Acquired temozolomide resistance in human glioblastoma cell line U251 is caused by mismatch repair deficiency and can be overcome by lomustine

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Abstract
Purpose Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults. While the alkylating agent temozolomide (TMZ) has prolonged overall survival, resistance evolution represents an important clinical problem. Therefore, we studied the effectiveness of radiotherapy and CCNU in an in vitro model of acquired TMZ resistance.

Methods We studied the MGMT-methylated GBM cell line U251 and its in vitro derived TMZ-resistant subline, U251/TMZ-R. Cytotoxicity of TMZ, CCNU, and radiation was tested. Both cell lines were analyzed for MGMT promotor status and expression of mismatch repair genes (MMR). The influence of MMR inhibition by cadmium chloride (CdCl₂) on the effects of both drugs was evaluated.

Results During the resistance evolution process in vitro, U251/TMZ-R developed MMR deficiency, but MGMT status did not change. U251/TMZ-R cells were more resistant to TMZ than parental U251 cells (cell viability: 92.0% in U251/TMZ-R/69.2% in U251; \( p = 0.032 \)) yet more sensitive to CCNU (56.4%/80.8%; \( p = 0.023 \)). The effectiveness of radiotherapy was not reduced in the TMZ-resistant cell line. Combination of CCNU and TMZ showed promising results for both cell lines and overcame resistance. CdCl₂-induced MMR deficiency increased cytotoxicity of CCNU.

Conclusion Our results confirm MMR deficiency as a crucial process for resistance evolution to TMZ. MMR-deficient TMZ-resistant GBM cells were particularly sensitive to CCNU and to combined CCNU/TMZ. Effectiveness of radiotherapy was preserved in TMZ-resistant cells. Consequently, CCNU might be preferentially considered as a treatment option for recurrent MGMT-methylated GBM and may even be suitable for prevention of resistance evolution in primary treatment.

Keywords Glioblastoma · Antineoplastic drug resistance · Temozolomide · Lomustine · Mismatch repair

Abbreviations
7AAD 7-Amino-actinomycin D
AxV Annexin V
CCNU Lomustine
CdCl₂ Cadmium chloride
DMEM Dulbecco’s Modified Eagle Medium (Medium für Zellkultur)
GBM Glioblastoma multiforme
Gy Gray
MD Mean difference
MGMT O⁶-Methylguanin-DNA-methyltransferase
MMR Mismatch-repair
SF Survival fraction
TMZ Temozolomide

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Glioblastoma (GBM) is the most common malignant primary tumor of the brain in adults and associated with a particular poor prognosis [1]. The current standard of care includes surgery, radiotherapy, and the monofunctional alkylating agent temozolomide (TMZ). Although TMZ has improved overall survival, most patients still develop tumor recurrence within a period of 7 months [2]. Therapy failure is often due to resistance evolution processes against TMZ, one of the major obstacles in GBM treatment [3].

Amongst DNA adducts created by monofunctional agents like TMZ, O6-methylguanine assumedly is the most important lesion mediating TMZ toxicity. These adducts are repaired by O6-methylguanin-DNA-methyltransferase (MGMT); if MGMT is absent, base mispairing triggers repetitive but unsuccessful mismatch repair (MMR) leading to subsequent DNA strand breaks, cell cycle arrest, and apoptosis [4, 5]. Resistance to TMZ at the time of diagnosis is mostly due to high levels of MGMT [6, 7]. In contrast, tumors with methylated MGMT promoter are very sensitive to TMZ, but resistance almost inevitably develops during treatment. Several authors suggested MMR deficiency being responsible for acquired TMZ resistance in MGMT-methylated tumors [6, 8–13] and strategies to restore the effect of MMR system have been postulated to improve the effect of TMZ [14].

Aside from TMZ, lomustine (CCNU) is another alkylating agent with proven efficacy in GBM therapy [15, 16]. While a proficient MMR is vital for the expression of TMZ cytotoxicity, it is supposedly inversely related to CCNU toxicity as interstrand links caused by bifunctional agents such as CCNU are repaired by MMR. MMR deficiency indeed was shown to increase sensitivity to bifunctional agents in different MMR-deficient non-glioma cell lines [17–20].

Clinical trials showed promising results for the combination of CCNU and TMZ in patients with newly diagnosed GBM, with the largest clinical benefit found for MGMT-methylated patients [21, 22]. In addition, CCNU monotherapy demonstrated good efficacy for recurrent cases [15]. However, the underlying mechanisms have not been elucidated to date.

