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PDE5 Inhibitors Enhance Tumor Permeability and Efficacy of Chemotherapy in a Rat Brain Tumor Model

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Abstract

The blood-brain tumor barrier (BTB) significantly limits delivery of therapeutic concentrations of chemotherapy to brain tumors. A novel approach to selectively increase drug delivery is pharmacologic modulation of signaling molecules that regulate BTB permeability, such as those in cGMP signaling. Here we show that oral administration of sildenafil (Viagra) and vardenafil (Levitra), inhibitors of cGMP-specific PDE5, selectively increased tumor capillary permeability in 9L gliosarcoma-bearing rats with no significant increase in normal brain capillaries. Tumor-bearing rats treated with the chemotherapy agent, adriamycin, in combination with vardenafil survived significantly longer than rats treated with adriamycin alone. The selective increase in tumor capillary permeability appears to be mediated by a selective increase in tumor cGMP levels and increased vesicular transport through tumor capillaries, and could be attenuated by iberiotoxin, a selective inhibitor for calcium-dependent potassium (K_{Ca}) channels, that are effectors in cGMP signaling. The effect by sildenafil could be further increased by simultaneously using another BTB “opener”, bradykinin. Collectively, this data demonstrates that oral administration of PDE5 inhibitors selectively increases BTB permeability and enhance anti-tumor efficacy for a chemotherapeutic agent. These findings have significant implications for improving delivery of anti-tumor agents to brain tumors.

Keywords

brain tumor; PDE5 inhibitor; drug transport; drug delivery; blood-brain barrier; blood-brain tumor barrier

1. Introduction

The prognosis for patients with malignant glioma remains poor [8,14,19]. One of the barriers to effective treatment of malignant brain tumors is limited transport of antitumor therapeutics across brain tumor capillaries [2,19]. Brain capillary endothelium and its contiguous cells, pericytes and astrocytes, are the structural and functional components of the blood-brain barrier

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(BBB). The blood-brain tumor barrier (BTB), which includes the microvessels supplying brain tumors, retains many characteristics of the normal BBB that significantly impedes adequate delivery of chemotherapeutics into brain tumors [6,17,30].

The BTB, however, has structural and functional characteristics that are different from those of the normal BBB. In particular, there are uniquely overexpressed receptors, ion channels and enzymes that regulate vascular permeability [5,7,21,24,31,34]. Pharmacologically modulating these unique components of the BTB can selectively increase transport of anti-tumor therapeutics across tumor capillaries and into brain tumors. For example, we have previously shown that bradykinin (BK) and its analog, RMP-7, an agonist to the bradykinin-2 receptor (B2R), selectively increase drug transport across the BTB in rat brain tumor models and humans [5,21,24,34]. However, increased transport across the BTB mediated by RMP-7 is transient and dependent on B2R expression in individual brain tumors [21,34,46]. High enough doses of RMP-7 were not achieved in phase II and III clinical trials to improve the efficacy of chemotherapy, due to the dose limiting side effect of hypotension [23]. Additionally, prolonged infusion of BK leads to B2R internalization and subsequent loss of the sensitivity of BTB transport in response to BK [35]. We therefore sought to identify additional molecular targets that could be utilized to consistently and selectively modulate transport of therapeutics across the BTB.

Cyclic guanosine monophosphate (cGMP) is an important intracellular second messenger that has been implicated in the regulation of vascular tone and permeability [20,28,39]. cGMP is made from GTP in a reaction catalyzed by guanylyl cyclases, and is degraded to 5'-GMP by phosphodiesterases (PDE) [3,4]. Modulation of PDE activity, which can affect the levels of intracellular cGMP, may result in alteration of the permeability of capillaries including microvessels in brain tumors [45]. Sildenafil (Viagra), vardenafil (Levitra) are selective inhibitors of type 5 PDE (PDE5) that increase intracellular cGMP levels [11,12] and are FDA approved oral treatments for erectile dysfunction in men. Here we sought to determine whether these marketed PDE5 inhibitors could increase BTB permeability and thereby improve the efficacy of chemotherapeutic treatment of brain tumors.

In this study we tested the hypothesis that cGMP signaling is involved in the transport of compounds across the BTB. In particular, we investigated whether oral PDE5 inhibitors at doses well tolerated in humans could selectively increase BTB permeability in rat brain tumor models. The ultrastructure of brain tumor capillaries after PDE5 inhibitor treatment was also examined. Further, we examined whether oral PDE5 inhibitors given in combination with a chemotherapeutic could improve the survival of the animals bearing a malignant glial tumor. The findings from this study support the use of PDE5 inhibitors as a novel therapy to selectively increase drug transport to malignant brain tumors.

