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Original Article

Efficacy of local delivery of ardipusilloside I using biodegradable implants against cerebral tumor growth

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Abstract: Ardipusilloside I (ADS-I) is a natural compound that can be isolated from the Chinese medicinal herb *Ardisiapusilla* A.DC, and has been reported to inhibit the growth of glioblastoma cells in cultures. This study was designed to test its efficacy by the delivery using biodegradable implants against glioblastoma *in vivo*. ADS-I was incorporated into polymer microspheres, which were prepared by a mixture of poly (D, L-lactic acid) and poly (D, L-lactic-co-glycolic acid) polymers and then fabricated into wafers. The anti-glioma activities of ADS-I-loaded wafers were examined by methylthiazol tetrazolium (MTT) assay in cultured rat C6 glioma cells, and by magnetic resonance imaging (MRI) and survival monitoring in C6 glioma-bearing rats. Here, we showed that ADS-I-loaded wafers sustained ADS-I release *in vitro* for 36 days in Higuchi model of kinetics, and had the same cytotoxic activity as ADS-I in the solution against the growth of C6 glioma cells in cultures. In C6 glioma-bearing rats, ADS-I wafer implants inhibited tumor growth in a dose-dependent matter, and were more effective than the same dosage of ADS-I in the solution. The tumor suppression efficacies of ADS-I wafer implants were positively correlated with an increase in tumor cell apoptosis and prolonged animal survival, and were associated with a decrease in vascular endothelial growth factor, C-reactive protein, tumor necrosis factor-α and interleukin-6, and an increase in interleukin-2 expression. In conclusion, this study demonstrates significant efficacy of local delivery of ADS-I using polymer implants against glioma tumor growth *in vivo*, suggesting the potential of ADS-I-loaded wafers for glioma treatment.

Keywords: Ardipusilloside I, glioblastoma, interstitial therapy, implant wafer, anti-tumor activities

Introduction

Glioma is the most common intracranial malignant tumors in the brain and spinal cord, and remains a challenge in the treatment of this type of cancer due to its highly proliferative, infiltrative and invasive behaviors [1-3]. Many traditional Chinese medicinal herbs have been received much attention for the development of anti-cancer therapies owing to their remarkable anti-tumor activities. For example, ardipusilloside I (ADS-I), a triterpenoid saponin, is isolated from medicinal herbal *Ardisiapusilla* A.DC (*Myrsinaceae*), and has been demonstrated to be a promising anti-cancer agent [4]. It has been reported that ADS-I induces cell death of human glioblastoma cells through FasL/Fas-signaling pathway or activation of autophagy [5, 6], and also effectively inhibits the growth of NCI-H460 cells via regulating Bcl-2, Bax and vascular endothelial growth factor receptor (VEGFR) [5]. In addition, ADS-I can restrict the invasion, metastasis and angiogenesis of tumor cells [4, 7, 8]. However, all these studies were performed using *in vitro* experimental systems, and the low oral bioavailability and hemolysis effect of ADS-I still remain an obstacle to its potential for clinical applications. The drug delivery systems specifically encapsulating ADS-I have not been reported as of yet, and its efficacy against tumor growth in vivo and the mechanisms of its action have not been fully investigated.

ADS-I is metabolized through the deglycosylation pathway in the gastrointestinal tract of

