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Anticancer drug candidate CBL0137, which inhibits histone chaperone FACT, is efficacious in preclinical orthotopic models of temozolomide-responsive and -resistant glioblastoma

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Background. The survival rate for patients with glioblastoma (GBM) remains dismal. New therapies targeting molecular pathways dysregulated in GBM are needed. One such clinical-stage drug candidate, CBL0137, is a curaxin, small molecules which simultaneously downregulate nuclear factor-kappaB (NF-κB) and activate p53 by inactivating the chromatin remodeling complex, Facilitates Chromatin Transcription (FACT).

Methods. We used publicly available databases to establish levels of FACT subunit expression in GBM. In vitro, we evaluated the toxicity and effect of CBL0137 on FACT, p53, and NF-κB on U87MG and A1207 human GBM cells. In vivo, we implanted the cells orthotopically in nude mice and administered CBL0137 in various dosing regimens to assess brain and tumor accumulation of CBL0137, its effect on tumor cell proliferation and apoptosis, and on survival of mice with and without temozolomide (TMZ).

Results. FACT subunit expression was elevated in GBM compared with normal brain. CBL0137 induced loss of chromatin-unbound FACT, activated p53, inhibited NF-κB–dependent transcription, and was toxic to GBM cells. The drug penetrated the blood–brain barrier and accumulated in orthotopic tumors significantly more than normal brain tissue. It increased apoptosis and suppressed proliferation in both U87MG and A1207 tumors. Intravenous administration of CBL0137 significantly increased survival in models of early- through late-stage TMZ-responsive and -resistant GBM, with a trend toward significantly increasing the effect of TMZ in TMZ-responsive U87MG tumors.

Conclusion. CBL0137 targets GBM according to its proposed mechanism of action, crosses the blood–brain barrier, and is efficacious in both TMZ-responsive and -resistant orthotopic models, making it an attractive new therapy for GBM.

Keywords: CBL0137, curaxin, Facilitates Chromatin Transcription, glioblastoma, temozolomide.

Glioblastoma (GBM) is the most prevalent and aggressive primary brain tumor, with a dismal 5-year survival rate of about 5%.[1] Standard-of-care therapies, including surgery, radiation, and temozolomide chemotherapy, have brought median survival to almost 15 months. However, this figure has stalled. Novel drugs, which can be given as monotherapy or in combination with current therapies, are greatly needed.

Nuclear factor-kappaB (NF-κB) and p53 are often dysregulated in GBM. NF-κB, a pro-survival transcription factor, is constitutively activated in a large proportion of GBM.[2] Inactivation or mutation of the tumor suppressor p53 is one of the most common molecular abnormalities in gliomas.[3] Antimalarial drugs, such as quinacrine, are known to activate p53 while suppressing NF-κB without inducing genotoxicity.[4] The antimalarial drug chloroquine has even shown efficacy in the clinical setting when combined with conventional therapy.[5,6] CBL0137 is a clinical candidate from a novel class of anticancer agents, curaxins, with a structure different from the tested antimalarials but with similar effects on p53 and NF-κB.[7]

CBL0137 was discovered in a cell-based screening of small molecule libraries for activators of p53 and inhibitors of NF-κB.[7] CBL0137 binds DNA but does not cause any sort of

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chemical modifications in DNA and therefore lacks genotoxicity.[7] However, CBL0137 binding to DNA leads to functional inactivation of the **Fa**cilitates **C**hromatin **T**ranscription (FACT) complex, a chromatin remodeling complex involved in transcription, replication, and DNA repair.[7] FACT is composed of 2 subunits, structure-specific recognition protein (SSRP1) and suppressor of Ty-16 (SPT16), and is expressed in early embryogenesis and in undifferentiated progenitors and stem cells. It is virtually undetectable in differentiated cells and tissues of most mammalian organs, but its expression is elevated in multiple types of tumors.[8,9] In the presence of CBL0137, FACT, which normally binds histone oligomers in a transient manner, is found in a very high affinity, stable complex with chromatin, which interferes with its dynamic activity. Thus, in treated cells, FACT is lost from the nucleoplasm and trapped in chromatin, resulting in the inhibition of FACT-dependent transcription, including NF-κB–mediated transcription. Additionally, chromatin trapping of FACT leads to casein kinase 2 (CK2)–dependent phosphorylation and activation of p53.[7]

In this study, we establish that FACT (SSRP1 and SPT16 subunits) is elevated in GBM compared with normal brain, with the classical and proneural GBM subtypes, as defined by Verhaak et al,[10] overexpressing the most. Furthermore, we show that CBL0137 inhibits FACT in human GBM cell lines. Consequently the drug upregulates p53 and downregulates NF-κB transcriptional targets, as predicted by the proposed mechanism of action of CBL0137.[7] CBL0137 crosses the blood–brain barrier and is toxic to GBM cells in vitro and in vivo. The drug is efficacious in temozolomide (TMZ)-responsive and -resistant orthotopic models when administered at 3 different time points during tumor progression. Additionally, the combination of CBL0137 and standard-of-care TMZ shows a clear trend toward increasing survival significantly over TMZ monotherapy in the TMZ-responsive U87MG model. These results suggest that CBL0137 may have clinical benefit to patients with GBM, either sensitive or resistant to TMZ.

Materials and Methods

Analysis of FACT Subunit mRNA Expression

SSRP1 and SPT16 mRNA expression in human normal brain and GBM samples was compared using publicly available mRNA expression datasets and 2 data-mining platforms: (i) IST Online (MediSapiens) with built-in tools for transstudy and transtechnology normalization[11]; and (ii) the ONCOMINE database, which allows comparison of samples within separate datasets.[12] Differences in subunit expression among 4 GBM subtypes[10] were evaluated from The Cancer Genome Atlas (TCGA) dataset using CBioPortal.[13] Further relationships between subunit expression and O^6-DNA methylguanine-methyltransferase (MGMT) methylation status were also investigated. *P*-values were calculated using ANOVA for the IST Online data and the unpaired *t*-test for the ONCOMINE and CBioPortal data (GraphPad Prism 6 software).

Cell Culture

U87MG (American Type Culture Collection) and A1207 (a gift from Stuart Aaronson, Mt Sinai Hospital, New York, NY) were cultured in Dulbecco's modified Eagle's medium, high glucose (Gibco/Life Technologies) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) buffer (Life Technologies).

