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Triple combination of lomustine, temozolomide and irradiation reduces canine glioma cell survival in vitro

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Abstract

Background: Combined chemoradiation offers a promising therapeutic strategy for dogs with glioma. The alkylating agents temozolomide (TMZ) and lomustine (CCNU) penetrate the blood-brain barrier, and doses for dogs are established. Whether such combinations are clinically advantageous remains to be explored together with tumour-specific markers.

Objective: To investigate if triple combination of lomustine, temozolomide and irradiation reduces canine glioma cell survival in vitro.

Methods: We evaluated the sensitising effect of CCNU alone and in combination with TMZ-irradiation in canine glioma J3T-BG cells and long-term drug-exposed subclones by using clonogenic survival and proliferation assays. Bisulphite-SEQ and Western Blot were used to investigate molecular alterations.

Results: TMZ (200 μM) or CCNU alone (5 μM) reduced the irradiated survival fraction (4 Gy) from 60% to 38% ($p = 0.0074$) and 26% ($p = 0.0002$), respectively. The double-drug combination reduced the irradiated survival fraction (4 Gy) more potently to 12% ($p < 0.0001$).

After long-term drug exposure, both subclones show higher IC_{50} values against CCNU and TMZ. For CCNU-resistant cells, both, single-drug CCNU ($p = 0.0006$) and TMZ ($p = 0.0326$) treatment combined with irradiation (4 Gy) remained effective. The double-drug-irradiation combination reduced the cell survival by 86% ($p < 0.0001$), compared to 92% in the parental (nonresistant) cell line. For TMZ-resistant cells, only the double-drug combination with irradiation (4 Gy) reduced the cell survival by 88% ($p = 0.0057$) while single-drug treatment lost efficacy.

Chemoresistant cell lines demonstrated higher P-gp expression while *MGMT*-methylation profile analysis showed a general high methylation level in the parental and long-term treated cell lines.

Conclusions: Our findings indicate that combining CCNU with TMZ-irradiation significantly reduces canine glioma cell survival. Such a combination could overcome current challenges of therapeutic resistance to improve overall patient survival.

KEYWORDS
brain tumour, chemoradiation, dog, in vitro, *O-6-methylguanine-DNA methyltransferase* (*MGMT*)

1 | INTRODUCTION

In dogs, gliomas represent the second most common brain tumour of all primary intracranial tumours, and especially brachycephalic breeds are more frequently affected (Snyder et al., 2006; Song et al., 2013). Radiotherapy (IR) provides good tumour control with improved or even normal quality of life (Debreuque et al., 2020; Dolera et al., 2018; Rohrer Bley et al., 2021; Schwarz et al., 2018). However, in about a third of those patients, the tumour recurs or progresses within a year, and can include cerebrospinal fluid drop metastasis (Bentley et al., 2021; Debreuque et al., 2020; Dolera et al., 2018; Schwarz et al., 2018). This indicates that a resistant subpopulation of tumour cells survive treatment and induce tumour progression. Further therapy is required to target these cells and co-treatment with temozolomide (TMZ) is a promising option.

The chemotherapeutic drug TMZ is commonly used together with irradiation for gliomas in human medicine. TMZ improves radiation efficacy and provides higher survival rates (Stupp et al., 2005). By alkylating the targeted DNA, it causes the DNA strands to break, induces apoptosis and activates signal cascades, causing cell cycle arrest and sensitisation to IR (Gustafson & Bailey, 2020). Cancer drug resistance, however, is a considerable problem which limits the effectiveness of current cancer therapies (Holohan et al., 2013). Intrinsic DNA-repair mechanisms diminish the treatment outcome of alkylating drugs (Jiapaer et al., 2018), and the 0^6-methylguanine-DNA methyltransferase (MGMT) appears to be specifically capable of removing DNA damage (Kaina & Christmann, 2002; Perazzoli et al., 2015).

Adding TMZ to irradiation results in a survival advantage only when the *MGMT* gene promoter is silenced by methylation (Chinot et al., 2007). An even longer survival time has been observed by the addition of lomustine (CCNU) to TMZ-irradiation in young human glioblastoma patients (Herrlinger et al., 2019). CCNU, like TMZ, is an alkylating agent and in addition has an inhibitory effect on enzymes through carbamylation, which is attributed to the isocyanate group. It is unknown whether the carbamylation shows a clinical effect compared to the overall impact of CCNU (Weller & Le Rhun, 2020). Further, CCNU was proved an effective option in overcoming acquired TMZ resistance due to mismatch repair (MMR) deficiency (Stritzelberger et al., 2018).

In a recent study, TMZ in combination with IR had an additive effect in different canine glioma cells (Tresch et al., 2021). Both, TMZ and CCNU, penetrate the blood-brain barrier, and therapeutic dosing has been determined for dogs (Gustafson & Bailey, 2020; Marconato et al., 2020). The use of CCNU as well as TMZ for canine brain tumours has been clinically investigated but has not resulted in a survival advantage. These studies, however, only used either maximally tolerated doses as single treatments and at longer drug intervals (Moirano et al., 2018), or subtherapeutic doses with or without radiation therapy (Dolera et al., 2018; Van Meervenne et al., 2014). Furthermore, the maximally tolerated dose of TMZ in dogs has only recently been established (Marconato et al., 2020), and response of canine glioma has not yet been tested at this dose. It therefore remains to be seen if dogs with glioma might benefit from treatment combinations of IR with chemotherapy. Therefore, we evaluated the benefit of adding CCNU to TMZ-irradiation (triple treatment) in the canine glioma cell line J3T-BG. Moreover, we generated drug-resistant cell lines to investigate possible alterations in DNA methylation and protein levels which could be pivotal for the development of resistance.

2 | MATERIALS AND METHODS

2.1 | Cell line validation statement and culture conditions

In vitro chemoradiation was performed on the canine glial cell line J3T-BG while methylation analysis was performed on J3T-BG, SDT3G and G06A. All three cell lines were originally derived in the Paul C. and Borghild T Petersen Brain Tumor Laboratory (UC Davis, USA) by Dr. Dan York and by Dr. Peter J. Dickinson, as described in York et al. (2012). The J3T-BG cell line is a subclone of the J3T cell line, originally developed by Dr. Michael E. Berens. All cell lines were cultured as previously described (Tresch et al., 2021).