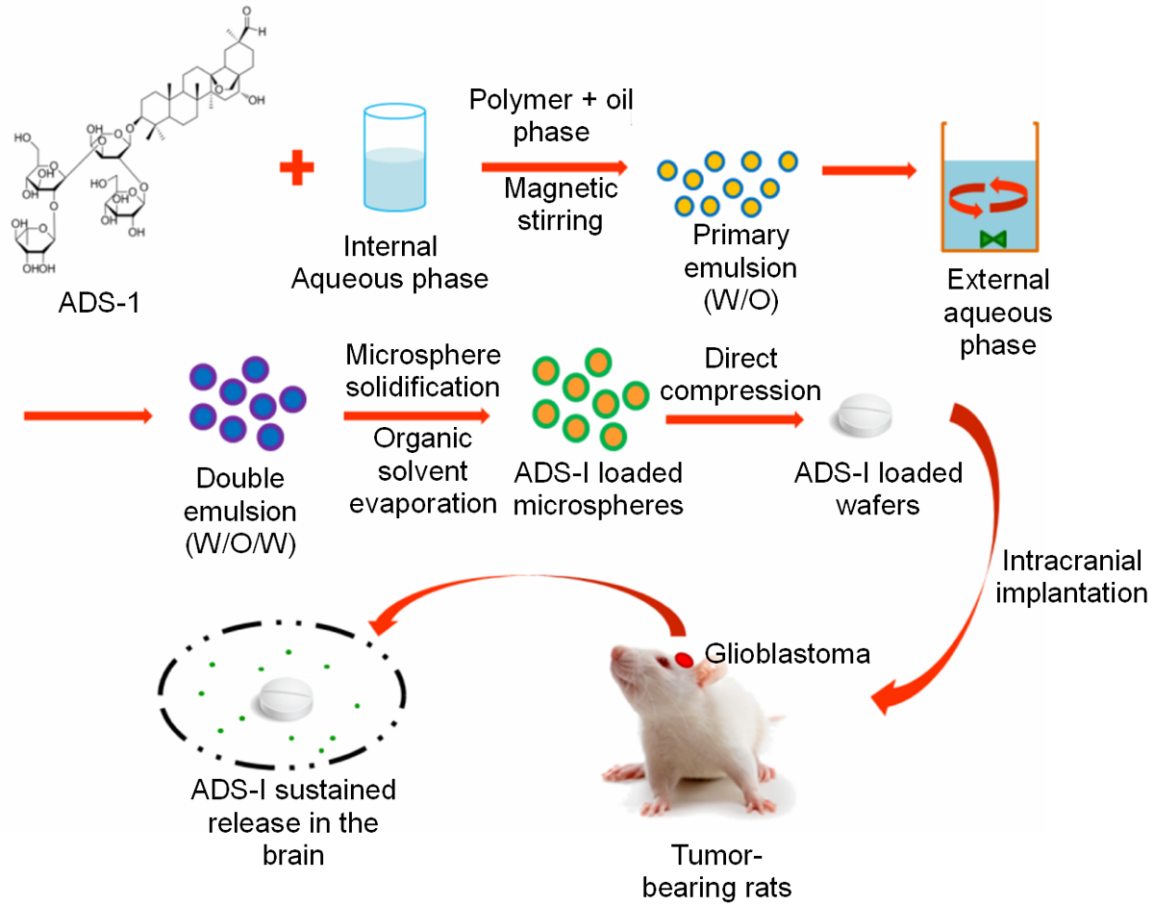

Figure 1. A simple diagram of experimental procedure: ADS-I preparation and intracranial implantation.

rats when it is orally administrated, and its half-life in the plasma after intravenous administration is 5.61 h [9]. Thus, the present study was designed to develop an efficacious delivery vehicle for ADS-I, and to evaluate its potency and mechanisms of its anti-tumor activity in a glioma rat model. There are many disadvantages of oral administration of natural compound triterpenoid saponin, such as hydrolysis and enzymolysis [10], and of intravenous injection, such as hemolysis of blood cells [11], and also it is limited to the brain by the blood brain barrier if it is used for brain tumor treatment, the potential of microspheres-derived poly (D, L-lactic acid) (PLA) and poly (D, L-lactic-co-glycolic acid) (PLGA) polymers for locally delivery of ADS-I for glioma treatment was tested in rats. Both PLA and PLGA are biocompatible and biodegradable, and most importantly, are FDA approved for a long-acting drug delivery system for many therapeutic agents, such as PLA for controlled release of leuprolide in prostate cancer treatment [12] or 25-hydroxyvitamin D3 in the treatment of diabetic periodontitis [13], and PLGA for 1,3-bis(2-chloroethyl)-1-nitrosourea release against 9L gliosarcoma [14] or L-dopa-alpha-lipoic acid for the treatment of Parkinson's disease [15]. Apart from the advantages of maintaining higher concentrations of therapeutic agents at the diseased site and of minimizing systemic toxicity, these polymeric formulations may provide a sustained drug release by modulating the type, ratio and molecular weight of polymers [15, 16].

In the present study, ADS-I was firstly entrapped into PLA/PLGA microspheres by water in oil in water (W/O/W) double emulsion/solvent evaporation technique as described previously [17]. Then ADS-I-loaded microspheres were compressed into wafers. The physicochemical properties of ADS-I wafers were examined in terms of encapsulation efficiency (EE), drug loading (DL) and *in vitro* release. And the effects of ADS-I wafers on the growth of glioma C6 cells both in cultures and in rats were also

examined. The experimental protocol was presented in **Figure 1**. The objective of this proof-of-principle study was to provide preclinical evidence for the future development of ADS-I as an anti-glioma agent.

Materials and methods

Reagents, cells and animals

ADS-I (98.36% purity) was prepared by Dr. Xiao-Juan Wang's laboratory in the Department of Pharmacy at the Fourth Military Medical University (Xi'an, China), and ADS-I solution was prepared by dissolving in 2% dimethylsulfoxide (DMSO) and 25% polyethyleneglycol (PEG) (1:1, v/v) mixture. PLA (M_w: 10 kDa) and PLGA (50/50, M_w: 10 kDa) were purchased from Daigang Biomaterial Co. Ltd. (Jinan, China), polyvinyl alcohol (PVA, degree of hydrolysis 88%, average M_w 1.7 kDa) from Shanghai Jiachen Chemical Industry Co. Ltd. (Shanghai, China), Peregal O (M_w 1.2 kDa) from (Xingtai Shengda Auxiliaries Co., Ltd, Xingtai, China), dichloromethane (DCM) and methanol of analytical grade from Fisher Scientific (Pittsburgh, PA, USA), methylthiazol tetrazolium salt (MTT) from Amersco (Framingham, MA, USA), Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from HyClone Laboratories (Logan, UT, USA), and penicillin-streptomycin and tryspin solutions from Solarbio (Beijing, China). All other chemicals were of analytical grade and were used without further purification.

Rat C6 glioma cells were provided by the cell bank of Chinese Academy of Science (Shanghai, China). This cell line was cultured in DMEM medium, supplemented with 10% FBS and 1% antibiotics (100 IU/ml penicillin and 100 μg/mL streptomycin) at 37°C in a 5% CO_2 atmosphere. Male Sprague-Dawley rats (7-9 weeks old; bodyweight: 200 ± 20 g) were purchased from the Experiment Animal Research Center at the Fourth Military Medical University. All animal procedures were performed in accordance with the protocols approved by the Animal Care and Use Committee of the Fourth Military Medical University according to the Chinese Council on Animal Care guidelines.

ADS-I wafer preparation

ADS-I loaded microspheres were first prepared by W/O/W double emulsion/solvent evaporation method as described previously [17]. Briefly, polymer (approximately 700 mg) was dissolved in 10 mL of DCM at a final concentration of 70 mg/mL. The polymer solution was emulsified with 2.5 mL of ADS-I solution [80 mg of ADS-I in 2% DMSO and 25% PEG (1:1, v/v) mixture]. The emulisification was completed by a magnetic stirring for 60 s at a speed of 750 rpm. The resultant primary emulsion (W/O) was then added dropwise into a 1000 ml of aqueous solution containing 0.5% PVA and 1% NaCl using a FJ-200 homogenizer (Shanghai, China) to produce a double W/O/W emulsion. Then the suspension was magnetically stirred for 2 h at room temperature to allow the organic solvent elimination and microsphere solidification. Finally, the products were collected by centrifugation at 2500 rpm for 10 min, and subsequently washed three times with de-ionized water. The products were then lyophilized for 3 d. One hundred milligrams of optimized microspheres (an average 10.22% of ADS-I loading) were compressed into wafers using Tablet Press (DP30A, Beijing, China) at 4 t/m^2 for 5 s at room temperature. The wafers were 3 × 1 mm^2 in size with a flat surface, and stored at 0°C prior to use.

