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RESEARCH PAPER

Small-molecule inhibition of prostaglandin E receptor 2 impairs cyclooxygenase-associated malignant glioma growth

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Background and Purpose: An up-regulation of COX-2 in malignant gliomas causes excessive synthesis of PGE_2, which is thought to facilitate brain tumour growth and invasion. However, which downstream PGE_2 receptor subtype (i.e., EP_1–EP_4) directly contributes to COX activity-promoted glioma growth remains largely unknown.

Experimental Approach: Using a publicly available database from The Cancer Genome Atlas research network, we compared the expression of PGE_2 signalling-associated genes in human lower grade glioma and glioblastoma multiforme (GBM) samples. The Kaplan–Meier analysis was performed to determine the relationship between their expression and survival probability. A time-resolved FRET method was used to identify the EP subtype that mediates COX-2/PGE_2-initiated cAMP signalling in human GBM cells. Taking advantage of a recently identified novel selective bioavailable brain-permeable small-molecule antagonist, we studied the effect of pharmacological inhibition of the EP_2 receptor on glioma cell growth in vitro and in vivo.

Key Results: The EP_2 receptor is a key $G\alpha_s$-coupled receptor that mediates COX-2/PGE_2-initiated cAMP signalling pathways in human malignant glioma cells. Inhibition of EP_2 receptors reduced COX-2 activity-driven GBM cell proliferation, invasion, and migration and caused cell cycle arrest at G0–G1 and apoptosis of GBM cells. Glioma cell growth in vivo was also substantially decreased by post-treatment with an EP_2 antagonist in both subcutaneous and intracranial tumour models.

Abbreviations: CD31, cluster of differentiation 31; EP receptor, PGE_2 receptor; GBM, glioblastoma multiforme; LGG, lower grade glioma; PGES, PGE synthase; TCGA, The Cancer Genome Atlas

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Conclusion and Implications: Taken together, our results suggest that PGE_2 signalling via the EP_2 receptor increases the malignant potential of human glioma cells and might represent a novel therapeutic target for GBM.

1 | INTRODUCTION

Gliomas constitute approximately 80% of all primary malignant brain tumours in humans, and 82% of these cases are classified as the World Health Organization Grade IV tumour—glioblastoma multiforme (GBM; Omuro & DeAngelis, 2013). The current standard treatment for GBM is exclusively limited to surgical resection, followed by radiotherapy and chemotherapy with temozolomide (Stupp et al., 2005). However, even with these combined therapies, the prognosis of GMB remains poor with a median overall survival of just under 15 months, and less than 10% of patients survive over 5 years (Alexander & Cloughesy, 2017; Omuro & DeAngelis, 2013; Stupp et al., 2009). Among comprehensive factors rendering GBM particularly difficult to treat is that most anti-tumour agents including immunotherapeutic drugs cannot reach the tumour sites due to insufficient brain penetration (Alexander & Cloughesy, 2017; Beduneau, Saulnier, & Benoit, 2007; Mellinghoff & Gilbertson, 2017; Omuro & DeAngelis, 2013). Developing new therapeutics with adequate efficacy for this most lethal and devastating brain condition is an urgent unmet need (Alexander & Cloughesy, 2017; Mellinghoff & Gilbertson, 2017).

Although the molecular mechanisms underlying glioma growth remain largely unclear, mounting evidence over the past decade suggests that inflammation within the brain, or neuroinflammation, contributes to many forms of brain cancer (Sowers, Johnson, Conrad, Patterson, & Sowers, 2014). As a chief pro-inflammatory mediator, **COX-2** is often up-regulated in intracranial tumours (Joki et al., 2000; Patti et al., 2002) and has been shown to promote the growth, migration, angiogenesis, and immune evasion of malignant gliomas (Qiu, Shi, & Jiang, 2017; Xu, Wang, & Shu, 2014). However, COX inhibition for glioma treatment by non-steroidal anti-inflammatory drugs or selective COX-2 inhibitors (Coxibs) has been discouraged by their well-documented toxicity to the cardiovascular and cerebrovascular systems (Grosser, Yu, & Fitzgerald, 2010) and by the results of several recent population studies and clinical trials, which lack consistency (Qiu et al., 2017). The untoward consequences of COX-2 inhibition inspired us to postulate that targeting the downstream prostanoid receptors might offer more therapeutic specificity than simply shutting down the entire COX cascade (Qiu et al., 2017). As a major enzymatic product of COX-2 within the brain, **PGE_2** directly mediates inflammatory processes during the pathogenesis of tumours and other chronic conditions and facilitates the disease progression presumably via acting on four GPCRs—PGE_2 receptors **EP_1**, **EP_2**, **EP_3**, and **EP_4** (Jiang, Qiu, Li, & Shi, 2017; Wang & Dubois, 2010). In the present study we have used our recently developed novel selective brain-permeable small-molecule antagonists to identify the EP receptor type that is involved in the malignant glioma growth as a novel target, and also determined the therapeutic effects of these antagonists in multiple malignant glioma models.

What is already known

- COX-2 is often elevated in human gliomas and facilitates gliomagenesis.
- PGE_2 is a key effector that mediates COX activity-promoted glioma growth.

What this study adds

- The EP_2 receptor is a leading G_s-coupled receptor that mediates PGE_2-initiated cAMP signalling in human malignant gliomas.
- Activation of EP_2 receptor contributes to COX activity-driven glioma cell proliferation, invasion, and migration.
- EP_2 receptor inhibition decreases the glioma growth in both subcutaneous and intracranial tumour models.

What is the clinical significance

- PGE_2 signaling via EP_2 receptors increases the malignant potential of human glioma cells.
- Pharmacological inhibition of EP_2 receptors represents an emerging strategy to treat malignant gliomas.

2 | METHODS

2.1 | Cell culture

Human glioma cell lines—LN229 (ATCC Cat # CRL-2611, RRID: CVCL_0393) and SF767 (RRID:CVCL_6950)—and the COX-2-overexpressing cells derived from these lines were generously provided by Dr. Kaiming Xu and Dr. Hui-Kuo G. Shu from the Winship Cancer Institute of Emory University and authenticated by genetic profiling using polymorphic short tandem repeat loci (ATCC, 2011; Xu et al., 2014). The cells were cultured in DMEM (Gibco, Waltham, MA, USA) supplemented with 10% (v:v) FBS (HyClone, Pittsburgh, PA, USA) and penicillin (100 $U \cdot ml^{-1}$)/streptomycin (100 $\mu g \cdot ml^{-1}$; Gibco) in a humidified incubator at 37°C with 5% CO_2.

2.2 | PGE_2-pathway gene expression in gliomas

Gene expression data for the PGE_2 signalling pathway-associated enzymes and receptors including two COXs, three **PGE synthases (PGESs)**, and four EP receptors were obtained using a publicly

available database from The Cancer Genome Atlas (TCGA) research network derived from the UCSC Xena Browser (http://xenabrowser.net) on October 1, 2018. The database has information of 530 lower grade glioma (LGG) patients and 631 GBM patients. The pancan-normalized RNAseq data that allow comparison of gene expression across multiple TCGA cancer types (Goldman et al., 2015) are available for all 530 LGG and 172 GBM samples.

2.3 | Kaplan–Meier analysis of survival probability with gene expression

RNAseq and clinical data were acquired using TCGA combined LGG/GBM dataset (n = 525 for LGG and 165 for GBM) downloaded from the UCSC Xena Browser. Survival analyses were performed with Kaplan–Meier estimator and post hoc log-rank test using OriginPro software (OriginLab, RRID:SCR_014212).

2.4 | Multiple correlation analysis of gene expression

For correlation analysis of multiple gene expression in human glioma samples, RNAseq data of TCGA combined LGG/GBM dataset (n = 702) were downloaded from the UCSC Xena Browser and analysed using Pearson correlation coefficient.

2.5 | Cell-based cAMP assay

Cytosol **cAMP** was measured using a cell-based homogeneous time-resolved FRET (TR-FRET) method (Cisbio Bioassays, Codolet, France; Jiang et al., 2010; Jiang, Van, Ganesh, & Dingledine, 2018). The assay is based on the generation of a strong FRET signal upon the interaction of two fluorescent molecules: FRET donor cryptate coupled to anti-cAMP antibody and FRET acceptor d2 coupled to cAMP. Endogenous cAMP produced by cells competes with labelled cAMP for binding to the cAMP antibody and thus reduces the FRET signal. Human GBM cells were seeded into 384-well plates with 40 µl of complete medium (4,000 cells per well) and grown overnight. The medium was completely withdrawn and 10 µl of Hanks' Balanced Salt solution (HBSS; HyClone) supplemented with 20 µM **rolipram** was added into the wells to block PDEs that metabolize cAMP. The cells were incubated at room temperature for 30 min and then treated with vehicle or compound for 5–10 min before incubation with PGE_2 for 40 min. The cells were lysed in 10 µl lysis buffer containing the FRET acceptor cAMP-d2, and 1 min later, another 10 µl lysis buffer with anti-cAMP-cryptate was added (Cisbio Bioassays, Cat # 62AM4PEC). After incubation for 1 hr at room temperature, the FRET signal was measured by a 2103 Envision Multilabel Plate Reader (PerkinElmer) with an excitation at 340/25 nm and dual emissions at 665 and 590 nm for d2 and cryptate (100-µs delay) respectively. The FRET signal was expressed as F665/F590 × 10^4.

2.6 | Radioligand binding assay

The EP_2 receptor binding assay for TG6-10-1 was performed as previously described for TG4-155, allowing comparison for binding affinity (Jiang et al., 2012). In brief, cell membrane homogenates (20 µg of protein) were incubated with 3 nM **[^{3}H]-PGE_2** in the absence or presence of compound TG6-10-1 in a buffer containing 10 mM MES/KOH (pH 6.0), 10 mM $MgCl_2$, and 1 mM EDTA, at room temperature for 2 hr. Nonspecific binding was determined in the presence of 10 µM PGE_2. Following incubation, the samples were filtered rapidly under vacuum through glass fibre filters (GF/B, Packard) presoaked with 0.3% polyethyleneimine and rinsed several times with ice-cold 50-mM Tris–HCl using a 96-sample cell harvester (UniFilter, Packard). The filters were dried then counted for radioactivity in a scintillation counter (TopCount, Packard) using a scintillation cocktail (MicroScint 0, Packard). The results are expressed as % of remaining radioligand. A dose–response curve was generated, and IC_{50} and K_i values were calculated with OriginPro.

2.7 | Pharmacokinetics

Pharmacokinetic (PK) study was performed at SRI International as previously described (Jiang et al., 2013). To minimize the experimental variation that might be introduced by using animals from different strains, genders, or ages, female C57BL/6 mice (5–9 weeks) from Charles River (Wilmington, MA, USA) (Strain Code # 027) were used. All animals were housed under a 12-hr light/dark cycle with food and water ad libitum. At 0.5, 1, 2, 4, and 8 hr after administration of TG6-10-1 (10 mg·kg^{-1}, p.o.), blood samples (300 µl) were collected from the retro-orbital sinus or by cardiac puncture once per animal; the animals were anaesthetized with isoflurane for these procedures and the depth of anaesthesia was typically examined by toe pinch. The brain samples were collected at 1 and 2 hr after TG6-10-1 administration; animals were killed with an overdose of sodium pentobarbital. Plasma and brain homogenates were extracted and analysed for compound concentrations.

