Inhibition of Nuclear Factor-κB Activity by Temozolomide Involves O⁶-Methylguanine–Induced Inhibition of p65 DNA Binding

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Abstract
The alkylating agent temozolomide, commonly used in the treatment of malignant glioma, causes cellular cytotoxicity by forming O⁶-methylguanine adducts. In this report, we investigated whether temozolomide alters the activity of the transcription factor nuclear factor-κB (NF-κB). Temozolomide inhibits basal and tumor necrosis factor α (TNFα)–induced NF-κB transcriptional activity without altering phosphorylation or degradation of inhibitor of κB-α. Inhibition of NF-κB is secondary to attenuation of p65 DNA binding, not nuclear translocation. Inhibition of DNA binding is shown both in vitro, with gel shift studies and DNA binding assays, and in vivo at κB sites. Consistent with inhibition of NF-κB activity, temozolomide reduces basal and TNFα–induced κB-dependent gene expression. Temozolomide also inhibits NF-κB activated by inducers other than TNFα, including lipopolysaccharide, doxorubicin, and phorbol 12-myristate 13-acetate. The inhibitory action of temozolomide on NF-κB is observed to be maximal following pretreatment of cells with temozolomide for 16 h and is also seen with the S₉,₁-type methylating agent methylsaccharose. The ability of temozolomide to form O⁶-methylguanine adducts is important for inhibition of NF-κB as is the presence of a functioning mismatch repair system. Activation of NF-κB with TNFα before administration of temozolomide reduces the cytotoxicity of temozolomide, whereas 16-h pretreatment with temozolomide resensitizes cells to killing. This work shows a mechanism whereby O⁶-methylguanine adducts formed by temozolomide lead to inhibition of NF-κB activity and illustrates a link between mismatch repair processing of alkylator–induced DNA damage and cell death. [Cancer Res 2007;67(14):6889–98]

Introduction
The monofunctional alkylating agent temozolomide is routinely used for the treatment of malignant glioma (1). Although temozolomide induces the formation of several DNA adducts (2), cytotoxicity results primarily from the formation of O⁶-methylguanine lesions (3, 4). The importance of O⁶-methylguanine in mediating cell killing was shown by studies with O⁶-alkylguanine–DNA alkyltransferase (AGT; ref. 5), a protein that specifically removes alkyl groups from the O⁶ position of guanine (6). Following DNA replication, O⁶-methylguanine mispairs with thymine, leading to activation of the mismatch repair (MMR) system. However, as the repair process targets the newly synthesized DNA strand (7), the methylated adduct is left intact. One theory proposes that subsequent futile cycles of repair eventually result in cell death (8), whereas a second hypothesis maintains that it is the damage repair machinery itself that directly signals apoptosis (9). Although there is extensive evidence linking O⁶-methylguanine and MMR to cell death, the cytotoxic pathways downstream of MMR processing continue to be elucidated (10–13).

The transcription factor nuclear factor-κB (NF-κB) plays a prominent role in resistance to cell killing (14, 15) and consists of five structurally related proteins, the most abundant form consisting of the heterodimer of p50 (NF-κB1) and p65 (RelA; ref. 16). Each NF-κB subunit contains an NH₂-terminal Rel homology domain (RHD) that, among other functions, is important for DNA binding and dimerization (17). In unstimulated cells, NF-κB is sequestered in the cytosol bound to inhibitor of κB (IκB) proteins. Following stimulation, IκBα, a well-described IκB protein, is phosphorylated by the IκB kinase (IκK) complex and thus marked for ubiquitination and proteosomal degradation. Degradation of IκBα results in the release of the NF-κB subunits which translocate into the nucleus and bind specific DNA sequences in the promoter region of NF-κB–regulated genes (18). Although NF-κB is controlled primarily by IκB protein degradation, transcriptional activity can be regulated at multiple different sites (18–20). In this regard, several chemotherapeutic agents have been reported to modulate the NF-κB activation pathway (21, 22), with some enhancing and others inhibiting the overall activity.

In this study, we examined the effect of temozolomide on NF-κB transcriptional activation. We find that incubation with temozolomide for >6 h results in inhibition of NF-κB by an IκB–independent mechanism. Temozolomide inhibits the DNA binding ability of p65 without affecting its nuclear translocation. Both low cellular AGT activity and the presence of an intact MMR system are necessary for inhibition of NF-κB by temozolomide.