2. Results

2.1. Expression of PDE1, PDE5 and PDE10

We first examined, by using real-time PCR, mRNA levels of PDEs in 9L gliosarcoma cell line that was used to generate the brain tumor model in this study. mRNA of three PDEs, PDE1, PDE5 and PDE10 were detected. However, mRNA levels of PDE5 were much higher than those of PDE1 and PDE10 (Supplemental Figure 1). PDE5 mRNA was also highly detected in other brain tumor cell lines such as GL26, U87, RG2, and importantly, a human microvessel endothelial cell line as well as human brain tumor samples available in this laboratory (data not shown).

2.2. Effects of Sildenafil and Vardenafil on BTB permeability (K_i)

We previously reported a high correlation between the initial transport, K_i , of [^{14}C] sucrose and the initial transport of water soluble chemotherapeutic agents including [^{14}C] carboplatin [32] and [^{11}C] methotrexate [22] in rat brain tumor models. [^{14}C] sucrose was therefore used in this study to examine the BTB permeability as reported before [24,32]. The effects of two marketed PDE5 inhibitors, sildenafil, and vardenafil on the initial blood to brain or blood to tumor transport, K_i , was studied and compared with that of BK, which increases BTB transport [21,34].

Consistent with previous reports, intravenous infusion of BK (120 $\mu\text{g/kg/min}$ for 15 minutes) significantly increased the K_i ($16.56 \pm 1.03 \mu\text{l/g/min}$ versus 8.26 ± 0.89 , $p < 0.001$) as compared to the untreated controls. The K_i was also significantly increased in the rats treated with 50 mg/kg sildenafil ($15.03 \pm 3.21 \mu\text{l/g/min}$, $p < 0.01$ versus the control), and vardenafil significantly increased the K_i at various doses with the maximal effect at 10 mg/kg ($20.05 \pm 3.59 \mu\text{l/g/min}$, $p < 0.001$ versus the control) (Figure 1A). BK resulted in a 2.4-fold increase in BTB permeability while sildenafil and vardenafil caused increases of 1.8-fold and 2.7-fold, respectively. Transport of the tracer in normal brain was not affected by the treatments (data not shown). Iberiotoxin, a selective K_{Ca} channel antagonist, abolished the vardenafil-induced BTB transport increase (Figure 1B), suggesting that the effects by PDE5 inhibitors are mediated, at least in part, through the K_{Ca} channels.

We found that K_i values remained elevated between 60 and 105 minutes after oral administration of sildenafil (50 mg/kg) and 45 to 105 minutes after vardenafil (10 mg/kg) (Figures 2A and 2B). Transport across the BTB into tumor tissues reached the maximum at 60 and 75 minutes after administration of sildenafil and vardenafil, respectively. A much shorter duration (5 – 20 minutes) of K_i elevation has been reported for BK infusion [21,34].

To determine any possible benefit of combination treatment, 9L tumor-bearing rats were given by gavage sildenafil or vardenafil with or without a 15-minute intravenous BK (120 $\mu\text{g/kg/min}$) infusion. The combination of BK and sildenafil treatment resulted in an increase in transport across the BTB at 45 minutes after the treatment as compared to either sildenafil or BK alone ($p < 0.001$) (Figure 3A). However, combining vardenafil with BK did not produce an increase in tumor transport (data not shown). The combination of sildenafil and BK did not increase transport in normal brain (Figure 3B).

2.3. Animal Physiologic Parameters

Mean-arterial blood pressures were decrease approximately 30% secondary to the femoral infusion of BK. The sildenafil (5–100 mg/kg) or vardenafil (1–20 mg/kg) caused a reduction in mean-arterial blood pressure of only 10%. Arterial blood pH, carbon dioxide, and partial pressure of oxygen were not changed significantly by the femoral infusion of BK or by the oral administration of sildenafil or vardenafil.

2.4. cGMP Levels in the Plasma and in 9L Tumors of Rats after Oral Administration of PDE5 Inhibitors

To test whether the effect of PDE5 inhibition K_i is related to cGMP signaling, we measured the levels of cGMP in the plasma and tumor tissue from 9L tumor-bearing rats. Plasma cGMP levels significantly increased at 30, 60, and 90 minutes ($54.96 \pm 25.13 \text{ pg/ml}$, $p < 0.05$; $79.20 \pm 37.36 \text{ pg/ml}$, $p < 0.05$; $30.13 \pm 17.82 \text{ pg/ml}$, $p < 0.05$, respectively) after oral administration of vardenafil as compared to no treatment controls ($0.72 \pm 0.48 \text{ pg/ml}$), with the peak concentration at 60 minutes (Figure 4A). Immunohistochemistry was performed to determine cGMP levels within the tumor (Figure 4B). The semi-quantitative measurement of cGMP levels using Zeiss AxionVision software in untreated tumor-bearing rats showed that the

normal brain contralateral to the tumor had very low levels of cGMP while the tumor tissue had increased cGMP-immunopositive staining (Figure 4B and 4C). Vardenafil treatment further increased immunostaining in the tumor tissues. The increase was apparent at 30 and 60 minutes after the drug treatment, and returned to the baseline at 90 minutes. Interestingly, although there was a trend for increase, we did not observe a significant increase by vardenafil treatment in immunofluorescent signal for cGMP in normal brain contralateral to the tumor. These results are consistent with those of transport studies, indicating that PDE5 inhibitors have selective effects on the transport across the BTB in tumors compared to the normal brain.