2.2 | Drugs

CCNU and TMZ were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Both were dissolved in DMSO to generate a stock solution of 100 mM or 50 mM, respectively, and aliquoted before storage at −20°C. The aliquots containing TMZ stock solution were used within one month.

For clonogenic and proliferation assays, the drugs (and DMSO solvent control) were diluted in medium to a final concentration of 200 μM for TMZ and 1–10 μM for CCNU before cell treatment.

2.3 | Generation of drug-resistant subclones of J3T-BG

The generation of resistant cell lines was described before (Stritzelberger et al., 2018; Yip et al., 2009). The glioma cell line J3T-BG was treated with 200 μM TMZ, 5 μM CCNU or 0.4% DMSO solvent control. The cells were cultured in TC flasks T25, seeded at a concentration of 100.000 cells per flask and allowed to adhere overnight. The cells were treated every 24 h for 5 consecutive days with fresh medium

containing the drug or DMSO (solvent control). Afterwards, the exposure to fresh medium was repeated every third day to a total of 3 weeks. The parental cell line was denoted as J3T-BG/DMSO and the new resistant cell lines as J3T-BG/CCNU and J3T-BG/TMZ, respectively. Before starting the experiments, cells were cultured in drug-free medium for one passage. Otherwise, drug-resistant cells were maintained in drug-free medium under the same incubator conditions.

2.4 | Irradiation

IR was performed with a 6 MV linear accelerator (Clinac iX, Varian, Palo Alto, USA) with a source-surface distance (SSD) of 100 cm and a dose-rate of 600 monitor units per minute as described before (Tresch et al., 2021).

2.5 | Clonogenic cell survival assay

Cells were seeded in 10 cm Petri dishes and incubated to adhere overnight. Depending on the irradiation dosage, 200 cells (0 Gy), 300 cells (2Gy), 400 cells (4 Gy) and 4000 cells (8 Gy) were seeded per 10 cm dish. Cells were first treated with the chemotherapeutic drugs (CCNU or/and TMZ) at different doses or DMSO as control and subsequently irradiated. The incubation time was 24 h and varied maximally by ±30 min. To compare the influence of different incubation times, additional experiments including a reduced incubation time with cytostatics of 1 h were performed.

Directly after IR, the medium containing the drug or solvent control was removed and substituted with drug-free medium. After a total of 7–9 days, surviving colonies were fixed and stained for subsequent calculation of the survival fraction as previously described (Franken et al., 2006; Tresch et al., 2021).

2.6 | Proliferation assay

A total of 1000 cells were seeded in 100 μL medium per well of a 96-well plate and incubated overnight. Cells were further incubated for 24 h with CCNU (10 μM), TMZ (200 μM), in combination (double-drug treatment) or with DMSO as solvent control and subsequently irradiated with 0, 4 or 8 Gy. Cells remained thereafter incubated with drug-containing medium for the duration of the experiment. The impact of different treatments on cell proliferation 48 h after IR was analysed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) as described before (Tresch et al., 2021).

2.7 | Genomic DNA isolation and methylation analysis

Cell lysates of the canine glioma cell lines (J3T-BG, G06A and SDT3G) and the drug-resistant subclones (J3T-BG/CCNU and J3T-BG/TMZ) were generated with the PureLink™ Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's protocol. In case of the long-term treated cells, DNA lysates were generated directly after treatment, after the first passage, and weekly for two weeks. Total cell concentration was measured with the EVE™ Automatic Cell Counter (NanoEntek Inc., Seoul, Sud Korea) and ranged from 5×10^5 to 9.1×10^6 cells/mL.

Total DNA (200–500 ng) for each specimen was treated with sodium bisulphite using the EZ DNA Methylation-Lightning Kit (Zymo Research Europe, Freiburg, Germany), according to the manufacturer's protocol. Target enrichment for 12 genes (*MGMT*, *MLH1*, *MSH2*, *MSH3*, *BRCA1*, *ATM*, *XRCCS*, *FEN1*, *ODZ1*, *HIF1a*, *CASP8*, *CDK1*) was performed using a two-step PCR protocol as previously described (Morandi et al., 2020; Tresch et al., 2021). In brief, a first round of PCR served to amplify the regions of interest with tagged primers, and a second PCR with 8 cycles was used to insert barcodes and P5/P7 Illumina Adaptors (Nextera™ Index Kit (Illumina, San Diego, CA, USA). The coordinates of the regions of interest and primer sequences are described in Table S1 considering the reference genome canFam3 (UCSC, Genome Browser).

The web tool MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) was used for primer design accounting for a set of specific CpGs in the promoter region for each gene selected for this study.

After purification and quantification of the library, sequencing was conducted on MiSeq sequencer (Illumina, San Diego, California), according to the manufacturer's protocol. Each next-generation sequencing (NGS) experiment was designed to allocate at least 1000 reads/region, with an expected depth of coverage of at least 1000×.

FASTQ files were processed in a Galaxy Project environment following a pipeline as described previously (Tresch et al., 2021). In brief, after filtering for quality >Q30 and for read lengths (>80 bp), FASTQ files were then mapped by BWAmeth to generate BAM (Binary Alignment Map) files, which were then in turn processed by MethylDackel using CanFam3.1 as reference genome. The output files assigned the exact methylation level for each investigated CpG position (Baker et al., 2018). The data were analysed using methylation plotter (Mallona et al., 2014).

2.8 | Western blot

Western Blot analysis was used to assess the protein expression levels and was performed as previously described (Tresch et al., 2021). In brief, cell lysates were generated using RIPA lysis buffer and protein concentration was measured with the Pierce™ Protein Assay Kit (Thermo Fischer, Waltham, MA, USA). Total protein of 50 μg was separated by SDS-Page on a 4–15% gradient gel (Bio-Rad) and transferred on a PVDF membrane using a transfer apparatus (Transfer-Blot Turbo Transfer System, Bio-Rad, Hercules, CA, USA). Blotting membranes were blocked with 5% nonfat milk in TBST (Bio-Rad, Hercules, CA, USA), incubated overnight with an antibody against P-gp (C219, #MA1-26528, 1:1000 Invitrogen, Waltham, MA, USA) and detected following incubation with an anti-mouse IgG, HRP-linked secondary antibody (#7076S, 1:1000, Cell Signaling). For loading control,

a β-actin antibody (8226, 1:1000, Abcam) was used. Pierce™ ECL Western Blotting Substrate (Thermo Fischer Scientific, Waltham, MA, USA) was used for chemiluminescent detection with the Fusion Solo S Edge – Chemiluminescence Imaging System (Witec AG, Sursee, Switzerland).