Materials and Methods
Reagents and cells. Temozolomide and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, NIH. Methylsaccharose, lipopolysaccharide, doxorubicin, phorbol 12-myristate 13-acetate (PMA), N-acetylcysteine, DTT, 3-aminobenzamide, and O⁶-benzylguanine were obtained from Sigma Chemical Co. Temozolomide and methyl-BCNU were dissolved in DMSO (final concentration <0.1%, v/v); BCNU was dissolved in 1% ethanol. Human glioblastoma cell lines U87MG and T98G and colon cancer HCT116 cells were purchased from American Type Culture Collection and cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and
streptomycin (100 μg/mL) at 37°C and 5% CO₂. HCT116-ch3 cells were a kind gift from Dr. C.R. Boland (Baylor University Medical Center, Dallas, TX). TK6 and MT1 cells, a gift from Dr. Giancarlo Marra (Institute of Molecular Cancer Research, University of Zurich, Zurich, Switzerland), were grown in RPMI 1640 with 10% FCS.

Stable transfection. The construct pcDNA-AGT has previously been described (23). H80 glioma cells were transfected with pcDNA-AGT or with pcDNAs by electroporation. Briefly, cells were cultured to 40% to 60% confluence and electroporated using the Electro Square Porator T820 (Genetronics, Inc.). Parameters used for electroporation were as follows: 500 V/cm, 10 pulses of 1.0 ms each in X-VIVO 10 + 1% human serum albumin. Electroporated cells were plated, grown for 24 h, and then cultured for 12 to 14 days in the presence of 250 μg/mL genetin. Individual colonies were grown in genetin-containing medium for preparation of cell extract as previously described (24).

AGT activity assay. Extracts were prepared from H80 stable transfectants by homogenization in 50 mmol/L Tris (pH 7.5), 0.1 mmol/L EDTA, and 5 mmol/L DTT buffer. Samples were sonicated for 1 min and centrifuged at 14,000 × g for 30 min. The assay for AGT activity was done as described (5) and measured as the removal of O6-[3H]methylguanyline from a 3H-labeled methylated DNA substrate (18 Ci/mmol) after incubation with tissue extract at 37°C for 30 min. Results were expressed as femtomoles of O6-methylguanyline released from DNA per milligram of protein.

Luminescence assay. Cells (5 × 10³) were plated overnight and cotransfected with the NF-κB luciferase reporter immunoglobulin-B (Ig-B-Luc) (or activator protein AP-1) and the Renilla reniformis, pRL-TK, expression vectors (ratio of 10:1 Ig-B-Luc/pRL-TK) using the SuperFectin transfection kit (Qiagen). After 24 h, these were pretreated with chemotherapeutic agent (or 0.1% DMSO, vehicle) and then with 10 ng/mL human tumor necrosis factor α (TNFα) as indicated. NF-κB (or Egr1) and Renilla luciferase activities were measured with the Dual-Luciferase Reporter assay system (Promega Corp.) 5 h after TNFα stimulation. Relative luminescence was calculated as the ratio of firefly luminescence to Renilla luminescence.

Cell fractionation and electrophoretic mobility shift assay. Cells were grown and treated as indicated. They were then pelleted by centrifugation at 1,000 rpm for 5 min at 4°C and resuspended in ice-cold buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL leupeptin, 5 μg/mL aprotinin]. Following the addition of 25–10% NP40, the suspension was vortexed and centrifuged at 14,500 rpm for 5 min at 4°C; the supernatant was designated as the cytoplasmic fraction. Nuclear pellets were resuspended in 50 mL of ice-cold buffer B [20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, 25% glycerol, 1 μg/mL leupeptin, 5 μg/mL aprotinin] and centrifuged at 14,500 rpm for 5 min. The supernatant was used as the nuclear fraction and protein concentration determined by the Bradford method. Electrophoretic mobility shift assay (EMSA) was done with 10–μg nuclear protein using the Promega gel shift assay system and 32P-labeled NF-κB or [activator protein AP-1] (AP-1) consensus oligonucleotide. Nuclear extract was also preincubated with 100-fold excess unlabeled NF-κB (specific competitor) or AP-1 consensus sequence (non-specific competitor). Supershift studies were done by preincubation with anti-p65, anti-p50, or anti-c-Rel antibody (Active Motif) for 30 min.

Dissociation of NF-κB from IκB proteins was done in cytoplasmic fractions as described (27). Five micrograms of cytosolic protein were adjusted to 0.2% sodium deoxycholate (DOC; w/v) and incubated on ice for 15 min. The solution was then adjusted to 0.2% NP40 (v/v) and then incubated for an additional 15 min on ice before being analyzed.

