BET protein inhibition sensitizes glioblastoma cells to temozolomide treatment by attenuating MGMT expression

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Article

Keywords: BET inhibitors, epigenetics, glioblastoma, MGMT, synergistic combination therapy, resistance to therapy, DNA repair

Posted Date: August 1st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1832996/v1

Abstract

Bromodomain and extra-terminal tail (BET) proteins have been identified as potential epigenetic targets in cancer, including glioblastoma. These epigenetic modifiers link the histone code to gene transcription that can be disrupted with small molecule BET inhibitors (BETi). With the aim of developing rational combination treatments for glioblastoma, we analyzed BETi-induced differential gene expression in glioblastoma derived-spheres, and identified 6 distinct response patterns. To uncover emerging actionable vulnerabilities that can be targeted with a second drug, we extracted the 169 significantly disturbed DNA Damage Response genes and inspected their response pattern. The most prominent candidate with consistent downregulation, was the O-6-methylguanine-DNA methyltransferase (*MGMT*) gene, a known resistance factor for alkylating agent therapy in glioblastoma. BETi not only reduced *MGMT* expression in GBM cells, but also inhibited its induction, typically observed upon temozolomide treatment. To determine the potential clinical relevance, we evaluated the specificity of the effect on *MGMT* expression and MGMT mediated treatment resistance to temozolomide. BETi-mediated attenuation of *MGMT* expression was associated with reduction of BRD4- and Pol II-binding at the *MGMT* promoter. On the functional level, we demonstrated that ectopic expression of *MGMT* under an unrelated promoter was not affected by BETi, while under the same conditions, pharmacologic inhibition of MGMT restored the sensitivity to temozolomide, reflected in an increased level of g-H2AX, a proxy for DNA double-strand breaks. Importantly, expression of MSH6 and MSH2, which are required for sensitivity to unrepaired O6-methylGuanin-lesions, was only briefly affected by BETi. Taken together, the addition of BET-inhibitors to the current standard of care, comprising temozolomide treatment, may sensitize the 50% of patients whose glioblastoma exert an unmethylated *MGMT* promoter.

Introduction

New avenues have to be taken to improve the outcome of patients with glioblastoma (GBM) who have a median survival of less than 2 years. No major improvements have been made since 2005, when TMZ was introduced [1], despite numerous efforts with targeted agents or immunotherapies that have shown some efficacy in other solid tumors. The striking failures of these single agent therapies [2] have incited the exploration of rational combination therapies that synergistically induce tumor vulnerabilities, sensitizing the cells to treatment. The development of drugs targeting epigenetic modifiers, such as Bromodomain and extra-terminal tail (BET) proteins, holds new opportunities [3, 4]. Overexpression of proto-oncogenes in cancer has been associated with increased binding of BET proteins to their promoter region and respective active enhancer elements [5]. BET proteins are epigenetic readers that recognize acetylated lysines on histone tails and recruit proteins to the transcriptional complex, thereby connecting the histone code to gene transcription. This interaction can be targeted by small-molecule BET inhibitors (BETi) that specifically bind to the tandem domains of BET proteins and strip BET proteins from the chromatin, thereby inhibiting gene expression [5]. Treatment of cancer cells with BETi, such as the tool drug JQ1, disturb cancer relevant pathways that may uncover vulnerabilities targetable with a second drug as we and others have reported previously [6, 7].

Based on the fact that genotoxic treatments show some efficacy in GBM, such as combined chemo-radiotherapy with the alkylating agent temozolomide (TMZ), the current standard of care [8], we set out to uncover potential BETi-induced vulnerabilities in the DNA damage response (DDR). Previous work, reporting on opportunities to target DDR in cancer, provides a valuable resource to identify potentially synergistic drugs [9].

Here, we report on the potential of BETi to modulate the DDR in GBM cells, including the gene encoding the O6-methylguanin transferase (*MGMT)*. MGMT expression is a known resistance factor to TMZ treatment [10] as it repairs the most toxic lesion, O6-methylguanin, thereby blunting the treatment effect [11]. We demonstrate that BETi specifically down regulates endogenous *MGMT* expression in GBM cells, sensitizing them to TMZ therapy, without compromising the mismatch repair (MMR) system that is essential for sensitivity to alkylating agent therapy [12, 13]. These findings provide evidence that the addition of BETi in combination with TMZ may overcome treatment resistance in patients, whose GBM harbor an unmethylated *MGMT* promoter by directly inhibiting *MGMT* expression.

