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Interleukin-24 overcomes temozolomide resistance and enhances cell death by down-regulation of O^6-methylguanine-DNA methyltransferase in human melanoma cells

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Abstract

Melanoma is the most malignant of skin cancers, highly resistant to chemotherapy and radiotherapy. Temozolomide, a promising new derivative of dacarbazine, is currently being tested for treatment of metastatic melanoma. Resistance to alkylating agents such as temozolomide correlates with increased expression of DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT). Interleukin-24 (IL-24; *mda-7*) is a tumor suppressor cytokine that selectively inhibits tumor cell growth by inducing apoptosis and cell cycle arrest in melanoma cell lines and solid tumors. This tumor-selective activity has been observed in multiple preclinical animal models and in clinical trials. In this study, we analyzed the ability of Ad-IL-24 and its protein product, IL-24, to overcome temozolomide resistance in human melanoma cells. We have shown that Ad-IL-24 via exogenous IL-24 protein induces combinatorial synergy of temozolomide-induced cell killing in temozolomide-resistant melanoma cells by inhibition of MGMT. Neutralizing antibodies against IL-24 or its receptors significantly blocked the apoptotic activity of IL-24 + MGMT treatment. We show that accumulation of functional p53 is essential for IL-24-induced down-regulation of MGMT. Using either MGMT small interfering RNA, p53 small interfering RNA, or a p53 dominant-negative mutant to block MGMT protein expression resulted in increased sensitization to temozolomide. However, MGMT blockade in combination with IL-24 + temozolomide resulted in loss of combinatorial synergy, indicating that MGMT expression is required for the reversal of temozolomide resistance in melanoma cells. This study shows that IL-24 can play a significant role in overcoming temozolomide resistance and that the clinical efficacy of temozolomide may be improved by using a biochemotherapy combination with IL-24. [Mol Cancer Ther 2008;7(12):3842–51]

Introduction

Melanoma is a common skin cancer resulting in high morbidity and mortality. Current standard therapy for metastatic melanoma is adjuvant therapy with IFN-α or interleukin (IL)-2; however, response rates have not exceeded 15% to 20%. Clinical studies using chemotherapeutic agents as monotherapy or in combination with biotherapies have not improved response rates (1). At the present time, dacarbazine is the only Food and Drug Administration-approved chemotherapeutic for melanoma and is being tested in combination with other chemotherapies; however, the toxicity of these regimens is high. Temozolomide (Temodar) is also being tested as a promising chemotherapeutic treatment (2). Given the limitations of the current therapies for metastatic melanoma, there is an urgent need for developing novel treatments that can overcome acquired drug resistance.

Temozolomide is an oral methylating agent derivative of dacarbazine. At physiologic pH, temozolomide degrades into 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide, the active metabolite of dacarbazine. Temozolomide has been approved for treatment of resistant anaplastic astrocytomas and is being tested for treatment of metastatic melanoma (3–5). A phase III study comparing it to dacarbazine in advanced metastatic melanoma reports significantly longer progression-free survival, improved quality of life, and higher systemic exposure to 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide for the temozolomide arm. Temozolomide has the advantage of crossing the blood-brain barrier and thus may prevent or treat central nervous system metastases (4, 6). Regional therapy in an animal model of advanced melanoma comparing intra-arterial delivery of alkylating agents showed that temozolomide was more effective than melphalan (7).

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The mechanism of action of temozolomide is primarily mediated by DNA alkylation, although it can also inhibit other enzymes (esterase and glyoxalase; refs. 8, 9). Three repair mechanisms counteract the action of temozolomide: the primary mechanism involves O^6-methylguanine-DNA methyltransferase (MGMT), although DNA mismatch repair and poly(ADP-ribose) polymerase also contribute. High levels of MGMT are reported in brain and other tumors and correlate with resistance to temozolomide (10–12). Methylation of the MGMT promoter inhibits its repair activity and correlates with reduced MGMT protein expression in gliomas and B-cell lymphomas (13, 14). Additionally, low or absent MGMT expression correlates with improved overall and disease-free survival in B-cell lymphoma and glioma patients (14, 15). Confirmation for the regulation of temozolomide activity by MGMT comes from reports that show temozolomide can decrease the activity of MGMT (16, 17), and inhibition of MGMT enhances temozolomide cytotoxicity (18, 19).

