Adenosine A2A Receptor Activation Enhances Blood–Tumor Barrier Permeability in a Rodent Glioma Model

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ABSTRACT

The blood–tumor barrier (BTB) limits the entry of effective chemotherapeutic agents into the brain for treatment of malignant tumors like glioblastoma. Poor drug entry across the BTB allows infiltrative glioma stem cells to evade therapy and develop treatment resistance. Regadenoson, an FDA-approved adenosine A2A receptor (A2AR) agonist, has been shown to increase drug delivery across the blood–brain barrier in non–tumor-bearing rodents without a defined mechanism of enhancing BTB permeability. Here, we characterize the time-dependent impact of regadenoson on brain endothelial cell interactions and paracellular transport, using mouse and rat brain endothelial cells and tumor models. In vitro, A2AR activation leads to disorganization of cytoskeletal actin filaments by 30 minutes, downregulation of junctional protein expression by 4 hours, and reestablishment of endothelial cell integrity by 8 hours. In rats bearing intracranial gliomas, regadenoson treatment results in increase of intratumoral temozolomide concentrations, yet no increased survival noted with combined temozolomide therapy. These findings demonstrate regadenoson’s ability to induce brain endothelial structural changes among glioma to increase BTB permeability. The use of vasoactive mediators, like regadenoson, which transiently influences paracellular transport, should further be explored to evaluate their potential to enhance central nervous system treatment delivery to aggressive brain tumors.

Implications: This study provides insight on the use of a vasoactive agent to increase exposure of the BTB to chemotherapy with intention to improve glioma treatment efficacy.

Introduction

Drug delivery to the central nervous system (CNS) is influenced by molecular size, charge, and hydrophobicity, as well as multidrug-resistant protein substrate activity. When tumor cells are present within the brain, the formidable blood–tumor barrier (BTB) greatly limits potential chemotherapeutic agents from crossing into the CNS. The BTB results from newly formed brain tumor capillaries and differs from the blood–brain barrier (BBB) by exerting a more “leaky” phenotype in certain areas (1). The BBB is mainly formed by endothelial cells, pericytes, and astrocytic end feet allowing tight regulation of small molecules and fluid exchange between the blood and the brain (2). Heterogeneous BTB permeability is mainly conferred by lack of tight junctions and astrocytic end feet in contact with endothelial cells (1, 3, 4). Specifically, in the case of glioblastoma (GBM), the invasive potential of glioma cells and glioma stem cells (GSC) allow them to proliferate at distant sites from the tumor where the BTB resembles the more impermeable BBB. Consequently, the BTB remains a major obstacle in GBM therapy by limiting effective therapeutic agents to reach the primary tumor site and often the most invasive tumor cell–infiltrating areas (1, 4).

Disruption of the BBB at the endothelial cell level has been a common goal to increase permeability to therapeutic agents in various CNS diseases (5). Techniques to overcome the BTB in brain tumor therapy include osmotic disruption, focused ultrasound, convection-enhanced delivery, bolus injection of therapeutic agents directly into the tumor bed, surgically implanted chemotherapy infused biodegradable wafers, nanoparticle carriers, peptide-based delivery, and pharmacologic inhibition of efflux pumps coupled with chemotherapy (6).

Limitations of these methods include high expense, required surgical expertise, and/or inability to provide durable responses. In contrast, the use of pharmacologic agents to transiently open the BTB is a noninvasive technique to improve concomitant chemotherapeutic delivery with low toxicity and lower risk of secondary edema and hemorrhage. However, previous studies evaluating vasoactive mediators such as Bradykinin analogues, histamine, and alkylglycerol have shown variability in drug delivery efficacy in tumor-bearing rodents and patients (7).

About a decade ago, regadenoson, an FDA-approved adenosine A2A receptor (A2AR) agonist used for cardiac stress testing, was proven to induce cerebral vasodilation causing transient headaches in 26% of patients receiving it (8, 9). This effect is thought to be secondary to high A2AR expression on the surface of brain endothelial cells (10, 11).
Regadenoson is highly selective to A2AR and causes rapid vasodilatation with a short half-life between 2 to 4 minutes (8). Regadenoson is more selective to adenosine A1 receptor (A1R) by 10-fold over A2AR in mice and selective only for A2AR in rats and humans (12–14). Previous studies showed that regadenoson dampens P-glycoprotein (P-gp) expression and decreases tight junction expression on rodent brain endothelial cells (15). In addition, regadenoson increases paracellular transport of 70 kDa dextran and of temozolomide, a common GBM chemotherapeutic agent, in non–tumor-bearing rodents (16–19).

While GBM is the most common and lethal malignant brain tumor in adults, standard chemoradiotherapy using temozolomide with surgery only provides a two-year survival rate of 26.5% (20). Thus, ongoing preclinical and clinical studies are focused on improving disease prognosis, with limited toxicities. A recent phase 0 clinical trial with recurrent GBM patients sought to evaluate the effect of regadenoson on temozolomide intratumoral entry via brain extracellular fluid sampling (21). Out of 5 patients, two complained of headaches and testing in this subgroup demonstrated a significant increase in their intratumoral temozolomide concentrations when concomitantly treated with regadenoson. The three other patients demonstrated no difference in intratumoral temozolomide concentrations nor experienced headaches following concomitant regadenoson administration. Such interpatient differences were attributed to (i) postoperative standard-of-care dexamethasone coadministration, which is known to tighten the BTB and maintain its integrity and (ii) proposed varied A2AR activation and efflux transporter activity within tumor and healthy brain vasculature (21, 22). This variability between patients with GBM led us to investigate the details of regadenoson’s impact on increasing BTB permeability for enhancement of chemotherapy delivery in glioma models.