In vitro ADS-I release assay

The *in vitro* release of ADS-I from the wafer was estimated in a release medium - phosphate buffered saline (PBS) containing 0.9% Peregal O (pH 7.2). A piece of ADS-I wafer (12 mg) was suspended in 7 ml of the release medium with constant shaking at 110 rpm at 37°C, respectively. At indicated time points the wafer was collected from the release medium, washed with ultra-pure water, followed by vacuum-drying for 48 h. The remaining ADS-I in the wafer was determined using HPLC analysis as described below. The amount of ADS-I release to the release medium was calculated by the subtraction of the remaining ADS-I in the wafer from 1.23 mg (10.23% of 12 mg, the total ADS-I in the wafer before incubation with the release medium).

High-performance liquid chromatography (HPLC) analysis of ADS-I

The content of ADS-I in the wafer was determined using HPLC. Briefly, a dry piece of wafer was dissolved in 3 mL of DCM. After complete solubilization by a 15 min-ultrasonication, ADS-I was extracted using methanol, in which 5

mL of methanol was added, and the mixture was subjected to ultrasonic treatment for another 15 min so as to extract all the drugs from DCM into methanol. The insoluble portion was removed by centrifugation and filtration. The amount of ADS-I in the methanol was determined using HPLC (LC 20A, Shimadzu Corporation, Kyoto, Japan), coupled with an evaporative light scattering detector (ELSD 3300, Alltech Associates, Deerfield, USA), a LabSolutions software (Shimadzu Corporation, Kyoto, Japan), and a Welch materials INC C_{18} column (4.6 mm × 250 mm, 5 μm). The column temperature was kept constant at 25°C, and the mobile phase was consisted of 75% methanol and 25% H_2O with the flow rate of 1.0 mL/min. The drift tube temperature for ELSD was set at 82°C, and the nebulizing gas flow rate was 2.0 l/min. The amount of ADS-I in the sample was calculated based on the measurement of ADS-I standards.

MTT assay

ADS-I was released from wafers by incubation with the release medium – PBS containing 0.9% Peregal O (pH 7.2), and its concentration in the medium at each time point (day 1, 2, 3, 5, 15 and 36) was determined using HPLC as described above. The equal amount of ADS-I in the solution (ADS-I solution) was used as a control at each time point for the comparison of the anti-proliferative activity of ADS-I in the release medium. The *in vitro* anti-proliferative activity of the released ADS-I from ADS-I wafers as compared to controls (the release medium with blank wafers, and the same amount of ADS-I in the solution) was determined in cultured C6 glioma cells using MTT assay. Briefly, cells were seeded in 96-well plates at the density of 10^4 cells per well. After 24 h of incubation at 37°C under 5% CO_2, 100 μL of the testing solution was added to each well, and cell cultures were grown for another 72 h. After further incubation with MTT (20 μL, 5 mg/mL) for 4 h, cells were dissolved in 150 μL DMSO per well, and the optical density (OD) or absorbance at 490 nm was measured with a microplate reader (Bio-RAD instruments, USA). The anti-proliferative activity was presented as the percent of reduction in cell viability, which was calculated by: Anti-proliferative activity = $(OD_0 - OD_x)/OD_0 \times 100\%$, where OD_0 represented the OD measurement of untreated cell cultures, and OD_x the OD of drug-treated cell cultures.

C6-glioma rats model

C6 cells were harvested for implantation by trypsinization and washed with sterile PBS, followed by suspending in iced-cold PBS at 2×10^7 cells/mL until injection. Male Sprague-Dawley rats were anesthetized with intraperitoneal (IP) injection of 0.5% pentobarbital sodium (1 mL/100 g) and meperidine (2 mg/100 g). Animals were then shaved and placed on a stereotactic head holder. After disinfection and incision of the skin, 10 μL of C6 cell suspension were stereotaxically implanted in the bregma using the following coordinates: 1 mm behind coronal suture, 3 mm right lateral, and 4 mm depth. The rate of infusion was 1 μL/min. The needle was left in place for 5 min after cell infusion and removed slowly. After tumor cell implantation, the burrholes were filled with bone waxes and the scalp wound closed with polyamide thread suture. Rats were removed to a clean cage with free access to food and water, and given an intramuscular injection of penicillin for 3 days to prevent infection. The inoculation date was designated as 0 day.

Tumor size measurement using magnetic resonance imaging (MRI)

Seven days after the tumor cell implantation (day 7), rats were anesthetized with pentobarbital sodium (1 mL/100 g) and examined using a 3.0 T MR scanner (MAGNETOM Trio, Siemens AG, Erlangen, Germany). Both coronal T1 SE sequences (TR = 8.6 ms, TE = 4.0 ms, FOV 220 × 220 mm^2, slice thickness 1.5 mm, matrix size 256 × 256, giving a Voxel size of 0.3 × 0.3 × 1.5 mm^3) and T2 SE sequences (TR =3000.0 ms, TE = 85.0 ms, FOV 220 × 220 mm^2, slice thickness 1.5mm, matrix size 256 × 256, giving a Voxel size of 0.3 × 0.3 × 1.5 mm^3) were used to acquire MR images of the whole brain.

The tumor size was determined using a standard viewing and reformatting software (RadiAnt DICOM Viewer). The X, Y tumor diameters were measured in T1-weighted images, whereas Z diameter was determined by counting the number of images containing tumor and multiplying with the image thickness (1.5 mm) in T2 imaging. The tumor size (V) was calculated as described previously [18]: V = 4/3 × pi × (X × Y × Z/8), where the pi was 3.14.