IL-24 (MDA-7) was originally identified as melanoma differentiation-associated gene-7 (*mda-7*), a cytokine-tumor suppressor located within a cluster on chromosome 1q32 that encodes IL-10, IL-19, and IL-20 (20). IL-24 protein expression is lost during melanoma tumor progression; virtually all metastatic melanomas lack IL-24 expression (21). IL-24 functions as a pro-Th1 cytokine in human peripheral blood mononuclear cells and induces secretion of IL-6, IFN-γ, and tumor necrosis factor-α (22). Adenovirus-mediated IL-24 gene delivery (Ad-IL-24) induces apoptosis selectively in cancer cells while sparing normal cells (23, 24). This tumor cell-specific growth-inhibitory effect has also been shown using multiple *in vivo* animal models and has been observed in human clinical trials (25–27). The tumor suppressor activity of IL-24 is independent of the status of other tumor suppressor genes such as p53, Rb, p16, or Ras (24, 28). IL-24 regulates many proliferative control mechanisms in tumor cells and can down-regulate antiapoptotic proteins (Bcl-2/Bcl-xL) and up-regulate proapoptotic proteins (Bax and Bak); this effect is not seen in normal cells (29, 30). IL-24 also regulates p38 mitogen-activated protein kinase signaling in melanoma and glioma cells (31). Many studies have established IL-24 as a promising therapeutic candidate with potent antitumor, antiangiogenic, and cytokine activities. In this study, we test the effect of IL-24 on overcoming resistance to temozolomide in melanoma and have identified the role of MGMT in overcoming chemoresistance by IL-24. We also explored the mechanism by which IL-24 can reverse resistance to temozolomide.

Materials and Methods

Cell Culture and Reagents

All melanoma cell lines were obtained from the American Type Culture Collection and maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum (Life Technologies), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and HEPES (Life Technologies). The cells were screened routinely to verify lack of *Mycoplasma* contamination and used in the log phase of growth. 3,4-Dihydro-3-methyl-4-oxoimidazo [5,1-*d*]-as-tetrazine-8-carboxamide (temozolomide; Schering-Plough) was dissolved in PBS and used at concentrations ranging from 0 to 6,400 μmol/L, although most studies employed 200 μmol/L temozolomide. Monoclonal anti-IL-24 antibody was prepared as described previously (32). Rabbit Ser^{15} phospho-p53 antibody and SignalSilence p53 small interfering RNA (siRNA) kit were purchased from Cell Signaling Technology. β-Actin monoclonal antibody and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling kits were purchased from Oncogene Research Products. Antibodies for IL-20R1 and IL-22R1 were purchased from R&D Systems. MGMT siRNA, p53 siRNA, and control siRNA; transfected medium; antibodies for MGMT, p53, p21, phospho-Akt, phospho-extracellular signal-regulated kinase, and phospho-mitogen-activated protein kinase; and all other primary and secondary antibodies were purchased from Santa Cruz Biotechnology. Cell viability was analyzed by trypan blue exclusion assay. Cells were trypsinized and an aliquot was suspended 1:1 (v/v) with 0.4% trypan blue. Total cell numbers and cell viability counts were assessed using a hemocytometer by light microscopy.

Gene Transfer

Replication-deficient human Ad5 carrying the *IL-24* gene was described previously (24). The *IL-24* gene was linked to an internal CMV-IE promoter followed by a SV40 polyadenylation sequence. The same adenoviral vector containing the sequence for expression of luciferase (Ad-luc) was used as control virus. Cells were plated 1 day before infection. Target cells were infected with adenoviral vectors (Ad-IL-24 or Ad-luc) using 1,000-3,000 viral particles (vp) per cell (50-150 plaque-forming units/cell). Experimental conditions were optimized to achieve IL-24 protein expression in >70% of cells based on results of immunohistochemical staining. The transfections of MGMT and p53 siRNA were executed according to the Santa Cruz Biotechnology provided siRNA transfection protocol. MGMT plasmid (ORF Clone that contains full-length of *Homo sapiens* MGMT cDNA) was purchased from OriGene Technologies, amplified, and transfected into A375, a MGMT-negative cell line, using Lipofectamine 2000 according to the standard procedure described above. Transfected cells were incubated at 37°C for 18 to 48 h before testing for transgene expression. The cells were then passaged at a 1:10 dilution into fresh growth medium 24 h after transfection and maintained in selective medium (containing 400 μmol/L G418) for stable clone selection. This MGMT-expressing A375 subclone was named ''A375M.'' To establish a MGMT knockdown cell line from a MGMT highly expressing melanoma cell line, MGMT-targeted short hairpin RNA (shRNA) and control vectors encoding a neomycin selectable marker (purchased from SuperArray Bioscience) were used to transfect MeWo melanoma cell line according to the manufacturer's protocol. Western blot analysis was used to evaluate MGMT expression. The pEGFP-N1 plasmid was provided by Dr. Roger Bryan Sutton (Department of Neuroscience

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and Cell Biology, University of Texas Medical Branch at Galveston). The pP53wt and pP53mut (p53 codons 22-23 were mutated from Leu-Trp to Gln-Ser) vector constructs were provided by Drs. Kishor K. Bhakat and Sankar Mitra (Sealy Center for Molecular Sciences, University of Texas Medical Branch at Galveston; ref. 33). MeWo cells were seeded in 6-well plates with antibiotics-free medium to 80% confluence. MeWo cells were transfected with 2.5 μg/well pEGFP-N1, pP53wt, or pP53mut by Lipofectamine 2000 reagent using the methods recommended by the manufacturer (Invitrogen).

