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Decoy receptor DcR1 is induced in a p50/Bcl3-dependent manner and attenuates the efficacy of temozolomide

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Abstract

Temozolomide is used widely to treat malignant glioma but the overall response to this agent is generally poor. Resistance to DNA damaging drugs such as temozolomide has been related to the induction of anti-apoptotic proteins. Specifically, the transcription factor NF-κB has been suggested to participate in promoting the survival of cells exposed to chemotherapy. To identify factors that modulate cytotoxicity in the setting of DNA damage, we used an unbiased strategy to examine the NF-κB-dependent expression profile induced by temozolomide. By this route, we defined the decoy receptor DcR1 as a temozolomide response gene induced by a mechanism relying upon p50/NF-κB1. A conserved NF-κB binding sequence (κB-site) was identified in the proximal promoter and demonstrated to be required for DcR1 induction by temozolomide. Loss-of-function and gain-of-function studies reveal that the atypical IκB protein, Bcl3, is also required for induction of DcR1 by temozolomide. Mechanistically, DcR1 attenuates temozolomide efficacy by blunting activation of the Fas receptor pathway in $p53^{+/+}$ glioma cells. Intracranial xenograft studies show that DcR1 depletion in glioma cells enhances the efficacy of temozolomide. Taken together, our results show how DcR1 upregulation mediates temozolomide resistance, and provide a rationale for DcR1 targeting as a strategy to sensitize gliomas to this widely used chemotherapy.

Keywords

Glioblastoma multiforme; DcR1; temozolomide; NF-κB; Bcl3

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Introduction

Over the past decade, the oral alkylating agent temozolomide has become the standard chemotherapeutic for the management of malignant glioma. Specifically, the addition of temozolomide to ionizing radiation improves overall patient survival by approximately two months (1). Despite the clinical success of temozolomide, it is clear that many patients respond poorly to this agent at least in part due to the intrinsic resistance of tumor cells to damage-induced cell death. Temozolomide causes cytotoxicity by forming O^6-methylguanine (O^6-MeG) adducts that mismatch with deoxythymidine residues and induce apoptosis following processing by the mismatch repair (MMR) system (2). While the repair protein O^6-methylguanine DNA methyltransferase (MGMT) plays a prominent role in resistance to temozolomide (3,4), downstream factors that regulate induction of apoptosis are also important in the overall response.

Nuclear factor-κB (NF-κB) is a pivotal factor in the cytotoxic response to DNA damage (5). Although DNA double-strand breaks (DSBs) activate NF-κB by a well elucidated nuclear to cytoplasmic pathway (6), temozolomide and other replication stress-inducing agents modulate NF-κB signaling in a more complex, promoter-specific manner (7-9). The NF-κB family of proteins is comprised of five subunits: p50 (NF-κB1, p105), p52 (NF-κB2, p100), p65 (relA), relB, and crel that appear in their mature form as dimers (10). In general, NF-κB dimers are retained in the cytoplasm through interaction with the inhibitor-kB (IkB) proteins and, following translocation to the nucleus, mediate their effect by binding to specific consensus elements (κB-sites) in the promoter regions of genes. While all NF-κB subunits contain a conserved N-terminal rel homology domain (RHD), only p65, relB and crel have a C-terminal transactivation domain (TAD). p50 is a ubiquitously expressed NF-κB subunit that is targeted by the temozolomide-induced DNA damage response (8). Despite the lack of a TAD, p50 can induce NF-κB-dependent gene expression by associating with other rel subunits or co-regulator proteins. Bcl3 is one such NF-κB co-regulator that was originally identified at the t(14;19) chromosomal translocation in chronic lymphocytic leukemia patients (11,12). Bcl3 was subsequently found to be an atypical IkB protein that modulates NF-κB transcriptional activity in conjunction with p50- or p52-containing dimers (13). In this capacity, Bcl3 has been reported to induce the expression of anti-apoptotic factors such as Bcl2 or Hdm2 (14,15).

Apoptosis in response to temozolomide is reported to involve both the intrinsic, or mitochondrial, pathway and the extrinsic, or receptor-induced, response (16,17). The extrinsic pathway is initiated by receptor-ligand binding resulting in caspase-mediated cell death (18). TNF receptor superfamily members such as Fas receptor (Fas, CD95, Apo-1) and TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1, DR4) or TRAIL-R2 (DR5) mediate killing via the extrinsic response. In addition to these death receptors, two decoy receptors, DcR1 (TRAIL-R3, TRID, TNFRSF10C) and DcR2 (TRAIL-R4, TRUNDD), have been described. Decoy receptors do not induce cell death as they lack critical components that are necessary for apoptosis signaling (19). DcR1, a model decoy receptor, is found preferentially in untransformed cells (20) and attenuates apoptosis by competing with DR4 and DR5 or by forming an inactive heteromer with other death

receptors (21). DcR1, like other TRAIL receptors, is a p53 target gene with an intronic p53-binding site (BS) that is induced by DNA damaging agents (22).

In the current study, using an unbiased genome-wide expression analysis, we identify DcR1 as a factor significantly induced by temozolomide in a p50-dependent fashion. Mechanistic studies demonstrate that DcR1 is co-regulated by p50 and Bcl3 via a novel κB-site, and *in vitro* and animal studies demonstrate that depletion of DcR1 sensitizes gliomas to cytotoxicity by temozolomide. Together, these findings support the observation that temozolomide induces apoptosis via the death receptor pathway and suggest that targeting DcR1 is a strategy that can potentially enhance the anti-glioma effect of temozolomide clinically.

Materials and Methods

Cell lines, reagents and plasmids

Human U87, A172, T98 and U251 glioblastoma cells were purchased from American Type Culture Collection and authenticated by routine morphological and growth analysis and also by western blotting. Cells were cultured as previously described (8). U87 glioma cells expressing sh-p105 or sh-control were also previously described (8). pCMV-p50 was previously described (8), and used for experiments in Figure 4. HA-p50 was cloned from the template, p50 cFlag pcDNA3 (Addgene plasmid 20018), obtained from Dr. Stephen Smale, following excision of the Flag and insertion of an HA tag. The Bcl3 expression construct, Bcl3-pFlag-CMV2, was a kind gift from Dr. Albert Baldwin (University of North Carolina).