We hypothesized that acquired resistance to TMZ in MGMT-methylated GBM cells is mediated by MMR deficiency and, therefore, might be accompanied by increased sensitivity to CCNU. We, therefore, sought to investigate the combination and the differential effects of TMZ and CCNU in the human GBM cell line U251 and a TMZ-resistant line U251/TMZ-R with regard to the role of MMR and MGMT.

**Materials and methods**

**Cell lines and primary culture**

The human glioblastoma cell line U251 was obtained from Cell Line Service (CLS; Eppelheim, Germany). Cells were maintained and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Darmstadt, Germany) and cultured at 37 °C in a 5% CO₂ incubator.

**Drugs**

TMZ, CCNU, and CdCl₂ were obtained from Sigma-Aldrich (St Louis, USA). TMZ was dissolved in dimethyl sulfoxide (DMSO), CCNU in ethanol, and CdCl₂ in sterilized water. Stock solutions were stored at −20 °C.

**Generation of a TMZ-resistant cell line**

U251 cells were cultured in 75 cm² cell flasks (Cellstar; Greiner BioOne, Nürtlingen, Germany) and allowed to adhere overnight. Cells were treated with 100 μM TMZ. Cell treatment was repeated every 24 h for 5 consecutive days. After those 5 days, exposure to the fresh TMZ was repeated every 3 days to a total of 3 weeks. This procedure has been previously described for U251 [13].

**Treatment with drugs and irradiation**

Cells were seeded in 25 cm² flasks at a density of 2.0 × 10⁵ cells. The chemotherapeutics were added 48 h later. CdCl₂ was added 2 h before chemotherapy treatment for pre-incubation according to a protocol introduced by Yamauchi et al. [23]. 1 h after chemotherapy treatment, cells were irradiated with 2 Gy, corresponding to the daily dose employed in clinical practice [24]. Irradiation was performed with an X-ray generator (120 kV, 22.7 mA, variable time; GE Inspection Technologies, Ahrensburg, Germany).

**Cell death detection**

We used APC Annexin V (AxV)/7-amino-actinomycin D (7AAD) staining and flow cytometry to investigate TMZ and CCNU-induced cell death. After harvesting the cells 72 h after treatment by trypsinization, cell suspension (100 μl, 1 × 10⁵ cells) and 5 μl of AxV and 7AAD (both BD Biosciences, Franklin Lakes, USA), respectively, were combined with 400 μl Ringer solution (B. Braun
Melsungen AG, Melsungen, Germany) and incubated at 4 °C for 30 min. Cell death was determined using flow cytometry (Gallios, Beckman Coulter, Brea, USA) and its associated Kaluza 1.3 Software (Beckman Coulter, Krefeld, Germany). For each sample, a minimum of 2 × 10⁴ events was assayed. Experiments were performed at least thrice with two replicates per run. AxV/7AAD-double-negative cells were considered to be viable cells, AxV-positive/7AAD-negative cells early apoptotic cells, and AxV/7AAD-double-positive cells late apoptotic/necrotic cells [25–27].

Cell cycle analysis

Cell cycle distribution was analyzed by Hoechst 33342 staining (BD Biosciences, Franklin Lakes, USA) and flow cytometry. 72 h after treatment, cells were harvested. 2 ml of cell suspension (± 2 × 10⁶ cells) were combined with 10 ml of 70% ethanol (Carl Roth, Karlsruhe, Germany) at 4 °C for at least 2 h to fix the cells. Following incubation, cells were resuspended in 1 ml Ringer solution, combined with 3 μl of Hoechst, and incubated at 4 °C for 20 min. In each sample, 2 × 10⁵ cells were assayed using flow cytometry. The cell cycle phase distribution was determined with the Kaluza 1.3 software.

Clonogenic assay

The effects of irradiation were determined with clonogenic assays. Cells were plated in 60-mm dishes (Nunc Thermo Fisher, Waltham, USA) with 300–1600 cells per dish. After 6–12 h, cells were treated with drugs and irradiated with increasing doses of 2, 4, 6, 8, and 10 Gy (see above). After incubation for 10–14 days in drug-free fresh, medium cells were fixed with methylene blue for 30 min. Subsequently, colonies containing >50 cells were counted. The survival fraction (SF) was calculated as follows: SF at a given condition = colonies counted of the given condition/(cells seeded of the given condition × plating efficiency/100). Plating efficiency = percentage of untreated cells seeded that grow into colonies.