2.5. Vesicular Density and Tight Junction Integrity in Brain Capillaries after Oral Administration of Vardenafil

Our previous studies [18,31] indicate that increased vesicular transport is an important cellular mechanism for enhanced drug delivery via biochemical modulation (e.g. BK treatment) of BTB. Here we investigated vesicular density and tight junction integrity in BTB after oral treatment of vardenafil in rat tumor models. Vesicular formation was similar between normal and tumor capillary endothelium (Figure 5A). The vesicular formation was unchanged in the normal capillaries by vardenafil compared with PBS treated group (data not shown). However, vardenafil treatment dramatically increased vesicular formation in the tumor capillary endothelium (Figure 5A). This was further indicated by quantitative analysis of vesicle numbers in the capillary endothelium. The vesicular density and cumulative area of vesicles in vardenafil treated groups were significantly larger than those of PBS treated groups (Figure 5B). It was noticed that the effect by vardenafil on vesicular density lasted at least 3 hours. Reaction product for blood borne HRP was seen on the luminal surface membrane and within endothelial vesicles, endosomes, multivesicular bodies, and tumor cells nearby (data not shown). As expected, the tight junction integrity, which can be reflected by cleft index and cleft area [31,32], in tumor capillary endothelium was worse compared to that in normal brain tissue (Figure 6). However, vardenafil treatment did not result in any changes in tight junction integrity.

2.6. Survival Study

In order to determine if the permeability increase in brain tumors by the PDE5 inhibitors can be translated to improve efficacy of chemotherapy, the chemotherapeutic, adriamycin, was administered in 9L brain tumor-bearing rats, with and without vardenafil. Fischer rats were implanted intracranially with 1×10^5 9L cells, and treated for three days beginning at day 4 after tumor implantation with oral administration of vardenafil (10 mg/kg) followed by tail-vein injection of adriamycin (2 mg/kg). Log-rank analysis of the Kaplan-Meier survival curves showed a significant increase ($p < 0.05$) in survival in the rats treated with vardenafil and adriamycin together as compared to the untreated, vardenafil alone- or adriamycin alone-treated rats (Figure 7A). The mean survival time for the rats treated with vardenafil combined with adriamycin was significantly longer (53 ± 4 days) compared to those of saline (32 ± 2 days), vardenafil alone- (35 ± 1 days) and adriamycin alone-treated rats (42 ± 2 days). As expected, adriamycin alone also significantly increased the survival of 9L brain tumor-bearing rats when compared to saline ($p < 0.05$) (Figure 7A).

In addition to monitoring survival, images of tumors in the rats were obtained and the tumor sizes were calculated by using the IVIS system at day 4 after tumor implantation and day 9 after the last treatment. At day 4 after implantation, the size of the tumor was similar among all of the 4 groups (data not shown). However, after 9 day treatment, the saline control, vardenafil-treated and adriamycin-treated groups had relatively larger intracerebral tumors [$(2.21 \pm 1.27) \times 10^6$, $(3.14 \pm 2.04) \times 10^6$, $(1.03 \pm 0.28) \times 10^6$, respectively] compared to the group treated with the combination of vardenafil and adriamycin [$(4.75 \pm 0.45) \times 10^5$] (Figure 7B). In a separate experiment 9 days after the last treatment, histology staining confirmed that

the tumor sizes of the animals receiving the combination treatment were smaller than those of the other three groups. The tumor was more restricted for the combination treatment group (supplemental Figure 2). The animals in the combination treatment group showed no symptoms of dying while some animals in the other 3 groups showed neurological symptoms and start dying at the time of sacrifice.

3. Discussion

In this study, we sought to understand whether cGMP signaling is involved in determining the rate of transport of compounds across the BTB and particularly whether the pathway could be modulated by inhibition of PDEs, which are key enzymes determining intracellular cGMP levels, to improve efficacy of chemotherapy for brain tumors. We observed that: PDE5 is highly expressed in 9L tumor cells, brain capillary endothelial cells, as well as other tumor cell lines. Oral administration of the PDE5 inhibitors vardenafil and sildenafil selectively increased cGMP levels and vesicular transport in tumors and increased the rate of transport of [^{14}C] sucrose, a radioactive trace, from blood to brain tumor tissue. Importantly, vardenafil when given in combination with adriamycin significantly improved the survival and reduced the tumor size in the brain-tumor bearing rats. Collectively, this study is the first demonstration that oral administration of PDE5 inhibitors such as vardenafil and sildenafil increases the rate of transport of compounds across the BTB and improves the efficacy of adriamycin in treatment of brain tumors in a rat model.

