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Laboratory Investigation

Lactacystin exhibits potent anti-tumor activity in an animal model of malignant glioma when administered via controlled-release polymers

Federico G. Legnani[1,3,†], Gustavo Pradilla[1,2,†], Quoc-Anh Thai[1], Alessandro Fiorindi[5], Pablo F. Recinos[1], Betty M. Tyler[1], Sergio M. Gaini[3], Francesco DiMeco[1,4], Henry Brem[1,2] and Alessandro Olivi[1,2]
[1]*Department of Neurosurgery, School of Medicine, Johns Hopkins University;* [2]*Department of Oncology, School of Medicine, Johns Hopkins University;* [3]*Department of Neurosurgery, School of Medicine, University of Milan, Italy;* [4]*Istituto Nazionale Neurologico "Carlo Besta", Milan, Italy;* [5]*Department of Neurosurgery, Ospedale Ca' Foncello, Treviso, Italy*

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Summary

Lactacystin, a proteasome-inhibitor, has been shown to induce apoptosis of experimental gliomas *in vitro*. However, its systemic toxicity prevents further clinical use. To circumvent this problem, lactacystin can be delivered intratumorally. We tested the efficacy of lactacystin incorporated into controlled-release polymers for treating experimental gliomas. 9L-gliosarcoma and F98-glioma cell lines were treated with lactacystin (10–100 μg/ml) for 72 h *in vitro*. Cell-viability was measured with MTT-assays. Toxicity of lactacystin/polycarboxyphenoxypropane-sebacic-acid (pCPP : SA) polymers was tested *in vivo* using Fischer-344 rats intracranially implanted with lactacystin polymers loaded from 0.1 to 2% lactacystin by weight. The efficacy of 1, 1.3, 1.5 and 1.7% lactacystin/pCPP : SA polymers was determined in Fischer-344 rats intracranially challenged with 9L and treated either simultaneously or 5 days after tumor implantation. Lactacystin was cytotoxic in 9L cells, causing a 16 ± 8% growth inhibition at 10-μg/ml that increased to 78 ± 4% at 100-μg/ml. Similarly, lactacystin inhibited growth of F98 by 18 ± 8% at 10-μg/ml and 74 ± 2% at 100-μg/ml *in vitro*. Polymers released lactacystin for 21 days and intracranial implantation in rats neither generate local nor systemic toxicity at doses lower than 2%. Treatment with lactacystin/pCPP : SA polymers with loading concentrations of 1.0, 1.3, and 1.5% prolonged survival of animals intracranially challenged with 9L when polymers where inserted in the day of tumor implantation. In conclusion, lactacystin exhibits potent cytotoxic-activity against 9L and F98 *in vitro*, it can be efficiently incorporated and delivered using controlled-release polymers, and at the proposed concentrations lactacystin polymers are safe for CNS delivery and prolong survival in the 9L model.

Introduction

Lactacystin is a microbial product that inhibits cell proliferation[1]. Its anti-neoplastic activity is dependent on inhibition of the ubiquitin proteasome proteolysis pathway (UPP) which controls cellular protein turnover[2]. Lactacystin selectively inhibits the proteasome on neoplastic cells [3–5] and appears to enhance the activity of chemotherapeutical agents [6] and radiosensitizers [7]. Lactacystin has been shown to trigger apoptotic cell death in several human glioma cell lines [8].

Recently published clinical studies have shown that proteasome inhibitors are effective in the treatment of both solid and hematological malignancies. Phase I and II clinical trials established that the proteasome inhibitor bortezomib could be administered with acceptable and manageable toxicity [9,10] and that it was efficacious in the treatment of various malignancies such as multiple myeloma [11], non-small cell lung carcinoma [9], and non-Hodgkin's lymphoma, among others [12]. Systemic administration of lactacystin for the treatment of brain tumors, however, would require very high doses to achieve penetration through the blood brain barrier (BBB), an approach likely to generate severe toxicity.

Local delivery of drugs using controlled-release polymers has been a safe alternative for administration of chemotherapeutical agents to malignant brain tumors. Controlled-release polymers bypass the BBB, increase local concentrations of the delivered agents and prevent systemic toxicities [13]. Local delivery of carmustine using biodegradable polymers (Gliadel®) prolongs survival of patients with brain tumors and received regulatory approval for both recurrent and newly diagnosed malignant gliomas.

