Dissecting the role of novel EZH2 inhibitors in primary glioblastoma cell cultures: effects on proliferation, epithelial-mesenchymal transition, migration, and on the pro-inflammatory phenotype

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Abstract

Background: Glioblastoma (GBM) is the most lethal and aggressive malignant primary brain tumor in adults. After surgical resection of the tumor, the patient typically should be subjected to chemotherapy (temozolomide, TMZ) and concomitant radiotherapy. Since the TMZ treatment does not lead to complete remission and often develops resistance, the identification of efficacious therapeutics is strongly pursued. Among the epigenetic players, the H3K27 methyltransferase (MT) EZH2 (enhancer of zeste homologue 2) has been found overexpressed or mutated in several human cancers including gliomas, and its overexpression is associated with poor outcome in GBM. Two EZH2 inhibitors (EZH2i), UNC1999 and GSK343, suppressed GBM growth in vitro and in vivo indicating that EZH2i can be potential drugs against GBM.

Results: Two new EZH2i, MC4040 and MC4041, were designed, prepared, and tested by us to determine their effects in primary GBM cell cultures. MC4040 and MC4041 displayed single-digit micromolar inhibition of EZH2, 10-fold less potency against EZH1, and no activity towards other MTs. In primary GBM cells as well as in U-87 GBM cells, the two compounds reduced H3K27me3 levels, and dose- and time-dependently impaired GBM cell viability without inducing apoptosis and arresting the cell cycle in the G0/G1 phase, with increased p21 and p27 levels. In combination with TMZ, MC4040 and MC4041 displayed stronger, but not additive, effects on cell viability. The potent clinical candidate as EZH2i tazemetostat, alone or in combination with TMZ, exhibited a similar potency of inhibition of GBM cell growth when compared to MC4040 and MC4041. At the molecular level, MC4040 and MC4041 reduced the VEGFR1/VEGF expression, reversed the epithelial-mesenchymal transition (EMT), and hampered cell migration and invasion attenuating the cancer malignant phenotype. Treatment of GBM cells with MC4040 and MC4041 also impaired the GBM pro-inflammatory phenotype, with a significant decrease of TGF-β, TNF-α, and IL-6, joined to an increase of the anti-inflammatory cytokine IL-10.

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Introduction

Glioblastoma (GBM) is the most common and aggressive malignant primary brain tumour in adults [1]. The standard treatment consists of surgical resection of the tumour, followed by chemotherapy and concomitant radiotherapy [2]. To date, the sole drug in use against GBM is the alkylating agent temozolomide (TMZ). Despite such combined and articulated regimen, the current therapeutic strategy is not a cure, and the prognosis for GBM patients remains poor with a median survival around 15 months and a 2-year survival rate of about 27%. Additionally, the intrinsic or acquired resistance to TMZ reduces the therapeutic success. Several different factors obstacle the development of successful treatments against GBM, including its intrinsic aggressive and infiltrative nature, and the high inter- and intra-tumour variability at the histopathological, genetic and epigenetic levels [3, 4]. Therefore, alternative and/or complementary therapeutic targets and strategies need to be found. In the last years, efforts have been done to this aim, and researchers started also to evaluate the possibility to use immunotherapy; however, no success has been yet recorded [5]. A growing number of literature evidences prove that epigenetic mechanisms are implicated in the development and progression of GBM [6–8].

To date, numerous molecules have been synthesized and evaluated in preclinical and clinical settings as competitive, catalytic EZH2 inhibitors (Fig. 1) [14], and some of them including GSK126, tazemetostat and CPI-1205, are being tested in clinical studies [14]. Moreover, few of them (UNC1999 and GSK343, an analogue of GSK126) have been tested against GBM in vitro and in vivo, proving the relevance of EZH2 as target to fight in this disorder [15, 16].

Recently, through a pruning strategy applied on known indazole-containing EZH2 inhibitors (EZH2i) (such as EPZ005681 and UNC1999, Fig. 1), our group reported the novel pyrazole-based EZH2i MC3629 (Fig. 1), potent at low micromolar doses and able to reduce H3K27me3 levels, to arrest cell proliferation and to induce autophagy in a panel of cancer cell lines (SK-N-BE neuroblastoma, MDA-MB-231 breast cancer, and K562 leukaemia cells) [17]. When tested in Sonic Hedgehog (SHH) medulloblastoma (MB) stem-like cells, MC3629 impaired cell proliferation and self-renewal inducing apoptosis [18]. In MB xenografted mice, MC3629 showed a significant decrease of tumour volume, a reduction of stemness and cell proliferation, and induction of apoptosis [18]. Mimicking the benzo-cracking approach followed for MC3629, starting from the structures of known indole-based EZH2 inhibitors (such as GSK126, EI1, and CPI-1205, Fig. 1) we designed and synthesized a series of pyrrole dimethylpyridone-containing compounds to be tested against EZH2. Among them, MC4040 and MC4041 (Fig. 1) were tested in GBM cells including primary GBM cell cultures, to determine their antiproliferative effects alone and in combination with temozolomide. To dissect the molecular mechanisms of these new EZH2 inhibitors, their efficacy in mitigating the GBM malignant phenotype has been determined through the reduction of VEGFR1/VEGF expression, reversion of the EMT process, inhibition of invasive phenotype, and decrease of inflammatory cytokines levels.

Materials and Methods

Chemistry

Melting points were determined on a Buchi 530 melting point apparatus and are uncorrected. 1H NMR (nuclear magnetic resonance) spectra were recorded at 400 MHz on a Bruker AC 400 spectrometer; chemical shifts are
reported in δ (ppm) units relative to the internal reference
tetramethylsilane (Me₄Si). EIMS spectra were re-
corded with a Fisons Trio 1000 spectrometer; only
molecular ions (M+) and base peaks are given. All com-
pounds were routinely checked by TLC (thin layer chro-
matography), 1H NMR and 13C NMR spectra. TLC was
performed on aluminium-backed silica gel plates (Merck
DC, Alufolien Kieselgel 60 F254) with spots visualized by
UV light. All solvents were reagent grade and, when ne-
necessary, were purified by standard methods. The concen-
tration of solutions after reactions and extractions involved the use of a rotary evaporator operat-
ing at reduced pressure of ca. 20 Torr. Organic solutions
were dried over anhydrous sodium sulphate. Elemental
analysis has been used to determine the purity of the de-
scribed compounds, that is > 95%. Analytical results are
within ± 0.40% of the theoretical values. All chemicals
were purchased from Sigma-Aldrich Chimica, Milan
(Italy), or from Alfa Aesar, Karlsruhe (Germany), and
were of the highest purity.