Immunostaining

To assess MMR protein expression, immunostaining and subsequent image analyses were performed following a standard protocol [28] with slight modifications. The following mouse monoclonal antibodies were used: anti-MLH1, anti-MSH6, anti-PMS2 (all BD Biosciences), and anti-MSH2 (Merck Millipore, Darmstadt, Germany). Primary antibodies were applied at a dilution of 1:100 and Alexa labelled secondary antibodies (Invitrogen; Life Technologies GmbH, Darmstadt, Germany) at 1:400. Images were captured by fluorescence microscopy (Leica DM 6000). Overlays were built using an image-processing software (Biomas 3.3 10/2004 MSAB). MLH1 foci were counted as previously described [28, 29].

Pyrosequencing for promoter status determination

In both cell lines, quantitative methylation analyses of the MGMT promotor were performed by pyrosequencing (PyroMark Q24 MGMT-Kit [Qiagen]) in the Institute of Neuropathology, Erlangen.

Statistical analysis

If not indicated otherwise, results are expressed as the mean ± standard deviation (SD) of three independent experiments. Statistics were performed with IBM SPSS Statistics 22.0 for Windows (IBM Corporation, New York, USA) using the two-sided t test and the non-parametric Mann–Whitney U test. Significant differences with a p value of ≤0.05 are marked as *, very significant differences (p ≤ 0.01) as **, and highly significant differences (p ≤ 0.001) as ***.

Results

U251/TMZ-R was more resistant to TMZ than U251

Cytotoxicity of 500 μM TMZ for 72 h was determined by analyzing cell viability, apoptotic, and necrotic cell death using AxV/7AAD staining (Fig. 1a). Following exposure to TMZ, the percentage of viable U251/TMZ-R cells (AxV−/7AAD−) was significantly elevated compared to parental U251 cells (92.0 ± 2.0% for U251/TMZ-R and 69.2 ± 8.8% for U251, p = 0.03). Both early apoptotic cells (AxV+/7AAD−) and late apoptotic/necrotic cells (AxV+/7AAD+) were notably decreased in U251/TMZ-R compared to U251 (mean difference (MD) in apoptosis: 0.8 ± 0.3 vs. 2.7 ± 0.5%; p = 0.02; MD in necrosis: 5.9 ± 1.5 vs. 22.3 ± 6.63%; p = 0.04) (Fig. 1a, b).

Cell cycle distribution after exposure to 500 μM TMZ was analyzed by Hoechst 33342 staining (Fig. 2a). TMZ induced a G2/M block in parental U251 cells (G2/M fraction; Controls: 11.0 ± 1.8%, TMZ: 45.3 ± 15.4%; p = 0.05) and a distinct reduction of cells in G1 phase (76.5 ± 1.6% vs. 34.7 ± 7.6%; p = 0.03). U251/TMZ-R cells, however, did not accumulate in G2/M after TMZ treatment, and the cell cycle distribution remained largely unchanged (Fig. 2b).

Growth inhibition was assessed using colony formation assay. TMZ suppressed colony formation in U251, but not
in U251/TMZ-R (SF: 41.3 ± 11.6 vs. 100.0 ± 2.8%; p = 0.003).

**U251/TMZ-R cells did not develop cross resistance to CCNU and cytotoxicity of CCNU was enhanced in U251/TMZ-R**

Cytotoxicity of CCNU was analyzed using AxV/7AAD staining and flow cytometry as described for TMZ (Fig. 1a). After exposure to 38.5 μM CCNU for 72 h, cell viability (AxV−/7AAD−) was decreased in U251/TMZ-R compared to U251 (56.4 ± 6.5 vs. 80.8 ± 4.6%; p = 0.02). Late apoptosis/necrosis (AxV+/7AAD+) was notably increased in U251/TMZ-R (30.7 ± 5.7 vs. 12.3 ± 3.3%; p = 0.04). Thus, cells were very sensitive to CCNU-induced cell death with an even more cytotoxic effect of CCNU in U251/TMZ-R cells than in U251 cells (Fig. 1b, c). CCNU induced G2/M arrest (Fig. 2b) and suppressed colony formation in both cell lines (data not shown), strengthening the conclusion that TMZ-R cells were not cross-resistant to CCNU.
Combination of CCNU and TMZ led to increased cytotoxicity in both cell lines and overcame resistance

