

Research Paper

Local Controlled Delivery of Anti-Neoplastic RNase to the Brain

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Received January 1, 2009; accepted April 11, 2009; published online May 5, 2009

Purpose. Antineoplastic RNase proteins, also known as Amphibinases, have been shown effective against various solid tumors but were found selectively neurotoxic to Purkinje cells in the cerebellum. This work describes the use of a waxy biodegradable poly(ricinoleic-co-sebacic acid) for the local controlled delivery of cytotoxic amphibinases in the parietal lobe of the brain in an attempt to overcome cerebellar neuronal toxicity while affecting glioma cells.

Methods. Amphibinase analogues were encapsulated in poly(ricinoleic-co-sebacic acid) formulations using mix-melt technology and loaded onto surgical foam. *In-vitro* release was monitored by BCA colorimetry and by RNase specific bioactivity. The implants were inserted into rat brains bearing 9L glioma to assess toxicity and efficacy.

Results. The various formulations showed extended linear release for several weeks with minimal burst effect. Best *in-vivo* efficacy was obtained with ACC7201 containing implants, resulting in the extension of the median survival from 13 to 18 days with 13% long-term survivors.

Conclusion. Antineoplastic proteins were released from a *p*(SA-RA) polyanhydride implants in a controlled manner, providing efficacy against 9L glioma, while evading neurotoxicity in the cerebellum. The controlled release of Amphibinases forms the potential for a new therapy against brain tumors.

KEY WORDS: amphibinase; glioma; polymer; ranpirnase; RNase.

INTRODUCTION

The standard protocol for the treatment of glioblastomas calls primarily for surgical debulking followed by either administration of Gliadel or oral temozolomide in combination with radiation therapy (1,2). Local drug delivery in the brain at the site of the tumor is an approach that has been well studied and approved by the US FDA and other regulatory agencies worldwide, showing the feasibility and enhanced efficacy of a variety of therapeutic agents when released from a biodegradable polyanhydride wafer implanted in the parietal lobe of the brain (3–9). In this work, the controlled local delivery of anti-neoplastic RNases as a potential therapy against brain cancer is investigated.

RNases derived from oocytes of Northern Leopard frogs (*Rana pipiens*), have been shown effective against a

variety of cancers (10). A number of these proteins, generally known as Amphibinases, have been investigated in preclinical and clinical studies (11–14), the best known among them being Ranpirnase, also referred to as Onconase®. Ranpirnase shows cytostatic or cytotoxic activity *in-vitro* in many different cell lines, being resistant to the mammalian ribonuclease inhibitor (RI) (14). *In-vivo* inhibition of tumor growth has been reported in various animal models, including multidrug resistant tumors. The exact pathway by which Onconase exerts its cytotoxic or cytostatic effects in neoplastic cells is still unknown. As ATP is required for the uptake, Ranpirnase is suggested to be actively transported into the cell by receptor mediated endocytosis. Once in the cytosol, tRNA is degraded, following G1-phase cell cycle arrest and subsequent apoptosis (14). There is some evidence that the cytotoxic action of Ranpirnase occurs by influencing the expression of cyclin dependent kinases. Other reports point to the cell's dependency on Ca²⁺-activated potassium channels to counter Ranpirnase toxicity (14). Though cytotoxic effects of Ranpirnase and related RNases have always been associated with their catalytic activity in cleaving RNA in general (15), a recent study suggests the cytotoxic effects could be the result of a more specific action in the cleavage of siRNA (16). In the study it was suggested that the catalytic activity was likely to occur within the RNA-induced silencing complex (RISC) of the RNA interference (RNAi) mechanism. The IC₅₀ values for cytotoxicity of Ranpirnase towards a number of different tumor cell-lines varied in the range from 10⁻⁶ to 10⁻⁸ M (10,14).

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When administered systemically, Amphibinases have shown only very few toxic side effects including spermatogenic cytotoxicity and embryo toxicity (17).

Unlike their high tolerance when used systemically, various Amphibinases, and in particular Ranpirnase, have displayed significant neurotoxicity when injected intracranially (18,19). The toxicity was shown to be dose-dependent and aimed specifically at Purkinje cells in the cerebellum with a reported IC_{50} concentration of 4.0×10^{-7} M. Loss of Purkinje neurons lead to symptoms, generally known as the Gordon phenomenon, including ataxia, muscular rigidity, paralysis, and tremor that may eventually lead to death (19).

Albeit their narrow therapeutic window, we hypothesized that the potential anti-tumor effects of Amphibinases in the brain could still be investigated, if the compounds were released in a controlled manner from a biodegradable depot, implanted in the frontal lobe, somewhat removed from the cerebellum. The hypothesis is based on the assumption that a slow continuous release of the active compound creates locally a diffusion-based concentration gradient, high enough in the frontal lobe to have a therapeutic effect and well below the threshold of toxicity in the cerebellum. In order to achieve this envisioned concentration gradient, the local delivery system must show a constant rate of release to maintain a therapeutic level, without any burst release that would cause concentrations in the cerebellum to reach toxic IC_{50} values.