Material & Methods

Clustering procedure for trajectory analysis of RNA-seq data and time

The RNA-seq data reported in Gusyatiner et al. [6] served as input and was obtained in the GBM derived sphere line LN-2683GS upon treatment with 1mM JQ1 or DMSO over a time course of 48h with 3 biological replicates. Differential expression analysis used a model with full interaction between treatment and time (edgeR package). Bonferroni corrected p-value (FWER) from log-likelihood ratio test for generalized linear models (GLM) and averaged log2-counts per millions (CPM) by genes as measuring expression level were used to identify genes significantly associated with JQ1-treatment, yielding 4 712 genes (FWER < 0.1 and $\log_2(\text{CPM} + 1) > 1$) [6]. Afterwards, their temporal trajectories were classified in function of their response pattern to the treatment by a two-step procedure. The first step consisted of randomly selecting 500 genes to establish the optimal number of temporal patterns. The Fréchet distance [14, 15] was used to compare the trajectories of the genes and Principal coordinate analysis (PCO [16]) on a pairwise distance matrix was performed to reduce the data noise. The three first components were used to partition the 500 selected genes by K-means. Several partitions forming a cascade from a small (k = 2) to a large number (k = 10) of groups were created. The Calinski-Harabasz (*Calinski's*) criterion [17] was used to define the optimal number of clusters. Averaged profiles (reference profiles) were computed for each cluster. In the second step, the *re-clustering* of all genes was provided by the computation of the Fréchet distance between each averaged profile and each gene. The corresponding cluster was attributed according to the minimal distance to the reference/averaged profiles. The procedure is illustrated in the Supplementary Fig. 1. Enrichment analyses based on hypergeometric test, associated with Bonferroni correction of the p-value for multiple testing, were performed by cluster for DDR pathways defined by

Pearl et al. [9]. All analyses (e.g. “cascade” K-means and Fréchet distance), differential expression analysis, enrichment analysis and graphical representation related to longitudinal clustering were performed in R (URL http://www.R-project.org) [18] and the R packages vegan, longitudinalData, edgeR, clusterProfiler and ade4.

Cell Culture

Patient-derived GBM sphere (GS) lines LN-2683GS and LN-4372GS, and the adherent cell lines LN-18, LN-229, LN-340, and LN-382 were established and molecularly characterized in our laboratory [19, 20, 21] according to institutional directives, approved by the Ethics Committee of the Canton de Vaud (CER-VD, protocol F25/99). T98G was obtained from ATCC. All lines were regularly tested mycoplasma-free (MycoAlert Kit Lonza, Cat. LT07-418), and authenticated in 2022 by STR fingerprinting at the Forensic Genetics Unit of the University Center of Legal Medicine, Lausanne and Geneva [22]. Adherent cell lines were grown in Dulbecco’s modified Eagle medium (DMEM, Glutamax Gibco™, Life Technologies, Cat. 61965-026) with 5% Fetal Bovine Serum (Hyclone). GS lines were maintained under neural stem cell culture conditions in DMEM/F12 (Life Technologies, Cat. 31331-028) containing B27 supplement and growth factors, as previously described [23]. The transduced cell lines were continuously maintained under respective selection.

Molecular Cloning

LN-229MGMT^{ind}_C12 was derived from LN-229 upon transduction with a Tet-ON inducible system for *MGMT* as previously described [24], and maintained under Blasticidin (Thermofisher, R21001) selection at 10 [µg/ml]. *MGMT* was induced with doxycycline (Dox, Sigma Aldrich, D9891-1G) at 100 [ng/ml].

Production and delivery of lentiviral particles

For the production of LN-340shMSH6#1^{ind}_C8 and LN-340shMSH6#2^{ind}, and the respective LN-340shCTRL^{ind}, we obtained TRIPZ Dox-inducible Lentiviral shRNA targeting h*MSH6* as E. coli glycerol stock cultures (Horizon Discovery Ltd. Clone Id: V2THS_258239 & Clone Id: V2THS_82749), and the non-targeting TRIPZ shRNA designed with minimal homology to known mammalian genes (Horizon Discovery Ltd. Catalog ID:RHS4743), respectively. The replication-incompetent lentiviral particles were produced according to the manufacturer’s protocol (Dharmacon™ Trans-Lentiviral packaging kits, Cat. TLP5912). After an incubation of 16h the transfection mix was removed and 14ml DMEM 5% FBS were added. After 48h, the virus containing supernatant was harvested, passed through a 0.22µM filter and complemented with 10µg/ml Polybrene (Sigma-Aldrich, TR-1003-G), and added to the target cells for 24h. The medium was replaced with fresh DMEM 5% FBS, and after 24h incubation cells were subjected to selection by adding 5µg/ml Puromycin (Catalog Number P8833, Sigma-Aldrich). Expression of the shRNAs were induced by treatment with Dox at 500 [ng/ml].

RNA Extraction and qRT-PCR

Total RNA isolation and qRT-PCR were performed as described previously [23] using primers compiled in Supplementary Table S1. The expression levels were normalized to *GAPDH*.

Protein Extraction and Western Blot

Cells were collected by centrifugation for GS-lines and by scraping for adherent cells. Westerns were done as described [23] and probed with respective antibodies: anti-α-Tubulin (Sigma, T5168, 1:10 000), anti- β-Actin Bioconcept, 8H10D10. 1:10 000), anti-MGMT (R&D systems, AF3794-SP, 1:4 000), anti-MSH6 (Cell Signaling, #5424S, 1:4 000), anti-MSH2 (Cell Signaling, #2017S, 1:4 000). Membranes were washed 5min x3 in TBS-T at RT, followed by incubation at RT for 1h with the following secondary antibodies (according to primary antibody specifics): anti-rabbit (Promega, W4011, 1:5 000), anti-mouse (Thermofisher, 31430, 1:5 000), anti-goat (Thermofisher, 31402, 1:5 000).