NF-κB DNA binding assay. Five micrograms of nuclear sample or 10 ng of purified recombinant p65 protein (Active Motif) were added to each well and binding assay done according to the manufacturer’s instructions (TransAM NF-κB Assay, Active Motif). For competition, 20 pmol of wild-type or mutated consensus oligonucleotide were added before the sample. Absorbance value was read at 450 nm following primary and secondary horseradish peroxidase (HRP)-conjugated antibody administration.

Western blotting. Twenty micrograms of nuclear, cytosolic, or cellular lysate were subjected to SDS-PAGE. Following electrophoresis, immobilized-P membranes (Millipore Corp.) were probed with primary rabbit polyclonal antibody against IκBα, phospho-Ser53-IκBα, NF-κB-p65, phospho-Ser59-p65 (Cell Signaling Technology, Inc.), MS29 (Santa Cruz Biotechnology), p65 COOH terminus (AB1694), and monoclonal anti-AGT antibody (Chemicon) diluted 1:1,000 overnight at 4°C. Antirabbit immunoglobulin G (IgG) HRP-linked secondary antibody (Cell Signaling Technology) was used at 1:1,000 dilution. Immunoreactive bands were detected by SuperSignal ECL (Pierce) and exposed to Kodak X-Omat film.

Indirect immunofluorescence imaging. U87 cells grown on glass chamber slides were treated and then washed in PBS followed by 20-min fixation with 4% paraformaldehyde. Permeabilization was done with 1% Triton X-100 in PBS for 10 min. Fixation and permeabilization were done at room temperature. Cells were then washed, blocked with 2% goat serum, and incubated with 20 μg/mL anti-NF-κB-p65 (Santa Cruz Biotechnology) overnight at 4°C. Cells were washed before incubation with affinity-purified, FITC-conjugated goat anti-secondary antibody (1:100; Santa Cruz Biotechnology) for 45 min. The samples were counterstained with 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI) solution for 15 min. Following washing, cells were mounted with Citifluor medium and imaged using a Zeiss Axiosplan microscope with 63× oil immersion objective (numerical aperture, 1.4); Excitation (Ex) and emission (Em) wavelengths for FITC and DAPI were Ex 480 nm, Em 525 nm, and Ex 360 nm, Em 460 nm, respectively.

Results

Temozolomide inhibits NF-κB-dependent transcription. To evaluate the action of temozolomide on NF-κB, we used a NF-κB-responsive luciferase reporter (25), which is 100% inhibited by the specific NF-κB inhibitor IκBα. Bcl-2 inhibitor IκBα (BMS-345541; ref. 33; data not shown). In U87 cells, both basal and TNFα-induced NF-κB activities are inhibited by temozolomide with an IC₅₀ of 75 μmol/L.

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Inhibition of NF-κB by Temozolomide

(Fig. 1A; 0.1% DMSO, vehicle, has no significant effect on NF-κB luciferase; data not shown). Cell viability assay shows that treatment with 10 ng/mL TNFα and 100 μmol/L temozolomide results in only 10% decrease in survival at the time point tested in the luciferase assay (data not shown). Temozolomide has no effect on an Egr1-promoter luciferase construct (Fig. 1B), showing that the action of temozolomide is not due to a nonspecific effect on the luciferase cDNA. Time course studies show that inhibition of NF-κB by temozolomide is maximal after 16 h (Fig. 1C). Temozolomide also inhibits TNFα-induced NF-κB activity in U251 glioma cells (data not shown). In addition, temozolomide attenuates NF-κB activated by inducers other than TNFα, including lipopolysaccharide, doxorubicin, and PMA (Supplementary Fig. S1). These results indicate that incubation of glioma cells with clinically relevant doses of temozolomide (peak patient plasma temozolomide level in clinical trials is ~ 100 μmol/L; ref. 34) for >6 h results in inhibition of NF-κB activity.

To examine if temozolomide inhibits NF-κB–dependent transcription in vivo, we evaluated mRNA levels of several NF-κB–regulated genes. There is an increase in COX2, IκBα, and MnSOD mRNA 1 and 3 h after TNFα stimulation and of X-linked inhibitor of apoptosis (XIAP) 3 h after TNFα (Fig. 1D). Pretreatment with 100 μmol/L temozolomide inhibits both basal and TNFα-induced mRNA expression of these genes and of Bcl-XL, findings consistent with the luciferase reporter studies.