In addition to linear release characteristics and biocompatibility with brain tissue, the delivery system must be suitable for proteins without losing bioactivity during manufacturing. Several polyanhydrides based on co-polymers of sebacic acid with either 1,3-bis(*p*-carboxyphenoxy)propane (CPP) or with fatty acid dimers (FAD) were found to be biocompatible and have been used successfully to deliver specific chemotherapeutic agents in the brain (1,2,5,20). However, incorporation and release of proteins from these polymers resulted in short release times and loss of activity due to heat and use of incompatible solvents. In this work a member of a newly discovered class of waxy poly(ester-anhydrides) has been used for making an implantable device for the release of Amphibinases. The implants were made under very mild conditions, requiring only mixing at room temperature for drug incorporation without need for additional solvents. The polyesteranhydride is based on sebacic acid (SA) and ricinoleic acid (RA) at a monomer weight ratio of 3:7. The polymer is pasty at room temperature and gels upon contact with water (21–27). Its chemical structure is depicted in Fig. 1.

The objective of this study was to examine the use of this polymeric implantable system for safe and effective delivery of amphibinases for treating brain tumors. Three amphibinases—Ranpirnase, Ranpirnase conjugated with epithelial

growth factor (ACC7201), and Amphinase—were formulated in the biodegradable implant and examined for their *in-vitro* release, biocompatibility and efficacy in rat brains bearing a 9L glioma tumor model.

MATERIALS AND METHODS

Materials

Poly(sebacic acid-co-ricinoleic acid) in a ratio of 3:7 (poly(SA-RA)3:7) was synthesized as previously described (21,22), with a molecular weight of $M_w=6,000$ Da and $M_n=4,000$; $T_m=25-30^\circ\text{C}$, $PI=1.5$. The Amphibinases, Ranpirnase, Amphinase (M_w of both are 11.8 kDa), and ACC7201, a conjugate of Ranpirnase with epithelial growth factor (EGF) at a ratio of 2:1 ($M_w=30.1$ kDa), were provided by Alfacell Inc, NJ. Tetraglycol was purchased from Aldrich-Sigma (St. Louis, MO) and solvents were obtained from Fisher Scientific (Pittsburgh, PA). Total Protein Assay was purchased from Pierce, Rockford, IL; RNase Alert Kit from Ambion, Austin, TX. Gelfoam gelatin sponges, size 4, were purchased from Upjohn Co. (Kalamazoo, MI).

Thermal analysis of the polyanhydrides was performed on a Mettler TA 4000-DSC differential scanning calorimeter, calibrated with zinc and indium standards, at a heating rate of $10^\circ\text{C}/\text{min}$. Molecular weights of the polymers were estimated on a gel permeation chromatography (GPC) system consisting of an isocratic HPLC pump (Waters, MA), Waters 2410 Refractive Index Detector, Rheodyne (Coatati, CA) injection valve with a 20- μL loop and Breeze software (Waters, MA). Samples were eluted with CHCl_3 through a linear Styragel HR2 column (Waters, 7.8×300 mm, $10 \mu\text{m}$ pore size) at a flow rate of 1 mL/min. The molecular weights were determined relative to polystyrene standards (Polyscience, Warrington, PA) with a molecular weight range of 1,100 to 7,920 Da.

In-Vitro Cytotoxicity

9L glioma cells were obtained from the Brain Tumor Research Center (San Francisco, CA). 9L cells were seeded in 96 well-plates and maintained in DMEM medium with 10% fetal bovine serum. For assay, cells were seeded at 3,000 cells per well and incubated for 24 h at 37°C . Ranpirnase, ACC7201 or Amphinase at various concentrations were added to the wells ($n=10$) and the plate was returned to the incubator. After 72 h, cytotoxicity was measured using the thiazolyl blue tetrazolium (MTT) assay. MTT at 5 g/mL was added to wells followed by incubation for 2 h. The medium was removed and 50 μL of DMSO were added and plates were scanned in 96-well plate reader at $\lambda=570$ nm.

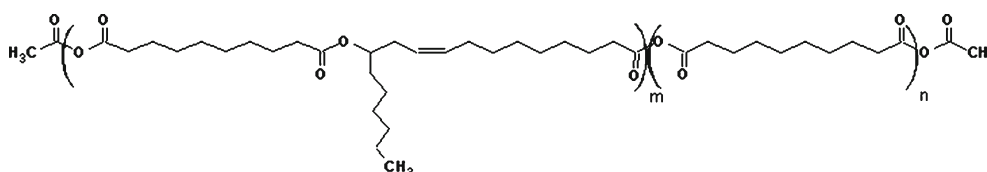


Fig. 1. Structure of poly(sebacic acid-co-ricinoleic acid anhydride) (poly(SA-RA) 3:7).

Polymer Formulation

In a typical formulation, containing 4% protein *w/w* polymer, poly(SA-RA) 3:7 (187.5 mg) was melted by placing the polymer in an oven set at 70°C. The molten polymer was then left at room temperature to cool to an ambient temperature. Addition of tetraglycol (195 μ L) to lyophilized protein powder (7.5 mg) resulted in a very fine, almost clear suspension. This suspension was subsequently mixed into the molten polymer forming a viscous opaque mixture. The protein-polymer formulation was absorbed in pieces of Surgical Gelfoam at a ratio of 30 mg polymer formulation per 1 mg of gelfoam: a pre-weighted piece of gelfoam (4 mg), measuring approximately 2×2×10 cm, was impregnated by placing it directly in the molten polymer-protein formulation until fully saturated. The piece of saturated gelfoam was placed on dry ice for the polymer to harden and form a waxy implantable material. The waxy material was weighed again, and four pieces were cut, weighing 31 mg (30 mg polymer formulation with 1 mg of gelfoam). For formulations containing less than 2% of the bioactive protein *w/w* polymer, BSA was added to keep the total protein load at 2% *w/w*. The protein mixtures were prepared by lyophilization of aqueous solutions containing the bioactive compound and BSA at the required ratios. For formulations containing higher protein loadings the ratio between polymer and tetraglycol was kept constant as more protein was mixed in, still resulting in finely, well dispersed suspensions.