RNA interference and stable transfectants

The following siRNA constructs were obtained from Dharmacon: siGENOME Human Bcl3, si-p53 (M-3329-03), si-DcR1 (sc-40235) and si-scrambled control (D-001210-03-05). Also, si-p50 (sense: GUCACUCUAACGUAUGCAAUU) and si-control (sense: CCUACGCCACCAAUUUCGUUU) were obtained from Santa Cruz. All siRNA constructs were transfected using Oligofectamine (Invitrogen).

To make cells stably expressing sh-DcR1, PAGE-purified oligos (sense: GATCCGCTGAAGAGACAATGAACATTCAAGAGATGTTCATTGTCTCTTCAGCTTTTTTACGCGTG and antisense: ATTCACGCGTAAAAAAGCTGAAGAGACAATGAACATCTCTTGAATGTTCATTGTCTCTTCAGCG) or scrambled control, were obtained from IDT and annealed. Oligos were ligated into the BamHI and EcoRI sites of the retrovirus: pSIREN-RetroQ-DsRed (Clontech). For retroviral production, sh-control and sh-DcR1 vectors were co-transfected with pCMV-VSV-G into Plat-GP cells using Xtreme gene according to manufacturer's protocol (Roche). After 48 hours, the supernatant was cleared using a 0.45 μm syringe and concentrated using Clontech Retro-X at 3.5 ml per 1 ml of viral supernatant. The virus was collected by centrifugation at 1500 g for 45 minutes. The pellet was resuspended in regular media with 20 μl polybrene and added to U87 cells. Cells were split after 48 hours and maintained in regular media. 80- 90 % infection efficiency was determined by expression of Ds-Red, and knockdown of DcR1 verified by mRNA and protein analysis.

Immunoblot and electrophoretic mobility shift assay (EMSA)

Immunoblotting was performed using whole cell lysate as previously described (23). Primary antibodies used include: anti-Bcl3 (Santa Cruz, sc185), anti-p21 (Santa Cruz, sc397), anti-p50 (Santa Cruz, sc7178), anti-GAPDH (Santa Cruz, sc-137179), anti-p53 (Santa Cruz, sc71818), anti-DcR1 (R & D Systems, 398600), anti-HA (Covance, MMS-101R). Alexa-Fluor 680 and Alexa-Fluor 800 fluorescent dye-conjugated secondary antibodies (Invitrogen) were used for visualization with Odyssey Infrared system (LICOR Biosciences). EMSA was performed as previously described (8) with competition using cold specific and non-specific probes and supershift with anti-p50. The κB probe sequence is shown in Figure 4.

Quantitative real-time polymerase chain reaction (qPCR) and quantitative chromatin immunoprecipitation (qChIP)

qPCR was performed following total mRNA isolation as described (8). *DCR1* was normalized to *GAPDH* and relative *DCR1* mRNA expression is shown as the average of three experiments performed in triplicate. Primers used for *DCR1* are: sense, CACCAACGCTTCCAACAATGAACC and antisense, TCCGGAAGGTGCCTTCTTTACACT.

qChIP was also performed as described (8) following IP with anti-p50 or anti-Bcl3 antibodies. Control IP was performed using anti-Histone H1 and anti-mouse IgG. qPCR was performed using promoter-specific primers for human DcR1 that span the region encompassing the putative κB-site (sense, CCTCGACCATGCAAAGGGT and antisense, ACAGAATGAAGGACACAGGGG) and the change in DNA enrichment for each IP condition determined relative to input DNA. To control for non-specific binding, the anti-p50 or anti-Bcl3 data was subtracted from the anti-H1 results (anti-IgG showed no binding) as previously described (8). The PCR product was also run on a gel for a semi-quantitative analysis of the data.

Flow cytometric analysis of apoptosis and DcR1 expression

Analysis of apoptosis was performed as described (24) at 72 or 96 hours following treatment. For DcR1 surface expression, cells were treated with temozolomide and trypsinized, washed with blocking buffer (PBS + 1% BSA) and centrifuged at 1500 RPM. The cell pellet was divided into 2 groups and incubated with anti-DcR1 (Abcam), or anti-IgG control. Pellets were incubated with Alexa-fluor-conjugated secondary antibody and analyzed on an LSRII Flow Cytometer (BD Biosciences). Samples were run in triplicate and data analyzed using FlowJo (TreeStar Inc.).

Luciferase reporter and luciferase assay

For construction of a DcR1 luciferase reporter, we amplified a segment of DNA containing the *DCR1* proximal promoter and part of intron 1 using fresh human genomic DNA with the following primers: GATAGACACTTGACTGGGGGAC and GAAGAACTGGGTC. After cloning the 1.232 kbp segment into the pCR II Topo Vector (Invitrogen), the *DCR1* sequence was liberated using XhoI and HindIII and ligated into the pGl4.20 luciferase

vector. Site-directed mutagenesis, using the QuickChange Lightning II mutagenesis kit, was used to mutate the κB and p53 BSs to the sequences indicated in Figure 4.

For luciferase assay, cells were co-transfected with the indicated reporter and *Renilla reniformis* and relative luciferase calculated after treatment with temozolomide as described (23). All experiments were performed in triplicate.

DcR1 cDNA cloning and lentiviral infection

The coding region of *TNFRSF10C* (NM_003841) was amplified (sense: AGCAGGCTCCGAATTCGCCACCATGGCCCGGATCCCCAAGACCCTA and antisense: AAGCTGGGTCGAATTCTCAAGCGTAATCTGGAACATCGTATGGG TAAACAAACACAATCAGAAGCACAATTAG) using genomic DNA from HEK293T cells. An HA tag was added to the C-terminus for immunoblot detection. This cDNA was then inserted into the pLVX-Puro vector (Clontech) and lentivirus generated using the packaging system from Addgene (pMD2.G and psPAX). U87 cells were then infected with HA-DcR1 lentivirus and selected with puromycin for 5 days.

Clonogenic and trypan blue assays

Assays were performed essentially as described (8,24). FAS neutralizing antibody (FNAb) was obtained from Millipore (#05-338). For clonogenic assay with FNAb, U87 cells were transfected with *DCR1*, or control, siRNA and after 48 hours replated. Cells were then treated with 25 μM temozolomide and 1 μg/ml FNAb (or vehicle). FNAb was re-administered once 16 hours later and colony number counted at 2 weeks. Data show the change in surviving fraction in temozolomide treated samples in the presence and absence of FNAb in cells expressing the different siRNAs. Experiment was performed in triplicate and repeated. Clonogenic assay was performed after treatment of U87 cells with 30 ng/ml FAS ligand in the presence and absence of FNAb.