Drug Preparation

CBL0137 was provided by Incuron and dissolved in dimethyl sulfoxide (DMSO) for in vitro and in Captisol (CyDex Pharmaceuticals) for in vivo studies. Temozolomide (Sigma-Aldrich) was dissolved in DMSO (MediaTech) for in vitro studies and to a final concentration of 0.625 mg/mL in 10% DMSO in physiological saline for in vivo studies. The volume was adjusted so that a 25-g mouse would receive a dose of CBL0137 in 200 μL. Human recombinant tumor necrosis factor (TNF) protein (R&D Systems) was reconstituted in sterile phosphate buffered saline and used in vitro at a concentration of 10 ng/mL.

Protein Analysis

Cells were treated with CBL0137 (0.6 or 2.0 μM) alone or in combination with 200 μM TMZ for 1 or 16 h. For MGMT analysis, untreated cells were used. Cell lysis and western blotting were done as described.[7] The following antibodies were used for western blotting: p53 (D01), Hdm2, NF-κB, MGMT (C-20), ß-actin, and glyceraldehyde 3-phosphate dehydrogenase (V-18) from Santa Cruz Biotechnology; and SSRP1 and SPT16 from BioLegend. De-identified patient biopsy samples of GBM and normal brain, within multicancer tissue microarrays (TMAs), were provided by the Pathology Resource Network of Roswell Park Cancer Institute and immunostained for SSRP1 according to methods described previously.[9]

Cytotoxicity Assay

Cells were plated in a 96-well plate at 3000 cells/well (U87MG) or 1000 cells/well (A1207). Serial dilutions of CBL0137 were added 24 h later for 24 h. Cell viability was assessed after 72 h incubation in drug-free medium using CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Promega). Data were plotted and the half-maximal inhibitory concentration (IC_{50}) determined using fit to spline and lowess analysis (GraphPad Prism 6). Experiments were performed in duplicate or triplicate.

Real-Time PCR

Cells (2×10^6) were treated for 2 h with 0.01% DMSO, 2 μM CBL0137, 200 μM TMZ, or their combination in the absence or presence of 10 ng/mL TNF. RNA was isolated and expression of interleukin (IL)-8 and inhibitor of kappaB alpha (IκBα) was determined using real-time PCR as described previously.[14] ANOVA was used to determine statistically significant differences across groups ($P < .05$, GraphPad Prism 6). Experiments were performed in triplicate.

Orthotopic Models

All in vivo studies were approved by the Roswell Park Cancer Institute Institutional Animal Care and Use Committee. Eight- to 9-week-old male athymic nude mice (Harlan Laboratories) were implanted orthotopically with 5×10^5 A1207 or U87MG according to the methods outlined in the Supplementary material.

Tissue Accumulation Studies

Three to 5 animals per group were inoculated with U87MG or A1207 cells as described above. CBL0137 administration was initiated on day 14 and given in 2 dosing regimens: 25 mg/kg by oral gavage (p.o.) on days 14–21 (total 200 mg/kg) or 90 mg/kg intravenously (i.v.) on days 14 and 21 (total 180 mg/kg). A control animal for each dose was given Captisol vehicle. Twenty-four hours after the final dose, animals were euthanized and perfused transcardially with saline. Tumors and normal brain tissue from the corresponding contralateral brain hemisphere were removed and frozen in liquid nitrogen. CBL0137 was extracted and liquid chromatography–tandem mass spectrometry was performed as stated in the Supplementary material. Data were analyzed by unpaired *t*-test (GraphPad Prism 6).

Proliferation and Apoptosis Studies

Twelve animals were inoculated with U87MG or A1207 cells, as described above. Three mice with each type of tumor were treated with 70 mg/kg CBL0137 or Captisol-based vehicle i.v. on days 14, 18, and 22. Animals were euthanized 24 h after the last dose and perfused with saline and then 4% paraformaldehyde. Brains were removed, post-fixed for 24 h in 4% paraformaldehyde, processed, and stained for proliferation using the Ki67 rabbit monoclonal SP6 antibody (RM-9106-S1, Thermo Scientific) or apoptosis using the Tumor TACS In Situ Apoptosis Detection Kit (Trevigen). Staining, digital image acquisition, and analysis are supplied in the Supplementary material section. Data were compared using the unpaired *t*-test (GraphPad Prism 6).

Survival Studies

Six to 8 animals per group were inoculated orthotopically as outlined above. CBL0137 was administered in the following dosing regimens: (i) 25 mg/kg p.o. 5 days on/2 days off (maximum tolerated dose [MTD] for orally administered), (ii) 35 mg/kg i.v. every fourth day (q4d), (iii) 70 mg/kg i.v. q4d (MTD for q4d schedule), or (iv) 90 mg/kg i.v. once per week (MTD for 1×/wk schedule) for 4 weeks. Treatment was initiated on day 1, 7, or 14 after U87MG cell inoculation and day 1, 14, or 28 after A1207 cell inoculation. Temozolomide (5 mg/kg) was given in a 5 days on/2 days off regimen, for 2 cycles. Control animals received Captisol vehicle i.v. Mice were weighed 3 times per week. Animals losing 15% or more of their original body weight had drug delivery suspended until regaining weight to <10% difference from starting weight. Mice were euthanized when they showed evidence of tumor burden such as anorexia, hunched posture, and lack of ambulation or at 90 days. Survival data were plotted on a Kaplan–Meier curve and analyzed using the Mantel–Cox log-rank test (GraphPad Prism 6).

Results

FACT Level Is Higher in GBM than Normal Brain Tissue and Is Differentially Expressed in GBM Subtypes

Comparison of SSRP1 and SPT16 mRNA expression in the publicly available database of ~350 samples, IST Online,[11] revealed significantly higher levels in GBM than normal brain tissue ($P <$ .0001 for SSRP1 and SPT16; Fig. 1A). Similar analysis of samples from the Sun[15] and TCGA datasets using ONCOMINE[12] corroborated this mRNA expression data (Fig. 1B and C, respectively). A high proportion of tumor cells stained positive for SSRP1, while only a very rare cell from the cortex of normal brain stained positive in patient TMAs (Fig. 1D).

Analysis of SSRP1 and SPT16 mRNA expression, from the dataset of TCGA using CBioPortal[13] in 4 different subtypes of GBM[10] revealed that SSRP1 expression was significantly higher in the classical and proneural than in the mesenchymal and neural subtypes. SPT16 expression was significantly elevated in the classical subtype (Supplementary Fig. S1A and B). Further investigation into the association of subunit expression with MGMT methylation status showed no significant difference in SSRP1 or SPT16 mRNA expression between methylated and unmethylated tumors (Supplementary Fig. S1C and D).