2.8 | Western blot analysis

Cells were homogenized on ice in RIPA buffer (25 mM Tris·HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a mixture of protease and phosphatase inhibitors (Roche Applied Science, Penzberg, Germany). The homogenates were centrifuged (12,000× g, 15 min, 4°C), and protein concentration in the supernate was measured by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein samples were resolved by 4–15% SDS-PAGE and electroblotted onto PVDF membranes (Millipore, Burlington, MA, USA). Membranes were blocked with 5% non-fat milk at room temperature for 2 hr then incubated overnight at 4°C with primary antibodies: rabbit anti-COX-2 (1:1,000, Abcam, Cat # ab15191) or mouse anti-GAPDH (1:1,000, Calbiochem, Cat # CB1001). This procedure was followed by incubation with HRP-conjugated secondary

antibodies (1:3,000; Santa Cruz Biotechnology, goat anti-rabbit IgG-HRP secondary antibody Cat # sc-2004, goat anti-mouse IgG-HRP secondary antibody Cat # sc-2005) at room temperature for 2 hr. The blots were developed by enhanced chemiluminescence (Pierce) and scanned (Jiang et al., 2015; Jiang et al., 2019).

2.9 | PGE_2 measurement

The PGE_2 levels in culture medium were measured by ELISA (Arbor Assays, Cat # K051). After each treatment of the cells, 50-μl culture medium was taken for PGE_2 measurement according to the manufacturer's protocol. The OD generated from each well was measured in a microplate reader (Molecular Devices) at 450 nm. A standard curve for PGE_2 was run with each experiment.

2.10 | Cell proliferation assay

Human GBM cells were seeded in six-well plates (1×10^4 cells per well) and treated with TG6-10-1 (10 μM) or **celecoxib** (10 μM) for 96 hr. The cells were then stained with 0.1% crystal violet for 1 min and counted under a microscope in five random fields per well at a magnification of 200×.

2.11 | Cell invasion assay

Cells were seeded into culture inserts with pore size of 8 μm that were pre-coated with 150-μl Matrigel (1/2.5 dilution in serum free DMEM, Corning). After 48 hr, cells that moved across the insert membrane were stained with HEMA 3 stain, set and counted under a microscope in five random fields per well at a magnification of 200×, as previously described (Jiang & Dingledine, 2013b).

2.12 | Wound healing assay

Human GBM cells were grown to confluence in 12-well plates, and a uniform scratch was created using a sterile pipette tip (~1 mm in diameter). After being washed to remove the debris, cells were cultured in medium with reduced FBS (1%). The scratch area was monitored by microscopy for 48 hr.

2.13 | Analysis of cell cycle progression

Cell cycle analysis was performed by flow cytometry as described previously (Qiu et al., 2015). In brief, human GBM cells were seeded in six-well plates (3×10^5 cells per well) and treated with TG6-10-1 (10 μM) or celecoxib (10 μM) for 72 hr. The cells were then harvested and fixed with ice-cold 70% ethanol for 30 min and stained with propidium iodide (50 $\mu g \cdot ml^{-1}$) and DNase-free RNase (100 $\mu g \cdot ml^{-1}$) for 15 min in the dark. Cell cycle analysis was performed in a BD FACSCalibur Cell Analyzer (BD Biosciences, RRDI:SCR_000401). Fluorescence intensity was measured using the excitation wavelength of 488 nm and FL2 detector with 585-nm filter. Data were analysed using ModFit LT software (ModFit LT, RRID:SCR_016106).

2.14 | Apoptosis assay

Human GBM cells were seeded in six-well plates (3×10^5 cells per well) and treated with TG6-10-1 (10 μM) or celecoxib (10 μM) for 72 hr. The cells were harvested and stained with annexin V-FITC and propidium iodide for 15 min in the dark. Flow cytometry acquisition with BD FACSCalibur Cell Analyzer was used to measure fluorescence intensity in FITC (FL1, 533 nm) and propidium iodide (FL2, 585 nm) channels. The early apoptotic cells (annexin V-positive only) and late apoptotic cells (annexin V- and propidium iodide-positive) were quantified and analysed with the FlowJo software (FlowJo, RRID:SCR_008520).

2.15 | Animals for tumour models

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*. Athymic nude mice (4–5 weeks, female) from Envigo (Hsd:Athymic Nude-*Foxn1*^{nu}) were used in this study to avoid any possible complexities caused by comparing results across strains/genders/ages. Mice were housed in ventilated shoebox cages (13–1/4″ long × 7–1/8″ wide × 5–7/16″ deep; ≤ 4 mice per cage) in a controlled environment at 22–24°C and humidity 60–62%, under a 12-hr light/dark cycle with food and water ad libitum. Soft bedding and environmental enrichment in the cages were provided to help to reduce stress and anxiety in the animals and to improve their welfare. Every effort was made to minimize animal suffering. For all survival surgeries including the s.c. tumour model, intracranial tumour model, and bioluminescence imaging, mice were under general anaesthesia induced by isoflurane (2–5%) through an EZ-150C vaporizer (E-Z Systems). All animal procedures followed the institutional and IACUC guidelines of the University of Cincinnati and the University of Tennessee Health Science Center.

2.16 | Subcutaneous tumour model

To create s.c. tumours, mice were injected with human GBM cells in 200 μl of HBSS (~3×10^6 cells/tumour, s.c.) while the animals were anaesthetized with isoflurane to enable an accurate site of injection. After solid tumours appeared (0.5 cm × 0.5 cm), mice were then randomized and continuously treated with either vehicle (10% PEG 200, 0.5% methylcellulose) or TG6-10-1 (10 $mg \cdot kg^{-1}$, b.i.d.) by oral gavage for 4 weeks. Tumour growth was monitored by measuring tumour volume daily using the formula: $V = (\pi/6) \times [(A + B)/2]^3$ (A = longest diameter; B = shortest diameter). Four weeks after injection, mice were killed under isoflurane anaesthesia, and tumours were immediately dissected out for further analyses. It should be noted that mice did not show any locomotion, lethargy, or any other behavioural abnormality before they were killed for tumour collection.

2.17 | Immunohistochemistry

The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*. Subcutaneous tumours were harvested, fixed, paraffin embedded, and sectioned (8 μm). The tumour sections were permeabilized with 0.25% Triton X-100 at room temperature for 15 min, then blocked with 10% goat serum in PBS, followed by incubation in primary antibodies at 4°C overnight: rabbit anti-COX-2 (1:500, Abcam, Cat # ab15191); rabbit anti-KI-67 (1:200, Biocare Medical, Cat # CRM 325B); and mouse anti-cluster of differentiation 31 (CD31) FITC (1:200, eBioscience, Cat # 11-0311-85). The sections were washed with PBS and incubated with goat anti-rabbit secondary antibody Alexa Fluor 488 (Invitrogen, Cat # A-11034) at room temperature for 2 hr and DAPI (10 $\mu g \cdot ml^{-1}$ in PBS) for 10 min. Slides were mounted using DPX Mountant (Electron Microscopy Sciences). Images were obtained using EVOS FL Auto Cell Imaging System (Invitrogen). The fluorescence intensity was quantified using ImageJ software developed at the National Institutes of Health (NIH, RRID:SCR_003070).

2.18 | Intracranial tumour model

Mice were anaesthetized with isoflurane through a vaporizer and placed in a stereotaxic frame. Human glioma cells labelled with luciferase (5×10^5 in 3 μl or 8×10^5 in 5 μl of HBSS, as indicated) were inoculated into striatum of the right hemisphere (coordinates from bregma: AP = 1.0 mm; ML = +2.0 mm [right], DV = −3.0 mm). After the surgery, mice were treated with **buprenorphine** (0.05 $mg \cdot kg^{-1}$, s.c.) twice daily for pain control for three consecutive days. After recovery from the surgery, mice were randomized and continuously treated with either vehicle (0.5% methylcellulose, 10% PEG 200) or TG6-10-1 (10 $mg \cdot kg^{-1}$, p.o., b.i.d.) for 4 weeks. Bioluminescence imaging was performed once a week for 4 weeks by a blinded investigator to monitor tumour growth in vivo using an IVIS imaging system (Xenogen). Mice were anaesthetized with isoflurane, administered through a vaporizer, and injected with luciferin substrate (150 $mg \cdot kg^{-1}$, i.p.), followed by the bioluminescence imaging 5–10 min later. The criteria for humane endpoint used in this study included: (a) over 20% body weight loss; (b) impaired locomotion; and (c) neurological symptoms such as ataxia, lethargy, paresis, and paralysis. Animals were killed, by isoflurane inhalation immediately followed by decapitation, when any of these symptoms was observed, and these mice were included in the survival study. Mouse brains harbouring tumours were harvested, fixed, sectioned (8 μm), and visualized by haematoxylin and eosin staining.

2.19 | Experimental blinding and randomization

Experimental blinding was performed to reduce the risk of bias in this study whenever possible; the drugs used for treating animals were prepared by people who did not carry out the treatments. In addition, all animals were randomized before they were treated. Results from at least seven different animals were acquired for all experimental protocols and data analyses in this study.

2.20 | Statistical analysis

The data and statistical analyses comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2015; Curtis et al., 2018). The PGE_2 dose–response curves were generated, and EC_{50}s were calculated using OriginPro software (OriginLab, RRID:SCR_014212). Datasets that have passed the Kolmogorov–Smirnov normality test for Gaussian or log-Gaussian distribution were subject to the appropriate statistical tests. Statistical analyses were performed using Prism (GraphPad Software, RRID:SCR_002798) by one-way/two-way ANOVA with post hoc Bonferroni/Dunnett's test or Student's *t*-test as indicated. Survival analyses were performed using Kaplan–Meier estimator with post hoc log-rank test. $P < 0.05$ was considered statistically significant. Data are presented as mean ±SEM. No statistical analysis was undertaken if a dataset had a group size (n) < 5.

2.21 | Chemicals and drugs

PGE_2 (CAS # 363-24-6), celecoxib (CAS # 169590-42-5), and **TG4-155** (CAS # 1164462-05-8) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Rolipram (CAS # 61413-54-5), forskolin (CAS # 66575-29-9), and luciferin (CAS# 103404-75-7) were from Sigma-Aldrich (St. Louis, MO, USA). TG6-10-1 (CAS # 1415716-58-3) was kindly provided by Thota Ganesh or obtained from MedChem Express (Monmouth Junction, NJ, USA) (Ganesh, Jiang, & Dingledine, 2014; Ganesh, Jiang, Yang, & Dingledine, 2014; Jiang et al., 2019). Compounds from different sources and batches were compared for consistency in potency and selectivity.

2.22 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY2017/18 (Alexander, Christopoulos, et al., 2017; Alexander, Fabbro, et al., 2017; Alexander, Kelly, et al., 2017).

3 | RESULTS

3.1 | COX/PGE_2 signalling correlates with the malignancy of human gliomas

COX-2 is often highly expressed in various tumour tissues, and its activity has been well known to increase tumour aggressiveness via generating PGE_2 (Wang & Dubois, 2010). The biosynthesis of PGE_2 involves two steps: (a) Arachidonic acid is converted by either

constitutive COX-1 or inducible COX-2 to PGH_2; and (b) short-lived PGH_2 is further quickly catalysed to PGE_2 by tissue-specific isomerases—PGESs comprising microsomal PGES-1 (mPGES-1), mPGES-2, and cytosolic PGES (Hirata & Narumiya, 2011; Samuelsson, Morgenstern, & Jakobsson, 2007). TCGA research network has pinpointed a number of core pathways and gene mutations in gliomas, providing valuable insights into the disease biology (Brennan et al., 2013; Cancer Genome Atlas Research, 2008). To study COX downstream PG signalling pathways in malignant gliomas, we began with examining the gene expression data in human glioma samples using a publicly available database from TCGA network derived from the UCSC Xena Browser (http://xenabrowser.net). We first compared the gene expression of all five PGE_2 biosynthesis enzymes and four EP receptors (EP_1–EP_4) in human LGG (n = 530) and GBM (n = 172) samples. The pancan-normalized RNAseq data were used, allowing comparison of gene expression in different TCGA cancer types (Goldman et al., 2015). In line with previous findings, COX-2 in GBM was considerably induced when compared to LGG (P < 0.05; Figure 1a). Surprisingly, the expression of COX-1, the conventionally constitutive COX isozyme, was also higher than that in LGG (P < 0.05; Figure 1a). However, the difference of inducible COX-2 expression between GBM and LGG was about 50% higher than that of COX-1 (Figure 1a). Among the three PGES isozymes, mPGES-1 is inducible and functionally coupled to COX-2 (Samuelsson et al., 2007). Compared to the other two isomerases, mPGES-1 showed significantly higher expression in GBM than in LGG (P < 0.05; Figure 1a). Consistently, the higher COXs and mPGES-1 were expressed in gliomas, the lower the probabilities of patient survival were n = 690, P = 8.882E-16, 6.615E-6, and 1.118E-3 for COX-1, COX-2, and mPGES-1 respectively (Figure 1b).

