Carbonic Anhydrase XII Inhibitors Overcome P-Glycoprotein-Mediated Resistance to Temozolomide in Glioblastoma

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Abstract

The role of carbonic anhydrase XII (CAXII) in the chemoresistance of glioblastoma is unexplored. We found CAXII and P-glycoprotein (Pgp) coexpressed in neurospheres derived from 3 of 3 patients with different genetic backgrounds and low response to temozolomide (time to recurrence: 6–9 months). CAXII was necessary for the Pgp efflux of temozolomide and second-line chemotherapeutic drugs, determining chemoresistance in neurospheres. Psammaplin C, a potent inhibitor of CAXII, resensitized primary neurospheres to temozolomide by reducing temozolomide efflux via Pgp. This effect was independent of other known temozolomide resistance factors present in the patients. The overall survival in orthotopic patient-derived xenografts of temozolomide-resistant neurospheres, codosed with Psammaplin C and temozolomide, was significantly increased over temozolomide-treated ($P < 0.05$) and untreated animals ($P < 0.02$), without detectable signs of systemic toxicity. We propose that a CAXII inhibitor in combination with temozolomide may provide a new and effective approach to reverse chemoresistance in glioblastoma stem cells. This novel mechanism of action, via the interaction of CAXII and Pgp, ultimately blocks the efflux function of Pgp to improve glioblastoma patient outcomes. Mol Cancer Ther; 17(12); 2598–609. © 2018 AACR.

Introduction

Glioblastoma (GB) is the most common and lethal adult primary brain tumor. The standard-of-care treatment comprises surgery, followed by radiotherapy and chemotherapy, then maintenance chemotherapy. Chemotherapy is based on the drug temozolomide (TMZ). With treatment, the increase in median survival rate for all patients is 2 months, whereas the median overall survival is 12 to 15 months (1). In the subset of GB patients with chemotherapy (IHC). This trend led us to hypothesize that CAXII and Pgp (http://www.proteinatlas.org/ENSG00000074410-CA12/pathology) are highly expressed in 3D culture of GB cells (neurospheres, giving rise to GB niches, e.g., SC-enriched and Pgp (http://www.proteinatlas.org/ENSG00000085563-ABCB1/pathology) proteins were poorly detectable by immunohistochemistry (IHC). This trend led us to hypothesize that CAXII and Pgp may be coexpressed in specific GB niches, e.g., SC-enriched, with CAXII maintaining optimum pH for Pgp activity.

CAXII is overexpressed in aggressive GB (10) and is a negative prognostic factor in infiltrating astrocytoma (11). CAXII is also highly expressed in 3D culture of GB cells (neurospheres, NS, ref. 12), a mimic of cancer-derived GB SC. The therapeutic implications surrounding CAXII have been poorly investigated in GB or GB SC. Here, we demonstrate that CAXII mediates resistance to TMZ in GB SC in a Pgp-dependent manner. We show that the combination of a CAXII inhibitor and TMZ...
substantially improves TMZ efficacy against GB SC in GB NS patient-derived xenographs (GB-NS-PDX), and that this effect is independent of known factors of TMZ resistance.

Materials and Methods

Reagents and plasticware

Plasticware for cell cultures was obtained from Falcon (Becton Dickinson). Electrophoresis reagents were obtained from Bio-Rad Laboratories. The protein content of cell lysates was assessed using a BCA kit from Sigma Chemicals Co. Doxorubicin, etoposide, topotecan, and irinotecan were from Sigma Chemicals Co. Unless specified otherwise, all reagents were purchased from Sigma Chemicals Co.

Compounds synthesis and CAXII inhibition

Compounds were synthesized as in Supplementary Information (Scheme S1). CAXII activity was measured as detailed previously (13).

Cells

Primary human GB cells (CV17, 010627, No3) were obtained from surgical samples from Neurosurgical Units, Universities of Torino and Novara after written-informed consent, and were used within passage 5. The samples were designated as patient#1, patient#2, and patient #3. Researchers performing the experiments were unaware of the genetic background or clinical outcome of the patients. The study was performed in accordance with the Declaration of Helsinki and was approved by the Bio-Ethical Committee of University of Torino (#ORTO11WNST). The histologic diagnosis of GB was performed according to World Health Organization guidelines. MGMT methylation was detected by methylation-specific PCR and capillary electrophoresis (14). EGFR amplification, IDH1/2 and TP53 mutations, and 1p/19q codeletion were examined as described in ref. 15. Cells were cultured as differentiated/adherent cells (AC) or NS as previously described (17). Materials.

