In vitro studies on radiation and temozolomide in human glioma

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CHAPTER 4

Differential radiosensitising potential of temozolomide in *MGMT* promoter methylated glioblastoma multiforme cell lines

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Abstract

Purpose: To investigate the radiosensitising potential of temozolomide (TMZ) for human glioblastoma multiforme (GBM) cell lines using single-dose and fractionated γ-irradiation.
Methods and Materials: Three genetically characterized human GBM cell lines (AMC-3046, VU-109, and VU-122) were exposed to various single (0-6 Gy) and daily fractionated doses (2 Gy per fraction) of γ-irradiation. Repeated TMZ doses were given before and concurrent with irradiation treatment. Immediately plated clonogenic cell survival curves were determined for both the single-dose and the fractionated irradiation experiments. To establish the net effect of clonogenic cell survival and cell proliferation, growth curves were determined, expressed as the number of surviving cells.
Results: All three cell lines showed *MGMT* promoter methylation, lacked MGMT protein expression, and were sensitive to TMZ. The isotoxic TMZ concentrations used were in a clinically feasible range of 10 μmol/L (AMC-3046), 3 μmol/L (VU-109), and 2.5 μmol/L (VU-122). Temozolomide was able to radiosensitiser two cell lines (AMC 3046 and VU-122) using single-dose irradiation. A reduction in the number of surviving cells after treatment with the combination of TMZ and fractionated irradiation was seen in all three cell lines, but only AMC 3046 showed a radiosensitising effect.
Conclusions: This study on TMZ-sensitive GBM cell lines shows that TMZ can act as a radiosensitiser and is at least additive to γ-irradiation. Enhancement of the radiation response by TMZ seems to be independent of the epigenetically silenced *MGMT* gene.

Introduction

Glioblastoma multiforme (GBM) is the most frequent and aggressive primary brain tumour in adults. Despite standard treatment consisting of surgery and postoperative radiotherapy, patient survival remains poor, owing to radio- and chemoresistance of the tumour. Recently it was demonstrated that the combination of temozolomide (TMZ) with standard daily fractionated irradiation therapy followed by adjuvant TMZ improves the prognosis [30]. Since then, TMZ has become part of the standard therapy for patients with newly diagnosed GBM.

The benefit of TMZ is most prominent for tumours with a methylated O^6-methylguanine-DNA methyltransferase (*MGMT*) promoter. Methylation of the *MGMT* promoter has been associated with longer overall survival of GBM patients treated with radiotherapy and TMZ compared with radiotherapy alone [14, 15]. However, this study also showed relative long-term survivors - both in the methylated and unmethylated GBM groups - treated with radiotherapy plus TMZ [15]. This might indicate interaction between TMZ and irradiation.

Temozolomide is a chemotherapeutic prodrug that transforms under physiological conditions into its active unstable methylating metabolite 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC). Methylation of the DNA by MTIC results in O^6-methylguanine adducts, which are considered to be responsible for the cytotoxic effect of TMZ [4, 33, 39]. O^6-methylguanine adducts can result in futile attempts of the mismatch repair system, leading to DNA double-strand breakage and eventually cell death [18, 22].

O^6-methylguanine-DNA methyltransferase is a cytoprotective DNA repair protein that can remove the methyl group from the O^6 position of guanine. Therefore, presence of this repair protein may undo (in part) the cytotoxic effect caused of alkylating agents, hence resulting in tumour resistance [2, 11, 17, 27]. Hypermethylation of the CpG islands in the promoter region of the *MGMT* gene has been found to be associated with transcriptional silencing [7, 25, 38] and a good clinical response to alkylating agents in glioma patients [6, 8, 14, 15, 24].

Only a few studies have investigated the radiosensitising potential of TMZ for glioma cell lines using different treatment protocols. In some cell lines an enhancement of the radiation effect was demonstrated, whereas the remaining cell lines showed no interaction, merely an additive effect [5, 16, 36, 40]. Although TMZ is given concomitantly with and after radiotherapy in clinical treatment protocols, detailed information about the possible interaction between TZM and radiation is not yet available. The above-mentioned studies suggest that TMZ has either an additive or a radiosensitising effect on GBM cells.