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP-qPCR was largely performed following the iDeal ChIP-seq kit for Transcription Factors (Diagenode, cat. C01010170). Briefly, proteins from 20M T98G cells were cross-linked to DNA in a 15cm petri dish by adding fresh Paraformaldehyde (PFA) (Lucerna, cat. 15714) to a final concentration of 1% for 15 minutes at RT. Fixation was quenched with Glycine for 5 minutes at RT. Fixed cells were then washed with cold PBS, and nuclei were extracted via cell membrane lysis. Using 1.5ml Bioruptor® Microtubes with caps (Diagenode, cat. C30010016), chromatin was sonicated at a density of 1.5M cells/ 100µl complete Shearing buffer iS1b with Bioruptor Pico (Diagenode, Serial Number P-181503) for 12 cycles (30s “ON”, 30s “OFF”) in order to obtain fragments between 100bp and 600bps. The chromatin was briefly centrifuged for 15s, and subsequently, the supernatant was centrifuged for 10min at 4°C at 16 000g. An aliquot of 50µl of the supernatant was kept for shearing assessment, and the sample was stored at -80°C for subsequent immunoprecipitation. The chromatin was decross-linked with 1µl of proteinase K 20mg/ml (Life Technologies, cat. AM2546) overnight at 65°C. DNA was extracted by adding one volume of phenol/chloroform/isoamyl alcohol (25/24/1) to the sample and mixed vigorously for 30s. Samples were centrifuged at RT for 5 minutes at 16 000g. The aqueous phase was carefully removed and transferred to a new tube. 2.5 volumes of ice-cold 100% ethanol, 0.5 volumes of sodium acetate and 1µl of GlycoBlue™ Coprecipitant (Invitrogen, cat. AM9515) were added to the samples and incubated at -80°C for 2h. Samples were centrifuged for 30 minutes at 4°C at 16 000g. The supernatant was carefully removed and the DNA pellet was washed with 300µl with 70% ethanol. Samples were centrifuged for 10 minutes at 4°C at 16 000g. The supernatant was removed, and the DNA pellet was air-dried. The pellet was dissolved in 30µl TE elution buffer and DNA was quantified with the Qubit™ dsDNA HS Assay Kit (Thermofisher, cat. Q32851). 300-600ng of DNA were analyzed on a 1.5% agarose gel to determine fragment sizes. Samples with fragments between 100bp and 600bps were used for subsequent magnetic immunoprecipitation. Frozen sheared chromatin pellets were incubated overnight at 4°C under constant rotation with the corresponding ChIP reaction mix. Each ChIP reaction mix corresponds to 1 immunoprecipitation of interest: anti-BRD4 (Bethyl Laboratories Inc., A301-985A50, 2µg/IP), anti-Pol II (Cell Signalling Technology, CST14958, 1µg/IP), anti-CTCF (Diagenode Kit, 2µg/IP), anti-IgG (Diagenode Kit, 1µg/IP). Subsequently, immunoprecipitated DNA was eluted, decross-linked, and purified according to

protocol. DNA was quantified with Qubit™ dsDNA HS Assay Kit for quality control purposes only. Shearing assessment was performed by adding 50µl TE elution and RNAse A (Thermo Fisher Scientific, cat. EN0531) for 1h at 55°C. Immuno-precipitated DNA and corresponding INPUT were analyzed by qPCR analysis with primers of interest [19, 25] (Supplementary Table S1). Finally, the relative amount of immune-precipitated DNA compared to INPUT DNA (% of recovery) was calculated.

Immunofluorescence analysis for γ-H2AX

Target cells were seeded on open µ-Slides (chambered coverslip) with 8 wells (Vitaris, 80826) (between 2 500-3 000 cells/well). Cells were treated with JQ1 (APExBIO, No. A1910) and Dox for 5 days prior TMZ (Sigma-Aldrich, T2577) and O6-Benzylguanine (O6BG; Sigma-Aldrich, 19916-73-5) treatments. Subsequently, cells were incubated for 48h. Cells were fixed with 4% PFA (Lifetechnologies, cat. 28908) for 15 minutes at RT, followed by permeabilization with 0.3% Triton-X for 15 minutes at RT. Cells were blocked at RT for 1 hours in blocking buffer (5% Donkey Serum, 0.5% BSA, 0.3% Triton-x-100). Cells were incubated overnight at 4°C with γ-H2AX AB (Cell signaling, 2577, 1:800 in blocking buffer). Secondary antibody Alex Fluor 647 (Thermofisher, A31573, 1:300 in blocking buffer) was added to cells for 1 hour at room temperature. Image acquisition was performed with Zeiss LSM 880 Airyscan at 40x magnification with oil. Fifteen images per condition were acquired and further analyzed with the Cell Profiler software Version 3.1.9. γ-H2AX was quantified as integrated intensity using an optimized images acquisition software pipeline.

Cell viability analysis

Cells were seeded into 48-well plates and treated for 5 days with JQ1, followed by 3 shots of TMZ at an interval of 6h, using a clinically relevant dose of 100µM, according to a previously reported schedule [26], and 1 shot of 10µM O6BG. After 96h, cells were stained with the CyQUANT Direct Cell Proliferation Assay Kit (Thermoscientific, cat. C35011). Following 1h incubation, cells were scanned, and fluorescence was measured with the SpectraMax® M Series Multi-Mode Microplate Reader. For the experiments depleting MSH6 with the inducible sh-M*SH6*-constructs, cells were pretreated for 5 days with Dox at 500 [ng/ml], followed by the same treatment scheme described above.