In-Vitro Release

Pieces of gelfoam loaded with the Amphibinase protein/polyanhydride formulation of 30 mg were suspended in PBS (1 mL) and placed at 37°C. At specified time intervals, the PBS release medium was removed and replaced with fresh medium. Levels of released protein in the PBS were measured by the copper reduction-dependent Total Protein Assay or by determining specific RNase activity with the RNase Alert Kit. The following modification of the protocol of the RNase Alert Kit was made to enable quantitative measurements: 25 μ L of labeled RNA (2.5-fold excess over that required by the manufacturer's instructions) was added to the reaction mixture of the RNase sample (80 μ L) and RNase Alert Lab Test buffer (10 μ L). Leaving the plate at room temperature the samples were measured within 2 min of adding the labeled RNA, using a fluorescence 96-well plate reader (Perkin-Elmer, Shelton, CT) equipped with FI Winlab 4.00.02 software. Samples were always analyzed alongside calibration samples in triplicate. The plate was read multiple times during a period of several hours following the enzymatic reaction. However, it was found that data collected within 2 min after addition of the labeled RNA appeared most reliable based on the calibration curve. Using internal standards, no further kinetic analysis was done.

In additional experiments release medium, collected at specific time points, from the incubation of a polymeric formulation containing either polymer only, polymer with tetraglycol, or a formulation with Ranpirnase (2% *w/w* polyanhydride), was incubated with 9L cells. After 72 h the cell viability was measured using a MTT test as described above.

In-Vivo Safety Studies

Blank polymer formulations and formulations with increasing protein content (*w/w* polymer) were prepared as described and kept on dry ice before implantation. The implantation was conducted according to the method as previously reported (6). Briefly, female Fischer 344 rats, weighing 150–200 g (purchased from Charles River, Wilmington, MA) were anesthetized with an intraperitoneal injection (i.p.) (3 mL/kg) of a mixture of ketamine hydrochloride, 25 mg/mL, xylazine, 2.5 mg/mL, and 14.25% ethyl alcohol in 0.9% NaCl. After anesthetization, the head was shaved and sterilized with povidone-iodine and a burr hole was created. The formulation was then implanted into the brain cavity. After hemostasis was obtained, the scalp incision was closed with surgical staples and buprenorphine was injected intraperitoneally (0.3 mL/kg) for pain medication. Although we did not sterilize the formulations, a clean environment was kept during the preparation and implantation, similar to previous studies (6). The rats were kept in standard facilities with free access to food and water. After surgery, each rat was examined daily for general appearance, behavioral changes, and neurological deficits. Upon death, the brain was harvested and fixed in 10% formalin. A cross-section, including the cerebellum and cerebral hemisphere containing the implant, was prepared and stained with hematoxylin and eosin (H&E).

Intracranial Efficacy

The rodent 9L gliosarcoma was excised from the flanks of carrier rats and cut into 1-mm³ pieces, which were then implanted into the brain similar to the polymer implant procedure as described above (6). In the group receiving simultaneous implantation of tumor and therapeutic protein-loaded polymer, the piece tumor tissue was implanted first and then the polymeric formulation was inserted adjacent to it. Various polymer concentrations (*w/w*) were examined. In additional experiments the polymeric formulation was implanted 5 days after implantation of the tumor piece.

Postmortem, the brains were removed and placed into 10% formalin. Cross-section slides of the site of implantation as well as of the cerebellum were stained with H&E. Additionally, samples of brain tissue with or without 9L tumor were stained for EGFR expression with EGFR-specific antibody (28).

RESULTS

Formulation: Preparation and Analysis

Release of Ranpirnase, ACC7201 and Amphinase from Poly(SA-RA) 3:7 formulations have been measured using a BCA total protein assay or an RNase specific activity assay. Typical release profiles are shown in Figs. 2 and 3. Controlled release of RNases was followed using a BCA total protein assay. Fig. 2 shows continuous release profiles of Ranpirnase and Amphinase from poly(SA-RA) 3:7. From formulations with initial protein loadings up to 4% *w/w* protein, the controlled release did not show any significant burst effects in the first 24 h. At 2% *w/w* protein loading, 40% of the total

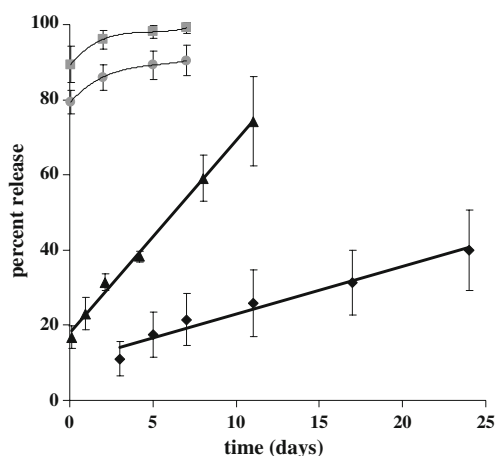


Fig. 2. Protein release from poly(RA:SA)7:3. Polymer (187.5 mg) was melted and cooled to ambient temperature. Protein was dispersed in Tetraglycol and mixed with the molten polymer. Surgical gelfoam was saturated with the formulation and put on dry ice to harden. Pieces of 30 mg were cut, weighed and suspended in PBS at 37°C for controlled release measurements. Release was analysed with Total Protein Assay (Pierce). *Black diamonds* 2% Ranpirnase w/w polymer; *black triangles* Amphinase in ratio 4%, *gray circles* 10%, and *gray squares* 20% w/w polymer.