Table 1. Model fitting of release mechanism of ADS-I loaded wafers

Model	Equation	r
Higuchi	$Q = 0.192\ t^{1/2} - 0.156$	0.990
Weibull	$\ln\ln[1/(1-Q)] = 1.037\ln t - 2.751$	0.930
Zero-order dynamic model	$Q = 0.026\ t + 0.135$	0.979
First-order dynamic model	$\ln Q = 0.057t - 1.696$	0.815

Q stands for cumulative release percentage, r stands for correlation coefficient, and t stands for time (d).

Treatment of orthotopic tumor bearing rat with ADS-I wafer implants

Seven days after tumor cell inoculation (day 7), rats were randomly divided into four groups (12 rats in each group): *Group* 1, blank wafer implants (control group); *Group* 2, ADS-I wafer implants (0.77 mg); *Group* 3, ADS-I wafer implants (1.53 mg), and *Group* 4, ADS-I solution group. The wafers were implanted into the brain of rats after anesthesia, in which the rats were fixed on the stereotaxic instrument and drilled a hole (3 mm diameter) into the skull in the bregma at the same location as the tumor was grafted, followed by implanting the wafers into the subdural and sealing the holes with bone waxes, the scalp wound was closed with polyamide thread suture. The rats in the fourth group received a single dose of intratumoral injection of 153 μL ADS-I solution (containing 1.53 mg ADS-I). Fourteen days (day 14) after implantation, the tumor sizes in the rats in each group were measured again using MRI imaging analysis as described above.

Histopathological analysis

Four tumor-bearing rats were randomly selected from each group at day 14 (after MRI imaging analysis). After anesthetized, the rats were perfused intracardially with a 4% paraformaldehyde solution, and their brain tissues were fixed in the same solution overnight. The fixed tissues were then processed for paraffin embedding, and the sections were stained hematoxylina and eosin (H&E). The remaining eight rats in each group were kept for survival monitoring.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

The apoptotic cells in tumor tissue sections were detected using TUNEL assay the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer's instructions. In brief, the sections of tumor tissues were deparaffinined and dehydrated, followed by permeabilization with 20 μg/mL proteinase K and 0.2% Triton X-100 in PBS. The slides were then labeled with a TdT reaction mixture for 90 min and were mounted with a mounting solution containing 4',6-diamidino- 2-phenylindole (DAPI) (Vectorshild, Vector Laboratories, Burigame, CA). The apoptotic cells (green) and cell nuclei (blue) were examined using a confocal microscopy (LSM 710; Carl Zeiss Q10, Germany).

The enzyme-linked immunosorbent assay (ELISA)

The tumor-associated indices (TAI) or factors, C-reactive protein (CRP), interleukin 2 (IL-2), interleukin 6 (IL-6), vascular endothelial growth factor (VEGF) and tumor necrosis factor-α (TNF-α), in the peripheral blood of rats were measured using commercial ELISA kits according to the manufacturer's protocol (Biosource, Camarillo, CA, USA). Blood samples were collected from the orbit and then centrifuged at 3000 rpm for 10 min at 4°C. The supernatants were collected for the determination of TAI.

Survival assay

C6 glioma-bearing rats (eight rats per group) were randomly selected for survival study. Without any further treatment, these rats freely accessed laboratory chow and water, and were monitored daily. When severe neurological damage appeared due to the tumor growth, rats were euthanized.

Statistical analysis

The statistical analyses were performed using GraphPad Prism statistics software (GraphPad Software, Inc., La Jolla, CA). Analysis of variance (ANOVA) or *t*-test was used for comparing the difference between experimental groups. Survival curves were estimated by means of the Kaplan-Meier method and the differences among the curves were compared using the log-rank test. All results were expressed as mean ± standard derivation (SD). The p value of < 0.05 was considered as significant.

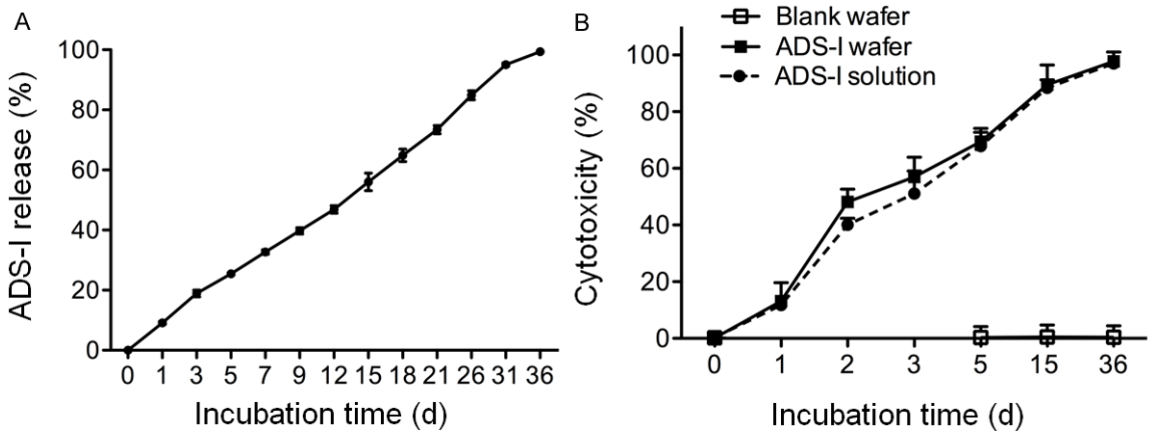

Figure 2. *In vitro* ADS-I release from ADS-I-loaded wafer and its cytotoxicity in cultured C6 cells. For each determinant, a piece of ADS-I wafer (12 mg containing 1.23 mg of ADS-I) was suspended in 7 ml of PBS containing 0.9% Peregal O (pH 7.2). At each indicated time point, the remaining ADS-I in the wafer was determined using HPLC analysis, and was used to calculate the amount of ADS-I release to the release medium. A. The percentage of ADS-I release from the ADS-I wafer. Data are represented as mean ± standard deviation (SD) of three determinants. ADS-I was released from the wafer in a time-dependent manner ($p < 0.0001$, one-way ANOVA, n = 3). B. The cytotoxicity of ADS-I released from ADS-I-loaded wafers was determined in C6 cell cultures using MTT assay. The same amount of ADS-I in the solution (ADS-I solution) and the same volume of the release medium from blank wafer group at each time point were used as controls. Data are represented as mean ± SD of three determinants. The cytotoxicity of released ADS-I was increased in a time/dose-dependent manner ($p < 0.0001$, one-way ANOVA, n = 3), and was not significantly different from that of ADS-I solution ($p = 0.06$, two-way ANOVA).