Synthesis of 1-(3-bromophenyl)-2,5-dimethyl-1H-pyrrole
(1)
A solution of 2,5-hexanedione (8.50 mmol, 1.2 eq, 1 mL)
in glacial acetic acid (3 mL) was prepared in a flame dried
sealed tube, and 3-bromoaniline (7.08 mmol, 1.0 eq, 1.22
g, 0.77 mL) was added to the solution. The reaction solu-
tion was stirred at 100 °C for 2 h. After this time the reac-
tion was complete, the acetic acid was removed in vacuo
(azeotrope with toluene) and the residue was purified by
silica gel chromatography, eluting with the n-hexane-
chloroform (60:1) mixture. The product was obtained as a
white solid (5.31 mmol, 1.33 g, 75%). m.p. 84–85 °C (n-
hexane). 1H-NMR (CDCl₃, 400 MHz, δ; ppm): δH 1.96
(6H, s, C(2)CH₃, C(5)CH₃), 5.82 (2H, s, C(3)H, C(4)H pyr-
role), 7.08–7.11 (1H, ddd, J = 7.6 Hz, 1.8 Hz, 0.8 Hz, aro-
matic proton), 7.26 (1H, t, J = 8 Hz, aromatic proton), 7.32
(1H, t, J = 1.6 Hz, aromatic proton), 7.45-7.48 (1H, ddd, J
= 8 Hz, 1.8, 1.2 Hz, aromatic proton) ppm. MS (EI) m/z
[M]+: 249.02. The reported data are in good agreement
with the literature [19, 20].

General procedure for the synthesis of the intermediates
2a,b. Example: Synthesis of 1-(3-(2,5-dimethyl-1H-pyrrol-
1-yl)phenyl)piperidine (2b)
In a flame dried sealed tube, 1-(3-bromophenyl)-2,5-di-
methyl-1H-pyrole 1 (1.59 mmol, 1.0 eq, 0.40 g), piperi-
dine (3.20 mmol, 2 eq, 0.27 g, 0.316 mL), palladium(II)
acetae (Pd(OAc)₂) (0.024 mmol, 1.5% mol, 5.4 mg), tri-
tert-butylphosphonium tetrafluoroborate (PH(tBu)₃BF₄)
(0.0192 mmol, 1.2% mol, 5.6 mg), and potassium tert-
butoxide (3.59 mmol, 2.25 eq, 0.404 g) were added in se-
quence and suspended in dry toluene (3.0 mL). The sys-
tem was degassed (N₂) and left under stirring at 80 °C
for 16 h. After this time, the reaction was quenched by
adding ethyl acetate, and it was filtered over celite pad.
The filtrate was concentrated in vacuo and the crude
was purified by silica gel chromatography eluting with the n-hexane-
ethanol (5:1) mixture, to give the 1-(3-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)piperidine 2b as
a colourless oil (1.27 mmol, 0.32 g, 80%). 1H-NMR (d₆-
DMSO, 400 MHz, δ; ppm): δH 1.59-1.60 (6H, m, piperi-
dine protons), 1.97 (6H, s, C(2)CH₃, C(5)CH₃ pyrrole),
3.17-3.20 (4H, m, piperidine protons), 5.70 (2H, s,
C(3)H, C(4)H pyrrole), 6.56 (1H, dd, J = 7.6 Hz, 2.0 Hz, aromatic proton), 6.69 (1H, t, J = 2.0 Hz, aromatic proton), 6.97 (1H, dd, J = 7.6 Hz, 2.0 Hz, aromatic proton), 7.29 (1H, t, J = 7.6 Hz, aromatic proton), 7.29 (1H, t, J = 7.6 Hz, aromatic proton) ppm. MS (EI) m/z [M]+: 254.18.

Chemical and physical characterization of 4-(3-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)morpholine (2a): light yellow oil (yield 82%)

1H-NMR (d6-DMF, 400 MHz, δ; ppm): δH 1.97 (6H, s, C(2)CH3, C(5)CH3 pyrrole), 3.16 (4H, t, J = 11.0 Hz, morpholine protons), 3.73 (4H, t, J = 11.0 Hz, morpholine protons), 5.76 (2H, s, C(3)H, C(4)H pyrrole), 6.63 (1H, dd, J = 8.2 Hz, 2.0 Hz, aromatic proton), 6.73 (1H, t, J = 2.0 Hz, aromatic proton), 6.99 (1H, dd, J = 8.2 Hz, 2.0 Hz, aromatic proton), 7.32 (1H, t, J = 8.0 Hz, aromatic proton) ppm. MS (EI) m/z [M]+: 256.16.

General procedure for the synthesis of pyrrole-3-carboxylic acids (3a,b). Example: Synthesis of 2,5-dimethyl-1-(3-morpholinophenyl)-1H-pyrrole-3-carboxylic acid (3b): m.p. 178–180 °C (toluene/acetonitrile)

1H-NMR (d6-DMF, 400 MHz, δ; ppm): δH 1.56 (6H, m, piperidine protons), 1.94 (3H, s, C(5)CH3 pyrrole), 2.21 (3H, s, C(2)CH3 pyrrole), 3.19-3.21 (4H, m, piperidine protons), 6.20 (1H, s, C(4)H pyrrole), 6.59 (1H, d, J = 7.6 Hz, aromatic proton), 6.75 (1H, bs, aromatic proton), 7.30 (1H, dd, J = 8.2 Hz, 2.0 Hz, aromatic proton), 7.53 (1H, t, J = 8.0 Hz, aromatic proton), 11.51 (1H, bs, COO-H) ppm. MS (EI) m/z [M]+: 298.17.

General procedure for the synthesis of pyrrole amides (MC4040 and MC4041). Example: Synthesis of N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-2,5-dimethyl-1-(3-morpholinophenyl)-1H-pyrrole-3-carboxamide (MC4040).