In this study we tested the cytotoxic activity of lactacystin in 9L gliosarcoma and F98 glioma cell lines *in vitro*, and incorporated lactacystin into controlled-release polymers to asses the safety and efficacy of intracranially implanted lactacystin polymers in the brain of animals challenged with 9L gliosarcoma.

† These authors equally contributed in all aspects of this study.

Material and methods

Tumor cell lines

Rat 9L gliosarcoma cell line was obtained from Dr. M. Barker at the University of California at San Francisco Brain Tumor Research Center (San Francisco, CA). The F98 glioma cell line was obtained from Dr. R. Barth (Ohio State University, Columbus, OH). Cells were maintained in 10-ml of Dulbecco's Modified Eagle Medium (DMEM) with fetal bovine serum media, and replaced when reached an appropriate density according to the suggestions of the provider.

Efficacy in vitro

The cytotoxic activity of lactacystin (purchased from A.G. Scientific, Inc., San Diego, CA) was evaluated *in vitro* against 9L gliosarcoma and F98 glioma cells using the MTT assay. Five thousand 9L cells/well and 5000 F98 cells/well were plated in 96 well plates each, and incubated for 24 h at 37 °C, with a total volume of 100-μl/well. Treatment with lactacystin was administered at concentrations of 10, 25, 50, 75 and 100 μg/ml and percentages of cell viability were calculated using the MTT assay 72 h later. The MTT assay is described elsewhere [14]. Briefly, after experiment completion, supernatant from each of the wells was aspirated and replaced with 0.5 mg/ml MTT solution (prepared in DMEM) at 130-μl/well. Cell plates were then incubated at 37 °C for 4 h at the end of which the MTT solution was aspirated and then replaced with 150-μl/well of reagent grade dimethyl sulfoxide (DMSO). Cells viability was measured with an ELISA plate reader (Beckman Coulter, model AD 340, Fullerton CA) at a wavelength of 570 nm.

Polymer preparation

The matrix poly[bis(p-carboxyphenoxy)propane-sebacic acid] (pCPP : SA), was supplied by Guilford Pharmaceuticals Corp. (Baltimore, MD). pCPP : SA, a biodegradable polymer, was used in combination with lactacystin 0.1, 0.5, 1, 1.3, 1.5, 1.7 and 2% by weight (w : w). pCPP : SA and lactacystin were combined and dissolved in methylene chloride at room temperature. This solution was dried in a vacuum desiccator for 4–5 h and stored at −20 °C. The polymers were pressed with a steel mold into disks measuring 3-mm in diameter and 1-mm in thickness, with a final weight of 10-mg each.

Pharmacokinetics in vitro

Pharmacokinetics of lactacystin incorporated into pCPP : SA matrix were determined with a Spectronic Genesis 5 Spectrophotometer (Spectronic Instruments, Rochester, NY) by comparison with a standard absorbance curve using a methodology previously established in our laboratory and described in detail elsewhere [15]. Briefly, triplicate samples of pCPP : SA polymers (10 mg wafers) loaded with lactacystin at concentrations of 1, 1.3, 1.5 and 1.7% were suspended in separate glass vials each containing 1-ml of Dulbecco's Phosphate-Buffered Saline (Invitrogen Co., Grand Island, NY 14072) (PBS) at 37 °C. Polymers were sequentially transferred to fresh 1-ml aliquots of PBS at several time points up to 504 h (21 days), samples were collected and spectrophotometrical absorbance was recorded.

Animals

Female Fischer 344 rats, weighting 150–200 g, obtained from Harlan Sprague Dawley (Indianapolis, IN) were used in this study; all animals were housed in standard animal facilities with free access to Baltimore city water and rodent chow. All animal experiments were performed in accordance with the policies and principles of laboratory care of The Johns Hopkins University School of Medicine Animal Care and Use Committee.

Experimental design

Animal toxicity study

Female Fisher 344 rats (n = 15, 3 animals per polymer concentration) were intracranially implanted with 10 mg wafers of 0.1, 0.5, 1.0, 1.5, and 2% lactacystin loaded pCPP : SA polymers. Animals were evaluated pre-operatively, and daily for 120 days to determine signs of local and systemic toxicity. Animals were euthanized at 120 days and their organs were harvested and fixed in 10% paraformaldehyde for histopathological analysis.