In vitro cultured GB cells (CV17, 010627, No3) were obtained from surgical samples from Neurosurgical Units, Universities of Torino and Novara after written-informed consent, and were used within passage 5. The samples were designated as patient#1, patient#2, and patient #3. Researchers performing the experiments were unaware of the genetic background or clinical outcome of the patients. The study was performed in accordance with the Declaration of Helsinki and was approved by the Bio-Ethical Committee of University of Torino (#ORTO11WNST). The histologic diagnosis of GB was performed according to World Health Organization guidelines. MGMT methylation was detected by methylation-specific PCR and capillary electrophoresis (14). EGFR amplification, IDH1/2 and TP53 mutations, and 1p/19q codeletion were examined as described in ref. 15. Cells were cultured as differentiated/adherent cells (AC) or NS as previously described (16), with minor modifications (17). For AC, DMEM supplemented with 1% v/v penicillin-streptomycin and 10% v/v FBS (Lonza) was used. For NS, DMEM-F12 medium was supplemented with 1 mol/L HEPES, 0.3 mg/mL glucose, 75 µg/mL NaHCO3, 2 mg/mL heparin, 2 mg/mL BSA, 2 mmol/L progesterone, 20 ng/mL EGF, and 10 ng/mL b-FGF. AC were obtained from dissociated NS cells, centrifuged at 1,200 g for 5 minutes, and seeded in AC medium. In vitro clonogenicity and self-renewal and in vivo tumorigenicity were reported in ref. 3. Cell phenotypic characterization is detailed in the Supplementary Materials. Mycoplasma spp contamination was assessed by PCR every 3 weeks; contaminated cells were discharged.

Immunoblotting

Protein extracts (20 µg) from whole-cell lysate were subjected to SDS-PAGE and probed with the following antibodies: anti-CAXII (goat, #ab219641; Abcam), anti-CAX (rabbit, #ab15086; Abcam), anti-Pgp (mouse, clone C219; Millipore), and anti-caspase 3 (mouse, clone C33, GenTex). Plasma membrane–associated proteins were evaluated in biotinylation assays (7). Anti-β-tubulin (rabbit, # ab6046; Abcam) and anti-pancadhcerin (mouse, clone CH-19; Santa Cruz Biotechnology Inc.) antibodies were used to confirm equal protein loading in whole-cell and plasma membrane–associated extracts. In coimmunoprecipitation experiments, 100 µg of plasma membrane–associated proteins were immunoprecipitated with anti-CAXII and anti-CAIX antibodies, using PureProteome protein A and protein G Magnetic Beads (Millipore).

Flow cytometry

Cells (5 × 106) were resuspended in culture medium containing 5% v/v FBS, incubated with anti-CAXII (Abcam) or anti-Pgp (mouse, clone MRK16; Kamiya) antibody, followed by the secondary Alexa488-conjugated antibody (Abcam), fixed with 4% v/v paraformaldehyde, and analyzed by the Guava easyCyte flow cytometer (InCyte software, Millipore). Control experiments included incubation of cells with nonimmune isotypic antibody, followed by secondary antibody.

Proximity ligation assay

The CAXII–Pgp interaction was measured with the DuoLink In Situ Kit (Sigma Chemicals Co), as per the manufacturer’s instructions. The method employs mouse anti-human Pgp (mouse, clone UIC-2, Millipore) or rabbit anti-human CAXII (#102344; NovoPro) antibodies. Cell nuclei were counterstained with 4’-6-diamidino-2-phenylindole (DAPI). Cells were examind using a Leica DC100 fluorescence microscope (Leica microscope). A minimum of five fields were examined for each experimental condition.

Confocal microscope analysis

NS cells (1 × 105) were seeded onto glass coverslips and collected by cyto-spinning. Cells were fixed using 4% paraformaldehyde for 15 minutes, washed with PBS, and incubated for 1 hour at room temperature with an anti-human CAXII (NovoPro) or an anti-Pgp (Millipore) antibody. Samples were washed 5 × with PBS and incubated for 1 hour with tetrarmethylrhodamine isothiocyanate– or fluorescein isothiocyanate-conjugated secondary antibodies (Sigma Chemicals Co.), respectively, then washed with PBS 4 × and deionized water 1 ×. Cells were examined using a Leica TCS SP2 AOP confocal laser-scanning microscope. The number of yellow pixels, indicative of a Pgp-CAXII interaction, was calculated using the JACoP plug-in of the ImageJ software (https://imagej.nih.gov/ij) and expressed as a percentage of the total green pixels (corresponding to Pgp) measured over a total of five fields per experiment.

Pgp ATPase activity

The assay was performed on Pgp-enriched membrane vesicles as detailed in ref. 18. The rate of ATP hydrolysis, an index of Pgp catalytic cycle and a necessary step for substrate efflux, was measured. Results were expressed as nmol hydrolyzed phosphate (Pi)/min/mg proteins.

Doxorubicin and TMZ accumulation

Doxorubicin content was measured fluorimetrically (7). The results were expressed as nmol doxorubicin/mg cell proteins. TMZ content was measured by liquid scintillation counting in cells incubated with 10 µmol/L [3H]-temozolomide (0.7 μCi/mL; Moravek Biochemical Inc.) for 24 hours. The results were expressed as nmol [3H]-temozolomide/mg cell proteins.

Lactate dehydrogenase release

The extracellular release of lactate dehydrogenase (LDH), considered an index of cell damage, was measured as detailed...
previously (3). The extracellular LDH activity was calculated as a percentage of the total LDH activity in the dish.