In a flame dried round bottom flask, 2,5-dimethyl-1-(3-morpholinophenyl)-1H-pyrrole (0.43 mmol, 1.0 eq, 0.13 g) was added to the reaction solution, and the reaction was stirred under stirring at room temperature for 40 min. After this time, 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one hydrochloride (MC4041) (0.37 mmol, 1.1 eq, 69 mg) was added to the solution and the reaction was stirred under N2 at 80 °C for 4 h. After this time, the reaction was complete, and it was quenched by addition of 3 mL of a saturated aqueous solution of NaCl. The product precipitated, and it was filtered and rinsed with distilled water. The solid was dried and further purified by silica gel chromatography eluting with the chloroform:methanol (35:1) mixture, to give MC4041 (0.18 mmol, 76.7 mg, 53%). m.p. 178-180 °C (acetonitrile).

Chemical and physical characterization of N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-2,5-dimethyl-1-(3-piperidin-1-yl)phenyl)-1H-pyrrole-3-carboxamide (MC4041)

1H-NMR (d6-DMF, 400 MHz, δ; ppm): δH 1.54–1.60 (6H, m, piperidine protons), 1.92 (3H, s, C(5)CH3 pyrrole), 2.11 (3H, s, CH3 dimethylpyridine), 2.18 (3H, s, CH3 dimethylpyridine), 2.18 (3H, s, CH3 dimethylpyridine), 2.21 (3H, s, CH3 dimethylpyridine), 2.21 (3H, s, CH3 dimethylpyridine), 2.31 (4H, t, J = 4.6 Hz, morpholine protons), 3.72 (4H, t, J = 4.6 Hz, morpholine protons), 4.22 (2H, d, J = 5.2 Hz, -CH2-NHCO-), 5.84 (1H, s, dimethylpyridone CH), 6.26 (1H, s, C(4)H pyrrole), 6.64 (1H, d, J = 8 Hz, aromatic proton), 6.76 (1H, s, aromatic proton), 7.03 (1H, d, J = 7.2 Hz, aromatic proton), 7.34-7.39 (2H, m, aromatic proton and -CH2-NHCO-), 11.47 (1H, bs, -NH- pyrrole) ppm. MS (EI) m/z [M]+: 434.23.

Chemical and physical characterization of N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-2,5-dimethyl-1-(3-piperidin-1-yl)phenyl)-1H-pyrrole-3-carboxamide (MC4041):
CH₃ dimethylpyridone), 2.21 (3H, s, C(2)CH₃ pyrole), 3.18-3.20 (4H, m, piperidine protons), 4.22 (2H, d, J = 5.2 Hz, -CH₂NHCO-), 5.86(1H, s, dimethylpyridone CH), 6.25 (1H, s, aromatic proton), 6.55 (1H, d, J = 7.6 Hz, aromatic proton), 6.70 (1H, bs, aromatic proton), 7.01 (1H, aromatic proton), 7.34 (1H, t, J = 8 Hz, aromatic proton), 7.40 (1H, t, J = 5.2 Hz, -CH₃NHCO-), 11.48 (1H, bs, -NH- pyridone). m.p. 135-137°C (benzene); Yield = 55%. MS (EI) m/z [M]+: 432.25.

Biochemistry

**EZH2/PRC2 complex assay**

The EZH2 substrate (0.05 mg/mL core histone) was added in the freshly prepared reaction buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% DMSO). The PRC2 complex [complex of human EZH2, human EED, human SUZ12, human AEBP2, and human RbAp48] was delivered into the substrate solution and the mixture was mixed gently. Afterwards, the tested compounds dissolved in DMSO were delivered into the enzyme/substrate reaction mixture by using Acoustic Technology (Echo 550, LabCyte Inc. Sunnyvale, CA) in nanolitre range, and 3H-SAM was also added into the reaction mixture to initiate the reaction. The reaction mixture was incubated for 1 h at 30°C and then it was delivered to filter-paper for detection. The data were analysed using Excel and GraphPad Prism software for IC₅₀ curve fits.

**EZH1/PRC2 complex, G9a, PRMT1, and DNMT1 assays**

The appropriate methyltransferase (MT) substrate (0.05 mg/ml core histone for EZH1 complex, 5µM histone H3 (1-21) peptide for G9a, 5 µg histone H4 for PRMT1, and 0.001 mg/ mL poly(dI-dC) for DNMT1) was added in freshly prepared reaction buffer (50 mM Tris-HCl (pH 8.5), 5 mM MeCl₂, 50 mM NaCl, 0.01 % Brij35, 1 mM DTT, 1 % DMSO). The MT enzyme was delivered into the substrate solution and the mixture was mixed gently. Afterwards, the tested compounds dissolved in DMSO were delivered into the enzyme/substrate reaction mixture by using Acoustic Technology (Echo 550, LabCyte Inc. Sunnyvale, CA) in nanolitre range, and 3H-SAM was also added into the reaction mixture to initiate the reaction. The reaction mixture was incubated for 1 h at 30°C and then it was delivered to filter-paper for detection. The data were analysed using Excel and GraphPad Prism software for IC₅₀ curve fits.

**Molecular modelling**

Molecular docking simulations were run on MC4041 and MC4040 modelled conformation to gather information on their likely binding modes into EZH2. To this, eight experimental structures of human PRC2 in complex with several inhibitors (pdb entry codes: 4w2r, 5ch2, 5ij7, 5ls6, 5ch1, 5hy1, 5ij8 and 6b3w) were retrieved from PDB [22] (www.rcsb.org). PRC2 co-crystallized complexes were structurally inspected and experimentally missing residues were modelled by means of the programme MODELLER ver 9.19 [23]. To avoid insertion of too unfolded parts, only 10 of the about 80 initial missing residues were manually modelled. The internal gaps were all automatically filled similarly as described in the MODELLER manual. The modelled complexes were then subjected to single-point energy geometry optimization in explicit water and the minimized complexes. Cross-docking experiments were performed with the programme PLANTS [24] using the ChemPLP scoring functions. Chemical compounds were parameterized with AM1-BCC [25] partial charges and GAFF v2.1 [26] Lennard-Jones and valence parameters, using AmberTools 17 antechamber and parmtop files [27, 28]. The OPC [29] water model and AMBER14SB [30] forcefield were used during complexes minimizations. By means of molecular mechanics/generalized Born surface area (MMGBSA) method [31], the protein-ligand binding free energies were evaluated. MarvinSketch 17.1.16 (http://www.chemaxon.com) was used for drawing the ligand structures, which were then converted in 3D-Structures with OpenBabel 2.4.1 [32] employing MMFF94 [33] force field.