Production and Treatment with Human IL-24

Ad-IL-24 was transfected (1,000 vp/cell, 96 h) into 10 L wave bioreactor containing 1,700,000 HeLa cells/mL grown in serum-free medium and supernatant was concentrated 10 times by tangential flow filtration (100K Pellicon II membranes were purchased from Millipore; feeding pressure, ~8 psi) followed by diafiltration (feeding pressure, ~8 psi, with 4 volumes PBS) to ~35 μg/mL IL-24. Cells were treated with purified IL-24 protein at 0 to 39 ng/mL.

Other Treatments

For combination temozolomide treatments, 2×10^5 cells were plated and allowed to attach overnight. The next day, cells were treated with either Ad-IL-24, Ad-Luc (both at 0-2,000 vp/cell, as indicated in each figure), temozolomide (200 μmol/L), or a combination of these. Three or 4 days after treatment, cells were harvested and processed to determine percent cell death, changes in protein expression, or apoptosis as described in each subsection.

Immunoblotting Assay

Immunoblotting was done using standard procedures as described elsewhere (34). Briefly, 1×10^5 cells per well were plated in 6-well tissue culture plates (Corning) and treated. Four days later, cells were rinsed in PBS, scraped, and lysed (lysis buffer and protease inhibitor cocktail was purchased from BD Biosciences). Protein concentration was determined using a modified Bradford assay (protein concentration assay reagent was purchased from Bio-Rad Laboratories), and proteins were separated by SDS-PAGE in 4% to 20% Tris-glycine gels (Invitrogen). Proteins were then transferred to a nitrocellulose membrane (Invitrogen) using standard procedures (70 V, 1.5 h, 4°C) and visualized using enhanced chemiluminescence (GE Healthcare Bio-Sciences) after incubation with primary or secondary antibodies.

Fluorescence-Activated Cell Sorting Analysis

Cell surface receptor subunits IL-20R1 and IL-22R1 were examined by flow cytometry following protocols described by Santa Cruz Biotechnology. Briefly, 1×10^5 cells per well were plated in 6-well tissue culture plates (Corning); 4 days later, a monolayer of cells was detached by adding 0.2% trypsin-EDTA/PBS, washed once with ice-cold PBS, pelleted, and resuspended into 0.1 mL of 1% fetal bovine serum in PBS and incubated with either anti-IL-20R1, anti-IL-22R1 (R&D Systems), or normal IgG control antibody (Santa Cruz Biotechnology) for 60 min at room temperature. Cells were then washed and incubated in FITC-conjugated secondary antibody (Santa Cruz Biotechnology) in 1% fetal bovine serum in PBS for 30 min on ice. The cells were washed three times with 0.1% Tween 20 in PBS, pelleted, and resuspended in 500 μL of 1% paraformaldehyde and data were acquired and analyzed.

Apoptosis was determined via fluorescence-activated cell sorting analysis and FragEL DNA Fragmentation Detection Kit (EMD Chemicals) was done according to the manufacturer's protocol. The cells were analyzed by flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences). A minimum sample population of 10,000 cells was used for each analysis.

Statistical Analysis

All studies were repeated two to three times as indicated using triplicate samples for cell counting analyses. Data are mean ± SD and were considered significant when $P < 0.05$. The statistical significance of the experimental results was evaluated using the Student's t test. Coeffects of two drugs were analyzed by isobologram method: fitting two-way ANOVA model with the percentage of dead cell as the response. Fixed effect of IL-24 protein, temozolomide, and the interaction was categorized to combinatorial synergy or nonsynergistic interaction.

Results

Temozolomide Sensitivity Inversely Correlates to Endogenous MGMT Levels in Melanoma

