Epigenetic targeting of glioma stem cells: Short-term and long-term treatments with valproic acid modulate DNA methylation and differentiation behavior, but not temozolomide sensitivity

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Received October 28, 2015; Accepted January 20, 2016

DOI: 10.3892/or.2016.4665

Abstract. Glioblastoma (GBM) is the most aggressive tumor of the central nervous system. GBM is a fatal tumor, incurable by conventional therapies. One of the factors underlying tumor recurrence and poor long-term survival is the presence of a cancer stem-like cell population, termed glioma stem cells (GSCs), which is particularly resistant to chemotherapy and radiotherapy and supports tumor self-renewal. The aim of the present study was to evaluate the impact and difference in effects of short-term and long-term treatments with valproic acid (VPA), a histone deacetylase inhibitor, on seven GSC lines. We investigated for the first time the changes in the genome-wide DNA methylation profile and the differentiation behavior of GSCs induced by short-term and long-term VPA treatments. Moreover, we verified VPA sensitivity after long-term VPA pretreatment and, notably, the results provide evidence of a subpopulation more resistant to further VPA treatments. Finally, since short-term VPA treatment induced a reversal of the MGMT methylation status, we aimed to sensitize GSCs to temozolomide, the drug commonly used for this tumor, using this regimen. The overall data highlighted the heterogeneous behavior of GSC lines that is representative of tumor heterogeneity in GBM. The VPA effects were variable among these cell lines in terms of pro-differentiating ability and DNA methylation switch. Here, we attempted to identify a suitable therapy for the eradication of the stem cell subpopulation, which is mandatory to achieve an effective treatment for this tumor. Differentiation-inducing and epigenetic therapies are the most promising approaches to affect the multiple properties of GSCs and, finally, defeat GBM.

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Abbreviations: GBM, glioblastoma multiforme; GSCs, glioma stem cells; VPA, valproic acid; HDACi, histone deacetylase inhibitor; TMZ, temozolomide

Key words: glioblastoma, glioma stem cells, epigenetic therapy, valproic acid, differentiation therapy, DNA methylation profile, temozolomide

Introduction

Glioblastoma multiforme (GBM) is the most aggressive tumor of the central nervous system and represents over 70% of all brain malignancies. GBM is incurable by conventional therapeutic strategies (1). In fact, despite recent advances in surgery, radiotherapy and chemotherapy with temozolomide (TMZ) (2), the median survival of glioma patients remains poor and amounts to ~14 months. One of the factors underlying tumor recurrence and poor long-term survival is the presence of a cancer stem-like cell population, termed glioma stem cells (GSCs), that is particularly resistant to chemotherapy and radiotherapy and supports tumor self-renewal (3). Another explanation for drug resistance can be ascribed to intratumor heterogeneity due to the development of independent clones derived from a unique progenitor (4,5).

Increasing evidence suggests the role of epigenetic events, apart from genetic alterations, in the development and/or progression of several types of tumors, such as gliomas (6-8). The potential reversibility of epigenetic alterations represents an attractive approach to 'reset' the abnormal cancer epigenome by using epigenetic drugs such as histone deacetylase inhibitors (HDACi). Most recent data suggest the role of this class of drugs in tumor growth inhibition, promotion of apoptosis and induction of differentiation (9,10). Total eradication of the stem subpopulation may be achieved by using

pro-differentiative drugs, which are able to induce differentiation of GSCs affecting their self-renewal capabilities (11). Thus, HDACi can induce de-repression of genes epigenetically silenced in cancer (12,13) and involved in different cellular processes, including cell cycle control, differentiation, DNA repair and apoptosis (14).

Valproic acid (VPA), apart from being an anticonvulsant and mood stabilizing drug, is also an HDACi that has shown potent antitumor effects in a variety of *in vitro* and *in vivo* glioma studies (15-18). In recent years, the discovery of the ability of VPA to affect TMZ sensitivity in GBM cell lines suggests the use of this drug as a chemosensitizing agent (19-21).

In the present study, we investigated and compared for the first time the effects of short-term and long-term VPA treatments on cell morphology, differentiation behavior and DNA methylation profile of GSCs. Moreover, we examine whether long-term VPA treatment induces chemoresistance to VPA. Finally, we utilized VPA to chemosensitize the GSC subpopulation to TMZ action.

Materials and methods

Cell lines and cell culture conditions. The seven GSC lines used in this study (GBM2, GBM7, GBM04, G144, G166, G179 and GliNS2) were isolated from patients affected by GBM (22,23) and, in 2013, our research group characterized their cytogenomic and epigenomic profiles (24). Cells were cultured in neural stem cell-specific medium, and their stem-like properties were periodically monitored, as previously described (22-24). Cells were cultured in an adherent culture condition using 10 μg/ml laminin (Invitrogen) in a proliferation permissive medium composed of Dulbecco's modified Eagle's medium (DMEM)/F-12 and Neurobasal 1:1 (Invitrogen), B-27 supplement without vitamin A (Invitrogen), 2 mM L-glutamine, 10 ng/ml recombinant human bFGF and 20 ng/ml recombinant human EGF (Miltenyi Biotec), 20 UI/ml penicillin and 20 μg/ml streptomycin (Euroclone).

Drug treatments. Valproic acid (sodium salt; Sigma) was dissolved in sterile water to a stock concentration of 0.5 M. Temozolomide (Sigma) was dissolved in sterile DMSO at the final concentration of 0.05 M. All drugs were stored at -20˚C.

In vitro treatments were performed using 2 mM VPA for 24, 48,72, 96 h and 14 and 30 days; with regard to TMZ, we administered 50, 100, 200 or 400 μM TMZ for 48 or 72 h.

Cell viability by MTT assay. The methyl-thiazolyl tetrazolium (MTT) assay was performed to evaluate VPA efficacy in VPA long-term treated (30 days) cells compared with cells that were not previously treated (naïve). We also performed the cell viability analysis on naïve cells treated with the combination of 2 mM VPA plus several concentrations of TMZ (50, 100, 200 and 400 μM) for 96 h. The combined treatment was divided into a pretreatment phase (only VPA for 48 h) and a combination phase (VPA plus TMZ for an additional 48 h).

Cells were seeded at a density of $4x10^4$ cells/well in a 96-well-plate in 100 μl of culture medium and incubated at 37˚C. On the following day, we added VPA or TMZ at the selected final concentrations. After 24, 48, 72 and 96 h of treatment, 100 μl of 1 mg/ml MTT solution (Sigma) was added to each well and incubation was carried out for 3 h at 37˚C. Therefore, formazan granules were solubilized in absolute ethanol. The dye absorbance was measured spectrophotometrically at a 595-nm wavelength with the FLUOstar Omega microplate reader (BMG Labtech). The percentage of metabolic activity inhibition was determined by comparing the absorbance values of the treated cells with those of the untreated controls: (Treated cell absorbance/untreated cell absorbance) x 100. Results are the mean values of two independent experiments performed in quadruplicate.

Trypan blue dye exclusion assay. GBM2 ($0.25x10^6$) or G144 cells ($0.5x10^6$) were seeded in T25 flasks and then treated with different pharmacological regimens: i) 2 mM VPA for 96 h; ii) 50 μM or iii) 200 μM TMZ for 72 h; iv) 50 μM or v) 200 μM TMZ for 72 h after pretreatment with 2 mM VPA for 96 h. Then, cells were harvested and counted to evaluate the number of live cells and cell mortality, using trypan blue dye to discriminate live from dead cells. The results reported are the mean values of three different experiments performed at least in triplicate.

Cooperative index. In order to evaluate the effects of the combined VPA-TMZ treatments, the cooperative index (CI) (26) was calculated comparing the sum of the metabolic activity reduction percentages obtained for each single agent to the percentages obtained upon combined treatments (CI = VPA% + TMZ%/combined treament cell%). CI values <1 indicate a synergistic effect, CI values =1 indicate an additive effect, while CI values >1 indicate an antagonistic effect.

Morphological analysis. To evaluate the cellular morphological changes after VPA treatment, cells were seeded in serum-free medium at $3x10^3$-10^4 cells/ml, depending on the cell growth rate, specific for each GSC line. After 24 h, cells were treated with 2 mM VPA concentration for 14 and 30 days. Every three days the culture medium was changed and the drug was administered again. The morphological changes were evaluated by phase contrast microscopic observation, comparing VPA-treated and untreated cells. Representative images were captured for each cell line and for each treatment.