Cell cycle analysis

LN-340 cells were seeded (0.25M/10cm petri dish per condition). Following 5 days pretreatment with JQ1 or DMSO, cells were treated with TMZ and O6BG. After 48h cells were washed with PBS and fixed with ice-cold 70% ethanol overnight at 4°C. Subsequently, cell pellets were washed with ice-cold PBS and treated with 1ml Propidium Iodide solution (20µg/ml final concentration) (Sigma-Aldrich, P4864-10ml) and RNAse A. Following at least 4 hours incubation at 4°C (protected from light), cells were filtered through 5 ml Round Bottom Polystyrene Test Tubes, with Cell Strainer Snap Cap (Falcon®). Stained and filtered cells were immediately processed with the Gallios II Beckman Coulter (Flow Cytometry Facility - University of Lausanne). Cell cycle distribution was analyzed with the FlowJo software.

Statistical analysis

Statistical analysis of the experiments was executed using GraphPad Prism 9 Software. The responses to the treatment over the time course (48h) were tested by two-way ANOVA, including the interaction term between time and treatment and using the Geisser-Greenhouse correction for variance heterogeneity. The analyses were completed by Dunnett's multiple comparisons test as post hoc tests. The differences of means among the treatments, JQ1, TMZ and O6BG were tested by three-way ANOVA including the interactions between the treatments (first and second order interaction effects). Additive mixed model with interactions between treatments was used when the groups were unbalanced. The Tukey's multiple comparisons tests were used as post hoc tests. Two-group comparison tests were performed by two-tailed ratio paired t-test including correction for variance heterogeneity. The comparison of several groups was provided by one-way ANOVA completed by Dunnett T3 multiple comparisons tests. Statistical significance is defined according to p-values, indicated by the asterisk symbol (*) in the Figures: (*) $p < 0.05$, (**) $p < 0.01$, (***) means $p < 0.001$. (****) means $p < 0.0001$. Data are shown as mean values. Error bars represent Standard Deviation (SD), unless indicated otherwise.

Results

BET protein inhibition disturbs DNA damage response signaling pathways in glioblastoma

In an effort to leverage clinically relevant pathways disturbed by BETi for druggable targets, we analyzed differential gene expression data obtained in a GBM-derived sphere line, LN-2683GS, treated with the tool drug JQ1. The cells treated with 1μM JQ1 over a time course of 48h underwent extensive transcriptome changes as reported [6]. Significant association with JQ1-treatment was observed for 4 712 genes (adjusted p-value by Bonferroni correction, < 0.1 and $\log_2(CPM + 1) > 1$), whereof 169 were annotated as DDR genes as defined by Pearl et al. [9]. To identify JQ1-response patterns we determined the optimal number of gene clusters using K-means and obtained 6 clusters, as visualized in a *Calinski's criterion* graphic and a corresponding heatmap (Supplementary Fig. 1). The comparison of the original K-means and the *re-clustering* method exhibited a similar classification for the 500 training datasets. The percentage of good classification was equal to 95% with a kappa value of 0.94. Two clusters showed JQ1-induced gene expression over time (clusters 1 and 2), and two clusters displayed consistent downregulation (clusters 4 and 5), while two clusters displayed transient down- (cluster 3) or upregulation of expression (cluster 6), respectively. The 169 DDR genes (Supplementary Table 1) were distributed among all six JQ1-response patterns (cluster 1, 23 genes; cluster 2, 35 genes; cluster 3, 51 genes; cluster 4, 16 genes; cluster 5, 34 genes; cluster 6 and 10 genes) as visualized in a heatmap (Fig. 1). Enrichment analyses of DDR pathways by cluster, showed only one significant association between cluster 3 and MMR genes (adjusted p-value by Bonferroni correction < 0.001). The annotated list of all 169 retained DDR genes, their expression levels by treatment and time point, cluster affiliation, Fréchet distance, and pathway information, based on Pearl et al. [9], is available in Supplementary Table S2.

A direct effect of BETi may be expected among rapidly downregulated genes by stripping BET proteins from their binding sites. Inspection of the clusters 4 and 5 revealed *MGMT* among the consistently downregulated genes, following expression pattern 4, as visualized in Fig. 1. The consistent downregulation by JQ1, identified *MGMT* as a prime target which opens the opportunity to sensitize GBM with an unmethylated *MGMT* promoter to TMZ. Patients with *MGMT* unmethylated GBM basically show no benefit from TMZ therapy [10]. Given the fundamental role of a fully functional MMR system for sensitivity of cells to O6-methylguanin lesions in MGMT-deficient cells, we paid attention to the modulation of key genes involved in the MMR system. As mentioned above, the MMR genes were significantly enriched in cluster 3, and comprised among others *MSH6*, *MSH2* and *MLH1* that have been associated with acquired treatment resistance to TMZ in recurrent gliomas when mutated or silenced otherwise [27]. Even though the expression of *MSH6*, *MSH2* and *MLH1* was transiently downregulated upon JQ1 treatment at early time points (6h and 12h), it was restored to baseline 24h after initiation of JQ1-treatment, suggesting that BETi does not compromise MMR in this sphere line (Fig. 1, Supplemental Table S2).