CBL0137 Inhibits FACT Function and Modulates p53 and NF-κB Activity

SSRP1 and SPT16 proteins were detected in U87MG and A1207 human GBM cell lines by western blotting. Within one hour of CBL0137 treatment, the levels of both subunits in the soluble protein fraction were reduced or eliminated (Fig. 1E).

In both cell lines, p53 expression markedly increased upon incubation with both 0.6 and 2.0 μM CBL0137. Expression of the p53-responsive gene, Hdm2, was increased in response to the lower dose of CBL0137 but almost completely disappeared upon incubation at the 2 μM dose, which is in line with the previously published bell-shaped curve of p53 activation by CBL0137.[7] TMZ neither interfered with the effects of CBL0137 on FACT and p53 nor caused any effects itself (Fig. 2A and B).

Due to inhibition of FACT, which is required for effective transcriptional elongation, CBL0137 treatment causes reduction in transcription of NF-κB–dependent genes.[7] Both cell lines showed some basal IL-8 transcription. A 2-hour incubation with the NF-κB activator, TNF, caused a 700-fold increase in the IL-8 mRNA level in A1207 cells ($P = .006$; Fig. 3A) and a 7-fold increase in U87MG cells ($P <$ .0001; Fig. 3B). CBL0137 ameliorated this induction in both cell lines ($P = .007$ and $P <$ .0001, respectively), while TMZ moderately reduced the TNF-induced IL-8 expression in the U87MG cells ($P <$ .0001). The combination of CBL0137 and TMZ reduced IL-8 expression in both cell lines, but not more than the level of reduction by CBL0137 alone (Fig. 3A and B).

Changes in NF-κB–regulated IκBα transcription followed a similar pattern to the changes in IL-8. Incubation with TNF increased transcription significantly over the basal level in both A1207 (12-fold; Fig. 3C) and U87MG (4-fold; Fig. 3D) ($P = .0002$ and $P <$ .0001, respectively). CBL0137 alone decreased the TNF-induced increase in both lines ($P = .0011$ and $P <$ .0001) and TMZ only reduced IκBα induction in the U87MG line

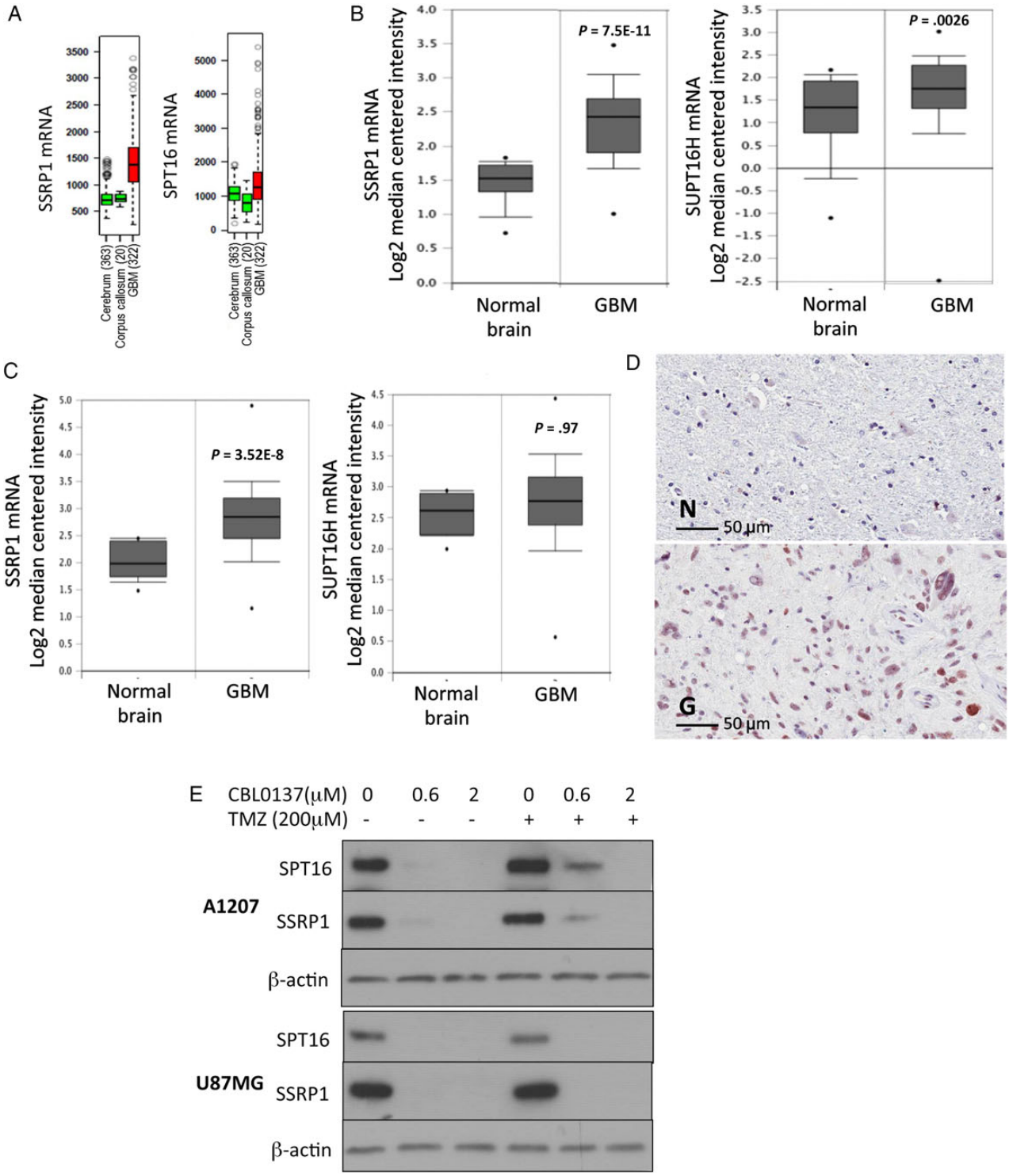

Fig. 1. FACT subunit expression in glioblastoma. (A) Box-whisker plots of SSRP1 and SPT16 mRNA levels in samples of normal brain and GBM (numbers in parentheses = number of samples of each type, from the IST Online database). (B and C) Box-whisker plots of SSRP1 and SPT16 mRNA levels from the Sun (B) and TCGA (C) brain datasets in the ONCOMINE database. (D) Representative images of SSRP1 staining of patient samples of normal brain (denoted N) and GBM (denoted G). SSRP1 positive cells are brown. Scale bar = 50 μm. (E) Protein expression of SSRP1 and SPT16 in A1207 and U87MG human glioblastoma cells 1 h after treatment with CBL0137 alone and in combination with TMZ as shown by western blotting. Data were compared with ANOVA for (A) ($P < .0001$ for both SSRP1 and SPT16) and an unpaired *t*-test for (B and C).

