 3 weeks, enabling estimation of daily drug release over that time. Time intervals of incubation were varied slightly in a repeat experiment with a second polymer disk.

In vivo kinetics

Experimental Design. Two of three groups of rats were implanted simultaneously with both intracranial and intraperitoneal FAD:SA polymers. One of the pair of polymers was empty while the other was loaded with 20% (wt:wt) 4HC. The intracranial polymer was loaded with 4HC in the first group ($n = 14$) and empty in the second ($n = 7$), whereas the intraperitoneal polymer was empty in the first group and loaded with 4HC in the second group. The third group was injected intraperitoneally with cyclophosphamide ($n = 8$). At specified times after implantation, anesthesia was induced with xylaket. Then 1 cm^3 of blood was withdrawn via cardiac puncture and mixed with the reagent solution for later determination of blood 4HC levels. The rat was then sacrificed via a lethal dose of pentobarbital (nembutal). After recovery of the whole brain and supracollicular transection, the remaining brain, consisting mainly of the cortex, was divided in half sagittally. The polymer was removed. After each half of the brain was weighed and homogenized in 3 cm^3 of sterile water, 1 cm^3 of the homogenate was aspirated and mixed with the reagent solution. The reagent was kept in the refrigerator, wrapped in aluminum foil, before and after processing.

When reporting the results, ipsilateral hemisphere referred to the side implanted with polymer, whether empty or loaded with 4HC.

Animals. Male Fischer 344 rats weighing 150–250 gm were obtained from Sprague–Dawley and maintained, 4–5 rats to a cage, with free access to water and Certified Rodent Chow.

Anesthesia Each rat was initially anesthetized with 0.5–0.6 cm^3 of xylaket intraperitoneally. Each 100 cm^3 of the xylaket stock solution contained a filter-sterilized mixture of 25 cm^3 of ketamine for anesthesia, 2.5 cm^3 of xylazine for sedation, 14.5 cm^3 of 100% ethanol, and 58 cm^3 of normal saline.

Surgery

Intracranial. The general technique of intracranial implantation of polymer has been described previously [5, 13]. Briefly, under aseptic conditions, the

polymer disk was inserted intracranially through a 3 mm burr hole drilled 3 mm parasagittally and 5 mm posterior to the coronal suture on the left. The Weck microsurgical knife (Edward Weck Inc., Princeton, NJ, USA) was used to make a dural incision and a 3 mm transcortical niche for the polymer. The polymer was inserted and the skin was closed with staples.

Abdomen. The abdomen was shaved and prepared with 70% ethanol and povidone–iodine solution. A 5 mm long lower midline, vertical incision was made to the peritoneum. The polymer disk was inserted in the right lower quadrant. The incision was closed with 3-O vicryl sutures.

Quantitation of Drug Levels

7-Hydroxyquinoline Assay. The 7-hydroxyquinoline assay is a method for the measurement of 4HC levels in both blood and brain tissue which combines reagent extraction with HPLC separation and fluorometric quantification [14]. It measures the aldophosphamide and 4-hydroxycyclophosphamide degradation products of cyclophosphamide and its derivatives, such as 4HC.

Calculations. The 4HC levels were calculated in micromolar units during the assay. For the brain tissue, 4HC levels in units of μmoles/gm brain tissue were generated using the following formula:

$$\mu\text{moles/gm brain} = \frac{(\mu\text{moles/L}) \times (\text{L}/10^3\ \text{ml})}{(1/3 \times \text{gm brain wt})}.$$

It was assumed that each hemisphere of brain tissue was distributed uniformly into the 3 cm^3 of sterile water during the homogenization.

RESULTS

General

Qualitatively, the physical integrity of the polymer changed during the 3 weeks in which drug release was measured. By day 4 of the *in vitro* experiments, the polymer appeared soft, swollen and deformed. By day 6, the disk was disintegrating and the pieces adhered to the container whenever there was contact. By day 21, there was minimal remnant polymer at the end of the needle to transfer to buffer solutions. These characteristics are in contrast to the *in vivo* experiments where the disk essentially retained its original shape after 3 weeks and had a paler and drier appearance than the original disk.

In vitro kinetics

In Fig. 1 is shown the time course of *in vitro* 4HC release from the drug-loaded polymer disk, expressed as percentage of drug released. Two polymers measuring 2 × 1 mm and weighing, respectively, 12.1 mg and 10.5 mg were used. The two profiles were parallel and reproducible. Figure 2 is an expanded plot of the initial 12 hours of Fig. 1(a), fitted by linear regression. This phase was strongly linear ($r = 0.998$). Over the following 12–24 hr, there was a steady rate of drug release to a peak level of 20–30% of the total drug initially incorporated into the polymer. Subsequently, and over the following 3 weeks, there was minimal increase in the total quantity of drug released. After 3 weeks, less than 30–40% of the drug originally loaded on the polymer was recoverable in our assay.