Efficacy of lactacystin incorporated into pCPP : SA wafers

First experiment. Fischer 344 rats (n = 17) were randomized into 2 experimental groups. Animals in group 1 (n = 9) received 9L intracranial tumor only serving as controls, animals in group 2 (n = 8) received 1% lactacystin/PCPP : SA polymers simultaneously with the tumor implant (day 0) (Table 3).

Second experiment. In two separate trials, Fischer 344 rats (n = 48) were randomized into 3 experimental groups. Animals in group 1 (n = 16) received intracranial 9L tumor only, animals in group 2 (n = 16) received 1.5% lactacystin/pCPP : SA polymers on day 0, animals in group 3 (n = 16) received 1.5% lactacystin/pCPP : SA polymers on day 5.

Third experiment. Female Fischer 344 rats (n = 32) were randomized into 3 experimental groups. Animals in group 1 (n = 8) received intracranial 9L tumor only, animals in group 2 (n = 8) received 1.3% lactacystin/pCPP : SA polymers on day 0, and animals in group 3 (n = 8) received 1.7% lactacystin/pCPP : SA polymers on day 5.

After surgery, each rat was examined daily for behavioral changes and neurological deficits. Upon death, the brains were harvested and fixed in 10% formalin for at least 2 weeks. The area within the cerebral

hemisphere containing the neoplasm was sectioned and stained with hematoxylin and eosin to confirm the presence of tumor.

Surgical technique

In the initial phase of the efficacy experiment, 9L gliosarcoma was excised from the flanks of carrier rats and cut into 1-mm^3 pieces. Fischer 344 rats were anesthetized, and their heads were shaved and prepared with a povidone-iodine solution. A midline scalp incision was made followed by identification of the coronal and sagittal sutures. A burr hole measuring 3 mm in diameter was made at a point 3 mm lateral to the sagittal suture and 5 mm posterior to the coronal suture. The dura, cortex, and underlying white matter were resected with gentile suction until the brainstem was visualized. In the experimental arm a 1 mm^3 tumor piece was placed into the resection cavity followed by placement of the lactacystin-loaded polymer. Surgical staples were used to close the scalp incision.

Statistical analysis

Statistical analysis was performed using SPSS version 8.0 for windows (Chicago, IL). Cytotoxicity, determined *in vitro* by the MTT assay, was analyzed using the Student's *t*-test; values are expressed as mean ± standard error of the mean (SEM). Kaplan–Meier curves for the efficacy studies were analyzed based on survival algorithms and significance was determined by the log rank and Kruskal–Wallis tests. A probability value of <0.05 was considered significant for all tests.

Table 1. Dose inhibition of rodent cell lines induced by incubation with lactacystin for 72 h

Dose of lactacystin (μg/ml)	Growth (%)	± SEM	*P* value
9L gliosarcoma			
0	100	11	
10	84	8	0.109
25	60	4	0.002
50	53	6	0.0009
75	46	4	0.0002
100	22	3	< 0.0001
F98 glioma			
0	100	5	
10	82	8	< 0.05
25	54	2	< 0.0001
50	39	3	< 0.0001
75	26	1	< 0.0001
100	26	2	< 0.0001

Results

Efficacy in vitro

Lactacystin was cytotoxic in 9L cells, causing 16 ± 8% growth inhibition at 10 μg/ml ($P = 0.109$), which increased to 78 ± 4% at 100 μg/ml ($P < 0.0001$). Similarly, treatment of F98 cells inhibited growth by 18 ± 8% at 10 μg/ml ($P < 0.05$), which increased to 74 ± 2% at 100 μg/ml ($P < 0.0001$) (Figures 1a and b, and Table 1).

Pharmacokinetics in vitro

The rate of lactacystin released from pCPP : SA polymers was measured *in vitro* over a 21 day period. One,