Immunofluorescence. The immunofluorescence assays were performed on untreated and 2 mM VPA-treated GSC cultures for 14 and 30 days using rabbit anti-CD133 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-nestin (1:50; Millipore, Billerica, MA, USA), rabbit anti-glial fibrillary acidic protein (GFAP, 1:200; Dako), rabbit anti-βIII-tubulin (1:100; Covance) and goat anti-myelin basic protein (MBP, 1:50; Santa Cruz Biotechnology) as primary antibodies. Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit (1:200; Molecular Probes, Eugene, OR, USA) was used as the secondary antibody. Alexa Fluor 647-conjugated phalloidin (1:200; Molecular Probes) was used to visualize the actin filaments. Propidium iodide (PI) was used to counterstain the nuclei.

Briefly, untreated cells were placed onto slides by means of Cytospin, while VPA-treated cells spotaneously adhered to the slides. Cells were washed with Dulbecco's modified phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min and treated for 10 min with 0.1 M glycine (in PBS). The slides were incubated for 30 min at room temperature (RT)

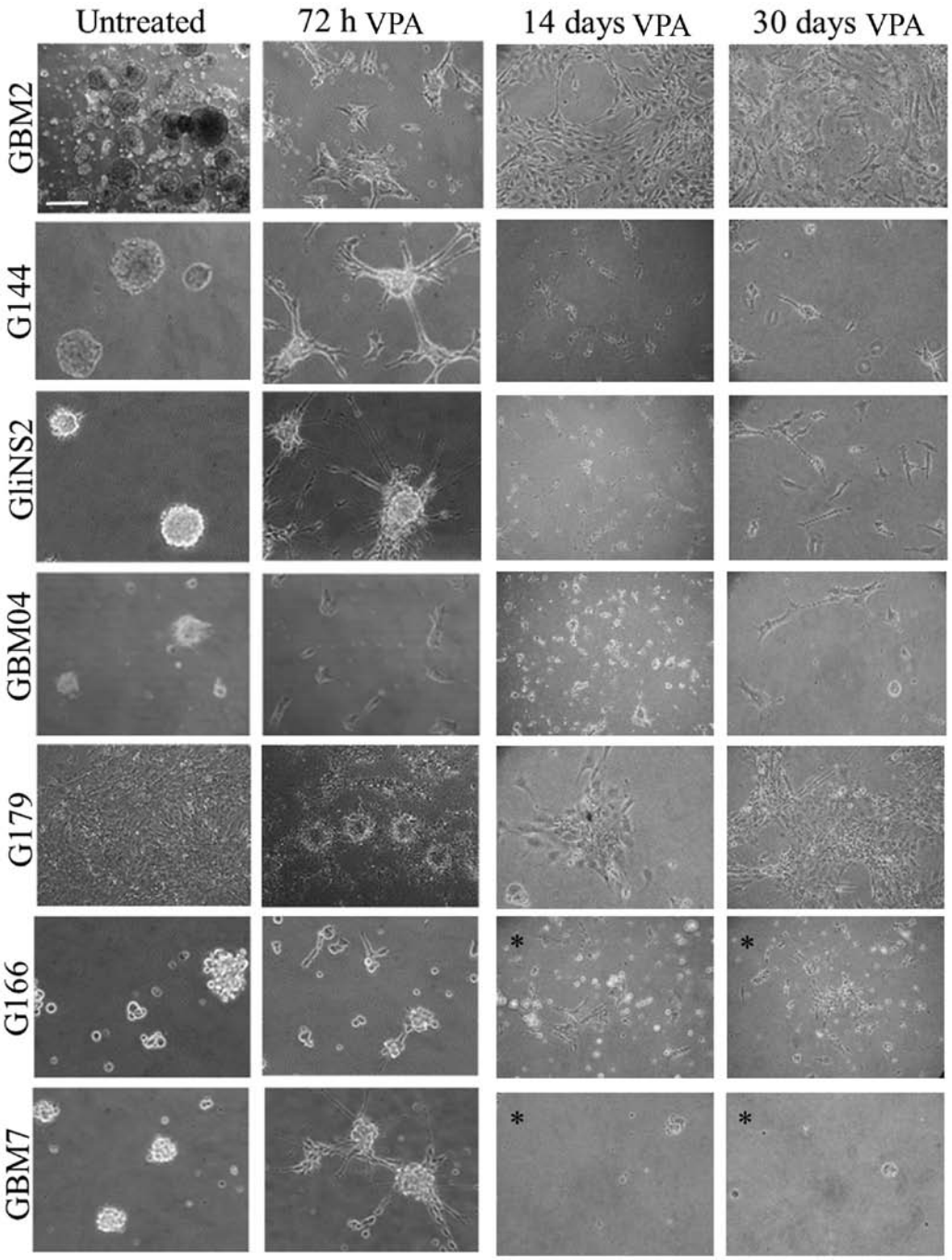

Figure 1. Analysis of cell morphology after valproic acid (VPA) administration. Representative images were captured by phase contrast microscopy of seven glioma stem cell (GSC) lines folllowing treatment with 2 mM VPA for 14 and 30 days. *G166 and GBM7 cells were treated with 1 mM VPA. Scale bar, 100 μm.

in blocking solution [5% bovine serum albumin (BSA), 0.6% Triton X-100 in PBS] and treated for 30 min with 70 U/mg RNAse (1:30; Sigma-Aldrich, Milan, Italy) in blocking solution. Cells were incubated with the primary antibodies at 4˚C overnight. Then, the slides were rinsed with washing buffer (0.3% Triton X-100 in PBS) and incubated with secondary fluorescent antibodies, phalloidin and 2.5 mg/ml PI for 1 h at RT. The cells were then washed with PBS and coverslips were mounted using polyvinyl alcohol mounting medium (Fluka Analytical, Milan, Italy). Fluorescent cell preparations were examined using a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA, USA).

In order to perform a quantitative-like analysis, the number of immunereactive cells for each marker was counted, evaluating at least 100 cells/sample over different areas of the slide.

MeDIP-Chip. GBM2 and G144 cell lines were treated with 2 mM VPA for 96 h and 30 days and then DNA extraction from the control and treated cultures was performed using the Wizard Genomic DNA Purification kit (Promega, Milan, Italy), according to the manufacturer's protocol.

Methylated DNA immunoprecipitation and chip hybridization were performed as previously described (24) following the guidelines of the Agilent Microarray Analysis of Methylated

Table I. Analysis of stemness and differentiation marker expression in GSC lines.