**In cell studies**

**Cell culture and treatments**

Three human GBM primary cell cultures (GL1, GL2, GL3) were established from surgical tissues of three different patients, and in addition, one human normal dermal fibroblast primary culture (HF) was obtained. All the primary cell cultures were prepared as previously described [34]. Additionally, a human GBM cell line U-87 MG (ATCC HTB-14), here indicated as U-87, was utilised. The U-87 cell line was grown in EMEM medium supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, 50 U/mL penicillin-streptomycin, 1 mM non-essential amino acid, 1 mM sodium pyruvate. GL1, GL2, GL3 and HF were grown in DMEM medium supplemented with 10% FCS, 2 mM glutamine, 50 U/mL penicillin-streptomycin. MC 4040 and MC 4041 were solubilized in dimethylsulfoxide (DMSO) (Sigma-Aldrich) at 10 mM stock solution and utilized to final concentrations from 10 to 50 µM. Control cells were treated with equivalent amounts of DMSO in each experiment. Stauroporine (Sigma-Aldrich) was dissolved in DMSO in a 1 mM stock solution and used at 5 µM for 6 h. Tazemetostat was solubilized in a 10 mM DMSO stock solution and utilized at 25 µM. Temozolomide was...
solubilized in a 100 mM DMSO stock solution and utilized at 100 μM.

Cytotoxicity assay
To determine cytotoxicity, sulforhodamine B colorimetric assay was performed: 1.5 × 10⁴ cells were plated on 96 well plates, grown for 24 h and treated with different concentrations of MC4040 and MC4041 (10, 25, and 50 μM), with tazemetostat (25 M) and/or with temozolomide (100 μM) for 48, 72, 96 and 120 h. Control cells were treated with equivalent amounts of DMSO. Cells were then fixed with 50% trichloroacetic acid for 1 h at 4 °C and stained for 30 min at room temperature with 0.4% sulforhodamine B in 1% acetic acid. Excess dye was removed by washing four times with 1% acetic acid. Protein-bound dye was dissolved in 10 mM TRIS pH 10, and optical density (OD) was determined at 510 nm using a microplate reader.

Flow cytometry analysis
GLI1 and U-87 cells were seeded in 60-mm culture plates and grown for 48 h untreated or treated with 25 μM MC4040 and MC4041. The cells were harvested by trypan-blue, washed twice with cold PBS, and fixed in 70% ethanol at 4 °C overnight. After fixation, the cells were rinsed with PBS and incubated with 10 mg/mL RNase A and 1 mg/mL propidium iodide (Sigma-Aldrich) at room temperature for 2 h in the dark. The cell cycle distribution was measured and analysed by FACS Calibur flow cytometry using the Cell Quest analysis programme to detect the distribution of subpopulations through the cell cycle. Each experiment was performed in triplicate.

Western blot analysis
Cell lysates were obtained scraping the cells in lysis buffer 1% Triton, 0.1% SDS, 150 mM NaCl, 50 mM TRIS-HCl pH 7.4, 2 mM EDTA plus protease inhibitor cocktail tablet (Roche Applied Sciences) for 30 min at 4 °C. The lysates were then centrifuged at 12,000 rpm for 15 min at 4 °C. The protein concentration was evaluated by Bio-Rad Protein Concentration Assay. Samples of lysate (100 μg) were separated by molecular weight on 10 or 12% SDS-PAGE and then transferred into a nitrocellulose membrane. Blots were blocked for 1 h at room temperature in 5% non-fat dry milk and then incubated overnight at 4 °C with rabbit polyclonal anti-H3K27me3 antibodies (cell signalling technology) diluted 1:1000 in 5% BSA, 0.1% Tween 20 TRIS-buffered saline, or alternatively for 1 h at room temperature with all the other primary antibodies in TRIS-buffered saline. The primary antibodies were the following: mouse anti-Parp1 (1:500 diluted) (Santa Cruz Biotechnology); rabbit anti-p21 Waf1/Cip1 (1:1000 diluted) (Cell Signaling); mouse anti-p27 (1:500 diluted) (Santa Cruz Biotechnology); rabbit anti-β-actin (1:1000 diluted) (Sigma-Aldrich) rabbit anti-E-cadherin (1:1000 diluted) (Gene Tex); rabbit anti-N-cadherin (1:1000 diluted) (Gene Tex); rabbit anti-MMP-2 (1:1000 diluted) (Gene Tex); rabbit anti-MMP-9 (1:500 diluted) (Biomol); mouse anti-TGF-β (1:500 diluted) (Santa Cruz Biotechnology); rabbit anti-TNF-α (1:1000 diluted) (Elabscience); mouse anti-IL-6 (1:500 diluted) (Santa Cruz Biotechnology); mouse anti-IL-10 (1:500 diluted) (Santa Cruz Biotechnology). Blots were washed in TRIS-buffered saline with 0.1% Tween 20 and then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:5000 diluted) (Sigma-Aldrich). The filters were then developed by enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Thermo Scientific) using Kodak X-Omat films. The densitometry and quantitation of the bands was performed using Image J software.

Immunofluorescence
The cells were grown directly on Labteck chamber slides (Nunc) for 48 h and then used untreated or treated for 48 h with 25 μM MC4040 or MC4041. The cells were then washed with PBS with Ca/Mg and fixed with absolute cold methanol for 5 min (only for nestin staining) or with 3% buffered paraformaldehyde for 20 min at 4 °C for all other antibodies. The cells were incubated with 3% bovine serum albumin for 1 h at room temperature and then with anti-glial fibrillary acidic protein (GFAP) rabbit antibody (1:200 diluted, Abcam) or anti-vascular endothelial growth factor (VEGF) mouse antibody (1:100 diluted, Santa Cruz Biotechnology) or anti-VEGF-receptor1 (VEGFR1) mouse antibody (1:100 diluted, Abcam) for 1 h at room temperature; with anti-nestin rabbit antibody (1:200 diluted, Abcam) overnight at 4 °C. Cells were then washed twice with PBS with Ca/Mg and then incubated with the secondary anti-rabbit antibody Alexa Fluor 594 conjugated (Invitrogen, 1:400 diluted) or anti-mouse antibody Alexa Fluor 488 conjugated (Invitrogen, 1:200 diluted) for 1 h at room temperature. The cells were finally washed twice with PBS with Ca/Mg, mounted with Prolong Antifade reagent (Life Technologies) and analysed by a fluorescence microscope (Olympus BX52); imagine acquisition and processing were conducted by IAS 2000 software.