Previous studies have indicated that endogenous MGMT levels are related to temozolomide resistance (10, 11, 18, 35). In order to explore temozolomide resistance mechanisms, we examined the endogenous MGMT levels of five human melanoma cell lines (A375, MeWo, WM35, SK-MEL-28 and WM1341) by Western blot assay. WM35 cells have no detectable MGMT protein, A375 cells express low levels of MGMT protein, and MeWo, SK-MEL-28 and WM1341 express relatively high levels of MGMT (Fig. 1, *top*). We examined the growth inhibitory effect of temozolomide on the five melanoma cell lines using increasing concentrations up to 800 μmol/L. There was a clear distinction between temozolomide-sensitive and temozolomide-resistant cell lines (Fig. 1, *bottom*). Temozolomide, at the concentration of 200 μmol/L induced cell death in temozolomide-sensitive melanoma cells, A375 and WM35 (29% and 23% cell death respectively), while the resistant cells showed minimal death: MeWo (4%), SK-MEL-28 (4%) and WM1341 (5% cell death). Temozolomide at 800 μmol/L killed about 90% of A375 and WM35 cells, which express low MGMT levels. In contrast, cells expressing high endogenous MGMT (MeWo, SK-MEL-28 and WM1341) did not show more than 20% killing at this temozolomide dose (Fig. 1, *bottom*). Therefore, cells with high levels of endogenous MGMT (MeWo, SK-MEL-28 and WM1341) were classified as temozolomide-resistant, but cells with low levels of endogenous MGMT (A375 and WM35) were temozolomide-sensitive. In subsequent studies we use the temozolomide-resistant cells, MeWo, SK-MEL-28 and WM1341 which displayed IC_{50} values of

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1,563, 1,111, and 1,508 μmol/L, respectively and compared them to the sensitive cell lines WM35 and A375, which had IC_{50} values of 281 and 313 μmol/L, respectively.

Ad-IL-24 Overcomes Resistance to Temozolomide and Enhances Cell Death by Down-Regulation of MGMT via p53 Induction

To examine whether IL-24 expression modulates temozolomide-induced cell killing, we treated melanoma cells with Ad-IL-24 and temozolomide. Temozolomide-resistant (MeWo, SK-MEL-28, WM1341) and temozolomide-sensitive (A375, WM35) melanoma cells were exposed to PBS control, Ad-luc control virus, Ad-IL-24, temozolomide, Ad-luc + temozolomide or Ad-IL-24 + temozolomide. The combination of Ad-IL-24 and temozolomide produced combinatorial synergy in cell killing in all three temozolomide-resistant cells but not in temozolomide-sensitive cells; for example, in SK-MEL-28 cells, Ad-IL-24 treatment induced 17.2 ± 1.6% killing and temozolomide caused 5.1 ± 0.5% killing when used as single agents, however, the combination of Ad-IL-24 + temozolomide killed 30.9 ± 2.0% cells (Fig. 2A). This synergistic effect was not observed with the Ad-luc + temozolomide combination. Treatment with Ad-luc killed 7.8 ± 1.6% of SK-MEL-28 cells, and the Ad-luc + temozolomide combination generated only 9.6 ± 0.2% killing, indicating IL-24 and not adenovirus was responsible for the enhanced temozolomide-induced killing. Combinatorial synergy was induced by Ad-IL-24 + temozolomide in all three temozolomide-resistant melanoma cells (MeWo, SK-MEL-28, WM1341), indicating that Ad-IL-24 could partially overcome temozolomide resistance. Ad-IL-24 also enhanced the killing activity of temozolomide in temozolomide-sensitive (A375 and WM35) cells. In A375, Ad-IL-24 alone or temozolomide alone killed 18.1 ± 1.3% and 12.3 ± 1.5% of the treated cells respectively; however, the combination of Ad-IL-24 and

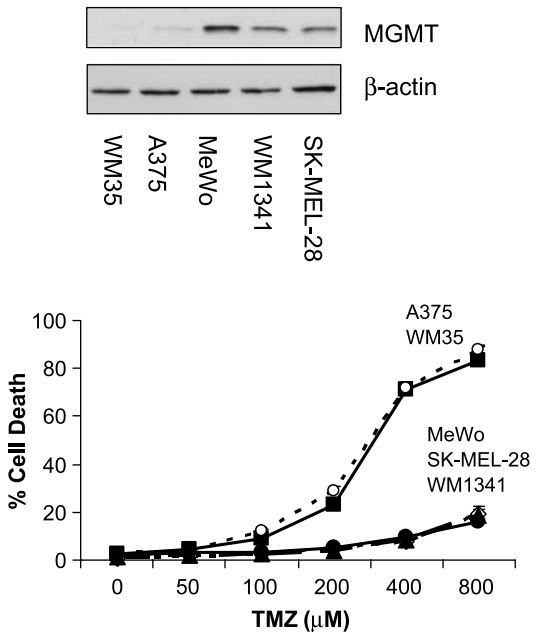

Figure 1. Comparison of endogenous MGMT levels and temozolomide sensitivity among human melanoma cells. *Top,* melanoma cells (A375, WM35, MeWo, SK-MEL-28, and WM1341) were harvested and cellular lysates were examined for MGMT expression using rabbit anti-MGMT polyclonal antibody. *Bottom,* triplicate samples of A375, WM35, MeWo, SK-MEL-28, and WM1341 cells were treated with up to 800 μmol/L temozolomide for 96 h. Results of cell counting by trypan blue exclusion assay. Mean ± SD of three independent experiments.