Cell line	Treatment	CD133 (%)	Nestin (%)	βIII Tub (%)	GFAP (%)	MBP (%)
GBM2	Untreated	62.96	75.44	19.27	63.81	100.00
	2 mM VPA 72 h	56.48	53.77[b]	36.45[a]	64.81	94.96[a]
	2 mM VPA 14 days	43.39[a]	100.00[c]	100.00[c]	100.00[c]	0.00[c]
	2 mM VPA 30 days	100.00[c]	100.00[c]	100.00[c]	100.00[c]	93.00[a]
	RPMI 10% FCS 14 days	97.09[c]	100.00[c]	100.00[c]	8.26[c]	100.00
	RPMI 10% FCS 30 days	78.38[a]	68.90	100.00[c]	5.93[c]	100.00
G144	Untreated	78.38	91.35	45.79	82.46	100.00
	2 mM VPA 72 h	67.86	87.85	13.79[c]	80.90	96.82
	2 mM VPA 14 days	32.00[c]	0.00[c]	100.00[c]	0.00[c]	0.00[c]
	2 mM VPA 30 days	0.00[c]	0.00[c]	18.00[c]	32.71[c]	0.00[c]
	RPMI 10% FCS 14 days	100.00[c]	100.00[a]	0.00[c]	100.00[c]	100.00
	RPMI 10% FCS 30 days	100.00[c]	100.00[a]	100.00[c]	100.00[c]	100.00
G166	Untreated	7.34	78.30	25.92	83.90	100.00
	2 mM VPA 72 h	1.92	53.70[b]	43.52[a]	81.31	100.00
	1 mM VPA 14 days	0.00[a]	0.00[c]	99.21[c]	100.00[c]	100.00
	1 mM VPA 30 days	0.00[a]	0.00[c]	0.00[c]	0.00[c]	0.00[c]
	RPMI 10% FCS 14 days	100.00[c]	54.37[b]	100.00[c]	100.00[c]	100.00
	RPMI 10% FCS 30 days	98.30[c]	79.17	100.00[c]	100.00[c]	100.00
G179	Untreated	100.00	99.17	100.00	98.21	0.00
	2 mM VPA 72 h	96.32	100.00	100.00	100.00	0.00
	2 mM VPA 14 days	100.00	73.56[c]	100.00	100.00	0.00
	2 mM VPA 30 days	100.00	0.00[c]	100.00	100.00	100.00[c]
	RPMI 10% FCS 14 days	100.00	96.48	98.20	100.00	95.28[c]
	RPMI 10% FCS 30 days	100.00	94.07	100.00	100.00	100.00[c]
GliNS2	Untreated	100.00	79.63	71.43	93.64	100.00
	2 mM VPA 72 h	55.50[c]	88.46	62.96	89.32	90.74[b]
	2 mM VPA 14 days	28.15[c]	0.00[c]	95.93	0.00	0.00[c]
	2 mM VPA 30 days	0.00[c]	100.00[c]	100.00	0.00	0.00[c]
	RPMI 10% FCS 14 days	90.48[a]	100.00[c]	100.00	100.00	100.00
	RPMI 10% FCS 30 days	93.14[a]	97.17[b]	100.00	100.00	100.00
GBM04	Untreated	86.29	96.58	76.78	86.08	0.00
	2 mM VPA 72 h	100.00[c]	97.33	88.28[c]	44.17[c]	0.00
	2 mM VPA 14 days	11.29[c]	0.00[c]	54.20[c]	100.00[c]	76.03[c]
	2 mM VPA 30 days	0.00[c]	0.00[c]	0.00[c]	0.00[c]	100.00[c]
	RPMI 10% FCS 14 days	100.00[c]	100.00	100.00[c]	100.00[c]	96.36[c]
	RPMI 10% FCS 30 days	100.00[c]	100.00	100.00[c]	88.24	100.00[c]

Expression of markers was evaluated in untreated and valproic acid (VPA)-treated cells for 14 and 30 days. The table also shows data derived following a 72-h VPA treatment as previously described by our group (25); all the other data are original. Cells cultured in RPMI supplemented with 10% FCS were considered as a positive control of differentiation. Fisher's exact test was used for statistical analysis (treated vs. untreated cells). [a]P<0.05; [b]P<0.001; [c]P<0.0001.

DNA Immunoprecipitation protocol (version 1.0; Agilent Technologies, Santa Clara, CA, USA).

The raw data, expressed as combined z-score values, were analyzed according to the methodological approach conceived by Dr Ravid Straussman (27). This method allowed us to classify the methylation status of each CpG island (CGI) as methylated, unmethylated, or undetermined (CGIs with uncertain methylation status). Then, we decided to consider only data referred to CGIs located in gene promoters for the subsequent analysis due to the well-known biological effects of methylation in these regions. When a promoter contained two or more CGIs, we averaged the related combined z-score values in order to establish the correct methylation status. Finally, we compared data from the untreated and VPA-treated samples to highlight the methylation changing promoters.

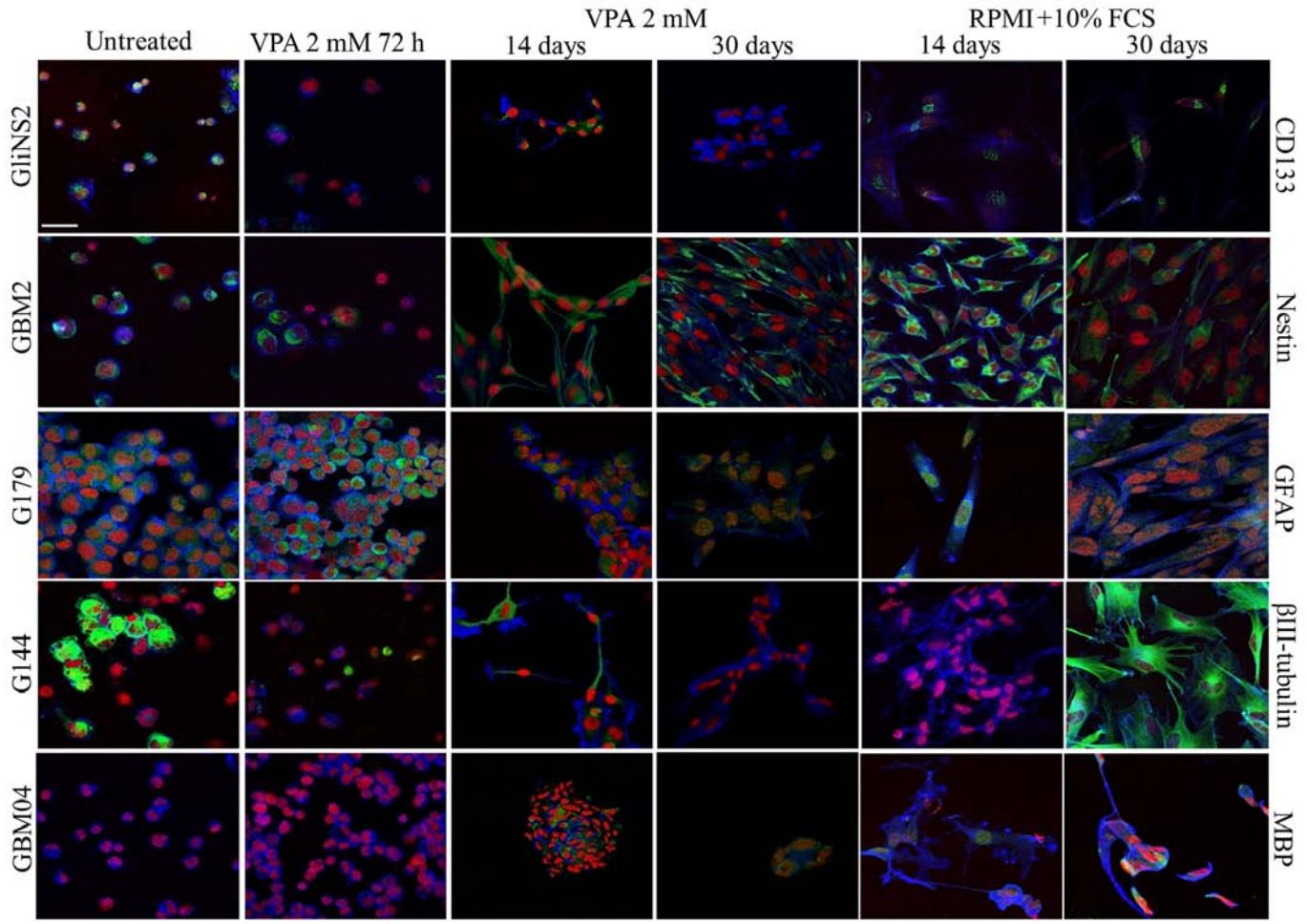

Figure 2. Representative images of the immunofluorescence analysis performed on untreated glioma stem cells (GSCs) and GSCs treated for 14 and 30 days with 2 mM valproic acid (VPA). Cells cultured for 14 and 30 days in 10% FBS RPMI medium represent a positive control of the differentiation capability. Cells were immunostained with antibodies against stemness (CD133, Nestin) and differentiation (GFAP, βIII tubulin, MBP) markers (green), phalloidin (blue) and propidium iodide (red). Images were captured using confocal microscopy. G166 cells were treated with 1 mM VPA. For GBM7, the prevailing cytotoxic effect of long-term VPA treatment did not allow to conduct the experiment. Scale bar, 100 μm.

Bioinformatic analysis. The Gene Ontology (GO) analysis was performed, as previously described (24), using GOstat software (http://gostat.wehi.edu.au/) (28).

Statistical analysis. Statistical analysis was carried out performing Fisher's exact and Student's t-tests. The critical level of significance was set at $P<0.05$.

Results

Effect of short- and long-term VPA treatments on GSC morphology. In a previous study, we evaluated the effect on GSC morphology induced by VPA after 24, 48 and 72 h (25). We revealed that short-term VPA treatment severely modified cell morphology. In this study, we extended the evaluation to 14 and 30 days of treatment.

