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Chapter 5

Valproic acid sensitises human glioma cells for temozolomide and γ -radiation

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Abstract

Temozolomide (TMZ) is given in addition to radiotherapy in glioma patients, but its interaction with the commonly prescribed anti-epileptic drug valproic acid (VPA) is largely unknown. Induction of DNA demethylation by VPA could potentially induce expression of the O⁶-methylguanine-DNA-methyl-transferase (MGMT) protein causing resistance to TMZ and thereby antagonising its effect. Therefore, this study investigates the interaction between VPA, TMZ and γ -radiation. Two glioma cell lines were used that differ in TMZ sensitivity caused by the absence (D384) or presence (T98) of the MGMT protein. VPA was administered before (24/48h) or after (24h) single doses of γ -radiation; or, after 24h, VPA treatment was accompanied by a single dose of TMZ for another 24h. For trimodal treatment the combination of VPA and TMZ was followed by single doses of γ -radiation. In both cell lines VPA caused enhancement of the radiation response after preincubation (DMF_{0.2} 1.4 and 1.5), but not after postirradiation (DMF_{0.2} 1.1 and 1.0). The combination of VPA and TMZ caused enhanced cytotoxicity (DMF_{0.2} 1.7) in both the TMZ-sensitive cell line (D384) and the TMZ-resistant cell line (T98). The combination of VPA and TMZ caused a significant radiation enhancement (DMF_{0.2} 1.9 and 1.6) that was slightly more effective than that of VPA alone. VPA does not antagonise the cytotoxic effects of TMZ. Preincubation with VPA enhances the effect of both γ -radiation and TMZ, in both a TMZ-sensitive and a TMZ-resistant human glioma cell line. VPA combined with TMZ may lead to further enhancement of the radiation response.

Introduction

The current standard therapy for patients with a newly diagnosed glioblastoma multiforme (GBM) consists of surgery, followed by radiotherapy and temozolomide (TMZ). Compared to radiotherapy alone, radiotherapy with concomitant and adjuvant TMZ improved both the median survival and the five-year overall survival [38, 39].

TMZ is an alkylating agent that adds methyl groups to the O⁶ position of guanine that eventually leads to cell death [12, 24, 25]. The DNA repair protein O⁶-methylguanine-DNA-methyl-transferase (MGMT) is an important mechanism of resistance to TMZ due to its ability to remove the methyl group from the O⁶ position of guanine [3, 19]. Transcriptional silencing by methylation of the promoter of the *MGMT* gene is a favourable prognostic factor for patients with a GBM treated with radiotherapy and TMZ, as compared to patients treated with radiotherapy alone [22, 23]. Moreover, others and we have demonstrated enhancement of the radiation response by treatment with TMZ in human glioma cells [6, 28, 40, 42].

Valproic acid (VPA) is a commonly prescribed anti-epileptic drug for the treatment and prevention of seizures in brain tumour patients. Besides its anti-seizure property, VPA is reported to inhibit cell proliferation and induce cell differentiation and apoptosis [20, 32]. Furthermore, VPA is an effective inhibitor of histone deacetylase (HDAC) [20, 32, 35], which is involved in modulating chromatin structure and gene expression.

Different HDAC inhibitors enhance the radiation response both *in vitro* and *in vivo* in various tumour cell types [4, 8, 15, 16, 26, 30, 31]. VPA has also shown to enhance the radiosensitivity of a variety of tumour cell types [2, 7, 13, 27], including glioma cell lines [5, 9].

However, less information is available about the interaction of VPA with (γ -) radiation and TMZ. A cause for concern is the DNA demethylation action of VPA [14] that may influence the availability of the DNA repair protein MGMT. Induction of the MGMT protein due to loss of methylation of the *MGMT* gene promoter might affect the sensitivity of tumour cells to TMZ. If VPA antagonizes the effect of TMZ, it would be contraindicated in GBM patients on radiotherapy plus TMZ.