Among the four EP receptors, EP_1 is $G\alpha_q$-coupled to mediate the mobilization of cytosolic Ca^{2+} and activation of PKC; EP_2 and EP_4 are associated with $G\alpha_s$ that activates AC, resulting in cAMP-dependent signalling pathways; EP_3 is mainly coupled to $G\alpha_i$, and its activation down-regulates the cytosolic cAMP (Hirata & Narumiya, 2011). PGE_2 has been reported to regulate tumourigenesis through all four EP receptors, depending on their presence in different tumour cells (Jiang & Dingledine, 2013a; Wang & Dubois, 2010). Indeed, EP receptors appeared to be differentially expressed in gliomas, as GBM had more EP_1, EP_2, and EP_4 but lower EP_3 expression compared to LGG (P < 0.05; Figure 1a). In line, glioma patients with tumours expressing low levels of $G\alpha_q$-coupled EP_1 receptors or $G\alpha_s$-coupled EP_2/EP_4 receptors showed significantly higher probabilities of survival than those with high EP_1, EP_2, or EP_4-expressing tumours (n = 690, P = 1.976E-8, 3.331E-16, and 1.110E-16 for EP_1, EP_2, and EP_4, respectively; Figure 1b). Conversely, the expression of $G\alpha_i$-coupled EP_3 receptors showed a significant positive correlation with the probability of survival in these glioma patients (n = 690, P = 1.266E-14; Figure 1b). These interesting findings together suggest that GBM-up-regulated EP_1, EP_2, and EP_4 receptors might up-regulate—whereas GBM-down-regulated EP_3 receptors might down-regulate—the malignancy of gliomas.

Previous findings suggest that the EP_1 receptor might contribute to glioma cell proliferation (Matsuo, Yoshida, Zaitsu, Ishii, & Hamasaki, 2004); the EP_4 receptor is involved in glioma resistance and progression after radiation treatment (Cook et al., 2016; Oliver, Olivier, & Vallette, 2016). In contrast, little has been known about the role of EP_2 receptor in the development of malignant gliomas except its possible involvement in the radiation resistance of GBM cells in vitro (Brocard et al., 2015), motivating us to investigate its relevance to glioma growth. Interestingly, LGG patients with tumours expressing low levels of EP_2 receptors had significant higher probability of survival than those with high EP_2-expressing tumours (n = 525, P = 0.0071); likewise, GBM patients showed an overt trend of increased survival as EP_2 receptor expression decreases in their tumours (n = 165, P = 0.1470; Figure 1c). In addition, the expression levels of EP_2 receptors and the inducible forms of COX and PGES in gliomas significantly correlate to each other (n = 702, P = 0; Figure 1 d and Table 1), indicating an elevated COX-2/mPGES-1/PGE_2/EP_2 signalling as a whole in tumours from patients with high-risk gliomas. EP_2 receptor activation has been reported to induce tumour-promoting cytokines, chemokines, and growth factors, thereby amplifying inflammation, reinforcing tumour micro-environment, and expediting tumour growth (Jiang & Dingledine, 2013b; Ma, Aoki, Tsuruyama, & Narumiya, 2015). In line, there were significant positive correlations between EP_2 receptors and the majority of these inflammatory and tumoural mediators (23 out of 25) that might potentially promote tumour proliferation, survival, migration, invasion, angiogenesis, and immune evasion in human gliomas (n = 702; Table 2; Poon, Sarkar, Yong, & Kelly, 2017; Yeung, McDonald, Grewal, & Munoz, 2013). Our findings based on TCGA data analyses of a large patient population together led us to test a hypothesis that PGE_2 signalling via the EP_2 receptor is involved in COX activity-mediated glioma growth.

3.2 | PGE_2 mediates $G\alpha_s$-dependent signalling via EP_2 receptors in human malignant glioma cells

To further investigate the PGE_2/EP_2/cAMP signalling in malignant gliomas, we treated the human GBM cell lines LN229 and SF767 with PGE_2 or forskolin, a direct activator of the **adenylyl cyclases** that is commonly used to indicate the maximal capability of the cells to produce cAMP. The cytosol cAMP levels were measured by a TR-FRET assay, in which a reduction of FRET signal indicates an increase of cAMP level. We found that PGE_2 induced cAMP accumulation in both GBM cell lines to a degree similar to that of forskolin (P < 0.05; Figure 2a). The GBM cell response to PGE_2 stimulation was concentration-dependent and virtually maximized with 0.1 and 1 μM of PGE_2 for SF767 and LN229 cells respectively (Figure 2b).

We previously reported a small-molecule compound TG4-155 that is among the first-generation selective EP_2 antagonists with high potency and selectivity (Figure 2c; af Forselles et al., 2011; Ganesh, Jiang, Shashidharamurthy, & Dingledine, 2013; Jiang et al., 2012). To improve its PK properties, a trifluoromethyl group was incorporated into its methylindole ring, leading to the creation of a novel analogue

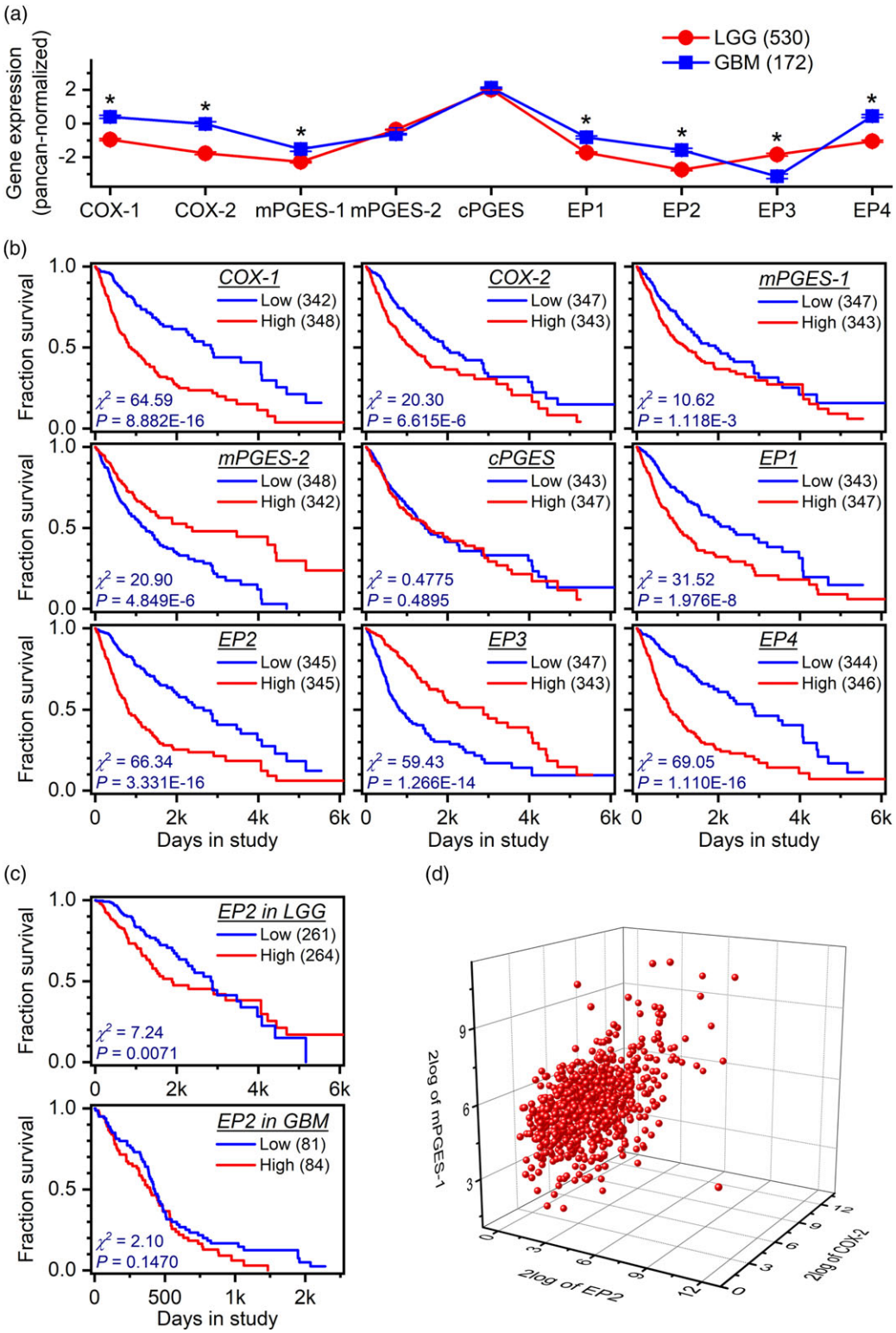

FIGURE 1 COX-2/mPGES-1/PGE_2/EP_2 receptor signalling is highly associated with human malignant gliomas. (a) The PANCAN-normalized expression of PGE_2 signalling pathway-associated genes including COX-1, COX-2, mPGES-1, mPGES-2, cPGES, EP_1, EP_2, EP_3, and EP_4 receptors in human lower grade glioma (LGG) and glioblastoma multiforme (GBM) tissues (n = 530 for LGG and 172 for GBM, $^*P < 0.05$, two-way ANOVA and post hoc Bonferroni test for multiple comparisons). (b) The relationship between survival probability of glioma patients (n = 690) and the expression of PGE_2 signalling pathway-associated genes in their tumours was shown by Kaplan–Meier estimator with post hoc log-rank test. (c) The relationship between the EP_2 receptor expression and survival probability of LGG patients (top, n = 525) or GBM patients (bottom, n = 165) was shown by Kaplan–Meier estimator. (d) Pearson's correlation coefficient analysis was performed to show the 3D relationship of expression levels of COX-2, mPGES-1, and EP_2 receptors in human glioma tissues (n = 702). The gene expression and survival analyses were based on LGG and GBM datasets of The Cancer Genome Atlas (TCGA) network derived from the UCSC Xena Browser

TABLE 1 Expression correlation of COX-2, mPGES-1, and EP_2 receptors in gliomas

	COX-2	mPGES-1	EP_2 receptor
COX-2	$R = 1$	$R = 0.3544$ $P = 0$	$R = 0.4346$ $P = 0$
mPGES-1	$R = 0.3544$ $P = 0$	$R = 1$	$R = 0.3276$ $P = 0$
EP_2 receptor	$R = 0.4346$ $P = 0$	$R = 0.3276$ $P = 0$	$R = 1$

The expression relationship of COX-2, mPGES-1, and EP_2 receptors was determined by Pearson's correlation coefficient analysis, based on TCGA dataset of GBM and LGG from the UCSC Xena Browser ($n = 702$). GBM: glioblastoma multiforme; LGG: lower grade glioma; mPGES-1: mPGES PGE synthase 1; TCGA: The Cancer Genome Atlas.