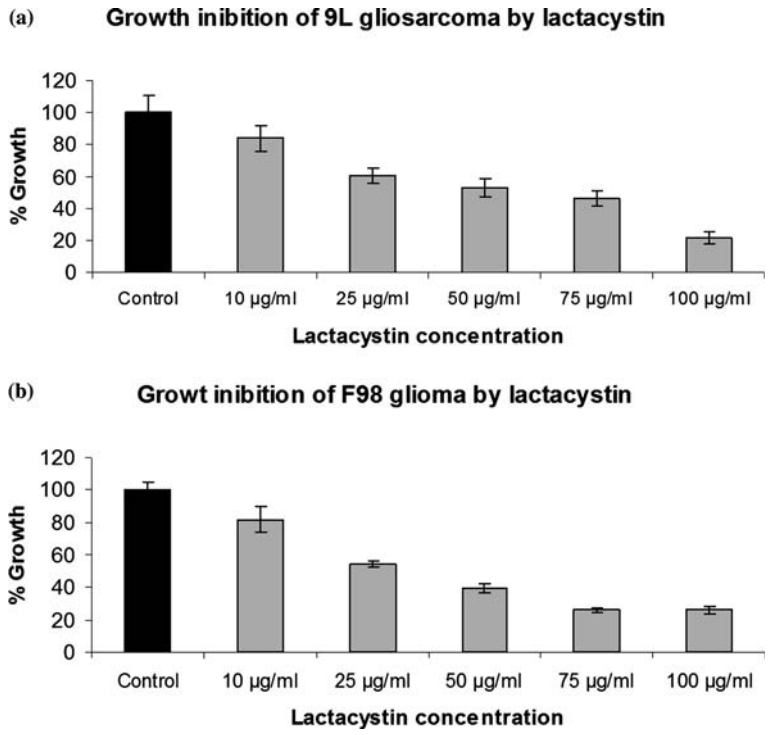

Figure 1. Cytotoxicity of lactacystin on 9L and F98 cells *in vitro*. (a) 9L gliosarcoma. Significant growth inhibition of 9L was achieved at 100, 50 and 25 μg/ml, but not at 10 and 1 μg/ml when compared to control. (b) F98 glioma. Lactacystin showed significant decrease in F98 cell viability at all concentrations tested.

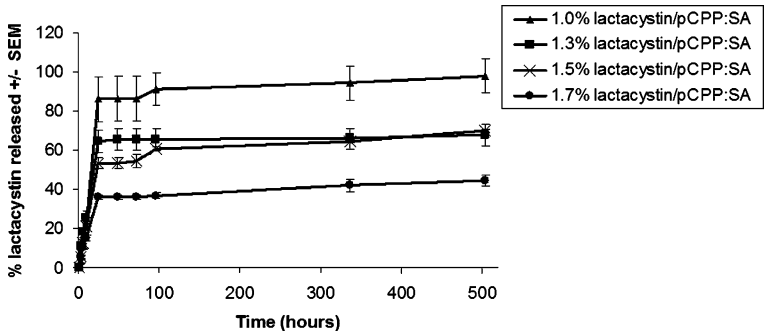

Figure 2. Pharmacokinetics. The release rate of lactacystin from pCPP : SA 1, 1.3, 1.5 and 1.7% (w : w) measured over 19-days *in vitro*. Sustained-release of lactacystin was observed with all polymer formulations.

1.3, 1.5 and 1.7% lactacystin/pCPP : SA polymers (w : w) released 98 ± 9, 68 ± 6, 70 ± 1 and 44 ± 3% of the loaded lactacystin, respectively (Figure 2).

Animal toxicity study

Toxicity was present in all animals treated with 2%-lactacystin polymers 3 days post-implantation, and consisted of hemorrhagic necrosis and marked inflammation surrounding the implantation site. Animals treated with 1.5% lactacystin polymers showed an early death on day 4, and 67% long term survivors (Figure 4). Animals treated with lower doses did not exhibit toxicity (Figure 3, Table 2).

Animal efficacy study

First experiment. Treatment with 1.0% lactacystin/pCPP : SA polymers on day 0 statistically prolonged survival of animals intracranially challenged with 9L when compared to control and generated 12.5% long-term survivors. Whereas the median survival of the controls was 13 days (mean 11 days, range 11–16 days), the median survival of the 1.0% lactacystin/pCPP : SA-treated animals was 16 days (mean 31 days, range 14–120 days, $P = 0.024$) (Figure 5a, Table 3).
Second experiment. Treatment with 1.5% lactacystin/pCPP : SA polymers on day 0 and on day 5 after tumor implantation significantly extended the survivals of animal challenged with 9L when compared to controls.

Table 2. Local and systemic toxicity of lactacystin polymers intracranially implanted in rats

Dose (%)	Median survival (days)	Survival at 120 days (%)	CNS toxicity	Systemic toxicity
Toxicity of lactacystin/pCPP : SA polymers				
0.1	120	100	No	No
0.5	120	100	No	No
1.0	120	100	No	No
1.5	120	66.6	No	No
2.0	3	0	Yes	No

The median survival of the animals treated with 1.5% lactacystin/pCPP : SA polymers on day 0 was not reached since 56% of the animals were long-term survivors (mean survival 76 days, $P = 0.0004$). These animals were euthanized on day 120 for confirmation of tumor presence. Animals treated with 1.5% lactacystin/pCPP : SA polymers on day 5 had a median survival of 14 days, and a mean survival of 22 days ($P = 0.024$). Control animals had a mean and median survival of 12 days (Figure 5b, Table 3).