The present study investigates the interaction of VPA on TMZ and γ -radiation. The experiments were performed using two established glioma cell lines that differ in TMZ sensitivity due to the presence or absence of the MGMT protein.

Methods and Materials

Cell lines

Two established glioma cell lines, D384 (astrocytoma grade III) [1] and T98 (GBM), were cultured at 37°C in a humidified atmosphere using (Leibovitch) L15 medium supplied with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin (all from Invitrogen, Breda, The Netherlands). The MGMT protein was detected in the T98 but not in the D384 cell line, which explains the

difference in TMZ sensitivity [41]. The D384 cell line was kindly provided by Dr. C.H. Langeveld, (Dept. of Neurology, VU University Medical Center Amsterdam, The Netherlands).

Experimental procedures

Experiments were conducted on exponentially growing cells that were seeded before the start of treatments. VPA (Sigma-Aldrich, Zwijndrecht, The Netherlands) was prepared freshly and dissolved in complete culture medium. TMZ (Schering-Plough RS, Houten, The Netherlands) was prepared freshly and dissolved in dimethyl sulfoxide (Sigma-Aldrich) before dilution in culture medium. Final concentrations of the solvent did not affect cell proliferation or clonogenic cell survival. A schematic presentation of the treatment protocols showing the timing of administration of various treatments is presented in Figure 1. Cells were irradiated at room temperature by single doses of γ -radiation (2-6 Gy) from a ^{60}C source (Gammacell 220; Atomic Energy of Canada, Mississauga, Ontario, Canada). Cell survival was assessed by the clonogenic capacity of single cells.

Clonogenic assay

Following the experimental treatments, cells were dispersed with trypsin/EDTA (Invitrogen) and plated in adequate numbers. The amount of seeded cells (500-50000 per 25 cm² culture flask) depended on the estimated survival. After an incubation of 10-12 days, the developing cell colonies were fixed with 100% ethanol and stained with 5% Giemsa solution (Merck, Darmstadt, Germany). All colonies in the 25 cm² culture flasks containing 50 cells or more were counted and considered as cells with an unaffected clonogenic capacity. Average plating efficiencies (PE = total number of colonies formed / total number of cells seeded) for D384 and T98 cells were 0.63 ± 0.06 and 0.50 ± 0.030 , respectively.

Survival (S) data after dose (D) of radiation, drugs alone or combinations thereof are presented after correction for the PE of relevant control cells as surviving fractions [SF= S(D)/S(0)]. Thus, raw data for each individual survival curve were normalised for the survival of the associated pre-/postirradiation treatment, presenting the effect of radiation treatment alone. Radiation survival data were fitted by a weighted, stratified, linear regression according to the linear-quadratic (LQ) formula $S(D)/S(0)=\exp(-\alpha D+\beta D^2)$ as described by Franken et al. [17] and constructed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). All the radiation curves showed a relationship between radiation dose and survival ($p < 0.05$) [17]. Experiments were repeated at least twice, except for the cell line T98 in Figure 4b and Table 2. The dose-modifying factor (DMF), the ratio of the radiation dose of the single treatment and the radiation dose for combinations with VPA, TMZ or both, was calculated at a SF level of 0.2.