**Cell viability**

Cell viability was evaluated using an ATPLite kit (PerkinElmer). The results were expressed as a percentage of viable cells in each experimental condition versus untreated cells (considered 100% viable). To calculate the combination index (CI), NS were incubated with TMZ and compound 1, alone and then in combination, over the range of concentrations 10^{-10} to 10^{-5} mol/L. CI values were calculated using CalcuSyn software (www.biosoft. com/v/calcusyn.htm).

**Generation of Pgp- and CAXII-knocked out clones**

Five \( \times \) 10^6 cells were transduced with 1 \( \mu \)g CRISPR pCas vectors (Origene) targeting ABCB1/Pgp or CAXII, respectively, or with 1 \( \mu \)g nontargeting vector (Origene), following the manufacturer’s instructions. Stable KO cells were selected from medium containing 1 \( \mu \)g/mL puromycin for 4 weeks.

**In vitro plasma stability**

Compound 1 was spiked into mouse plasma (Animal Resource Centre) to a concentration of 1,000 ng/mL (DMSO/acetonitrile concentrations 0.2/0.4% v/v) at 37°C for 4 hours. At various time points, plasma samples were snap-frozen and analyzed by LC-MS (Micromass Xevo triple quadrupole mass spectrometer, Waters Co.) relative to calibration standards (1 and diazepam as internal standard). The average concentration of test compound was expressed as a percentage of compound remaining relative to the sample quenched at 5 minutes.

**In vitro metabolic stability**

Metabolic stability was performed by incubating 1 \( \mu \)mol/L compound 1 with 0.4 mg/mL mouse liver microsomes (Xenotech) at 37°C, adding an NADPH-regenerating system, and subsequently quenching with acetonitrile (containing diazepam as internal standard) at 2, 30, and 60 minutes. A species scaling factor was used to convert the in vitro clearance (CL_in) to an in vivo CL_out (19). Hepatic blood clearance and hepatic extraction ratio \((E_{H})\) were calculated as described (20). \(E_{H}\) was used to classify compounds as low \((<0.3)\), intermediate \((0.3–0.7)\), high \((0.7–0.95)\), or very high \((>0.95)\) extraction compounds.

**In vitro cytochrome P450 (CYP) stability**

Compound 1 (0.25 to 20 \( \mu \)mol/L) was incubated with CYP substrate in human liver microsomes (batch #1410230; Xenotech LLC) at 37°C. The total organic solvent concentration was 0.47% v/v. The reactions were initiated by adding a NADPH-regenerating system and quenched with ice-cold acetonitrile containing analytical internal standard (0.15 \( \mu \)g/mL diazepam). Metabolite concentrations were determined by UPLC-MS (Waters/Micromass Xevo TQD triple-quadrupole) relative to calibration standards prepared in quenched microsomal matrix. The inhibitory effect of compound 1 was assessed based on the reduction in the formation of the specific CYP-mediated metabolite relative to a control for maximal CYP enzyme activity.

**In vivo tumor growth**

In dose-dependent experimental sets, \( 1 \times 10^6 \) AC or NS cells, mixed with 100 \( \mu \)L Matrigel, were injected subcutaneously in female BALB/c nu/nu mice (weight: 19.6 g ± 2.4; Charles River Laboratories Italia, Calco). Animals were housed (5 per cage) under 12-hour light/dark cycles in a barrier facility on HEPA-filtered racks and were fed with an autoclaved diet. Tumor dimensions were measured daily with calipers and growth calculated using the equation \((L \times W^2)/2\), where \(L\) = tumor length and \(W\) = tumor width. When the tumor reached a volume of 50 mm^3, animals were randomized (10 animals/group) and treated over 2 cycles of 5 consecutive days (days: 1–5; 11–15 after randomization) as detailed in Supplementary Fig. S6.

Animals were euthanized by injecting zolazeepam (0.2 mL/kg) and xylazine (16 mg/kg) intramuscular (i.m.) at day 30. Hemocromocytometric analyses were performed with a UniCel DxH 800 Coulter Cellular Analysis System (Beckman Coulter) on 0.5 mL of blood collected immediately after euthanizing, using commercial kits from Beckman Coulter Inc.

In a second experimental set, \( 1 \times 10^6 \) NS cells, stably transected with the pGL4.51[luc2/CMV/Neo] vector encoding for luciferase (Promega Corporation), mixed with 150 \( \mu \)L sterile physiological solution, were stereotactically injected into the right caudate nucleus into 6- to 8-week-old female BALB/c nu/nu mice (weight: 20.3 g ± 2.4), anesthetized with sodium phenobarbital (60 mg/kg) intraperitoneally (i.p.). Tumor growth was monitored by in vivo bioluminescence (Xenogen IVIS Spectrum, PerkinElmer) at days 6, 14, and 24, after implantation. At day 7, animals were randomized (6 animals/group) and treated with 2 cycles of 5 consecutive days (days: 7–11; 17–21 after randomization) as indicated in Fig. 5. Animals were euthanized at day 30. Brains were fixed in 40 \( \mu \)g/mL paraformaldehyde at 4°C overnight. Tumors were excised, and the volume was determined using calipers. Tumor sections were fixed overnight in 4% paraformaldehyde and stained with hematoxylin and eosin or immunostained for CAXII (Abcam), anti-Pgp (Millipore), Ki67 (mouse, clone KiSS; Millipore), and cleaved Ap(Asp)75 caspase 3 (rabbit, #9661; Cell Signaling Technology Inc.), followed by a peroxidase-conjugated secondary antibody (Dako). Stained sections were examined with a Leica DC100 microscope. In parallel, tumor tissue was homogenized for 30 seconds at 15 Hz, using a TissueLyser II device (Qiagen), and clarified at 12,000 \( \times \) for 5 minutes. Protein (10 \( \mu \)g) from tumor lysates was used for the immunoblot analysis of Pgp, as reported above. In a third experimental set, animals with orthotopic tumors were monitored after the treatment detailed in Fig. 5. Animals were euthanized when they showed signs of significantly compromised neurological function or loss of body weight >20%. Overall survival was defined as the time interval between tumor implant and euthanasia.