In this study, we demonstrate the timing and mechanism of action of A2AR activation by regadenoson on endothelial cell–cell junctional interactions, cytoskeletal organization, and enhanced CNS paracellular permeability in intracranially injected glioma rodent models. We establish that short-term desensitization of A2AR, but not A1R, by regadenoson results in transient rearrangement of endothelial actin cytoskeleton and disruption of cell–cell junctional proteins, allowing for altered endothelial barrier electrical integrity. We further show that regadenoson administration causes an increase in intratumoral concentrations of temozolomide in rats intracranially injected with GSCs, but not in tumor-bearing mice. Yet with repeat dosing of combined regadenoson and temozolomide, glioma-bearing rats did not exhibit a survival advantage. Collectively, these studies bring insight into the use of vasoactive modulators, like regadenoson, to improve efficient delivery of poorly BTB permeable agents to high-grade CNS tumors. These studies also delineate the importance of studying receptor species differences, as it relates to drug responsivity, in an effort, to optimize preclinical model use for future clinical applications.

Materials and Methods

Animals
C57BL/6J mice were purchased from The Jackson Laboratory (JAX-000664) and SCID mice [8 weeks old, obtained from Charles River Laboratories (#NCI SCID/Ncr, strain code: 561]. Athymic nude female rats (nu/nu) were from an in-house colony at Oregon Health Science University (OHSU) or Envigo RMS Inc, between the size of 250–300 g. The care and use of the rats were approved by the Institutional Animal Care and Use Committee (IACUC) and were under the supervision of the Department of Comparative Medicine at OHSU or the NIH, National Institute of Neurological Disorders and Stroke. Food and water were supplied to all rats ad libitum. The mouse and rat study protocols were approved by the Animal Care and Use Committees of OHSU and the NIH and the use of all study animals conformed to the rules outlined in the NIH Guide for the Care and Use of Laboratory Animals. All mice and rats were housed in standard facilities and provided free access to rodent chow and water.

Cell lines
Immortalized mouse brain endothelial cells (bEnd.3, endothelial polyoma middle T antigen transformed) were obtained from ATCC (#CRL-2299). The bEnd.3 cells were derived from a 6-week-old mouse endothelioma giving them tumor-like characteristics and making them an optimal in vitro model for studying the BTB (23, 24). Cells were grown in DMEM supplemented with 10% FBS, 1% antibiotic–antimycotic (Gibco). C57BL/6 mouse primary brain microvascular endothelial cells (mBMEC) were obtained from Cell Biologics (#C57–6023). Primary mBMECs were grown in complete mouse endothelial cell medium containing 0.5 mL of each: VEGF, endothelial cell growth supplement, heparin, EGF, hydrocortisone, 5 mL of each: 1-glutamine, antibiotic–antimycotic solution, and 25 mL FBS (#M1168-Kit). Rat brain microvascular endothelial cells (RBMEC) were obtained from Cell Applications (#R841–75a). RBMECs were grown in rat brain endothelial cell media (#R819–500). GSC cell lines, GSC923 and GSC827, were patient derived from GBM tissues surgically removed and maintained by the Neuro-Oncology Branch laboratory at NCI per NCI Institutional Review Board–approved protocol (NCI 02C–0140). GSCs were cultured as described previously (25). Briefly, cells were grown as spheroids in NRE medium consisting of Neurobasal-A medium, N2 and B27 supplements (Invitrogen), 1 µg/mL basic fibroblast growth factor (bFGF), 1 µg/mL EGF, and 1 mmol/L L-Glutamine. All cells were maintained at 37°C under a humidified environment containing 5% of CO2.

Reagents and antibodies
Mouse siRNA for transient knockdown of Adora1 (Mm_Adora1_10 FlexiTube siRNA: S010917136) and Adora2a (Mm_Adora2a_6 FlexiTube siRNA: S010917150; Mm_Adora2a_8 FlexiTube siRNA: S010917164) were purchased from Qiagen. A negative control siRNA was obtained from Sigma. The medium affinity green fluorescent calcium binding dye Fluo-8 AM was purchased from Abcam. Ionomycin and cyclopiazonic acid (CPA) were kindly provided by Zayd Khaliq (NIH, Bethesda, MD). Antibodies used for immunoblotting and immunostaining are listed in Supplementary Table S1. Alexa Fluor 488-phallolidin was purchased from Thermo Fisher Scientific (#AB12379). Regadenoson was acquired from Astellas Pharma, CA ENBA (A1R agonist) was from Tocris (catalog no. 3576), whereas CGS21680 (A2AR agonist), UK432097 (A2AR antagonist), and SCH442416 (A2AR antagonist) were graciously offered by Dr. Kenneth Jacobson (NIH, Bethesda, MD). Temozolomide was supplied from Sandoz/Novartis.

Establishment of brain endothelial cell monolayer
Prior to each experiment, bEnd.3 cells and RBMECs were grown for five days until confluence to ensure appropriate BBB phenotype. For immunofluorescence assays, bEnd.3 cells were plated on chamber slides precoated with 0.1 mg/mL of poly-1-lysine (ScienCell), whereas RBMECs were plated on coverslips precoated with attachment factor solution (Cell Applications).
Calcium influx assays
Cells were incubated with Fluo-8 AM following manufacturer’s protocol. Using a Zeiss LSM 780 confocal microscope, time series images were recorded every second over 300 seconds. Treatment was added approximately 30 seconds after recording started. Fluorescence intensity of stacked images of 20 cells per field per sample were quantified with ImageJ after removal of background fluorescence. Ionomycin and CPA were, respectively, used as positive and negative controls (26, 27).

Total RNA isolation, cDNA synthesis, and qPCR
Cells were collected in TRIzol reagent and total RNA was extracted per manufacturer’s protocol (Thermo Fisher Scientific). Complementary DNA (cDNA) was obtained by reverse transcription of 1 μg of total RNA. Gene expression was quantified by qPCR using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). DNA amplification was carried out using a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific). Primer sets were obtained from Integrated DNA Technologies and sequences are listed in Supplementary Table S2. Gene expression was measured by the difference between the mean Ct values of the target gene and those of Gapdh (Agi). The relative expression was obtained by calculation of 2^(-ΔΔCt) for each sample and compared with Gapdh.

Transfection method and RNA interference
bEnd.3 cells were transfected with 10 nmol/L of siRNA against Adora1, Adora2a, a combination of Adora1 and Adora2a, or a negative control siRNA using Lipofectamine 2000 (Thermo Fisher Scientific). We have tested multiple siRNA sequences and the ones with highest knockdown efficiency after 24 hours of transfection. Specific gene knockdown was evaluated by qRT-PCR as described above.