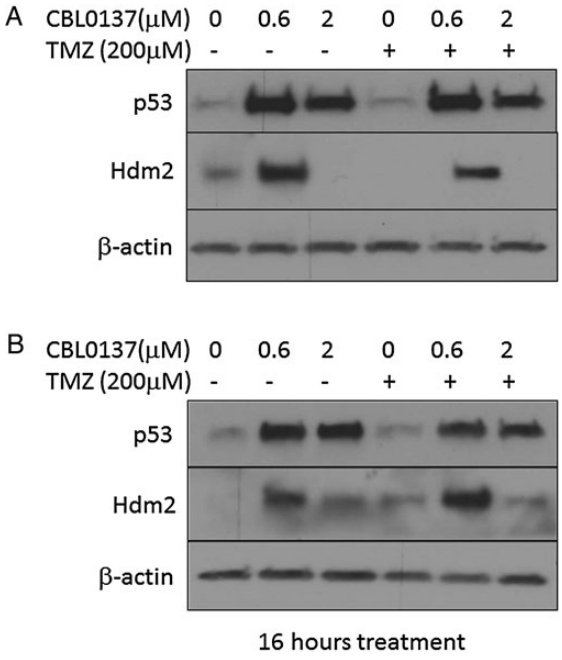

Fig. 2. Effect of CBL0137 alone or in combination with TMZ on protein levels of p53 and Hdm2 assessed using western blotting of lysates of A1207 (A) or U87MG (B) cells 16 h after treatment.

($P < .0001$) (Fig. 3D). Interestingly, without TNF induction, CBL0137 alone significantly decreased even the basal levels of IκBα transcription in U87MG cells ($P = .0044$; figure not shown).

CBL0137 Accumulates in Tumor to a Greater Extent than Normal Brain Tissue

An important requirement of potential anti-GBM agents is the ability to penetrate the blood–brain barrier. To test if CBL0137 is delivered to the brain upon systemic administration, we injected U87MG and A1207 cells orthotopically into nude mice. CBL0137 was administered using 2 regimens: daily oral gavage of 25 mg/kg for 8 days (total dose of 200 mg/kg) and 2 weekly i.v. doses of 90 mg/kg (total dose of 180 mg/kg). Both regimens of administration are currently being tested in phase I clinical trials: oral administration in patients with advanced solid cancers in the Russian Federation and i.v. in patients with advanced solid cancers and refractory lymphomas in the US (https://www.clinicaltrials.gov/ct2/show/NCT01905228). Brain and tumor tissues were collected 24 h after the last administration from mice perfused with saline, to reduce tissue contamination with blood. CBL0137 was present in normal brain after both oral and i.v. administration, with a >6-fold increase when changing from oral to i.v, even though the dose delivered i.v. was slightly lower than the oral dose. The amount detected in normal brain after i.v. administration was 8-fold and 3.5-fold greater than the in vitro IC_{50} for A1207 and U87MG, respectively. Importantly, CBL0137 accumulated at significantly higher levels in tumor versus normal brain tissue in both A1207 and U87MG orthotopic GBM models (Fig. 4A and B, respectively). We also observed that i.v. administration resulted in a significantly higher accumulation of CBL0137 in tumor tissue compared with oral therapy.

CBL0137 Is Toxic for GBM Cells In vitro and In vivo

CBL0137 was incubated with GBM cell lines in vitro for 24 h and cell viability was assessed at 72 h. CBL0137 caused a dose-dependent reduction in viability of GBM cells, with A1207 ($IC_{50} = 0.635$ μM) being 3 times more sensitive than U87MG ($IC_{50} = 2.045$ μM).

We then tested the ability of CBL0137 to cause toxicity to GBM cells in vivo. We treated mice with orthotopic GBM tumors with 3 doses of CBL0137 at 70 mg/kg i.v. every 4 days starting at day 14, to allow the tumors to reach an appreciable size for histology, and collected tumor tissues 24 h after the last dose. There was a low level of apoptosis ($3.74\% \pm 0.43$; $0.74\% \pm 0.13$) and a high level of proliferation ($63.45\% \pm 1.4$; $65.99\% \pm 1.77$) in control A1207 and U87MG tumors, respectively. CBL0137 tripled the apoptotic index in A1207 tumors ($P = .011$; Fig. 5A) and significantly reduced the proliferative index ($P = .04$; Fig. 5B). In U87MG, CBL0137 doubled the apoptotic index ($P = .0074$; Fig. 5A) and decreased the proliferation index by more than 1.5 times ($P = .001$; Fig. 5B). Thus we observed that CBL0137 is toxic for GBM cells in vitro and in vivo.

CBL0137 and TMZ Are Efficacious Alone and in Combination in the Orthotopic U87MG Model

We used the TMZ-sensitive cell line, U87MG, to evaluate survival after treatment with CBL0137 alone and in combination with TMZ, initiated at 3 different time points (days 1, 7, and 21) after tumor inoculation representing low, moderate, and large tumor burden, respectively. For the low tumor burden model, we administered CBL0137 p.o. (25 mg/kg daily) or i.v. (70 or 35 mg/kg, q4d). The 70 mg/kg CBL0137 i.v. regimen increased survival significantly over controls and 35 mg/kg CBL0137 i.v. treatment ($P < .05$ and $P < .01$, respectively; Fig. 6A). CBL0137 did not show efficacy when given orally or i.v. at 35 mg/kg. TMZ had a similar effect to 70 mg/kg CBL0137. CBL0137 at 70 mg/kg, in combination with TMZ, increased survival significantly over monotherapy ($P < .05$). However, the combination wasn't statistically significant compared with TMZ monotherapy, despite the separation in survival curves (median survival 56.5 days for 70 mg/kg CBL0137 i.v. + TMZ vs 41 days for TMZ alone). The low dose CBL0137 combination with TMZ was not better than TMZ alone (Fig. 6A). Since the 35 mg/kg dose showed no efficacy and the higher 70 mg/kg dose regimen showed no toxicity when combined with TMZ, 70 mg/kg i.v. dosing was continued through the delayed initiation experiments in the U87MG model.