After 14 and, particularly, 30 days of exposure, the pro-differentiating effect of VPA was evident: most of the cells were star-shaped and displayed neurite-like processes (Fig. 1). G166 and GBM7 cells showed a high number of dead cells, demonstrating the predominant cytotoxic effect of VPA or the induction of apoptosis following terminal differentiation. Therefore, these two cell lines were treated with a lower drug concentration (1 mM) for 14 and 30 days. While G166 cells showed important morphological modifications at this lower concentration treatment, the GBM7 cell line was still extremely sensitive, showing a prevalence of cell death.

Differentiation behavior of GSCs in response to short- and long-term VPA treatment. The pro-differentiation ability of VPA was evaluated analyzing the expression of markers of stemness (CD133 and Nestin) and differentiation (GFAP, βIII-tubulin and MBP) by immunofluorescence in the untreated and 2 mM VPA-treated cells for 14 and 30 days. We compared these data with those we had obtained after VPA short-term treatment (25).

The summary of the immunofluorescence results is reported in Table I, and representative images are shown in Fig. 2. After 14 and 30 days of VPA exposure, 100% of the GBM2 cells maintained nestin, βIII-tubulin and GFAP expression. Regarding CD133 and MBP markers, we observed a significant increase in cells expressing these two proteins with the prolongation of the treatment time. The G144 cell line manifested a reduction in cells expressing both stemness and differentiation markers after 30 days compared to the control cells. An unusual behavior was observed for GFAP, for which

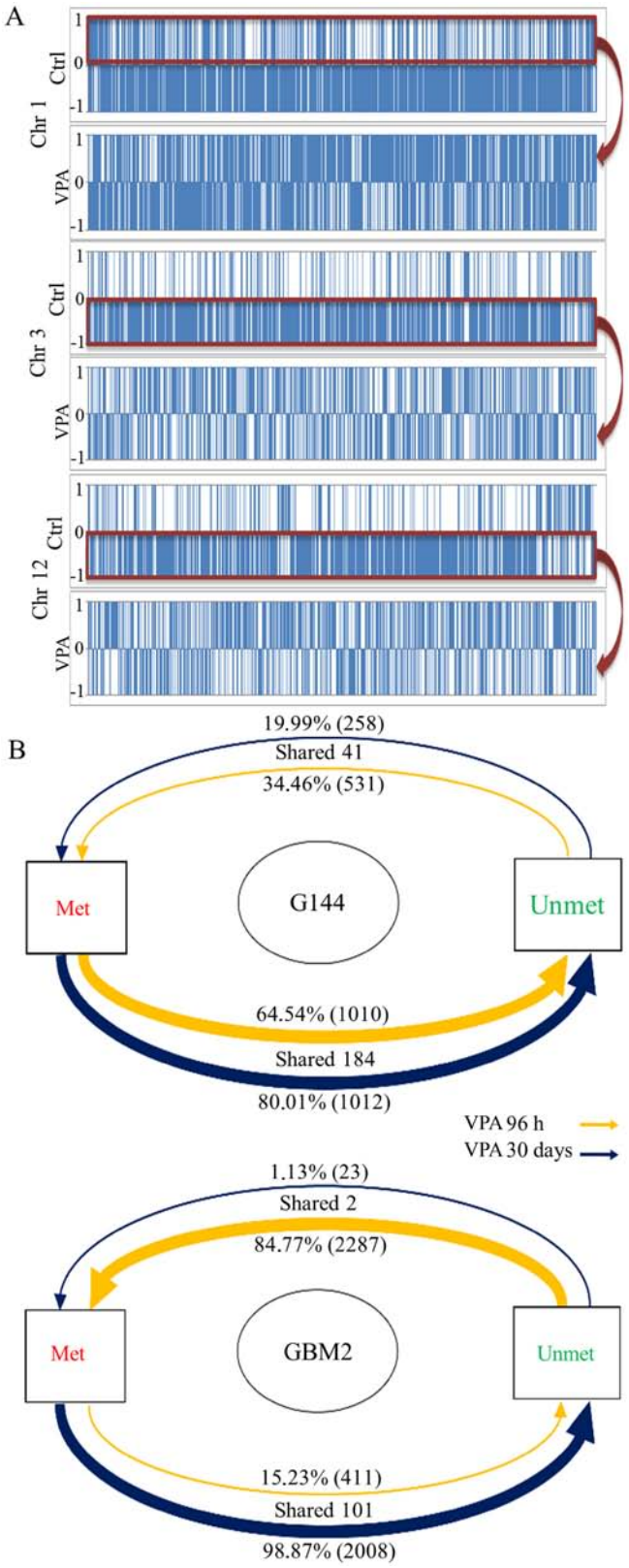

Figure 3. (A) Methylation profile changes of chromosomes 1, 3 and 12 in glioma stem cells (GSCs) treated with 2 mM valproic acid (VPA) for 96 h. Each blue bar represents an oligonucleotide probe. In particular, bar 1 indicates methylated probes, while bar -1 represents unmethylated probes. These raw data, taken as examples, show that VPA treatment induced serious changes in the CGI methylation status. (B) Percentages of modified methylation status promoters in CTRL vs. VPA 96 h and CTRL vs. VPA 30 days. Yellow and blue arrows represent the percentages of modified methylation gene promoters (Unmet→Met; Met→Unmet) following VPA treatments for 96 h and 30 days, respectively. Note the different behaviors of the two GSC lines at 96 h of VPA and, instead, a similar trend at 30 days of VPA.

there was a decrease in cells expressing GFAP after 14 days, while this number increased after 30 days, but remained significantly below the untreated control. The G166 cell line showed a very high number of cells expressing differentiation markers after 14 days of treatment; after 30 days these values were drastically decreased. In contrast, few cells were positive for both Nestin and CD133 after 14 days of treatment. The GliNS2 cell line showed a significant reduction in CD133, GFAP and MBP immunoreactive cells after 14 and 30 days of treatment. Contrariwise, the percentage of cells positive for βIII-tubulin was increased after both treatment times. An unusual behavior was observed for Nestin as after 14 days almost all cells were negative, while after 30 days nearly all cells were positive. After 30 days, the GBM04 cell line exhibited an enrichment of cells expressing MBP, probably indicating the capability to induce oligodendrocyte differentiation, while all other markers were negative. Almost 100% of G179 cells retained the expression of CD133, βIII-tubulin and GFAP after 14 and 30 days. The longest treatment caused a significant decrease in cells expressing Nestin; in contrast, a strong increase in cells expressing MBP was observed after this specific time.

In our previous study (25), we showed that GSCs expressed both stemness and differentiation markers at variable levels in untreated and 72 h VPA-treated samples. This behavior persisted also after VPA long-term treatments in 3 out of 6 cell lines (GBM2, G179 and GliNS2). Notably, the others did not show immunoreactive cells for stemness markers after 30 days of treatment.

Methylation profiles of GSCs after short- and long-term VPA treatments. We investigated the effect of 2 mM VPA administration for 96 h and 30 days on the CGI methylation status of two GSC lines characterized by a different susceptibility to this drug. In particular, the GBM2 cell line showed a reduced viability after a 72 h VPA treatment and co-expressed stemness and differentiation markers also after 30 days of VPA exposure, while the G144 cell line did not display any reduction in cell viability, but it seemed able to differentiate terminally after long-term VPA exposure.

Following observation of the raw data of the three chromosomes taken as examples (Fig. 3A), it is clear that VPA severely affected CGIs within the gene promoters. The results of the two treatments were matched with the respective untreated control cells at 96 h and 30 days of culture in order to normalize the spontaneous fluctuations of the methylation status due to *in vitro* expansion, and finally the two regimens were compared to each other.

We calculated the percentage of genes that did not change their methylation status after treatment (Table II, 'unchanged' columns), and the percentage of genes that, instead, had a modified methylation status after the regimen (Table II, 'modified' columns).