We investigated TMZ sensitivity and the interaction between TMZ and irradiation in human glioma cell lines. Data are presented for three long-term primary TMZ-sensitive glioma cell lines that were exposed to either single or fractionated doses of γ-irradiation in combination with protracted TMZ administration. Alongside genetic characterization of the cell lines, cell proliferation, MGMT expression and *MGMT* promoter methylation status were investigated to obtain more insight into the interaction of TMZ with γ-radiation.

Materials and Methods

Primary cell culture

Seven cell lines (AMC 3046, AMC 3344, VU-28, VU-98, VU-109, VU-110, and VU-122) were isolated from tumour samples obtained from patients undergoing surgery for a GBM (World Health Organization Grade 4 astrocytoma). Tumour samples were partly snap-frozen and stored at -80°C, whereas another part was used for preparation of a primary cell line, as described by Gerlach et al. [10]. Cell lines were cultured at 37ºC in a 7% CO_2 humidified atmosphere using Dulbecco's modified Eagle's medium (D-MEM) supplied with 20% fetal calf serum and 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin (all from Invitrogen, Groningen, The Netherlands). The cells used for the experiments had already undergone >100 passages. Patients gave written informed consent for the surgical, radiotherapeutic and experimental procedures.

Genetic analysis

The genetic profiles were determined by screening three cell lines (AMC 3046, VU-109, and VU-122) and their corresponding tumours for genetic changes that frequently occur in GBM [19, 37]. Genomic DNA from cell lines, tumour samples, and blood leukocytes was extracted according to standard procedures. The loss of heterozygosity status of the chromosomal regions investigated, *TP53* status and *EGFR* amplification level of the three cell lines and their corresponding tumour were determined according to the outlines, and the references therein, as described previously [34].

MGMT protein expression and promoter methylation status

Promoter methylation status for MGMT was determined using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) and methylation-specific polymerase chain reaction (MS-PCR) as described by Nygren et al. [21]. Protein expression for MGMT was determined using Western blot analysis. Protein samples (50 μg) from whole-cell lysates were separated on a NuPAGE Novex Bis-Tris 4-12% gel (Invitrogen) and electro-transferred onto a polyvinylidene fluoride membrane (Millipore, Amsterdam, The Netherlands). Antibodies used were mouse monoclonal antibody against human MGMT (clone MT 3.1; Neomarkers/Lab Vision Corporation, Fremont CA), incubation 1:1000 overnight at 4°C, and goat antimouse horseradish peroxidase polyclonal antibody (DAKO Cytomation, Glostrup, Denmark), incubation 1:1000 for 1 h at room temperature. Proteins were visualized, using an ECL plus system (Amersham Bioscience, Buckinghamshire, United Kingdom), by exposure of the membrane to a hyperfilm.

Experimental procedures

Experiments were performed on exponentially growing cells. Cells were seeded 3 days before the start of treatment. Temozolomide (Schering-Plough RS, Utrecht, The Netherlands) was dissolved freshly before every treatment in dimethyl sulfoxide and diluted in culture medium. For repeated 24-h exposures the medium with TMZ was refreshed daily. Final concentrations of the solvent did not affect cell proliferation or survival. Clonogenic cell survival after 24-h exposure of TMZ was assessed by adding TMZ (0-500 μmol/L) to the cells 4 hours after plating. Subsequently, medium was replaced with fresh normal medium for the remaining part of the clonogenic assay.

For combination treatment consisting of radiation and TMZ, isotoxic concentrations of TMZ for clonogenic cell survival were used for the cell lines. The isotoxic concentrations chosen resulted in a 50% reduction of clonogenic cell survival and had minor effects on cell proliferation. Repeated exposures of TMZ were chosen according to van Rijn et al. [36]. The cell lines were treated for 96 h (repeated 24-h exposures) with isotoxic concentrations of TMZ and then exposed to single doses of γ-radiation (1-6 Gy). Other experiments using fractionated radiation followed a 5-day treatment schedule combining repeated 24 h exposures of TMZ with daily doses of γ-radiation (2 Gy/24 h). Cells were plated for clonogenic assay immediately after each single (after 96 h) and every 2-Gy fractionated radiation dose (at the start and after 24, 48, 72 and 96 h). Cells were irradiated at room temperature by γ-radiation from a ^{60}C source (Gammacell 200; Atomic Energy of Canada, Mississauga, Ontario, Canada). Cell survival after treatment was determined by the clonogenic assay.