BET protein inhibition reduces MGMT expression and prohibits its induction upon temozolomide treatment

Next, we monitored downregulation of *MGMT* in several GBM sphere and cell lines with endogenous MGMT expression on the RNA and protein level in response to treatment with JQ1, TMZ, or both. The JQ1 concentrations were adapted to the sensitivity of the individual cell and sphere lines (0.1-1µM), and TMZ was used at a clinically relevant dose of 100µM [26, 28]. The *MGMT* expression was significantly affected by treatment over the time course of 48h for the cell lines LN-340, LN-2683GS and LN4372GS ($p = 0.0051$, $p < 0.0001$ and $p = 0.0095$ respectively, Fig. 2A, Supplementary Table S3).

The results confirmed rapid downregulation of *MGMT* expression upon JQ1 treatment as measured over a time course of 48h (Fig. 2A). Moreover, *MGMT*-induction generally observed upon TMZ treatment alone, was prohibited by JQ1, and the expression levels were kept significantly below the baseline over the time course. This behavior also translated to the protein level, although with a delay (Fig. 2B). Substantial MGMT depletion with JQ1 treatment alone was observed after 72h of treatment, with a more pronounced effect after 120h. TMZ treatment alone also showed a decrease of MGMT protein after 24h, compatible with the suicide reaction of MGMT after transfer of the methyl group that leads to ubiquitination and proteasome-mediated degradation, requiring *de novo* synthesis [29]. These results were consistent across all the different GBM cell lines and sphere lines tested (Fig. 2). In the following experiments testing TMZ-related effects, cells were pretreated 120h with JQ1 (or DMSO, control) to allow for JQ1-mediated depletion of MGMT.

BET protein inhibition reduces BRD4 occupancy at the MGMT promoter region

To investigate whether *MGMT* expression is directly regulated by BRD4, we performed chromatin immuno precipitation followed by quantitative PCR (ChIP-qPCR) analysis for BRD4 binding in the *MGMT* promoter

region. T98G cells that exert relatively high levels of endogenous MGMT on the RNA and the protein level (Supplemental Figure S2), were treated for 2h with JQ1 [1μM], and relative BRD4 occupancy was determined by ChIP-qPCR using previously described primer sets [19, 25]. The two regions interogated are located within the CpG island of the promoter and the first exon and the analysis demonstrated a significant difference in BRD4 occupancy at both *MGMT* promoter regions tested (both locations, F2, F3, $p < 0.5$, two-tailed ratio paired t-test, with correction for variance heterogeneity, Fig. 3). The JQ1-associated decrease in BRD4 binding was supportive of a direct regulatory effect on *MGMT* expression. Furthermore, we also evaluated associated changes of RNA polymerase II (Pol II) occupancy, a marker for active transcription. In line with the observed decrease in *MGMT* expression, we detected a significant difference in Pol II occupancy (F2, $p < 0.0001$; F3, $p = 0.0377$), with decreased binding at both *MGMT* promoter regions tested, suggesting attenuation of the *MGMT* transcription process.

Overall, our results support that *MGMT* transcription is attenuated upon JQ1 treatment due to BRD4 depletion at the promoter region.

BET protein inhibition modulates TMZ-induced DNA damage in an MGMT-dependent manner

In light of the postulated direct downregulation of *MGMT* upon BETi in GBM lines, we investigated the role of BETi in modulating the TMZ-induced DNA damage response. We treated the MGMT expressing GBM cell line LN-340 with TMZ alone or in combination with JQ1 [0.1μM], and monitored γ-H2AX levels, a marker for DNA double-strand breaks (DSBs)[30]. The formation of γ-H2AX foci is among the first steps that initiates the recruitment of DNA repair proteins. Cells were pretreated for 120h with JQ1 or DMSO to allow for JQ1-mediated depletion of the MGMT protein, and γ-H2AX was measured 48h after treatment with TMZ, quantifying immunofluorescence determined by confocal microscopy. In absence of JQ1, we observed that LN-340 showed no difference in γ-H2AX levels upon TMZ treatment alone, as compared to DMSO control, while a significant difference was observed upon treatment with the MGMT-specific pharmacologic inhibitor O^6-benzylguanine (O6BG) (adjusted p-value < 0.01, Dunnett T3 multiple comparisons tests following one-way ANOVA), restoring sensitivity to TMZ (Fig. 4A, Supplementary Table S4). Indeed, LN-340 cells treated with O6BG in combination with TMZ showed a robust increase in the rate of DSBs as compared to TMZ treatment alone. This is in line with the MGMT-mediated resistance in this cell line. In contrast, when treating the cells in combination with JQ1, a significant difference (adjusted $p < 0.01$) of γ-H2AX levels was observed upon TMZ treatment, as compared to TMZ alone, indicating that depletion of MGMT upon JQ1 treatment led to an increase in DSB formation following TMZ treatment (Fig. 4A). However, no interaction was observed between O6BG and JQ1, hence the addition of O6BG in JQ1 treated cells did not further sensitize cells to TMZ, suggesting that MGMT protein levels were already low from JQ1 treatment.