2.9 | Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (San Diego, CA, USA). Means, standard deviations (SD) and plots were used to assess the data for spurious observations and distributional assumptions.

To determine the half-maximal inhibitory concentration (IC_{50}), the drug concentrations were log transformed and a four-parameter nonlinear regression analysis fitting the HillSlope from the data (variable slope model) was performed. The four parameters were bottom, top, $logIC_{50}$ and HillSlope. Basal response was subtracted, and the bottom was set to a constant value of 0. The IC_{50} values of the resistant cell lines were then compared to the parental cell line using the unpaired *t*-test.

Treatment efficacy was compared using either the nonparametric unpaired *t*-test, or in the case of more than two groups, the one-way ANOVA with the Tukey's post hoc test was applied.

Linear-Quadratic (LQ) predictions were obtained by normalising the data to their control and fitting the equation $\ln(S) = -\alpha D - \beta D^2$ (α and $\beta \geq 0$), where *S* represents the survival fraction and *D* represents the dose.

The methylation data were analysed using methylation plotter (Mallona et al., 2014) and statistical significance was calculated using Kruskal–Wallis test.

Data summaries are expressed as the mean ± standard deviation (SD). For single statistical tests, significance was set at 0.05. For multiple comparisons, the overall error rate was set at 0.05.

3 | RESULTS

3.1 | CCNU increases cytotoxic effects of irradiation in canine glioma cells

First, to determine the optimal CCNU concentration for further combination experiments, we tested different concentrations of 1–100 μM CCNU using the colony formation assay. While 1 μM CCNU only minimally reduced cell clonogenicity, a concentration of 10 μM had already a strong impact on clonogenicity, even without IR (Figure 1a and c). With a concentration of 5 μM CCNU alone, we observed a reduced colony formation compared to the DMSO-treated control group ($p = 0.0004$; Figure 1b). Reduction in clonogenic cell survival in CCNU-treated cells was enhanced with IR at dose of 4 and 8 Gy. IR alone (4 Gy) reduced survival fraction to 57% but in combination with 5 μM CCNU, only 16% ($p < 0.0001$) of the seeded cells survived treatment (Figure 1b).

In the proliferation assay, a higher CCNU concentration resulted in decreased proliferation in a dose-dependent manner (Figure 1d). At doses of 5, 10 and 20 μM, cell survival was reduced to 82% ($p = 0.3465$), 67% ($p = 0.0366$) and 49% ($p = 0.0019$), respectively. However, in combination with IR, no additional reduction in proliferation was observed. Additionally, effect of lomustine (CCNU) alone and together with irradiation (4 and 8 Gy) on clonogenic cell survival fitting for linear-quadratic model is shown as Figure S1.

To evaluate the effect of drug exposure time, we performed clonogenic assays with drug exposure of 1 h versus 24 h prior to IR. The longer incubation time with CCNU reduced clonogenic cell survival to 61%, compared to 77% following 1 h of exposure ($p = 0.0584$) (Figure S2A). When combined with 4 Gy irradiation, no difference was observed between exposure duration as both incubation times strongly reduced clonogenic survival to 21%.

When repeated with exposure to TMZ, the longer incubation time reduced clonogenic cell survival to 61%, compared to 93% after 1 h of exposure ($p = 0.00069$) (Figure S2B). When combined with 4 Gy irradiation, clonogenic cell survival decreased further to 23% after 24 h ($p = 0.0576$) and to 36% after 1 h of exposure ($p = 0.8952$). Again, for combined treatment, no significant differences were evident with the longer exposure ($p = 0.4717$).

3.2 | Triple treatment (TMZ/CCNU/IR) further reduces cell survival in comparison to TMZ/IR

To compare the effect of CCNU and TMZ combination on clonogenic cell survival, we used 5 μM of CCNU and 200 μM of TMZ (Tresch et al., 2021) alone or in combination, with or without IR (Figure 2a and b).

Interestingly, without irradiation, the double-drug combination was stronger than TMZ ($p = 0.0003$) or CCNU ($p = 0.0007$) alone in reducing clonogenic survival (Figure 2a). Together with 4 Gy irradiation alone, TMZ-IR reduced surviving fraction to 38% ($p = 0.0074$) and CCNU-IR to 26% ($p = 0.0002$). Strikingly, the combined use of TMZ, CCNU and 4 Gy irradiation (triple treatment) further reduced the survival fraction to just 12% ($p < 0.0001$). Double-drug treatment together with irradiation also further reduced clonogenic survival compared to TMZ-IR (4 Gy: $p = 0.0018$; 8 Gy: $p = 0.0095$) but not to CCNU-IR (4 Gy: $p = 0.0836$; 8 Gy: $p = 0.5079$). Additionally, we performed the assay with the dose of 2 Gy, which is relevant for treatment of certain types of tumours. Similarly to other doses, also at 2 Gy the triple combination further reduced clonogenic cell survival in comparison to single treatments (Figure S3).