In vivo Kinetics

Intracranial Polymeric Release. *In vivo* release was followed over a 3 week period and plotted on a semi-logarithmic (base = 10) scale to facilitate showing a wider time range (Fig. 3). Intracranial drug levels exhibited an initial small peak in concentration, followed by a larger peak occurring between 5–20 days. As shown in Figs 3 and 4, the ipsilateral brain levels were higher than the contralateral, whereas the blood levels were consistently lower than those measured intracerebrally. A fifth order polynomial, fitted

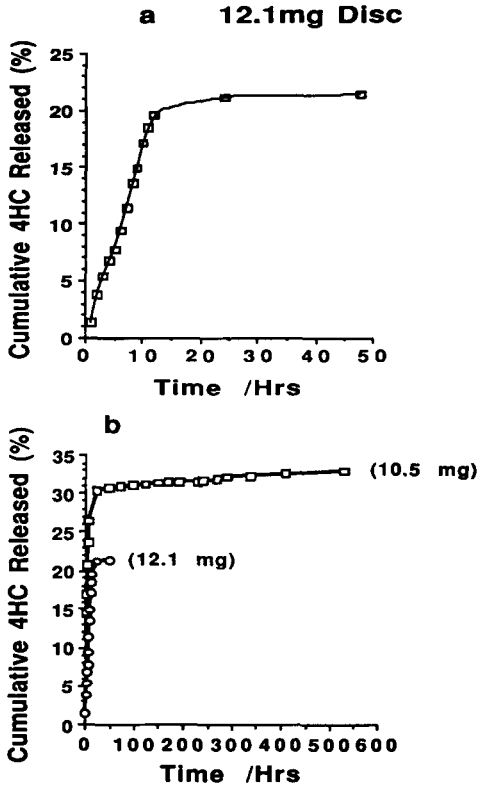

FIGURE 1. *In vitro* release kinetics of two separate disks of 20% 4HC/FAD:SA (1:1) measuring 2 × 1 mm (diameter × thickness) and weighing, respectively, 12.1 mg and 10.5 mg. In (a), the time scale is expanded to show release over the first 2 days, while (b) is a superimposition of the release profiles of the two disks. (b) Shows the extended release over 3 weeks and reveals a parallel release profile for the separate disks. Note that the measurable long-term release was less than 35% of the initial amount of drug impregnated in the polymer (see text).

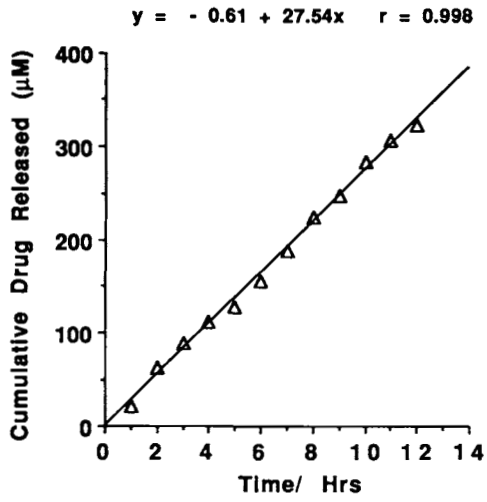

FIGURE 2. *In vitro* kinetics. The drug release over the initial 12 hours of the plot in Fig. 1(a) was replotted after performance of linear regression. The linear relationship ($r = 0.998$) is consistent with a surface erosion mode of control of polymeric release during that time window.

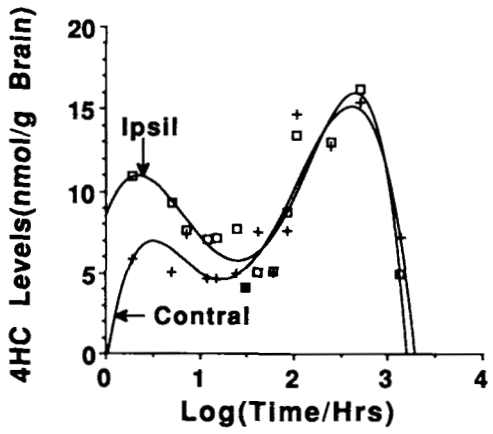

FIGURE 4. *In vivo* kinetics: superimposed plots of ipsilateral and contralateral drug levels from Fig. 3, each fitted with polynomial regression (fifth order). Drug levels of the ipsilateral and contralateral brain were measured after simultaneous implantation of 20% 4HC/FAD:SA (1:1) intracranially and empty FAD:SA (1:1) intraperitoneally. During the first 10 hr, both ipsilateral and contralateral hemispheres showed a local peak in drug level, suggesting early burst release. A second peak at days 5–20 probably reflected subsequent saturable kinetics (see text). Drug levels were generally higher in the ipsilateral hemisphere than the contralateral.