amount of protein was released from the polyanhydride over a period of 25 days. In the first 24 h a very modest release of 11% was observed. The release profile was found to be linear ($R^2=0.964$). At 4% w/w loaded formulation with Amphinase, an initial release of 17% of the total loading was observed. Over 11 days, 74% of the total protein content was released in a linear fashion ($R^2=0.995$). By increasing the loading of protein a faster release rate was observed. In formulations containing 10% or 20% Amphinase, almost 80% of the total protein content was released within the first 24 h. The remaining protein was released in a controlled manner over a period of several days.

Controlled release of Ranpirnase, ACC7201 and Amphinase has also been established on the basis of RNase specific activity. In formulations where initial RNase concentrations were lower than 2% w/w, BSA was used to maintain an initial total protein concentration in the formulation of 2% w/w. Therefore, release of RNases from those formulations that also contained BSA could only be determined using the specific RNase activity assay. Short term release of EGF-bound Ranpirnase (ACC7201) at 0.5% w/w with 1.5% w/w BSA in poly(SA-RA) 3:7 implants was investigated. Typical data indicated a time-controlled release curve. Using the specific RNase activity assay the overall profile was linear, similar to the release of Ranpirnase from this polymer formulation, releasing about 30% of the ACC7201 over 7 days, with 10% release in the first 24 h.

Amphinase containing formulations showed initial releases in the first 24 h of 10% to 20% (Fig. 3B). The investigated protein loadings were based on formulations that were tested for toxicity and efficacy as well.

In-Vitro Cytotoxicity

Antineoplastic activity of the various amphinases has been tested *in-vitro* on a 9L glioma brain tumor cell line. All amphinases showed a potent cytotoxic effect at sub-micromolar concentrations. As shown in Fig. 4, linear correlations were found when plotting the cell viability *versus* log(concentration). Concentrations yielding 50%-growth inhibition, the IC_{50} values, were calculated for the different compounds and presented in Table I.

When release medium, collected from the incubation of a polymeric formulation containing Ranpirnase (2% w/w polyanhydride) for a 12-h period, was incubated with 9L cells, MTT test showed a 92% growth inhibition. In contrast, release medium obtained from incubating a control polymeric formulation resulted in about 5% growth inhibition for p(SA-RA) only. When tetraethylenglycol was added to the formulation 44.5% growth inhibition was found. Incubation

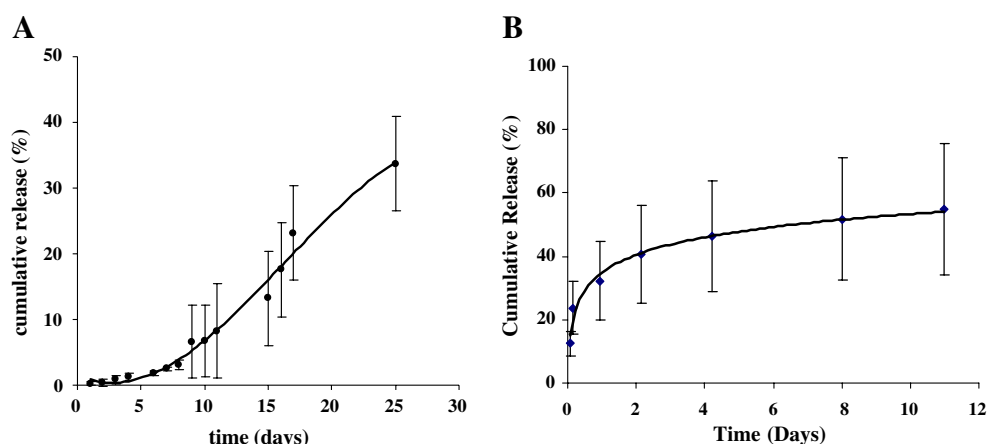


Fig. 3. Protein bioactivity from controlled release. Ranpirnase in ratio of 2% w/w polymer (A), Amphinase in ratio of 4% w/w polymer (B). Polymer (50 mg) was melted and cooled to ambient temperature. Protein (1 or 2 mg respectively) was dispersed in 52 μ l Tetraglycol and mixed with the molten polymer. Surgical gelfoam was saturated with the formulation and put on dry ice to harden. Pieces of 30 mg were cut and weighed. Three pieces were suspended in 1 mL PBS at 37°C for controlled release measurements. At specific time intervals the buffer was exchanged with fresh buffer and the controlled release of the different RNases was measured by specific RNase activity using the RNase Alert kit.