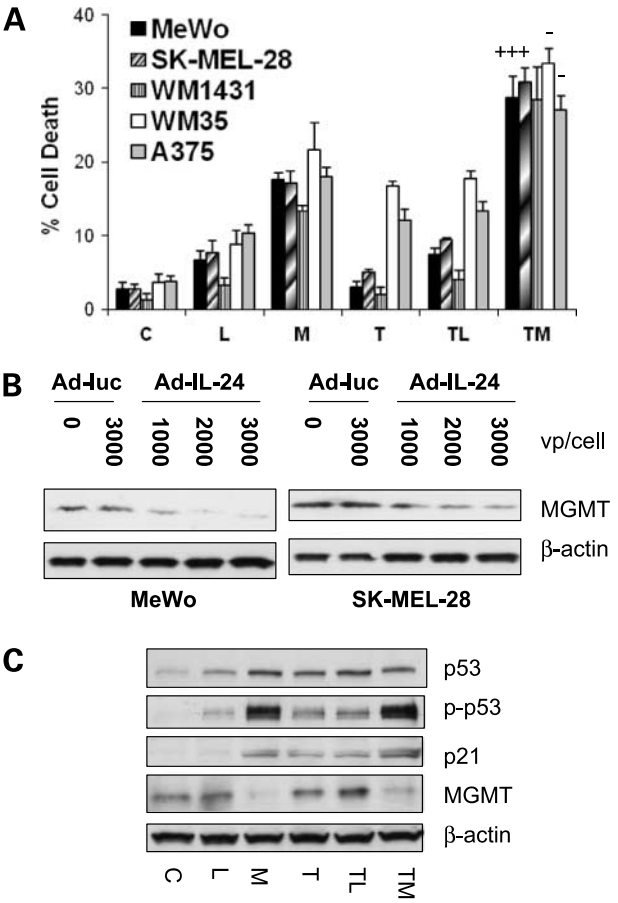

Figure 2. Reversal of temozolomide resistance by Ad-IL-24 occurs via down-regulation of MGMT. **A,** Ad-IL-24 enhanced temozolomide-induced killing and overcame temozolomide resistance in melanoma cells. Temozolomide-resistant cell lines (MeWo, SK-MEL-28, and WM1341) and temozolomide-sensitive cell line (A375 and WM35) were treated with either PBS control (*C*), 200 μmol/L temozolomide (*T*), 2,000 vp/cell Ad-IL-24 (*M*), 2,000 vp/cell Ad-luc control (*L*), temozolomide + Ad-luc (*TL*), or temozolomide + Ad-IL-24 (*TM*) for 72 h. Results of cell counting by trypan blue exclusion assay. Mean ± SD of three independent experiments. Killing activity of temozolomide + Ad-IL-24 was significantly greater than that of temozolomide in all melanoma cells ($P < 0.01$). Combinatorial synergy (+) or without this effect (−) was statistically analyzed. **B,** Ad-IL-24 down-regulates MGMT. MeWo and SK-MEL-28 cells were treated with 3,000 vp/cell Ad-luc or varying doses (1,000-3,000 vp/cell) Ad-IL-24 for 72 h. Western blot analysis of MGMT was done as in Fig. 1A. **C,** Ad-IL-24 activates p53 pathway. Temozolomide-resistant (MeWo) cells were treated with either PBS control, 200 μmol/L temozolomide, 2,000 vp/cell Ad-IL-24, 2,000 vp/cell Ad-luc control, temozolomide + Ad-luc, or temozolomide + Ad-IL-24 for 48 h and cytosol proteins were analyzed by Western blot for p53, phospho-p53, $p21^{WAF1/CIP1}$, and MGMT. β-Actin was used a loading control.

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temozolomide induced 27.2 ± 1.8% cell death. The killing manner of Ad-IL-24 and temozolomide in WM35 was similar to A375.

We also evaluated cell proliferation. In SK-MEL-28 cells, 200 μmol/L temozolomide alone inhibited 8.4 ± 1.6% proliferation compared with controls, and Ad-IL-24 induced 38.8 ± 3.6% growth inhibition; however, treatment with the combination of Ad-IL-24 and temozolomide inhibited 53.1 ± 2.5% proliferation (data not shown).

Previous studies have shown that IFNβ is able to inhibit MGMT mRNA expression and also to stimulate IL-24 mRNA in human melanoma (23, 36). To test whether Ad-IL-24 can regulate MGMT protein expression, we treated MeWo and SK-MEL-28 cells with Ad-luc control vector or Ad-IL-24. Ad-luc did not inhibit MGMT; in contrast, Ad-IL-24 suppressed MGMT in a dose-dependent manner (Fig. 2B).