via nonlinear regression, was the best mathematical characterization of intracerebral release of 4HC from the FAD:SA polymer over the 3 week time range (Fig. 4).

Intraperitoneal Polymeric Release. We compared the levels of 4HC degradation products attainable in the brain and blood after simultaneous implantation of empty polymer intracranially and loaded polymer intraperitoneally. Only an early peak in concentration was demonstrable (Fig. 5). Drug levels quickly decreased to relatively low levels. The contralateral

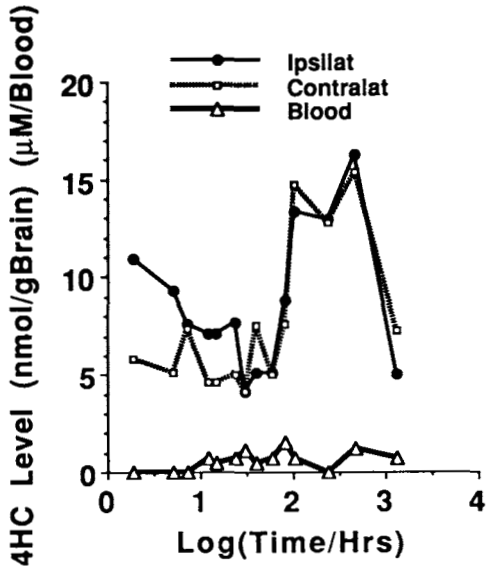

FIGURE 3. *In vivo* kinetics. Drug levels in blood, and in ipsilateral and contralateral hemispheres were plotted semi-logarithmically over 1,500 hr. The curves reveal a peak drug level during the first 10 hr, and a higher, sustained, second peak in the approximate time range of 10^2–10^3 hr (5–40 days). Blood levels were substantially lower.

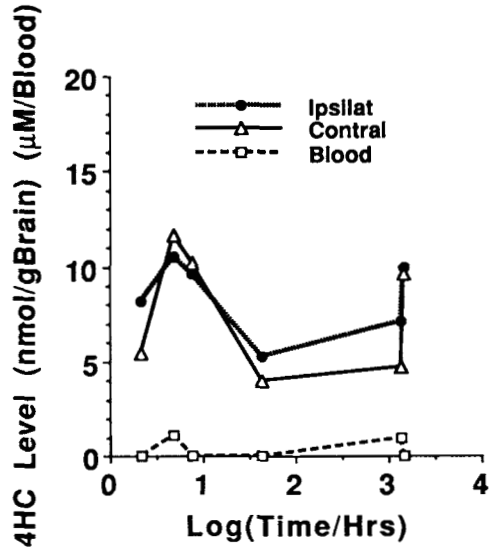

FIGURE 5. *In vivo* 4HC release from intraperitoneally implanted 20% 4HC-loaded FAD:SA (1:1). These plots again reveal an early peak in drug concentration in both hemispheres. However, in this instance, there was early tapering of drug levels over the first 2 days. Blood levels were negligible throughout the period of observation.

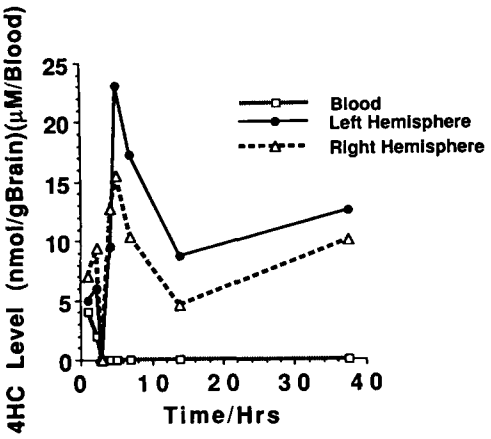

FIGURE 6. 4HC metabolic products in the blood and left and right hemispheres of the brain after intraperitoneal (systemic) injection with 100 mg/kg cyclophosphamide. Note that the abscissa is linear. Drug peaks were measurable approximately 5 hr after the injection.

hemisphere had levels lower than or equal to levels in the ipsilateral brain. Both sides of the brain had levels higher than those present in the blood.