The combination of CCNU (38.5 μM) and TMZ (500 μM) had stronger effects on cell viability and late apoptosis/necrosis than each single drug for U251 and U251/TMZ-R (Fig. 1b). The effects of CCNU+TMZ were even stronger in resistant cells (cell viability: 26.0 ± 2.7 vs. 39.7 ± 1.1%; p = 0.01, MD in necrosis: 60.6 ± 2.9 vs. 48.1 ± 0.6%; p = 0.02). Similarly, the combination of TMZ and CCNU led to an increase in G2/M arrest (Fig. 2b).

Inhibition of MMR increased sensitivity to CCNU in U251

Cells were pre-incubated with CdCl₂, an inhibitor of MMR, at a concentration of 2.5 μM, chosen for its minimally toxic effects (Fig. 3a). 72 h after exposure to 4.8 μM CCNU, cells pre-incubated with CdCl₂ showed significantly increased rates in late apoptosis/necrosis (3.3 ± 0.9 vs. 8.2 ± 1.0% for CCNU vs. CCNU + CdCl₂; p = 0.02) and decreased rates in cell viability (93.6 ± 1.8 vs. 86.5 ± 1.8%; p = 0.05, Fig. 3d).

Correspondingly, G2/M arrest after exposure to CCNU was significantly more distinct in cells pre-incubated with CdCl₂ (G2/M fraction: 15.7 ± 2.2 vs. 23.5 ± 1.4% for CCNU vs. CCNU+CdCl₂; p = 0.04, Fig. 3b). Although TMZ toxicity was slightly decreased in cells pre-incubated with CdCl₂ with regard to cell viability (60.7 ± 8.0 vs. 65.1 ± 1.0%; p = 0.64), late apoptosis/necrosis rate (30.1 ± 4.7 vs. 25.8 ± 2.6%; p = 0.53), and G2/M arrest (61.1 ± 2.3 vs. 58.1 ± 4.8%; p = 0.61), no significant effects were observed (Fig. 3c). Our results indicate that MMR inhibition sensitized cells to CCNU but not to TMZ.

Radio-sensitivity

To investigate effects of irradiation, clonogenic assays were performed. No significant differences between
parental and resistant cells were detected for doses of 2 and 6 Gy with regard to growth restriction. However, significantly reduced colony formation was observed in TMZ-resistant cells for radiation doses of 4, 8, and 10 Gy, although the observed effects were minimal (Fig. 4). Furthermore, we did not observe differences in radio-sensitivity between both cell lines with regard to cell viability, apoptosis, and cell cycle distribution (data not shown).

Molecular characterization of U251/TMZ-R

U251 and U251/TMZ-R were tested for MGMT promotor methylation by pyrosequencing and promotor methylation was detected in both cell lines (Fig. 5). When tested for expression of MMR proteins, MLH1 was only activated in U251 (17.7 ± 1.1 vs. 0.4 ± 0.0 foci/cell; p = 0.04). For MSH2, MSH6, and PML1, no countable foci were detected in both cell lines. Our results suggest that TMZ resistance acquired in U251/TMZ-R was not related to changes in MGMT status but to deficiency of the MMR protein MLH1 (Fig. 6).

Discussion

In our study, TMZ-resistant MGMT-methylated GBM cells showed increased resistance to TMZ-induced cell death, but intriguingly increased sensitivity to CCNU-induced cell death compared to parental U251 cells. Beyond that, the combination of CCNU and TMZ was more effective than each single agent in both cell lines regarding drug-induced cell death. It is especially interesting that combination of CCNU and TMZ was even more effective in resistant cells than in non-resistant parental cells.
Our observations are well supported by clinical trials. Herrlinger et al. investigated the combination of CCNU and TMZ in the single arm phase II UKT-03 trial. In an updated analysis after an extended follow-up, Glas et al. reported long-term survival especially in the subgroup that received intensified TMZ and CCNU dose [21, 22]. In the recurrent setting, the REGAL trial showed an impressive overall survival of 9.8 months in the arm that received CCNU alone [15].