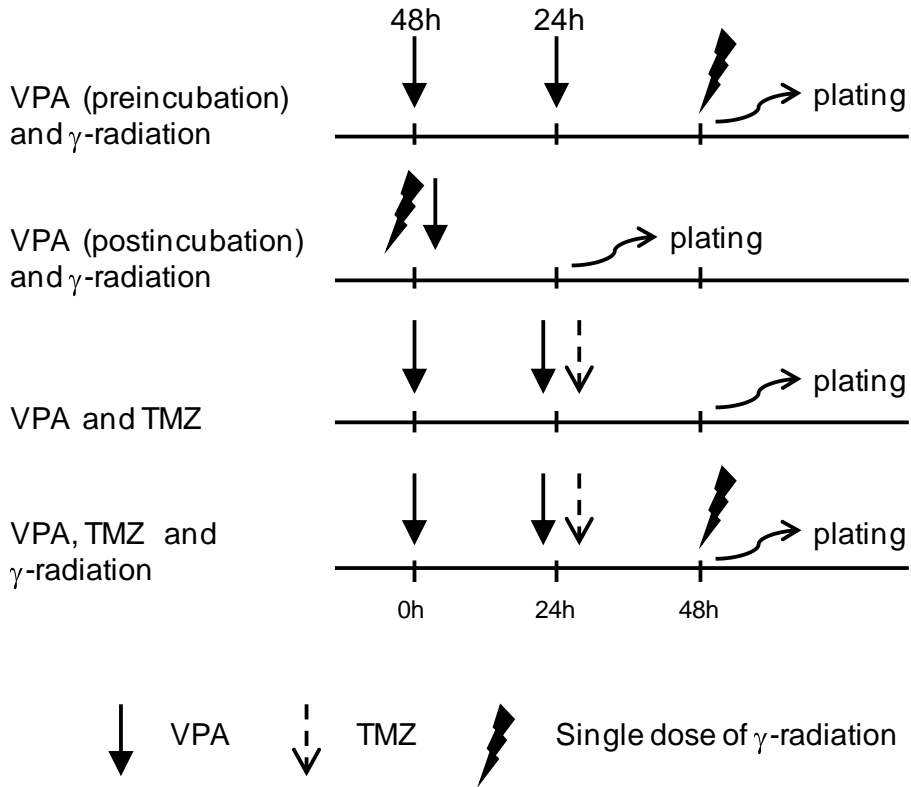


Figure. 1 Schematic presentation of the treatment protocols. Cells were incubated with VPA for 24 and 48h before irradiation (preincubation) or for 24h after irradiation (postirradiation, delayed plating). For combinations of VPA (48h) and TMZ (24h), cells were exposed to 24h of VPA followed by 24h coincubation of VPA and TMZ. For the trimodal combination, VPA (48h) and TMZ (24h) were added before treatment with γ -radiation. In the combinations with TMZ (with or without γ -radiation) a new dose of VPA was administered daily (2 x 24h).

Results

Effect of VPA on cell proliferation and clonogenic cell survival

Glioma cells were treated with different concentrations of VPA for 24h or 48h (D384: 0-2.5-5-7.5 mM; T98: 0-1-2.5-5 mM). Exposure to 24h of VPA in D384 cells resulted in minimal inhibition of cell proliferation (up to 20%). The 48h incubation with VPA resulted in a time and concentration-dependent inhibition of cell proliferation (up to 68%). VPA did not affect cell proliferation in T98 cells.

The clonogenic capacity after 24h exposure to VPA of D384 cells was minimally affected (and concentration independent), and a reduction in clonogenic capacity of approximately 24% was found in T98 cells with the higher concentrations. A stronger

and concentration-dependent reduction in clonogenic capacity was shown after 48h of VPA treatment in both D384 (up to 38%) and T98 cells (up to 35%).

Treatment with a dose of 4 Gy of γ -irradiation following exposure to VPA caused a significant decrease in the clonogenic capacity of both cell lines. The reduction in clonogenic capacity at the highest concentrations in D384 was 68% (24h) and 96% (48h), and for T98 it was 93% (24/48h). The maximum radiation enhancement with an acceptable VPA toxicity was 5 mM VPA in D384 and 2.5 mM VPA in T98. These VPA doses were used for further experiments. Average PE for D384 cells treated with 5 mM VPA were 0.89 ± 0.03 (24h) and 0.87 ± 0.02 (48h); average PE for T98 cells treated with 2.5 mM VPA were 0.77 ± 0.04 (24h) and 0.79 ± 0.04 (48h).