Real-time endothelial barrier function measurement
Assessment of brain endothelial barrier function was carried out using the Real-Time Cell Analyzer (RTCA) Dual-Plate (DP) Instrument of the xCELLigence system (ACEA Biosciences). Forty thousand cells were seeded per well onto an E-Plate 16 (ACEA Biosciences). Cells grew in complete culture medium for 16 to 24 hours. Complete medium was replaced by serum-free medium and starvation occurred for 3 hours to allow stabilization of the system. Treatment was then added to the cells and impedance measurement was recorded every 2 minutes for the first two hours, and every 5 minutes until 8 hours. Values are expressed in arbitrary units as Normalized Cell Index which is defined, at a time point, as (Zn-Z0)/nominal Z; where Zn is the cell–electrode impedance of the well when it contains cells and Z0 is the background impedance of the well with the media alone (28).

Cell viability assay
Endothelial cells were seeded in a 96-well plate at 4 × 10^4 cells/well for 24 hours. Cells were then treated for 8 hours with increasing doses of regadenoson (0.1, 1, 10 μmol/L). Viability was measured using CellTiter-Glo assay per manufacturer’s protocol (Promega). The luminescence was recorded by a luminometer (PolarStar Optima).

Electron microscopy
bEnd.3 cells were grown in a 6-well plate and treated with vehicle or regadenoson. Cells were washed with PBS before incubation for 1 hour at room temperature in electron microscopy (EM) fixative buffer containing 2% glutaraldehyde in 0.1 mol/L cacodylate buffer. Cells were dehydrated in 100% ethanol, embedded in situ in a pure epoxy resin, and cured in 55°C oven. The resin blocks were examined under inverted light microscope. Areas of interest for thin-sectioned EM analysis were cut off by a jeweler’s saw and glued on a blank block. Thin sections (80 nm) were made from the selected areas and mounted on 150-mesh copper grids. The grids were stained with aqueous uranyl acetate (0.5% w/v) and Reynold’s lead citrate and examined in H7650 (Hitachi) electron microscope equipped with a CCD camera (Advanced Microscopy Techniques Corp). Quantification of distance in cell gap and cell–cell junction lengths was made by measuring 100 distances of triplicates using prints of digital EM images. Distances were measured with a measuring device (American Map Corp) and converted to nanometers or micrometers.

Subcellular fractionation and immunoblotting
Proteins from whole-cell lysates were obtained using RIPA lysis buffer (Santa Cruz Biotechnology) and protease inhibitor cocktail (Santa Cruz Biotechnology). Subcellular fractions were obtained using the ProteoExtract Subcellular Proteome Extraction Kit (Millipore Sigma). Protein samples were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% nonfat dry milk solution in TBS/Tween-20, membranes were immunoblotted overnight with primary antibody in TBST containing 3% BSA and 0.05% NaN3, followed by horseradish peroxidase–conjugated secondary antibody and ECL detection. Further quantification of the bands was made using ImageJ software.

Immunocytochemistry
Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After blocking unspecific binding sites with PBS containing 1% BSA, cells were incubated with primary antibody overnight at 4°C followed by incubation with secondary antibody 1 hour at room temperature. Between each step, cells were washed three times with PBS for 5 minutes. Fluorescent images were acquired with a Zeiss LSM 710 confocal microscope.

Actin cytoskeleton staining
Cells were incubated with 1U Alexa Fluor 488-phalloidin in PBS-BSA 1% for 30 minutes at room temperature. DAPI-containing Vectashield antifade mounting medium (Vector Laboratories) and coverslips were added on the cells. Fluorescent images were acquired with a Zeiss LSM 710 confocal microscope. Quantification of fluorescence intensity was performed using ImageJ software.

IHC
Slices of 10 to 12 μm thickness were obtained from fresh-frozen mouse and rat brain tissue followed by fixation and permeabilization in acetone at −20°C for 10 minutes. For mice, unspecific binding sites were blocked for 20 minutes in PBS-containing 5% normal goat serum. Primary antibody was incubated overnight at 4°C in a humidified chamber. Tissue was then incubated 1 hour at room temperature with secondary antibody and DAPI. For rats, unspecific binding sites were blocked for 20 minutes in PBS-containing 5% normal donkey serum. Primary antibody Pecam1 (Supplementary Table S2) was incubated overnight at 4°C in a humidified chamber. Tissue was then incubated 1 hour at room temperature with secondary antibody. Subsequently, tissue was washed and blocked for 20 minutes in PBS-containing 5% normal goat serum. Primary antibody claudin-5 (Supplementary Table S2) was incubated overnight at 4°C in a humidified chamber. Tissue was then incubated 1 hour at room temperature with secondary antibody.
antibody and DAPI. Coverslips were mounted on tissue with DAKO fluorescent mounting media (Agilent). Fluorescent images (z-stacks) were acquired with a Zeiss LSM 780 confocal microscope. Background fluorescence was subtracted and quantification of maximum relative fluorescence intensity and tight junction areas were analyzed with ZEN software.

Mouse brain endothelial cell isolation

The brain endothelial cell isolation method was adapted from Swanson and colleagues (29) Five C57BL/6j mice were euthanized; their brains were removed, minced, and incubated in an enzymatic solution of 0.2 mg/mL Collagenase P (Sigma), 1.6 mg/mL Dispase (Sigma), and 0.1 mg/mL DNAse (Roche) in RPMI1640 for 10 minutes in a 37°C water bath. They were homogenized by pipetting, washed with PBS-FBS-EDTA and centrifuged. Pellets were resuspended in 35% Percoll solution and centrifuged at 1,700 rpm for 20 minutes to allow myelin removal. Pelleted brain cells were incubated with Fc block, IgG, and selection antibodies, CD31-PE (BioLegend) and CD45-FITC (BioLegend), as well as DRAQ5 and DAPI for live and dead cell selection. The endothelial cell population (CD31+/CD45−/DRAQ5+/DAPI−) was sorted in TRIzol and RNA was isolated using the RNEasy Micro Kit (QIAGEN).