Nanoparticle (NP) production and characterization

NPs were manufactured and characterized as previously described (25) by LNK Chemsolutions LLC, USA. SiRNA constructs were incorporated into NPs in a similar fashion to incorporation of other agents (25) and the product maintained in sterile conditions.

Animal studies

Six to seven-week old male nude mice (Harlan) were used in accordance with guidelines of the Institutional Animal Care and Use Committee of the University of Chicago. For intracranial studies, $5x10^5$ U87 cells were injected into the right striatum as previously described (24) and the animals randomized into 6 groups (n = 6 per group). 10 μL of either PBS or nanoparticles (NP) carrying si-Control or si-DcR1 were injected intracranially on day 4 and 7 following tumor inoculation. Mice were also treated with intraperitoneal (i.p.) injection of vehicle or temozolomide on day 4 (5 mg/kg), 7 (5 mg/kg) and 10 (2.5 mg/kg) (total temozolomide dose: 12.5 mg/kg). Animals were followed until terminal, sacrificed and brains harvested for verification of tumor.

For hindlimb studies, $7.5x10^6$ U87 cells were injected into the right hind limb and animals randomized into 6 groups (n = 5 per group) when tumors reached an average of 120 mm^3 (day 0). Mice were treated with daily intratumoral (i.t.) injection of 50 μl NPs containing either si-control or si-DcR1 for 5 consecutive days, and/or i.p. temozolomide (total dose: 12.5 mg/kg). Control animals were injected i.t. or i.p. with vehicle. Tumor volume was measured and documented every 2- 3 days.

Statistical Analyses

Results are expressed as mean ±SD and significance determined as $P < 0.05$ using a 2-tailed student's t-test.

RESULTS

TMZ induces DcR1 protein expression

To identify NF-κB-dependent factors that modulate the response to temozolomide, we performed an unbiased genome-wide expression analysis in isogenic glioma cells following treatment with temozolomide. U87 cells stably expressing sh-p105 or sh-control, described previously (8), were treated with temozolomide or vehicle and mRNA expression analyzed using the affymetrix Human Genome U133 Plus 2.0 Array. The expression data will be described in detail in a separate publication (Voce, D *et. al*. manuscript in preparation). *DCR1* (*TNFRSF10C*) was identified as one of a small set of genes significantly induced only in p50-proficient cells following treatment (Supplementary Figure S1A). Given the importance of DcR1 in attenuating death receptor-induced apoptosis, we examined this decoy receptor in the response to temozolomide. DcR1 is induced within 24 hours in U87 cells (Figure 1A) and the induction occurs in a concentration-dependent manner (Figure 1B). As DcR1 is a cell surface receptor, the change in DcR1 expression was quantified using flow cytometry and, consistent with the immunoblot analysis, DcR1 surface expression is induced by temozolomide (Supplementary Figure S1B). In addition, temozolomide induces expression of *DCR1* mRNA (Figure 1C). These results indicate that temozolomide up-regulates DcR1 in glioma cells.

DcR1 is induced in a p50 and p53 co-dependent manner

To verify the p50 dependence of DcR1, U87 cells expressing sh-p105 or sh-control were used. Consistent with the array findings, loss of p105/p50 blocks induction of DcR1 at the mRNA (Figure 2A) and protein level (Figure 2B). Moreover, knockdown of p105 also attenuates DcR1 surface expression (Supplementary Figure S1C). Importantly, re-expression of p50 in sh-p105 cells enables DcR1 induction, confirming that it is p50, and not p105, that is necessary for temozolomide-induced expression of DcR1 (Figure 2C). Of note, re-expression of p50 is possible because the sh-p105 targets the C-terminal of p105 (8). DcR1 is also a p53 target gene (22), and temozolomide increases expression of p53, and its downstream target p21, with a time course consistent with activation of DcR1 (Supplementary Figure S1D). Knockdown of p53 blocks the ability of temozolomide to induce *DCR1* mRNA in U87 cells (Figure 2D). In addition, consistent with p53-dependence, temozolomide does not induce DcR1 in either T98 or U251 glioma cells, both of which are p53 mutant, even at a concentration of 250 μM (Figure 2E). These results indicate that DcR1

is induced by temozolomide in a p50-dependent manner and also verify the importance of p53 in regulating DNA damage-induced DcR1.

DcR1 contains a conserved kB-site that binds p50

As our data indicate that DcR1 is transcriptionally regulated by NF-κB, we therefore searched the promoter and first intron of *DCR1* (NCBI Gene ID: 8794) for potential NF-κB consensus elements. Using the program, TFSEARCH (26), a 10-nucleotide sequence sharing 86% homology with the canonical kB-site was identified in the *DCR1* proximal promoter (Figure 3A). *DCR1* has only been described in primates (NCBI search *TNFRSF10C*) and an identical nucleotide sequence is found in other primate species (Figure 3B). Gel shift assay (EMSA) shows that the putative kB-site binds NF-κB *in vitro* and supershift analysis demonstrates that p50 is present within the NF-κB band (Figure 3C). Also, temozolomide does not alter the binding of NF-κB to the kB-site (Figure 3C). To examine whether p50 binds the *DCR1* promoter *in vivo*, ChIP assay was performed. p50 is recruited to the region of the *DCR1* promoter containing the kB-site, and consistent with the EMSA, temozolomide does not significantly change this (Figure 3D). A blot of the PCR product further verifies the enrichment of p50 in this region (Supplementary Figure S2A, right).

To examine the functional significance of this consensus site, we constructed a luciferase reporter bearing a 1.232 kbp region encompassing the proximal promoter, exon 1 and a segment of the first intron of *DCR1* (Figure 4A). Given information from previous DcR1 promoter/reporters (22,27), our reporter was constructed to contain the p53 BS shown to be important for DcR1 induction. Temozolomide induces expression from this reporter that peaks at 12 hours (Figure 4B). Mutation of the p53 BS or kB-site blocks basal and induced reporter activity (Figure 4C) demonstrating that the putative kB-site is necessary for activity of the reporter. We next examined whether this site is regulated by p50. Knockdown of p105/p50 blocks induction of activity from the wildtype (wt) reporter in response to temozolomide (Figure 4D). Moreover, when p50 is re-expressed in sh-p105 cells, a substantial increase in basal activity is seen that is further increased by temozolomide (Figure 4D, right). These results suggest that a kB-site in the *DCR1* promoter binds p50 and is required for p50-mediated induction by temozolomide.