TG6-10-1 (Ganesh, Jiang, & Dingledine, 2017), which is our current lead compound of this series of EP_2 antagonists (Figure 2c). The exploratory in vitro radioligand binding assay with [^{3}H]-labelled PGE_2 showed that TG6-10-1 bound to the human EP_2 receptor with a K_i of 175 nM (Figure 2d), demonstrating its selectivity and potency. After oral administration to mice (10 mg·kg^{-1}), TG6-10-1 showed improved plasma $t_{1/2}$ 1.8 hr and brain-to-plasma (B/P) concentration ratio 1.2 (Figure 2e; Ganesh, Jiang, & Dingledine, 2014; Ganesh, Jiang, Yang, & Dingledine, 2014), when compared to TG4-155 ($t_{1/2}$: 35 min; B/P: 0.3; Jiang et al., 2012).

Importantly, with this oral dose (10 mg·kg^{-1}), the concentration of TG6-10-1 in the brain was well above its Schild K_B for EP_2 receptors for more than 8 hr without affecting other EP receptors including EP_1, EP_3, and EP_4 (Figure 2e). In addition to PGE_2, COX activity also can lead to the syntheses of several other prostanoids—PGD_2, $PGF_{2\alpha}$, (prostacyclin) PGI_2, and TXA_2, which activate their receptors **DP_1**, FP, IP, and TP respectively (Du, Kemper, Qiu, & Jiang, 2016). Among these receptors, TG6-10-1, after an oral dose of 10 mg·kg^{-1} to mice, only briefly acted on the DP_1 receptor (<2 hr) in the brain (Figure 2e). However, the DP_1 receptor does not appear to be relevant to the malignancy of human gliomas, as evidenced by its similar expression levels in LGG and GBM tumours (Figure 2f). Moreover, DP_1 receptor expression in glioma has no correlation with the patient survival (Figure 2g). These PK and pharmacodynamic (PD) results suggest that any effect of TG6-10-1 on glioma cells should largely be attributed to its action on the EP_2 receptor.

The TR-FRET assay further revealed that the inhibition of EP_2 receptor by TG4-155 or TG6-10-1 suppressed the PGE_2-initiated cAMP production in human GBM cells in a concentration-dependent manner (Figure 2h). There was a trend of greater efficacy of TG6-10-1 in inhibiting cell response to PGE_2 stimulation than that of TG4-155 in both LN229 cells (90% vs. 77%) and SF767 cells (44% vs. 37%), while TG4-155 showed a higher potency than TG6-10-1 (Figure 2h), as it was previously reported in other EP_2-expressing cells (Kang et al., 2017). These results together suggest that a strong PGE_2/EP_2 signalling commonly exists in human malignant glioma cells and the current lead EP_2 antagonist TG6-10-1 has adequate PK and PD profiles for both in vitro and in vivo tests.

TABLE 2 Expression correlation between EP_2 receptors and glioma-promoting factors

Function	Factor	*R*	*P*
Microglia/ macrophage recruitment	CCL2	0.53676	0
	POSTN	0.53307	0
	CXCR4	0.52077	0
	CCL7	0.47822	0
	CXCL12	0.35387	0
	VEGF	0.35347	0
	CSF-1R	0.23988	1.20487E-10
	CSF-1	0.16131	1.75083E-5
	CX3CR1	0.07472	0.04782
	CX3CL1	−0.22975	7.31451E-10
Immunosuppression	IL-6	0.55876	0
	MIC-1	0.52185	0
	STAT3	0.42783	0
	TGF-β	0.42230	0
	MIF	0.01493	0.69283
Angiogenesis	IL-6	0.55876	0
	PECAM-1	0.55694	0
	VEGF	0.35347	0
	CXCL2	0.23762	1.81619E-10
	IGFBP1	0.11436	0.00241
Invasion	TGFBR2	0.60646	0
	MMP-9	0.52467	0
	MMP-1	0.48760	0
	MMP-2	0.39506	0
	CSF-1R	0.23988	1.20487E-10
	CSF-1	0.16131	1.75083E-5
	PTK2B	0.10822	0.0041
Proliferation	IL-1β	0.31699	0
	MKI67	0.14996	6.64602E-5

The expression relationship between EP_2 receptors and essential tumour-promoting cytokines, chemokines, and growth factors in human glioma tissues was examined by Pearson's correlation coefficient analysis, based on TCGA combined dataset of GBM and LGG from the UCSC Xena Browser ($n = 702$). **CCL2**: chemokine (C-C motif) ligand 2; **CCL7**: chemokine (C-C motif) ligand 7; **CSF-1**: colony stimulating factor 1; **CSF-1R**: colony stimulating factor 1 receptor; **CX_3CL1**: chemokine (C-X3-C motif) ligand 1; **CX_3CR1**: CX3C chemokine receptor 1; **CXCL2**: chemokine (C-X-C motif) ligand 2; **CXCL12**: chemokine (C-X-C motif) ligand 2; **CXCR4**: chemokine (C-X-C motif) receptor type 4; IGFBP1: insulin-like growth factor-binding protein 1; **MIC-1**: macrophage inhibitory cytokine-1; MKI67: antigen KI-67; **MIF**: macrophage migration inhibitory factor; PECAM-1: platelet endothelial cell adhesion molecule 1 or cluster of differentiation 31 (CD31); POSTN: periostin or osteoblast-specific factor-2 (OSF-2); **PTK2B**: protein TK2β; **TGFBR2**: TGF β receptor II.

3.3 | EP_2 receptor inhibition suppresses GBM cell proliferation, migration, and invasion

Development of the novel selective ligands for EP_2 receptor provides an unprecedented opportunity to study this receptor in tumour cells via an inhibitory strategy. To investigate the PGE_2 signalling in malignant gliomas, human GBM cell lines LN229 (Figure 3a) and SF767 (Figure 3b) were transfected with COX-2 cDNA, generating cell lines that stably overexpress COX-2 (LNCOX-2 and SFCOX-2) to mimic the up-regulated COX activity in gliomas (Xu et al., 2014). COX-2 overexpression in these cells led to an increase of PGE_2

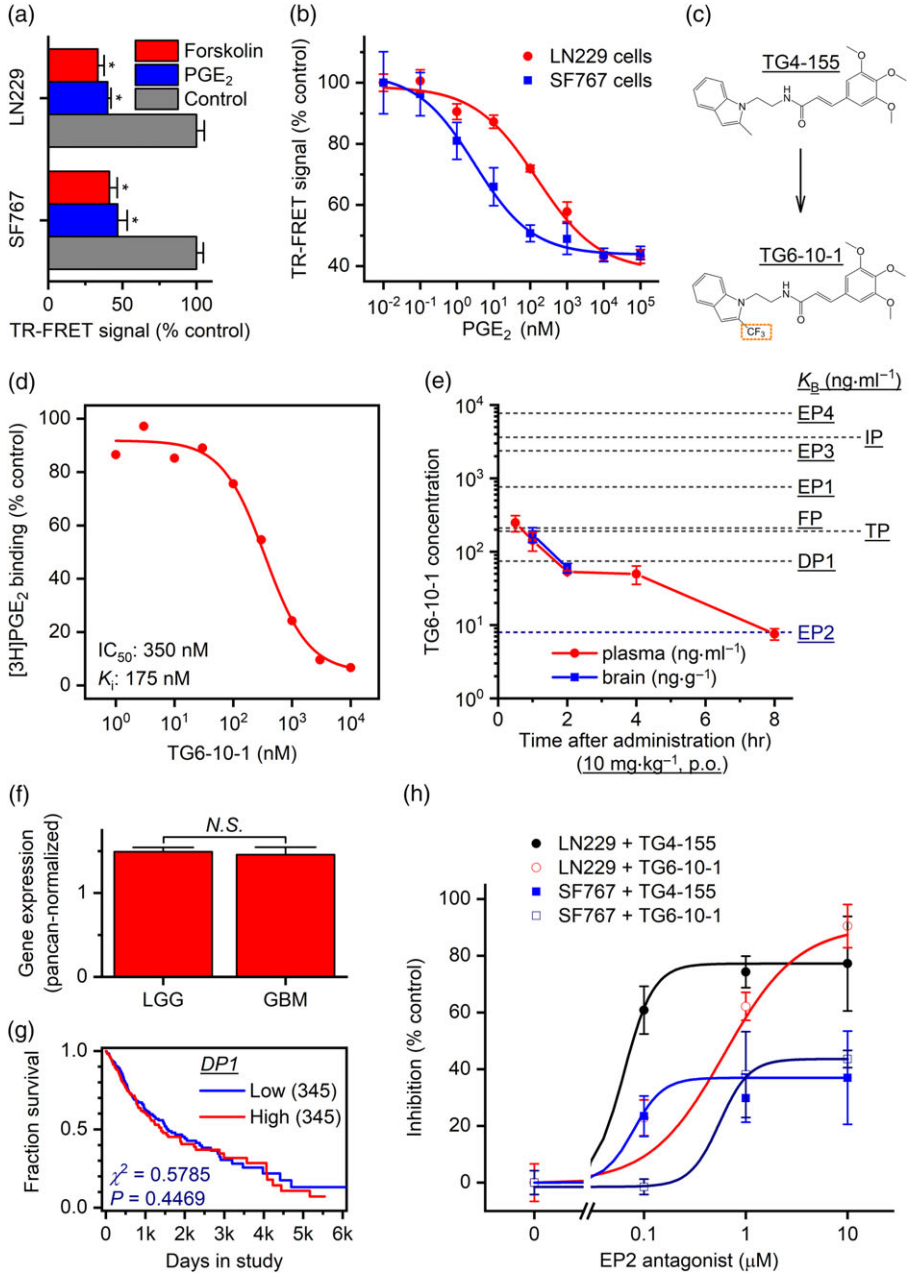

FIGURE 2 PGE_2 mediates cAMP signalling in human malignant glioma cells via EP2 receptor. (a) Stimulation with PGE_2 (10 μM) or forskolin (100 μM) induced the biosynthesis of cAMP in human GBM cells LN229 and SF767, measured by a cell-based assay using the time-resolved FRET (TR-FRET; n = 5, $^*P < 0.05$ compared with control group, one-way ANOVA and post hoc Dunnett's test). Data are shown as mean + SEM. (b) PGE_2 increased cAMP in GBM cells in a concentration-dependent manner. The PGE_2 EC_{50}s: 150 nM for LN229 and 3 nM for SF767 cells. Note that the cell response was approximately maximized with 0.1 and 1 μM PGE_2 for SF767 and LN229 cells respectively. Exploratory data are shown as mean ± SEM (n = 4). (c) Chemical structures of EP_2-selective antagonist TG4-155 and the current lead compound TG6-10-1. (d) Radioligand binding assay was performed to evaluate the affinity of TG6-10-1 to the human EP_2 receptor by measuring its inhibition of binding of [^{3}H]-PGE_2 (3 nM) to the cell membrane homogenates (Jiang et al., 2012). The average of two independent measurements of radio-labelled PGE_2 binding was plotted against the increasing concentrations of TG6-10-1, and the error bars were not displayed. The compound showed an IC_{50} of 350 nM and a K_i of 175 nM on the human EP_2 receptor. (e) With systemic administration to mice (10 mg·kg^{-1}, p.o.), compound TG6-10-1 showed a plasma terminal $t_{1/2}$ of 1.8 hr and brain-to-plasma concentration ratio of 1.2 (Ganesh, Jiang, & Dingledine, 2014; Ganesh, Jiang, Yang, & Dingledine, 2014). Exploratory data are shown as mean ± SEM (n = 3 mice per time point). The compound Schild K_B values for eight canonical prostanoid receptors are also indicated: 74.6, 762, 7.98, 2,380, 7,740, 202, 3,640, and 193 ng·ml^{-1} for DP_1, EP_1, EP_2, EP_3, EP_4, FP, IP, and TP receptors respectively. (f) The PANCAN-normalized expression of PGD_2 receptor DP1 in human LGG and GBM tissues (n = 530 for LGG and 172 for GBM, *N.S.*: not significant, Student's t-test). Data are shown as mean + SEM. (g) The relationship between survival probability of glioma patients (n = 690) and the expression of the DP_1 receptor gene in their tumours was shown by Kaplan–Meier estimator with post hoc log-rank test (P = 0.4469). (h) Inhibition of cAMP production in human GBM cells LN229 and SF767 by TG4-155 and TG6-10-1 in response to stimulation of PGE_2 (1 μM for LN229 cells; 0.1 μM for SF767 cells). Data were normalized and presented as a percentage of maximum response; points represent mean ± SEM (n = 6)