Temozolomide does not inhibit TNFα-induced IκBα degradation. The primary regulatory point of NF-κB activity is at the level of IκB protein degradation. Temozolomide has minimal effect on total IκBα levels after 16 h (Fig. 2A, compare lanes 1 and 5) whereas TNFα treatment results in phosphorylation and almost complete degradation of IκBα by 5 min (Fig. 2A, lane 2). Pretreatment with temozolomide, at a dose and duration that inhibits NF-κB transcriptional activity, does not alter TNFα-induced phosphorylation or degradation of IκBα (Fig. 2A). The lack of inhibitory effect of temozolomide on TNFα-induced IκBα phosphorylation was confirmed with an IκB activity assay showing no inhibition of IκB kinase activity by concentrations of temozolomide up to 250 μmol/L (data not shown). These data

![Figure 1](https://example.com/f1.png)

**Figure 1.** Temozolomide inhibits NF-κB–dependent transcription. A, U87 cells were cotransfected with NF-κB-luciferase (IκB-Luc) and Renilla reniformis expression vectors. After 24 h, cells were treated with temozolomide (TMZ; 16-h pretreatment) and/or 10 ng/mL TNFα for an additional 5 h. Columns, mean fold change of relative luminescence from triplicate samples (untreated sample = 1); bars, SD. *, P < 0.05. B, U87 cells were cotransfected with IκB-Luc (white columns) or pEGR-660 (black columns) and Renilla. Cells were untreated or treated with 100 μmol/L temozolomide (16 h pretreatment) and/or 10 ng/mL TNFα. C, U87 cells were untreated or treated with 100 μmol/L temozolomide for the times indicated either alone or followed by TNFα, as in (A). **, P < 0.001. D, RT-PCR in U87 cells. Cells were untreated or treated with 100 μmol/L temozolomide (16 h pretreatment) and/or 10 ng/mL TNFα. Columns, fold change in RNA expression of indicated genes compared with control. Representative of at least three separate experiments showing similar fold change.
show that temozolomide does not act at the level of IκBα to inhibit NF-κB activity.

Temozolomide inhibits TNFα-induced NF-κB on EMSA. We next investigated whether temozolomide alters nuclear lysate binding to a NF-κB consensus probe using EMSA. TNFα stimulation results in an increase in NF-κB binding that is maximal after 30 min (Fig. 2B). Pretreatment with temozolomide for 16 h reduces TNFα-induced NF-κB at all time points tested and, consistent with the luciferase studies, inhibition is only evident with temozolomide pretreatment for >6 h (Fig. 2C, left). Analysis of the subunit composition of the translocated NF-κB reveals that both an anti-p65 antibody and an anti-p50 antibody shift the NF-κB band whereas a c-Rel antibody has no effect (Fig. 2C, right). The residual NF-κB present following incubation with an anti-p50 antibody that is shifted with an anti-p65 antibody likely represents the p65 homodimer band. EMSA with AP-1 consensus sequence shows no inhibition by temozolomide of TNFα-induced AP-1 binding (Fig. 2D), suggesting that the inhibition of NF-κB on EMSA is not a nonspecific effect.

6-Methylguanine and MMR mediate inhibition of NF-κB by temozolomide. Inhibition of NF-κB by temozolomide is seen following incubation with temozolomide for an extended period (Fig. 1C), suggesting that some metabolite or intermediate is involved. Because O6-methylguanine is the primary cytotoxic lesion formed by temozolomide, we evaluated the role of O6-methylguanine in inhibition of NF-κB using cells with different AGT activities. In U87 and U251 cells, both with low AGT activity (35, 36), 100 μmol/L temozolomide results in almost 100% inhibition of TNFα-induced NF-κB (Fig. 1A, and data not shown). However, in T98G glioma cells, which have high AGT activity (36), there is minimal inhibition of NF-κB by 100 μmol/L temozolomide and only 50% inhibition by 250 μmol/L temozolomide (Fig. 3A), a finding also seen on EMSA (Fig. 3B). To confirm the role of O6-methylguanine, we used pharmacologic and genetic studies targeting AGT. Pretreatment of T98 cells with O6-benzylguanine, an irreversible AGT inhibitor (5), increases inhibition of TNFα-induced NF-κB by temozolomide in both luciferase reporter studies (Fig. 3A) and EMSA (Fig. 3B, lanes 6–9). AGT was then stably expressed in H80 glioma cells using wild-type AGT, pcDNA-AGT (H80AGT), or empty vector, pcDNA3 (H80pcDNA3), and AGT activity was assessed. AGT activity is below the limit of detection and 1,358 fmol/mg of protein in H80 pcDNA3 and H80 AGT cells, respectively (Fig. 3C, inset). In parental H80 cells (no detectable AGT activity) and H80pcDNA3 cells, 100 μmol/L temozolomide significantly inhibits TNFα-induced NF-κB reporter expression (P < 0.01; Fig. 3C). However, in H80AGT cells, concentrations as high as 250 μmol/L temozolomide have no inhibitory effect on TNFα-induced NF-κB. Furthermore, the addition of O6-benzylguanine enhances the inhibition of NF-κB by temozolomide in H80AGT cells but not H80pcDNA3 cells [Fig. 3D; O6-benzylguanine alone has no effect on the NF-κB reporter (Fig. 3A)].