Because Ad-IL-24 enhanced cell death, after treatment with temozolomide, in temozolomide-resistant melanoma cells (Fig. 2A) and because sensitivity to temozolomide is related to MGMT, mismatch repair, and p53 status (6, 10, 37), we next examined whether Ad-IL-24 (a MGMT inhibitor) also regulates p53. MeWo cells (with a p53 point mutation in the 72th amino acid mutated from Glu to Lys yet it with normal p53 function) treated with Ad-IL-24 increased p53 expression compared with cells treated with Ad-luc (Fig. 2C). To assess function of the induced p53, we evaluated the expression of p21, phospho-p53 (Ser^{15}), and MGMT. Ad-IL-24 induced p53 phosphorylation with concomitant increases in p21 in both temozolomide-resistant cell lines. p27 was also induced by Ad-IL-24 in both lines (data not shown). Treatment with the combination of Ad-IL-24 + temozolomide increased phospho-p53 and p21 compared with cells treated with Ad-luc + temozolomide. Treatment with Ad-IL-24 + temozolomide potently inhibited MGMT in MeWo, a temozolomide-resistant cell line (Fig. 2C). p53 activation, p21 increase, and MGMT inhibition were also observed in another temozolomide-resistant cell, SK-MEL-28, a 245G/S point mutation of p53 and overexpression of p53 protein (data not shown).

IL-24 Specifically Down-Regulates MGMT via IL-20R/IL-22R Receptors in Human Melanoma Cells

Previous studies showed that Ad-IL-24 induces both intracellular and secreted IL-24 protein and that both forms can induce tumor cell death (24, 34, 38). To investigate whether exogenous IL-24 is involved in the down-regulation of MGMT, we treated SK-MEL-28 cells with IL-24. Western blot data showed that MGMT was inhibited by IL-24 in a dose-response manner (Fig. 3A, *top*). When MeWo cells were treated with IL-24, MGMT was also inhibited in the same manner (data not shown). Analysis results shown in Fig. 3A (*bottom*). Cell death after treatment with a combination of temozolomide + IL-24 + anti-IL-24 antibodies was significantly decreased (35.2% and 20.9% in MeWo and SK-MEL-28, respectively) in a dose-dependent manner compared with cells treated with temozolomide + IL-24 + IgG (44.9% and 36.9% in MeWo and SK-MEL-28, respectively). Because Ad-IL-24 inhibits MGMT and induces p53 (Fig. 2C), we asked whether IL-24 protein has the same function. We treated MeWo and SK-MEL-28 cells with IL-24, temozolomide, or both IL-24 + temozolomide to evaluate MGMT, p21, and p53 protein expression. Western blot results show that IL-24 strongly induced p53 and p21 expression, whereas MGMT expression was completely inhibited (Fig. 3B). In contrast, temozolomide was a weak inducer of p53 and p21 and did not reduce MGMT expression. The combination of IL-24 + temozolomide, like IL-24 treatment, resulted in complete inhibition of MGMT in both cell lines.

Our previous studies show that exogenous IL-24 binds specific receptors to mediate its biological effects (34, 38). To evaluate whether IL-24-mediated down-regulation of MGMT involves IL-20R/IL-22R receptors, we treated two melanoma lines expressing high levels of endogenous MGMT (MeWo and SK-MEL-28) with IL-24 in combination with or without IL-24 neutralizing antibodies. Western blot analyses showed that, when the cells were treated with IL-24, MGMT protein levels were suppressed by 40% in SK-MEL-28 and 70% in MeWo (Fig. 3C), whereas either anti-IL24 antibody or antibodies against the IL-24 receptors were able to block the inhibitory effect on MGMT of IL-24. Treatment with single anti-IL-20R1 or anti-IL22R1 antibodies also reversed IL-24 inhibition of MGMT although to a lesser extent than the combination of the two (data not shown).

IL-24 Enhanced Temozolomide-Induced Cell Killing and Apoptosis via IL-20R/IL-22R Receptors in Human Melanoma Cells

Because IL-24 reduced MGMT protein expression via IL-20R1 and IL-22R1 receptor binding (Fig. 3C), we wanted to evaluate IL-24-induced cell death in melanoma cells. Thus, MeWo and SK-MEL-28 cells were treated with IL-24, temozolomide, or combinations of IL-24 + temozolomide as well as specific neutralizing antibodies for IL-20R and IL22R. IL-24 killed both melanoma cells, and cotreatment with anti-IL-20R1, anti-IL22R1, or a combination of the two significantly inhibited ($P < 0.01$) IL-24-mediated cell killing (Fig. 4A). Temozolomide (200 μmol/L) monotherapy induced similar activity against MeWo and SK-MEL-28 cells (5.3 ± 1.1% and 7.3 ± 0.8% cell death, respectively). IL-24 monotherapy induced cell death in both MeWo and SK-MEL-28 (16.7 ± 1.1% and 18.7 ± 1.1%, respectively) cells. The temozolomide + IL-24 combination showed combinatorial synergy in cell killing against both MeWo cells (37.8 ± 2.8%) and SM-MEL-28 cells (34.0 ± 0.5%). Therefore, IL-24 enhances temozolomide-induced killing to MeWo and SK-MEL-28 cell and shows combinatorial synergy to both MeWo and SK-MEL-28 cells (Fig. 4A).