Animal care and experimental procedures were approved by the Bio-Ethical Committee of the Italian Ministry of Health (#122/2015-PR).

**Statistical analysis**

All data in the text and figures are provided as mean ± SD. The results were analyzed by a Student t test, using Statistical Package for Social Science (SPSS) software (IBM SPSS Statistics v.19). The Kaplan–Meier method was used to calculate overall survival of mice. The log-rank test was used to compare the outcome of the treatment groups, using MedCalc software (v.17.4). \( P < 0.05\) was considered significant. Data analysis was performed blinded.

**Results**

GB-derived NS coexpress CAXII and Pgp

The clinical, genomic, and phenotypic data for the 3 GB patients of this study are provided in Table 1 and Supplementary
Table 1. Patient clinical, pathologic, and genetic data

<table>
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<th>Patient #1</th>
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<td>Surgery + radiotherapy + chemotherapy</td>
<td>Surgery + radiotherapy + chemotherapy</td>
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<td>1p/19q codeletion</td>
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</table>

NOTE: Anagraphical, pathologic, clinical and genetic data of patients of samples were used in the study. Radiotherapy: 60 Gy (30 fractions). Chemotherapy: 75 mg/m² TMZ, per os, daily, concurrently to radiotherapy, followed by 200 mg/m² TMZ, per os, days 1 to 5, every 28 days, 6 cycles. Postrecurrence therapy: radiotherapy: 60 Gy (30 fractions); chemotherapy: 80 mg/m² carmustine (BCNU), days 1 to 3, every 8 weeks, 3 cycles. Time to recurrence: time between the surgery and the appearance of tumor relapse at MRI. Overall survival: time between diagnosis and patient death. Fully methylated: promoter methylation of both alleles; partially methylated: promoter methylation of one allele. Amplified: > 2 copies of EGFR genes; not amplified: < 2 copies of EGFR gene. IDH: isocitrate dehydrogenase.

Table S1. The MGMT promoter status is partially methylated in patient #1, unmethylated in patient #2, and fully methylated in patient #3 (Table 1). We generated primary cultures from patients #1 to #3 and grew them as AC or NS.

NS had comparable levels of CAIX and CAXII protein, whereas only CAIX was detected in AC (Fig. 1A). NS had higher levels of CAIX and Pgp on the cell surface than AC (Fig. 1B). CAXII coimmunoprecipitated with Pgp (Fig. 1C).

The results of the PLA demonstrated that CAXII and Pgp are physically associated in the NS plasma membrane (Fig. 1D). The quantification of CAXII:Pgp colocalization, based on confocal laser-scanning microscopy, indicated that 58.67 ± 1.1% of NS Pgp interacted with CAXII in the plasma membrane of NS (Fig. 1E).

The expression of Pgp and CAXII was independent of the different culture conditions between AC and NS (Supplementary Fig. S1).

The addition of CAXII inhibitors to NS reduces Pgp activity and increases retention and cytotoxicity of chemotherapeutic drugs

A compound panel comprising Psammamplin C (1), its derivatives (2–4), and the non-CAXII inhibitor control compound (Fig. 2A; ref. 5) was tested for the ability to indirectly reduce Pgp activity in NS. The more potent the CAXII inhibitor (lower Ki, Supplementary Table S2), the higher the reduction of Pgp activity (Fig. 2B). The Pgp substrate doxorubicin accumulated to a greater extent (Fig. 2C) and exhibited greater toxicity (Fig. 2D and E) in AC than in NS. NS were refractory to doxorubicin. These characteristics were unchanged by compounds 1–5 in AC. In NS compounds 1 or 3, the two most potent CAXII inhibitors restored the intracellular doxorubicin concentration to a level comparable with AC (Fig. 2C), partially restored the release of LDH induced by doxorubicin (Fig. 2D), and reduced cell viability (Fig. 2E).

Comparable effects were observed with the chemotherapeutic drugs etoposide, topotecan, and irinotecan. These drugs are known substrates of Pgp (ref. 21; Supplementary Fig. S2A–S2F). As compound 1 was the most effective in restoring the effects of Pgp substrates in GB NS, it was selected for further characterization.