Because methylation of guanine mediates the inhibition of NF-κB by temozolomide, we investigated whether inhibition of NF-κB is a finding common to agents that alkylate guanine. Whereas the S9-type methylating agent methylnitrosourea dose-dependently inhibits NF-κB activity (Fig. 4A), the chloroethylating agent BCNU, administered at a dose that is almost 10 times more cytotoxic than 100 μmol/L temozolomide (37, 38), has no inhibitory effect on TNFα-induced NF-κB in either H80pcDNA3 cells (Fig. 4B) or H80AGT cells (data not shown). These data suggest that O6-alkylation in general does not lead to inhibition of NF-κB.

We also evaluated whether temozolomide-induced formation of N3-methyladenine, an adduct that reportedly inhibits NF-κB via
poly(ADP-ribose) polymerase 1 (PARP-1) activation (39), plays a role in inhibition of NF-κB by temozolomide. Although temozolomide activates PARP-1 (40), inhibition of PARP-1 with 3-aminobenzamide does not reverse the inhibitory effect of temozolomide on NF-κB (data not shown).

O6-Methylguanine lesions mismatch with thymine, leading to activation of MMR; we therefore evaluated whether inhibition of NF-κB transcriptional activity by temozolomide requires a functional MMR system. NF-κB activation was studied in the MMR-deficient human colon adenocarcinoma cell line HCT116 and the paired MMR-proficient cell line HCT116+ch3, which contains one copy of chromosome 3 bearing the hMh1 gene (41). Whereas 100 μmol/L temozolomide inhibits TNFα-induced NF-κB activity in HCT116+ch3 cells, there is no significant inhibition

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**Figure 3.** O6-Methylguanine mediates inhibition of NF-κB by temozolomide. A, C, and D, NF-κB–dependent luciferase assay. Columns, mean fold change relative to control of triplicate samples; bars, SD. A, T98 cells were treated with temozolomide for 16 h, 10 ng/mL TNFα, and/or 50 μmol/L O6-benzylguanine (BG; given 1 h before temozolomide). *, P < 0.01. B, T98 cells were treated with temozolomide for 16 h, 10 ng/mL TNFα, and/or 100 μmol/L O6-benzylguanine (1 h before temozolomide) and NF-κB EMSA done. C. H80 parental and H80pDNA3 and H80AGT cells were treated with temozolomide for 16 h and/or TNFα as indicated. *, P < 0.01. Inset, AGT activity determined as described in Materials and Methods (activity is in femtomoles per milligram of protein). D, H80AGT and H80pDNA3 cells were treated with 100 μmol/L temozolomide for 16 h, O6-benzylguanine (1 h before temozolomide), and 10 ng/mL TNFα as in (B). Representative of three separate experiments with similar results.
seen in HCT116 cells ($P > 0.2$; Fig. 4C). To confirm the importance of MMR, we studied the paired lymphoblastoid cells, TK6 and MT1 (42). Consistently, 25 μmol/L temozolomide inhibits NF-κB activity in MMR-proficient TK6 cells but concentrations as high as 100 μmol/L temozolomide have no effect on NF-κB in MMR-deficient MT1 cells (Fig. 4D). These data, considered together, indicate that it is the processing of $O^6$-methylguanine adducts by the MMR system that leads to the inhibition of NF-κB.