As 5 μM of CCNU did not significantly inhibit cell proliferation (Figure 1d), we chose a concentration of 10 μM CCNU to test the effect of triple treatment on glioma cell proliferation. Without irradiation, all drugs significantly reduced proliferation compared to DMSO-treated cells (Figure 2b). 10 μM CCNU alone or the double-drug treatment reduced proliferation more potently than TMZ alone. Together with 4 Gy ($p = 0.019$) and 8 Gy ($p = 0.043$), the triple treatment achieved significant reduction in proliferation compared to the exclusively irradiated control cells. Similarly, triple treatment further decreased

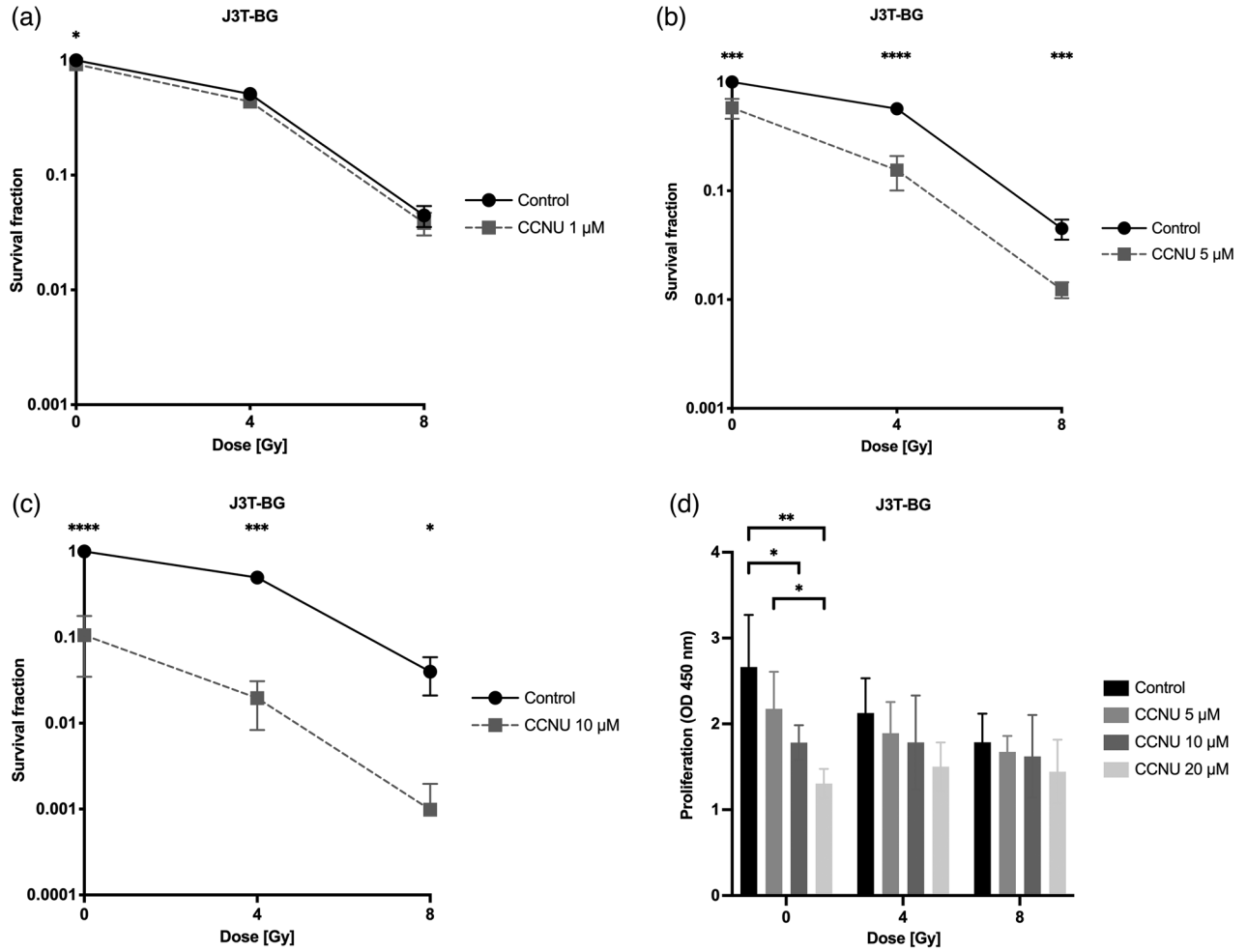

FIGURE 1 Effect of lomustine (CCNU) alone and together with irradiation (4 and 8 Gy) on clonogenic cell survival (a–c) and cell proliferation (d) in the canine glioma cell line J3T-BG. (a–c) Clonogenic cell survival assay with 1, 5 and 10 μM CCNU ($n = 3$–4). Statistical significances were calculated with the unpaired *t*-test. (d) Proliferation assay with 5, 10 and 20 μM CCNU ($n = 3$). Cell viability was measured 48 h after treatment. Statistical differences were calculated using the one-way ANOVA with the Tukey's post hoc test. Mean ± SEM of four independent experiments is shown. *p* Values < 0.05 are labelled with an asterisk (*), < 0.01 with two asterisks (**), < 0.005 with three asterisks (***) and < 0.001 with four asterisks (****).

proliferation in comparison to single-drug TMZ-IR ($p = 0.0208$) and CCNU-IR ($p = 0.0281$) when combined with 8 Gy. A similar trend was observed when combined with 4 Gy, however this was not found to be statistically significant.

3.2.1 | Resistant cell lines show higher IC_{50} values for CCNU and TMZ

Initially, we tested if the long-term exposed cell lines indeed developed resistance against both cytostatics. We investigated the inhibitory effect of both TMZ and CCNU and calculated the IC_{50} values for these compounds (Table S2). Logarithmic curves for all 3 cell lines are displayed in Figure 3.

CCNU concentrations from 0.625–1600 μM were used in the treatment of the parental J3T-BG, CCNU-resistant and TMZ-resistant cell lines resulting in IC_{50} values of 11.52, 16.62 and 14.99 μM, respectively.

For the IC_{50} values for TMZ, a range from 6.25 to 3200 μM was used, resulting in IC_{50} values of 292.6, 373.8 and 328.3 μM for J3T-BG, J3T-BG/CCNU and J3T-BG/TMZ.

The two resistant cell lines showed higher IC_{50} values following treatment with both alkylating agents, suggesting cross-resistance towards CCNU after TMZ therapy and towards TMZ after CCNU therapy. However, no significant difference in CCNU or TMZ IC_{50} was observed for the drug-resistant cell lines compared to the parental cell line (J3T-BG).

In order to further evaluate whether the cell lines truly developed resistance, we analysed the levels of p-glycoprotein (P-gp), also known as multidrug resistance protein (MDR1) (Gros et al., 1986). In our analysis, we found the protein in all cell lines, parental and DMSO-treated as well as drug-resistant (Figure 3b). The signal of P-gp was stronger in cells treated with either cytostatics. Interestingly, in cells treated with TMZ, the signal decreased over time. These results confirm that induction of P-gp protein might be a part of the resistance mechanism against CCNU and TMZ.