Anti-IL-22R1 and anti-IL20R1 treatments induced a modest reduction in cell death triggered by temozolomide + IL-24 treatment in both MeWo and SK-MEL-28 cells, whereas nonspecific normal mouse IgG had no effect. Cotreatments with anti-IL-20R1 + IL-24 + temozolomide induced 12.4 ± 2.2% and 16.8 ± 0.5% cell death in MeWo

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and SK-MEL-28, respectively; cotreatments with anti-IL-22R1 + IL-24 + temozolomide induced 16.1 ± 0.4% and 21.4 ± 0.9% cell death in MeWo and SK-MEL-28, respectively. Combining neutralizing antibodies against both receptors further reduced killing to levels comparable with single-agent treated controls.

Induction of cell cycle arrest and apoptosis by IL-24 and temozolomide, used as single agents, has been well documented (22, 39, 40). To evaluate the role of IL-24 in the activation of programmed cell death pathways when temozolomide-resistant melanoma cell lines (MeWo and SK-MEL-28) are treated with a combination of IL-24 and temozolomide, we performed terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assays (fluorescence-activated cell sorting assays after DNA fragmentation labeling). Treatment with IL-24 caused 10.7 ± 1.4% and 8.1 ± 0.4% apoptosis in MeWo and SK-MEL-28 cells; treatment with temozolomide induced 7.5 ± 2.1% and 2.6 ± 0.2% apoptosis, respectively. Addition of temozolomide to IL-24 resulted in combinatorial synergy of melanoma cell killing, and IL-24 relieved the resistance to temozolomide in both cell lines ($P < 0.01$), inducing a higher apoptotic index compared with that of IL-24 or temozolomide single-agent treatment (Fig. 4B).

p53 Plays a Critical Role in IL-24 Reversal of Resistance to Temozolomide by Inhibition of MGMT

To examine if blockade of MGMT modifies killing by the combination of temozolomide and IL-24, we next transfected MGMT siRNA or control siRNA into MeWo cells. Analysis of lysates from the transfected cells showed that MGMT expression was significantly reduced, whereas p53 and p21 expression were not altered, by MGMT siRNA (Fig. 5A, *left*). A375 is a wild-type p53 cell line. We confirmed inhibition of MGMT expression using A375M cells transfected with MGMT-targeted shRNA (Fig. 5A, *middle*). Treatment with temozolomide induced increased cell death in MGMT shRNA-transfected (36.9 ± 2.7%) MeWo and (42.9 ± 2.9%) A375M cells compared with control shRNA-transfected cells (6.7 ± 1.0% and 6.4 ± 2.2% for MeWo and A375M, respectively), indicating that MGMT shRNA-transfected cells were more sensitive to temozolomide ($P < 0.01$; Fig. 5A, *right*). The combination of temozolomide + IL-24 induced combinatorial synergy in control shRNA-transfected cells (45.3 ± 2.1% in MeWo and 39.3 ± 1.7% in A375M). However, the synergistic cell death effect in IL-24 + temozolomide-treated cells disappeared on transfection with MGMT shRNA (38.4 ± 2.9% in MeWo and 43.1 ± 4.5% in A375M), indicating that the reversal of temozolomide resistance by IL-24 was abrogated when MGMT was inhibited (Fig. 5A, *right*).