Previously, we demonstrated that BK increases transport across the BTB in rat brain tumor models via a mechanism involving cGMP [45] which is made from GTP in a reaction catalyzed by guanylyl cyclases and is degraded to 5'-GMP by PDEs [3,4]. Inhibition of PDEs may thus be utilized to increase BTB permeability. Currently, there are more than 11 PDE isoforms identified, most of which have been detected in the brain [27]. We detected PDE5 mRNA in 9L tumor cells that we used to generate the brain tumor model in this study, with less expression of PDE1 and PDE10. In addition, PDE5 expression was also detected in a human microvessel endothelial cell line, other brain tumor cell lines such as GL26, U87, and RG2 and human brain tumor samples available in this laboratory (data not shown). As our findings suggest that PDE5 and other PDEs could serve as drug targets for selectively increasing transport of chemotherapeutic agents across the BTB, the expression of PDEs in brain tumors and BTB microvessels need to be further characterized.

Sildenafil and vardenafil are oral drugs that are currently used to treat erectile dysfunction in men. They selectively inhibit the activity of cGMP-specific PDE5 with different potencies [11]. In the present study, oral administration of vardenafil and sildenafil selectively increased transport of [^{14}C] sucrose to brain tumors. Vardenafil appeared to have a greater effect than sildenafil. This may reflect a higher potency for vardenafil to inhibit PDE5 as described in a previous study [11]. It should be noted that the doses of sildenafil and vardenafil used in our animals were comparable to the dose range clinically approved for erectile dysfunction in man, and they did not result in any detectable side effects in the rats. The two drugs appeared to be at least if not more effective in increasing drug transport into tumors as compared to the infusion of high dose BK (in this study we used 1000-times the BK dose used in previous human studies), which caused significant hypotension in rats (30% decrease in mean arterial blood pressure). Neither sildenafil nor vardenafil increased the transport of tracers into normal brain, thereby reducing potential toxicity of increased delivery of chemotherapeutic drugs to normal brain tissue.

BK increases transport into tumor in rat brain tumor models [21,24,25,34]. However, BK has a short biological half-life because of proteolytic inactivation [1,29], and its effect on transport into tumors is diminished within 15 minutes after intra-carotid infusion [21,34], which makes

the clinical use of BK or its analog RMP-7 difficult. We found that transport of tracers into tumors remained elevated for a much longer period after oral administration of sildenafil or vardenafil in comparison to BK. The extended period of BTB opening by PDE5 inhibitors could facilitate a greater accumulation of anti-tumor therapeutic agents in malignant brain tumors when administration is optimized with the pharmacokinetics of the agents.

In the absence of a PDE 5 inhibitor, higher levels of cGMP were detected in brain tumors than in the normal brain, consistent with the higher baseline permeability in tumors. Oral administration of vardenafil further increased cGMP levels in tumor tissue. cGMP has been reported to be involved in determining brain tumor permeability [45]. Our data suggests that the increase rate of transport into tumor after treatment with PDE5 inhibitors is mediated via elevated cGMP levels. The observation that vardenafil elevated cGMP levels much more in the tumor tissue than in the normal brain is consistent with the selective effects of the drug on K_i increase in tumors. In this study, the increased tumor permeability induced by PDE5 inhibitors could be abolished by a selective K_{Ca} channel antagonist, iberiotoxin. cGMP can activate cGMP-dependent protein kinase (PKG) which subsequently stimulates K_{Ca} channels [15]. It has been reported in an animal brain tumor model that K_{Ca} channels are highly expressed in tumor microvessels compared to normal brain and their activation results in increased vesicular transport of drugs from blood to tumor tissue [31].

Three major cellular mechanisms have been suggested to account for increased BBB permeability: increased vesicular transport, increased opening of tight junctions of endothelial cells, and increased transcellular penetration [10,43]. We investigated whether increased drug transport across the BTB by PDE5 inhibitors is associated with any change in vesicular transport and tight junction integrity. Our data demonstrate that vardenafil treatment significantly increase vesicle formation without any effect on tight junction integrity in tumor capillary endothelium without alteration of vesicular density in normal brain capillary endothelium. Further studies are required to determine the mechanism of transendothelial vesicular pathway mediated by vardenafil. For example, there are several transcytosis pathway in endothelium for substances from blood to brain such as receptor-mediated transport, carrier-mediated transport, and active efflux transport. Caveolae is most abundant in continuous capillary endothelia [36,38,40] and could be a suitable means for the transfer of anti-cancer drugs or other therapeutics from blood to brain [13,16]. Filipin, caveolae-mediated vesicle inhibitor, can be used to determine whether vardenafil mediate caveolae dependent vesicular formation.