Invasion assay
Invasion assay was performed with Bio Coat Matrigel Invasion Chambers (Corning), consisting of inserts with an 8 μm pores size membrane that was previously treated with Matrigel matrix. For invasion assay 2.5 × 10³/mL cells were plated in serum-free medium plus vehicle DMSO or in serum-free medium plus 20 μM MC4040 and MC4041 in the insert chamber, the lower chamber instead contained only complete medium (with FCS).
After 48 h of culture at standard conditions, the inserts were washed with PBS with Ca/Mg and fixed by 100% methanol for 20 min at 4 °C, then washed twice with PBS with Ca/Mg and stained for 20 min at room temperature with crystal violet. The inserts were then mounted on a slide with glycerol and the cells that migrated through the filter pores to the lower side of the membrane were counted by an optical microscope (Olympus BX52). Imagine acquisition and processing were conducted by IAS 2000 software.

Statistical analysis and graphic programmes

All results were analysed by ANOVA, and the significance was evaluated by the Tukey HSD post hoc test (Honestly Significant Difference). All figures were elaborated with Adobe Photoshop CS5 and all graphs with Graph Pad Prism 5.0.

Results

Synthesis of MC4040 and MC4041

The synthesis of MC4040 and MC4041 started with the preparation of the pyrrole key intermediate 1 through a Paal-Knorr reaction (Scheme 1). More in detail, 2,5-hexanedione was treated with 3-bromoaniline in the presence of acetic acid to afford the 1-(3-bromophenyl)-2,5-dimethyl-1H-pyrrole 1, which underwent a Buchwald-Hartwig reaction by using morpholine or piperidine, palladium (II) acetate (Pd(OAc)₂) as catalyst and tri-tert-butyolphosphonium tetrafluoroborate (PH(tBu)₃BF₄) as ligand, providing 2a and 2b, respectively, in good yields (Scheme 1) [35]. The subsequent Friedel-Craft acylation performed on 2a,b with trichloroacetyl chloride gave the corresponding crude 3-trichloroacetyl pyrroles, which were directly converted into the related 3-pyrrolecarboxylic acids 3a,b (Scheme 1). Further coupling of the acids 3a,b, activated with O-(benzotriazole-1-y1)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) with the 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one hydrochloride 4 [21] in the presence of triethylamine yielded the final compounds MC4040 and MC4041 (Scheme 1).

Assessment of EZH2 inhibition activity and selectivity against other methyltransferases

The newly synthesized compounds have been screened in a 10-dose IC₅₀ mode with 2-fold serial dilution starting from 200 μM solutions, in an in vitro enzymatic assay against a human five component PRC2 complex, containing EZH2, EED, SUZ12, RbAp48, and AEBP2, to evaluate their ability to inhibit the EZH2 catalytic activity. The assay was performed using core histone as substrate and L-SAM as co-substrate. S-Adenosyl-L-homocysteine (SAH), GSK126 and tazemetostat were used as reference compounds. Compounds MC4040 and MC4041 proved to inhibit EZH2 displaying IC₅₀ values in the low micromolar range (Table 1). Both compounds proved to inhibit also the PRC2-EZH1 complex in vitro, displaying however 10-fold higher IC₅₀ values (Table 1). When tested against other SAM-dependent methyltransferases such as G9a (another lysine methyltransferase), PRMT1 (an arginine methyltransferase), and DNMT1 (a DNA methyltransferase), no inhibition was recorded for both compounds up to 200 μM. Only MC4041 showed a slight PRMT1 inhibition (11.9%) at 200 μM.

Molecular modelling studies

Molecular modelling studies were performed to highlight any difference in the binding mode of the two novel compounds. Cross-docking experiment revealed that the programme PLANTS is suitable to perform docking experiments for potential ligands on PRC2 as a docking accuracy of 90% was achieved [36]. MC4040 and MC4041 lowest energy docking conformations were associated with the PRC2 protein obtained from the

Scheme 1 Reagents and conditions: (a) glacial acetic acid, sealed tube, 100 °C, 1.5 h (35-80%); (b) Pd(OAc)₂, Pd(tBu)₃BF₄, potassium tert-butoxide, dry toluene, sealed tube, N₂, 80 °C, 4 h (76-80%); (c) trichloroacetyl chloride, dry 1,2-dichloroethane, sealed tube, 0 °C to 70 °C, 1 h; (d) I. 2 N KOH (aq), EtOH, r.t. to 60 °C; II. 2 N HCl (aq) (54-73%); (e) triethylamine, TBTU, dry DMF, N₂, r.t., 4 h (52-75%)

MC4040 X= O
MC4041 X= CH₂
6b3w complex. Visual inspection of MC4040 and MC4041 docked conformations revealed that although structurally very similar, the two molecules only partially share their binding modes (Fig. 2). In more detail, while the two pyridone and the carboxamide moieties are completely overlapped, the rest of the two molecules assume conformations that tend to gradually separate. In particular, MC4040 and MC4041 pyridone moieties interact with Gly520, Trp51, Phe583, Ala584, Asn585 and Tyr623 residues (Fig. 2), constituting a sub-pocked that lays in the EZH2 SAM binding site (not shown). Hydrogen bonds (not shown) are possible among Gly520 oxygen and the inhibitors’ pyridone NHs (distance O\textsubscript{Gly520}···N\textsubscript{inhibitors} = 2.886 Å) and among Trp521 amidic NH and inhibitors’ pyridone carbonyl oxygens (distance N\textsubscript{Trp521}···O\textsubscript{inhibitors} = 3.029 Å). The central pyrrole-3-carboxamides portions, almost overlapped, also share the same interactions, and are trapped in a rounded hydrophobic cage formed mostly by Ile109, Met110, Tyr111, Phe665, Thr678 and Arg685 side chains (see magenta surfaces in Fig. 2). Regarding the phenylpiperidine and phenylmorpholine portions of MC4040 and MC4041 inhibitors, the docked conformations indicate the MC4040 morpholine group to be stuck in a sub-pocked defined by Ser112, His213, Val657, Thr678, and Arg679. Differently, the piperidine of the MC4041 pose was shifted and buried in a hydrophobic environment surrounded by His3, Arg236, Tyr658, and Tyr661.