Both 70 mg/kg i.v. CBL0137 and TMZ monotherapies increased survival significantly over vehicle controls ($P < .0001$) when administration was delayed for 7 days. TMZ monotherapy was more efficacious than CBL0137 ($P < .01$). Combination therapy increased survival significantly over CBL0137 monotherapy ($P < .01$) and approached significance against TMZ monotherapy ($P = .058$). Again, there was a large separation in survival curves between the combination treatment (median 65.5 days) and TMZ monotherapy (median 49.5 days) (Fig. 6B).

Both 70 mg/kg i.v. CBL0137 and TMZ monotherapies initiated 21 days after tumor inoculation, when the tumor was very large, still increased survival significantly over vehicle controls ($P < .01$ and $P < .001$, respectively). Combination therapy

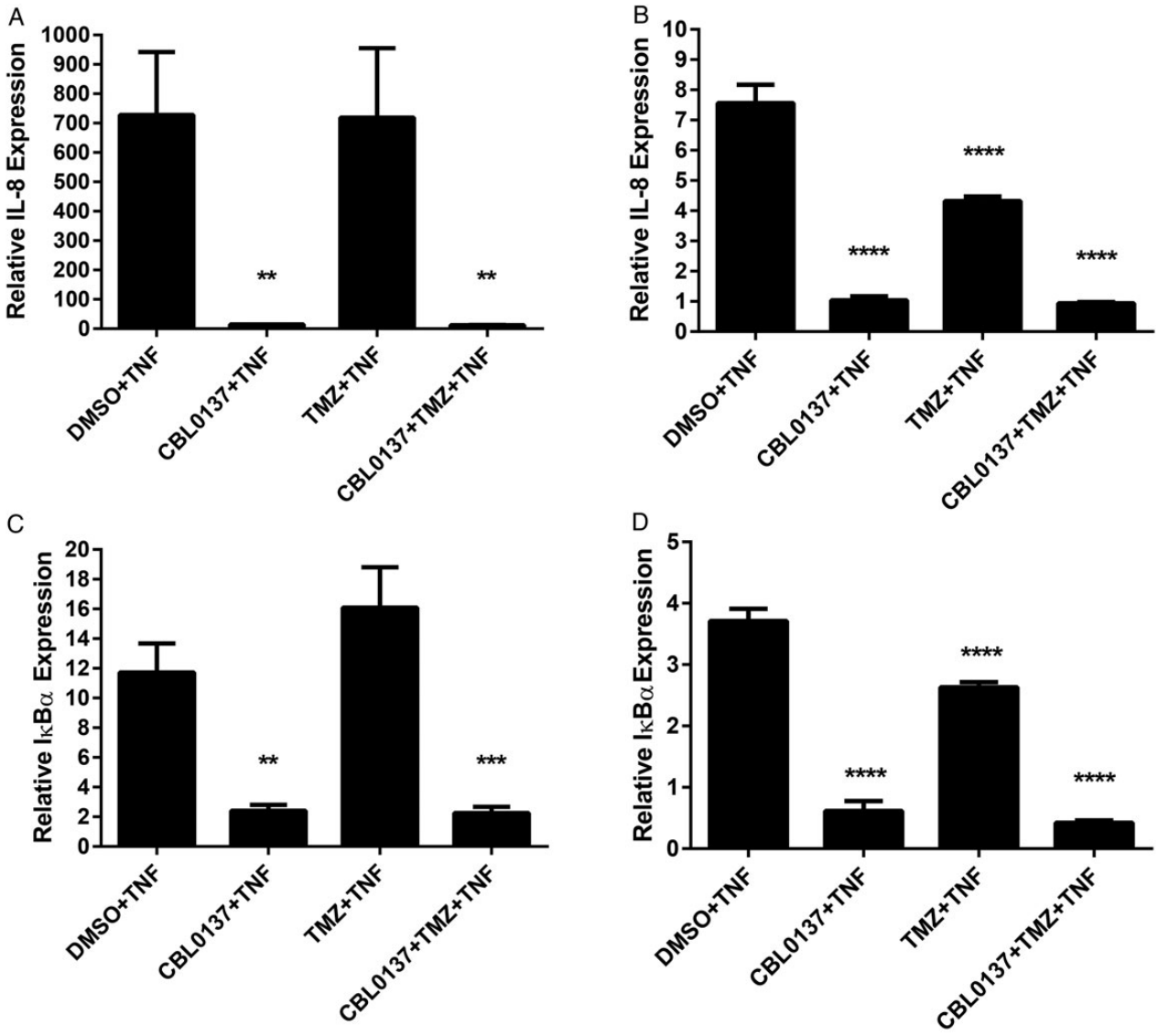

Fig. 3. Analysis of the effect of CBL0137 or TMZ alone or in combination on TNF-induced expression of NF-κB targets, IL-8 and IκBα. A1207 and U87MG human glioblastoma cells were treated with DMSO vehicle, CBL0137 alone, TMZ, or a combination of CBL0137 and TMZ in the presence of TNF to evaluate the effects of treatment on induced expression of IL-8 (A and B) and IκBα (C and D). All expression levels (±SEM) are relative to basal levels (DMSO vehicle alone) in A1207 (A and C) and U87MG (B and D) cells. ANOVA was used to determine statistically significant differences across groups (**$P < .01$, ***$P < .001$, ****$P < .0001$).

increased survival significantly over CBL0137 monotherapy ($P < .05$), but not over TMZ monotherapy (Fig. 6C).

CBL0137 Is Efficacious in the Orthotopic A1207 Model

For the A1207 model we used different initiation times (days 1, 14, and 28) than for U87MG, which reflected the longer survival of animals with the A1207 tumor. We evaluated 2 different i.v. dosing regimens, 70 mg/kg q4d and 90 mg/kg once per week, since both timing schedules are being considered for clinical use. As with the U87MG model, oral CBL0137 administration did not improve survival. However, i.v. CBL0137 initiated 1 day after tumor inoculation for both dosing schemes prolonged survival significantly over controls ($P < .001$ and $P < .0001$, respectively; Fig. 6D). There was no difference between the 2 i.v. dosing regimens, so 70 mg/kg q4d was used for delayed drug initiation studies for consistency, as the more frequent dosing was beneficial in the drug combination studies in the U87MG model. TMZ alone had no effect on survival and did not alter the effect of CBL0137 when given in combination. Moreover, we detected MGMT expression in A1207 cells using western blotting (Supplementary Fig. S2), which is known to be associated with TMZ resistance. These data indicated that A1207 is a TMZ-insensitive model. Therefore, we did not include TMZ in the delayed initiation experiments.