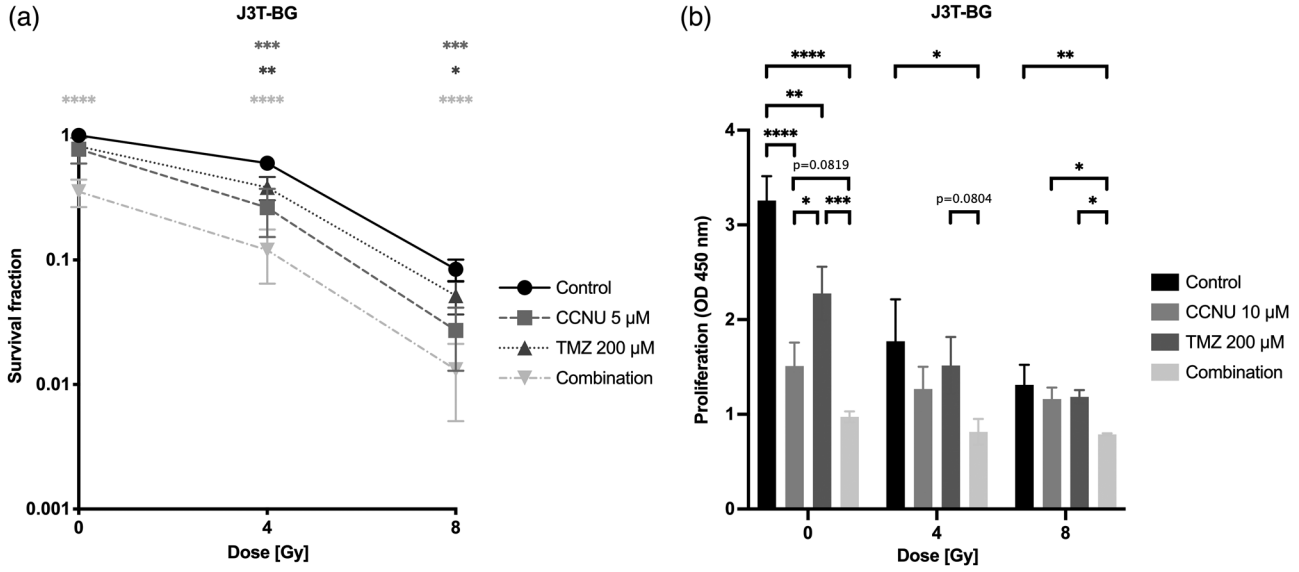

FIGURE 2 Effect of double-drug combination together with radiation therapy on clonogenic cell survival and cell proliferation in the canine glioma cell line J3T-BG. (a) Clonogenic cell survival assay with 5 μM CCNU and 200 μM TMZ ($n = 3$). (b) Proliferation assay with 10 μM CCNU and 200 μM TMZ. Cell viability was measured 48 h after treatment ($n = 3$). Mean ± SEM of at least three experiments performed independently is shown. Statistical differences were calculated using the one-way ANOVA with the Tukey's post hoc test. p Values < 0.05 are labelled with an asterisk (*), < 0.01 with two asterisks (**), < 0.005 with three asterisks (***) and < 0.001 with four asterisks (****).