The two cell lines did not show substantial differences between each other in the 'unchanged' category, in which the class of unmethylated genes was the most highly represented. Instead, in the 'modified' category they behaved somewhat differently: in the GBM2 cell line a high percentage of genes switched from an unmethylated to a methylated status after 96 h (23.27%), while a similar percentage of genes moved in the opposite direction after 30 days (21.73%). Conversely, in the

Table II. Percentages of gene promoters with unchanged or modified methylation status in CTRL vs. VPA 96 h, CTRL vs. VPA 30 days and VPA 96 h vs. VPA 30 day comparisons.

		Unchanged methylation status promoters (%)			Modified methylation status promoters (%)		
Cell line	Comparisons	Methylated	Unmethylated	Total unchanged	Met→Unmet	Unmet→Met	Total modified
G144	CTRL vs. VPA 96 h	17.96	64.56	82.52	11.82	5.66	17.48
	CTRL vs. VPA 30 days	24.76	60.48	85.24	11.81	2.95	14.76
	VPA 96 h vs. VPA 30 days	13.91	63.88	77.79	9.59	12.62	22.21
GBM2	CTRL vs. VPA 96 h	16.93	54.60	71.53	5.20	23.27	28.47
	CTRL vs. VPA 30 days	14.94	63.07	78.01	21.73	0.26	21.99
	VPA 96 h vs. VPA 30 days	10.38	54.99	65.37	29.95	4.68	34.63

The 'Methylated' and 'Unmethylated' columns represent the percentages of gene promoters that maintained, respectively, the methylated or unmethylated status. The 'Met→Unmet' and 'Unmet→Met' columns show the percentages of gene promoters with modified methylation status, respectively, from methylated to unmethylated and vice versa. VPA, valproic acid.

G144 cell line VPA produced a comparable change in regards to both short- and long-term treatments, since an analogous percentage of genes was found in both directions (11.82 and 5.66% after 96 h, 11.81 and 2.95% after 30 days). These data can also be appreciated by comparing the two regimens with each other directly. Indeed, the percentages of the 'modified' category were similar in the G144 cell line (9.59 and 12.62%), reflecting the homogeneity of both short- and long-term VPA treatments (Table II, row 3). On the contrary, in the GBM2 cell line the percentage of the 'modified' category 96 h versus 30 days was very different (29.95 vs. 4.68%) (Table II, row 6).

In order to evaluate the methylation changes specifically caused by VPA treatment and not by fluctuations due to *in vitro* expansion, we chose the genes that showed a change in the same direction following both treatments, thus assuming that the changes occurred during the short-term treatment and were maintained until the second time-point, i.e., the change was stable. Judging from the fraction of the genes with modified methylation status that were shared at the two treatment times, the changes made by VPA in the G144 cell line, although less relevant, appeared more stable and maintained than those recorded in the GBM2 cell line, where few genes retained the same methylation profile in the two treatments (Fig. 3B). Indeed, a large fraction of genes changed from unmethylated to methylated status after 96 h of VPA, but after 30 days of VPA a similar number of genes returned back from a methylated to an unmethylated status (Fig. 3B). With regard to the G144 cell line, we observed that the majority of 'modified' genes underwent a variation of the methylation pattern from methylated to unmethylated status in both treatments. These behaviors could be assessed to the different initial VPA sensitivities of the two cell lines and to a sort of resistance mechanism acquired during long-term VPA treatment by the GBM2 cell line.

Gene Ontology analysis revealed that two functional categories were more represented after short- and long-term VPA treatments in both cell lines: '*development and differentiation*' and '*metabolism*' (Fig. 4). Interestingly, VPA treatments induced in both cell lines a general trend to the unmethylated status for the '*neural differentiation and nervous system process*' category. Once again, the G144 and GBM2 cell lines showed differences related to the effect of long-term treatment. Indeed, in the GBM2 cell line the gene list of the 'switched from unmethylated to methylated status' did not show any statistically significant GO terms after long-term treatment (Fig. 4).

Finally, we investigated whether the short- and long-term treatments influence the methylation status of specific genes involved in critical signaling pathways responsible for GBM initiation, migration and invasion, such as WNT, SHH, PDGFR and EGFR pathways (29,30). First of all, following analysis of the methylation status of several critical genes in the two untreated cell lines, we observed, for example in the 96 h controls, that they showed several homologies such as the unmethylated status of many tumor-suppressor genes (TSC1, NF2, SMAD4 and MEN1, EXT1, GPC3 and RB1), but for other genes the results were discrepant. For example, PTEN and E-cadherin (CDH1) were methylated in the GBM2 cell line and unmethylated in the G144 cell line, while CTNNB1, several WNT ligands and RUNX1 were methylated in G144 and unmethylated in GBM2 (Table III).

In the second instance, we considered the methylation status of the same genes after the drug treatments. The general effect attributable to VPA in both cell lines was towards an unmethylated status of the genes in all pathways considered. Furthermore, a greater number of genes showed this switch (from methylated to unmethylated status) after 30 days of VPA treatment, compared to 96 h (Table III).

VPA resistance in VPA long-term treated cells. To confirm the hypothesis of the acquisition of a resistant phenotype after VPA long-term treatment by the GBM2 cell line, we performed analysis of the cell viability by MTT assay on naïve and VPA-pretreated G144 and GBM2 cells.

The results showed that G144 cell viability after VPA treatment was generally not modified by the pretreatment for

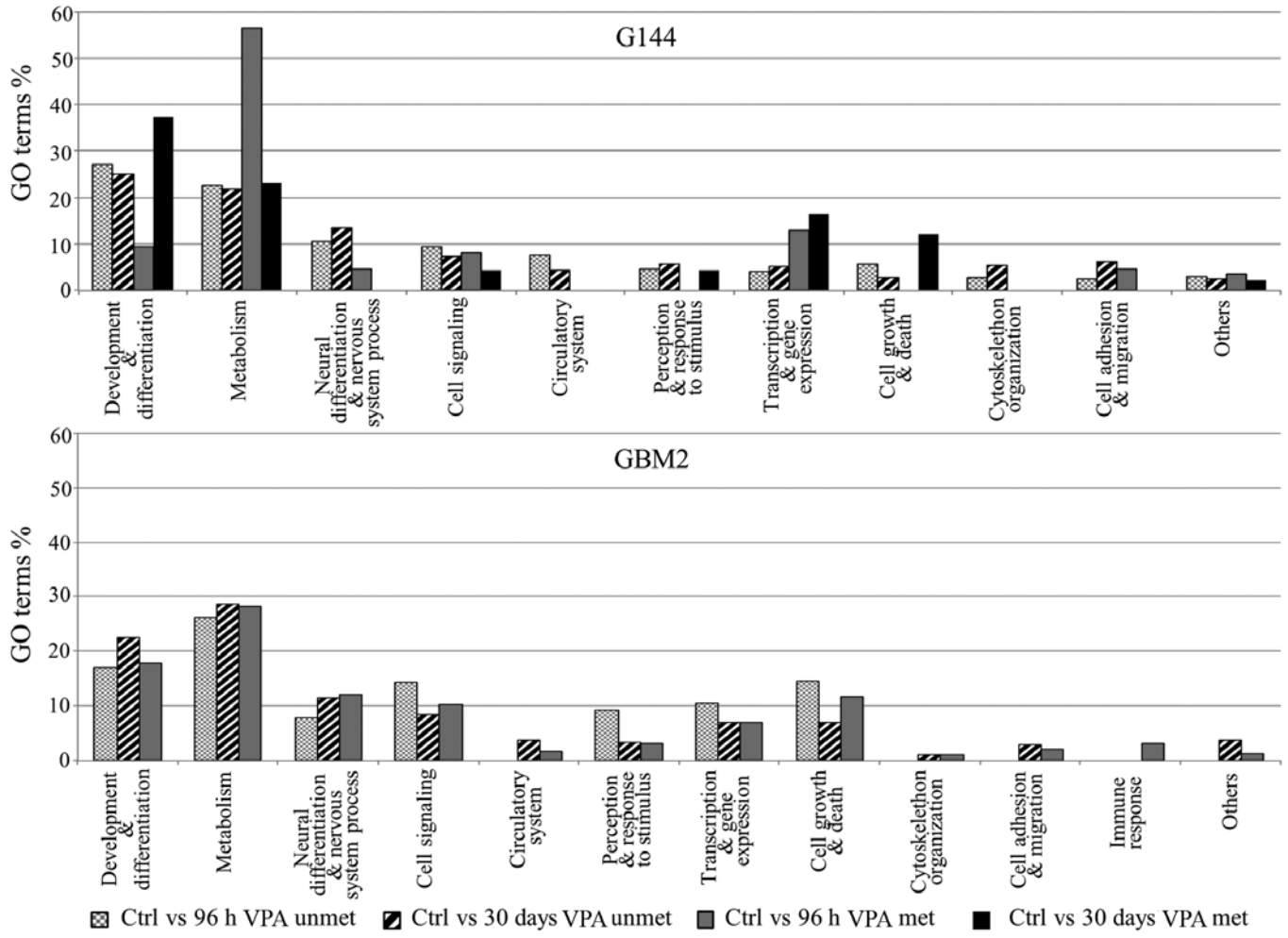

Figure 4. Gene functional groups with valproic acid (VPA)-influenced DNA methylation status after short- and long-term treatments. The functional annotation analysis of genes with modified methylation status after short- and long-term VPA treatments was performed using the GOstat software. The statistically significant GO terms obtained from the Gene Ontology analysis were grouped in cancer relevant categories. In the graph, we show a percentage related to each category. This value is the ratio between the GO term number of a specific category and the totality of the statistically significant GO terms.