Human glioma rodent model brain temozolomide concentrations

Female SCID mice were injected into the right caudate putamen (striatum) with 500,000 GSCs (GSC923 or GSC827) using a stereotactic device (coordinates, 2 mm anterior and 2 mm lateral from bregma, and 2.5 mm depth from the dura). Establishment of the tumors occurred after approximately 5 weeks and was verified through hematoxylin and eosin (H&E) staining. Establishment of the tumors occurred after approximately 5 weeks and was verified through hematoxylin and eosin (H&E) staining. Five days of consecutive treatment began on week 10, in sterile water, orally, temozolomide (10 mg/kg, orally), and temozolomide (50 mg/kg, orally) and regadenoson (0.0005 mg/kg, i.v., 1 hour (Sterile water, orally), temozolomide (10 mg/kg, orally), and temozolomide (50 mg/kg, orally) and regadenoson (0.0005 mg/kg, i.v., 1 hour (Sterile water, orally), temozolomide (50 mg/kg, orally) and regadenoson (0.0005 mg/kg, i.v., 1 hour post-temozolomide). After temozolomide treatment, approximately 35% Percoll solution and centrifuged at 1,700 rpm for 20 minutes to allow myelin removal. Pelleted brain cells were incubated with Fc block, IgG, and selection antibodies, CD31-PE (BioLegend) and CD45-FITC (BioLegend), as well as DRAQ5 and DAPI for live and dead cell selection. The endothelial cell population (CD31+/CD45−/DRAQ5+/DAPI−) was sorted in TRIzol and RNA was isolated using the RNEasy Micro Kit (QIAGEN).

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Survival analysis

Male, athymic nude rats were injected into the right caudate putamen (striatum) with 1 × 106 GSC827 cells using a stereotactic device. Establishment of the tumors occurred after approximately 3 weeks and was verified through hematoxylin and eosin (H&E) staining. Five days of consecutive treatment began on week 10, in which rats were randomized into 3 treatment groups (n = 5–6): control (sterile water, orally), temozolomide (10 mg/kg, orally), and temozolomide (10 mg/kg, orally) and regadenoson (0.0005 mg/kg, i.v., 1 hour post-temozolomide). Animals were then monitored for clinical end-point criteria including hunched posture, labored breathing, impaired mobility, and 15% weight loss. Once endpoint was reached, duration of survival was noted. Brains were extracted and flash frozen in optimal cutting temperature for preservation before they were subsequently sliced and stained for H&E to verify tumor establishment.

Statistical analysis

Results are reported as means ± SEM of three or more experimental replicates. Significant differences between groups were determined with unpaired Student t test. P values smaller than 0.05 were considered significant. Analysis was performed using GraphPad Prism software. Differences are indicated as *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; and ns, not significant.

Results

Regadenoson impacts rodent brain endothelial barrier function and allows for transient disruption of cell–cell interactions

We first evaluated the levels of adenosine receptor expression in bEnd.3 cells, mBMECs, and rat brain RBMECs by qPCR. We found that they all expressed higher levels of A2AR and A2BR than A1R, and almost no A3R (Fig. 1A). We noted that bEnd.3 cells expressed higher levels of A1R and A2AR compared with mBMECs and RBMECs. This data corroborates previous RNA-sequencing data showing very low expression of A1R and high expression of A2AR in mouse brain endothelial cells compared with other brain cell types (10, 11). We next assessed regadenoson’s impact on endothelial cell barrier function, using the xCELLigence real-time cell analysis assay that records impedance. We observed that regadenoson decreased the bEnd.3 and RBMEC endothelial barrier cell index with an IC50 of approximately 13 μmol/L and 7 μmol/L, respectively, at 30 minutes (Supplementary Fig. S1A and S1B). We observed a significant decrease in bEnd.3 and RBMEC cell index (between 35% and 75%) after 30 minutes of exposure to 10 μmol/L regadenoson (Fig. 1B). By approximately 3 hours, cell index increased by about 15% and slightly continued to increase until 8 hours (Fig. 1B). At 8 hours, the difference in bEnd.3 cell index was not significant between vehicle and regadenoson, suggesting transient barrier disruption with a normalization of endothelial cell–cell integrity (Fig. 1B). It appeared that regadenoson’s impact on RBMEC cell index was sustained for a longer period of time (Fig. 1B). To ensure functional changes were due to biological activity of regadenoson, we observed no change in viability of bEnd.3 cells and RBMECs over the course of 8 hours with increasing doses (Fig. 1C). Interestingly, we observed that regadenoson had no impact on mBMEC endothelial barrier when compared with vehicle-treated cells (Fig. 1B). Therefore, we did not use mBMECs for further analysis. Furthermore, to evaluate the specificity of A2AR activation in the modulation of endothelial barrier integrity, we observed decreased bEnd.3 cell index occurring through selective activation of A2AR when using a specific agonist (CGS21680), compared with an A1R agonist (CI-ENBA), which had no impact on cell index (Supplementary Fig. S2A). In addition, regadenoson-mediated decreased cell index was abrogated with pretreatment of bEnd.3 cells with increasing doses of an A2AR antagonist (SCH442416; Supplementary Fig. S2B).

We observed on a confluent monolayer of bEnd.3 cells that 30 minutes of regadenoson exposure induced tight junction disruption as seen by breaks in electron density at the cell–cell junctions compared with vehicle control (Fig. 1D). We measured no differences in the distance between adjoining bEnd.3 cells (cell gaps; Fig. 1E). However, we observed a decreased percentage of junctional interactions after treatment with regadenoson, which was represented as the length of
electron dense material divided by the length of cell–cell membrane based on the Hubner cell–cell coverage equation (Fig. 1F; ref. 31). These results confirm the ability of regadenoson to rapidly and transiently modulate rodent brain endothelial cell–cell interactions.