CAXII inhibition enhances TMZ cytotoxicity in NS by reducing Pgp activity

TMZ, a substrate and downregulator of Pgp (4, 22), decreased Pgp expression in NS (Fig. 3A; Supplementary Fig. S3A). TMZ consistently reduced the amount of Pgp that coimmunoprecipitated with CAXII (Fig. 3B; Supplementary Fig. S3B) and the activity of Pgp (Fig. 3C). Pgp expression (Fig. 3A; Supplementary Fig. S3A) and the interaction of Pgp and CAXII (Fig. 3B; Supplementary Fig. S3B) were unchanged by compound 1; however, Pgp-ATPase activity was reduced, even more so when in combination with TMZ (Fig. 3C).

Consequently, in NS compound 1 increased TMZ accumulation (Fig. 3D), cell necrosis (Fig. 3E), apoptosis (Fig. 3F), and reduced viability in the presence of TMZ (Fig. 3G), to the extent observed in Pgp-KO NS clones (wherein the levels of CAXII were unaltered) or in AC (Fig. 3D–G; Supplementary Fig. S3C and S3D). In Pgp-KO NS clones, compound 1 did not enhance the effect of TMZ on reduced cell viability (Fig. 3G), suggesting that Pgp is the ultimate—although indirect—target of compound 1.

The isobologram analysis in NS (Supplementary Fig. S4A–S4C) indicated a CI of TMZ and compound 1 equal to 0.08838 for patient #1, 0.07017 for patient #2, and 0.1775 for patient #3.

CAXII-KO NS clones had the same levels of Pgp in whole-cell (Fig. 4A; Supplementary Fig. S5A) and plasma membrane extracts (Fig. 4B; Supplementary Fig. S5B) than in NS or NS treated with TMZ. However, when plasma membrane extracts of CAXII-KO NS clones were immunoprecipitated with an anti-CAXII antibody, Pgp was undetectable in the immunoprecipitated extracts (Fig. 4C; Supplementary Fig. S5C), confirming a strong and specific interaction between the two proteins. CAXII-KO NS clones had lower Pgp-ATPase activity than wild-type NS (Fig. 4D) even when the levels of Pgp were the same. TMZ further reduced Pgp-ATPase activity in CAXII-KO NS clones (Fig. 4D) and produced the same phenotypic response as in AC, namely TMZ accumulation (Fig. 4E) and cytotoxicity (Figs. 4F–H; Supplementary Fig. S5D).
Figure 1.
CAXII and Pgp are coexpressed and associated in GB-derived NS. Primary GB cells derived from 3 patients (#1, #2, #3) were cultured as ACs or as NS. A, Cells were lysed and immunoblotted with the indicated antibodies. The figure is representative of one out of three experiments. B, Cell surface expression of CAXII and Pgp was detected by flow cytometry in replicate. The histograms are representative of one out of three experiments. C, Plasma membrane extracts were immunoprecipitated (IP) with anti-CAXII or anti-CAIX antibodies, then immunoblotted (IB) with an anti-Pgp antibody. In a complementary experimental set, plasma membrane extracts were immunoprecipitated with an anti-Pgp antibody and immunoblotted with an anti-CAXII antibody, to confirm the specificity of the interaction between Pgp and CAXII. No Ab: #2 NS sample immunoprecipitated without antibody. An aliquot of the extracts before the immunoprecipitation was loaded and probed with an anti-pancaderin antibody, as control of equal protein loading. The figure is representative of one out of three experiments. D, Proximity ligation assay between CAXII and Pgp in patient #2 AC and NS. Bl: cells incubated without primary antibodies; Ab: cells incubated with primary antibodies. Blue: nuclear staining (DAPI); green: Pgp/CAXII interaction. The image is representative of one out of three experiments. A minimum of five fields/experiment were examined. Bar: 10 μm (10 × ocular lens; 63 × objective lens). E, Immunofluorescence detection of plasma membrane–associated CAXII and Pgp in nonpermeabilized NS from patient #2, by confocal microscope analysis. The image is representative of one out of three experiments. A minimum of five fields/experiment were examined. Bar: 10 μm (10 × ocular lens; 60 × objective lens).
Figure 2.
CAXII inhibition reduces Pgp activity and increases cytotoxicity of doxorubicin in GB-derived NS. **A**, Chemical structures of CAXII inhibitors used. For **B** to **D**: pooled data of patients #1, #2, and #3 are presented as mean ± SD (n = 3 independent experiments for each patient). Violet, orange, and blue circles represent the mean of technical replicates of patients #1, #2, and #3. **B**, Spectrophotometric measure of Pgp ATPase activity, detected in triplicates in NS, grown for 24 hours in fresh medium (-) or in medium containing 10 nmol/L compounds 1 to 5. **P** < 0.02: compound 4 vs. untreated (-) cells; **P** < 0.001: compounds 1 and 3 vs. untreated (-) cells (Student t test). **C**, Fluorimetric detection of doxorubicin (dox) accumulation, measured in duplicates in cells treated 24 hours with 5 μmol/L dox, alone (-), or in the presence of 10 nmol/L compounds 1 to 5. **P** < 0.05: NS treated with compound 4 vs. corresponding AC. **P** < 0.001: untreated NS or treated with compounds 2 and 5 vs. corresponding AC. **P** < 0.002: NS treated with compound 1 and 3 vs. untreated (-) NS (Student t test). **D**, Release of LDH, measured spectrophotometrically in duplicates, in cells grown for 24 hours in fresh medium (-) or in media containing 10 nmol/L compounds 1 to 5, in the absence or presence of 5 μmol/L dox. **P** < 0.05: treated AC/NS vs. corresponding “− dox” cells; **P** < 0.001: treated AC/NS vs. corresponding “− dox” cells; **P** < 0.002: NS treated with compound 1 and 3 vs. “+ dox” NS (Student t test). **E**, Viability of cells, measured by a chemiluminescence-based assay in quadruplicates, after 72 hours in fresh medium (-) or in media containing 10 nmol/L compounds 1 to 5, in the absence or presence of 5 μmol/L dox. **P** < 0.001: treated AC/NS vs. corresponding “− dox” cells; **P** < 0.001: NS treated with compound 1, 3, and 4 vs. “+ dox” NS (Student t test).
CAXII inhibition restores the efficacy of TMZ in tumors derived from resistant GB NS in vivo