Effect of γ -radiation prior to or following VPA exposure

Cells were exposed to VPA for either 24h or 48h prior to treatment with γ -radiation. Figure 2a shows that preincubation of D384 cells with 5 mM VPA caused an enhancement of the radiation response for both the 24h (DMF_{0.2} of 1.3) and 48h (DMF_{0.2} of 1.4) treatment. Treatment with VPA 48h prior to irradiation is not more effective than preincubation with 24h VPA. Figure 2b presents the results for the T98 cells. A clear enhancement of the radiation response is demonstrated after preincubation with 2.5 mM VPA for 24h and 48h (DMF_{0.2} of 1.7 and 1.5). No difference was found between the curves that were exposed to VPA for 24h and 48h prior to irradiation.

Furthermore, D384 and T98 cells were treated with γ -radiation followed by a 24h postirradiation treatment with 5 mM and 2.5 mM VPA, respectively: the results are shown in Figures 2c (D384) and 2d (T98). This postirradiation treatment with VPA did not affect the cytotoxic response of both cell lines to irradiation (D384, DMF_{0.2} of 1.1; T98, DMF_{0.2} of 1.0).

Effect of TMZ after VPA exposure

Cells were exposed to VPA for 48h of which the last 24h were coincubated with various concentrations of TMZ. D384 (5 mM VPA) and T98 (2.5 mM VPA) cells were treated with a selected range of TMZ concentrations, in accordance with their sensitivity to the drug.

As shown in Figure 3, the exposure to VPA made cells more sensitive to TMZ and caused enhancement of the cytotoxic response in both cell lines at concentrations higher than 5 μ M (D384, DMF_{0.2} of 1.7) and 125 μ M (T98, DMF_{0.2} of 1.7).