Although the overall interaction pattern of either MC4040 or MC4041 seem quite similar, the piperidine of compound MC4041 seems to establish a larger number of positive van der Waals interactions accounting at least in part for its higher IC\textsubscript{50} inhibition potency. By means of molecular mechanics generalized Born surface area (MMGBSA) method [37], the protein-ligand binding free energies were evaluated for all the minimized complexes (Table 2). Analysis of protein-ligand binding free energies (Table 2) showed that MC4041 binds to EZH2 with a better energetic profile than MC4040, in agreement with the experimental biochemical IC\textsubscript{50} values.

### Effects of MC4040 and MC4041 on GBM cell proliferation

At the beginning of our studies, we collected patient-derived GBM cells, establishing three primary cell cultures (GL1, GL2, GL3). First, the homogeneity of the neoplastic phenotype of the three human GBM primary cell cultures was determined. Each culture was characterized by immunofluorescence staining of two typical GBM markers: GFAP and nestin. 100 % of cells from GL1, GL2, and GL3 resulted positive for both markers (Fig. 3).

 Afterwards, we investigated the effects of the two novel EZH2 inhibitors on cell viability and replication in all the three GBM primary cultures (GL1, GL2, and GL3) and in the GBM cell line U-87. Additionally, a primary culture of human dermal fibroblasts, HF cells, was used as control of somatic not transformed cells. To this scope, GL1, GL2, GL3, U-87, and HF cells were treated for 24, 48, 72, or 96 h with 10, 25, or 50 μM doses of MC4040 or MC4041, separately. After 24 (data not shown) and 48 h of treatment, no effect or a slight decrease of cell viability was evident at the highest doses with both compounds. For longer incubation times (72 and 96 h), the decrease in cell viability became significant also at 10 μM (Fig. 4). Notably, none of the two inhibitors displayed any significant effect on HF proliferation, even after 96 h of treatment at the highest dose (Fig. 4). Based on these data, all the following experiments were performed using MC4040 and MC4041 at the concentration of 25 μM for 72 h.

### Compounds MC4040 and MC4041 reduce H3K27me3 levels in GBM cells

In order to confirm an effective inhibition of EZH2 by MC4040 and MC4041 in a cellular context, U-87, GL1 and HF were treated with DMSO (ctr), or with MC4040, or with MC4041 (both at 25 μM for 72 h), and the levels of H3K27me3 were analysed by western blot. Interestingly, H3K27me3 basal levels were upregulated in GL1 cells when compared to U-87 cells, while no H3K27me3 was detectable in dermal HF cells, as expected (Fig. 5). MC4040 and MC4041 treatment determined an evident downregulation of H3K27me3 in both GBM U-87 and GL1 cells (Fig. 5a), confirming the inhibitory control exerted by both molecules on EZH2. In a time-course experiment, MC4041 while showing no effect after 24 h treatment, reduced H3K27 trimethylation in a time-dependent manner over 72 h (Fig. 5b).

### MC4040 and MC4041 potentiate the effects of temozolomide on GBM cell proliferation

Temozolomide is the sole chemotherapeutic agent available for GBM treatment. Here, we compared the effects...
of EZH2 inhibition by MC4040, MC4041, or by the clinically studied inhibitor tazemetostat with those of temozolomide. Therefore, U-87 and GL1 were treated either with MC4040 (25 μM), MC4041 (25 μM), tazemetostat (25 μM), or temozolomide (100 μM) for 72, 96, and 120 h. As shown in Fig. 6, the effect of all the three tested EZH2 inhibitors on cell replication was comparable to the effect obtained with temozolomide at all the tested incubation times in both the GBM cell lines.

To check if EZH2 inhibitors in combination with temozolomide could potentiate its effects, we also performed a combination study with each of the three EZH2i in U-87 and GL-1 cells in the same time range observed for the single drug treatment. Pleasingly, the combination had much stronger, but not additive, effects on cell viability already after 72 h, reaching 60% reduction after 96 and 120 h (Fig. 6). Despite the higher potency of tazemetostat in biochemical assays, its effects on the viability of the tested cell lines were comparable with those of our two inhibitors, also when used in combination with temozolomide.

Table 2 MMGBSA calculated binding energies (ΔG) for MC4040 and MC4041

| Ligand   | IC50 (μM) | GBSA ΔG (kcal/mol) |
|----------|-----------|---------------------|
| MC4040   | 4.06      | -23.0432            |
| MC4041   | 1.06      | -30.4428            |

ML4040 and MC4041 arrest GBM cell cycle in G0/G1 phase without apoptosis induction

In further investigation, we decided to better define the mechanism at the basis of GBM cell proliferation arrest by MC4040 and MC4041. We started by checking whether these EZH2 inhibitors induced apoptosis, through the evaluation of poly ADP-ribose polymerase 1 (PARP-1) expression. As shown by western blot analysis, nor MC4040 neither MC4041 induced PARP-1 cleavage up to 120 h of treatment in GL1 cells (Fig. 7a). As a positive control, the treatment with staurosporine (ST), a strong inducer of apoptosis, determined typical PARP-1 cleavage in the same cell line. (Figure 7a).

Once determined that MC4040 and MC4041 did not induce apoptosis, we analysed the effects of these compounds on two negative regulators of cell cycle, p21 and p27, which are cyclin-dependent kinase inhibitors. We found that the basal expression of both p21 and p27 was quite low in GL1 and U-87 cells, while both markers were significantly upregulated after MC4040 treatment (25 μM, 72 h) in both cell lines (Fig. 7b). Additionally, flow cytometry cell cycle analysis revealed an accumulation of U-87 and GL1 cells in the G0/G1 phase after treatment with MC4040 or MC4041 (25 μM, 72 h) (Fig. 7c). These results suggested that MC4040 and MC4041 arrest cell proliferation at the G0/G1 phase by increasing p21 and p27 levels and do not induce apoptosis. Our
Fig. 3 Immunofluorescence of GL1, GL2 and GL3 for GFAP and for nestin.

Fig. 4 Cell viability of untreated (ctr) and MC4040- and MC4041-treated cultures expressed as percentage of alive cells. Treatments were performed at 48 h (black), 72 h (dark grey), and 96 h (light grey). *p < 0.05 and **p < 0.01
findings strengthen the existing literature evidences documenting a G0/G1 cell cycle arrest after EZH2 inhibition in GBM cells [10, 38].