Compound 1 was stable in Balb/c mice plasma (half-life >240 minutes; Supplementary Table S3) and showed low potential to inhibit major drug-metabolizing CYP P450 enzymes (Supplementary Table S4).

Following identification of the dosing schedule that maximally reduced the tumor growth of AC in vivo (Supplementary Fig. S6A) with significantly less effect against NS (Supplementary Fig. S6B and S6C), we coadministered compound 1 in mice bearing patient #2–derived NS at two dosages, 38 ng/kg and 3,800 ng/kg, the former according to the CAXII \( K_{\text{i}, \text{ther}} \) to limit hematoc/lymphatic clearance. Compound 1 did not reduce AC- or NS-derived tumor growth. When compound 1 was combined with TMZ, TMZ efficacy in AC-derived tumors was unchanged; however in NS-derived tumors, TMZ efficacy...
was enhanced in a dose-dependent manner (Supplementary Fig. S6B and S6C). Moreover, the combined treatment did not elevate hematopoiesis, liver, kidney, or muscle toxicity compared with TMZ-only treatment (Supplementary Table S5).

Figure 4. CAXII inhibitors reverse chemoresistance in GB-derived NS. NS were grown for 48 hours (A–G) or 72 hours (H) in fresh medium (−) or in medium containing 50 μM/L temozolomide (T) or 10 nM/L compound 1, alone or in association. D, E, F, and H: pooled data of patients #1, #2, and #3 are presented as mean ± SD (n = 4 independent experiments for each patient). Violet, orange, and blue circles represent the mean of technical replicates of #1, #2, and #3. AC were included as control of cells with undetectable CAXII levels. A. Patient #2 NS were grown in fresh medium (−), transduced with a nontargeting vector (scrambled vector; scr) or with two CRISPR pCas CAXII-targeting vectors (KO#1, KO#2), lysed, and immunoblotted with the indicated antibodies. The figure is representative of one out of three experiments. B, Plasma membrane extracts were probed with an anti-Pgp antibody, or an anti-pancadherin antibody, as control of equal protein loading. The figure is representative of one out of three experiments. C, Plasma membrane extracts from patient #2 CAXII-KO NS clones were immunoprecipitated (IP) with an anti-CAXII antibody and immunoblotted (IP) with an anti-Pgp antibody. No Ab: sample immunoprecipitated without antibody. An aliquot of the extract before the immunoprecipitation was loaded and probed with an anti-pancadherin antibody, as control of equal protein loading. The figure is representative of one out of three experiments. D, Spectrophotometric measure of Pgp ATPase, detected in triplicates in NS. *P < 0.05: T-treated vs. scrambled-treated (−) cells; **P < 0.01: KO1/KO2 or T+KO1/KO2 cells vs. scrambled-treated (−) cells; ***P < 0.001: T+KO1/KO2 cells vs. T-treated cells; ****P < 0.001: all experimental conditions vs. untreated (−) NS (Student t test). E, Intracellular content of TMZ, measured in duplicates after cell radiolabelling. ***P < 0.001: all experimental conditions vs. untreated (−) NS (Student t test). F, LDH release, measured spectrophotometrically in duplicates. ***P < 0.001: all experimental conditions vs. untreated (−) NS (Student t test). G, Patient #2 NS were lysed and immunoblotted for procaspase and cleaved caspase 3. The figure is representative of one out of three experiments. H, Cell viability measured by a chemiluminescence-based assay in quadruplicates. ***P < 0.001: all experimental conditions vs. untreated (−) NS (Student t test).
growth of scrambled-transduced tumors was not (Supplementary Fig. S6D). Compound 1, at the dosage of 3,800 ng/kg, enhanced the antitumor effect of TMZ in animals bearing scrambled-NS but not in animals bearing Pgp-KO NS (Supplementary Fig. S6D) that lack this indirect target of compound 1.