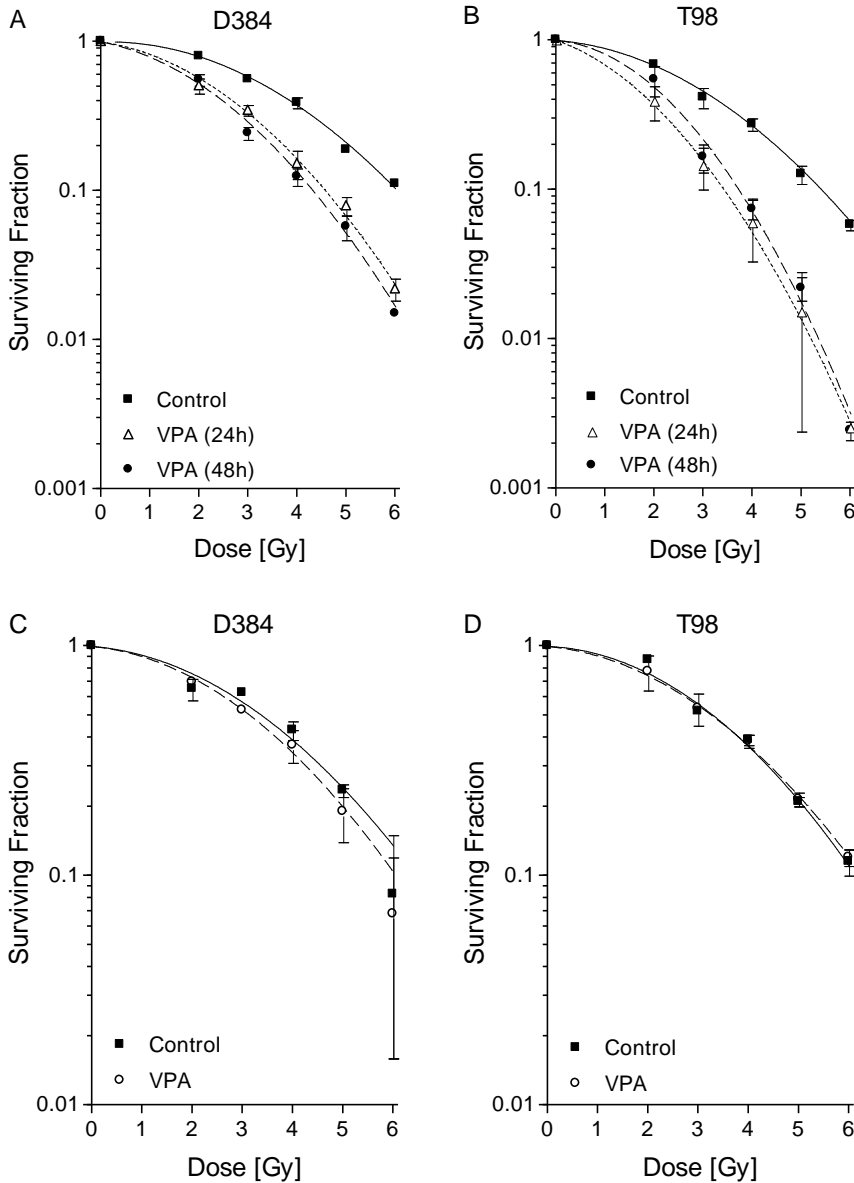


Figure 2 a, b Effect of γ -radiation after preincubation with 24h and 48h of VPA in D384 (**a**, 5 mM) and T98 (**b**, 2.5 mM) cells. Survival data are corrected for the plating efficiency of relevant preirradiation treatment (control, 24h VPA and 48h VPA). Symbols represent means \pm SEM (N=3). **c, d** Effect of γ -radiation with or without 24h VPA (5 mM D384; 2.5 mM T98) postirradiation in D384 (**c**) and T98 (**d**) cells. Survival data are corrected for the plating efficiency of relevant postirradiation treatment (control = 24h delayed plating and 24h VPA). Symbols represent means \pm SEM (N=2).

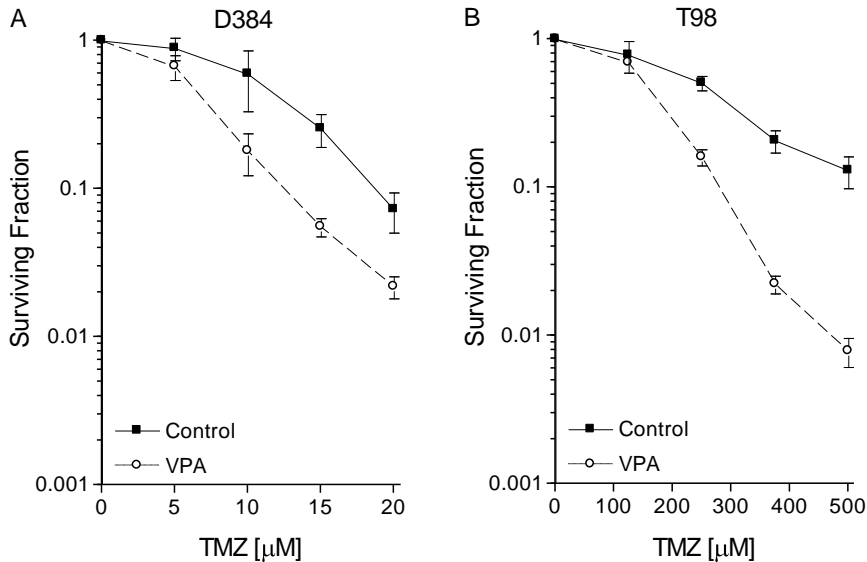


Figure 3 a, b Effect of TMZ following preincubation with VPA in D384 (a) and T98 (b) cells. Cells were exposed to VPA for 48h (5 mM D384; 2.5 mM T98) of which the last 24h were coincubated with various concentrations of TMZ. Survival data are corrected for the plating efficiency of relevant pretreatment (control and 48h VPA). Symbols represent means \pm SEM (N=2 D384; N=3 T98).