Results

ADS-I release of wafer implants in vitro

The solubility of ADS-I was first tested by dissolving 40 mg of ADS-I in 10 mL of PBS containing 0.9% of Tween-80, PEG400 or Peregal O. After 3 days of shaking (110 rpm/min) at 37°C, the solubility of ADS-I was 2.96 mg/mL (74%) with Tween-80, 1.05 mg/mL (26.25%) with PEG400, and 3.79 mg/mL (94.75%) with Peregal O, suggested that Peregal O gave the maximum solubility of ADS-I in the medium. The ADS-I release from the wafer implants was then characterized in a dissolution medium – PBS containing 0.9% Peregal O. As shown in **Figure 2A**, ADS-I was released from the wafer implants in a time-dependent manner. The release rate was 9.14 ± 0.51% at day 1, 25.45 ± 0.34% at day 5, 56.06 ± 2.9% at day 15, and 99.35 ± 0.08% at day 36 ($p < 0.0001$, one-way ANOVA, n = 3). The release mechanism of ADS-I wafer implants was analyzed by fitting the data to four different mathematical models: Higuchi, Weibull, the zero-order kinetics and the first-order kinetics models [19, 20]. The fitting outcome was listed in **Table 1**, which indicated that the drug release profile of ADS-I wafers was similar to Higuchi model, with correlation coefficients close to 1.

Anti-glioma effect of ADS-I wafer in cell cultures

The *in vitro* activity of ADS-I wafer on growth of C6 glioma cells was examined in cultures as compared with ADS-I solutions. In this experiment, ADS-I was released from the wafer first by dissolving in the release medium, and was collected at different time points (day 1, 2, 3, 5, 15 and 36) for the determination of its anti-glioma activity *in vitro*. As shown in **Figure 2B**, the release medium from ADS-I wafer showed a release time-dependent cytotoxic activity against the growth of C6 glioma cells in cultures, indicated by 13.2 ± 6.45% of growth inhibition at day 1, 57.0 ± 6.98% at day 3 and 97.74 ± 3.35% at day 36 ($p < 0.0001$, one-way ANOVA, n = 3), which was not significantly different from the growth inhibition of ADS-I solution ($p = 0.06$, two-way ANOVA), while no cytotoxicity was found in the release medium from control blank wafer. These data suggest that ADS-I released from the wafer in the release medium maintains the same cytotoxic activity as in ADS-I solution against C6 glioma cells in cultures.