30 days, as expected. In fact, this cell line was already resistant, as confirmed by the low VPA sensitivity of naïve cells (Fig. 5A).

With regard to the GBM2 cell line, we already observed a consistent cell viability reduction in the naïve cells, especially at 48 and 72 h of treatment (65 and 62%, respectively). VPA long-term pretreated GBM2 cells showed a reduced sensitivity to the drug when compared to the naïve GBM2 cells at all the time-points tested; the induction of a resistant phenotype in the GBM2 cell line, probably due to the long-term *in vitro* exposure to 2 mM VPA, was particularly evident at 48 h of treatment in which the cell viability of the 30 day-exposed cells reached 92% ($P<0.0001$) (Fig. 5A).

VPA treatment failed to sensitize GSCs to TMZ. In GBM, first-line treatment includes radical surgical resection followed by concurrent radiotherapy and chemotherapy, typically temozolomide (TMZ) (31). TMZ exerts cytotoxicity against GBM cells by creating O^6-methylguanine lesions, which leads to DNA fragmentation (32). The response to TMZ is favorably affected by the promoter methylation of the methylguanine-DNA methyltransferase (MGMT) gene. This enzyme removes, if expressed, the methyl groups added by TMZ, thereby preventing GBM cell death (33). In this study we evidenced that, in GBM2 cells, the MGMT promoter switched from an unmethylated to a methylated status after 96 h of VPA treatment (Table III). To verify whether VPA treatment was able to increase TMZ efficacy by virtue of this change in MGMT promoter methylation, we analyzed cell viability after 2 mM VPA (96 h) plus several doses of TMZ (48 h). Unexpectedly, the results showed that VPA exposure did not produced any relevant increase in TMZ efficacy in the GBM2 cell line. In fact, cell viability was reduced at all drug combinations in a statistically significant manner when compared to TMZ only treatments (except for VPA plus 400 μM TMZ), but no statistically significant difference was observed with the VPA single treatment ($P>0.05$) (Fig. 5B). Furthermore, CI values for the combined treatments clearly indicated the presence of antagonism between the two compounds (CI>1; Table IV).

The MGMT promoter in the G144 cell line was methylated and this status was not altered by VPA; this cell line could be virtually sensitive to TMZ. Surprisingly, the G144 cells were resistant to TMZ and this issue was not modified by VPA treatment, suggesting the presence of a complex pattern of drug resistance mechanisms (CI>1; Table IV) (Fig. 5B).

Table III. Changes in the methylation status of genes involved in several glioma-related pathways, in neural differentiation and in stemness maintenance after short- and long-term VPA treatment.

	G144		GBM2	
Pathway/genes	96 h Ctrl→VPA	30 days Ctrl→VPA	96 h Ctrl→VPA	30 days Ctrl→VPA
Receptor tyrosine kinase pathway				
ALK	U	U	U	U
EGFR	U	M→U	ND→U	U
PDGFRL	U	U	U	U
PDGFC	U→ND	M→U	U	U
PDGFD	M→U	U	U→M	M→U
PDGFB	U	M→U	U	M→U
SHC1	U	U	U	U
SHC2	M→U	M	U→ND	M→U
SHC3	U	U	U	U
SHC4	U	U	U	U
GRB2	U	U	U	U
SOS1	U	U	U	U
SOS2	U	U	U	U
RAS	U	M→U	U	U
RAF	U	U	U	M→U
MAP2K1	U	U→M	U	U
MAP2K2	U	M→ND	U	U
MAP2K3	U	U	U	U
MAP2K4	U	U	U	U
MAP2K5	U	U	U	U
MAP2K7	U	U	U	M→U
MAPK1	M→U	U	U	U
NF1	U	M→U	U	U
ABL1	U	U	U	U
ABL2	U	U→M	U	U
FES	U	M	U→M	M
JAK1	U	U	U	U
JAK2	U	U	ND→U	M→U
JAK3	M→U	M	M	M
STAT1	U	U	U	U
STAT2	U→M	U	U	U
STAT3	U	U	U	U
RB pathway				
CDKN2A	U	U	ND→M	M
CDK6	U	U	U	U
CCND1	M→ND	M	ND→M	M→ND
RB1	U	U	U	U
E2F1	U	U	U	U
E2F2	U	U	U	U
E2F3	U	U	U	U
E2F5	U→ND	U	U	U
E2F6	U→M	U	U	U
E2F7	U	U	U	U
E2F8	U	M	U→M	M
TFE3	U	U	U	U
p53 pathway				
TP53BP2	U	U	U	U

Table III. Continued.

	G144		GBM2	
Pathway/genes	96 h Ctrl→VPA	30 days Ctrl→VPA	96 h Ctrl→VPA	30 days Ctrl→VPA
p53 pathway				
TP53I3	U	U→M	U	M→U
TP53INP1	M	M	M	M→ND
TP53I11	U	M	U→M	M→U
TP53INP2	U	U	M→U	M→U
MDM2	M→ND	M	M→U	U
NOTCH1	U	U	U	U
PUMA	M→U	U→M	U	M→U
NOXA	M	M	U	U
BCL2	U	M→U	ND	M→U
BAX	U	M→U	U	U
P21	U	U	U	U
CDK6	U	U	U	U
CDK7	U	U	U	U
CDK8	M→U	U	U	U
CDK10	U	U	U	U
CCND1	M→ND	M	ND→M	M→ND
MSH2	U	M→U	M→U	M→U
BRCA1	U	U	M	M
PI3K/AKT pathway				
PDK2	U→ND	U	U	U
PTEN	U	U	M	M
PIK3R1	U	U	U→M	M
PIK3R2	U→M	U	U	U
PIK3R3	U	M→U	U	U
PIK3R4	U	U	ND→U	U
PIK3CA	M→U	U	U	U
PIK3C2B	U	U	U	U
PIK3CD	U	U	U	U
AKT1	U	U	U→M	M→U
AKT3	U	U	U	U
BCL2	U	M→U	ND	M→U
FOXO1	U	U	U	U
FOXO3	U	U→M	U	U
FOXO4	U	U	U	M→U
TSC1	U	M→U	U	U
RHEB	U	M→U	U	U
MTOR	U	U	U	U
4EBP1	U→M	U	U→ND	M→U
RAC3	U	M→U	ND→U	U
RAC1	M→U	M→U	U	U
NF2	U	U	U	U
SHH pathway				
SHH	U	U	U	U
EXT1	U	U	U	U
PTCH1	U	U	U	U
PTCH2	M	M	M→U	M→U
SMO	M→U	M→U	U	M→U
GLI1	U	U	U	U
GLI3	U	U	U	U
GLI4	U→M	U	U	U

Table III. Continued.