To further support our findings of a MGMT-dependent effect of BETi and to confirm the key role of MGMT in conferring resistance to TMZ in these GBM models, we used a Dox-inducible Tet-On system for *MGMT* in the GBM line LN-229. LN-229 does not express endogenous *MGMT*, due to promoter methylation [19],

and is known to be highly sensitive to TMZ treatment [31, 32, 33]. Induction of *MGMT* with Dox at 100ng/mL yielded MGMT protein levels comparable to MGMT-proficient cell lines (Supplementary Figure S3). We observed that LN-229MGMT^{ind}_C12 cells acquired a strong TMZ resistance phenotype upon MGMT induction (Fig. 4B, Supplementary Table S4). Expectedly, the use of the pharmacologic MGMT inhibitor (MGMTi) O6BG had a significant effect on γ-H2AX levels ($p < 0.01$), restoring TMZ sensitivity, reflected by increased DSBs. In contrast, no effect (adjusted $p > 0.5$) was observed with JQ1 treatment, hence, not sensitizing Dox-treated LN-229MGMT^{ind}_C12 cells to TMZ treatment in this context. This suggested that JQ1 was not able to interfere with ectopic *MGMT* expression, which is controlled by the Dox-inducible Tet-On promoter. Therefore, BETi did not influence ectopic MGMT expression or sensitivity to TMZ induced DSBs, whereas pharmacologic depletion of MGMT by O6BG treatment reversed the acquired TMZ resistance.

Altogether, our data have shown that JQ1 induces more DNA DSBs in TMZ treated GBM cells expressing endogenous *MGMT* as compared to TMZ alone.

BET inhibition attenuates glioblastoma viability upon TMZ treatment

The observed increase of DSBs suggested that treatment with JQ1 may reduce the viability of GBM cell lines with endogenous *MGMT* expression, in response to TMZ treatment. We treated LN-340 and T98G with JQ1 or TMZ alone or combined with JQ1, while both single agent treatments had a significant effect on cell viability in both cell lines (for both lines and both treatments, $p < 0.0001$, fixed effect from mixed model with interactions between treatments, Supplementary Table S5), we observed that the addition of JQ1 significantly sensitized cells to TMZ treatment (Fig. 5A), reflected in the significant interaction effect between JQ1 and TMZ ($P = 0.006$ and $p < 0.0001$, respectively). The specificity of the MGMT-mediated effect of JQ1-treatment was further tested using the pharmacologic inhibitor O6BG in the experiments, with or without JQ1, respectively. The addition of O6BG on its own had no effect on cell viability, whereas it sensitized the cells in combination with TMZ. However, O6BG did not further sensitize the cells to TMZ in presence of JQ1 (no significant interaction between O6BG and JQ1, $p = 0.2746$. In accordance, cell cycle analysis revealed that TMZ or JQ1 treatment alone did not alter the cell cycle profile compared to untreated cells. However, combinatorial treatment of JQ1 and TMZ increased S phase and G2/M phase cell cycle arrest in GBM cells as compared to controls (Fig. 5B). And the addition of O6BG alone, or in combination with JQ1 had no effect on the cell cycle, while in combination with TMZ it increased the proportion of cells in S and G2/M phase (Fig. 5B).

BET protein inhibition does not compromise the MMR system in glioblastoma

As aforementioned, a compromised MMR system generates resistance to TMZ treatment as it is essential for the cytotoxic effect O6meG lesions that remain unrepaired in absence of MGMT. Our differential gene expression analysis in LN-2683GS had shown that key MMR genes, were only transiently modulated by

JQ1 treatment and their expression was restored after 12h (Fig. 1). Treatment with JQ1 alone or in combination with TMZ did not significantly alter RNA or protein expression levels of *MSH6* and *MSH2* in LN-340 (Fig. 6A, 6B, Supplementary Table S6) or T98G (data not shown).

To determine the effect of a non-functional MMR pathway in conferring TMZ resistance in our experimental model, we transduced LN-340 with a Dox-inducible Tet-On shRNA against *MSH6* using 2 distinct sequences (Fig. 6D). Depletion of *MSH6* confirmed that TMZ resistance was independent of MGMT in this scenario, as pharmacologic inhibition of endogenous MGMT with O6BG was ineffective in restoring sensitivity to TMZ as measured by cell viability (adjusted p-values = 0.0022 and 0.0002 for the 2 sequences, three-way ANOVA including interaction between the treatments, TMZ, OB6G and JQ1, corrected for multiple testing by Tukey's test). On the contrary, cells transduced with the non-targeting Dox-inducible Tet-On shRNA did not change behavior upon doxycycline exposure and remained sensitized to TMZ upon O6BG treatment (Fig. 6C, Supplementary Table S7) (adjusted p-value = 0.127).

Finally, we demonstrated that the use of BETi in GBM cells does not negatively impact the MMR system that would result an undesirable TMZ resistance.

Discussion

Changes of the epigenetic landscape in tumors contribute to all hallmarks of cancer and have been recognized as promising targets for treatment. Encouraging preclinical results have been obtained with small molecule inhibitors targeting BET proteins that are epigenetic modifiers and have been associated with overexpression of cancer relevant pathways [5]. While some single agent efficacy has been observed in preclinical GBM models [34, 35, 36] the challenge is to find specific synergistic combination therapies [6, 7, 37, 38]. In the present study we aimed at identifying rational combination therapies by leveraging potential vulnerabilities emerging upon disturbing GBM cells with BETi in the context of DDR. The significantly modulated genes displayed six main gene expression response patterns to JQ1 treatment. This revealed *MGMT* as a consistently downregulated gene, rendering it a top candidate, due to its pivotal clinical relevance of conferring resistance to TMZ in GBM patients. This finding provides a potential novel therapeutic strategy to inhibit *MGMT* expression and sensitize patients with an unmethylated *MGMT* promoter to TMZ therapy, who normally have no benefit from such treatment [10].