Delaying CBL0137 administration until day 14 resulted in increased survival over controls ($P < .001$; Fig. 6E). This increased survival was still observed after a 28-day delay ($P < .05$; Fig. 6F),

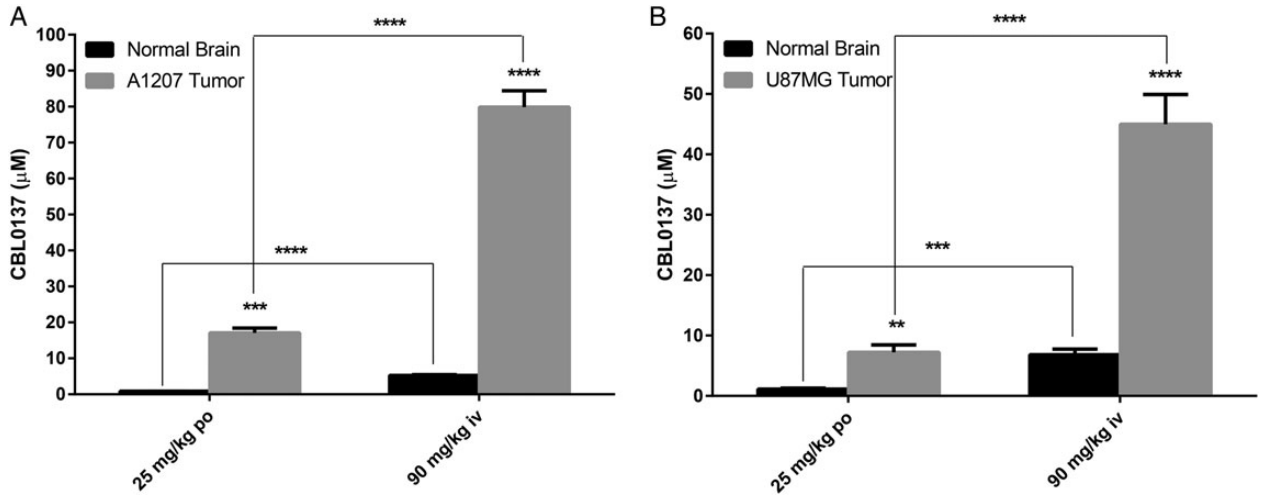

Fig. 4. Accumulation of CBL0137 in (A) A1207($n = 3–5$ each group) and (B) U87MG ($n = 5$ each group) orthotopic tumors and the contralateral normal brain tissue. CBL0137 was administered at either 25 mg/kg p.o. on days 14–21, or 90 mg/kg i.v. on days 14 and 21. Data represent the mean ± SEM and were compared with an unpaired *t*-test. (**$P < .01$, ***$P < .001$, ****$P < .0001$).