To further understand the cellular interactions at the endothelial barrier following A2AR activation, we investigated the expression alterations in junctional proteins. By assessing fluorescent immunocytochemistry of selected junctional proteins, we found that membrane expression of ZO-1 and VE-cadherin was disrupted by 4 hours and membrane morphology reorganized by 8 hours post regadenoson (Fig. 2A). Moreover, protein expression of claudin-5, occludin, and VE-cadherin was significantly decreased after 4 hours of incubation with regadenoson (Fig. 2B and C). Junctional expression was decreased by 30%, 48%, and 34% respectively (Fig. 2C). By 8 hours, overall junctional protein expression returned to baseline (Fig. 2B and C). Similarly, we observed disrupted ZO-1 and VE-Cadherin integrity in RBMECs during the first 4 hours of treatment and membrane morphology reorganized by 8 hours post regadenoson (Fig. 2D). Furthermore, we observed an increase in cytoplasmic localization of ZO-1 and VE-Cadherin over the first 4 hours when treated with regadenoson (Fig. 2D). There was no difference in the total protein expression of junctional protein (Fig. 2E and F). We have

Figure 1.
Regadenoson rapidly and transiently disrupts rodent brain endothelial cell-cell interaction. A, Relative gene expression of adenosine receptors in bEnd.3 cells, mBMECs, and RBMECs. Data are shown with mean ± SEM for three experimental samples. B, Top, representative endothelial monolayer functional assay of bEnd.3 cells, mBMECs, and RBMECs treated with vehicle or regadenoson using the xCELLigence system. Cell impedance was recorded every 2 minutes and reported as normalized cell index representing changes in cell–cell adhesion. Bottom, Quantification of drop in cell index over time after treatment with vehicle or regadenoson for three or more experiments. Data are represented as mean ± SEM. *, P < 0.05; **, P < 0.01 by Student t test. C, Viability of bEnd.3 cells, mBMECs, and RBMECs treated with increasing doses of regadenoson for 8 hours. Data are represented as mean ± SEM for three experimental samples. D, Representative EM images of cell–cell junctions in bEnd.3 cells treated with vehicle or regadenoson for 4 hours. Less electron-dense regions (arrows) represent disrupted cell-cell junctions. E, Quantification of cell gap, representing distance between cells at junctions. F, Schematic representation of cell–cell junctions percentage calculations, modified from Hubner, and colleagues 2018 (31). Quantification of percentage of cell–cell junctions, representing the overall length during which the cells are tightly adjoined. Data are represented as mean ± SEM of three independent experiments. ***, P < 0.001 by Student t test. All regadenoson treatments were at 10 μmol/L except where indicated in C.
Figure 2. Regadenoson transiently disrupts rodent brain endothelial cell junctional integrity. A, Representative immunocytochemistry images of tight junction protein ZO-1, and adherens junction protein VE-cadherin in bEnd.3 cells treated with vehicle or regadenoson for 0.5, 4, or 8 hours. Zoomed insets show the morphologic aspect of cell junctions and membrane organization. B, Representative immunoblots of tight junction proteins: claudin-5, occludin, and adherens junction protein: VE-cadherin in bEnd.3 cells treated with vehicle or regadenoson for 0.5, 4, or 8 hours. C, Quantification of junctional protein expression presented in B. Data are represented as mean ± SEM of three or more experiments. *, *P < 0.05; **, *P < 0.01 by Student t test. D, Representative immunocytochemistry images of tight junction protein ZO-1, and adherens junction protein VE-cadherin in RBMECs treated with vehicle or regadenoson for 0.5, 4, or 8 hours. Zoomed insets show the morphologic aspect of cell junctions and membrane organization. E, Representative immunoblots of tight junction proteins: claudin-5, occludin, and adherens junction protein: VE-cadherin in RBMECs treated with vehicle or regadenoson for 0.5, 4, or 8 hours. F, Quantification of junctional protein expression presented in E. Data are represented as mean ± SEM of three experiments. *, *P < 0.05 by Student t test. All regadenoson treatment was at 10 μmol/L.
observed disrupted ZO-1 expression at the membrane of bEnd.3 cells when treated with regadenoson or CGS21680 but not with CI-ENBA (Supplementary Fig. S2C). ZO-1 expression integrity was conserved when bEnd.3 cells were pretreated with the A2AR antagonist SCH442416 before incubation with regadenoson (Supplementary Fig. S2D). These results show that the impact of regadenoson on ZO-1 is dependent on A2AR activation. Collectively, these findings suggest that regadenoson enhances permeability by transient post-transcriptional modulation of endothelial barrier integrity. We have identified a specific time window between 30 minutes to 4 hours during which regadenoson impacts mouse brain endothelial cell–cell interactions.

**Regadenoson transiently disrupts rodent brain endothelial cytoskeleton**

Downstream activation of A2AR occurs through Gαs protein, which plays an important role in maintenance of intracellular calcium levels, as well as cytoskeletal and junctional organization (32). Cytoskeletal reorganization has previously been linked to junctional cell–cell adhesion by directly interacting with cytosolic portions of tight and adherens junction proteins (33). Hence, we demonstrated that F-actin cytoskeleton was disorganized after 30 minutes of exposure to regadenoson (Fig. 3A–C). Disruption of F-actin was sustained for at least 4 hours, and it completely reassembled by 8 hours (Fig. 3A–C).

Interestingly, F-actin fluorescent intensity was decreased by regadenoson in bEnd.3 cells but not in RBMECs (Fig. 3B–D). The cytoskeleton of bEnd.3 cells was also disorganized after 30 minutes with CGS21680 and returned to baseline organization after 4 hours, which was much faster than after regadenoson treatment (Supplementary Fig. S2E). To a lesser extent, we observed cytoskeleton disorganization with CI-ENBA at 30 minutes, which can be attributed to the fact that agonism of GPCRs can lead to cytoskeletal modulation (Supplementary Fig. S2E; ref. 34). In addition, pretreatment of bEnd.3 with SCH442416 abrogated the impact of regadenoson on endothelial cytoskeleton (Supplementary Fig. S2F).