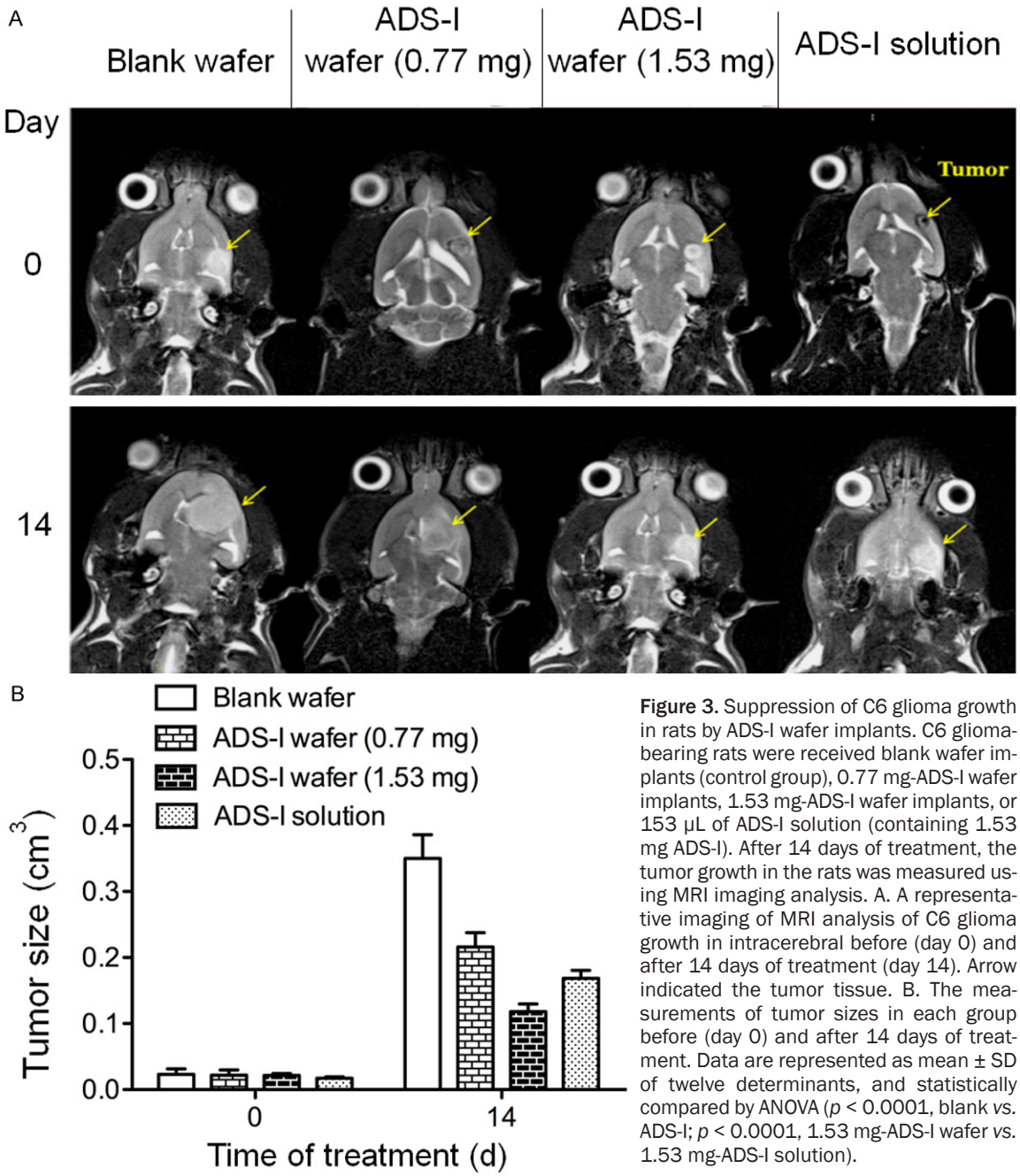

Figure 3. Suppression of C6 glioma growth in rats by ADS-I wafer implants. C6 glioma-bearing rats were received blank wafer implants (control group), 0.77 mg-ADS-I wafer implants, 1.53 mg-ADS-I wafer implants, or 153 μL of ADS-I solution (containing 1.53 mg ADS-I). After 14 days of treatment, the tumor growth in the rats was measured using MRI imaging analysis. A. A representative imaging of MRI analysis of C6 glioma growth in intracerebral before (day 0) and after 14 days of treatment (day 14). Arrow indicated the tumor tissue. B. The measurements of tumor sizes in each group before (day 0) and after 14 days of treatment. Data are represented as mean ± SD of twelve determinants, and statistically compared by ANOVA ($p < 0.0001$, blank *vs.* ADS-I; $p < 0.0001$, 1.53 mg-ADS-I wafer *vs.* 1.53 mg-ADS-I solution).

Suppression of glioma growth by ADS-I wafer implants in rats

The growth or tumor size of C6 glioma cells in rats was determined at both the beginning and the end of 14-day treatment using MRI imaging. As shown in **Figure 3**, ADS-I wafer implants significantly inhibited the tumor growth in a dose-dependent manner. The tumor size grew in blank wafer group from 0.0232 ± 0.0084 cm^3 at the beginning of the treatment to 0.35029 ± 0.03582 cm^3 at the end of the treatment (15-fold increase). The growth increase in this blank control was reduced to 9.88-fold (from 0.02188 ± 0.0081 cm^3 to 0.21614 ± 0.02161 cm^3) by the treatment of ADS-I wafer (0.77 mg), and further to 5.54-fold (0.0213 ± 0.003 cm^3 to 0.11807 ± 0.01159 cm^3) with ADS-I wafer (1.53 mg) ($p < 0.0001$, one-way ANOVA, n = 12), while the treatment with ADS-I solution at a dose of 1.53 mg also suppressed the tumor growth (0.01742 ± 0.0021 cm^3 to

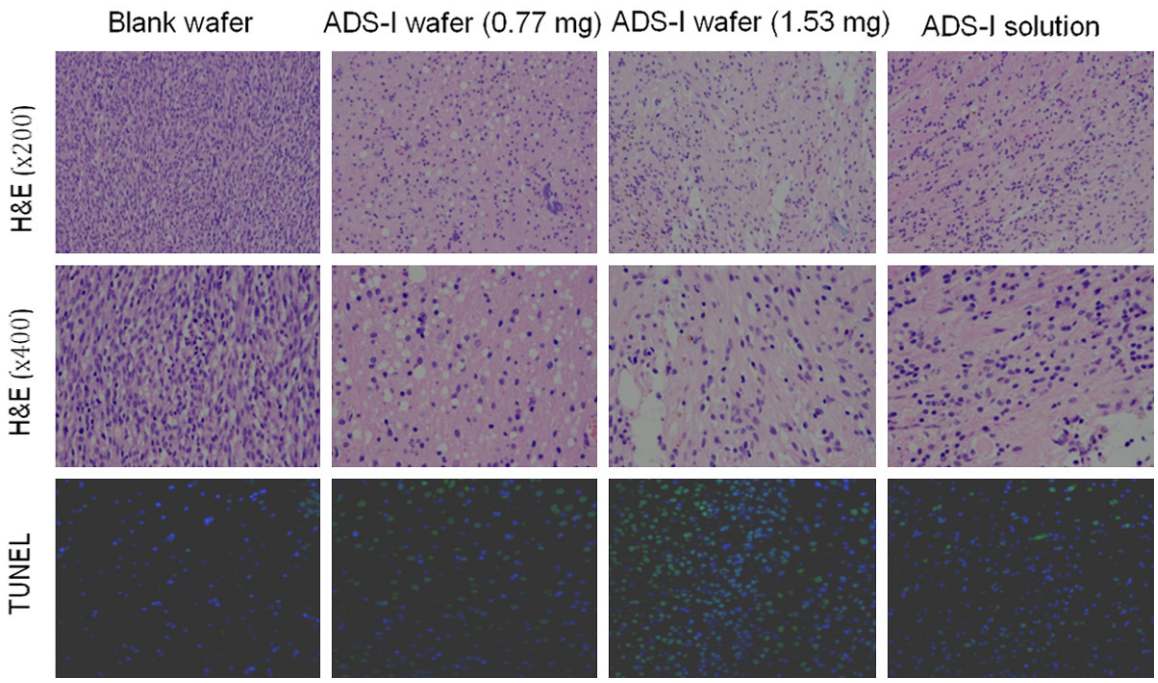

Figure 4. Histological analysis of anti-glioma activities of ADS-I wafer implants. Four tumor tissues were randomly selected from each group at day 14 (after MRI imaging analysis). After paraformaldehyde fixing and paraffin embedding, the sections were stained with H&E for histological examination of cell and tissue morphology and structures (upper and middle panels). Data are a typical microscopic imaging of tumor tissue sections under original magnification of both ×200 and ×400. Nuclei: blue, cytoplasm: pink. Apoptotic cells in tissues sections were detected using TUNEL assay (lower panel). Data are a typical fluorescent microscopic imaging of tumor tissue sections under original magnification of ×400. Nuclei: blue, apoptosis cells: green.