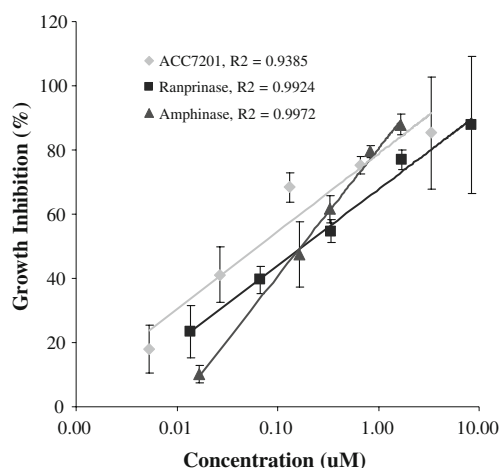


Fig. 4. *In-vitro* cell toxicity, dose–response correlations. 9L cells were seeded in 96-well plate at 3,000 cells per well and incubated for 24 h. The cells were then treated with Ranpirinase, ACC7201 or Amphipinase and incubated for 72 h. Growth inhibition was assessed using incubation with MTT.

of media of subsequent release periods, however, resulted in high growth inhibitions for all formulations, including polymer alone and for control formulations containing polymer and tetraethylenglycol. In all release media the pH turned acidic after 12 h as indicated by the media turning yellow.

***In-Vivo* Toxicity Studies**

In safety studies conducted in Fisher 344 rats ($n=3$), pieces of polymer in gelfoam (30 mg) were implanted intracranially. The implants, hardened on dry ice, were kept on dry ice until prior to implantation. We found that this procedure, besides form preventing the formulation to become too tacky to implant, by keeping the implants cold, also led to faster reaching hemostasis and significantly less bleeding. Severe neurotoxicity was observed for polymer formulations containing 5%, 1% and 0.1% Ranpirinase *w/w* polymer (Fig. 5A). Symptoms of Purkinje neuron toxicity consisted of hind paw paralysis, leading to animal death within 3 days. H&E staining of sagittal cross-sections, including tumor, cortex, and the cerebellum, revealed a significant loss of Purkinje cells along the granular layer in the cerebellum (as indicated by the white arrows in Fig. 5B). The formulation with 0.05% Ranpirinase *w/w* polymer was found to be the maximally tolerated dose that resulted in reduced loss of Purkinje cells and no animal deaths 30 days after implantation.

Amphipinase was tested at various loadings up to 20% *w/w*. At 10% and 20% *w/w* protein formulations several deaths were observed. H&E staining of the cerebellar tissue showed neuronal damage, similar in nature to the lesions caused by Ranpirinase. However, the overall amount of observed damage was less in comparison. (Fig. 5C). No deaths were observed with 5% *w/w* formulations up to 30 days after implantation.

Despite some Purkinje cell death, implantations of polymer formulations with ACC7201 in concentrations up to 4% *w/w* polymer did not result in any mortality. Fig. 5D shows an H&E staining of the cerebellum after exposure to

ACC7201 from a formulation containing 1% *w/w* of the protein, indicating limited damage to Purkinje cells.

***In Vivo* Efficacy**

For *in-vivo* experiments, in which rats were challenged with intracranially implanted 9L glioma, treatment with polymer implants containing 0.05% *w/w* Ranpirinase showed little to no prolongation in median survival, whether implanted directly with the tumor piece or 5 days after tumor implantation (Fig. 6A). The control group ($n=8$) had a median survival of 13 days. Groups treated with Ranpirinase-containing formulations ($n=8$) had a median survival of 14 days. Postmortem histochemical analysis by H&E staining of the brains from rats that died in efficacy experiments with 0.05% *w/w* Ranpirinase confirmed that these animals died from 9L glioma. Also, even though Ranpirinase-containing formulations at the maximally tolerated dose of 0.05% *w/w* polymer were implanted, damage to Purkinje cells was observed.

In an efficacy experiment testing 5% *w/w* Amphipinase, a modest increase in overall survival was observed. The median survival was increased from 14 days in the control group to 17 days in the animals treated with Amphipinase (Fig. 6B).

Median survival increased when animals were treated with an implant containing 4% *w/w* ACC7201. The median survival shifted from 12 days in the control group to 18 days in the group of animals treated with 4% *w/w* ACC7201 ($n=16$, $p<0.0001$) (Fig. 6B): Two out of 16 animals, treated with 4% *w/w* ACC7201 implants, were long-term survivors (>120 days).

It is assumed that part of the action of ACC7201 against tumor cells is due to targeting based on the EGF portion of the conjugate. EGFR-specific staining of brain tissue samples, with or without implanted 9L tumor, clearly show abundant, albeit inhomogeneous expression of the EGFR receptor in the tumor tissue. Except for local blood vessels, no EGFR expression could be found in Purkinje cells or their surrounding tissues (Fig. 7A, B).

DISCUSSION

Using the novel mixed polyester anhydride comprising sebacic and ricinoleic acids, poly(SA:RA) 3:7, we have shown that the different Amphibinases can be released effectively and in a controlled manner both *in-vitro* and *in-vivo* in the brain of rodents. No organic solvents were needed for the preparation of this formulation, because the protein could be dispersed in the polymer while in the molten state. In this manner, loss of bioactivity due to solvent–interface interaction with the protein was prevented. At physiological

Table I. IC₅₀ Values of Amphibinases on 9L Cells as Established Using the MTT Test