Third experiment. Treatment with 1.3% lactacystin/pCPP : SA polymers on day 0 was also efficacious in prolonging survival, however, treatment with 1.7% lactacystin/pCPP : SA polymers on day 5 showed no significant benefits. Animals treated with 1.3% lactacystin/pCPP : SA polymers on day 0 did not reach a median survival since 50% of the animals were long-

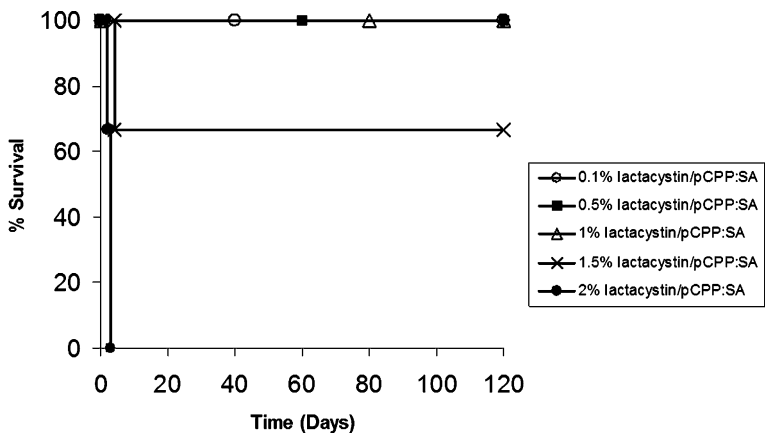

Figure 3. Kaplan–Meier animal survival curve. Healthy animals were implanted with different formulations of lactacystin polymers. Animals implanted with 0.1, 0.5 and 1.0% did not show neither local nor systemic toxicity up to the day of euthanasia (120 days after surgery); one animal implanted with 1.5% died prematurely, all animal implanted with 2% lactacystin polymer died due to local toxicity.

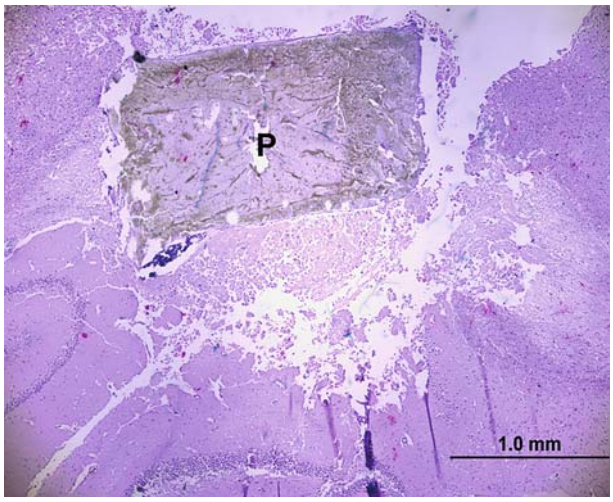

Figure 4. H&E stained section showing implantation of a 1.5% lactacystin/pCPP : SA polymer (P) in the brain of a rat. There are no appreciable signs of toxicity in the parenchyma surrounding the polymer.

term survivors (mean survival of 70 days, $P = 0.0004$). Animals treated with 1.7% lactacystin/pCPP : SA polymers on day 5 had a mean survival of 18 days and a median survival of 13 days ($P = 0.193$, Figure 5c, Table 3).

The macroscopic examination of all control brains showed evidence of tumor, asymmetry of the hemisphere, mass effect, midline shift and exophytic mass protruding though the craniotomy, the presence of tumor was confirmed with histology. Animals treated with lactacystin showed remarkably less midline shift, and mass effect, with no macroscopic evidence of exophytic tumor growth beyond the craniotomy (data not shown). Microscopic examination of the cross-sections confirmed presence of tumor in all animals, but the lesions appeared to be contained to the original site of implantation (Figure 6a and b).