Indeed, in combining BETi with TMZ, we demonstrated enhanced DNA DSB levels and reduced cell viability compared to single agent TMZ in GBM cells expressing endogenous MGMT. However, in light of the large number of the BETi-disturbed genes that contribute to the efficacy of the drug as anticancer agent through other mechanisms [39, 40], we sought to demonstrate the specificity of BETi to downregulate *MGMT* expression and prohibit its induction upon TMZ treatment. We observed reduction of BRD4 coverage at the promoter of *MGMT*, in concordance with a reduction of Pol II binding, and the associated decrease in *MGMT* expression levels. Moreover, ectopic expression of *MGMT* from an artificial promoter could not be attenuated by JQ1, while pharmacologic MGMT depletion restored sensitivity to TMZ. Taken together, this provides supportive evidence for a causal relationship of BETi in the depletion of *MGMT*.

At the same time we provided evidence that the MMR pathway is not affected by BETi which is highly relevant, as it would induce unwanted resistance to alkylating agents even in absence of MGMT. Inactivation of MMR is an important resistance mechanism rendering treatment with alkylating agents ineffective [41, 42]. Therefore, it is of note that the BETi response pattern of the MMR pathway exhibited only transient downregulation which we confirmed in several cell lines, exemplified for the key members *MSH6* and *MSH2*. The sensitivity of our *in vitro* model to attenuation of MMR, was illustrated by depleting MSH6 from the cells. Resistance to TMZ was observed, even upon pharmacologic inactivation of MGMT as expected, and reported by others [13, 27].

Small molecule BET inhibitors have similar features in *in vitro* models, allowing for mechanistic evaluations of the mode of action using a tool drug, like JQ1 as described in this study. And we have previously reported that BETi-specific responses can be measured in orthotopic GBM xenografts [6]. However, a major concern for combination therapies are overlapping toxicity. Previous attempts to specifically target MGMT comprised depletion of MGMT using pharmacologic inhibitors, such as pseudosubstrates like O6BG. However, they have failed in the clinic due to overlapping toxicity with TMZ and other alkylating agents [43, 44], Hence, the potential of the synergistic effect between the BETi-MGMT-mediated efficacy and cytotoxicity conferred through modulation of other, MGMT-unrelated cancer relevant pathways will be of importance for successful therapy without overt toxicity.

Clinical trials are currently ongoing for recurrent and progressive glioma and GBM, including a window of opportunity part for the evaluation of the drug in the patients' tumors (clinicaltrials.gov, CC-90010, NCT04047303). Furthermore, this drug is also under clinical investigation in combination with TMZ, with or without radiation therapy in patients with newly diagnosed glioblastoma (NCT04324840). These trials will provide information on the synergistic effect between BETi and TMZ in the context of the MGMT status.

Our study using a tool drug has provided novel mechanistic insights in support of the rational combination of BETi with TMZ in GBM. Investigation of BETi response signatures provides useful insights for the design of future clinical studies investigating novel combination therapies.

Declarations

Funding

This work was supported by the Swiss National Science Foundation (SNSF 31003A_182821) and the Swiss Cancer Research Foundation (KFS-4461-02-2018).

Acknowledgements

We thank the collaborators of the FACS and the Core Imaging Facility (CIF) of the University of Lausanne for excellent technical support.

Conflict of interest statement:

M.E.H. and O.G. received a research grant from Orion Pharma.

Authorship Contribution Statement.

A.T. and M.E.H. designed the study. A.T. performed the experiments, analyzed and interpreted the data, and wrote the manuscript with M.E.H. O.G. contributed the original experimental data and respective RNA-seq data analysis. P.B. provided the classification into treatment response patterns. M.B., R.L., and D.C. performed experiments and analyzed the data. M.M. provided study material. M.E.H. directed the study. All authors contributed to manuscript writing.

Ethics Statement:

The glioblastoma derived cell and sphere lines were established in our lab according to institutional directives, approved by the Ethics Committee of the Canton de Vaud, Lausanne, Switzerand (CER-VD, protocol F25/99; Brain tumor biobank, BB_031BBLGBT).

Funding Statement:

This work was supported by the Swiss National Science Foundation (SNSF 31003A_182821) and the Swiss Cancer Research Foundation (KFS-4461-02-2018).

Data Availability Statement

Due to patient privacy protection, the raw sequencing data will be made available upon request.

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Figures

Figure 1

Response patterns of DDR genes in LN-2683GS cells treated with 1 μM (+)-JQ1 or DMSO for 4, 12, 24, and 48h**.** The heatmap (**A**) illustrates the normalized expression of the DDR genes upon JQ1 treatment over a time course of 48h. The gene trajectories were classified into the 6 clusters of response to the treatment defined by the two-step procedure based on Fréchet distances. (B) The 6 averaged expression patterns of DDR genes are displayed upon clustering over the time course of treatment. (C) The expression profile of the *MGMT* gene, classified into cluster 4, is represented in function of the time and stratified by tretament (JQ1 in blue and DMSO in red). (D) Similarly, the expression profiles of MMR genes *MSH6*, *MSH2* and *MLH1* upon JQ1 (red) or DMSO (blue) treatment are shown over the time course of 48h, classified into cluster 3. The circles and squares correspond to the biological replicates and the means of the 3 independent biological replicates for each time point respectively. The standard deviations (SD) are represented by the vertical lines.