Induction of DcR1 by temozolomide requires Bcl3

Despite the p50/p53 co-dependence of DcR1, in A172 cells that are p53-wt (28) and have abundant p50, temozolomide only slightly induces expression of DcR1 (Figure 5A). To further study this observation, we looked at the cytogenetics of A172 cells and noted that these cells have a significant deletion near chromosome 19q13.2 (29), a region containing the locus of the NF-κB co-regulator, *BCL3*. As Bcl3 plays a prominent role in p50-dependent gene activation (30), we investigated whether DcR1 requires Bcl3 for induction by temozolomide. First, it is evident that although A172 cells do express Bcl3, they have substantially less than some other glioma cell lines (Figure 5B). Also, in U87 cells ChIP assay confirms that Bcl3 is bound to the *DCR1* promoter and its recruitment minimally altered by treatment (Supplementary Figure S2A). Knock-down of Bcl3 completely blocks the ability of temozolomide to induce *DCR1* mRNA (Figure 5C) and protein expression (Figure 5D). Moreover, depletion of Bcl3 blocks the ability of temozolomide to induce

activation of the *DCR1* promoter/intron 1 reporter (Figure 5E). To confirm the role of Bcl3, we obtained a Bcl3 cDNA construct (gift from Dr. Albert Baldwin). Over-expression of Bcl3 in A172 cells enables temozolomide to induce DcR1 expression (Figure 5F) an observation also noted in U87 cells (Supplementary Figure S2B). Together, these data indicate that temozolomide requires Bcl3 to induce DcR1 expression.

Induction of DcR1 blocks the cytotoxic effect of temozolomide

DcR1 attenuates apoptosis (21) suggesting that its induction by temozolomide acts to block killing. Depletion of DcR1 leads to a significant increase in apoptosis following temozolomide treatment as assessed by annexin V binding ($P < 0.05$, Figure 6A). As a specificity control, we also constructed U87 cell lines expressing shRNAs targeting *DcR1* or a scrambled sequence. Three DcR1 shRNA constructs were used and cell lines screened for knockdown of DcR1 using qPCR (Supplementary Figure S2C). Cells expressing sh-DcR1-3 were selected and depletion of DcR1 protein confirmed by immunoblot (Figure 6B, inset). Sh-RNA depletion of DcR1 augments induction of apoptosis by temozolomide (Figure 6B) supporting the siRNA data and confirming that the effect is not due to the specific siRNA used. In addition, as temozolomide induces cytotoxicity at late times following treatment, we examined cell viability over time using trypan blue dye exclusion. Temozolomide increases the percentage of non-viable cells over time and knock-down of DcR1 augments this effect beginning 72 hours after treatment (Supplementary Figure S2D). As DcR1 is not induced by temozolomide in U251 cells, we also examined cytotoxicity in these cells. Knockdown of DcR1 does not affect induction of apoptosis by temozolomide in U251 cells (Supplementary Figure S3A).

We next examined clonal survival. Depletion of DcR1 augments the decrease in survival induced by temozolomide in U87 cells (Figure 6C) and to a lesser extent in A172 cells (Supplementary Figure S3B), likely due to the lower level of DcR1 induction in A172 cells compared to U87 cells. By contrast, in U251 cells no significant change in clonal survival is seen in response to temozolomide following DcR1 depletion compared to control (Supplementary Figure S3C). To further study the importance of DcR1, we cloned a cDNA of human HA-tagged DcR1 and constructed an HA-DcR1 lentiviral expression vector. U87 cells were infected with this construct and stable expression of HA-DcR1 confirmed by immunoblot (Figure 6D). Over-expression of DcR1 significantly attenuates the decrease in clonal survival induced by temozolomide relative to vector control (Figure 6D). Taken together, over-expression and down-regulation studies indicate that DcR1 acts to attenuate the anti-glioma effect of temozolomide.

Given that DcR1 specifically blocks death receptor-induced cytotoxicity, we sought to examine whether DcR1 affects the temozolomide-induced death receptor response. It was previously noted that induction of apoptosis by temozolomide is attenuated by a FAS neutralizing antibody (FNAb) that blocks death receptor signaling (17). We used FNAb in temozolomide treated U87 cells that were initially transfected with siRNA targeting either DcR1 or a control sequence. While exposure to FNAb results in a 1.2-fold increase in clonal survival in temozolomide-treated cells expressing si-control, in cells depleted of DcR1 there is almost a 4-fold increase in survival in the presence of FNAb (Figure 6E). The observation

that blocking the death receptor response with FNAb has a more profound effect in the presence of DcR1 knockdown, suggests that DcR1 inhibits apoptosis induced by temozolomide specifically by affecting the death-receptor pathway. Importantly, we verified that in our hands FNAb does attenuate the decrease in survival induced by Fas ligand (FasL) (Supplementary Figure S4A). Moreover, although FNAb does not directly bind DcR1, both FNAb and DcR1 modulate apoptosis via the same receptor-mediated downstream signaling cascade (31). Consistent with DcR1 playing a role in the Fas response, while knockdown of DcR1 augments the effect of FasL (Supplementary Figure S4B), over-expression of DcR1 completely blocks the effect of FasL (Supplementary Figure S4C). Of note, depletion of DcR1 has much less profound effect than DcR1 over-expression likely because of the low basal DcR1 level. In sum, these findings indicate that induction of DcR1 blocks death receptor-mediated cytotoxicity in response to temozolomide.