Discussion

In this study we tested the cytotoxicity of the proteasome inhibitor lactacystin *in vitro* against 9L gliosarcoma and F98 glioma, incorporated lactacystin into controlled-release polymers, and determined its anti-neoplastic activity in an experimental model of malignant glioma. We fashioned implantable wafers composed of lactacystin and the biodegradable polymer pCPP : SA and found that lactacystin was released in a controlled fashion *in vitro*. Furthermore, intracranially implanted lactacystin-loaded polymers exhibited neither local nor systemic toxicity when incorporated at doses lower than 2% (w : w). Treatment with 1.0, 1.3 and 1.5% lactacystin polymers showed prolonged animal survival particularly when polymers were implanted on day 0 (simultaneously with the tumor implant) reaching statistical significance and long-term survivors in all the experiments. Furthermore, 1.5% lactacystin polymers were also efficacious when administered intracranially to animals with established tumors (day 5).

Lactacystin is a microbial product isolated from *Streptomyces lactacystinaeus* that inhibits cell proliferation and induces neurite outgrowth in a murine neuroblastoma cell line [1]. The anti-neoplastic activity of lactacystin is exerted primarily through inhibitory interactions with the proteasomes. Proteasomes are large multi-subunit proteases that are found in the cytosol (both free and attached to the endoplasmic reticulum) and in the nucleus of eukaryotic cells. Their presence and high abundance in these compartments reflects their central role in cellular protein turnover [2]. Proteasomes recognize, unfold, and digest protein substrates that have been marked for degradation by the

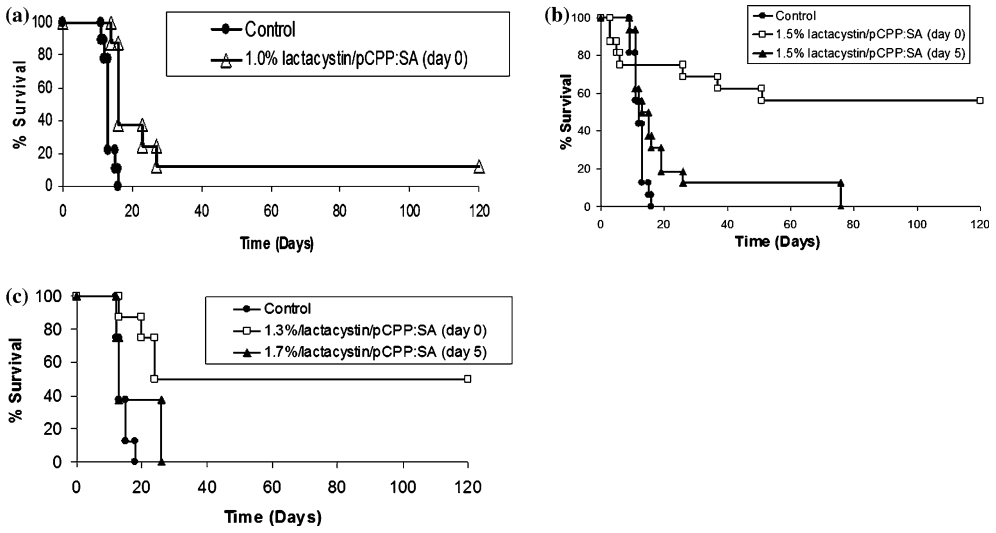

Figure 5. Kaplan–Meier animal survival curve. The efficacy of 1.0, 1.3, 1.5 and 1.7% lactacystin/pCPP : SA polymers was tested in the rat 9L gliosarcoma model using Fischer 344 rats. (a) Two groups received either a 1.0% lactacystin/pCPP : SA polymer on day 0 or tumor only. (b) Three groups of rats received either a 1.5% lactacystin/pCPP : SA polymer on day 0, a 1.5% lactacystin/pCPP : SA polymer on day 5, or tumor only. (c) Three groups received either a 1.3% lactacystin/pCPP : SA polymer on day 0, a 1.7% lactacystin/pCPP : SA polymer on day 5, or tumor only.