In orthotopic GB-NS-PDX, neither compound 1 nor TMZ alone reduced tumor growth, with the exception of tumors derived from patient #3, wherein the genetic profile and clinical history were suggestive of a more favorable response to TMZ (Table 1). The combination of compound 1 and TMZ significantly decreased tumor growth in all three GB-NS-PDX (Fig. 5A and B) and increased overall survival (Fig. 5C). Although TMZ reduced the expression of Pgp in CAXII-positive tumors (Fig. 5D–F), it did not reduce intratumor proliferation or increase apoptosis (Fig. 5D and E). Compound 1 did not change these parameters. The combined use of compound 1 and TMZ reduced Pgp expression in tumors (Fig. 5D–F) as it did in NS cultured in vitro (Fig. 3). The combination also rescued the antiproliferative and proapoptotic effects of TMZ, as demonstrated by the reduced intratumor-positive staining for Ki67 and by the increased activation of caspase 3 (Fig. 5D and E).

Discussion

We analyzed samples from 3 GB patients that experienced a variable but poor clinical response to TMZ. The patients had different genetic backgrounds however the NS-derived from all patients coexpressed CAXII and Pgp, suggesting a relationship that may represent an ancestral feature of GB SC, independent of genetic alterations or environmental conditions (such as different culture conditions). Notably, up to 60% of the Pgp in the plasma membrane of NS was found to interact with CAXII. This indicates that the enzymatic activity of CAXII may act to influence the microenvironment pH for the colocalized Pgp. We are currently investigating the mechanisms of upregulation of Pgp and CAXII expression in NS and the nature of interaction between these two proteins. To the best of our knowledge, ours is the first work showing an increased CAXII expression in GB NS–derived from primary tumors.

Until now, there have been no reports on the role of CAXII in the response to chemotherapy in NS. Based on our previous observations in Pgp-expressing solid cancer cell lines (7, 9), we hypothesized that CAXII inhibitors may reverse the Pgp-mediated drug resistance in GB NS, wherein Pgp activity is enhanced by CAXII activity. Even though CAIX is expressed in NS and is important in GB pathogenesis (23), our data indicate no significant role for CAIX in the chemoresistance of NS.

We recently synthesized Psammaplin C (compound 1 in this article), one of the most potent CAXII inhibitors ever reported (24). We synthesized a panel of related sulfonamides (2–4) and the control compound 5, which is identical to 1 but lacks the sulfonamide moiety. This panel enabled the structure–activity relationships between CAXII inhibition and indirect Pgp inhibition to be established. The strongest inhibitor of CAXII, compound 1, was the most effective in rescuing the cytotoxicity of all tested Pgp substrates: topoisomerase I/II inhibitors topotecan, irinotecan, etoposide, and doxorubicin. These drugs are under evaluation in clinical trials as second-line treatments for GB and in GB patients that are refractory to TMZ (25). Our findings suggest CAXII inhibitors may substantially enhance the efficacy of these agents, being particularly effective against GB NS, where improvement of current therapy is desperately sought.

Most importantly, compound 1 rescued the efficacy of TMZ, the first-line drug in GB treatment. TMZ fails to eradicate GB SC, owing to a combination of MGMT status, cell survival/anti-apoptotic pathways driven by EGF amplification, mutations in IDH1/2 and TP53, hypoaxia, niches rich of growth factors (2). The three patient-derived NS analyzed in this work had slight variations in their in vitro and in vivo sensitivity and clinical response to TMZ, likely as a consequence of their different genetic background. In general, however, NS from all patient samples were more resistant to TMZ than corresponding AC. The coadministration of compound 1 rescued the amount of TMZ, independent of MGMT status or other genetic alterations, suggesting that inhibition of CAXII may overcome Pgp-mediated resistance to TMZ.

Our findings in Pgp-KO and CAXII-KO NS support the hypotheses that (i) in addition to the MGMT methylation status and other known genetic alterations determining resistance to TMZ, the presence of Pgp plays a pivotal role in NS resistance to chemotherapy and (ii) CAXII inhibition overcomes this resistance by reducing Pgp activity. It is probable that the interaction of CAXII with Pgp sustains the activity of Pgp, and that interfering with CAXII by treatment with either compound 1 or genetic knockout significantly reduces ATPase activity. Notably, this genetic or pharmacologic inhibition does not alter the amount of surface Pgp. As Pgp mediates TMZ efflux (4), targeting CAXII increases the intracellular retention of TMZ to restore its cytotoxic effects.

Figure 5.