Knockdown of DcR1 enhances the effect of temozolomide in a glioma model

We next sought to examine DcR1 depletion in an animal glioma model. As there are no DcR1 inhibitors, blocking this factor requires a genetic approach. Stable sh-DcR1 cells do not form xenografts, therefore, we targeted DcR1 by encapsulating and delivering siRNA using a biodegradable nanoparticle (NP) vector previously described for the treatment of malignant glioma (25). NPs were fabricated to encapsulate either DcR1 siRNA (NP-si-DcR1) or control siRNA (NP-si-cntl). Exposure of U87 cells to NP-si-DcR1 results in a decrease in DcR1 protein expression relative to vehicle or NP-si-cntl (Figure 7A). In addition, NP-si-DcR1 results in a significant decrease in clonal survival in combination with temozolomide relative to that with temozolomide and NP-si-cntl (Figure 7B). To examine efficacy *in vivo*, intracranial U87 glioma xenografts were established and NPs administered by direct intracranial injection. A significant increase in animal survival is seen in mice treated with combination temozolomide and NP-si-DcR1 compared to either agent alone or to the combination of temozolomide and NP-si-cntl ($P < 0.01$, log-rank: TMZ + NP-si-DcR1 vs. TMZ + NP-si-cntl) (Figure 7C). A similar finding is also seen when hindlimb xenografts are treated with temozolomide and NPs ($P < 0.01$, TMZ + NP-si-DcR1 vs. NP-si-DcR1) (Supplementary Figures S4D). These findings indicate that in an established growing intracranial xenograft, depletion of DcR1 combines effectively with temozolomide to improve the anti-glioma effect.

Discussion

NF-κB plays a central role in regulating the cytotoxic response to DNA damage. To identify potential factors that modulate the cytotoxic effect of temozolomide, we examined the NF-κB-dependent expression profile induced by this agent in glioma cells. *DCR1*, a decoy receptor that attenuates death receptor-induced apoptosis, was identified as an NF-κB/p50-dependent gene significantly induced by treatment. Given that death receptor signaling is important for temozolomide-induced apoptosis (17), we examined the role of DcR1 in the response to temozolomide. Our findings demonstrate that DcR1 is induced by temozolomide in a manner dependent not only on p50 and p53, but also on the proto-oncogene, Bcl3. Moreover, we show that DcR1 attenuates the cytotoxic effect of temozolomide. In support of the ability of DcR1 to attenuate the anti-glioma response, examination of the Repository

for Molecular Brain Neoplasia Data (REMBRANDT) database indicates that patients with up-regulated *DCR1* expression do significantly worse than others (Supplementary Figure S5) (32).

Although it has previously been reported that DNA damage can induce DcR1 and that this response attenuates sensitivity to TRAIL (33), up-regulation of DcR1 has not previously been reported to directly block DNA damage-induced killing. In demonstrating that inhibition of DcR1 augments killing by temozolomide, our data support the hypothesis that temozolomide induces cytotoxicity, at least in part, via the death receptor pathway (17). This hypothesis is further suggested by the finding that FNAb has a greater inhibitory effect on the temozolomide-induced response (i.e. causes a greater increase in survival) when DcR1 is depleted than when DcR1 is present.

The NF-κB-dependence of DcR1 is supported by identification of a conserved κB-site within the proximal promoter of the *DCR1* gene. p50 binds this κB-site *in vitro* and is also recruited to the promoter region containing this sequence *in vivo*. Moreover, this consensus element is necessary for activation of a DcR1 promoter/reporter by temozolomide. Together, these findings support a functional role for the κB-site in regulating DcR1 expression in response to temozolomide. It is notable that the putative *DCR1* κB-site contains a conserved deoxythymidine (T) residue at the −1 position. Interestingly, we previously reported that phosphorylation of p50 by temozolomide results in inhibition of NF-κB binding to κB-sites with a −1C or G, while binding to κB-sites with a −1A or −1T is unaltered (9). Consistent with this observation, p50 binding to the *DCR1* κB-site (5'-GGGATGCCCC-3') is unchanged following temozolomide treatment. In addition, the NF-κB-dependence of DcR1 is also consistent with a previous study that noted that this gene is induced by crel and blocked by IkBα, respectively (34). Although a specific κB-site has not previously been reported for *DCR1*, functional consensus sequences have been identified for both *DR4* and *DR5* (35,36), death receptors that are highly homologous to *DCR1* (31).

DcR1 is induced in a p50-dependent manner; however this NF-κB subunit lacks a TAD and requires either a rel subunit or additional factor to induce NF-κB-dependent gene expression. The atypical IκB protein, Bcl3, is an important NF-κB co-regulator that contains two TADs and has been shown to induce NF-κB-dependent gene expression in conjunction with DNA bound p50 (30). We demonstrate that Bcl3 is necessary for efficient DcR1 expression in response to temozolomide. In support of this hypothesis, while loss of Bcl3 in U87 cells blocks the ability of temozolomide to induce DcR1, Bcl3 over-expression has the opposite affect, enhancing DcR1 induction. In addition, the findings that Bcl3 binds the *DCR1* promoter *in vivo* and that Bcl3 depletion blocks temozolomide-induced expression from the *DCR1* promoter/reporter support the hypothesis that this co-regulator directly modulates the expression of DcR1. From a mechanistic perspective, given the minimal change in p50 and Bcl3 recruitment to the *DCR1* promoter following treatment (Figure 3D and Supplementary Figure S2A), it is likely that temozolomide promotes DcR1 expression by inducing NF-κB post-translational modifications and/or by inducing recruitment of additional p50- or Bcl3-dependent co-factors (37).

DcR1, like its counterpart DcR2, was initially identified as a receptor preferentially expressed in normal tissues relative to cancer cells (20,38). However, it is evident that even in cancer cells DcR1 expression is inducible and attenuates DNA damage-induced cytotoxicity. Similarly, DcR2 is also induced by chemotherapeutics in malignant cells (39). These observations raise the possibility that targeting decoy receptors is a strategy that can potentially enhance the therapeutic effect of DNA damaging agents. Consistent with this hypothesis, we demonstrate that depletion of DcR1 enhances the cytotoxic effect of temozolomide. Moreover, by targeting DcR1 in an animal glioma model, we show that blocking expression of this receptor enhances the overall anti-glioma effect. Importantly, the *in vivo* effect of blocking DcR1 is demonstrated against a growing intracranial tumor whose cells were not previously manipulated to deplete DcR1, this finding highlights the potential of DcR1 as a viable clinical chemo-sensitizing target.

Despite the efficacy of RNA interference for targeted depletion of proteins, *in vivo* knock-down of genes is hampered by various factors that include among other things, the protection of the siRNA and efficient delivery to the target tissue (40). To improve these aspects, we encapsulated the DcR1 siRNA in a biocompatible NP vector that directly delivers its payload to the cytoplasm (25). In addition, NPs were injected directly into the brain to concentrate delivery to the tumor cells. While siRNA-mediated DcR1 depletion is good for proof-of-principle studies in an animal model, ultimately, delivery of a pharmacological inhibitor may be a more effective strategy for clinical use.