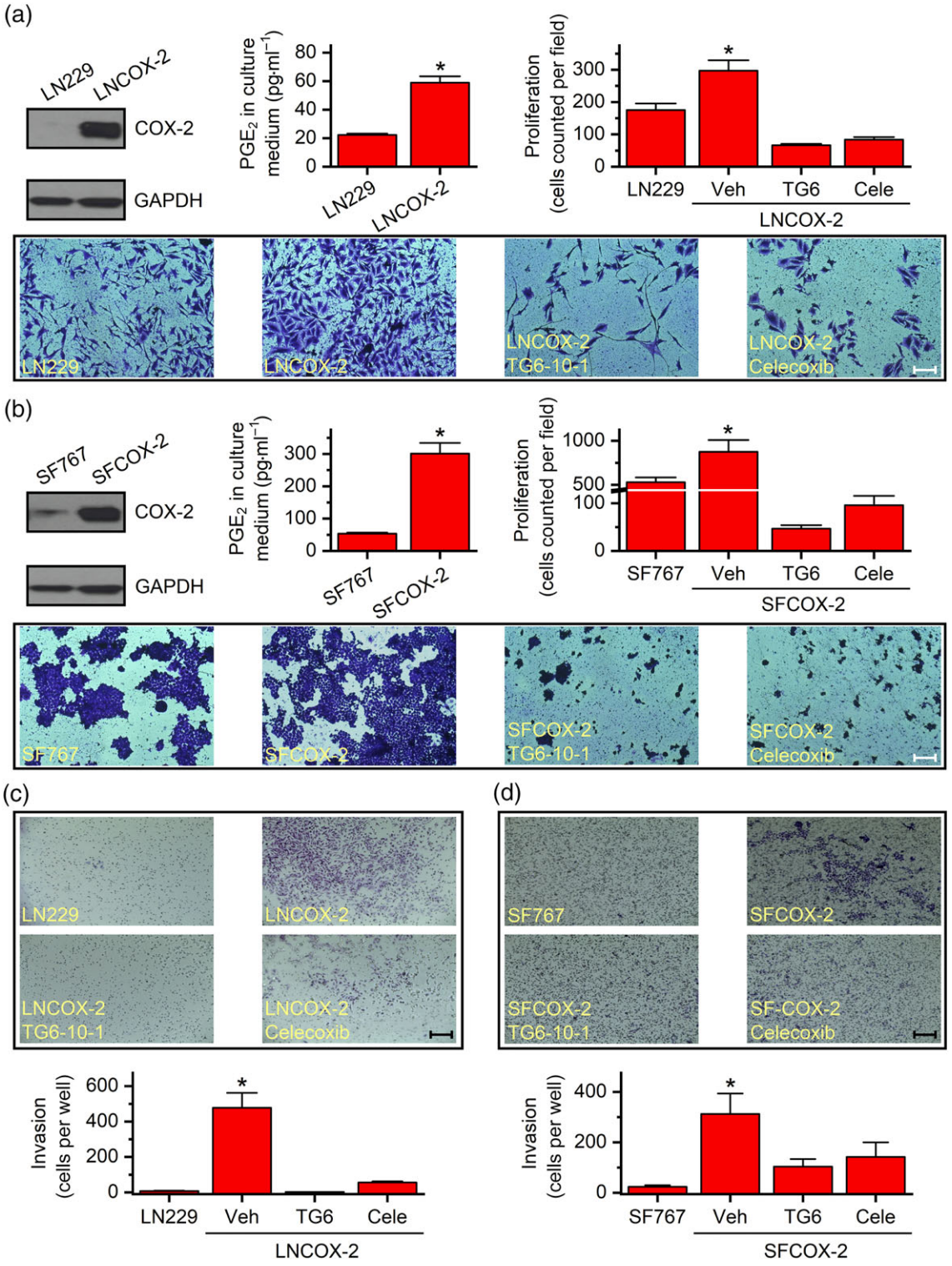

FIGURE 3 PGE_2 signalling via the EP_2 receptor mediates COX activity-driven human glioma cell proliferation and invasion. (a,b) GBM cells LN229 and SF767 that overexpress COX-2 (LNCOX-2 and SFCOX-2) were generated, and the COX-2 expression levels were detected by Western blot analysis. The COX-2 overexpression increased the PGE_2 levels in the culture medium, measured by ELISA (n = 5, $^*P < 0.05$, Student's t-test). COX-2 overexpression in GBM cells facilitated the cell proliferation, which was blocked by treatment with selective EP2 receptor antagonist TG6-10-1 or COX-2 inhibitor celecoxib (all 10 μM). Cells were visualized by crystal violet dye 72 hr after treatment and counted randomly (n = 5, $^*P < 0.05$ compared with LN229 or SF767 control group, one-way ANOVA and post hoc Dunnett's test). Data are shown as mean + SEM. Scale bar = 20 μm. (c,d) Overexpression of COX-2 in human GBM cell lines LN229 and SF767 facilitated the cell invasion, which was blocked by treatment with selective EP_2 receptor antagonist TG6-10-1 or COX-2 inhibitor celecoxib (all 10 μM). GBM cells that crossed the filter coated with Matrigel by 48 hr after treatment were visualized by HEMA 3 stain set and counted randomly (n = 5, $^*P < 0.05$ compared with control group, one-way ANOVA and post hoc Dunnett's test). Data are shown as mean + SEM. Scale bar = 50 μm

production and secretion into the culture medium, compared to cells transfected with vehicle only ($P < 0.05$; Figure 3a,b). Moreover, COX-2 overexpression markedly increased the proliferation of both LN229 and SF767 cells ($P < 0.05$), whereas treatment with selective EP2 receptor antagonist TG6-10-1 or COX-2 inhibitor celecoxib for 72 hr completely suppressed the COX-2-mediated GBM cell growth (Figure 3a,b).

To study the effect of EP_2 inhibition on GBM cell invasion, LN229 and SF767 cells were seeded into culture inserts that were precoated with Matrigel. After incubation for 48 hr, the cells that moved across the Matrigel-coated membrane were stained and counted. COX-2 overexpression in LN229 and SF 767 significantly promoted cell invasion ($P < 0.05$), which was largely prevented by treatment with TG6-10-1 or celecoxib (Figure 3c,d). We next examined the effect of EP_2 inhibition on GBM cell migration using the wound healing assay. COX-2 overexpression in GBM cells LN229 and SF767 markedly increased cell migration over a 48-hr detection period ($P < 0.05$ for LN229 at 24 and 48 hr; $P < 0.05$ for SF767 at 48 hr; Figure 4). The COX-2 activity-mediated cell migration was substantially attenuated by treatment with TG6-10-1 ($P < 0.05$ for LN229 at 24 and 48 hr; $P < 0.05$ for SF767 at 48 hr) or celecoxib ($P < 0.05$ for LN229 at 24 and 48 hr; $P < 0.05$ for SF767 at 48 hr; Figure 4). It appears that both TG6-10-1 and celecoxib powerfully inhibited the migration of the cells to a lower level than that of cells without COX-2 overexpression. This was likely caused by their blockade on the basal PGE_2/EP_2 signalling in these cells. The results from these in vitro assays together suggest that the COX-2-promoted tumourigenic activities in human GBM cells, including proliferation, migration, and invasion, can largely be attributed to EP_2 receptor activation by PGE_2.

3.4 | EP_2 receptor inhibition causes GBM cell cycle arrest and apoptosis

To determine whether EP_2 receptor inhibition by TG6-10-1 reduced the COX-2 activity-mediated tumourigenicity in glioblastoma cells due to cell cycle arrest or apoptosis, LNCOX-2 and SFCOX-2 cells were treated with either vehicle or TG6-10-1 for 72 hr and the cell cycle distribution was analysed by flow cytometry following cell staining with propidium iodide. Compared to vehicle-treated control cells, about 80–90% of TG6-10-1-treated LNCOX-2 and SFCOX-2 cells accumulated at G0/G1 ($P < 0.05$ for both LNCOX-2 and SFCOX-2; Figure 5a), concomitant with a depletion of cells from S phase ($P < 0.05$ for both LNCOX-2 and SFCOX-2; Figure 5a). Furthermore, there was an approximately threefold increase in the accumulation of both GBM cells in sub G1 phase following TG6-10-1 treatment (0.7% vs. 2.4% for LN229 cells; 0.3% vs. 0.8% for SF767 cells; Figure 5a), indicative of apoptosis caused by EP_2 receptor inhibition.

To further investigate the effect of EP_2 receptor inhibition on GBM cell death, LNCOX-2 and SFCOX-2 cells were treated with either vehicle or TG6-10-1 for 72 hr, stained with annexin V-FITC and propidium iodide, and then analysed by flow cytometry. Results showed that the treatment with TG6-10-1 induced apoptosis from 4% to 12% in LNCOX-2 cells ($P < 0.05$; Figure 5b, top) and from 5% to 17% in SFCOX-2 cells ($P < 0.05$; Figure 5b, bottom). In fact, both early apoptosis (indicated by annexin V-FITC positive cells only) and later apoptosis (indicated by annexin V-FITC and propidium iodide double positive cells) were induced by TG6-10-1 treatment (Figure 5 b). Taken together, our results suggest that EP_2 receptor inhibition by TG6-10-1 suppressed COX-2-mediated tumourigenic activities in human GBM cells via both cell cycle arrest and apoptosis.

3.5 | Inhibiting the EP_2 receptor blocks glioma cell growth in vivo

To determine the role of EP_2 receptors in glioma in vivo, we first used SF767 cells for growth as s.c. tumours in athymic nude mice. SF767 cells overexpressing COX-2 (SFCOX-2) were also used in order to detect any dose effect of COX-2/PGE_2 on tumour growth, as COX-2 overexpression in these cells led to a further increase of PGE_2 release when compared to cells transfected with vehicle only (Figure 3). Each animal was inoculated with SF767 and SFCOX-2 cells into two different sites for tumour formation, followed by oral treatment with either vehicle or TG6-10-1 (10 $mg \cdot kg^{-1}$) twice daily. After 4 weeks of treatment, tumours were then dissected out, weighed, and analysed. COX-2 overexpression overall increased the growth of SF767 cells-derived tumours ($P < 0.05$; Figure 6a–c), whereas TG6-10-1 treatment significantly reduced growth of s.c. tumours formed by SF767 or SFCOX-2 cells when compared to their vehicle-treated control group ($P < 0.05$; Figure 6a–c). It appears that the reduction effect by TG6-10-1 treatment on tumour growth was partially reversed by COX-2 overexpression in the tumour cells ($P < 0.05$; Figure 6a–c). These results suggest that the stimulatory effect of COX-2/PGE_2 on the tumour growth can largely be attributed to the EP_2 receptor activation. In line, TG6-10-1 treatment for 4 weeks on average decreased the weight of SF767 tumours by 63% ($P < 0.05$; Figure 6d) and the weight of the SFCOX-2 tumours by 57% ($P < 0.05$; Figure 6d). Other than the tumour burden, all mice were overall healthy without showing any locomotion, lethargy, or behavioural abnormalities; mice treated with vehicle or TG6-10-1 showed similar body weights throughout the 4-week treatment period (Figure 6e).