Clonogenic assay and statistical analysis of the data

After treatment the cells were processed for the clonogenic assay. A specified number of cells was seeded in 25-cm^2 tissue culture flasks and incubated at 37ºC in 7% CO_2 humidified atmosphere. During colony formation of the AMC 3046 and VU-109, a mixture of 50% normal culture medium and 50% conditioned medium from the standard glioma cell line D384 [1] was used to improve the quality and/or quantity of the colonies. After sufficient time the cells were fixed in 100% ethanol and stained with 5% Giemsa solution. Colonies containing ≥ 50 cells were counted and considered as cells with an unaffected clonogenic capacity. Average plating efficiencies were 24% (AMC 3046), 22% (VU-109), and 51% (VU-122). Average plating efficiencies after 96 h of TMZ were 13% (AMC 3046), 11% (VU-109), and 29% (VU-122).

Surviving fractions (SF) after dose (D) of radiation are presented after correction for the plating efficiencies of control cells. Single-dose radiation cell survival curves were constructed according to the linear-quadratic (LQ) formula $SF=\exp-(\alpha D+\beta D^2)$ using GraphPad Prism software (GraphPad Software, San Diego, CA) and analyzed using SPSS (Chicago, IL) statistical software for weighted linear regression [9]. Cell survival curves of different treatment groups were considered statistically different

when $p < 0.05$. Experiments were repeated at least 3 times and found to be reproducible.

Surviving cells

To establish the total number of surviving cells as a net effect of clonogenic cell survival and cell proliferation after fractionated irradiation in the absence or presence of TMZ, we plotted the number of surviving cells per 25-cm^2 tissue culture flasks [35]. The number of surviving cells was calculated by multiplying the total cell number per 25-cm^2 tissue culture flask (including surviving and ultimately nonsurviving cells) by the plating efficiencies of the matching treatment. The surviving cells are also shown corrected for the effect of TMZ alone.

Results

Intrinsic TMZ sensitivity

Figure 1 shows clonogenic cell survival after exposure for 24 h to TMZ for a panel of seven long-term GBM cell lines. The cell lines can roughly be assigned to either one of two groups: sensitive or resistant to TMZ. The sensitive group contains four cell lines and the resistant group contains three cell lines. Three cell lines (AMC 3046, VU-109, and VU-122), sensitive to TMZ in the clinically feasible dose range (< 50 μmol/L), were selected for combined treatment with irradiation.

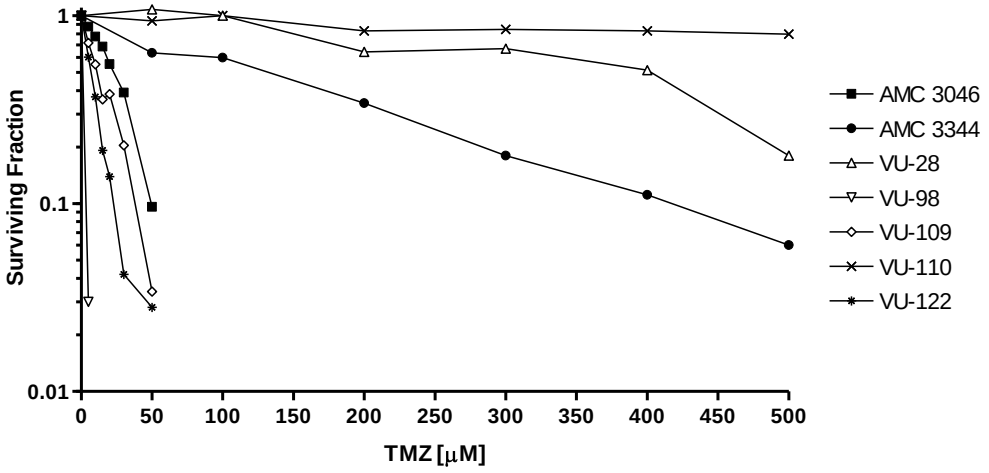

Figure 1. Clonogenic cell survival after 24-h temozolomide (TMZ) exposure of seven long-term primary glioblastoma multiforme cell lines (AMC 3046, AMC 3344, VU-28, VU-98, VU-109, VU-110, and VU-122).