Figure 2

BET protein inhibition reduces MGMT expression in GBM. (A) qRT-PCR analysis of relative *MGMT* gene expression at 6, 12, 24 and 48h after treatment. Each data point represents an independent experiment. Data were normalized to the respective DMSO treatment for each time point (baseline). Adjusted p-values (p) were determined by Dunnett's multiple comparisons test following two-way ANOVA using Geisser-Greenhouse correction for variance heterogeneity and including interaction between time and treatment. Error bars are SD. * ($p \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$). (B) Protein expression analysis of MGMT, β-Actin and α-Tubulin by western blot, 24, 48, 72 and 120h after treatment. One of 3 representative biological replicates is shown, full length Westerns are available in Supplemental Material.

Figure 3

BRD4 occupancy at the *MGMT* promoter region is reduced upon JQ1 treatment. T98G cells were treated for 2h with or without [1μM] JQ1. ChIP-qPCR for BRD4 and Pol II occupancy in the promoter region of *MGMT* interrogated at both F2 and F3 regions are represented as enrichment (%input). The experiment includes 7 independent experiments. P-values were determined by two-tailed ratio paired t-test including correction for variance heterogeneity. * ($p<0.05$), **** ($p< P \leq 0.0001$).

Figure 4

JQ1 modulates repair of TMZ-induced DNA damage. (A) Mean g-H2AX integrated intensity analysis was performed on LN-340 cells using immunofluorescence (IF). Cells were treated with JQ1 [250nM] and for 5 days. On day 5, cells were treated with O6BG [10μM] together with TMZ [100μM]. Two additional TMZ treatments were given every 6h, for a total of 3 TMZ treatments on day 5. End-point was set at 48h after TMZ treatments. Data represent the mean from 3 independent biological experiments. The adjusted p-values were provided by Dunnett T3 multiple comparisons tests following one-way ANOVA. * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($P \leq 0.001$). Error bars are SD. (B) Cells of GBM line LN-229MGMT^{ind}_C12 were treated according to the same schedule as in (A), but at a lower JQ1 concentration [100nM]. The experiments

were performed in absence and presence of doxycycline [100ng/ml] (Dox), respectively. Dox treatment induces ectopic expression of *MGMT* under the control of the Tet-ON promoter.

Figure 5

BETi sensitizes GBM to TMZ. (A) Cell viability was assessed in LN-340, and T98G. Cells were treated with JQ1 for 5 days at [250nM] and [100nM], respectively. On day 5, cells were treated with O6BG [10μM] alone, or together with TMZ [100μM]. Two additional TMZ treatments were given every 6h, for a total of 3 TMZ treatments on day 5. End-point was set at 96h after TMZ treatments. Data represent mean of 4 biological replicates Adjusted p-values were determined by Tukey's multiple comparisons tests following additive mixed model including interaction between the treatments (TMZ, OB6G and JQ1). Error bars are SD. * ($p \leq 0.05$), ** ($p \leq 0.01$), **** ($p < P \leq 0.0001$). (B) Analysis of the effect of JQ1 on the cell cycle profile of TMZ treated LN-340 cells was performed by FACS analysis using DAPI staining and subsequent flow cytometry cell cycle analysis using FloJo. Cells were treated as in (A) and analyzed 48h after TMZ treatment. Data represent 1 representative biological replicate.

Figure 6

BETi does not impair the MMR pathway in GBM. (A) qRT-PCR analysis of relative *MSH6* and *MSH2* gene expression at 6, 12, 24 and 48 hours after treatment. Each data point represents an independent biological replicate. Data were normalized to the respective DMSO treatment for each time point (baseline). Adjusted p-values (p) were determined by Dunnett's multiple comparisons test following two-way ANOVA using Geisser-Greenhouse correction for variance heterogeneity and including interaction between time and treatment. Error bars are SD. (B) Protein expression analysis of MSH6, MSH2 and β-Actin by WB at 24, 48, 72 and 120 hours after treatment. One of 3 biological replicates is shown. (C) Cell viability was performed on LN-340shMSH6#1^{ind}_C8, LN-340shMSH6#2^{ind} and LN-340shCTRL^{ind}. Cells were treated with Dox [500 ng/ml] for 5 days to induce expression of the respective shRNAs. On day 5, cells were treated with O6BG [10 μM] together with TMZ [100μM]. 2 additional TMZ treatments were given every 6 hours, for a total of 3 TMZ treatments on day 5. End-point was set at 96 hours after TMZ treatments. Data points represent independent biological replicates. Adjusted p-values were determined by Tukey's multiple comparisons tests following three-way ANOVA including interaction between the treatments (TMZ, OB6G and JQ1). (D) WB knockdown validation for the dox-inducible shRNAs systems. Error bars are SD. * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($P \leq 0.001$). Full length Westerns are available in Supplemental Material.

Supplementary Files

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