**MC4040 and MC4041 impair GBM aggressive and invasive phenotype by blocking neo-vascularization processes and reverting the EMT**

Afterwards, we investigated in depth the molecular effects of our described compounds on U-87 and/or GL1 cells, to determine whether they could affect GBM aggressiveness and invasiveness. GBM is characterized by a proangiogenic and proinflammatory microenvironment [5]. Typically, neoangiogenesis is supported by high levels of secreted vascular endothelial growth factor (VEGF) [39]. Together with its “classical” functions, VEGF is also increasing the number of infiltrative macrophages participating in the establishment of an immuno-suppressive microenvironment through the release of inhibitory cytokines [40]. Moreover, it has been shown that in the blood vessels of high-grade GBM the VEGF/VEGFR1 levels are higher when compared with lower grade gliomas [41]. Interestingly, VEGFR1 is expressed by endothelial cells during vessel formation and remodelling, but it does not have a relevant role in physiological angiogenesis in adults [42]. Accordingly, VEGFR1 signalling has been related to tumour growth, progression, and metastasis, being involved in apoptosis inhibition and chemoresistance induction [43, 44]. In time, the VEGF/VEGFR1 signalling cascade has been studied in G5M and it was proven an interesting target to hit. Hence, we questioned ourselves if our EZH2 inhibitors could indirectly hit also this important pathway. We determined the expression of VEGFR1 and VEGF in untreated and MC4040-treated U-87 cells, expressing detectable basal protein levels of VEGF and its receptor VEGFR1, and we found that MC4040 was able to strongly reduce the levels of both proteins (Fig. 8).

In parallel, the effect of MC4040 and MC4041 on epithelial to mesenchymal transition (EMT) was analysed determining, in GL1 and U87 cells, the levels of E-cadherin as epithelial marker, of N-cadherin as mesenchymal marker, and of matrix metalloproteinases (MMPs), specifically MMP2, MMP3, and MMP9, usually highly expressed in the majority of aggressive tumours and responsible for tumour invasiveness [15]. In these assays, MC4040 and MC4041 upregulated E-cadherin and downregulated N-cadherin, thus reverting the EMT process (Fig. 9a). Furthermore, MC4040 and MC4041 treatment decreased the expression of all the tested MMPs in U-87 and GL1 cells (Fig. 9a). In order to quantify the final readout of the effect of MC4040 and MC4041 on GBM cell invasiveness, we performed a Matrigel invasion assay. In both U-87 and GL1 cell lines the basal rate of motility was very high. The treatment with MC4040 and MC4041 markedly attenuated this malignant attitude (Fig. 9b, c), reducing the invasion capacity up to 4-fold. Taken together, our results

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**Fig. 5**

a Western blot of U-87, GL1 and HF treated with DMSO (ctr) or with 25 μM MC4040 and MC4041 for 72 h showing H3K27me3 and β-actin levels. The means from densitometry quantifications of three different experiments normalized to β-actin are indicated below each band. ND, not detectable. b Time-course experiment performed treating U-87 and GL1 cells with MC4041 (25 μM) for 24, 48, and 72 h. The levels of H3K27me3 are shown (mean from three different experiments) and have been normalized to β-actin.
demonstrate that our EZH2 inhibitors played an important role in reducing the angiogenic and invasive phenotype of GBM cells.

MC4040 and MC4041 display anti-inflammatory effects in GBM cells

Several different cancer types, including GBM, benefit of a chronically active inflammatory microenvironment, providing the proliferative and mutational incentive, necessary to enable the tumor to uncontrollable development. Histone methylation has been reported to epigenetically control the innate and adaptive immune responses [45]. Recently, several studies demonstrated that EZH2 plays a key role in inflammatory and autoimmune disorders. More in detail, EZH2 was reported to regulate cell adhesion and migration, with possible implications in leukocyte migration and immune responses [46], in production of inflammatory cytokines in dental pulp cells [47] or in macrophages [48], in regulation of microglial inflammatory gene expression [49], and in adaptive resistance to tumor immunotherapy [50]. The present evidences surely depict an articulated scenario in which EZH2 is a main regulator of inflammatory and immune processes at various levels.

To evaluate if EZH2 inhibition in GBM could impact on inflammation, and to gain further insight into the molecular mechanism of MC4040 and MC4041, we investigated on the expression patterns of the main inflammatory and anti-inflammatory cytokines in U-87 and GL1 cells in untreated and in MC4040- and MC4041-treated samples. As expected, both U-87 and GL1 expressed basally high levels of the pro-inflammatory cytokines TGF-β, TNF-α and IL-6, and very low levels of the anti-inflammatory cytokine IL-10 (Fig. 10). The treatment with either MC4040 or MC4041 determined a significant decrease of TGF-β, TNF-α, and IL-6 and a significant increase of IL-10 (Fig. 10). These data show...
that U-87 and GL1 cells have a strong pro-inflammatory phenotype, that could contribute to their aggressiveness and proliferation. Here we provided a first evidence of the possibility to attenuate this attitude by treatment with the EZH2 inhibitors MC4040 and MC4041.