Inhibition of cellular resistance pathways is an effective strategy to enhance the therapeutic effect of cytotoxic agents. Although blocking NF-κB can improve the anti-tumor effect of certain chemotherapeutics (41), this transcription factor has a variable role in cytotoxic signaling and is often required for cell death (8,42). Nevertheless, NF-κB promotes expression of many anti-apoptotic factors, suggesting that selective targeting of downstream NF-κB-dependent genes is a strategy that can be used to rationally enhance cytotoxicity. While temozolomide induces cell death primarily via the intrinsic apoptotic response in p53 mutant gliomas, in p53 wt tumors the exogenous pathway predominates (17). DcR1 represents an anti-apoptotic factor robustly induced by temozolomide that attenuates killing in p53 wt tumors. Given that virtually every patient diagnosed with malignant glioma is treated with temozolomide, targeting factors such as DcR1 can potentially make a significant impact on patient management specifically in tumors that are wt for p53.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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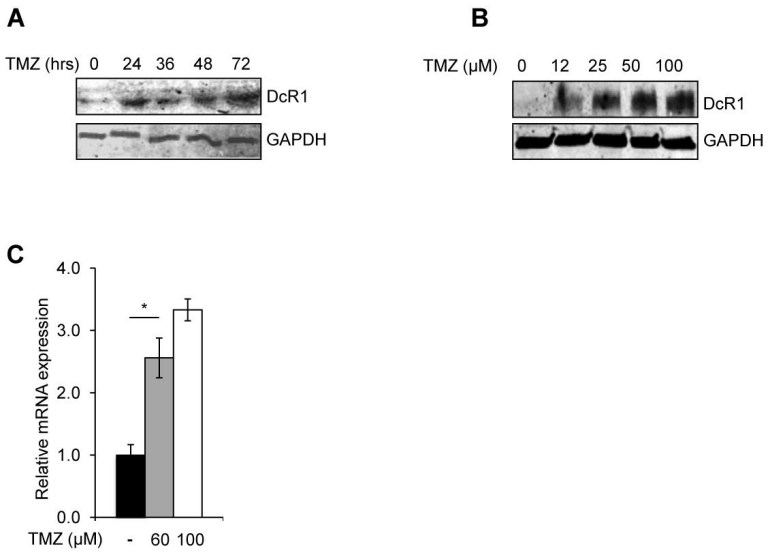

Figure 1.

Temozolomide induces DcR1 expression. A, immunoblot with anti-DcR1, or anti-GAPDH, antibody in U87 glioma cells at the indicated time following treatment with 100 μM temozolomide (TMZ). B, immunoblot with anti-DcR1 48 hours following treatment with TMZ. C, qPCR analysis of *DCR1* mRNA expression in U87 cells 16 hours following treatment with indicated concentration of TMZ. Data show mean *DCR1* transcript expression relative to *GAPDH* ± SD of triplicate samples from 3 separate biological experiments. *, $P < 0.05$.

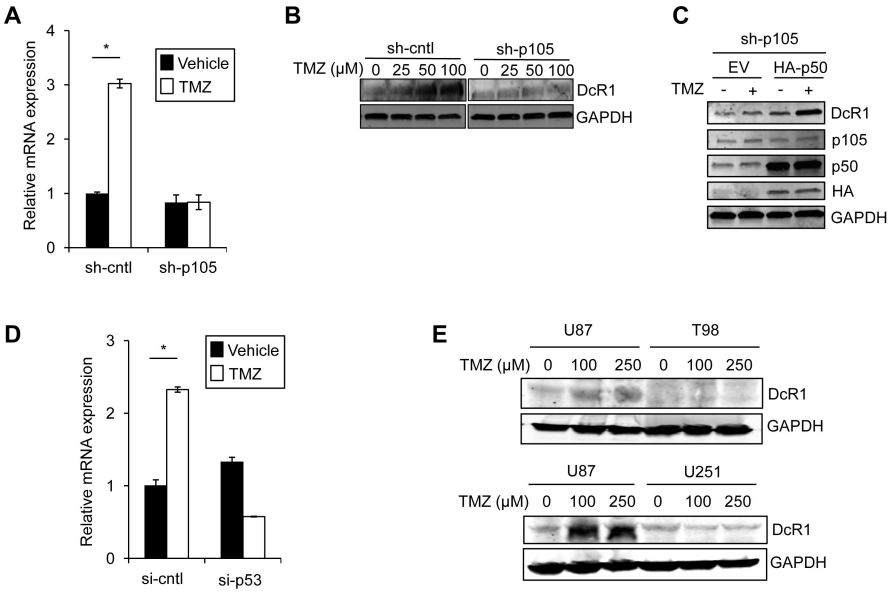

Figure 2.

DcR1 is induced by temozolomide in a p50 and p53 co-dependent manner. A, qPCR analysis of *DCR1* mRNA expression relative to *GAPDH*, ±SD from triplicate samples from 3 experiments using U87 cells stably expressing sh-control (cntl) or sh-p105, 16 hours after treatment with vehicle or 100 μM temozolomide (TMZ). B, immunoblot with anti-DcR1 or anti-GAPDH in cells from (A), 48 hours following treatment with vehicle or TMZ. C, immunoblot with the indicated antibody in U87-sh-p105 cells transfected with HA-p50 or empty vector (EV) following treatment with vehicle or 250 μM TMZ (24 hours). D, qPCR analysis of *DCR1* mRNA expression in U87 cells transfected with si-RNA following treatment with vehicle or 100 μM TMZ (16 hours). E, immunoblot with anti-DcR1 following treatment of the indicated glioma cell lines with TMZ for 48 hours. *, $P < 0.05$.