Compound 1 improves temozolomide efficacy against orthotopically implanted GB NS-derived tumors. A, Representative in vivo bioluminescence imaging of orthotopically implanted patient #2 NS, in animals treated with vehicle (ctrl), compound 1, and TMZ, as follows: (1) control group, treated with 0.2 ml saline injected intravenously (i.v.); (2) 1 group, treated with 3,800 ng/kg compound 1 i.v.; (3) TMZ group, treated with 50 mg/kg TMZ per os (p.o.); (4) TMZ+1 group, treated with 50 mg/kg TMZ p.o. + 5,800 ng/kg compound 1 i.v. (6 animals/group). B, Quantification of patient #1–3 NS-derived bioluminescence, taken as index of tumor growth. Data are presented as mean ± SD (6 animals/group). At day 24: **, P < 0.005 and ***, P < 0.001: TMZ+1 group vs. all the other groups of treatment; ***, P < 0.005 and ****, P < 0.001: TMZ+1 group vs. TMZ-group (Student t test). C, Overall survival probability was calculated using the Kaplan-Meier method. Patient #1 NS: P < 0.02: TMZ+1 group vs. all the other groups of treatment. Patient #2 NS: P < 0.002: TMZ+1 group vs. all the other groups of treatment. Patient #3 NS: P < 0.001: TMZ+1 group vs. ctrl and 1 group (log-rank test; not reported in the figure). D, Representative intratumor staining with hematoxylin and eosin (HE) or the indicated antibodies, from patient #2 NS-derived tumors. The photographs are representative of sections from 5 tumors/group of treatment. Bar = 10 µm (10× ocular lens, 20× objective). E, Quantification of immunohistochemical images, performed on sections with 111–94 nuclei/field. The percentage of proliferating cells was determined by the ratio Ki67-positive nuclei/total number (hematoxylin-positive) of nuclei using ImageJ software. The ctrl group percentage was considered 100%. The percentage of CAXII, Pgp, and caspase 1-positive cells was determined by Photoshop program. Data are presented as mean ± SD. ***, P < 0.001: TMZ+1 group vs. all the other groups of treatment; ****, P < 0.001: TMZ+1 group vs. TMZ-group. #, P < 0.005: TMZ vs. ctrl group (Student t test). F, Immunoblot analysis of the indicated proteins from tumors extracts of patient #2 NS (5 animals/group of treatment).
The strong synergism observed with TMZ and compound 1 further enforced the hypothesis that they are involved in the same pathway leading to inhibition of Pgp efflux activity. The ability of compound 1 to reduce Pgp activity together with its potency and selectivity for CAXII over other CAs contributes to make compound 1 highly effective against GB NS.

Furthermore, CAXII has minimal expression in healthy cells (https://www.proteinatlas.org/ENSG00000074410-CA12/tissue). This is a major advantage as targeting CAXII to indirectly reduce Pgp activity provides a selective GB SC–targeting tool and avoids the in vivo toxicity associated with using direct Pgp inhibitors (5). Furthermore, the in vitro results obtained from treatment of GB-SC with a combination of compound 1 and second-line chemotherapeutic drugs (all substrates of Pgp) may open the way for new combination therapies with the potential to lower the chemotherapy dose required to achieve significant GB reduction.

In line with the TMZ resistance observed in NS cultures and the clinical response of the corresponding patient to TMZ, two of the three GB-NS-PDX were refractory to TMZ. The third xenograft, generated from the patient with the most favorable genetic profile toward TMZ sensitivity, longest time to recurrence after TMZ treatment, and longest overall survival—was partially sensitive to TMZ. In accordance with the in vitro findings, tumor growth in compound 1–only treated GB-NS-PDX was not reduced; however, compound 1 in combination with TMZ significantly improved the antitumor activity over the TMZ-only cohort and increased the overall survival, likely as a consequence of the coexpression of CAXII and Pgp in these NS-derived tumors. Furthermore, the combination of TMZ and compound 1 reduced the intratumor level of Pgp and recapitulated the same cytotoxic events observed in NS cultures.

Recently, the combination of the CAIX/CAXII inhibitor SLC-0111 (100 mg/kg, daily over 14 days) with TMZ (100 mg/kg once every 7 days over 14 days) reduced GB growth compared with TMZ only. The authors speculate that the mechanism of SLC-0111 may be mediated by CAIX together with increased DNA damage (26). This study did not however have the benefit of the inactive probe/active compound combination (compound 5 and 1 in our study) to contribute evidence to support the hypothesis that CAIX was the predominant target of SLC-0111. Our work may provide an additional explanation for the effect of SLC-0111, correlating its efficacy with CAXII inhibition causing indirect inhibition of Pgp and increased intratumor retention of TMZ. Of note, compound 1 was effective at a substantially lower dosage than SLC-0111. In addition, compound 1 was devoid of toxicity and did not exacerbate TMZ side effects, suggesting an appropriate efficacy and safety window with this combination treatment.

In summary, we have investigated for the first time the expression and therapeutic implication of CAIX in the highly chemoresistant GB SC component of GB. We propose that CAXII and Pgp coexpression is a new hallmark of chemoresistance in GB NS. This relationship represents a previously unknown mechanism of TMZ resistance in GB-derived NS, wherein CAXII contributes to the Pgp-mediated resistance to TMZ and topoisomerase I/II inhibitors in patient-derived GB NS. The detection of CAXII in primary GB samples by routine IHC techniques may be difficult however as CAXII is restricted to the SC component that represents only a small portion of tumor bulk. This restricted distribution may limit the potential use of CAXII as a predictive marker of low TMZ response. CAXII may however represent an exciting new therapeutic target in GB patients resistant to TMZ and with a significant component of SC identified by pathology analysis. Pharmacologic inhibition of CAXII rescues the efficacy of TMZ, independently of genetic alterations commonly associated with TMZ resistance. Our results may form a basis to warrant clinical validation of a new combinatorial therapy, based on a CAXII inhibitor with TMZ and/or topoisomerase I/II inhibitor, as more effective treatments to eliminate GB SC compared with current treatment options.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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