Histological examination using immunostaining revealed that both SF767- and SFCOX-2-derived tumours expressed COX-2 protein, with a considerably higher COX-2 level in SFCOX-2 tumours (Figure 7a). Immunohistochemistry using KI-67 antibody showed a significant increase of proliferative index of COX-2-overexpressing tumours when compared to xenografts formed by SF767 parental cells ($P < 0.05$; Figure 7b). In alignment with the positive correlation between EP2 receptor and KI-67 expression in human gliomas ($R = 0.150$, $P = 6.646E\text{-}5$, Table 2), treatment with TG6-10-1 substantially decreased the proliferative index of both SF767 and SFCOX-2 tumours ($P < 0.05$; Figure 7b). Angiogenesis is a hallmark of malignant tumours and represents a cardinal feature of glioblastomas. We thus

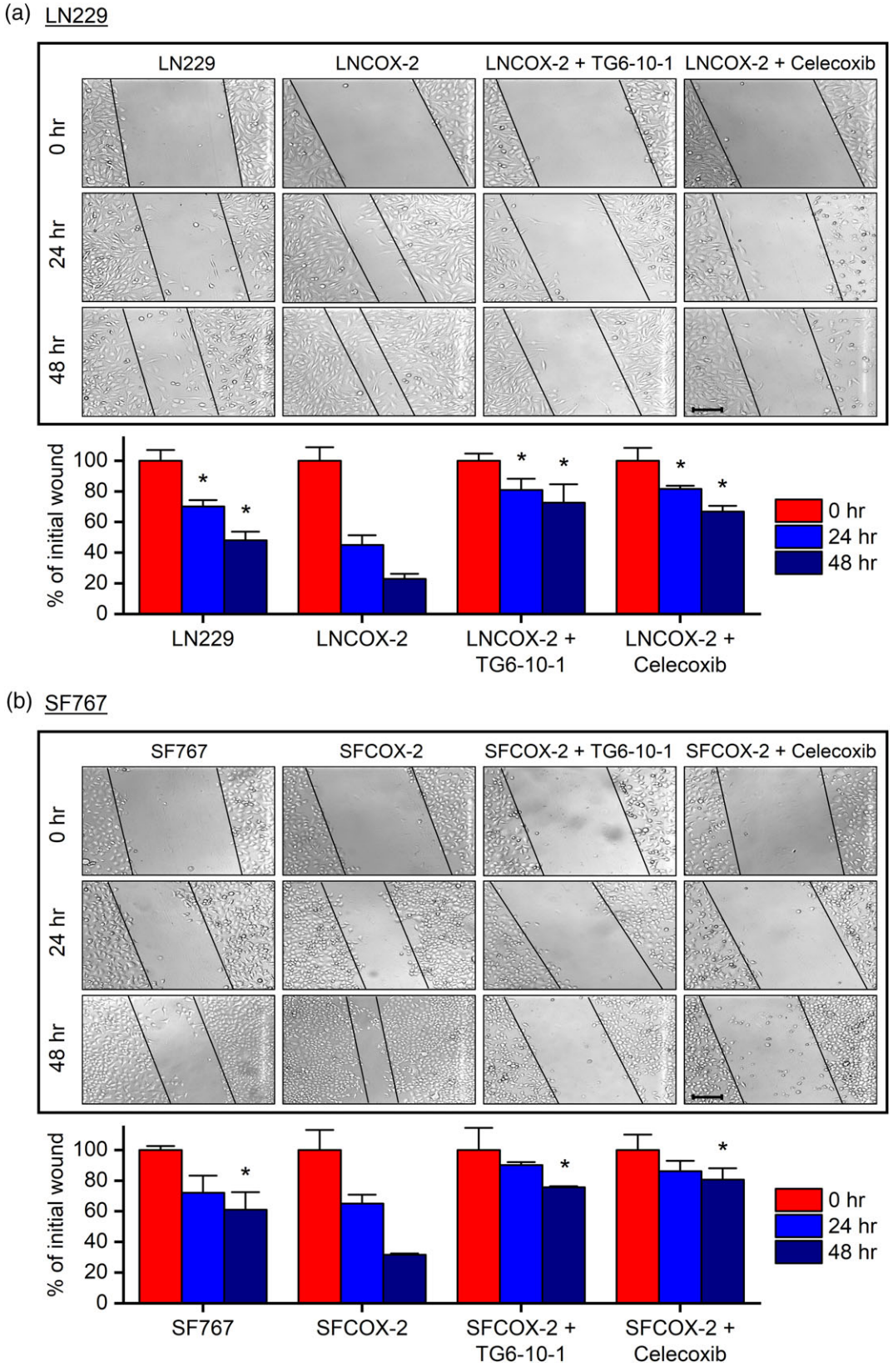

FIGURE 4 PGE_2 signalling via the EP_2 receptor mediates COX-2 activity-driven glioma cell migration. COX-2 overexpression increased the migration of GBM cells LN229 (a) and SF767 (b), evaluated by a wound healing assay at the time points indicated (24 and 48 hr). The COX-2 activity-mediated GBM cell migration was attenuated by treatment with selective EP2 receptor antagonist TG6-10-1 or COX-2 inhibitor celecoxib (all 10 μM; $n = 5$, $^*P < 0.05$ compared with COX-2 overexpression group, two-way ANOVA and post hoc Bonferroni test for multiple comparisons). Data are shown as mean + SEM. Scale bar = 50 μm

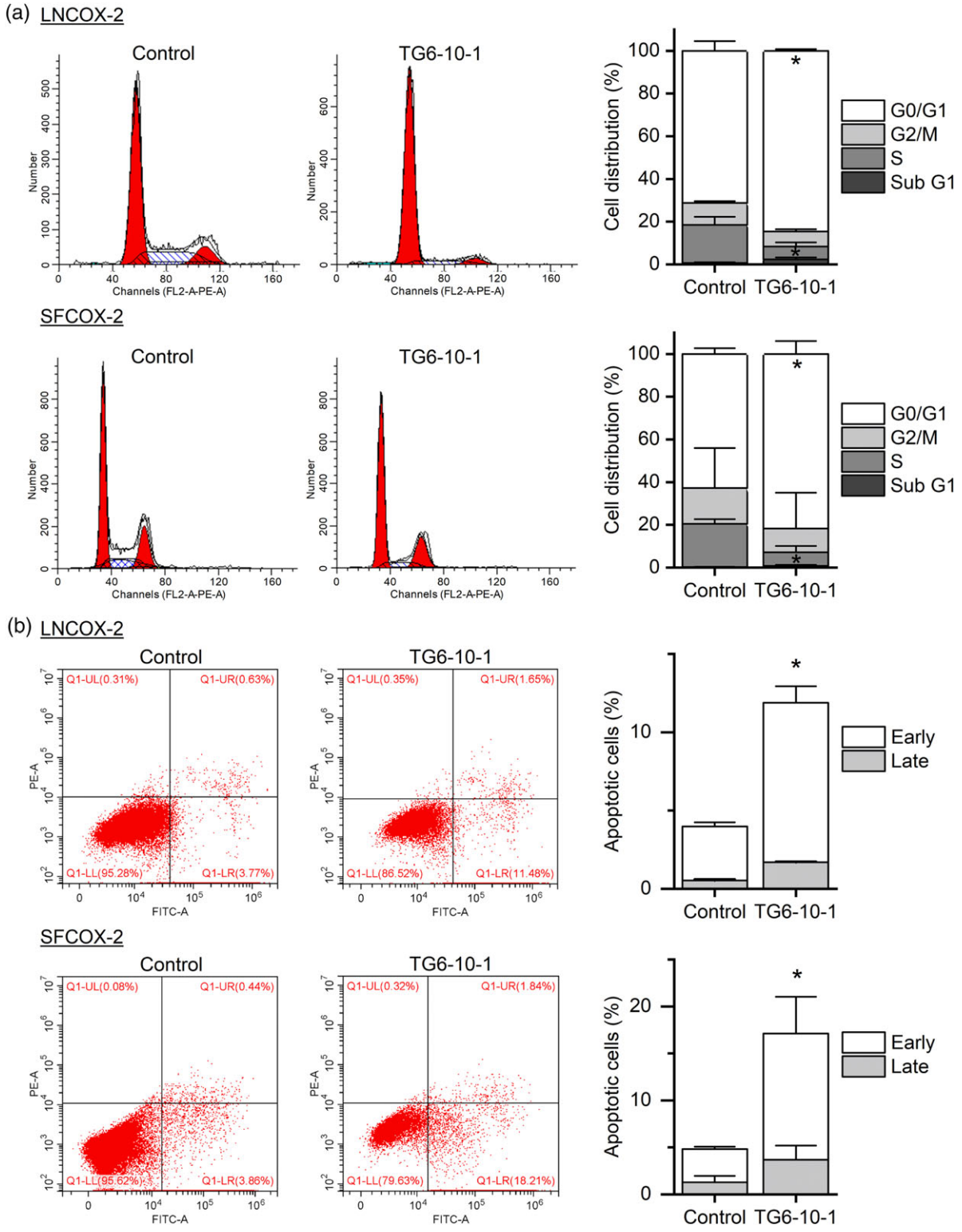

FIGURE 5 Cell cycle arrest and apoptosis of human GBM cells by an EP_2 receptor antagonist. (a) GBM cells overexpressing human COX-2, LNCOX-2 (top) and SFCOX-2 (bottom), were treated with TG6-10-1 (10 μM) for 72 hr, and the cell cycle phase distribution was evaluated by flow cytometry using propidium iodide. Bar diagram depicts the percentages of cells in different phases (G0/G1, G2/M, S, and sub G1) of cell cycle (n = 5, $^*P < 0.05$ compared with vehicle-treated control group, two-way ANOVA and post hoc Bonferroni test for multiple comparisons). (b) COX-2-overexpressing cells, LNCOX-2 (top) and SFCOX-2 (bottom), were treated with TG6-10-1 for 72 hr and stained by annexin V-FITC and propidium iodide, followed by flow cytometry acquisition. The lower right quadrant shows annexin V-positive cells (early apoptotic cells), and the upper right quadrant shows annexin V and propidium iodide double-positive cells (later apoptotic cells). The percentages of early and late apoptotic cells are represented in a histogram. Bar diagram depicts the percentages of early and late apoptotic cells (n = 5, $^*P < 0.05$ compared with vehicle-treated control group, two-way ANOVA and post hoc Bonferroni test for multiple comparisons). Data are shown as mean + SEM