Genetic analysis

Table 1 gives a summary of the genetic changes found for cell lines AMC 3046, VU-109, and VU-122 as well as their corresponding tumours. Among the investigated chromosome regions were those containing the tumour suppressor genes *CDKN2A* (9p21), *PTEN* (10q23), and *TP53* (17p13.1) and the putative tumour suppressor gene *DMBT1* (10q25). For every position and gene studied, the same genetic change was found for both the tumour and the corresponding cell line in all three cases. The only exception was loss of *EGFR* amplification in the cell line derived from the original tumour of VU-109. However, loss of additional extrachromosomal copies of *EGFR* due to absence of selective pressure occurs frequently during *in vitro* propagation [3, 23]. On the basis of the data presented in Table 1, all three cell lines were found to be genetically representative of the original tumour.

Table 1. Genetic characterization of the three cell lines and their corresponding tumours

	LOH									
Cell line	**1p36**	**9p21 *(CDKN2A)***	**10p15**	**10q23 *(PTEN)***	**10q25 *(DMBT1)***	**17p13.1 *(TP53)***	**19q13**	**22q13**	**Mutation of *TP53***	***EGFR* amplification**
AMC 3046	n/n	y/y	y/y	y/y	y/y	y/y	n/n	n/n	n/n	n/n
VU-109	n/n	n/n	y/y	y/y	y/y	y/y	n/n	n/n	y/y*	y/n
VU-122	y/y	n/n	y/y	y/y	y/y	ni	n/n	y/y	y/y[#]	n/n

Abbreviations: LOH = loss of heterozygosity; ni = noninformative; n = no LOH; y = LOH; x/y = results from tumour (x) and corresponding cell line (y).
* Codon 175: CGC → CAC (Arg → His); # Codon 135: TGC → TGG (Cys → Trp).

MGMT protein expression and promoter methylation status

To investigate the possible role of MGMT in the cell survival effects caused by TMZ in combination with γ-irradiation, the three cell lines (AMC 3046, VU-109, and VU-122) were tested for the expression of MGMT protein and the promoter methylation status of its gene by using MS-PCR, MS-MLPA, and Western blot analysis. All three cell lines contain a *MGMT* promoter region that is methylated for the most part (Fig. 2a) and do not show expression of the protein MGMT (Fig. 2b).

Clonogenic cell survival after single-dose irradiation with or without TMZ

To investigate the effect of the combination of TMZ and γ-irradiation on clonogenic cell survival, cells were exposed daily to TMZ before irradiation with γ-radiation (Fig. 3). Figure 4a shows a significant radiosensitising effect ($p < 0.05$) of TMZ for the AMC 3046 cells. The shoulder of the survival curve for irradiated cells disappears as a result of TMZ pre-treatment. This was also reflected by TMZ-induced changes in the α and β parameters of the linear quadratic model (Table 2). No radiosensitisation was found for VU-109 cells ($p = 0.054$; Fig. 4b), as demonstrated by the unchanged α and β parameters (Table 2). The VU-122 cells display a small but significant

radiosensitising effect of TMZ ($p < 0.05$; Fig. 4c), which is most obvious in the higher radiation dose range. This difference is reflected by an increase in the α parameter and no change in the β parameter (Table 2).

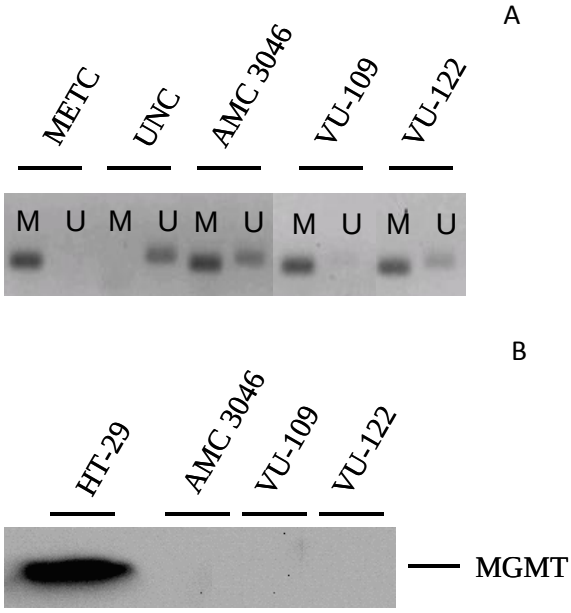

Figure 2. (a) Methylation-specific polymerase chain reaction results from AMC 3046, VU-109, and VU-122. M = methylated; METC methylated control; U = unmethylated; UNC unmethylated control. (b) Western blot analysis for the expression of MGMT in AMC 3046, VU-109, and VU-122. The established colorectal adenocarcinoma cell line HT-29 was used as positive control.