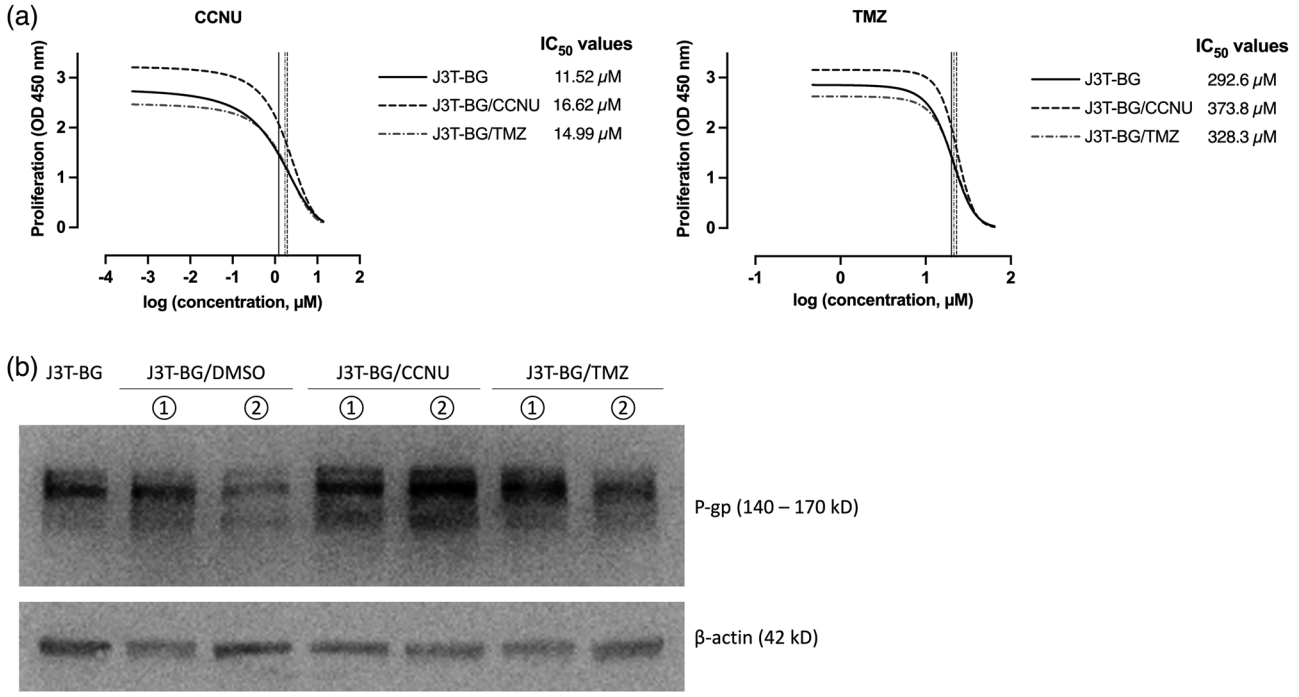

FIGURE 3 Determined IC_{50} values of temozolomide (TMZ) and lomustine (CCNU) and western blot analysis for the expression of *p-glycoprotein (P-gp/MDR-1)* in the control (J3T-BG), parental (J3T-BG/DMSO) and long-term drug-exposed (J3T-BG/CCNU and J3T-BG/TMZ) cell lines. (a) Curves were generated and IC_{50} values calculated with a four-parametric nonlinear regression analysis fitting the Hill Slope of four independent experiments ($n = 4$). The four parameters were bottom, top, logIC_{50} and Hill Slope. Basal response was subtracted, and the bottom was set to a constant value of 0. The IC_{50} values of the resistant cell lines were then compared to the control cell line using the unpaired t-test. No significant differences ($p < 0.05$) were observed. (b) Cell lysates were analysed by immunoblotting for P-gp and β-actin. For all samples, 50 μg of total protein were tested. A representative experiment of three experiments performed independently is shown. Numbers indicate cell lysates generated ① directly after long-term exposure and ② after 2 weeks maintained in drug-free medium.

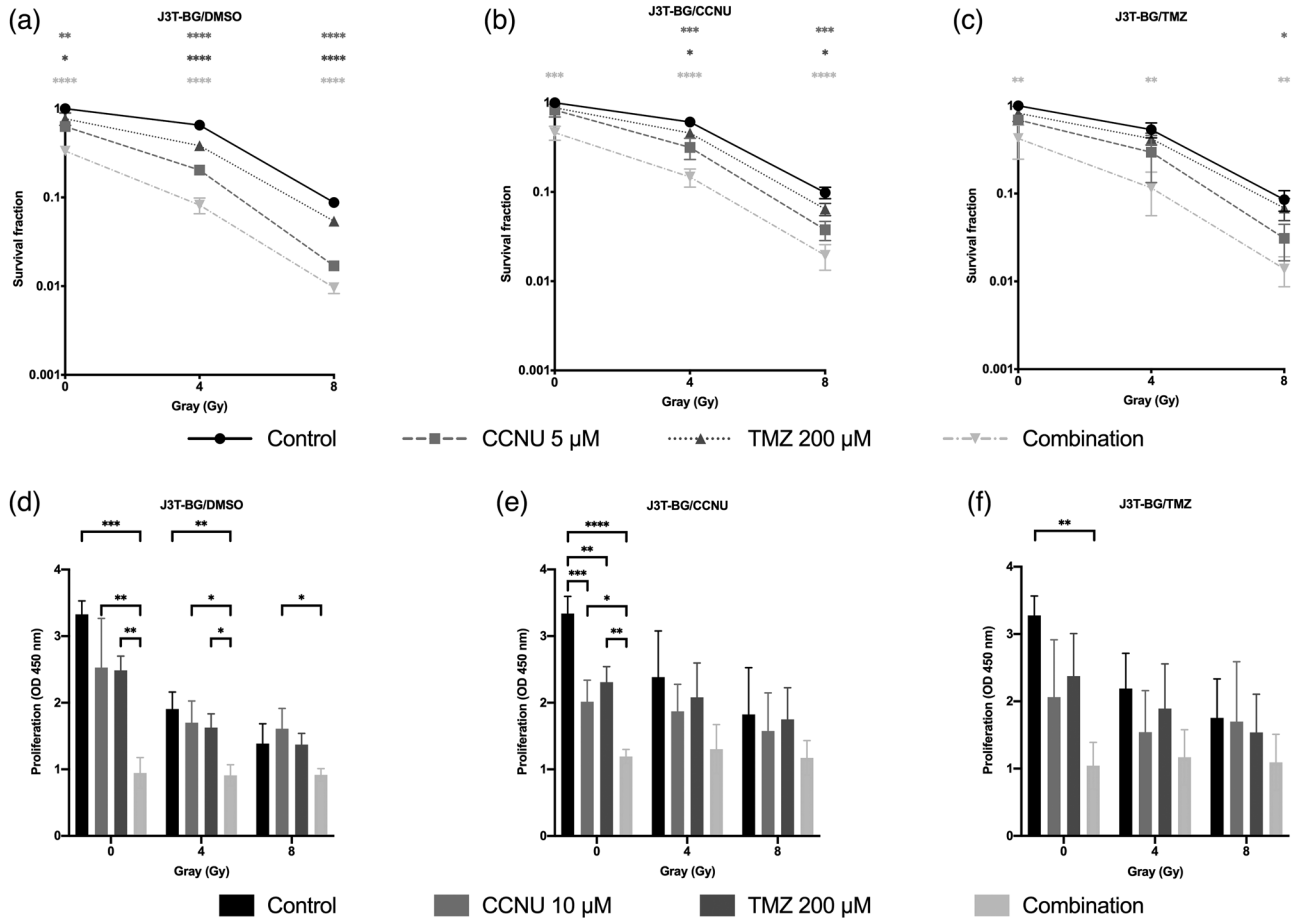

FIGURE 4 Effect of double-drug combination together with radiation therapy on clonogenic cell survival and cell proliferation in the resistant cell lines (J3T-BG/CCNU and J3T-BG/TMZ) compared to the parental cell line (J3T-BG/DMSO). (a–c) Clonogenic cell survival assay with 5 μM CCNU and 200 μM TMZ in the (a) J3T-BG/DMSO, (b) J3T-BG/CCNU and (c) J3T-BG/TMZ cell lines ($n = 3$). (d–f) Proliferation assay with 10 μM CCNU and 200 μM TMZ in the (d) J3T-BG/DMSO, (e) J3T-BG/CCNU and (f) J3T-BG/TMZ cell lines ($n = 3$). Cell viability was measured 48 h after treatment. Mean ± SEM of at least three experiments performed independently is shown. Statistical differences were calculated using the one-way ANOVA with the Tukey's post hoc test. p Values < 0.05 are labelled with an asterisk (*), < 0.01 with two asterisks (**), < 0.005 with three asterisks (***) and < 0.001 with four asterisks (****).