Systemic Injection of Cyclophosphamide. We compared levels of cyclophosphamide degradation products in the brain and the blood after intraperitoneal injection of the drug (100 mg/kg). As shown in Fig. 6, drug levels in the left and right cerebral hemispheres were parallel, with the latter lower than the former. Both were significantly higher than blood levels.

DISCUSSION

In vitro 4HC Release

The results (Figs 1 and 2) revealed an early and relatively rapid release of 20–30% of incorporated drug from the FAD:SA, followed by a slower rate of release through about 3 weeks of incubation. At that point, the polymer had essentially completely disintegrated: thus, we could not demonstrate more than 30–40% recovery of impregnated drug in this study. Since the polymer began to break down around day 4, it is possible that a significant percentage of polymer with incorporated drug was discarded with the 5 cm^3 aliquots of buffer solution as they were changed. It is also possible that the preactivated drug that was exposed after hydrolysis of the matrix decomposed spontaneously with time and, hence, was not measurable at its maximal level.

The linear profile of the release kinetics during the initial 12 hr is consistent with a predominance of surface erosion over diffusion in the control of polymeric release during this phase. The degradation products, consisting of sebacic acid and insoluble, oily fatty acid dimer, would eventually build up and produce an oil barrier around the polymer [15]. Thus, subsequent water entry would be significantly slowed, leading to a decrease in hydrolysis, matrix degradation, and drug release in the following 3 weeks (Fig. 1). However, it is difficult to interpret the release data after 4 days because of the loss of integrity of the polymer.

In vivo 4HC Release

The undulating, double-peak, polynomial release profile of 4HC from FAD:SA polymer is consistent with an early fast release, as predicted by the ***in vitro*** data, followed by diffusion over a wide spatial confine associated with ongoing, initial local utilization. During this time, drug would be degraded and taken up into the various intracranial compartments. Also, some portions of both intact drug and metabolic products would eventually be eliminated via extracranial routes. Such a high initial turnover rate would account for the early portion of the curve, when a high initial concentration is observed, followed by a fall in concentration. Presumably, saturation phenomena subsequently intervene, with elimination lagging behind drug release, which would lead to a gradual rise in tissue concentrations during the second phase of the curve, towards the higher level second peak.

The measured drug levels, *in vivo*, are dependent on several factors, one of which is the release of 4HC from FAD:SA. Based on the results of the *in vitro* experiment, we expect an early steady drug release followed by a slowed, sustained release, which may be due to the coating of polymer with oily fatty acid dimer degradation products. Other factors include the partitioning of released drug and the degradation products of both drug and polymer into the various intracerebral compartments, and the elimination of the drug via the systemic circulation. The intracerebral compartments involved in the distribution of released drug include the extracellular, intracellular, vascular and cerebrospinal fluid (CSF) spaces. Elevated drug levels in the contralateral hemisphere probably resulted from a combination of diffusion through the CSF space and recirculation through the vascular system. The details and mathematical modeling of polymeric intracranial drug release are available elsewhere [16].

Clearly, our observations indicate that the macroscopic integrity of the loaded polymer was different *in vivo* and *in vitro*. In this and other unpublished experiments, we have observed remnants of relatively intact polymer *in vivo* as long as 5 months after implantation. On the other hand, *in vitro*, the polymer in this and other studies [15] began to disintegrate by day 4, and was completely disintegrated by 3 weeks. This cannot be explained by differences in pH since the value (7.4) was similar in the two situations. *In vitro* polymeric disintegration probably resulted from the infinite sink conditions of our paradigm.

Another notable result of this study was the time at which the drug levels reached their peaks. This has obvious clinical implications, in terms of the optimal time window for tumor management by this particular controlled release route. This observation

is particularly significant because it is possible to modify the polymeric backbone to vary the periods of peak drug release [3, 9, 10].

In summary, we found that intracranial implantation of 20% 4HC loaded on FAD:SA (1:1) resulted in sustained, bilateral concentrations of 4HC during days 5–20. We did not observe this when the similarly loaded polymer was implanted intraperitoneally; nor could we show similar results after intraperitoneal injection of cyclophosphamide. Thus, we conclude from the present report that the FAD:SA (1:1), implanted intracranially, has the requisite properties to protect the otherwise unstable and labile hydrophilic 4HC adequately, allowing long-term, effective controlled release for localized cytotoxic and alkylating activity against established rat brain tumors.

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