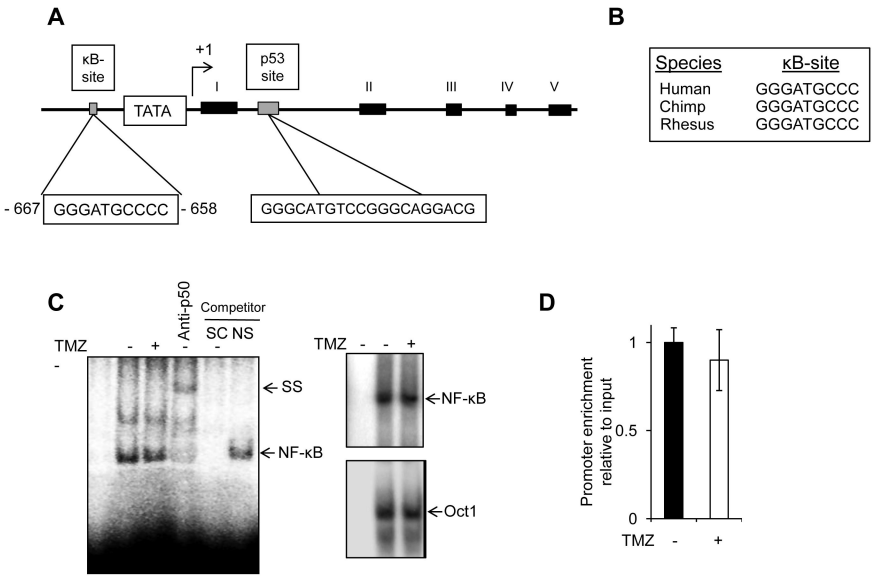

Figure 3.

DCR1 contains a conserved kB-site that binds p50. A, schematic representation of *DCR1* showing the putative κB-site and p53 BS. B, conservation of *DCR1* κB-site sequence in primates. C, EMSA with the DcR1 kB-site probe using nuclear extract from U87 cells treated with vehicle or 100 μM temozolomide (TMZ) (16 hours). Competition with specific and non-specific competitors (SC and NS), respectively. Supershift (SS) was performed using anti-p50. Oct1 EMSA (right) demonstrates equal lysate loading. D, qChIP using *DCR1* promoter-specific primers spanning the putative kB-site. Data show chromatin enrichment of p50 relative to input after controlling for non-specific binding using anti-histone H1 (positive control) and anti-IgG, normalized to vehicle, ± SD of 3 triplicate samples, repeated with similar results

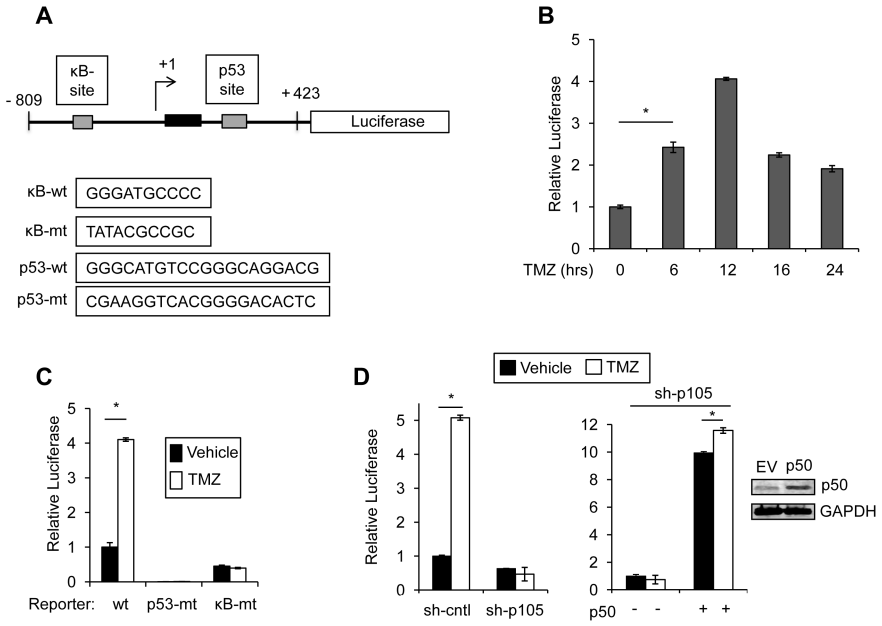

Figure 4.

The kB-site and p50 are required for activation of a *DCR1* promoter/intron 1 reporter by temozolomide. A, schematic representation of the 1.232 kbp luciferase reporter. B, luciferase expression relative to *renilla* in U87 cells using the wt-reporter following treatment with 100 μM temozolomide (TMZ) for the indicated time. C, relative luciferase activity from the indicated reporter following treatment of U87 cells with vehicle or 100 μM TMZ (12 hours). D, relative luciferase in sh-p105 and sh-control cells (left), and in sh-p105 cells transfected with empty vector (EV) or p50 (right) following treatment as in (C). Inset: immunoblot with anti-p50 antibody. Luciferase data represent mean ±SD of triplicate samples. *, $P < 0.01$.

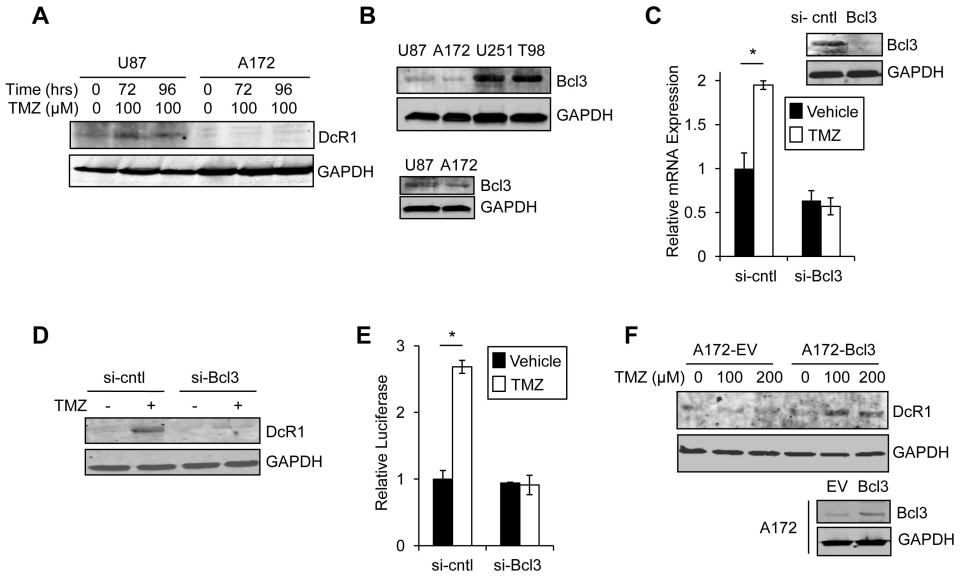

Figure 5.