Compound	IC ₅₀ (nM)
Ranpirinase	180
ACC7201	64
Amphipinase	172

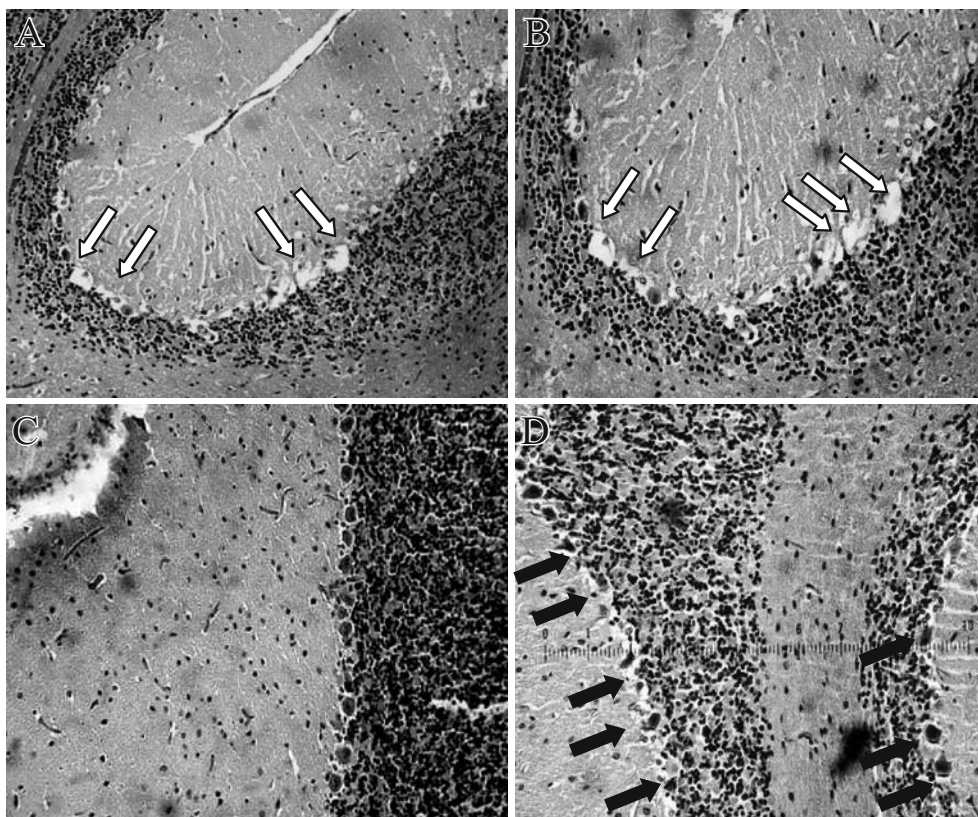


Fig. 5. *In-vivo* safety study. Postmortem H&E staining of cerebellum cross-sections, 3 days after polymer implantation (pieces of 30 mg) with **A** Ranpirnase 5% w/w and **B** ACC7201 conjugate 5% w/w poly(SA-RA) 3:7. White arrows indicate Purkinje cell deletion along the granular layer of the cerebellum. **C** H&E staining of Purkinje cells in cerebellum upon implantation of Amphinase 10% w/w poly(SA-RA)3:7. **D** ACC7201 1% w/w poly(SA-RA) 3:7. Pieces of formulation containing gelfoam were intracranially implanted. Black arrows point to intact Purkinje cells.

temperature the polymer formulation was of a waxy consistency. Among several different agents examined for their ability to disperse the protein in the molten polymer, tetraglycol was found to be the most suitable excipient, which kept the protein dispersed while being miscible with the liquid polymer. For making the implants, the optimal ratio of tetraglycol to polymer was 1:1 where a controlled release profile was still maintained.

Looking at values obtained by BCA total protein analysis, we found a general linear trend in the release profiles, where the release rate increased with the total protein loadings up to 4% w/w of the formulation. Higher loading percentages at 10% w/w or 20% w/w resulted in a burst, releasing about 80% of the total protein load within the first 24 h. The burst was followed by controlled release of only a few days. The linear release rate was shown in formulations with Ranpirnase and with Amphinase. The linear release profiles of the protein is thought to be the result of a number of factors, including diffusion based release, and release based on the degradation of the anhydride bonds in the mixed polyesteranhydride. The BCA assay is known to show some background in the presence of ethyleneoxide-based compounds such as PEG and tetraglycol. Although this has been accounted for using formulations without protein, the values at the onset of the release can still be higher as most of the tetraglycol was found to release in the first 24 h.

Ranpirnase and Amphinase are the smallest members of the ribonuclease (RNase) family. The compact quaternary structure of Ranpirnase and its distinctive fourth disulfide bond bridging the C-terminal cysteine contribute to the unusual thermal stability of the protein: the denaturation temperature was found to be 90°C, compared to 62.5°C for RNase A (14). It has been assumed, based on this extraordinary thermal stability of Amphinases, and the absence of organic solvents, that no activity would be lost during the processing of the formulations. In order to ascertain this assumption and to mitigate the effect of tetraglycol on the BCA assay, we used a modified RNase Alert Kit to quantify RNase based on specific activity. A slight difference was observed in the total release of both Ranpirnase and Amphinase when comparing the values obtained from the BCA total protein assay with those obtained from the RNase specific activity assay: total release of Ranpirnase found 39% versus Amphinase at 72%. Typical results of a short term release of EGF-bound Ranpirnase (ACC7201) at 0.5% w/w in a formulation with BSA in poly (SA-RA) 3:7 implants was found to occur in a similar time-controlled linear manner as the release of Ranpirnase.