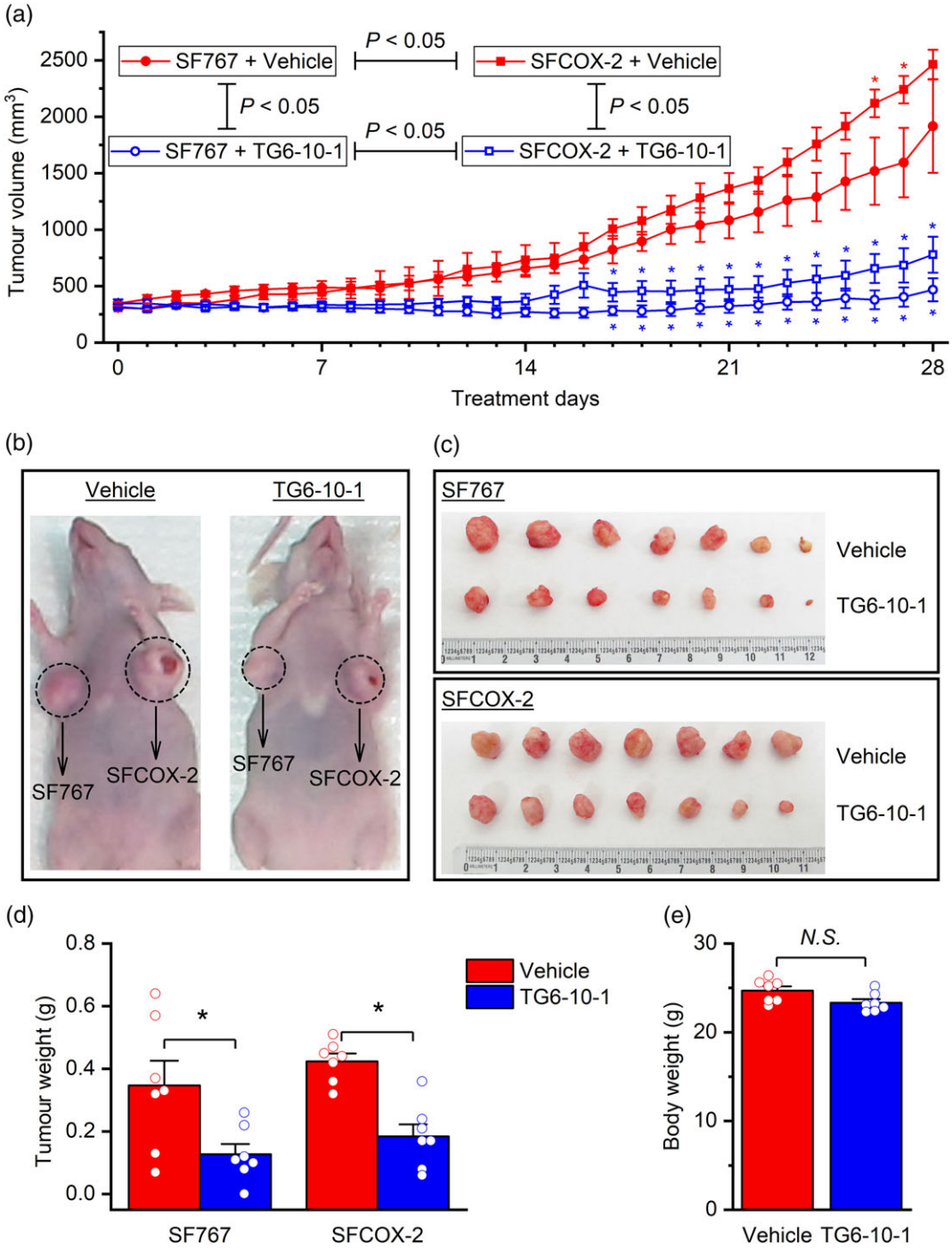

FIGURE 6 COX-2 mediates s.c. tumour growth through EP_2 receptors. (a) SF767 control cells and COX-2-overexpressing cells (SFCOX-2) were s.c. inoculated into athymic nude mice (two tumours per animal). After solid tumours had developed, vehicle (10% PEG 200, 0.5% methylcellulose) or a selective EP_2 antagonist TG6-10-1 (10 mg·kg^{-1}, p.o, b.i.d.) was administered for 4 weeks until tumour harvest. Tumour growth was monitored by measuring tumour volume daily using the formula: $V = (\pi/6) \times [(A + B)/2]^3$ (A = longest diameter; B = shortest diameter) and compared (n = 7, *P < 0.05 compared with vehicle-treated group, two-way ANOVA and post hoc Bonferroni test for multiple comparisons). Data are shown as mean ± SEM. (b) Representative digital photographs showing s.c. tumours formed by SF767 and SFCOX-2 cells in mice treated with vehicle or TG6-10-1. (c) Photographs showing tumours that were dissected out after 28-day treatment (n = 7). (d) All s.c. tumours were weighed for comparisons (n = 7, *P < 0.05 compared with vehicle-treated group, one-way ANOVA and post hoc Bonferroni test with selected pairs indicated). (e) Body weights of tumour-bearing mice before tumour harvest (n = 7, *N.S.*: not significant, Student's t-test). Data are shown as mean + SEM

were interested in determining the contribution of COX-2/PGE_2/EP2 signalling to the microvascular proliferation, which was evaluated here by immunostaining for the platelet endothelial cell adhesion molecule 1 or CD31, a biomarker commonly used to assess angiogenesis in tumours (DeLisser et al., 1997). Compared to the parental cell line-derived tumours, the COX-2-overexpressing tumours showed a

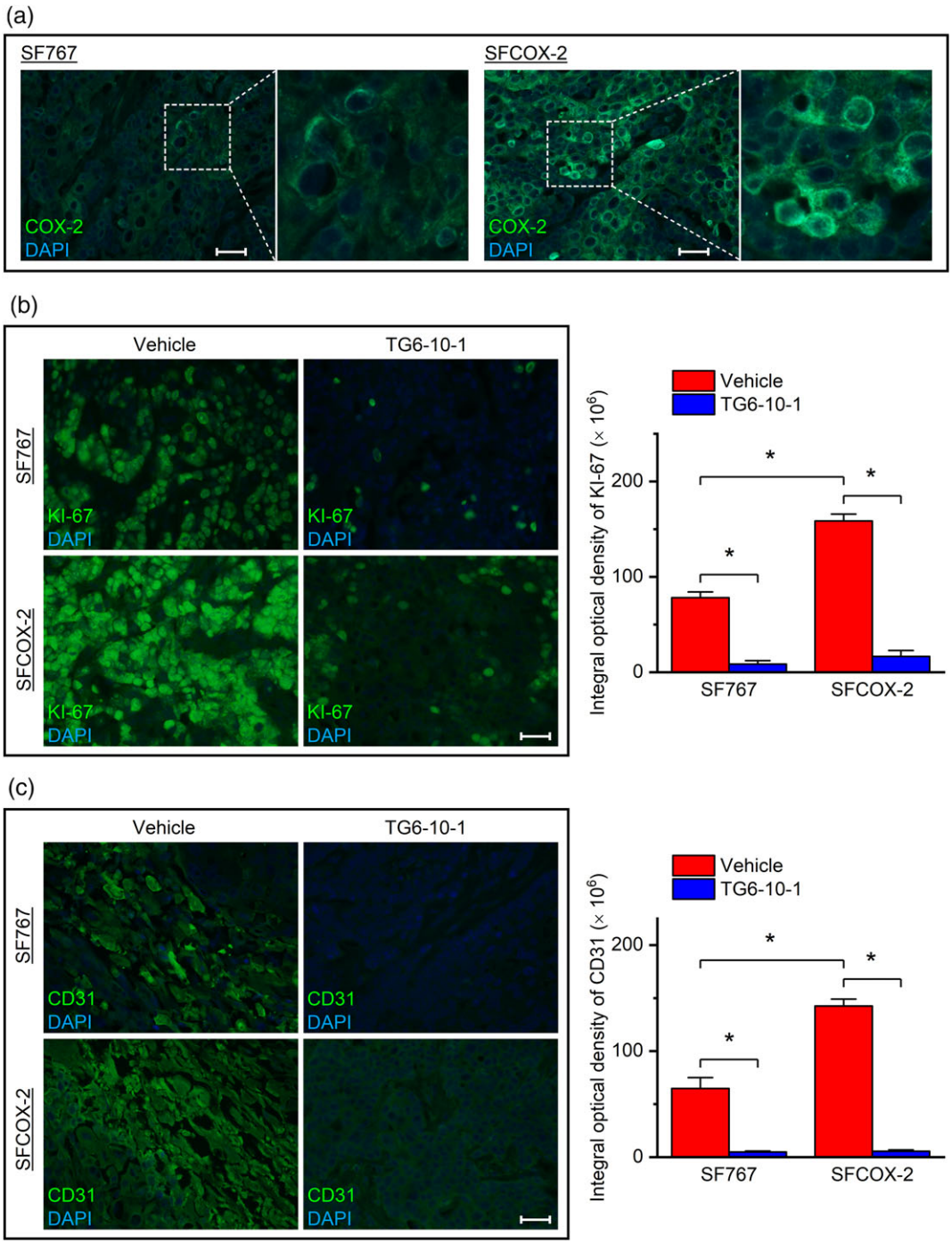

FIGURE 7 COX-2 increases the number of proliferating cells and microvessel density through EP_2 receptors. (a) Representative fields are shown to demonstrate COX-2 expression (green fluorescence) in s.c. tumours derived from SF767 cells (Left) and COX-2-overexpressing cells SFCOX-2 (Right). (b) Immunostaining for KI-67 (green fluorescence) was performed to identify proliferating cells in s.c. tumour tissues. KI-67 expression levels were measured by quantifying the fluorescence intensity using ImageJ software (n = 7, $^*P$ < 0.05, one-way ANOVA and post hoc Bonferroni test with selected pairs indicated). (c) Immunostaining for CD31 (green fluorescence) was utilized to indicate the microvessel density in s.c. tumour tissues. CD31 levels were assessed by quantifying the fluorescence intensity (n = 7, $^*P$ < 0.05, one-way ANOVA and post hoc Bonferroni test with selected pairs indicated). Note that nuclei within each tumour were counterstained with DAPI (blue fluorescence). Data are shown as mean + SEM. Scale bar = 50 μm

significant increase of microvascular proliferation indicated by CD31 expression (P < 0.05; Figure 7c). Treatment with TG6-10-1 largely decreased the CD31 expression in both SF767 and SFCOX-2-derived tumours (P < 0.05; Figure 7c). Together with the considerable expression correlation between EP2 and platelet endothelial cell adhesion molecule 1/CD31 in human gliomas (R = 0.557, P = 0, Table 2), our results suggest an important role for EP_2 receptor in COX-2 activity-associated angiogenesis of gliomas.

3.6 | Inhibiting EP_2 receptor suppresses orthotopic malignant gliomas

We next examined the effect of TG6-10-1 on orthotopic glioblastoma xenografts, as orthotopic tumours are considered to better recapitulate the original conditions particularly microenvironments than ectopic tumours owing to the fact that tumour cells are directly implanted into their organ of origin (Killion, Radinsky, & Fidler, 1998). To create orthotopic glioma tumours, luciferase-labelled LN229 cells were intracranially injected into the nude mice. Following the recovery from the surgery, the animals were treated with vehicle or TG6-10-1 (10 $mg{\cdot}kg^{-1}$) through oral garage twice daily for 4 weeks. The intracranial tumours were monitored weekly by bioluminescence using a Xenogen IVIS system. The bioluminescence results showed that the orthotopic tumours within the animal brains progressed aggressively, evident by the rapid increase of bioluminescent signalling over the 4-week detection period (Figure 8a), while TG6-10-1 treatment markedly suppressed the growth of the intracranial tumours compared to the vehicle treatment ($P < 0.05$ at Week 4; Figure 8b). Haematoxylin and eosin staining showed that the brain tumours treated with vehicle were visibly larger than those treated with TG6-10-1 harvested at the same time point (Figure 8c). Survival analysis using Kaplan–Meier estimator showed that treatment with TG6-10-1 for only 4 weeks prolonged survival of glioblastoma-harbouring mice (P = 2.835E-4; Figure 8d); the median survival times of tumour-harboring mice treated by vehicle (n = 20) and TG6-10-1 (n = 28) were 27 and 53 days respectively. Intriguingly, there was a sudden and continuous increase of mortality a few days after the treatment had been terminated (Figure 8d), suggesting a rapid tumour growth with EP_2 receptor reactivated in the absence of TG6-10-1. The collective results from s.c. and orthotopic xenograft models suggested that inhibiting PGE_2 signalling using EP_2 selective antagonist reduced the tumourigenicity of malignant glioma cells in vivo.

4 | DISCUSSION

Based on a series of comprehensive data analyses of gene expression and survival probability on cohort studies of LGG and GBM patients from TCGA research network, we hypothesized that PGE_2 signalling via the EP_2 receptor plays an essential role in COX activity-associated glioma growth. We tested this hypothesis using pharmacological and genetic approaches and demonstrated that EP_2 receptor inhibition by a novel brain-permeable small-molecular antagonist TG6-10-1 largely decreased the aggressiveness of malignant glioma cells both in vitro and in vivo. These findings establish the EP_2 receptor as an appealing target for novel therapeutics aimed at preventing and suppressing the development of malignant gliomas in humans.