Apart from repairing DNA adducts caused by monofunctional agents like temozolomide, MGMT also repairs the O6-chloroethylguanine residues induced by bifunctional agents such as CCNU; thus, elevated MGMT levels lead to cross resistance between both drugs [4, 30, 31]. When MGMT capacity is saturated by an excess of O6-methylguanine produced, TMZ treatment causes base-pair mismatches and replication errors, triggering repetitive but unsuccessful MMR. This leads to continuous DNA strand breaks, cell cycle arrest in G2 phase, and subsequent apoptosis in MMR-proficient cells [4, 5, 32]. In contrast, cells with MMR deficiency possess relative resistance to monofunctional agents such as TMZ and a correlation between MMR deficiency and GBM recurrence has been reported [10–12, 33]. In line with these previous studies, changes in MGMT promotor methylation were not involved in the acquisition of resistance in our study. Conversely, expression of the MMR protein MLH1 was strongly decreased in the resistant cell line. A decrease in MLH1 expression has been reported to occur early during acquisition of TMZ resistance in vitro and in vivo [12]. MLH1 expression was significantly decreased in recurrent GBM [33]. This proves the importance of MLH1 for a proficient MMR response in TMZ-sensitive GBM cells, supporting our results.

Our findings of increased CCNU toxicity in TMZ-resistant, MMR-deficient GBM cells are mechanistically supported by previous preclinical studies in non-glioma cell lines: Interstrand links caused by bifunctional agents like CCNU were shown to be repaired by MMR, leading to resistance to bifunctional agents [17–19]. To further examine the effects of MMR inhibition in U251, we used CdCl2, a substance targeting several proteins involved in MMR, at a concentration of 2.5 μM. At this concentration, CdCl2 effectively inhibits MMR [34, 35] and had minimally toxic effects (Fig. 3a). Sensitivity to CCNU was increased in cells pre-incubated with CdCl2 which is in line with...
with results from Yamauchi et al. who reported that BCNU-resistant leukemia cells were partially sensitized to CCNU as a result of CdCl₂-mediated MMR inhibition [23]. Although we would have expected decreased sensitivity to TMZ in cells pre-incubated with CdCl₂, this did not occur at a significant level in our experiments. This might be due to the low CdCl₂ concentration chosen on account of high toxicity levels; at a concentration of 100 μM, MMR efficiency is reduced by approximately 95%, but at 1 μM only by 2.7% [34]. A concentration of 2.5 μM might be just enough to cause MMR-deficiency-mediated sensitization to CCNU, yet not enough to induce TMZ resistance.

Aside from chemotherapy, the current standard of care includes radiotherapy [2]. Re-irradiation for recurrent GBM modestly increases overall survival [36, 37]. Accordingly, we did not detect meaningful differences in the effectiveness of radiotherapy between both cell lines. Both cell lines were sensitive to irradiation with dose-dependent growth restriction. The role of MMR in radiosensitization has been controversially discussed [11, 38, 39]. However, in our study, MMR deficiency did not affect radio-sensitivity in U251, confirming the importance of radiotherapy in recurrent GBM.

To the best of our knowledge, this preclinical investigation is the first to show that TMZ resistance mediated by MMR deficiency in MGMT-methylated GBM cells is accompanied by increased sensitivity to CCNU and to combined CCNU and TMZ. Radiosensitivity was preserved in resistant cells. These findings have important clinical implications as acquired TMZ resistance is one of the major obstacles in the treatment of GBM [3] and increased sensitivity to CCNU could be a work-around. As CCNU resistance is mediated by up-regulation and TMZ resistance by downregulation of MMR, we speculate that TMZ resistance evolution might even be preventable by concomitant CCNU administration. This may also explain the promising results for combined CCNU and TMZ in MGMT-methylated patients [21, 22].

Conclusion

This study showed promising results for CCNU in MMR-mediated TMZ resistance, indicating that further pre-clinical and clinical research is clearly warranted. Beyond that, our findings may provide the missing link between already well-described clinical and preclinical observations. Upcoming clinical trials like the German NOA-09 will provide further answers on the role of combined TMZ and CCNU.

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Author contributions

JS and FP performed the experiments mentioned in the “Materials and methods”, except for the pyrosequencing. RB performed pyrosequencing. JS, FP, LD were major contributors in writing the manuscript. RF provided materials and working space for the experiments we performed and revised the manuscript critically. All authors read and approved the final manuscript.

Compliance with ethical standards

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare that they have no competing interests.

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