Pathway/genes	G144 96 h Ctrl→VPA	G144 30 days Ctrl→VPA	GBM2 96 h Ctrl→VPA	GBM2 30 days Ctrl→VPA
WNT pathway				
GPC3	U→ND	M→U	U	U
WNT2B	U→ND	M	U	ND→U
WNT9A	M	M	U	U
WNT3A	M	M	U→M	M
WNT6	M→U	M→U	U	M→U
WNT10A	M→U	M	U→M	M
WNT7A	U	U	U	U
WNT5A	U	U	U	U
WNT2	U	M→U	U	M→U
WNT11	M→U	M→U	U	M→U
WNT3	M	M	M	M→ND
WNT9B	M	M→U	U	U
FZD7	U	M→U	U	U
FZD5	U	U	U	U
FZD9	U	M→U	M	M
FZD1	U	U	U	U
FZD6	U	M→U	ND→M	M
FZD8	U	U	U	U
FZD4	U	ND→M	U→M	M→ND
FZD10	M	M	M	M
FZD2	U	U	M→U	M→U
LRP6	U	U	U	U
GSK3β	U	U	U	U
β-catenin	M→U	ND→U	U	U
APC2	M→U	M→U	ND	M→U
AXIN1	M	M	M	M
AXIN2	U	U	U	U
CDH1	U	M	M	M→ND
α-catenin	U	ND→M	U	M→U
ACTB	U	U	U	U
ACTG1	M	M	ND→U	U
ACTA1	M→U	M	U	M→U
TCF7	M	M	M	M
TCF12	M	M	M	M
TCF25	M→U	M→U	U	M→U
TCF4	U	U	U	U
TCF15	U	M	U	M→U
C-MYC	U	U	U	U
BMP4	U	M	U→M	M→U
BMP/TGFβ pathway				
BMP2	U	U	U	U
BMP3	U	M	U	M→U
BMP4	U	M	U→M	M→U
BMP6	U→ND	U	U	U
BMP7	M→U	M→U	ND→M	M→ND
BMP8A	M	M	M	M→ND
BMP8B	M→U	M	ND→U	M→U
TGFβ1	M	M	U→M	M→ND
TGFβ2	M	M→U	U	U
TGFβR1	U	M→U	ND→U	M→U

Table III. Continued.

Pathway/genes	G144 96 h Ctrl→VPA	G144 30 days Ctrl→VPA	GBM2 96 h Ctrl→VPA	GBM2 30 days Ctrl→VPA
BMP/TGFβ pathway				
TGFβR2	U	U	M→U	U
SMAD2	U	U	U	M→U
SMAD3	U	U	U	U
SMAD4	U	U	U	U
RUNX1	M→U	U	U→M	M→U
MEN1	U→M	M	U→M	U
Neural differentiation				
BMPR1B	U	U	U→M	U
TUBB3	U	U	U	U
MAP2	M	U	U→M	U
OLIG1	U	M→U	U	U
OLIG2	M→U	M	M	M
OLIG3	M→ND	M	M	M→U
Stemness maintenance				
OCT4	M	M	M→U	M
NOTCH1	U	U	U→M	U
CD44	U	ND→U	U→M	ND→U
PROM1	U	U	U→M	U
NES	U	U	U	M→U
MGMT	M	M	U→M	U

U, unmethylated gene promoter; M, methylated gene promoter; ND, undetermined methylation status of the gene promoter.

Table IV. Cooperative index (CI) of VPA + TMZ combined treatments (MTT assay).

Cooperative index	VPA 2 mM GBM2	G144	G166	GBM04
TMZ 50 μM	1.33	1.35	1.25	1.33
TMZ 100 μM	1.31	2.01	1.22	1.37
TMZ 200 μM	1.50	2.21	1.43	1.54
TMZ 400 μM	1.71	445.80	1.90	1.51

CI values <1 indicate a synergistic effect; CI values =1 indicate an additive effect, while CI values >1 indicate an antagonistic effect. VPA, valproic acid; TMZ, temozolomide.

Considering the heterogeneity of GBM, we extended this analysis to two additional cell lines (GBM04 and G166). In both lines, all drug combinations displayed a statistically significant reduction in cell viability compared to the single treatments (except for VPA plus 200 μM TMZ in the G166 cell line), but the CI values clearly indicated that there was no synergism between the two drugs (CI>1; Table IV).

Moreover, we analyzed the percentages of live and dead cells by trypan blue dye-exclusion assay in the GBM2 and

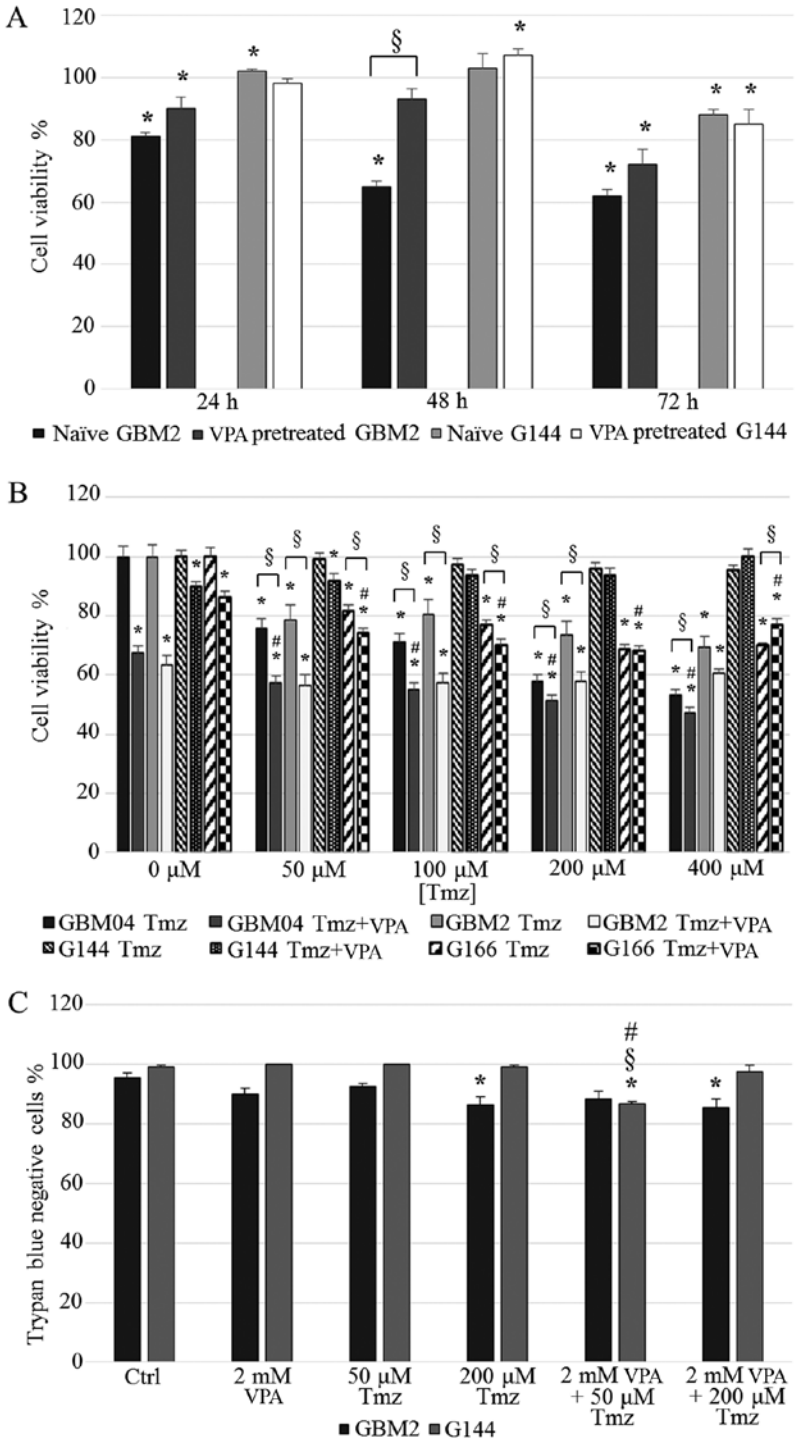

Figure 5. (A) Valproic acid (VPA) sensitivity in VPA pretreated and 'naïve' glioma stem cells (GSCs). (B) GSC viability after VPA or temozolomide (TMZ) single treatments and TMZ + VPA combined treatments. (C) Percentage of trypan blue-negative cells after VPA, TMZ and VPA + TMZ treatments compared to the untreated sample. (t-test: *P<0.05 in untreated vs. treated cells; §P<0.05 in TMZ vs. VPA + TMZ; #P<0.05 in VPA vs. VPA + TMZ).