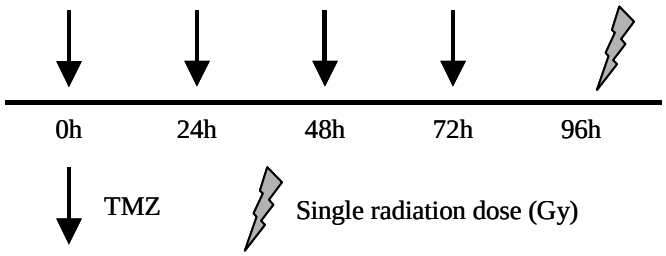

Figure 3. Schematic presentation of the treatment schedule for the single-dose irradiation experiments. Cells were treated with daily exposures of temozolomide (TMZ) for 96 h followed by a single radiation dose of 1-6 Gy.

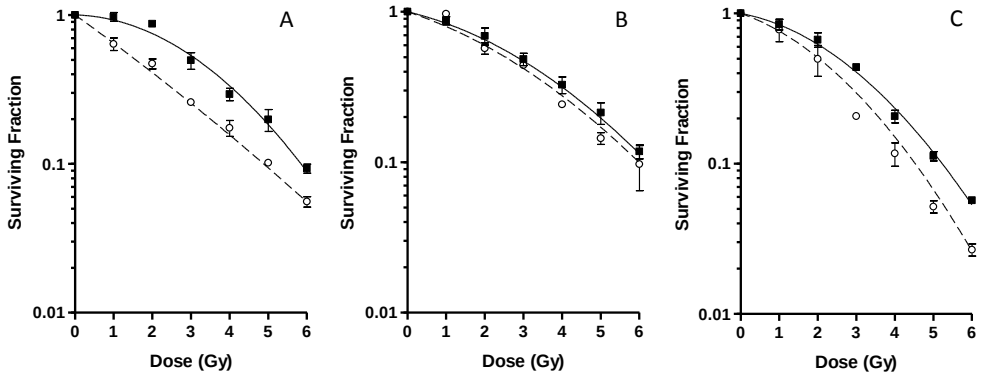

Figure 4. Single-dose radiation cell survival curves after repeated exposure to temozolomide and irradiation of AMC 3046 (a), VU-109 (b), and VU-122 (c). Cells were irradiated alone (solid squares) or after 96-h incubation with 10 μmol/L (AMC 3046), 3 μmol/L (VU-109), and 2.5 μmol/L (VU-122) TMZ (open circles). Symbols represent means ± S.E.M. of at least three independent experiments.

Table 2. Effect of TMZ on the α and β parameters (± SD) and α/β ratios of the LQ model of single-dose irradiated AMC 3046, VU-109, and VU-122 cells, with or without TMZ treatment

Cell line	LQ parameter	RT alone	RT + TMZ
AMC 3046	α (Gy^{-1})	0.014 ± 0.033	0.428 ± 0.025
	β (Gy^{-2})	0.065 ± 0.007	0.009 ± 0.005
	α/β (Gy)	0.22	47.56
VU-109	α (Gy^{-1})	0.140 ± 0.031	0.193 ± 0.038
	β (Gy^{-2})	0.037 ± 0.006	0.032 ± 0.008
	α/β (Gy)	3.78	6.03
VU-122	α (Gy^{-1})	0.111 ± 0.025	0.205 ± 0.047
	β (Gy^{-2})	0.063 ± 0.005	0.067 ± 0.010
	α/β (Gy)	1.76	3.06

Abbreviations: TMZ = temozolomide; LQ = linear-quadratic; RT = radiotherapy.

Cell proliferation after fractionated irradiation with or without TMZ

Conform clinical treatment protocols, consisting of a fractionated irradiation schedule rather than single-dose treatment, the three cell lines were investigated for their response to daily TZM combined with daily radiation doses, according to the schedule in Figure 5. From these experiments cell counts were obtained for all different treatments and all time points. Cell proliferation curves, including surviving and ultimately non-surviving cells, are presented for AMC 3046, VU-109, and VU-122 (Figs. 6a, 6b, and 6c respectively) for control, TMZ, irradiation, and the

combination. Cells keep proliferating although cells treated with the combination of fractionated irradiation and TMZ are inhibited slightly more relative to control and sole modalities.