3.3 | Effect of triple treatment (TMZ/CCNU/IR) on cell survival in TMZ/CCNU-resistant cell lines

To test the effect of long-term drug exposure on the cancer cells, we exposed the J3T-BG cell line to TMZ and CCNU for a prolonged period of time. For the purpose of our study, we call these cell lines hereafter 'resistant cell lines'. After long-term exposure to either cytostatic drug, we evaluated response to different concentrations of CCNU or TMZ in these resistant cell lines (J3T-BG/TMZ; J3T-BG/CCNU).

3.3.1 | Combination of CCNU and TMZ overcomes resistance and increases treatment efficacy in clonogenic cell survival assay

After IC_{50} testing, we re-evaluated the efficacy of triple combination on clonogenic cell survival and proliferation in the CCNU- or TMZ-resistant cell lines.

Without IR, only the combination of CCNU (5 μM) and TMZ (200 μM) compared to single-drug treatment had a significant inhibitory effect on clonogenic cell survival in resistant cell lines (Figure 4a–c). Whereas, when treated together with IR (4 and 8 Gy), the single drugs achieved significant inhibition in clonogenic survival in the CCNU-resistant cell line, while only CCNU-IR with 8 Gy showed this effect in the TMZ-resistant cell line. Importantly, the double-drug combination potently reduced irradiated survival (4 Gy) to 15% in CCNU-resistant ($p < 0.0001$) and 12% in the TMZ-resistant cell line ($p = 0.0057$). In the CCNU-resistant cell line, the triple treatment also remained superior to TMZ-irradiation with 4 ($p = 0.0004$) and 8 Gy ($p = 0.0032$). The same was seen in the TMZ-resistant cell line, where the triple treatment was stronger with 4 Gy ($p = 0.0315$) and with 8 Gy ($p = 0.0159$).

We also analysed these results by fitting them to the linear-quadratic model. Plots as well as a table containing α- and β-value together with enhancement factor can be seen in Figure S4. An additive effect was observed upon exposure to CCNU, TMZ and the drug com-

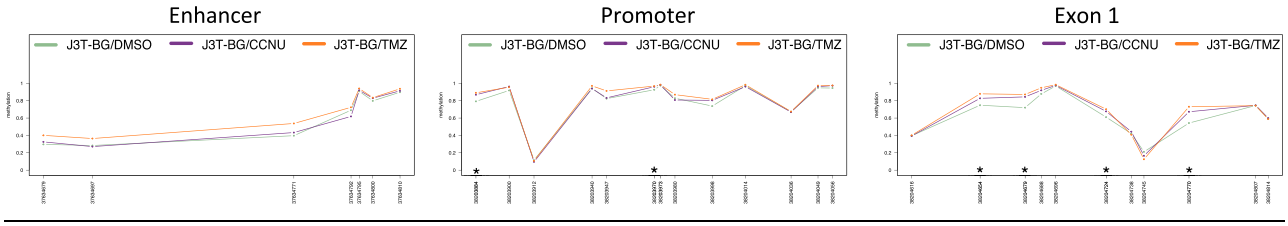

FIGURE 5 Methylation profile plots of MGMT enhancer, promoter and exon 1 of the parental (J3T-BG/DMSO) and the drug-resistant (J3T-BG/CCNU and J3T-BG/TMZ) cell lines. Each line represents the methylation mean (*y*-axis) for each CpG position (*x*-axis) for every cell line (four samples analysed per cell line). Asterisks highlights CpG's which show statistical differences ($p < 0.05$) as calculated by the nonparametric Kruskal–Wallis test.

bination in the parental J3T-BG cell line, while only CCNU and the drug combination remained additive in the CCNU-resistant cell line. In addition, only the drug combination together with 8 Gy remained additive in the TMZ-resistant cell line.

Comparing the survival fraction between the cell lines after chemoradiation revealed a higher survival fraction in the drug-resistant cells compared to the parental cell line, however, no significant differences were observed.

To evaluate the impact of the different treatment modalities on the proliferation ability of the resistant cell lines, we used the same settings as described above. In the absence of IR, double-drug treatment significantly reduced proliferation of all three cell lines in comparison to DMSO-treated cells (Figure 4d–f). Interestingly, in the CCNU-resistant cell line, single-drug treatment achieved a significant inhibitory effect on proliferation (CCNU: $p = 0.0007$; TMZ: $p = 0.0037$); however, the drug combination remained superior over CCNU ($p = 0.0139$) and TMZ alone ($p = 0.0022$). Together with 4 Gy irradiation, triple treatment did not significantly reduce proliferation in the resistant cell lines compared to exclusively irradiated cells, or compared to the single drugs alone (Figure 4e and f). This suggests that combination drug therapy is enough to overcome resistance.

3.4 | Methylation pattern

We have previously shown the methylation pattern of *MGMT* enhancer, promoter and exon 1 in J3T-BG as well as two further canine glioma cell lines, SDT3G and G06A (Tresch et al., 2021). In the current study, we further evaluated the methylation pattern of additional genes involved in DNA-repair pathways and cell survival in these three cell lines and in the J3T-BG cell lines that developed resistance against TMZ and CCNU. In the three regions of interest of the *MGMT* gene, the resistant cell lines showed a higher methylation rate in most CpGs compared to the DMSO-treated cell line (Figure 5). Especially in the promoter and exon 1, we observed significant differences in 2 and 4 CpG sites as indicated on Figure 5.

We also compared the methylation pattern in a number of genes in all three canine glioma cell lines (J3T-BG, SDT3G and G06A). Among these a different methylation rate was observed within five genes. Namely, the J3T-BG cell line showed a significantly higher methylation rate in the genes encoding for *MSH3* and *XRCC5*, whereas the same cell line had a lower methylation rate at one CpG in the *FEN1* gene (seen in the Figure S5). Multiple differences were detected across the individual canine glioma cell lines regarding the gene encoding for *ODZ1* (also known as *TENM1*), showing the highest methylation rate in the SDT3G and the lowest in the G06A cell line.

Methylation analysis revealed changes after drug treatment with alkylating agents in resistant cell lines. However, significant differences were only observed in the genes encoding for *MLH1*, *MSH3* and *XRCC5* (Figure S5).

In the other analysed genes (*CASP8*, *CDK1*, *ATM*, *HIF1* and *BRCA1)*, no differences in methylation patterns were found.