G144 cell lines treated with only VPA or TMZ, and VPA plus TMZ. The percentage of live GBM2 cells after the combined treatments was slightly reduced with respect to the single treatments and the differences were not statistically significant. With regard to the percentage of live G144 cells after the combined treatments, we observed a weak decrease only in the 2 mM VPA plus 50 μM TMZ combination compared to the control culture and single treatments (13.4%) (Fig. 5C).

Discussion

VPA is an approved drug for the treatment of epileptic seizures, and is effective for bipolar disorders and migraine. VPA is also an HDACi (34,35) that has shown potent antitumor effects *in vitro* and *in vivo* in a variety of cancers, including GBM (36,37). This drug is a chromatin remodeling agent, consequently able to strongly modify gene expression (38-40),

inducing cancer cell differentiation, apoptosis and growth arrest. We previously demonstrated that short-term VPA treatment is able to modulate the cancer stem-like properties of GSCs by its pro-differentiating ability, overcoming their perpetual stem cell state. In particular, VPA induced a sort of initial differentiation process and a sharp growth arrest (25).

The risk of GBM patients to experience epileptic seizures is in the range of 30-50% (41) and the majority are treated with VPA (42-44). For this reason, it is interesting to evaluate *in vitro* the potential effects of long-term VPA administration on GSCs, in order to also consider the application of this drug for anticancer purposes.

We firstly investigated the effects of long-term VPA treatment on cell morphology. As expected, the VPA pro-differentiating power was more marked after long-term treatment, with respect to short-term administration. Five out of seven GSC lines showed consistent morphological changes after 14 and 30 days (from rolling spheres to attached star-shaped cells with neurite-like structures), while in the G166 and GBM7 cell lines a cytotoxic effect or apoptosis prevailed following terminal differentiation. Overall, these results demonstrated that VPA induced several morphological variations, as previously described in other tumors (45): we speculated that the HDAC inhibitory action of VPA could trigger reactivation of genes involved in differentiation, usually silenced in cancer stem cells (12,13). In fact, for example, after 30 days of treatment, we identified a potentially reactivating methylation change of OLIG1 and OLIG3 promoters, which are involved in oligodendrocyte and neuronal differentiation (46,47), from the methylated to the unmethylated status in G144 and GBM2 cells, respectively (Table III).

The expression analysis of stemness and differentiation markers highlighted the variability of VPA pro-differentiating ability among the GSC lines; this issue is representative of GBM heterogeneity. In our previous study, the immunofluorescence analysis, performed after 2 mM VPA for 72 h, revealed the co-expression of stemness and differentiation markers (25). Therefore, in the present study, we investigated the expression of such markers after 14 and 30 days of treatment to verify the real capability of GSCs to terminally differentiate in the consequence of a long pro-differentiating stimulus. In particular, the G144 and GBM04 cell lines after 30 days expressed only one differentiation marker, GFAP or MBP respectively, suggesting peculiar differentiation behaviors towards the astrocytic and oligodendrocytic lineages. Conversely, the G166 cell line had a particular response; after 14 days it showed a high percentage of cells positive for the differentiation markers, while after 30 days, these cells did not express any one marker. We thus speculated that the G166 cell line is subjected to a transdifferentiation process (48). The analysis also revealed that 3 out of 6 cell lines maintained the co-expression of stemness and differentiation markers (GBM2, G179 and GliNS2), suggesting an impairment of any gene involved in the regulation of differentiation. Alternatively, resistance mechanisms to VPA may arise in these cell lines and VPA-resistant cells selectively grow during drug treatment (49,50).

In the second instance, we analyzed the DNA methylation profiles of the GBM2 and G144 cell lines after 96 h and 30 days of VPA treatment to study and compare the epigenetic impacts of the two pharmacological regimens. Specifically, we selected two cell lines showing deep differences in VPA responsiveness in order to verify whether these features were consequent to a drug-specific activity on chromatin remodeling. Overall, VPA strongly modified the DNA methylation pattern of these cell lines. Our data confirmed the described VPA property to modify the methyloma (51,52); it determines not only alterations in histone acetylation, but also, indirectly, changes in DNA methylation.

After 96 h of VPA treatment, the percentages of genes that modified their methylation status in the two cell lines could directly reflect their initial VPA sensitivity: we observed that 28.47% of genes had an altered methylation status in the GBM2 cell line, which is VPA sensitive, while few genes (17.48%) were altered in the G144 cells, a resistant cell line. Furthermore, the different VPA susceptibilities of the two cell lines were also evidenced by the different trends in the methylation change after short-term treatment. In fact, in GBM2 cells, a high percentage of genes switched from an unmethylated to a methylated status (23.27%), while the majority of 'modified' genes moved in the opposite direction compared with the G144 cells (11.82%). The latter also maintained this issue after the long-term regimen. In contrast, the GBM2 modifying trend was completely revolutionized and became similar to the G144 behavior. The long-term exposure probably promotes in the GBM2 cell line the acquisition of resistance and the clonal expansion of resistant cells. This hypothesis was confirmed by the MTT assay in the GBMS cells folloiwng a 30-day VPA pretreatment, which showed a reduced sensitivity to 2 mM VPA compared to the naïve cells.

Moreover, we observed by means of GO analysis that the '*development and differentiation*' and '*neural differentiation and nervous system process*' functional categories were the most represented ones, together with '*metabolism*', after VPA treatments in both cell lines. These data further confirmed the VPA pro-differentiating ability on the GSC lines.

Nevertheless, it is important to explore the function and the role of genes influenced by these changes in regards to the methylation status after VPA treatment. Thus, we decided to investigate the methylation status of genes involved in critical signaling pathways important in GBM, such as MGMT, before and after short- and long-term treatments. MGMT promoter methylation is currently considered a predictive biomarker for the responsiveness to chemotherapy with alkylating agents such as TMZ; it is associated with an increased survival of GBM patients treated with TMZ (33,53). In the present study, we found that VPA treatment for 96 h induced a methylation shift in the MGMT promoter only in the GBM2 cell line, from an unmethylated to a methylated status, while the long-term treatment did not modify the status. Conversely, G144 cells did not show any modification of MGMT promoter methylation after both regimens (methylated).

In consequence of these data, we finally verified whether a short-term VPA pretreatment is able to sensitize GSCs to TMZ. Here, we used VPA pretreatment, as a chemo-sensitizer, to augment TMZ sensitivity of the GBM2, G144, GBM04 and G166 cell lines. Unexpectedly, VPA did not produce any relevant increase in the TMZ efficacy in all cell lines in terms of metabolic activity and percentage of live cells. These data clearly indicate that the DNA methylation status of the MGMT promoter and its epigenetic switch induced by VPA

are not the only determinant factors to predict and induce TMZ chemosensitivity of GSCs. A complex and extensive chemo-resistance landscape characterizes these cancer cells and other molecular mechanisms, such as the overexpression of p-glycoprotein (54), are involved in TMZ resistance (55-58).

The development of new therapeutic approaches that selectively target GSCs is mandatory to defeat GBM. Specifically, differentiation-inducing therapies are promising treatments to affect GSC self-renewal ability. Furthermore, restoring the physiological epigenetic pattern could be a useful strategy to fight GBM chemoresistance, yet it is unable to be the unique and definitive resolution to overcome this devastating feature. Short-term VPA treatment could combine in a single pharmacological regimen both of these two therapeutic strategies. Unfortunately, long-term VPA treatment induced the development of a resistant phenotype and, although the therapeutic properties and side effects of VPA are known since the 1970s, the question of whether a long-term antineoplastic treatment with this drug may be harmful for patients has never been exhaustively answered (59).

However, the overall data obtained warrant further investigation into the VPA effects on this fatal tumor in order to benefit patient prognosis. Additional *in vitro* and *in vivo* studies are necessary to clarify various key aspects of this pharmacological approach.

Acknowledgements

The authors gratefully acknowledge Professor Austin Smith (University of Cambridge, Cambridge, UK), ISE (Integrated Systems Engineering) and Dr Antonio Daga (IRCCS-AOU San Martino-IST, Genova, Italy) for kindly providing us with the cell lines used in this study. This study was supported by FAR 2011 n.12-1-62 and n.12-155 from the University of Milano-Bicocca (to L.D. and A.B., respectively) and by FIRB project from the Ministry of University and Scientific Research (to M.L.).

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