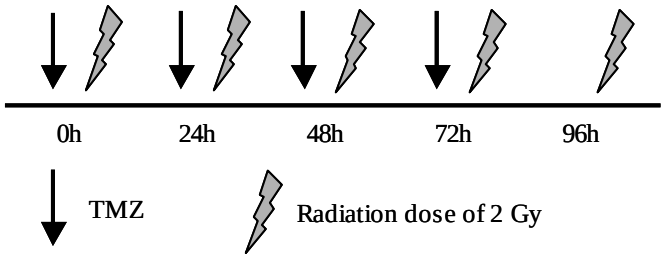

Figure 5. Schematic presentation of the treatment schedule for the fractionated irradiation experiments. Cells were treated with 5 fractions of 2 Gy at 24-h intervals with or without daily exposures of temozolomide (TMZ) for 96 h.

Surviving cells after fractionated irradiation with or without TMZ

The experiments shown in Fig. 6d-i were also done according to the treatment regimen shown in Figure 5. For every data point seen in these figures (6a-c), plating efficiencies were determined (data not shown). These plating efficiencies were processed with the cell proliferation data to obtain the amount of surviving cells. The surviving cells represent the cells that remain after treatment, taken into account both clonogenic cell death and cell proliferation. Without incorporation of cell proliferation, it is difficult to know or the total amount of tumour cells would grow, remain stable or decrease. The number of surviving cells per 25-cm^2 tissue culture flask was determined by multiplying the plating efficiencies (data not shown) by the total cell number (data from Figs. 6a-c). Figure 6d shows the number of surviving cells after fractionated irradiation for AMC 3046. The number of surviving cells increased after sham treatment as well as after repeated exposures of TMZ. The curve for irradiation alone (2 Gy/24 h) shows that clonogenic cell kill is almost compensated for by cell proliferation, resulting in a net effect of "growth arrest". However, the combination of fractionated irradiation plus TMZ causes a reduction in the number of surviving cells, which is more obvious with increasing numbers of fractions (Fig. 6d). Similar effects were found for VU-109 (Fig. 6e) and VU-122 (Fig. 6f), but the differences between irradiation alone and the combination treatment were less pronounced. However, all three cell lines benefit from the combined treatment of fractionated irradiation and TMZ, resulting in a decrease in the number of surviving cells. For analysis of the radiosensitising potential of TMZ, data from Fig. 6d-f (cells treated with fractionated irradiation with or without TMZ) were corrected for their controls (no treatment or TMZ alone). These data are shown in Fig. 6g-i. AMC 3046 cells show an enhanced effect of fractionated irradiation after TMZ treatment (Fig. 6g). This radiosensitising effect was not observed for VU-109 cells (Fig. 6h) and VU-122 cells (Fig. 6i).