Although the trends in total protein releases were found similar where using the RNase specific activity assay about 10% less protein release was measured, the profiles of the release did not completely match up as the release curves

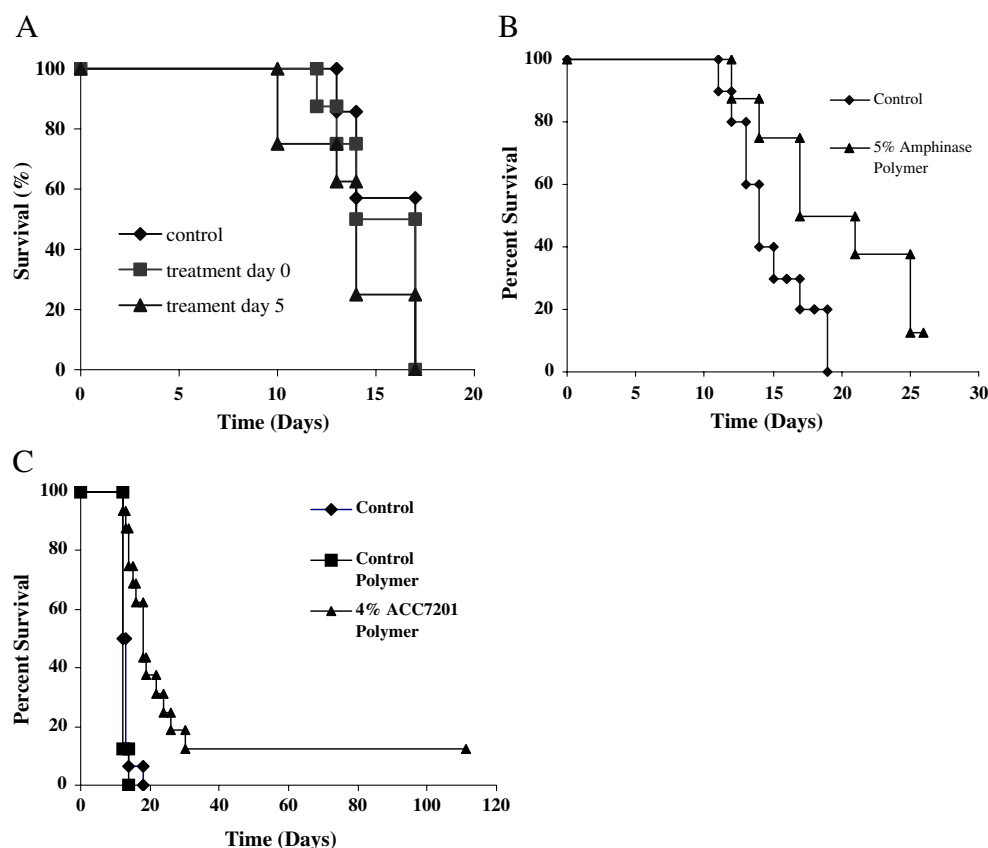


Fig. 6. Efficacy of locally delivered Ranpirnase, Amphinase and ACC7201 in the intracranial 9L glioma rodent model. Rodent 9L gliosarcoma was excised from the flanks of carrier rats and cut into 1-mm³ pieces, which were then implanted into the brain. The piece tumor tissue was implanted first and then the polymeric formulation was inserted adjacent to it. The polymeric formulation was implanted either simultaneously or 5 days after implantation of the tumor piece. Implants (30 mg) contained **A** Ranpirnase 0.05% w/w poly(SA-RA), *n*=8 per group; **B** Amphinase 5% w/w poly(SA-RA) 3:7, *n*=8 per group; and **C** ACC7201 4% w/w (*n*=16 per group).

obtained from RNase specific activity data were less linear in nature. Specifically differences were found in the onset of the release of Ranpirnase where the release seemed delayed looking at protein activity, compared to the initial release measured with the BCA kit. Part of these differences can

potentially be explained by effects of tetraglycol on the BCA kit that could not be accounted for using blank formulations. The delayed release of Ranpirnase in Fig. 3A and the faster tapering off of Amphinase in Fig. 3B could also be the result of loss in protein activity as for both proteins about 10% less

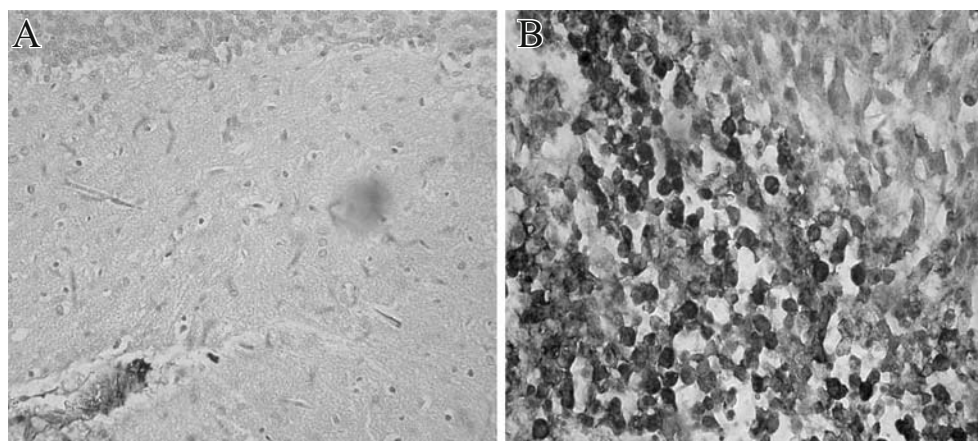


Fig. 7. EGFR expression: cerebellum *versus* 9L-glioma. Immunohistochemical staining for Epithelial Growth Factor Receptor (EGFR) in **A** cross section of cerebellum; **B** cross section of 9L glioma, implanted intracranially in the parietal lobe.

in total release was measured when using the RNase specific activity assay. It is unclear, however, why in the case of Ranpirnase this loss of activity happens at the onset of the release whereas in the case of Amphinase this loss of activity occurs after several days.