4 | DISCUSSION

In a recent Phase III trial, the drug combination was tested on newly diagnosed human glioblastoma patients with methylated *MGMT* promoter (Herrlinger et al., 2019). An extended survival time was found in patients additionally treated with CCNU, compared to patients who received TMZ-irradiation alone.

We have recently reported that TMZ reduces cell survival of canine glioma cell lines J3T-BG, G06A and SDT3G in combination with irradiation (Tresch et al., 2021). In the current study, CCNU alone as well as the investigated drug combination with or without irradiation had a stronger impact than TMZ with or without irradiation (Figure 2). A longer exposure to both, CCNU or TMZ alone led to a stronger reduction of clonogenic survival, which can be explained by the higher cytotoxicity of the drugs itself. However, no benefit of different incubation times with CCNU prior to IR could be shown, both incubation times resulted in a strong reduction of survival fraction after irradiation (Figure S2). No significant reduction was found after longer exposure with TMZ prior to IR. This is contrary to an earlier study observing an incubation dependent effect prior to IR for TMZ (Chalmers et al., 2009). However, they used a longer incubation time up to 72 h, whereas we pretreated our cells maximally for 24 h in the clonogenic assay.

Canine glioma cell lines that underwent chronic drug treatment showed higher IC_{50} values against cytostatic compounds, confirming

the onset of resistance (Figure 3a). In line with two prior reports of a TMZ-resistant human glioma cell line, we observed a slower growth in the TMZ-resistant cell line (Yamamuro et al., 2021) and an unchanged sensitivity towards irradiation in both resistant cell lines (Stritzelberger et al., 2018). Inversely, the CCNU-resistant cell line shows faster proliferation resulting in a higher baseline, when untreated. At the same time, Stritzelberger et al. (2018) could not observe a cross-resistance of the TMZ-resistant cell line towards CCNU and consider CCNU as primary therapy to prevent development of resistance. Yamamuro et al. (2021) recommend the use of nitrosoureas like CCNU as second line after TMZ-resistance acquisition. In the present study, we observed a cross-resistance after treatment with TMZ and CCNU and consider the double-drug treatment more beneficial as a first-line therapy instead of single use. The triple treatment with 4 Gy remained effective in both, which could even allow repeating the therapy (Figure 4b and c).

The doses used in our study were 200 μM of TMZ and 5–10 μM of CCNU. These are equivalent to 38.83 μg/mL and 1.17–2.34 μg/mL, respectively. For both lipophile drugs a high bioavailability is reported, whereby it must be noted that CCNU bioavailability cannot be correctly measured as it decomposes into two active metabolites in an aqueous solution (Kastrissios et al., 1996; Lee et al., 1985; Ostermann et al., 2004). Cerebrospinal fluid-plasma ratios of 20% are reported for TMZ (Ostermann et al., 2004; Portnow et al., 2009; Zhou et al., 2007). While this is less than we used in our experiments, in general higher drug concentrations are necessary under laboratory conditions (Lee et al., 1985; Ostermann et al., 2004).

Chemosensitivity depends on multiple factors and in terms of alkylating agents there may be preexisting (intrinsic) resistance like the DNA-repair enzyme *MGMT* or acquired resistance (Lee, 2016). TMZ treatment can induce higher expression of *MGMT* and/or mismatch repair (MMR) deficiency leading to higher resistance towards TMZ (Stritzelberger et al., 2018; Von Bueren et al., 2012; Yamamuro et al., 2021). While one study reported no association between P-gp expression and TMZ resistance (Perazzoli et al., 2015), we observed in the current study a higher expression in both resistant cell lines directly after long-term treatment (Figure 3b) that could be supported by another publication (Munoz et al., 2014). Nevertheless, the P-gp signal faded in the TMZ-resistant cell line 2 weeks after long-term exposure, presumably a consequence of absent drug exposure. However, as P-gp can mitigate drug efficacy, it will not be the only mechanism behind drug resistance in gliomas.

DNA methylation is one epigenetic mechanism that can regulate gene expression, with higher promoter methylation correlating with lower or no protein expression (Suzuki & Bird, 2008). Moreover, methylation pattern can be used as cancer biomarker and prognostic factor in tumour treatment (Koch et al., 2018). We have recently reported a high methylation level of the *MGMT* gene in the canine glioma cell lines J3T-BG, SDT3G and G06A (Tresch et al., 2021). Our drug-resistant cell lines showed an even higher methylation pattern within the *MGMT* gene (Figure 5), consequently, we do not expect *MGMT* expression after drug treatment as observed in other studies (Perazzoli et al., 2015). We therefore conclude that *MGMT* expression might not be the driving mechanism of resistance after drug treatment in our resistant cell lines.

4.1 | Limitations

To confirm a clinical benefit of chemoradiation on canine glioma, the results from our in vitro findings will need to be translated into clinical practice. Even if both drugs have established doses in dogs, hematopoietic and gastrointestinal side effects must be evaluated if given together (Newlands et al., 1992). Further, it must be clarified if efficient plasma and tumour tissue-levels can be achieved.

We also cannot rule out if resistance was stable over the time or changed during the lack of drug exposure (McDermott et al., 2014).

5 | CONCLUSION

Our findings show that the combination of CCNU, TMZ and IR enhance cytotoxic efficacy in canine glial tumour cells with methylated *MGMT* promoter. Translation of these findings for clinical use suggests that the triple treatment could serve as treatment option to improve local tumour control and reduce metastasis, even in cases of resistance to primary therapy or recurrent disease. Understanding potential biomarkers and their promoters methylation as potential prognostic markers should be further investigated.

AUTHOR CONTRIBUTIONS

Daniel Fuchs: conceptualisation; formal analysis; investigation; methodology; project administration; writing – original draft; writing – review & editing. Carla Rohrer Bley: funding acquisition; supervision; writing – original draft; writing – review & editing. Luca Morandi: investigation; methodology; writing – review & editing. Caterina Tonon: funding acquisition; methodology. Mathias S. Weyland: formal analysis; writing – review & editing. Katarzyna Nytko: Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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CONFLICT OF INTEREST STATEMENT

No conflict of interests to declare.

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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