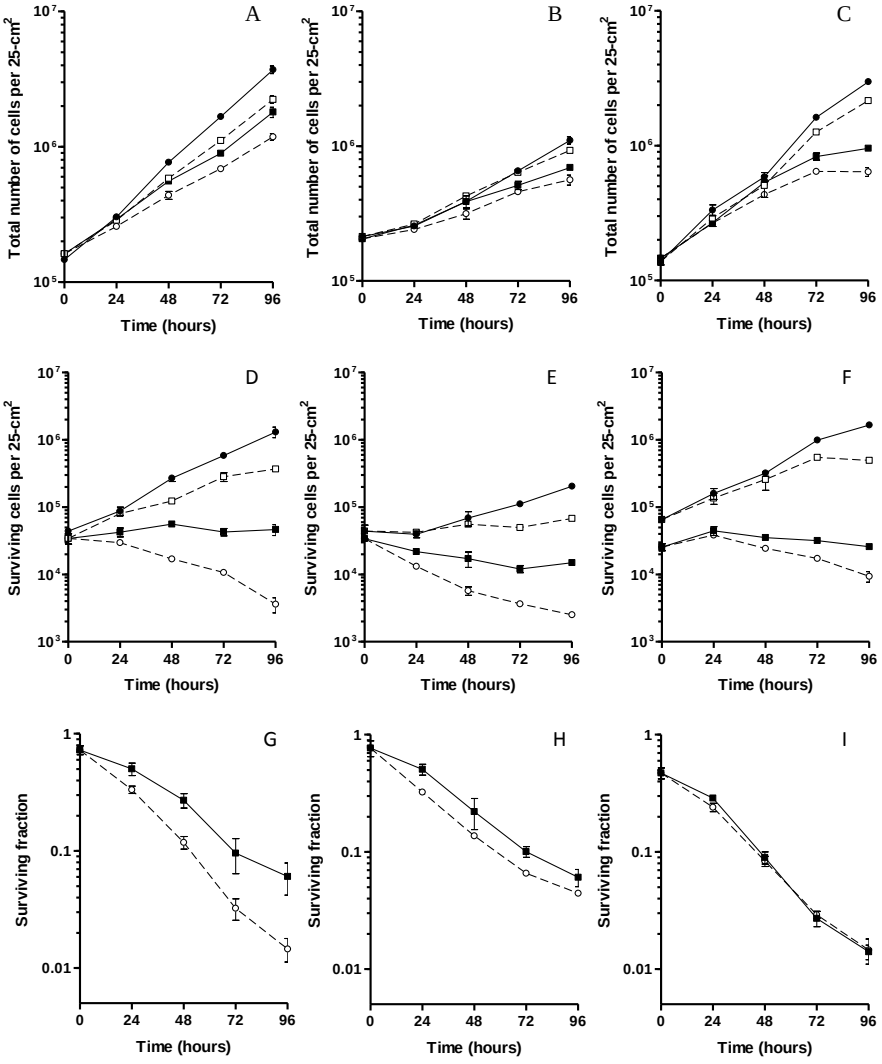

Figure 6. Effect of 10 μmol/L (AMC 3046), 3 μmol/L (VU-109), and 2.5 μmol/L (VU-122) temozolomide (TMZ) during fractionated irradiation. (a-c) The effect of TMZ on cell proliferation during fractionated irradiation showing the total number of cells per 25^2 cm for AMC 3046 (a), VU-109 (b), and VU-122 (c). Cell proliferation curves are shown for control (solid circles), TMZ (open squares), radiation (solid squares), and TMZ plus radiation (open circles). Symbols represent the mean ± SEM of two independent experiments. (d-f) Surviving cells per 25 cm^2 after combined treatment of TMZ and fractionated irradiation for AMC 3046 (d), VU-109 (e), and VU-122 (f). Curves represent control (solid circles), TMZ (open squares), radiation (solid squares), and TMZ plus radiation (open circles). Symbols represent the mean ± SEM of two independent experiments. (g-i) Surviving cells (data from 6d-f) corrected for the effect of TMZ alone. Cultures were irradiated only (solid squares) or after incubation with TMZ (open circles). Symbols represent means ± SEM of four independent experiments.

Discussion

This study demonstrates the potency of TMZ to enhance the radiation response in long-term primary GBM cell lines. Despite *MGMT* promoter methylation or the absence of the MGMT protein, the effect of TMZ is at least additive and can even be synergistic. Initially a panel of seven cell lines was tested for TMZ sensitivity. Further studies were performed on three long-term primary GBM cell lines, which were sensitive to TMZ at clinically achievable concentrations. Despite similar TMZ sensitivity and MGMT status, the three cell lines showed divergent responses to chemoradiation. Single-dose radiation experiments clearly showed radiosensitisation of TMZ in two cell lines. However, only one of these cell lines exhibited a radiosensitising effect after fractionated irradiation. This observation agrees with the findings of Hegi et al. [15] that a subpopulation of patients benefits from concomitant TMZ but that the majority of patients still fail to respond to treatment. The fraction size may be critical, and radiosensitisation might occur in the non-responsive cell lines when a different fractionation schedule is chosen.

Several studies demonstrated specific genetic alterations to be better or worse prognostic factors for survival of glioma patients [20, 28, 31, 32]. Patients with genomic deletions on chromosomes 9 and 10, which are typical for glioblastoma and are associated with poor prognosis, are reported to benefit significantly from TMZ in addition to radiotherapy [41]. Regarding the genetic analysis in our own study, the outcome for all three cell lines reveals mutual differences on at least 2 investigated chromosomal regions. Nevertheless, there does not seem to be a correlation between genetic alterations and *in vitro* treatment response, with a possible exception for the *TP53* gene. AMC 3046 and VU-122 may still be able to produce a functional p53 protein. The VU-109 cells however exhibit loss of one allele and carry a mutation in the other allele. Because wild-type p53 may be involved in TMZ-induced cytotoxicity [16, 26, 29], and p53 may play a key role in the cellular response to ionizing radiation [12, 13], further investigation of the p53 status is warranted.