### Temozolomide inhibits p65 DNA binding

Inhibition of NF-κB on EMSA without inhibition of IκBα degradation suggests that temozolomide inhibits either NF-κB nuclear translocation or DNA binding. To examine this, we first looked at the cellular distribution of NF-κB using immunofluorescence staining. p65 becomes concentrated within the nucleus following TNFα stimulation (Fig. 5A) and pretreatment with temozolomide does not alter this distribution pattern. To confirm the immunofluorescence data, Western blotting of nuclear and cytoplasmic fractions was done showing an increase in nuclear p65 following TNFα stimulation, a finding temporally consistent with IκBα degradation (Fig. 2A), and that pretreatment with temozolomide does not significantly alter this nuclear accumulation (Supplementary Fig. S2).

These results suggest that temozolomide inhibits NF-κB DNA binding. To examine this, nuclear extracts were isolated from TK6 cells that had been stimulated with TNFα and EMSA done following incubation with temozolomide. Temozolomide at 25 μmol/L inhibits NF-κB binding to the DNA probe without altering the level p65 protein (Fig. 5B, top). Similar inhibition of DNA binding is seen in U87 cells (Supplementary Fig. S3) and, consistent with the luciferase data, inhibition is only seen following incubation with temozolomide for 16 h (Supplementary Fig. S3, right). Inhibition of DNA binding by 16-h temozolomide treatment was also confirmed by an ELISA-based DNA binding assay in TNFα-stimulated nuclear extracts (Supplementary Fig. S4). The presence of DNA and MMR protein, both necessary for inhibition of NF-κB (Fig. 4), in the nuclear fraction extracts was verified by DNA gel electrophoresis (Supplementary Fig. S5, top) and Western blotting for MSH2 (Supplementary Fig. S5, right), respectively. In addition, DNA binding assay with purified recombinant p65 shows that temozolomide does not directly affect p65 DNA binding (Fig. 5C). To verify the role of MMR in the inhibition of DNA binding, EMSA of TNFα-stimulated nuclear extracts was done using MMR-deficient MT1 cells, showing that even doses as high as 100 μmol/L temozolomide do not inhibit NF-κB (Fig. 5B, bottom). Next, for further specificity controls, we examined the effect of temozolomide on NF-κB in cytoplasmic extracts following dissociation of NF-κB from IκBα proteins with DOC (27). As expected, temozolomide does not inhibit NF-κB DNA binding (as assessed by EMSA or binding assay) in the cytoplasmic environment (Supplementary Fig. S6) where there are insignificant levels of DNA or MMR proteins (Supplementary Fig. S5).

We next examined whether inhibition of DNA binding is also evident in vivo by evaluating recruitment of p65 to the promoters of the Bcl-X$_\text{L}$ and COX2 genes using chromatin immunoprecipitation (ChIP) assay. Untreated U87 cells have promoter-bound p65 at baseline, and incubation with temozolomide for 16 h inhibits DNA binding without affecting RNA polymerase II binding (Fig. 5D, top). Pretreatment with temozolomide also reduces TNFα-induced κB-element binding at 1 and 3 h (Fig. 5D, bottom). Similar inhibition of κB-element binding is seen in TK6 cells, whereas no effect of temozolomide on binding is noted in MT1 cells (data not shown). Serum starvation of U87 cells for 24 h considerably reduces basal p65 κB-element binding but does not block temozolomide-induced inhibition (Supplementary Fig. S7).

**Figure 4.** NF-κB is inhibited by $S_{\text{1}}$ methylating agents in the presence of active MMR. A to D, NF-κB–dependent luciferase assay. Columns, mean fold change relative to control of triplicate samples; bars, SD. A, U87 cells were pretreated with 10, 100, and 500 nmol/L methyl nitrosourea (MNU) for 16 h and/or stimulated with TNFα as indicated. B, HBF2MO5 cells were pretreated with BCNU for 16 h and/or stimulated with TNFα. C, HCT116 and HCT116+ch3 cells were untreated (UTC) or pretreated with 100 μmol/L temozolomide for 16 h and/or stimulated with 10 ng/mL TNFα as indicated. *, $P > 0.2$. D, TK6 and MT1 cells were pretreated with 2.5, 25, or 100 μmol/L temozolomide for 16 h and/or stimulated with TNFα. Representative of three separate experiments with similar results.
An important regulatory mechanism of NF-κB activity involves reversible p65 phosphorylation (19). Temozolomide does not significantly alter the TNFα-induced phosphorylation of p65 at either Ser536 (data not shown) or Ser276 (Supplementary Fig. S8). In addition, the antioxidants N-acetylcysteine and DTT do not significantly reverse temozolomide-induced NF-κB DNA binding or luciferase expression (Supplementary Fig. S9), suggesting that redox-sensitive residues are not involved in the inhibitory action of temozolomide.