Adriamycin was the anti-tumor agent used in the present study. Although adriamycin is one of the most effective agents against brain tumor cell lines *in vitro* [49], it has little effect *in vivo* as the BTB limits its ability to cross the BTB in brain tumor-bearing rodent models [19, 42] and in patients [41]. However, a significant increase in survival rate was achieved with intratumoral injection of adriamycin in patients with malignant glioma [47,48]. In our survival study, the treatment with a combination of oral vardenafil and adriamycin resulted in longer survival and smaller tumor size in the rats with a gliosarcoma. Our results may be extended to other chemotherapeutics. It should be pointed out that vardenafil did not show cytotoxicity effect itself nor did it enhance the effect by adriamycin against 9L tumor cells *in vitro* (data not shown), suggesting our observed *in vivo* effects by the combination were due to BTB or endothelial cell disruption. Further studies are required to test the mechanism in a well-established *in vitro* BBB or BTB model.

It has been shown that sildenafil increases angiogenesis in the ischemic regions of the brain of rats [50]. Our survival study showed that there is no difference in the survival days in control rats and rats treated with vardenafil, indicating vardenafil may not increase tumor infiltration. However, further studies need to be performed to find out whether PDE5 inhibitors could

induce neovascularization in the brain tumor. [^{14}C] sucrose, the tracer we used for Ki measurement, is similar in molecular weight (MW, 342.3), water solubility and ability to cross the BTB as many of the chemotherapeutics currently used to treat human tumors. We have previously reported similar Ki changes after biochemical modulation of the BTB for sucrose, carboplatin and methotrexate [22,31,32]. The water soluble adriamycin has a molecular weight (579.98) comparable to carboplatin (371.25) and methotrexate (454.44).

In conclusion, the present study demonstrates that oral administration of PDE5 inhibitors selectively increases transport across brain tumor capillaries and enhances the efficacy of chemotherapy in a rat brain tumor model. Currently, PDE5 inhibitors are clinically used to treat erectile dysfunction in men. Although the safety of application of these drugs to treat brain tumors requires evaluation, our findings have significant implications in improving drug delivery to brain tumors in patients.

4. Experimental Procedure

4.1. Animals and Materials

All animal experiments were conducted in accordance with policies set by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center and by NIH guidelines. Female Fischer rats, weighting 150–180 g, were used for this study. Bradykinin (BK) was obtained from the Sigma Co. (St. Louis, MO), sildenafil (Viagra) from Pfizer, Inc (New York, NY), vardenafil (Levitra) from the Bayer Pharmaceuticals Co. (West Haven, CT), and iberiotoxin from Sigma (Natick, MA). Adriamycin (doxorubicin hydrochloride) was obtained from Ben Venue Laboratories, Inc. (Bedford, OH). [^{14}C]-Sucrose (363 mCi/mmol) was obtained from ICN Biomedicals, Inc. (Dupont New England Nuclear, Boston, MA).

4.2. Intracerebral Tumor Implantation

9L gliosarcoma cells were kept frozen until use, and then thawed and maintained in a monolayer culture in DMEM medium with 10% FBS. The rats were anesthetized with intraperitoneal injections of ketamine (50 mg/kg)/xylazine (6 mg/kg), and immobilized in a stereotactic frame. The implantation (1×10^5 9L glioma cells) was conducted as described before [31,45].

4.3. Animal Preparation

For Ki study described below, six days after tumor implantation, the rats were anesthetized with ketamine/xylazine. One femoral vein was cannulated for administration of the appropriate drug and the radiotracer; one femoral artery was cannulated to withdraw arterial blood, and another femoral artery was cannulated to monitor systemic blood pressure. Body temperature was maintained at 37°C, and arterial blood gases, blood pressure and hematocrit were monitored. Animals that showed physiologic parameters outside a predetermined range during the procedures were eliminated from the study. The rats were treated with: (1) intravenous (i.v.) infusion of saline; (2) intravenous infusion of BK (120 $\mu\text{g/kg/min}$); (3) oral sildenafil; (4) oral vardenafil group; (5) oral sildenafil + i.v. BK; (6) oral vardenafil + i.v. BK; or (7) oral vardenafil + iberiotoxin (0.26 $\mu\text{g/kg/min}$).

4.4. [^{14}C] Sucrose Transport Studies

The method used to determine Ki, initial transport of a radioactive tracer from blood into tissue, has been described in our previous publications [24,31,32] with minor modifications. In brief, [^{14}C] sucrose was used as the radiotracer. Either BK or saline was infused into the femoral vein at a rate of 66.7 $\mu\text{l/min}$ for 15 minutes. For rats treated with oral PDE 5 inhibitors, the dose specified below was administered by oral gavage. Five minutes (ten minutes before decapitation) after the start of the intravenous infusion, or in the case of oral PDE 5 inhibitors

10 minutes before decapitation, 50 $\mu\text{Ci/kg}$ of [^{14}C] sucrose was injected as an intravenous bolus. Serum radioactivity was determined for 10 minutes after tracer injection. After completion of the experiments, the animals were euthanized by decapitation and the brains immediately removed and frozen.