Effect of γ -radiation after preincubation with VPA and TMZ

Different concentrations of TMZ were combined with VPA prior to treatment with a dose of 4 Gy γ -radiation to explore suitable conditions for clonogenic cell survival curves. Table 1 shows the effect of treatment with γ -radiation on clonogenic cell survival after preincubation with TMZ or the combination of VPA and TMZ. Clear radiation enhancement is shown after preincubation with VPA in both D384 and T98 cells. No radiation enhancement is found after preincubation with TMZ alone in D384 cells (Table 1). Preincubation with TMZ seems to sensitise T98 cells to radiation treatment and the effect appears to be concentration independent (Table 1). The contribution (of various concentrations) of TMZ to the trimodal treatment does not further enhance the radiation response caused by VPA alone in both D384 and T98 cells (Table 1).

Concentrations of 5 μ M TMZ (D384) and 125 μ M TMZ (T98) were chosen to investigate the effect of trimodal treatment with VPA, TMZ and γ -radiation on complete radiation survival curves. D384 and T98 cells were exposed to VPA and TMZ prior to treatment with γ -radiation (2-6 Gy). The cell survival data presented in Figure 4a show that TMZ had no effect on the radiation response in D384 cells (DMF_{0.2} of 1.1). A substantial enhancement of the radiation response was demonstrated in D384 cells after preincubation with VPA as a single agent (DMF_{0.2}

of 1.6) or combined with TMZ (DMF_{0.2} of 1.9). Figure 4b shows that TMZ did not affect the radiation response in T98 cells (DMF_{0.2} of 1.0). A clear radiation enhancement was caused by VPA as a single agent (DMF_{0.2} of 1.5) and by the combined drugs (DMF_{0.2} of 1.6). In both cell lines, preincubation with the combined drugs is slightly more effective on the radiation response than that of VPA alone (Figure 4a/b).

Plating efficiencies corresponding to the data (with 4 Gy) shown in Figures 4a/b are presented in Table 2. Although TMZ does not contribute to the enhancement of the radiation response in the trimodal treatment combination (Figures 4a/b), the uncorrected data in Table 2 show that the trimodal combination does benefit from TMZ with respect to overall cell death.

Table 1. Surviving fractions (means, N=2) of different treatment combinations with 48h VPA (5 mM D384; 2.5 mM T98), various concentrations of TMZ (24h) and 4 Gy γ -radiation in D384 and T98 cells. VPA and TMZ were given prior to irradiation. For combinations of VPA and TMZ, cells were exposed to 24h of VPA followed by 24h coincubation of VPA and TMZ. Survival data are corrected for the plating efficiency of the appropriate preirradiation treatment, presenting the effect of radiation treatment alone.

Cell line	Treatment	0 μ M TMZ	2.5 μ M TMZ	5 μ M TMZ	7.5 μ M TMZ	10 μ M TMZ
D384	TMZ + 4 Gy	0.32	0.39	0.32	0.21	0.27
	VPA + TMZ + 4 Gy	0.13	0.13	0.08	0.08	0.15

Cell line	Treatment	0 μ M TMZ	125 μ M TMZ	150 μ M TMZ	175 μ M TMZ	200 μ M TMZ
T98	TMZ + 4 Gy	0.45	0.13	0.14	0.15	0.14
	VPA + TMZ + 4 Gy	0.06	0.02	0.03	0.03	0.02

Table 2. Plating efficiencies of different treatment combinations of VPA, TMZ and 4 Gy γ -radiation in D384 (5 mM VPA; 5 μ M TMZ) and T98 (2.5 mM VPA; 125 μ M TMZ) cells. Data represent means of two independent experiments (D384) or means of duplicates in a single experiment (T98). VPA and TMZ were given prior to irradiation. For combinations of VPA and TMZ, cells were exposed to 24h of VPA followed by 24h coincubation of VPA and TMZ.