Pretreatment with temozolomide enhances combination temozolomide/TNFα–induced cytotoxicity. Activation of NF-κB by TNFα blocks cell killing (14) and temozolomide inhibits NF-κB only after pretreatment for many hours (Fig. 1C); thus, when TNFα is given at the same time as temozolomide, NF-κB remains activated. In U87 cells, there is an increase in cell viability when TNFα is administered at the same time as temozolomide compared with treatment with temozolomide alone, a finding not seen when TNFα is given 16 h after temozolomide.

Figure 5. Temozolomide inhibits TNFα-induced p65 DNA binding, not nuclear translocation. A, indirect immunofluorescence staining. U87 cells grown on chamber slides were left untreated (C) or pretreated with 100 μM temozolomide (16 h) and/or with 10 ng/mL TNFα for 30 min. After fixation, p65 was localized with anti-p65 primary antibody and FITC-conjugated secondary antibody and counterstained with DAPI (left column, DAPI; middle column, FITC; right column, merged). B, TK6 cells (left) and MT1 cells (right) were untreated or stimulated with 10 ng/mL TNFα and nuclear fractions isolated at 30 min. Samples were then treated with 25 μM temozolomide (and/or 0.1% DMSO; TK6 cells) or with the indicated concentration of temozolomide (MT1 cells) for 16 h and NF-κB EMSA was done. Supershift with anti-p65 or p50 antibody was done as indicated. Western blot of the TNFα-stimulated nuclear samples with anti-p65 antibody is shown. C, 10 ng of purified recombinant p65 protein, either untreated or incubated with 100 μM temozolomide and/or DMSO for the indicated times, were analyzed by NF-κB DNA binding assay. Wild-type (wt) or mutated (mut) NF-κB consensus oligonucleotide (20 pmol) was administered where indicated. Columns, mean fold change in DNA binding relative to untreated p65 (set to 1) of duplicate samples; bars, SD. Western blot with anti-p65 antibody. D, ChIP assay with Bcl-XL (top) and COX2 (bottom) promoter-specific primers. U87 cells were untreated or treated with 100 μM temozolomide for the times shown (left) or treated with 10 ng/mL TNFα (1 and 3 h) and/or 100 μM temozolomide 16 h pretreatment (right). Immunoprecipitation (IP) was done with the antibodies indicated. Representative of at least three separate experiments with similar results.
TNFα results suggest that TNFα administration of TNFα pretreatment was also examined by colony-forming assay. The data presented herein show for the first time an IκBα-independent mechanism for the inhibition of NF-κB activity by S-adenosylhomocysteine and COX2 inhibitors, it has previously been reported that, in melanoma cells, TNFα decreases NF-κB binding at the Bcl-XL and BAX promoters (43). Such differences in gene expression are consistent with promoter-driven differential regulation of subsets of NF-κB-dependent genes (20) and may be due to gene-specific variations in posttranscriptional p65 modification. Of note, one previous report briefly showed that temozolomide decreases NF-κB on EMSA (39); however, in this study, only the 48-h temozolomide treatment time point was evaluated and no analysis of NF-κB-dependent transcription was done.

Figure 6. Pretreatment with temozolomide enhances combination temozolomide/TNFα–induced cytotoxicity. A. U87 (left) and T98 (right) were untreated or treated with 25 to 100 μmol/L temozolomide alone and/or with 10 ng/mL TNFα. For combination treatment, TNFα was given 16 h after temozolomide (white columns) or at the same time as temozolomide (black columns). Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium assay 48 h following temozolomide administration. Columns, mean viability of triplicate samples relative to the untreated cells (set to 1); bars, SD. *, P < 0.02; **, P > 0.2. B. U87 cells were treated with temozolomide and TNFα alone or in combination: temozolomide was given 16 h before TNFα (Pre) or at the same time as TNFα (Same). Colony-forming assay was done. Columns, mean surviving fraction of duplicate samples relative to untreated; bars, SD. *, P < 0.05. Representative of three separate experiments.