To determine the dose response for the Ki, various doses of sildenafil (5, 25, 50 and 100 mg/kg) or vardenafil (1, 3, 10 and 20 mg/kg) were administered. The maximal effects on permeability by sildenafil and vardenafil were achieved at 50 mg/kg and 10 mg/kg, respectively, which were then employed for subsequent experiments. To study the duration of increased permeability induced by sildenafil or vardenafil, the Ki was determined at different time points from 30 to 105 minutes after oral administration of 50 mg/kg sildenafil or 10 mg/kg vardenafil.

4.5. Quantitative Autoradiography and Ki Calculation

The frozen brains were mounted onto pedestals with M1 embedding matrix, after which 20 μm coronal sections were cut with a cryostat. The sections were thaw-mounted onto slides, and autoradiographs were generated by exposing the sections along tissue-calibrated ^{14}C standards on a phosphor screen for 5 days. Quantitative analysis of the regional radioactivity for tumor and other brain areas was performed using a computer (Power Macintosh 7100) and Image 1.55 software (National Institutes of Health, Bethesda, MD) (Ningaraj et al, 2002-). The initial rate for blood-to-brain transfer (Ki) was calculated as described previously [7,31,32, 34].

4.6. Plasma cGMP Assay

Intracerebral inoculations of 9L tumor cells were performed as described above. On day 6 after tumor implantation, rats were treated with 10 mg/kg vardenafil by gavage. Different groups of rats were anesthetized 5 minutes before and at 30, 60, 90 and 120 minutes after treatment respectively and the femoral vein was cannulated. Blood was collected and the plasma was separated. The cGMP concentration in the plasma was determined using a commercially available ^{125}I -labeled cGMP assay kit (Amersham, Arlington Heights, IL). cGMP content was expressed as pmol/ml of plasma.

4.7. Brain cGMP Immunohistochemistry

Rats with a 9L tumor in the brain were decapitated before and at 30, 60, 90 and 120 minutes after vardenafil treatment. The procedures for immunohistochemistry was describe before [31] except that cGMP antibody was used in this study. Tissue sections were incubated with rabbit anti-cGMP diluted in 1:1000 in PBS. Localization of the cGMP antibody was accomplished by incubation of sections for 1 hour at room temperature with anti-rabbit IgG conjugated with fluorescent. The images were obtained by Zeiss fluorescece microscopy (Carl Zeiss MicroImaging, Inc., Thornwood, NY). The immunofluorescence intensity of cGMP staining was measured in 4 randomly chosen areas within the tumor, in the brain surrounding the tumor (BST; within 2 mm of the border of the tumor), and in contralateral cortex using Zeiss AxionVision software. Pictures of the tumor tissue were taken with a magnification of 20X under the same conditions.

4.8. Transmission Electron Microscopy (TEM)

TEM studies were performed as described before with modifications [18,31]. In brief, tumor implantation and animal preparation were conducted as described above. The rats were treated with vardenafil (10 mg/kg, p.o.) or PBS (n = 4) and sacrificed one (n = 3), two (n = 4), or three (n = 4) hours later. An additional group of rats (n = 3) was intravenously infused with BK (10 $\mu\text{g/kg/min}$ in PBS) for 10 minutes. Five minutes before sacrifice, the rats were intravenously

given a bolus of horse radish peroxidase (HRP) (200 mg/kg) which is the enzymatic tracer for transcytosis of blood borne protein through the BBB [9,18,33]. Ten minutes after HRP injection, rats were perfuse-fixed and the brains were removed and sectioned with a Leica VT1000S vibrating blade microtome (40 μ m sections). The sections were incubated to develop the substrate for HRP and then postfixed with 1% osmium tetroxide and 1% potassium ferricyanide. After dehydrated in ascending ethanol series and propylene oxide, the tissue was infiltrated with a mixture of resin and propylene oxide (3:1) and then with 100% resin. Epon embedded sections were examined on a microscope. Selected areas were cut and mounted on an epon block for thin sectioning [9]. The thin sections were cut with an ultramicrotome to a thickness of ~70 nm which was examined on a JEOL 1101 electron microscope (JEOL, Tokyo, Japan).