**Rapid desensitization of murine A2AR by regadenoson indicates the opportunity for its use as a BBB permeability agent in a specific time window**

A2AR is a G-protein coupled receptor (GPCR) where activation is known to lead to increased calcium influx (35). Therefore, we used a calcium influx assay to help determine the timeline of activation, desensitization, and recovery of A2AR in bEnd.3 cells with regadenoson and another A2AR selective agonist, UK432097 (36). We demonstrated that regadenoson and UK432097 induced calcium influx, confirmed with both a positive control, ionomycin, and a negative control, cyclopiazonic acid (CPA; Fig. 4A). A2AR was desensitized after short incubation of 2.5 minutes with

Figure 3.
Regadenoson transiently disrupt mouse brain endothelial cytoskeleton. **A,** Representative immunocytochemistry images of F-actin cytoskeleton filaments in bEnd.3 cells treated with vehicle or regadenoson for 0.5, 4, or 8 hours. Insets are enlarged representation of cytoskeletal morphology. **B,** Quantification of F-actin. Values are presented as normalized fluorescence intensity of 3–5 fields per sample of three independent experiments. **P < 0.01 by Student’s t test.** **C,** Representative immunocytochemistry images of F-actin cytoskeleton filaments in RBMECs treated with vehicle or regadenoson for 0.5, 4, or 8 hours. Insets are enlarged representation of cytoskeletal morphology. **D,** Quantification of F-actin. Values are presented as normalized fluorescence intensity of 3–5 fields per sample of three independent experiments. All regadenoson treatment was at 10 μmol/L.
regadenoson, as seen when bEnd.3 cells were unable to respond to UK432097 (Fig. 4B, short term — C). These findings correlate with the initial half-life of regadenoson that ranges between 2 to 4 minutes (37, 38). A2AR demonstrated cellular recovery by 4 hours after regadenoson administration, when stimulation with UK432097 increased calcium influx. (Fig. 4B, long term — C). After 4 hours, A2AR activation inducing calcium influx was still not as strong as initial activation but was significantly stronger than with only 2.5 minutes of exposure to regadenoson (Fig. 4B and C). Binding of a ligand to A2AR activates small protein Galpha, which is followed by phosphorylation of the receptor and subsequent internalization via recruitment of β-arrestin to the cell membrane (39). Hence, we observed significant differences in subcellular localization of β-arrestin, increasing at the membrane and decreasing in the cytosol after 4 hours of regadenoson, compared with loading controls, calnexin and GAPDH, respectively (Fig. 4D and E). β-arrestin is typically recruited to the membrane and is known to facilitate A2AR internalization and recycling through clathrin-coated pits (39). In bEnd.3 cells, β-arrestin levels returned to baseline both in the membrane and cytosol by 8 hours, supporting recovery of A2AR with an overall transient change in the membrane-cytosol ratio (Fig. 4D and E). Agonism of A2AR can modulate
MAPK/ERK pathway to impact tight junctions (40, 41). Therefore, we have measured decreased phosphorylation of ERK and upstream effector Raf-1 after 30 minutes of regadenoson treatment in bEnd.3 cells and RBMECs (Fig. 4F and G). These results suggest that the timing of A2AR turnover after regadenoson activation and availability is important for downstream modulation of intracellular signaling, potentially leading to cellular integrity changes at the BBB/BTB. These findings further support that morphologic changes induced by regadenoson occur through the MAPK–ERK pathway.

Regadenoson-mediated endothelial disruption occurs through an A2AR-dependent activation

Previous studies in mice have demonstrated the ability of regadenoson to serve as an agonist to both the inhibitory A1 and stimulatory A2A adenosine receptors (12). Studies show regadenoson binds specifically to A2AR in rats (13). Thus, we eliminated the possibility that regadenoson may agonize A1R in rats. To evaluate and confirm the selective effect of regadenoson on A1R and A2AR in mouse brain endothelium, we transiently transfected bEnd.3 cells with specific small interfering RNA (siRNA) sequences to knockdown Adora1, Adora2a, or both genes. We observed significant decreased Adora2a expression when siAdora2a was transfected alone or in combination with siAdora1 (Fig. 5A). We then analyzed the downstream impact of A2AR agonism by regadenoson, on brain endothelial cell integrity, by knocking down Adora1 and/or Adora2a. After 30 minutes of regadenoson exposure, we observed disorganization of F-actin cytoskeletal filaments when A1R was depleted, whereas we saw no change with depletion of either A2AR alone or both A1R and A2AR (Fig. 5B and C). In addition, after 4 hours of regadenoson exposure, we observed disrupted cell–cell

Figure 5.
Regadenoson mediated endothelial disruption occurs through an A2AR-dependent activation. A, Validation and efficacy of Adora1 and Adora2a gene knockdown. Normalized gene expression over GAPDH. Data are represented as mean ± SEM of five experiments. † † † † , P < 0.0001; † † † † † † , P < 0.0001 by Student t test. B, Representative immunocytochemistry images of F-actin filaments staining in bEnd.3 cells knocked down for Adora1 and/or Adora2a and treated with vehicle or regadenoson for 30 minutes. Insets are enlarged representation of cytoskeletal morphology. C, Quantification of F-actin. Values are presented as normalized fluorescence intensity of five fields per sample of three independent experiments. † † † † † † † , P < 0.0001; † † † † † † † † † † , P < 0.0001 by Student t test. D, Representative immunocytochemistry images of tight junction protein ZO-1 in bEnd.3 cells knocked down for Adora1 and/or Adora2a and treated with vehicle or regadenoson for 4 hours. Zoomed insets are the morphologic aspect of cell junctions and membrane organization. E, Representative immunocytochemistry images of VE-cadherin in bEnd.3 cells knocked down for Adora1 and/or Adora2a and treated with vehicle or regadenoson for 4 hours. Zoomed insets is the morphologic aspect of cell junctions and membrane organization. All regadenoson treatment was at 10 μmol/L.
Figure 6.
Regadenoson increases intratumoral temozolomide (TMZ) concentrations in glioma-bearing rats but not mice. A, Relative expression of adenosine receptors in freshly isolated mouse brain endothelial cells. Data are represented as mean ± SEM. N = 4. B, Representative IHC images of Claudin-5 and Pecam1 in non-tumor-bearing mice treated with vehicle or regadenoson for 30 minutes. C, Quantification of relative fluorescence intensity of the area occupied by Claudin-5 over Pecam1. Data are represented as mean ± SEM; n = 4 mice/group. D, Brain:plasma and tumor:plasma temozolomide concentrations, given alone or 30 minutes prior to regadenoson in SCID mice injected with GSC lines (GSC923 or GSC827). Data are represented as mean ± SEM; n = 3–8 mice/group. E, Brain:plasma and tumor:plasma ratios of temozolomide concentrations, given alone or 1 hour prior to regadenoson in rats with GSC827 intracerebral xenografts. Data are represented as mean (SD); n = 5 rats/group. F, Representative IHC images of Claudin-5 and Pecam1 in normal contralateral brain and GSC827 tumor tissue of rat treated with temozolomide alone or temozolomide with regadenoson. G, Quantification of relative fluorescence intensity of the area occupied by Claudin-5 over Pecam1. Data are represented as mean ± SD; n = 3–5 rats/group. H, Kaplan-Meier survival curves for treatment groups (control, temozolomide alone, temozolomide + regadenoson). Mice were given regadenoson 0.05 mg/kg (tail vein injection) and temozolomide 10 mg/kg (oral administration); rats were given regadenoson 0.0005 mg/kg (tail vein injection), temozolomide 50 mg/kg (oral administration) for pharmacokinetic analysis and temozolomide 10 mg/kg for survival.
Regadenoson increases intratumoral temozolomide concentrations in gliomas-bearing rats