Discussion

The data presented herein show for the first time an IκBα-independent mechanism for the inhibition of NF-κB activity by SN1-type methylating agents like temozolomide. The mechanism of inhibition involves attenuation of p65-κB-element binding. Although temozolomide reduces NF-κB binding at the Bel-XL and COX2 promoters, it has previously been reported that, in melanoma cells, temozolomide increases expression of the NF-κB–regulated chemokine CXCL8 (43). Such differences in gene expression are consistent with promoter-driven differential regulation of subsets of NF-κB–dependent genes (20) and may be due to gene-specific variations in posttranscriptional p65 modification. Of note, one previous report briefly showed that temozolomide decreases NF-κB on EMSA (39); however, in this study, only the 48-h temozolomide treatment time point was evaluated and no analysis of NF-κB–dependent transcription was done.

Temozolomide inhibits NF-κB activity through formation of O6-methylguanine adducts, a finding supported by experiments incorporating overexpression and inhibition of AGT. That cellular AGT level by itself is not responsible for the changes in NF-κB can be seen in experiments with BCNU, in that BCNU does not significantly inhibit NF-κB in either AGT-positive or AGT-negative cells (Fig. 4B, and data not shown), although AGT can efficiently repair O6-chloroethyguanine lesions (6). Interestingly, O6-methylguanine adducts have previously been reported to directly block NF-κB DNA binding suggests that temozolomide takes many hours to develop (Fig. 1C). Furthermore, when DNA binding is assessed with TNFα-stimulated nuclear extracts that are treated with temozolomide after isolation of the nuclei (Fig. 5B) and Supplementary Fig. S4), inhibition of NF-κB binding is observed despite the use of an unmethylated DNA probe. These in vitro experiments using nuclear extracts also indicate that the intermediates necessary for inhibition of NF-κB are already present within the nuclear contents and are activated following formation of O6-methylguanine.

Attenuation of NF-κB DNA binding suggests that temozolomide may exert its inhibitory action via an effect on the p65 RHD. In this regard, NH2-terminal cleavage of p65 represents one potential mechanism by which temozolomide could block NF-κB DNA binding. To investigate this, we evaluated p65 following treatment with temozolomide using an antibody that specifically recognizes the COOH terminus of the molecule. No cleavage fragment formation was seen (Supplementary Fig. S10), indicating that temozolomide does not cleave the p65 RHD to block DNA binding. We also observed no effect of temozolomide on the phospho-status of Ser276, an important residue in the RHD involved with NF-κB...
transactivation (46). In addition, treatment with reducing agents does not reverse the inhibition of NF-κB, suggesting that, unlike certain natural compounds (47), temozolomide does not block DNA binding via an effect on redox-sensitive residues. Several other sites in the RHD are known to be important for NF-κB DNA binding (17). Although temozolomide may target these sites, it is also possible that temozolomide acts at the COOH-terminal transactivation domain of p65, resulting in a molecular conforma-
tional change that then blocks DNA binding.

O6-Methylguanine adducts and MMR are not only necessary for the inhibition of NF-κB by temozolomide but also to the cytotoxicity of S9,1-type methylating agents. This finding raises the question of whether temozolomide-induced cell death is mediated by temozolomide-induced inhibition of NF-κB. Although there is no prior evidence to support this, our results, and the importance of NF-κB activation in promoting cell survival, are consistent with such a hypothesis. In this regard, we see that temozolomide administration results in reduced expression of the antiapoptotic genes Bcl-X and XIAP (Fig. 1D), a finding similar to reports that O6-methylguanine leads to inhibition of Bcl-2 (10), an NF-κB–responsive gene (48), and to activation of mitochondrial apoptosis cascade proteins (12). The requirement of MMR activity for inhibition of NF-κB is also reasonable in light of the central role played by MMR in DNA damage signaling (7, 49) and illustrates a potential mechanism by which MMR can target the removal of injured cells from the circulation. Interestingly, temozolomide-induced inhibition of NF-κB is blocked when the MMR system is disabled through a defect in either MutS α or MutL α (HCT116 cells; ref. 7). This finding suggests that inhibition of NF-κB by temozolomide is not mediated by a specific MMR protein but by MMR processing of O6-methylguanine in general.

The role of NF-κB in mediating cellular proliferation, oncogen-
esis, and resistance to therapy (15) has made it an important target in cancer therapy. Consequently, much investigation is currently focused on developing clinically useful inhibitors of this transcription factor (50). Understanding the mechanism by which temozolomide inhibits NF-κB can significantly increase our knowledge of the mechanism underlying MMR-induced damage signaling. Furthermore, exploiting this cellular damage response can potentially improve the design of rationally based combinatorial treatment strategies that are necessary for the successful management of this devastating disease.

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