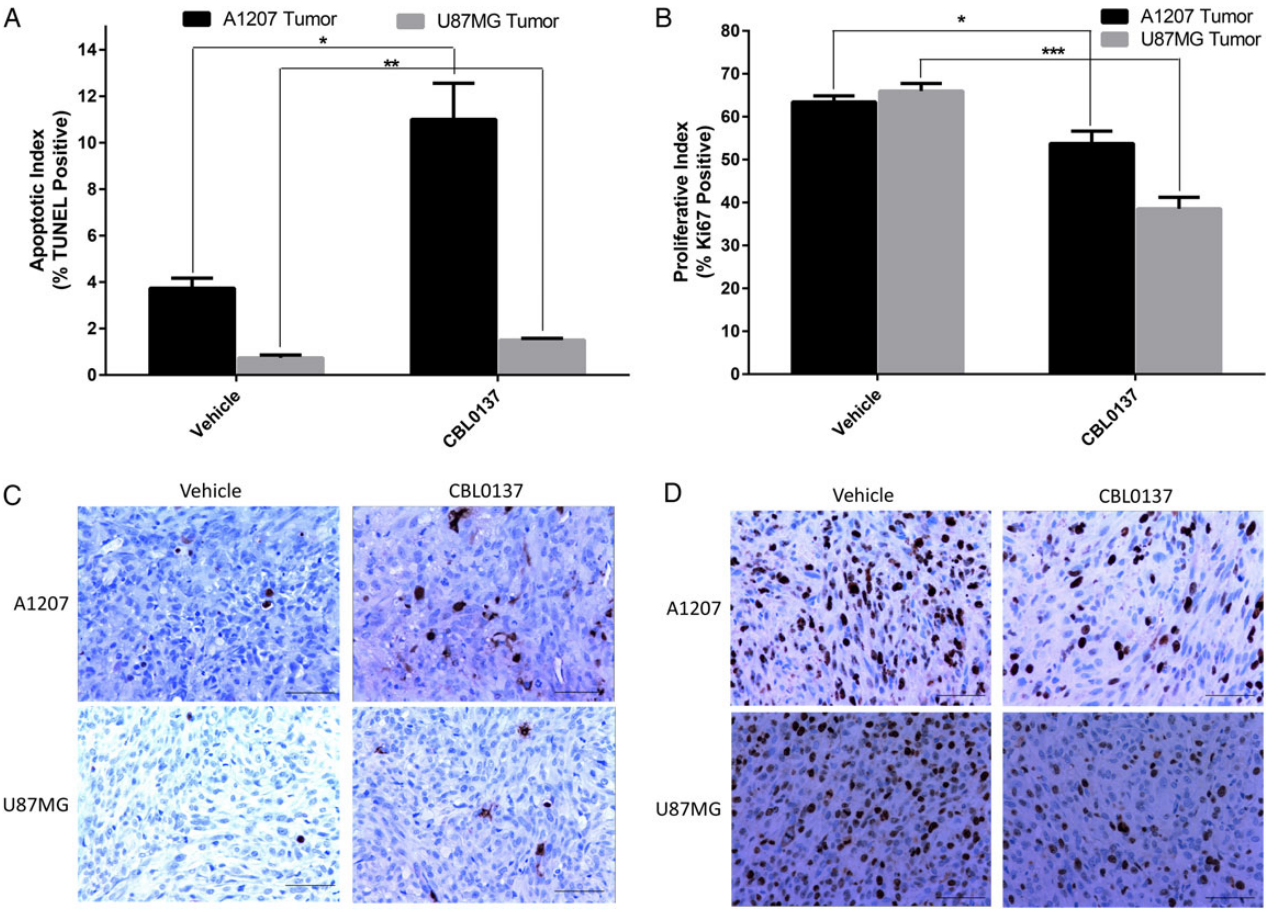

Fig. 5. Apoptotic (A) and proliferative (B) indices of A1207 and U87MG orthotopic tumors after administration of CBL0137 (70 mg/kg i.v.) or vehicle on days 14, 18, and 22 of tumor progression. Representative images of apoptotic (C) and proliferative (D) stains. Scale bar = 50 μm. Data represents the mean ± SEM and was compared with an unpaired *t*-test (*$P < .05$, **$P < .01$, ***$P < .001$). TUNEL, terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling.

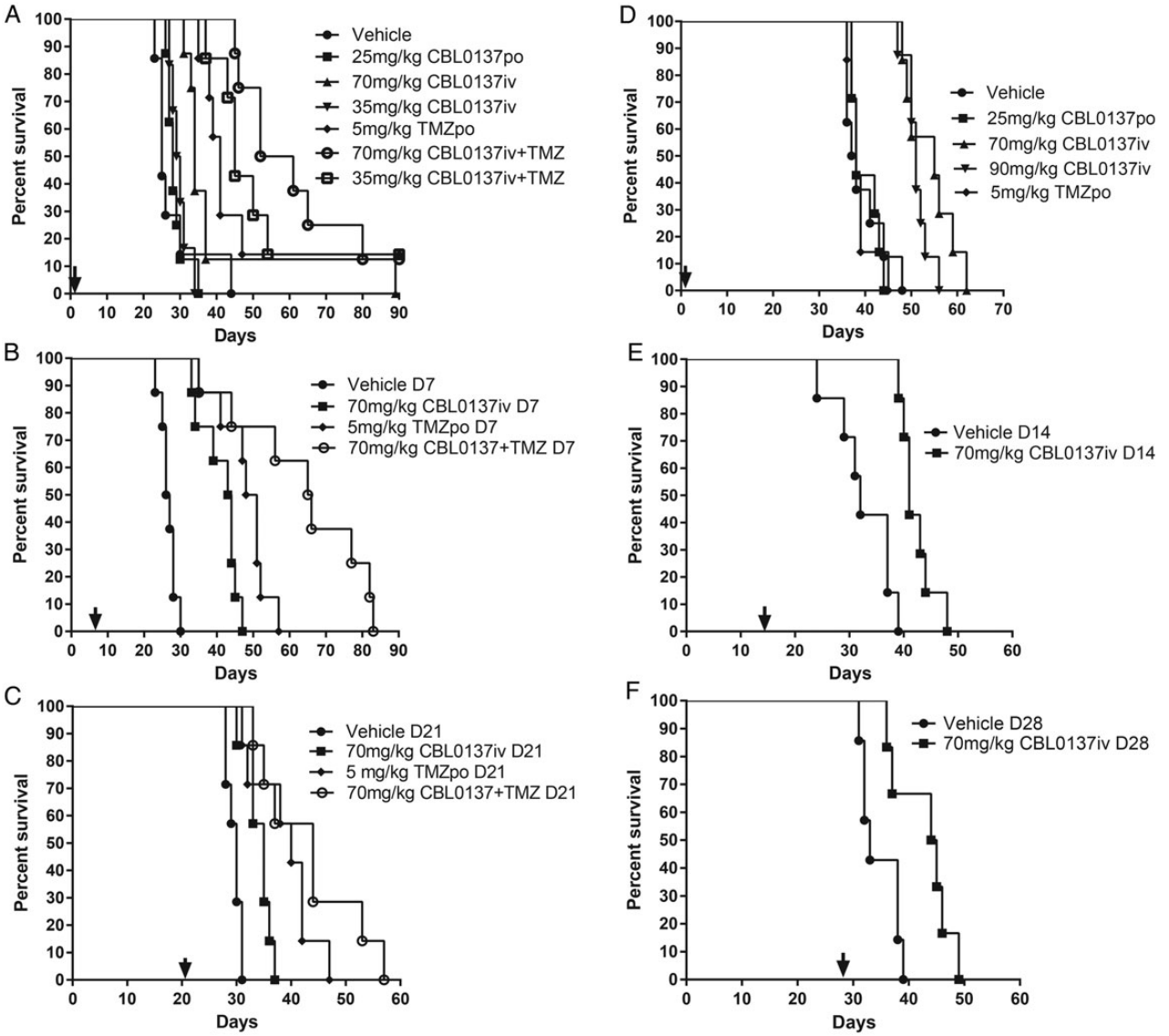

Fig. 6. Effect of CBL0137 alone, or in combination with TMZ (U87MG only), on the survival of mice bearing U87MG (A–C) or A1207 (D–F) orthotopic GBM tumors. CBL0137 was administered p.o. daily, i.v. q4d, or 1×/week (A1207 only) for up to 4 weeks. TMZ was administered in a 5-days-on/2-days-off schedule for 2 cycles. Drugs were administered in the doses indicated in the figure legends. Treatment commenced 1 (A), 7 (B), or 21 (C) days after U87MG inoculation and 1 (D), 14 (E), or 28 (F) days after A1207 inoculation. The arrow indicates drug administration start time for the experiment. $N = 6–8$ animals per group. Survival curves were compared with the Mantel–Cox log-rank test.

despite many animals receiving only one dose due to >15% weight loss.

Based on these results, CBL0137 is efficacious against both TMZ-sensitive and -resistant orthotopic GBM models, and TMZ enhances this effect in the TMZ-sensitive GBM model.