Table 3. Experimental groups – intracranial efficacy study in rats

Group	Treatment	Number of rats	Median survival	Mean survival	Survival range	Percentage of long term survivors	*P* value
Efficacy of lactacystin incorporated into pCPP : SA polymers							
Experiment # 1							
1	(Control) 9L intracranial tumor only	8	13	11	11–16	0	
2	1.0% lactacystin/pCPP : SA (day 0)	8	16	31	14–120	12.5	0.024
Experiment # 2							
1	(Control) 9L intracranial tumor only	16	12	12	9–16	0	
2	1.5% lactacystin/pCPP : SA (day 0)	16	NR	76	3–120	56	0.0004
3	1.5% lactacystin/pCPP : SA (day 5)	16	14	22	9–76	0	0.024
Experiment # 3							
1	(Control) 9L intracranial tumor only	8	13	14	12–18	0	
2	1.3% lactacystin/pCPP : SA (day 0)	8	NR	70	13–120	50	0.0004
3	1.7% lactacystin/pCPP : SA (day 5)	8	13	18	12–26	0	0.193

NR: Median survival not reached.

attachment of a ubiquitin moiety [16]. Within the proteasome, a specific subunit, the 26S is largely in charge of protein degradation. The 26S proteasome is a 2000 kDa multi-subunit cylindrical complex comprised of a 20S core catalytic component (the 20S proteasome), which is capped at one or both ends by a 19S regulatory component [17]. The 19S subunit recognizes and binds proteins specifically marked for degradation (polyubiquitinated proteins), these proteins are then unfolded and fed into the 20S core for final degradation [18].

The ubiquitin-dependent proteasome proteolysis pathway (UPP) is fundamental for degradation of cyclins and cell-cycle progression, for the generation of peptides presented on the cell surface by the major histocompatibility complex (MHC) class I molecules, and for the modulation of several transcriptional regulators [19,16,20–23]. The UPP can be inhibited by lactacystin [1,24], through several mechanisms that include cytochrome *c*-dependent and independent pathways, however, this inhibition varies among different cell types. Lactacystin binds specifically to the 20S proteasome β-type subunit X [1,25,26], and inhibits the hydrolysis of intracellular peptides normally carried out by the 26S complex and by the UPP [24]. Moreover, lactacystin exhibits selective proteasome inhibition on cells undergoing neoplastic transformation [3–5], it shows additive effects when combined with chemotherapeutical agents [6], sensitizes cell lines to radiotherapy [7] or chemotherapy [27,28], and may affect tumor growth by inhibiting angiogenesis [29].

Specific studies on experimental malignant gliomas have shown that lactacystin induces the stabilization

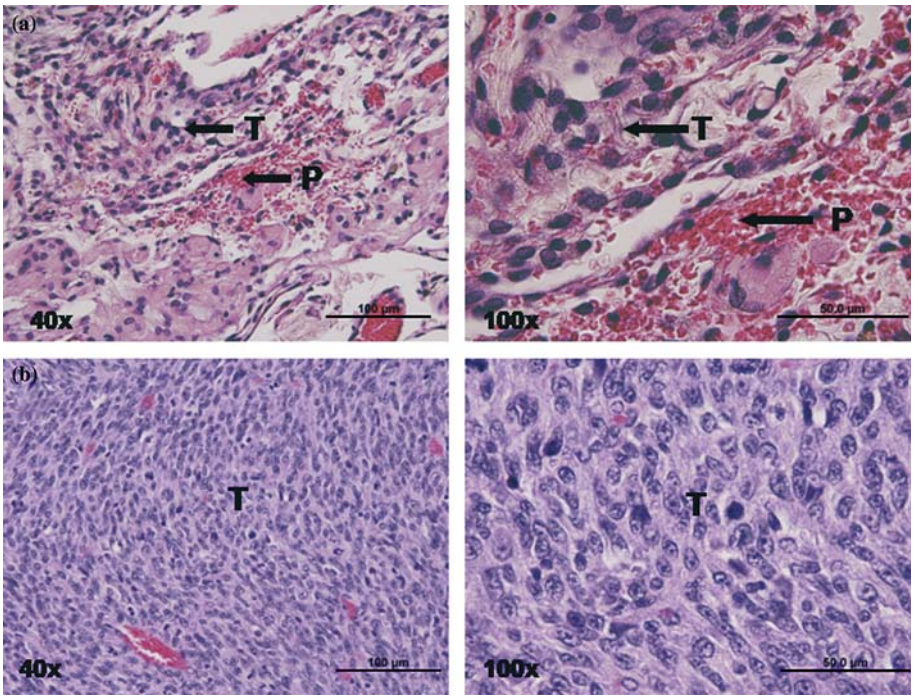

Figure 6. H&E stained coronal sections of the brain of a rat challenged intracranially with 9L gliosarcoma under 40x magnifications on the left and 100x on the right. (a) These sections were obtained from a long-term survivor rat implanted with 1.5% lactacystin/pCPP : SA polymer. The presence of tumor (T) was verified adjacent to the area of polymer implantation (P). (b) These sections were obtained from a control group rat and demonstrate a diffuse proliferation of 9L gliosarcoma.