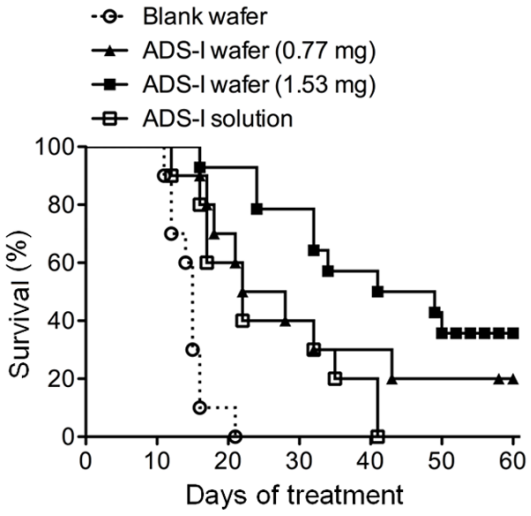

Figure 5. Prolongation of C6 glioma-bearing rats by ADS-I wafer implants. C6 glioma-bearing rats (eight rats per group) were randomly selected for survival monitoring without any further treatment. Data were statistically analyzed using Log-rank (Mantel-Cox) test. $p = 0.0002$ (Blank vs. 0.77 mg ADS-I wafer), $p < 0.0001$ (Blank vs. 1.53 mg ADS-I wafer), $p = 0.0008$ (blank vs. ADS-I solution) and, $p = 0.0048$ (1.53 mg ADS-I wafer vs. ADS-I solution.

0.16815 ± 0.01217 cm^3, 9.65-fold) as compared to control blank wafer group, but was significantly less effective than the ADS-I wafer (1.53 mg) ($p < 0.0001$, two-tailed *t*-test, n = 12).

Correlation of tumor growth suppression by ADS-I wafer implants with tumor cell inactive growth and apoptosis

After 14 days of treatment, the tumor tissues from each group were characterized by histological analysis. As shown in H&E stained sections (**Figure 4**, upper and middle panels), C6 glioma cells in rats treated with control blank wafer displayed typical fibroblast-like morphology, and active cell proliferation that was evidenced by a high density of nuclear staining. The density of the tumor cells in the sections of both ADS-I wafer groups was markedly reduced, suggesting that the tumor cell growth/proliferation in these rats receiving ADS-I wafer implants was significantly inhibited, whereas the density of nuclear staining in ADS-I solution group was higher than any of ADS-I wafer groups, but lower than control wafer group. Cell apoptosis

Table 2. Anti-tumor efficacy of each different ADS-I treatment in C6 glioma-bearing rats (n = 8)

Groups	Dose (mg)	MST (days)	Median (days)	ILS over blank wafer (%)	ILS over ADS-I solution (%)
Blank wafer	\	14.70 ± 2.386	15	\	\
ADS-I wafer	0.77	31.50 ± 2.830	25	114.29	23.53
ADS-I wafer	1.53	41.57 ± 3.423	45	182.79	63.02
ADS-I solution	1.53	25.50 ± 3.865	22	73.47	\

MST: mean survival time, ILS: increase in life span [(T/C-1) ×100%], where T and C stand for the treatment and control groups respectively.

in these tumor tissues was examined using TUNEL assay, as shown in **Figure 4** (lower panel), the positive stain of apoptotic cells was barely seen in the tissue sections of control blank wafer, and was significantly increased in ADS-I wafer groups, particularly in those receiving 1.53 mg that caused cell death mostly. Again, the treatment of ADS-I solution was associated with less cell apoptosis than those receiving the same dose of ADS-I in wafer implants.

Prolongation of glioma-bearing rat survival and changes of tumor-associated indices by ADS-I wafer implants

As shown in **Figure 5**, the mortality of the animals in blank wafer control group started at day 11, and all of the animals eventually died during the following 10 days with the median survival of 15 days (**Table 2**). As compared to this control group, the median survival or the increase in life span (ILS) was increased to 25 days or 114.29% by the treatment of ADS-I wafer implants (0.77 mg) (vs. blank wafer, p = 0.0002, Log-rank test), and to 45 days or 182.79% by the ADS-I wafer implants (1.53 mg) (vs. blank wafer, $p < 0.0001$, Log-rank test). The treatment with ADS-I solution (1.53 mg) also prolonged the median survival of rats to 22 days (p = 0.0008, Log-rank test) as compared to the blank wafer group, which however was not as effectively as seen in those treated with the same dose of ADS-I wafer implants (1.53 mg), indicated by that the survival in 1.53 mg wafer group were significantly longer than that in solution group (ADS-I wafer -1.53 mg vs. ADS-I solution, p = 0.0048, Log-rank test).

During the survival monitoring, TAI including CRP, IL-2, VEGF, IL-6 and TNF-α were measured using ELISA kits for further evaluating anti-glioma efficacies of each treatment as described previously [21, 22]. As shown in **Table 3**, as compared to control blank wafer group, IL-2 was elevated, while other markers (CRP, VEGF, IL-6 and TNF-α) were decreased in all of ADS-1-treated groups. The statistical comparisons suggested that these changes were closely correlated with the efficacy of each treatment in the suppression of tumor growth, indicated by that the statistically significant changes of all the markers (a decrease in CRP, VEGF IL-6 and TNF-α, and an increase in IL-2) were seen in ADS-I wafer (1.53 mg) group, and of three out of five (CRP, TNF-α and IL-2) in the low dose of ADS-I wafer (0.77 mg) group and only IL-6 in ADS-I solution.