4.9. Analysis of Vesicles and Tight Junction Integrity

The criteria of selecting microvessels, vesicles and tight junctions for quantitative analysis was reported as previously described [18,44]. Ten to fifteen profiles of capillaries sectioned transversely in each group were selected and photographed at low magnification (X6,000) for evaluation of their general features. Then four test zones of each endothelial cytoplasm were photographed at higher magnification (X60,000). Three to five vessels were sampled from each rat and each animal group consisted of 15 – 20 capillaries and a population of 60–80 test zones. Each test zone was analyzed for vesicle density (40–70 nm size of vesicle) and tight junction integrity using TEM Imaging Platform iTEM from Olympus soft Imaging System.

4.10. Real-time PCR

Total cellular RNA was extracted from 9L tumor cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. One microgram (1 μ g) total RNA was reverse-transcribed into cDNA using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). Targeting gene primers (PDE1b, PPR50677A; PDE5a, PPR45092A; PDE10a, PPR49833A.) were purchased from SuperArray (Frederick, MD). GAPDH is used as the internal control. Dural-color, real time quantitative RT-PCR was performed using SYBR Green method (Bio-Rad Laboratories, Hercules, CA). The mRNA expression level was normalized by GAPDH expression.

4.11. Survival Study

For the survival experiments, implantation of 9L gliosarcoma cells (9L/Luc) [37], which stably express luciferase, was performed using the same method as described above. Tumor-bearing rats were randomly divided into four groups as follows: (1) Control, saline-treated (N=8); (2) vardenafil-treated (10 mg/kg, orally, N=7); (3) adriamycin-treated (2 mg/kg, intravenously, N=8); and (4) vardenafil (10 mg/kg, orally) + adriamycin group (2 mg/kg, intravenously) (N=6). The rats received their treatments for three consecutive days beginning at day 4 after tumor implantation. In *Ki* studies, vardenafil was found to be more effective in increasing *Ki* than sildenafil, so vardenafil was used in survival studies. Based on the *Ki* studies, 10 mg/kg of vardenafil was chosen and administrated by gavage 45 minutes before adriamycin treatment. The dose of adriamycin (2 mg/kg, injected into tail vein) was based on our preliminary data and a previous publication [49]. The rats were monitored carefully for clinical signs attributable to brain tumor growth or until death. The efficacy of therapy was estimated by the median survival time of the animals.

4.12. Tumor Size Monitoring

The noninvasive bioluminescence imaging technology, Xenogen IVIS200 Image System (Xenogen Corporation, Alameda, CA), was used to monitor the antitumor effects of vardenafil and/or adriamycin in the survival studies [26]. After implantation of 9L cells that express

luciferase [37], tumors were allowed to grow untreated for 4 days, at which time the rats underwent initial imaging. The animals were given an aqueous solution of D-luciferin (150 mg/kg, administered intraperitoneally) 18 minutes prior to imaging. This time point was based on the results from our pilot studies. The rats were also imaged at day 9 after drug treatment. Data were analyzed using the Xenogen LivingImage software. In a separate experiment, other four groups of rats were given the same treatments and sacrificed at day 9 after the last treatment. The frozen brains were mounted and cut with a cryostat. The sections were then stained with hematoxylin and eosin (H&E) for histological examination.

4.13. Statistical Analysis

Results are expressed as mean $\pm$ standard error (SEM), where applicable. The statistical analyses of *Ki*, cGMP levels, vesicle numbers and survival time between different groups, with or without the drug treatment, were performed using ANOVA, followed by either unpaired Student's *t* test of parametric analysis or by the Mann-Whitney *U* test of nonparametric analysis. Kaplan-Meier plot was used to analyze survival. A *p*-value of less than 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Glossary

Abbreviations

BBB	blood brain barrier
BTB	blood-brain tumor barrier
BK	bradykinin
B2R	bradykinin-2 receptor
Kca	calcium-dependent potassium;
RMP-7	lobradimil
IBTX	iberiotoxin
SIL	sildenafil
VAR	vardeafil.

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Figure 1. Pharmacological modulation of tumor permeability by PDE5 inhibitors

A. Effects of oral PDE5 inhibitors on the rate of radioactive sucrose transport, K_i , into tumors. The PDE5 inhibitors were administered orally followed by permeability determination at the 60-minute time point. BK (120 $\mu\text{g/kg/min}$) was infused for 15 minutes with permeability determined at the 15-minute time point. Intravenous infusion of BK served as a positive control [5,7,21,24,31,34]. **B.** Effect of a selective K_{Ca} channel antagonist, iberiotoxin (IBTX), on transport induced by vardenafil. IBTX (0.26 $\mu\text{g/kg/min}$) was infused for 15 minutes with permeability determined during the 5 to 15-minute infusion interval. The calculation of regional K_i was described in the Methods. The data are presented as mean $\pm$ SEM. SIL,

sildenafil (50 mg/kg); VAR, vardenafil (10 mg/kg). * $p < 0.001$, significantly different from the saline-treated group.