and intracellular accumulation of c-Myc protein, and increases the expression of Fas-ligand (FasL), which trigger apoptotic cell death through activation of caspase-3 [8]. Proteasome inhibitors have the potential to affect normal tissue. Lactacystin, however, has shown a selectivity for neoplastic cells that appears to be related to the dependence of neoplastic cells on the chemotrypsin-like activity of the proteasome system, which is not required by normal cells to survive[3].

Malignant gliomas due to their increased rapid growth rate, aggressive nature, and high angiogenic activity have a high protein turnover rate and are highly dependent on the proteasome system, which makes them ideal targets for proteasome inhibitors such as lactacystin. The purpose of the present study was to test the anti-glioma activity of lactacystin *in vitro*, to develop a delivery system for local administration, and to test its efficacy in prolonging the survival of animals challenged intracranially with a lethal dose of 9L gliosarcoma.

In the present study, lactacystin exhibited potent cytotoxic activity when administered to malignant glioma cell lines *in vitro*. This effect was dose dependent and was consistent among the cell lines tested (Table 1). Moreover the LD_{50} was achieved at low doses (50 μg/ml in 9L-gliosarcoma cells and at 25 μg/ml in F98-glioma). The intrinsic physicochemical properties of lactacystin, however, prevent adequate penetration of the BBB at safe doses when administered systemically. Therefore, we incorporated lactacystin into controlled-release polymers to explore the benefit of local delivery. We used pCPP : SA, a polymeric matrix currently approved by the FDA for direct delivery of BCNU (carmustine), to clinically treat patients affected by newly diagnosed as well as recurrent malignant gliomas [30–34]. The lactacystin pCPP : SA formulation showed sustained release over time *in vitro* (Figure 2) and was safe for intracranial administrations at doses below 2% (w : w) (Table 2, Figure 3).

To determine the efficacy of the pCPP : SA/lactacystin formulation in treating experimental gliomas, we conducted three consecutive experiments that evaluated its activity in the 9L rat gliosarcoma model. The most efficacious formulation was 1.5% lactacystin polymers administered simultaneously with the tumor implant (day 0). Using this concentration median survival was not reached and we observed 56% long term survivors. Treatment with 1.5% lactacystin polymers was also efficacious in the treatment of established tumors, although the benefits observed were less when compared to those obtained when the polymers are implanted on day 0. These findings suggest that locally-delivered proteasome-inhibitors could be beneficial in controlling tumor growth if placed on the resection cavity during surgery, or when implanted intratumorally.

Proteasome inhibitors are a topic of increasing interest in clinical oncology. Growing evidence suggests significant therapeutic benefits of proteasome inhibition in preclinical studies and clinical trials have already been started to evaluate the effect of proteasome inhibitors in a wide variety of solid and hematological malignancies [35,36]. Proteasome inhibitors may also serve as adjuvant therapy with existing drugs to potentially overcome intrinsic, acquired, or induced chemoresistance. Indeed, malignant glioma cells that bear multiple genetic defects, including impaired repair mechanisms and faulty cell cycle checkpoint controls, are sensitive to the growth inhibitory actions of proteasome inhibitors both *in vitro* and *in vivo*. The characteristics of proteasome inhibitors make them appealing agents in primary or adjuvant malignant glioma therapy. In order to maximize glioma therapy, however, the highly selective and cytotoxic agents needed must be incorporated within an effective delivery system.

In summary, lactacystin exhibits potent anti-glioma activity *in vitro*, it can be efficiently incorporated into controlled-release polymers, and at the proposed concentrations lactacystin polymers are safe for CNS delivery and prolong survival in the 9L model. These findings support the role of proteasome-inhibitors in the treatment of malignant gliomas when administered using a local drug-delivery system.

Acknowledgement

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Address for offprints: Alessandro Olivi, M.D., Professor of Neurosurgery, Director Division of Neuro-oncology, Johns Hopkins University – School of Medicine, The Johns Hopkins Hospital – Meyer 9-100, 600 N Wolfe, Baltimore, MD 21287, USA; Tel.: +410-955-0703; Fax: +410-614-9877; E-mail: aolivi@jhmi.edu