Cell line	Control	4 Gy	TMZ + 4 Gy	VPA + 4 Gy	VPA + TMZ + 4Gy
D384	0.57	0.26	0.24	0.07	0.01
T98	0.5	0.13	0.06	0.03	0.01

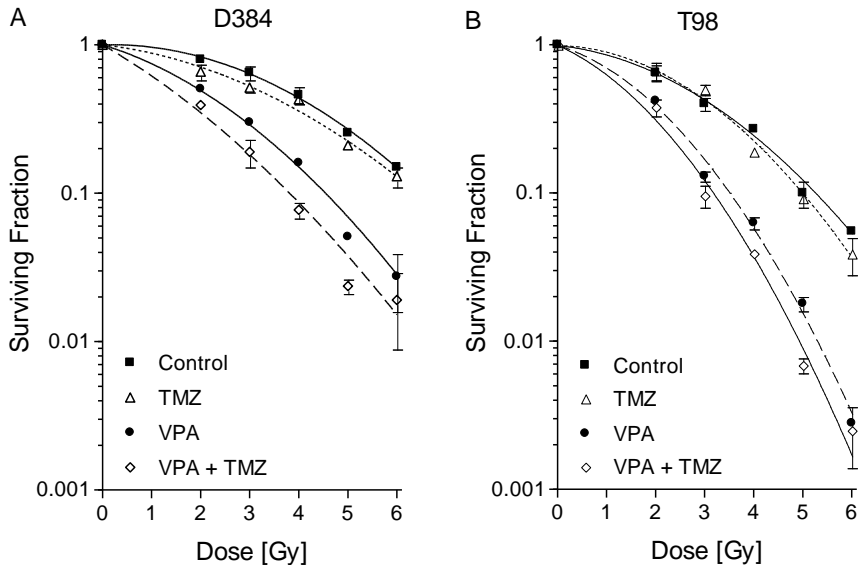


Figure. 4 a, b Effect of γ -radiation after preincubation with 48h VPA (5 mM D384; 2.5 mM T98), 24h TMZ (5 μ M D384; 125 μ M T98) or 48h VPA of which the last 24h were coincubated with TMZ in D384 (**a**) and T98 (**b**) cells. Survival data are corrected for the plating efficiency of relevant preirradiation treatment (control, 48h VPA, 24h TMZ or VPA and TMZ combined). Symbols represent means \pm SEM (N=2 D384) or means \pm SD (N=2 T98).

Discussion

The alkylating drug TMZ and the anti-epileptic drug VPA are independently known to sensitise for radiation in experimental glioma models [5, 6, 9, 28, 40, 42]. However, VPA (frequently prescribed in GBM patients) is a potential antagonist of TMZ [14] and may therefore be contraindicated in GBM patients on radiotherapy plus TMZ. This major clinical concern provided the rationale to investigate the interaction of VPA, TMZ and γ -radiation in two human glioma cell lines. These cell lines differ in sensitivity to TMZ due to the absence (D384) or presence (T98) of the MGMT protein [41]. Potential demethylation of the promoter region of the *MGMT* gene by VPA, could lead to inducing expression of the MGMT protein, and would cause cells to become (more) resistant to TMZ.

The present study does not support the hypothesis that VPA might antagonise the cytotoxic effects of TMZ. On the contrary, VPA significantly enhances the response to TMZ as well as to γ -radiation in both a TMZ-sensitive and a TMZ-resistant glioma cell line (Figures 2, 3 and 4). This effect is independent of the MGMT protein status. The combination of VPA and TMZ enhances the radiation response, although the contribution of TMZ to the radiation enhancement is minimal (Figure 4). However,

overall cell death is increased in the trimodal over bimodal treatment that seems to be caused not only by additive cytotoxic effects but also by sensitisation of the cytotoxic response to TMZ by pre-treatment with VPA (Table 1).