Solutions of the amphinases in cell medium in different concentrations were tested on cells in order to establish cytotoxic activity against the 9L glioma cell line and assess any dose response correlation. All three tested amphinases showed potent cytotoxicity against 9L glioma *in-vitro*. Based on the measured dose–response curves, IC_{50} values were calculated. Ranpirnase and Amphinase displayed similar IC_{50} values, whereas ACC7201 showed to be three times more potent. However, ACC7201, a Ranpirnase conjugate with EGF, contains two active Ranpirnase moieties that have to be accounted for.

We have tried to show cytotoxicity of Ranpirnase, when released from a 2% *w/w* polymer formulation. A significant difference in growth inhibition was seen when comparing the first 12-h release media of this formulation with the various control formulations, including controls that contained tetraglycol. *In-vitro* incubation of release media on 9L cells from of subsequent release periods, however, resulted already in high growth inhibitions for polymer alone. The release media at longer time points must contain ricinoleic acid, a breakdown product of the polyanhydride, which, at higher concentrations works as a surfactant causing cell damage. This assumption is also based on the fact that the pH in the release medium of polymer alone turned acidic after 12 h, as indicated by the cell medium pH indicator. Therefore no difference could be seen between control and Ranpirnase containing formulations in subsequent time points of the controlled release.

In the present study, we found that polymer formulations containing more than 0.1% Ranpirnase *w/w* polymer showed severe neurotoxicity in intracranial toxicity studies, conducted in rats. The toxicity expressed itself as what is known as the Gordon effect and was followed by animal death. Post mortem tissue staining showed that the toxicity was accompanied by an almost complete loss of Purkinje cells in the cerebellum. Unlike earlier reported findings (19), the Gordon effect was not reversed once its onset was observed. Intracranially delivered Ranpirnase at a concentration of 0.05% *w/w* polymer, did not affect animal survival, but partial toxicity to Purkinje cells was still observed since H&E staining of rat brains showed some Purkinje cell deletion (as indicated by the black arrows in Fig. 5D).

Amphinase showed a surprisingly lower specific neurotoxicity in the cerebellum as it may have a lower specific affinity for Purkinje cells. Still, intracranial bolus injections show the occurrence of the Gordon effect, similar to Ranpirnase. One death was observed in safety studies with a 10% *w/w* Amphinase polymer formulation and, therefore, efficacy studies were conducted with 5% *w/w* formulations. Based on the *in-vitro* release results, where it was shown that control of the release was lost in formulations containing 10% *w/w* protein or more, there is some evidence to suggest that the increase in mortality at higher loadings may be related to an increase in the burst release. The efficacy study showed a modest increase in the median survival ($p < 0.023$, Fig. 6). The

increase in median survival is thought to be the result of the combination of both its potency on 9L glioma cells *in-vitro*, being comparable to Ranpirnase, and its lower neurotoxicity enabling the increase of the total loading in the implant.

In-vivo safety studies with ACC7201 in concentrations up to 4% *w/w* polymer did not result in mortality, although slight toxicity to the cerebellum was observed. Unlike Ranpirnase, a 1% *w/w* ACC7201 formulation only slightly affected Purkinje cells (Fig. 5D). This increase in the maximally tolerated dose of ACC7201 was roughly 80 times higher (weight ratio) as compared to non-conjugated Ranpirnase. At higher concentrations of ACC7201 in a polymer formulation or as bolus intracranial injection, neuronal damage, similar to that caused by Ranpirnase, was observed as shown by histochemical staining in Fig. 5B. Part of the increase in tolerance for ACC7201 can be attributed to the EGF moiety in the conjugate. Many tumor cell lines over-express endothelial growth factor receptor (EGFR) making it a diagnostic marker in some cases (28). Except for the endothelium of blood vessels EGFR is generally not expressed abundantly in the brain, including the Purkinje cells. Although specific binding of ACC7201 to EGFR has not been verified in the present study, immunohistochemical staining confirmed abundant, though inhomogeneous, expression of EGFR in the 9L-tumor cell-line whereas Purkinje cells only weakly expressed the EGFR cell-surface receptor (Fig. 7A, B).

Locally delivering the 4% ACC7201 polymer formulation resulted in a modest but significant increase in survival with 13% long-time survivors in the treatment group ($p < 0.0001$, $n = 16$ per group; Fig. 6B). Specific targeting due to the difference in receptor expression may form an important factor in the success of ACC7201 to evade neurotoxicity and increase the median survival in addition to being locally released in a controlled manner.

CONCLUSION

We have been able to release anti-neoplastic proteins from a *p*(SA-RA) polyanhydride implant, establishing general linear release trends with low burst effects for formulations up to 4% *w/w* protein loading. Using mix-melt technology under mild conditions prevented any significant loss in protein activity. From these implants the various Amphinase analogues were delivered intracranially in a rodent model in a controlled manner, successfully evading neurotoxicity in the cerebellum. Efficacy studies showed a modest but promising increase in median survival in two formulations, containing 5% Amphinase or 4% ACC7201. It can be concluded that Amphinases provide a novel series of compounds with a potential for new therapies against brain tumors. The aspect of local controlled delivery of the Amphinases forms an enabling step in the realization of their full potential.

ACKNOWLEDGEMENTS

We acknowledge Alfacell Inc. for generously providing Ranpirnase. This work was supported in part by NIH NCDDG CA52857.

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