Bcl3 is required for induction of DcR1 by temozolomide. A, immunoblot with anti-DcR1 in U87 and A172 cells following treatment with temozolomide (TMZ). B, immunoblot with anti-Bcl3 antibody using the indicated glioma cells (40 μg protein). Lower blot: U87 and A172 cells using 80 μg protein. C, qPCR analysis of mean *DCR1* mRNA expression relative to *GAPDH*, ±SD of triplicate samples in U87 cells transfected with siRNA following treatment (TMZ: 100 μM). Inset: immunoblot with anti-Bcl3. D, immunoblot with anti-DcR1 in cells transfected and treated as in (C). E, relative luciferase expression following treatment with vehicle or 100 μM TMZ (16 hours) using the wt reporter in cells transfected as in (C), mean ±SD of triplicate samples shown. F, immunoblot at 48 hours with anti-DcR1 (upper) or anti-Bcl3 (lower) antibody in A172 cells transfected with empty vector (EV) or Bcl3 and treated as shown. *, $P < 0.05$.

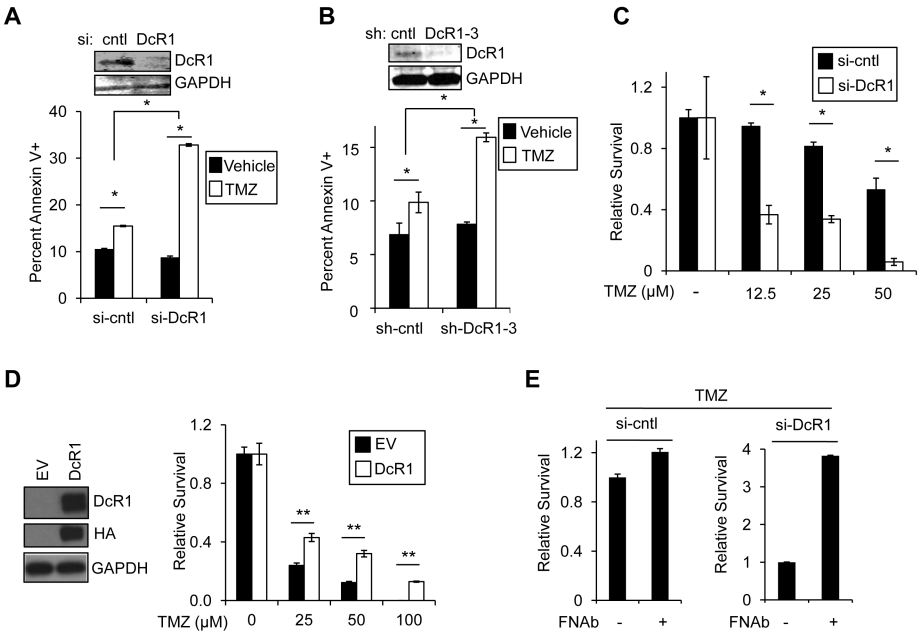

Figure 6.

Depletion of DcR1 sensitizes cells to temozolomide. A, annexin V binding in U87 cells treated as shown (TMZ: 100 μM, 72 hours) following transfection with the indicated siRNA. B, annexin V binding at 96 hours in U87 shRNA clones treated as shown (TMZ: 100 μM). Inset: immunoblot with anti-DcR1. C, clonogenic assay in U87 cells transfected with the indicated siRNA and treated with temozolomide (TMZ). D, clonogenic assay in U87 clones expressing HA-DcR1 or empty vector (EV) following treatment with TMZ. Inset: immunoblot with anti-DcR1 or anti-HA. E, clonogenic assay in U87 cells transfected with the indicated siRNA, treated with 25 μM TMZ and 1 μg/ml FNAb (twice) or vehicle. Data show survival following TMZ treatment in samples treated with FNAb relative to those without FNAb in each siRNA group. All data represent mean ±SD of triplicate samples, repeated with similar findings. *, $P < 0.05$. **, $P < 0.01$.

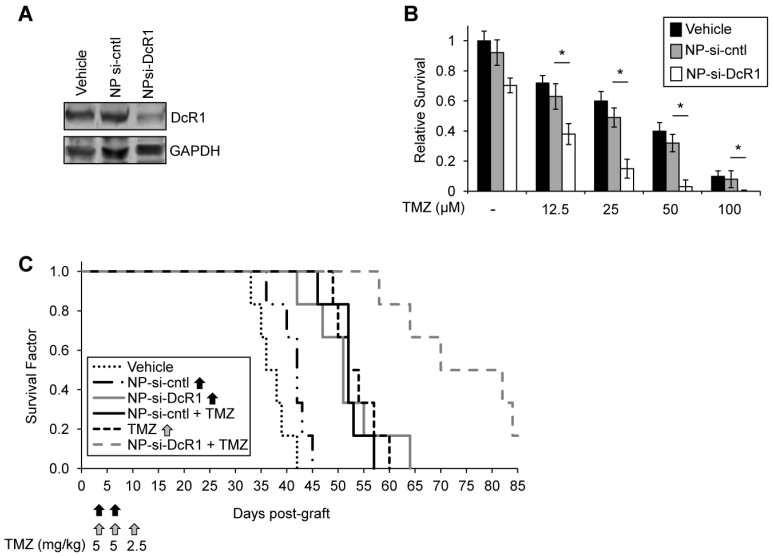

Figure 7.

Knockdown of DcR1 enhances the anti-glioma effect of temozolomide. A, immunoblot with anti-DcR1 antibody in U87 cells treated with vehicle or nanoparticles (NP) carrying the indicated siRNA. B, clonogenic assay in U87 cells treated with vehicle or the indicated NP and temozolomide (TMZ). *, $P < 0.05$. C, Kaplan-Meier survival curves of mice bearing intracranial gliomas (n= 6 mice per group) following treatment with TMZ (days 4, 7 and 10) at the concentrations indicated, and/or NPs carrying the indicated siRNA. $P < 0.01$, Log-rank: TMZ + NP-si-DcR1 vs. NP-si-DcR1 or TMZ + NP-si-cntl.