To further investigate the impact of regadenoson on the BBB, we first evaluated the integrity of junctional protein expression in non-tumor-bearing mouse brain. First, we observed that freshly isolated mouse brain endothelial cells express high levels of A2aR (Fig. 6A). In non-tumor-bearing mice, we observed a trend of decreased claudin-5 expression after regadenoson treatment, although not found to be statistically significant (Fig. 6B and C). To assess BBB permeability, we measured temozolomide concentrations in gliomas-bearing rodent models, comparing drug concentrations within GSCs-injected hemisphere to the normal contralateral brain hemisphere. We observed no significant difference in temozolomide plasma concentration, or the ratios of brain:plasma, and tumor:plasma concentrations in mice intracranially injected with human derived glioma stem cells (GSC923 or GSC827; Fig. 6D). On the basis of previous studies demonstrating a selectivity variation comparing mice with rats, we aimed to evaluate the effect of regadenoson on tumor-bearing rats (12). While we observed no difference in brain:plasma temozolomide concentrations with regadenoson cotreatment in rats intracranially injected with GSC827 tumor cells, we found a statistically significant 25.1% increase in tumor:plasma temozolomide concentrations with regadenoson cotreatment compared with TMZ alone (Fig. 6E). In addition, we found that brain:plasma concentrations in tumor-bearing rats were slightly lower (~0.6-fold) when compared with historical control studies evaluating temozolomide with and without regadenoson (18). This difference could be attributed to the presence of tumor cells, sampling time for temozolomide and/or animal morbidity due to tumor cell invasion, which could impact drug delivery. Increased BTB permeability was in part attributed to decreased expression levels of claudin-5 in tumor of rats cotreated with regadenoson compared with temozolomide alone and to normal contralateral brain with and without regadenoson, normalized to Pecam1, which was not different between the samples (Fig. 6F and G). Survival increase was observed in both treatment groups compared with control (Fig. 6H). However, concomitant treatment with Regadenoson and temozolomide did not provide survival benefit compared with temozolomide alone (Fig. 6H). Tumors in both glioma model results suggest a role for A2aR activation to selectively impact rat BTB and disrupt endothelial junctions resulting in increased intratumoral chemotherapy concentrations.

Discussion

In this study, we characterized the ability of regadenoson to transiently modulate mouse and rat brain endothelial cell morphology and render the BTB more permeable to the standard GBM chemotherapy agent, temozolomide. First, these findings allowed us to define the molecular and cellular alterations occurring over a short time course following activation of A2aR by regadenoson in vitro. These steps include that (i) regadenoson rapidly activates A2aR downstream signaling resulting in (ii) reorganized cytoskeletal morphology starting to occur after 30 minutes, accompanied by (iii) downregulation of specific tight and adherens junction proteins optimally occurring after between 30 minutes and 4 hours with suggested increased permeability of therapy agents, and that (iv) all cellular morphology returns to baseline after 8 hours (Fig. 7A).

Activation of GPCRs like A2aR have been directly associated with disruption of α-actinin and F-actin interactions, impacting junctional function (32, 42, 43). Because endothelial junctional integrity is essential to the impermeability and maintenance of the BBB, understanding the timing and mechanism of how to increase BTB permeability is critical for optimizing drug delivery for aggressive brain tumors (33, 44–46). The short half-life of regadenoson and its strong A2aR selectivity allow for rapid intracellular calcium influx, which is an important signal for relevant downstream cytoskeleton reorganization. The transient aspect of using this treatment method represents an important feature of BTB normalization after drug delivery, facilitating a brief increase in CNS permeability while potentially avoiding supplemental prolonged neurotoxicity such as headaches, acute hemorrhage, stroke, or further neurologic decline.

Second, we established that the impact of regadenoson on cytoskeleton and junctional cell–cell interaction in mouse brain endothelial cells specifically occurs through activation of A2aR and not A1R, ruling out the hypothesis that its binding to A1R could counterbalance its binding to A2aR in the endothelium. These findings compliment previous studies suggesting that activation of either receptor leads to the opening of the BBB (17). Binding of regadenoson to both receptors suggests counteractive effects, as A1R activates Gαs leading to decreased cAMP levels and vasoconstriction, whereas A2aR activates Gαi leading to increased cAMP levels and vasodilation (32). Furthermore, we showed very low expression of A1R and high expression of A2aR in mouse brain endothelial cells, suggesting that the effects of regadenoson on mouse brain endothelial cell–cell interactions and cell integrity depends exclusively on A2aR. These findings were supported by Adora1 and Adora2a knockdown studies where, in mouse cells, regadenoson was unable to impact cell-cell interaction when Adora2a was knocked down but not Adora1. Also, selective antagonism of A2aR blocked subsequent regadenoson treatment from disrupting junctional and cytoskeletal organization.