Discussion

CBL0137 represents a novel class of small molecules that simultaneously activate p53 and inhibit the NF-κB stress response pathway, without genotoxicity.[7] This drug candidate is effective in preventing and delaying mammary tumor onset and increasing survival of mice with tumors, without affecting normal organs and tissues.[16] It is also effective in preclinical models of pancreatic ductal adenocarcinoma, colorectal adenocarcinoma, renal cell carcinoma, and melanoma[7,14] and is being administered in clinical trials for advanced solid neoplasms (https://www.clinicaltrials.gov/ct2/show/NCT01905228). The proposed mechanism of action of CBL0137 is inactivation of the transcription elongation factor FACT, which blocks NF-κB transcription and activates p53, two factors often dysregulated in GBM. However, it is not dependent on p53.[7] FACT comprises 2 subunits, SSRP1 and SPT16. In this study, we demonstrated that mRNA expression of the SSRP1 and SPT16 subunits in GBM significantly exceeds that of normal brain tissue in the IST Online and ONCOMINE databases and is corroborated by immunohistochemical staining of patient samples. This difference in

expression of FACT suggests that normal brain would be an unlikely target for this drug compared with GBM, leading to few neurological side effects, making GBM an attractive target for CBL0137 therapy. Further breakdown of SSRP1 and SPT16 mRNA expression by GBM subtype[10] shows that patients with the classical and proneural subtypes may benefit the most from CBL0137 therapy, since they show significantly more SSRP1 and the classical subtype shows more SPT16 expression.

We found that CBL0137 treatment results in the loss of functional FACT subunits from the soluble protein fraction of cell lysates prepared from 2 human GBM cell lines, TMZ-responsive U87MG and TMZ-resistant A1207. The reduction in the levels of both FACT subunits is consistent with the proposed mechanism of chromatin trapping of FACT, as FACT is redistributed from the soluble portion to the chromatin-bound portion.[7] Upregulation of p53, with regulation of its responsive protein Hdm2, and downregulation of NF-κB downstream targets that we observed in GBM cells show that both lines have functional p53 and support the proposed mechanism of action of CBL0137 on p53 and NF-κB through FACT inhibition. Burkhart et al[14] saw similar results on downstream NF-κB–responsive genes in human pancreatic cancer cells after CBL0137 administration. It is possible that the suggested difference in reliance on the NF-κB pathway, as seen in the 100-fold difference in TNF-induced induction of IL-8 expression between A1207 and U87MG cells, may translate into a marker for patient response to CBL0137. It's not surprising that we observed increased apoptosis and decreased proliferation in the tumor models, given the increases in p53 and the decreases in NF-κB signaling. P53 is a well-known pro-apoptotic molecule, and others have seen increases in apoptotic markers[17] and decreases in proliferation in GBM upon downregulation of NF-κB.[18,19] GBM stem cells from patient samples which express FACT show an increase in apoptosis upon treatment with CBL0137.[20] These results further suggest that glioma is responsive to CBL0137 and that CBL0137 would be a beneficial treatment.

Our tissue accumulation studies demonstrate that CBL0137 crosses the blood–brain barrier, especially when administered i.v. Failure to penetrate the blood–brain barrier dooms many potential therapies of the CNS,[21] and the successful accumulation of CBL0137 in the brain, particularly the orthotopic GBM tumors, bodes well for the potential of this drug to treat CNS tumors. The accumulation of CBL0137 at higher levels in orthotopic GBM tumors than in normal brain is likely due to the greater abundance of DNA per gram of tissue in the hypercellular tumor compared with normal brain, to which CBL0137 binds. Clinically, the blood–brain barrier is heterogeneously leaky in gliomas,[21] which would serve to enhance the ability of CBL0137 to cross this barrier and inhibit FACT.

It is clear from our results that i.v. administration leads to greater tumor tissue accumulation than oral dosing, leading to greater bioavailability. The accumulation of CBL0137 after oral dosing is insufficient to affect survival; however, tumor accumulation after i.v. delivery is sufficient to demonstrate efficacy in both in vivo models, consistent with our in vitro toxicity data which predicted that CBL0137 would be toxic to U87MG and A1207 tumor cells. However, normal brain tissue accumulation did not cause observable neurotoxicity.

Combination therapy of CBL0137 with TMZ in the U87MG model was superior to CBL0137 monotherapy but not to TMZ monotherapy, despite an obvious separation in survival curves, especially when treatment was initiated 1 or 7 days after tumor inoculation. Perhaps larger sample sizes would bring the considerable difference observed between these survival curves to statistical significance. Others have found that inhibiting the NF-κB pathway increases the sensitivity of GBM, in particular U87MG, to TMZ.[22] P53 function may also be partly responsible for the increase in survival seen with combination therapy. Disruption of p53 function has been shown to render glioma cells resistant to TMZ.[23] Thus, the ability of CBL0137 to activate p53 in U87MG tumors may enhance the cellular response to the DNA damage caused by TMZ. Indeed, antimalarial compounds, which share the ability to activate p53 and inhibit NF-κB, have been shown to be cytotoxic to glioma cells.[24]

It is not surprising that TMZ was unable to increase survival in the A1207 model, since we found that A1207 expresses MGMT by western blot. MGMT expression is known to render the DNA O^6 alkylating agent TMZ ineffective because of its DNA repair function.[23,25] The fact that CBL0137 was efficacious, even in this TMZ-insensitive tumor, and that we found no significant difference in FACT subunit mRNA levels between methylated and unmethylated MGMT tumors leads us to believe that this therapy could be effective for patients with tumors resistant to TMZ, which made up over 50% of glioma patients in one study.[26]

The significant prolongation of survival, even when CBL0137 was given to animals with a large tumor burden, is impressive and encouraging. Since tumor progression was significant upon drug initiation, several lost a significant amount of weight after only one dose, which may or may not be attributable to CBL0137. Weight loss is a sign of tumor burden, and vehicle-treated animals survived a median of only 33 days in the U87MG model and 37.5 days in the A1207 model. It is likely that the weight loss was attributable to tumor burden and not drug toxicity at this late stage. Surprisingly, survival was increased in the large tumor burden groups with only a few doses of CBL0137, which attests to the potency of the drug. The results with a variety of tumor burdens in the orthotopic models suggest that starting either CBL0137 monotherapy or combination therapy with TMZ, in TMZ-sensitive tumors, early after debulking is best but that patients with moderate to large tumor burdens, where treatment options are limited, may benefit from this therapy as well.

Overall, the results obtained in these studies indicate that GBM is an attractive target for CBL0137 therapy. CBL0137 could provide clinical benefit to patients, regardless of TMZ responsiveness and at multiple stages of disease progression, from situations of minimal residual tumor after debulking to late-stage, large, or inoperable tumor, for which treatment options are limited.

Supplementary material

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

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