A radiosensitising effect of VPA in glioma cell lines has been reported earlier [5, 9]. Camphausen et al. showed enhancement of the radiation response in glioma cells in response to VPA exposure both before and after irradiation; VPA exposure before irradiation, without postirradiation exposure, caused less or no radiosensitisation [5]. Additional studies in mice showed that VPA starting 3 days before irradiation resulted in enhancement of the radiosensitivity of the xenografts [5]. Subsequently, this group investigated the effect of VPA exposure after irradiation, without pre-exposure, and found that exposure to VPA after irradiation of glioma cells induces a significant radiosensitisation [9]; however, combining VPA both before and after irradiation was more efficient in enhancing radiosensitivity [9].

The present study corroborates that VPA before irradiation positively affects the radiosensitivity of glioma cell lines. However, in contrast to the above-mentioned studies [5, 9], VPA did not enhance the radiation response when given after irradiation. These conflicting results might be due to the fact that different protocols were used to investigate VPA exposure after irradiation. During colony formation our cells were plated in VPA-free medium following a 24h postirradiation exposure to VPA, while in the other protocols medium-containing VPA was used [5, 9].

Besides its activity as an anti-epileptic drug, VPA is an HDAC inhibitor [20, 32, 35]. Inhibition of HDAC promotes histone acetylation that loosens up the chromatin structure, thereby increasing DNA accessibility. Relaxation of the chromatin structure by treatment with HDAC inhibitors increases the cytotoxicity of several anticancer drugs that target the DNA in a variety of tumour cell lines [29]. Studies on glioma cell lines have shown that VPA does increase sensitivity to chemotherapeutic drugs [10, 11]. VPA induces histone acetylation, resulting in an increased accessibility of the DNA; thereby, VPA could allow demethylases to erase methylation of specific genes [14]. If the promoter of the *MGMT* gene is demethylated by VPA, TMZ sensitive cell lines might turn into TMZ-resistant cell lines, thereby counteracting TMZ use during chemoradiotherapy of glioma patients.

However, we found no antagonistic effect of VPA on TMZ in glioma cells. On the contrary, we found a synergistic effect of VPA combined with TMZ. This effect may be caused by VPA loosening up the chromatin; thereby enabling TMZ increased accessibility to the DNA leading to more methylation, e.g. DNA damage. Our results are supported by others. Sasai et al. [36] combined VPA with 5-aza-2'-deoxycytosine (a DNA methyltransferase inhibitor and a demethylating agent in dividing cells); this combination failed to induce expression of the *MGMT* protein in glioma cell extracts. Also, Fu et al. [18] recently reported that combined treatment of VPA and TMZ had a synergistic effect on the induction of autophagic cell death in glioma cells both *in vitro* and *in vivo*.

The suggestion (arising from this and other studies) that VPA may be a clinically relevant sensitiser of TMZ is tempered by the *in vitro* experiments of Ständer et al; this group showed that anticonvulsant drugs fail to potentiate chemotherapy-induced cytotoxicity in human glioma cells at clinically obtained plasma levels [37]. However,

most *in vitro* studies use concentrations of VPA in a higher dose range, mostly from 1 mM up to 10 mM. Furthermore, the toxic effect of VPA combined with TMZ may give rise to concern about hematotoxic and hepatotoxic side-effects [21, 33, 34], although clinically relevant interactions have only rarely been observed [38].

Based on *in vitro* data, VPA does not antagonise the cytotoxic effect of TMZ and is therefore not contraindicated during chemoradiotherapy of glioma patients. Although promising as a sensitiser of both chemotherapy and radiotherapy, further studies with VPA are warranted. On the other hand it may be more worthwhile to investigate other more sensitive HDAC inhibitors in combination with TMZ and radiation to provide drugs that will be better and more suitable for future clinical application.

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