Third, we demonstrated that regadenoson enhanced temozolomide concentrations in glioma rodent models. Specifically, concomitant treatment of temozolomide and regadenoson led to decreased protein expression of claudin-5 specifically within the BTB of rats (Fig. 7B). However, regadenoson failed to increase CNS drug delivery in two mouse glioma models. In determining the reason for interspecies variabilities, we propose (i) higher binding selectivity of regadenoson to A1R in mice could potentially induce a counter effect coming from other cell types, like astrocytes and/or oligodendrocytes and (ii) the presence of glioma stem cell invasion altering vascular expression and binding of A2aR. Specifically, astrocytes express much higher levels of A1R than endothelial cells suggesting that binding of regadenoson on A1R in astrocytes has the potential to enhance the counteractive effect of vessel constriction, leading to failure of BBB disruption (10, 11). This would be specific to the mouse model since regadenoson is solely selective for A2aR in rats explaining the differential results (12). In addition, a previous study showed evidence of high expression of A2aR in human high-grade glioma cells (47). This could potentially influence regadenoson binding on BBB/BBT endothelial cells, depending on the glioma type, grade, and receptor expression levels. While we observe increased temozolomide concentration in the tumor of...
Figure 7.
Transient disruption of the BTB with regadenoson leads to increase in permeability to temozolomide (TMZ) in gliomas-bearing rats. A, Schematic timeline representation of in vitro A2AR activation on mouse endothelial cells by regadenoson leading to transient junctional disruption. 0 hour: binding of regadenoson to A2AR activates G<sub>as</sub> and subsequently results in calcium influx. 0.5 hour: desensitization of A2AR and cytoskeleton disorganization occurs after 0.5 hours. 4 hours: increase of β-arrestin levels at the membrane suggests internalization of A2AR, along with disruption of cell–cell junctional interaction. 8 hours: Cytoskeleton organization and junctional interaction reestablishes after 8 hours. B, Left, poorly permeable BTB characterized by normal levels of claudin-5 and normal entry of temozolomide to the tumor. Right, enhanced permeability amongst the BTB with regadenoson administration, characterized by decreased claudin-5 expression, represented by increased temozolomide within tumor tissue.

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gliomas-bearing rats, we saw no survival benefit. We may attribute this to multiple factors: (i) by using 10 mg/kg temozolomide treatment dose for survival studies versus 50 mg/kg temozolomide dose for brain: plasma concentration studies; it is unclear if a higher temozolomide dosing would have positively affected survival and/or induced higher toxicity resulting in shorter model survival; (ii) it is also unknown if animals develop resistance over time against regadenoson, because models survived long after drug administration; (iii) regadenoson enabled higher temozolomide entry at certain time points, but may have also facilitated higher temozolomide exit over time. Thus, continued studies are warranted to repeat regadenoson combination dosing with other impermeable agents, explore the potential effect with higher temozolomide dosing, evaluate additional human derived GSC-injected models and repeat pharmacokinetic studies linked with the expression/function of multidrug resistance proteins to inhibit viable agents from reaching malignant CNS tumor tissue.

This study has some limitations to consider. We predominantly focused on mouse brain endothelial cell biology using a mouse brain endothelioma cell line in vitro, as a model of BTB integrity because of its previously published easy translatability to preclinical studies (23, 48, 49). However, the BTB is more complex in vivo and remains a serious challenge for therapy of malignant glioma (1, 50–52). Furthermore, the BTB is also comprised of astrocytes and pericytes, which exert characteristics that contribute to maintenance, integrity, and impermeability of the barrier (53–55). Lack of pericyte and astrocyte coverage in the BTB alters its function, mainly at the center of GBM tumors (51). Usually, the BTB remains intact in the periphery of the tumor while being leaky to absent and nonfunctional in the tumor core. Thus, future studies are warranted to evaluate how regadenoson impacts astrocyte-pericyte–endothelial communication and BBB integrity in non–tumor-bearing animals as well as in GBM rodent models. Moreover, while previous studies have determined that temozolomide has approximately 20% brain penetration in patients, our findings showing an increase in tumor:plasma concentrations warrant evaluation of BTB impermeable agents (e.g., cisplatin, doxorubicin) with regadenoson to impair GBM tumor growth and invasion (56). In addition, because the use of a single dose of regadenoson has proven efficient to increase intratumoral concentrations temozolomide in only 40% of patients, further consideration on optimization of the treatment schedule, dosing, and means of delivery is warranted (21, 57). Specifically, it has been shown that repeat A2AR activation in a short-time window increased brain delivery of the large molecule 10 kDa-dextran in normal brain of mice and rats (17).

Another study demonstrated that regadenoson-loaded nanoparticles were efficient to open the BBB in a stroke mouse model (58). Further dosing and timing studies were performed to demonstrate the usefulness of regadenoson for CNS delivery of voltage-sensitive dye for brain neuronal activity monitoring (58, 59). In addition, a currently enrolling clinical trial is aimed at determining the optimal regadenoson dose to transiently disrupt the BBB in patients with high-grade glioma and allow for enhanced penetration of gadolinium during brain MRI (NCT03971734). MRIs with gadolinium are routinely used to measure size of space-occupying lesions, such as brain tumors, with BBB compromise. This type of imaging helps neuro-oncology teams tailor the best therapy and surgery options for each patient. Therefore, this study will inform on optimal timing of concomitant therapy with a vasoactive mediator for efficient chemotherapy delivery to tumor tissue. Detailing gadolinium permeability characteristics (Ktrans) will offer better understanding of drug entry and exit times with regadenoson. Combining the outcomes of this current clinical trial with our in vivo findings will generate additional studies that focus on the use of vasoactive mediators and/or GPCRs aimed at transiently disrupting endothelial cell integrity to improve overall treatment responses and impact disease survival.

Collectively, our data demonstrate that A2AR activation can modulate brain endothelial cell–cell interactions to influence CNS drug entry, specifically in tumor areas. We established both the molecular and cellular mechanisms involved in endothelial permeability after A2AR activation by regadenoson, leading to enhanced BTB permeability in glioma-bearing rats. Altogether, we provide a model that can be useful in studying the timing and impact of other propermeability agents on brain endothelium, as they relate to CNS drug delivery.

Authors’ Disclosures

No disclosures were reported.

Authors’ Contributions


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References


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