Figure 2. Time course of effects of oral PDE5 inhibitors on tracer transport into tumors
A. sildenafil treatment (50 mg/kg); **B.** vardenafil treatment (10 mg/kg). The PDE inhibitors were administered orally followed by transport determination at various time points. The regional *Ki* values were calculated as described in the Methods. The data are presented as mean $\pm$ SEM. SIL, sildenafil; VAR, vardenafil. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, significantly different from the saline-treated group.

Figure 3. Effect of the combination treatment with oral PDE5 inhibitors and intravenous BK infusion on transport into tumors

A. sildenafil with or without BK; **B.** the permeability at different brain areas by the combination treatment. The PDE5 inhibitor sildenafil (50 mg/kg) were administered by gavage at different time points with or without BK infusion (120 µg/kg/min for 15 minutes). BK, bradykinin; SIL, sildenafil; VAR, vardenafil; Cortex-Ips, ipsilateral cortex, Cortex-Contra, contralateral cortex. The data are presented as mean ± SEM. *** $p < 0.001$, significantly different from saline control group. τττ $p < 0.001$, significantly different from BK-treated group. ΦΦ $p < 0.01$, significantly different from sildenafil-treated group.

Figure 4. Effect of vardenafil on the cyclic GMP (cGMP) levels in the plasma and tissues

A. Effect of vardenafil on cGMP levels in the plasma of rats. Vardenafil (10 mg/kg) was administered orally and blood collected at 30, 60, 90 and 120 minutes later. cGMP levels were determined by ELISA-based analysis. **B.** Effect of vardenafil on cGMP levels in the 9L tumor. cGMP was detected by immunocytochemistry. Scale bar, 240 μ m. Brain tumors were removed 30, 60, 90 and 120 minutes after oral administration of vardenafil (10 mg/kg). The representative images for tumor and the normal tissue contralateral to the tumor (60 minutes after vardenafil) are also shown. Fluorescent microscopy was performed using anti-cGMP primary and FITC-conjugated secondary antibodies. **C.** Semi-quantification of immunofluorescent intensity of cGMP staining in brain and tumor tissues. The data are presented as mean $\pm$ SEM. VAR, vardenafil. * $p < 0.05$, significantly different from control group.

Figure 5. Vardenafil treatment increases vesicle formation in brain tumor capillary endothelium
A. Representative electron microscopic photographs of endothelium from the treatments of PBS (a, normal tissue; b, tumor tissue) and vardenafil (tumor tissue; c, 1 hour; d, 2 hours; e, 3 hours after treatment). Treatment of Vardenafil significantly increase the number of vesicles in the endothelial cytoplasm (arrowheads) compared to PBS treatment. Original magnification, 60,000X. **B.** Vesicular density of the endothelial cytoplasm (number of vesicles/ μm^2). Note that vesicular density is significantly increased after vardenafil treatment compared with control. * $p < 0.01$, ** $p < 0.001$ compared with the PBS control of tumor samples.

Figure 6. Morphometric evaluation of the opening degree of the tight junctions in endothelial cells from different treatments

A. Representative electron microscopic photographs of endothelium treated with PBS (a, normal; b, tumor) and vardenafil (tumor tissue; c, 1 hour; d, 2 hours; e, 3 hours after treatment). Red arrows indicate tight junction and white arrows indicate a cleft of tight junction. **B.** Cleft index (% of increase) measurement of tight junction in endothelial cells from different treatments. **C.** Cleft area index (%) measurement of tight junction in endothelial cells from different treatments. Cleft morphology (cleft index and cleft area) was altered in BTB capillaries compared with BBB capillaries, but was not changed as a result of vardenafil treatment.

Figure 7. Effect of vardenafil on adriamycin chemotherapy in 9L gliosarcoma-bearing rats

A. Effect of vardenafil on the survival rates of 9L gliosarcoma-bearing rats. The rats were treated with saline, vardenafil (10 mg/kg, oral), adriamycin (2 mg/kg, iv), or vardenafil (10 mg/kg, oral) plus adriamycin (2 mg/kg, iv). All rats were treated for three consecutive days beginning on the fourth day after tumor implantation (1×10^5 9L cells/rat). The Kaplan-Meier survival curves showed that the rats treated with vardenafil plus adriamycin survived significantly ($*p < 0.05$) longer than other three groups of rats. The rats treated with adriamycin alone also survived significantly ($*p < 0.05$) longer than the untreated and the vardenafil alone-treated rats. VAR, vardenafil. The data are presented as mean $\pm$ SEM. **B.** Effect of vardenafil on tumor size in 9L gliosarcoma-bearing rats. Rats were implanted with 9L tumor cells

containing the luciferase gene and treated with adriamycin (2 mg/kg, iv) with and without vardenafil treatment (10 mg/kg, oral) on days 4, 5 and 6 following implantation. Bioluminescence imaging was performed 9 days after the last treatment using the Xenogen IVIS 200 imaging system. Representative animals from the four groups of rats are shown.