Discussion

The anti-tumor activities of ADS-I have been demonstrated in a variety of tumor cells including human glioblastoma cells *in vitro*, but the *in vivo* efficacy of this compound against tumor growth has not reported as of yet. The present study demonstrated a local ADS-I delivery system using biodegradable PLA/PLGA polymer as a carrier for glioma treatment in a rat model. ADS-I encapsulation in polymer wafer did not affect its suppressive activity against rat C6 glioma cells, and treatment with ADS-I wafer implants more effectively inhibited the growth of C6 glioma cells in rats as compared to the same dosage of ADS-I in the solution. The therapeutic effects of ADS-I wafer implants was significantly associated with an increase in tumor cell apoptosis and the changes of TAI (a decrease in CRP, VEGF, IL-6 and TNF-α, and an increase in IL-2), resulting in prolongation of tumor-bearing rat survival.

Many polymer-based drug delivery systems, including chemically controlled (biodegradable), are currently explored for providing controlled release of a drug that has advantage of maintaining the drug concentration within a therapeutic band over both burst and pulsatile releases for a long period of time [23]. For the biodegradable drug delivery system, the poly-

Table 3. The expression of tumor-associated indices/factors (n = 12)

Groups	CRP (μg/mL)	IL-2 (ng/mL)	VEGF (pg/mL)	IL-6 (ng/mL)	TNF-α (ng/mL)
Blank wafer	1893.54 ± 172.42	1008.81 ± 105.77	1068.44 ± 247.05	147.39 ± 6.27	276.64 ± 38.21
ADS-I wafer (0.77 mg)	1634.32 ± 293.49*	1150.56 ± 214.65*	877.66 ± 151.05	100.93 ± 20.37	218.08 ± 39.80*
ADS-I wafer (1.53 mg)	1525.44 ± 267.16**	1193.64 ± 102.89*	765.53 ± 149.86*	89.40 ± 21.69*	211.26 ± 31.44*
ADS-I solution	1886.08 ± 131.52	1112.59 ± 130.4	902.00 ± 127.35	132.82 ± 26.20*	227.59 ± 47.34

The plasma levels of these tumor-associated factors were determined using ELISA kits. Statistical significant as compared to blank wafer group was determined by ANOVA. *$p < 0.05$; **$p < 0.01$.

mer requires hydrolytically or proteolytically labile bonds in their backbone or crosslinker [23], and PLA/PLGA polymers contain hydrolytic cleavage of ester bonds, suggesting that these polymers may be a promising drug delivery system for controlled release of a drug in the treatment of a disease in the central nervous system (CNS). Indeed, the brain biocompatibility of PLGA implant has been investigated using a rat model [24], which shows that PLGA polymer and their breakdown products are well tolerated by the brain tissue, and there are no adverse cellular reactions in the parenchymal tissue adjacent to the implanted polymer. Also, a 50:50 blend of PLA and PLGA has been used for sustained delivery of timolol maleate for treating glaucoma in humans [25]. Although the metabolism of ADS-I in the CNS has not been investigated as of yet, the present study reports a local, controlled ADS-I delivery using a mixture of PLA/PLGA polymers that significantly improves the efficacy of this drug in the treatment of glioma in rats as compared to that of directly intratumor injection (a burst release) of ADS-I in the solution, suggesting that PLA/PLGA polymer-based delivery system can release ADS-I at the tumor site and maintain its concentration within a therapeutic band (bioavailability) for a longer period of time. This study indicates the potential of ADS-I loaded PLA/PLGA for clinical use against glioma in future.

The mechanisms of ADS-I anti-tumor activity have been extensively examined in cell cultures; its anti-tumor effects are associated with the induction of cell death via apoptosis [7] and/or autophagy [6], inhibition of angiogenesis and interference of VEGFR signaling [7] and blockage of cancer cell invasion and metastasis [8]. Similar results are seen in this study *in vivo* as indicated by the correlation of tumor growth suppression by ADS-I wafer implants with tumor cell inactive growth and apoptosis in tumor-bearing rats (**Figures 3** and **5**).

In brain tumor, human glioma cells produce many different cytokines and growth factors, such as IL-6, VEGF and TNF-α, following stimulation with cellular stress (*i.e*. Fas ligation and hypoxia) [26-29], and up-regulation of these factors associates with brain tumor progression [30-32]. Similarly, CRP in patients with glioma is elevated [33], and its level is also associated with poor survival prognosis [34]. Tumor bearing patients, who respond to the treatment and have a significantly increase in IL-2 and significantly decreases in TNF-α and IL-6, have a significantly longer median survival versus patients who did not respond to treatment [35]. In this experimental study, we assessed peripheral blood levels of these tumor-associated cytokines (IL-2, IL-6, TNF-α), inflammation-responding or acute-phase protein CRP, and growth factor VEGF in tumor-bearing rats receiving treatment with ADS-I wafer in order to further confirm its efficacy. IL-2 is a cytokine produced by T cells during the immune response [36, 37]. In ADS-I treatment groups, particularly those with the high dose (1.53 mg) of wafer implants, there are significantly higher levels of IL-2 than blank wafer group, which is probably related to the stimulation of T cell response to the dead tumor cells, resulting in increasing IL-2 production, whereas the decrease of IL-6, TNF-α, and VEGF may be due to the reduction of tumor mass - the source of these factors and their associated CRP, which however remains further investigation.

In conclusion, this study demonstrated the success of the local delivery of ADS-I using PLA/PLGA polymer implants that represented a feasible method for prolonging ADS-I release in vivo. Encapsulation of ADS-I in PLA/PLGA polymer wafer did not attenuate its anti-tumor activity. Moreover, treatment with ADS-I wafer implants was able to suppress brain tumor growth and/or to induce tumor cell death, resulting in the prolongation of tumor-bearing animal survival, indicating the feasibility of

ADS-I-loaded wafer implants for glioblastoma treatment.

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Disclosure of conflict of interest

None.

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