The results of the fractionated experiments were predicted by visual examination of the shoulder areas of the single dose irradiation curves and confirmed by mathematical analysis using the LQ model, to obtain the radiosensitivity parameters α and β. The shoulder of the cell survival curve in TMZ-treated AMC 3046 cells disappears compared to irradiation alone. This phenomenon is reflected by the changes in the α and β parameters and a large difference between the α/β ratios (Table 2). Therefore, fractionation in the absence of TMZ allows cells to repair sublethal damage more effectively, resulting in higher survival compared with single dose radiation. Because of the radiosensitising effect of TMZ after 2-Gy irradiation exposure, there is further benefit from additional TMZ in a fractionation schedule with repeated 2 Gy fractions (Fig. 6g). No enhanced sensitivity to TMZ during fractionated irradiation of the VU-109 cells was found (Fig. 6h). The calculated LQ parameters of the single-dose cell survival curves with or without TMZ for VU-109 were not significantly different (Table 2).

Addition of TMZ to single-dose irradiation did not affect the shoulder of the VU-122 cell survival curves (Fig. 4c). Because the α parameter was increased while the β parameter was not affected, a more than additive effect of TMZ was to be expected. However, the α/β ratios were only slightly different (Table 2) in accordance with a limited radiosensitising effect. This effect of TMZ occurs only at higher doses of radiation (Fig 4c). Accordingly, when 2-Gy doses were used in the fractionation experiments, no radiosensitisation was found (Fig. 6i). Radiosensitisation might be obtained by hypofractionation (e.g. in a schedule consisting of 3-Gy fractions instead of 2-Gy fractions). Our data show that the radiosensitising potential of TMZ should be studied using both fractionated irradiation schedules and protracted TMZ exposure, which reflects more common clinical practice. Additional information can be obtained from single-dose radiation cell survival curves from which the LQ repair parameters can be estimated in order to predict the sensitivity of cells to fractionated irradiation.

There are few *in vitro* studies of the radiosensitising potential of TMZ. Only the human astrocytoma cell line D384 and the primary human GBM cell lines UN10 and UN11 demonstrate an enhanced radiation effect after pretreatment or concurrent TMZ [5, 36]. All other cell lines tested, using different treatment protocols and concentrations, revealed at best additive effects [5, 16, 36, 40]. Together with the results from our own study, in which two cell lines show a radiosensitising effect, it seems that radiosensitisation can be found for cell lines sensitive to TMZ and/or lacking expression of the MGMT protein after pre-treatment or concurrent TMZ (data D384 not shown). However, the cell lines VU-109 and U251 [36], which also lack MGMT expression (data U251 not shown), showed no enhancement of the radiation response. The human colorectal adenocarcinoma cell line Mawi [40] and the human primary GBM cell lines UN12 and UN13 [5], all with MGMT protein expression, did not exhibit an interaction of TMZ and irradiation. TMZ was only additive after concurrent and adjuvant treatment of the cell lines UN12 and UN13, and Mawi revealed an antagonistic effect by concurrent TMZ. Furthermore, it may be of importance to mention the difference in concentration of TMZ used for these cells lines without MGMT protein expression. The TMZ concentration in the study of Chakravarti et al. [5] was relatively high (100 μmol/L). Neither AMC 3046, VU-109, VU-122 (Fig. 1) nor D384 and U251 cells [36] survived a single dose of 100 μmol/L TMZ for 24 h. In addition, U373 glioma cells show similar sensitivity to TMZ as the glioma cells we used [40]. Furthermore, TMZ sensitivity and radiosensitisation by TMZ do not seem to depend on the absence of MGMT protein alone. Further research directed toward the role of p53 and the mismatch repair system, in particular in TMZ-resistant cell lines, might reveal more about the underlying mechanism involved in TMZ sensitivity and its interaction with radiation.

Conclusion

Clinical studies show that radiotherapy combined with TMZ for GBM patients improves survival, particularly for *MGMT* methylated tumours. This suggests a

radiosensitising potential of TMZ. Our results show that the relationship between TMZ sensitivity and radiosensitisation is not that straightforward. The contribution of TMZ to irradiation is at least additive and can even be synergistic. However, radio-enhancement by TMZ is probably not predicted by *MGMT* promoter methylation or the absence of the MGMT protein.

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