**Discussion**

Several different factors still obstacle the development of a proper cure for GBM, that remains the most common and aggressive malignant primary brain tumour in adults. To date, the sole chemotherapeutic agent in use against GBM is the alkylating drug temozolomide. The standard GBM treatment consists of surgical resection of the tumour, followed by chemotherapy and concomitant radiotherapy [2]. It is worth to point out that the current available treatment is not a cure. Hence, there is an urgent need for alternative and/or complementary therapeutic strategies and targets to hit. The epigenetic mechanisms underlying GBM development and progression are being elucidated and clarified. The SAM-dependent histone methyltransferase EZH2 has been found upregulated in gliomas and correlated with an aggressive phenotype [10, 11], while its depletion by RNA interference resulted in anti-glioma effects [12]. Importantly, some known EZH2 inhibitors have been already tested against GBM models in vitro and in vivo, confirming the biological evidences assessing the relevance of EZH2 as target in this disorder [16]. Here, we report the effects on GBM, including primary GBM cell cultures, of two novel EZH2 inhibitors, MC4040 and MC4041, containing a N-phenylpyrrole core decorated with a cyclic amine (morpholine, MC4040, or piperidine, MC4041), often present in known EZH2i, and carrying the 2-pyridone moiety known to be crucial for the EZH2 inhibiting activity [51]. When tested against a human five-component PRC2 complex, MC4040 and MC4041 displayed single-digit micromolar IC$_{50}$ values against EZH2-PRC2 with a 10-fold selectivity over EZH1-PRC2. Importantly, the two novel compounds proved to be selective for EZH2/EZH1 over a panel of SAM-dependent methyltransferases including DNMT1, G9a, and PRMT1, displaying no inhibition of these targets up to 200 $\mu$M. Molecular docking and MMGBSA calculations helped us to shed light on the differences in the binding mode of the two newly synthesized compounds, explaining also the difference in activity recorded in biochemical assays. The two novel compounds have been tested on the primary GBM cell cultures GL1, GL2, and GL3 as well as on the GBM cell line U-87, displaying a dose- and time-dependent reduction of cell viability, without significant effect on human normal dermal fibroblast (HF) primary culture. Interestingly, the effects of the two newly synthesized EZH2 inhibitors were comparable to those of the clinically studied inhibitor tazemetostat, tested at the same doses and times. The MC4040- and MC4041-mediated EZH2 inhibition in U-87 and GL1 cells was confirmed by the decrease of the H3K27me3 levels, basally increased in both cell lines.
Additionally, the combination of EZH2 inhibition with the standard GBM chemotherapy (temozolomide) was tested. The combination yielded much stronger, but not additive, effects with a 60% reduction of cell viability after 72 h. We have also found that MC4040 and MC4041 impair GBM cell viability by arresting cell proliferation at the G0/G1 phase by increasing p21 and p27 levels without inducing apoptosis. Our findings are in agreement with the existing literature evidences, documenting a G0/G1 cell cycle arrest after EZH2 inhibition in GBM cells [10, 38]. Additionally, we validated EZH2 as a valuable target to attenuate GBM malignant and aggressive phenotype. Hence, MC4040 and MC4041 treatment in GBM cells reduced VEGFR1 and VEGF expression, upregulated E-cadherin and downregulated N-cadherin and MMPs, reverting the EMT process. Accordingly, in a Matrigel invasion assay, the treatment with MC4040 and MC4041 markedly attenuated GBM cells motility, reducing the invasion capacity up to 4 folds respect to untreated cells. Noteworthy, to the best of our knowledge, we have shown for the first time that EZH2 inhibition impairs the pro-inflammatory GBM phenotype, that usually sustains and strengthens tumour development and aggressiveness. Specifically, the treatment of U-87 and GL1 cells with MC4040 or MC4041 determined a significant decrease of all the three tested pro-inflammatory TGF-β, TNF-α, and IL-6, as well as a significant increase of the anti-inflammatory IL-10 cytokines expression. Currently, we are working to shed light on the precise mechanism through which EZH2 inhibition impairs GBM aggressiveness and how it can impact on tumour-mediated immunomodulation and inflammation. We wish that our findings will stimulate and motivate new researches in this field, clarifying the complex molecular pathways and mechanisms at the bases of EZH2-mediated effects in GBM.

Conclusions

In conclusion, the present study describes the design and synthesis of two novel and selective EZH2 inhibitors able to reduce H3K27 methylation levels in U-87 and GL1 GBM cells. MC4040 and MC4041 dose- and time-dependently impaired GBM cell viability arresting the cell cycle in the G0/G1 phase and potentiated the effects of temozolomide. We proved that EZH2 inhibition by MC4040 and MC4041 attenuates cancer
Fig. 9  
a Western blot of EMT and invasion related proteins: E-cadherin, N-cadherin, MMP-2, MMP-3, and MMP-9. MC4040 and MC4041 were used at 20 μM for 48 h. The densitometry quantitation is indicated for each band normalized for actin band. 
b Matrigel invasion assay of U-87 and GL1 cells untreated (ctr) and treated with 20 μM MC4040 or MC4041 for 48 h. 
c Migrated cells were counted in five random fields. Three independent experiments were performed, and the results are presented with the mean ± SD. **p < 0.01

Fig. 10  
Western blot of U-87 and GL1 untreated (ctr) and treated with 20 μM MC4040 or MC4041 for 48 h for TGF-β, TNF-α, IL-6, IL-10, and actin. The densitometry quantification is indicated for each band normalized for actin band.
malignant phenotype by reducing VEGFR1/VEGF expression, reverting the EMT process and inhibiting invasiveness. To the best of our knowledge, here, we show for the first time that targeting EZH2 in GBM displays anti-inflammatory effects, further strengthening the relevance of this target in this cancer type.

Abbreviations
AEBP2: Adipocyte enhancer-binding protein 2; BSA: Bovine serum albumin; DNMT: DNA methyltransferase; EZH1: Enhancer of zeste homolog 1; EZH2: Enhancer of zeste homologue 2; FCS: Foetal calf serum; G3a (EHTM2): Euchromatic histone-lysine N-methyltransferase 2; GBM: Glioblastoma; GFAP: Glial fibrillary acidic protein; H3K27: Lysine 27 of the histone 3; H3K27me3: Trimethylated histone-lysine methylation; HF: Human fibroblasts; IL: Interleukin; MB: Medulloblastoma; MDA-MB-231: breast cancer cell line; MVNGBSA: Molecular mechanics generalized Born surface area; MMP: Matrix metalloproteinase; MT: Methyltransferase; NMR: Nuclear magnetic resonance; OD: Optical density; PARP: Poly ADP-ribose polymerase; PBS: Phosphate-buffered saline; PDB: Protein data bank; PMSF: Phenylmethanesulfonyl fluoride; PRC2: Polycomb repressive complex 1; PRMT1: Protein arginine methyltransferase 1; RbAp48: Retinoblastoma-associated protein 48; SAH: S-Acetylguanidino-L-homocysteine; SAM: S-adenosyl-L-methionine; SDS: Sodium dodecyl sulphate; SHH: Sonic Hedgehog; ST: Staurosporine; SUZ12: Polycomb repressive complex 2 subunit; TAZ: Tazemetostat; TBI/α: (Benzotriazole-1-yl)N,N,N',N'-tetramethyluronium tetrafluoroborate; TGF-β: Transforming growth factor β; TLC: Thin layer chromatography; TMZ: Temozolomide; TNF-α: Tumor necrosis factor α; VEGF: Vascular endothelial growth factor; VEGFR1: Vascular endothelial growth factor receptor 1

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All the authors approve the described studies and consented to participate.

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