COX-2 is often elevated in glioma tissues and its expression level is highly correlated with aggressive aspects of the tumours, such as tumour grade (Joki et al., 2000), proliferation rate (Prayson, Castilla, Vogelbaum, & Barnett, 2002), and poor prognosis (New, 2004), and thus was once widely considered as a favourable therapeutic target for CNS tumours. However, the notion of blocking the COX cascade to interrupt glioma progression has recently been dampened by contradicting outcomes from several population studies and a number of discontinued clinical trials (Qiu et al., 2017). These discouraging results are not unanticipated, since COX activity leads to the synthesis of five types of prostanoids that in turn can activate a total of nine GPCRs, implementing a myriad of pro- and anti-tumourigenic actions (Hirata & Narumiya, 2011; Ma et al., 2015; O'Callaghan & Houston, 2015; Wang & Dubois, 2010). The past decade has also witnessed a mounting recognition of the undesirable effects of chronic COX-2 inhibition on the microvascular systems (Grosser et al., 2010), leading to the voluntary withdrawal of two legendary COX-2 inhibitor drugs—rofecoxib and valdecoxib. The monumental lesson from COX-2 saga inspired us to explore the downstream prostanoid receptors as the next-generation therapeutic targets (Jiang et al., 2017; Jiang & Dingledine, 2013a). In this work, we presented evidence that targeting EP2 is a feasible strategy to suppress malignant gliomas and might provide more therapeutic specificity than inhibiting COX-2 without destructing other prostanoid signalling pathways that might be beneficial and are also governed by COX activity.

Many anti-tumour drugs fail to show adequate effects on intracranial tumours due to their inability to cross the blood–brain barrier (Alexander & Cloughesy, 2017; Beduneau et al., 2007; Mellinghoff & Gilbertson, 2017; Omuro & DeAngelis, 2013). As the current lead compound in this series of EP_2 antagonists, TG6-10-1 has a favourable brain-to-plasma ratio (1.2) after oral administration in mice, allowing the compound to accumulate in the intracranial tumour sites. Although TG6-10-1 has relatively short in vivo $t_{1/2}$ (1.8 hr), its concentration in the brain is steadily above its EP_2 receptor K_B for more than 8 hr, suggesting a long duration of action on EP_2 receptors following each oral dose. Intriguingly, temozolomide also has an in vivo $t_{1/2}$ of 1.8 hr showing survival benefits in GBM patients as an adjuvant treatment (Stupp et al., 2005; Stupp et al., 2009). With the dose used in this study (10 $mg{\cdot}kg^{-1}$, p.o.), the maximal in vivo concentration of TG6-10-1 is well below its K_B values for other prostanoid receptors including EP_1, EP_3, EP_4, FP, IP, and TP; the TG6-10-1 brain concentration is only briefly above the K_B for the DP_1 receptor that is irrelevant to glioma, demonstrating its EP_2 receptor specificity in vivo. The adequate PK and PD properties of TG6-10-1 reinforce its candidacy as a potential therapeutic agent for malignant gliomas.

PGE_2 has been reported to regulate tumourigenesis through all four EP receptors, among which the $G\alpha_s$-coupled EP_2 and EP_4 receptors have been well studied for their potential roles in the development and progression of tumours including those of the lung, head and neck, prostate, colon, ovary, breast, skin, and liver (Jiang & Dingledine, 2013a; Jiang & Dingledine, 2013b; Ma et al., 2015; Ma, Kundu, Collin, Goloubeva, & Fulton, 2012). It appears that EP_2 and EP_4 receptors are commonly expressed in most tumours and likely work synergistically to promote cancer cell activities as they share much of the downstream G protein-dependent and independent signalling pathways (Jiang & Dingledine, 2013a; Ma et al., 2012). Interestingly, recent studies suggest that PGE_2 via EP_4 receptors might up-regulate tryptophan-2,3-dioxygenase, thereby mediating the

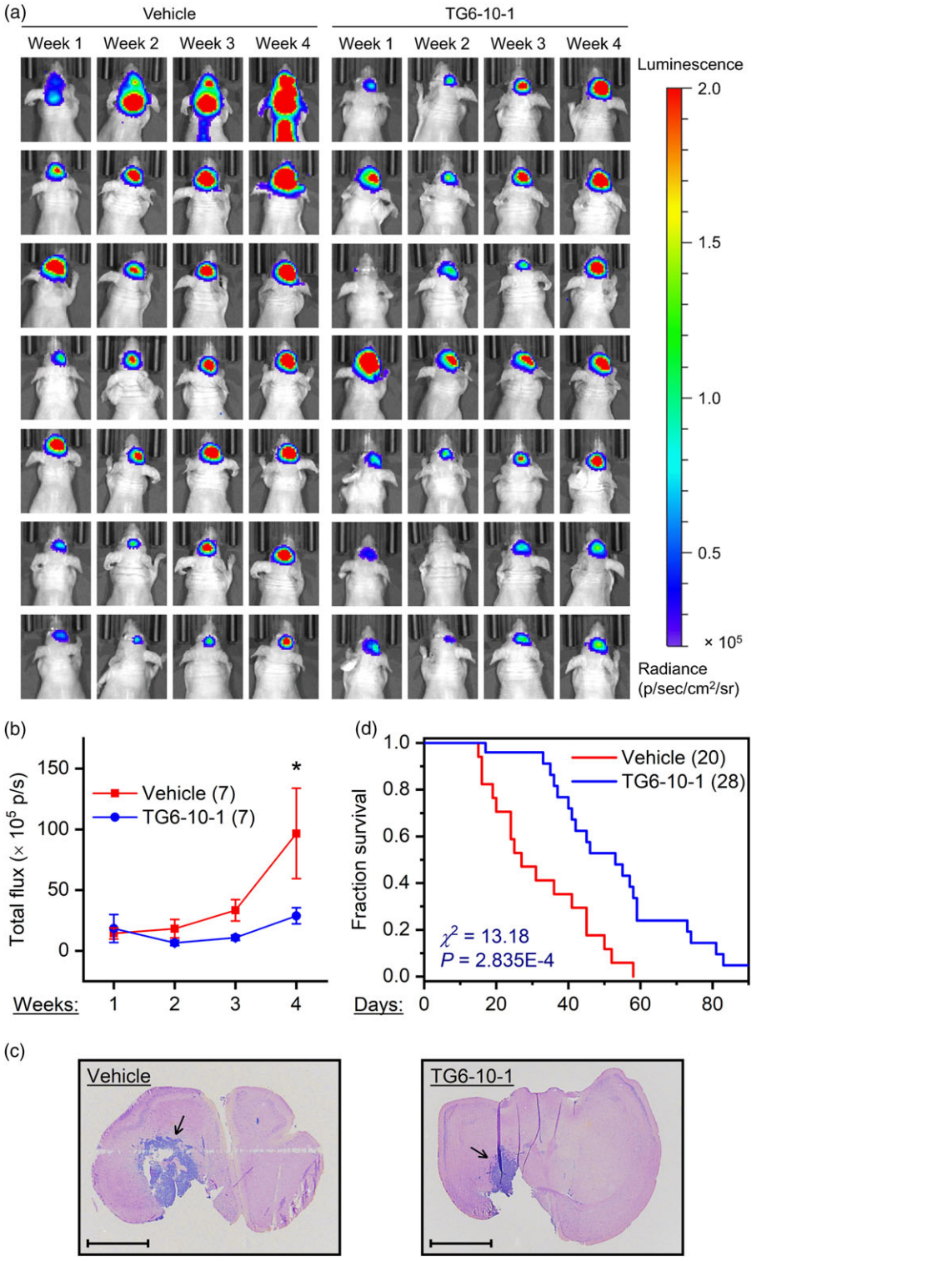

FIGURE 8 EP_2 receptor inhibition suppresses orthotopic malignant glioma growth. (a) Human GBM cells LN229 labelled with luciferase were implanted into the right striatum of athymic nude mice (5×10^5 cells per mouse). Vehicle (10% PEG 200, 0.5% methylcellulose) or a selective EP_2 antagonist TG6-10-1 (10 $mg \cdot kg^{-1}$, p.o, b.i.d.) was administered for 4 weeks. Tumour growth was monitored weekly by bioluminescence using Xenogen IVIS imaging system. Note that all implanted mice developed intracranial tumours that progressed aggressively ($n = 7$). (b) Quantification of bioluminescence signal to indicate the intracranial tumour growth ($n = 7$, $^*P < 0.05$ compared with vehicle-treated group, two-way ANOVA and post hoc Bonferroni test for multiple comparisons). (c) Representative H&E stained coronal sections of mouse brains harbouring tumours derived from LN229. Arrows denote brain tumours. It is noted that intracranial tumours were physically big and distorted the brain structure, causing the bewildering orientation of the sections. Scale bar = 2 mm. (d) Survival rates of animals that harboured intracranial tumours derived from LN229 cells (8×10^5 cells per mouse) and received vehicle ($n = 20$) or TG6-10-1 ($n = 28$) for 28 days ($P = 2.835E\text{-}4$, Kaplan–Meier survival analysis with post hoc log-rank test)

tumour-associated immunosuppression (Ochs et al., 2015), and contribute to GBM cell self-renewal and resistance to radiation therapy through inducing Id1, a functional biomarker for cancer stem cell-like cells with self-renewing capability (Cook et al., 2016). However, whether pharmacological inhibition of the EP_4 receptor can also suppress the growth of malignant gliomas in vivo and improve the GBM survival remains to be determined in the future.

Inflammation engaging tumour-derived COX activity has emerged as a major contributor to tumourigenesis through multiple mechanisms: promoting reactive mediators for cancer cell growth including cytokines, chemokines, and growth factors (Mantovani, Allavena, Sica, & Balkwill, 2008); regulating angiogenesis via acting on VEGF receptors (Gately & Li, 2004); and creating immunosuppressive microenvironments that allow tumour cells to escape immunosurveillance (Zelenay et al., 2015). The molecular mechanisms whereby PGE_2/EP_2 receptor signalling promotes gliomagenesis remain unknown; however, we previously found that EP_2 receptor activation induces pro-but not anti-tumoural cytokines (Jiang & Dingledine, 2013b). In line, we showed here a positive correlation between EP_2 receptor and a series of essential tumour-promoting cytokines, chemokines, and growth factors in human malignant gliomas (Poon et al., 2017; Yeung et al., 2013), such as CCL2, CCL7, CXCL2, CXCL12, CXCR4, IL-1β, IL-6, TGF-β, MMPs, and VEGF, and GBM cell cycle arrest and apoptosis caused by EP_2 receptor inhibition. In addition, treatment with EP_2 antagonist TG6-10-1 decreased the CD31 levels in tumours, indicative of a role of EP_2 receptors in promoting microvascular proliferation of gliomas. Whether EP_2 receptor activation is also involved in the generation of immunosuppressive microenvironments remains an important topic for the future study. Nonetheless, the present findings provide proof-of-concept evidence that PGE_2 signalling via EP_2 receptor represents a novel molecular target for GBM treatment through anti-inflammatory mechanisms and reinforce the notion that selective EP_2 antagonists are promising candidates for non-steroidal anti-inflammatory drug alternatives for chemoprevention of CNS cancer.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

J.Q., Q.L., Z.S., and J.J. participated in the research design; J.Q., Q.L., K. A.B., X.Y., Y.D., E.Z., Y.Y, and J.J. conducted the experiments; J.Q., Q. L., J.J.Y., Z.S., and J.J. performed the data analyses; J.Q., Y.Y., Z.S., and J.J. wrote the manuscript. All authors reviewed and revised the final version of manuscript and approved manuscript submission.

DECLARATION OF TRANSPARENCY AND SCIENTFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers, and other organisations engaged with supporting research.

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