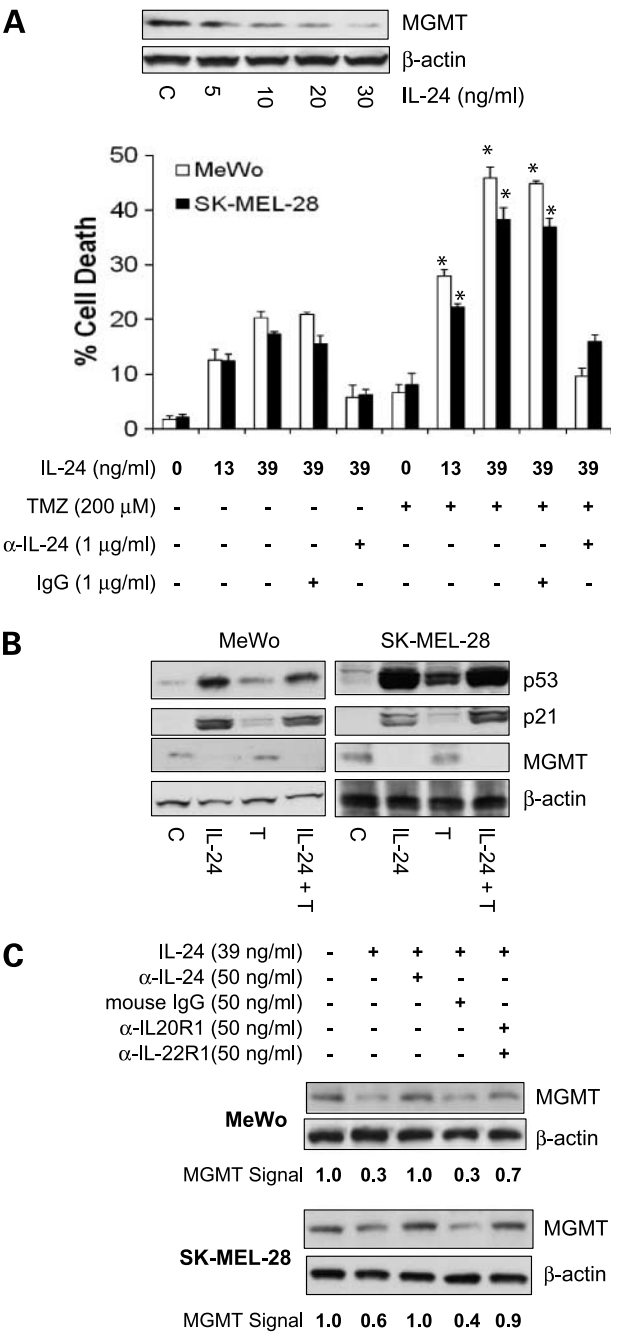

Figure 3. Inhibition of MGMT by IL-24 occurs via IL-24 receptors. **A,** IL-24 enhanced temozolomide-mediated cell death by inhibition of MGMT. SK-MEL-28 was treated with PBS (*C*) or varying concentrations of IL-24 as indicated for 72 h and MGMT was examined by Western blotting. β-Actin was used a loading control (*top*). MeWo and SK-MEL-28 cells in triplicate were treated with various concentrations (0, 13, or 39 ng/mL) of IL-24, 200 μmol/L temozolomide, normal mouse IgG, anti-IL24 monoclonal antibody, or combinations as indicated. After 96 h incubation, cell death was analyzed using trypan blue assay. Mean ± SD of three independent experiments. *, $P < 0.01$, compared with temozolomide-mediated killing (*bottom*). **B,** IL-24 down-regulates MGMT by induction of p53. MeWo and SK-MEL-28 cells were treated with PBS control (*C*), 39 ng/mL IL-24, 200 μmol/L temozolomide (*T*), or both temozolomide + IL-24 (*IL-24 + T*) for 72 h and MGMT, p21, and p53 were examined by Western blotting. β-Actin was used a loading control. **C,** IL-24 down-regulation of MGMT occurs via IL-24 receptors. MeWo and SK-MEL-28 cells were treated with PBS control, IL-24, IL-24 + anti-IL-24, or IL-24 + anti-IL-20R1 and/or IL-22R1 as indicated for 72 h. MGMT was examined by Western blotting. β-Actin was used a loading control. The Western blot signal for MGMT was quantified and indicated.

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To confirm that MGMT expression is dependent on p53 status, we used p53 siRNA from different sources to block p53 expression and examined both MGMT expression and killing mediated by temozolomide + IL-24. Western blot analysis showed partial inhibition of p53 expression using p53 siRNA and inhibition of $p21^{WAF1/CIP1}$ expression (Fig. 5B, *left*). We confirmed p53 knockdown in p53 siRNA-transfected A375M cells (Fig. 5B, *middle*). Evaluation of cell death revealed that temozolomide-induced cell death increased in p53 siRNA-transfected MeWo (29.9 ± 0.9%) and A375M (44.5 ± 2.1%) cells compared with that in control siRNA-transfected cells (7.0 ± 1.6% in MeWo and 8.4 ± 1.8% in A375M; $P < 0.01$). The combination of temozolomide + IL-24 induced similar percent cell death in control siRNA-transfected MeWo (44.8 ± 2.6%) and A375M (43.2 ± 5.0%) cells compared with p53 siRNA-transfected cells MeWo (39.1 ± 3.5%) and A375M (34.2 ± 1.2%) cells, indicating that the temozolomide sensitization induced by IL-24 was mediated by p53-dependent activation of MGMT (Fig. 5B, *right*).

A previous study has shown that the transcriptional activity of p53 is inhibited when a p53 plasmid with mutated residues at positions 22 and 23 ($Leu^{22}Gln$ and $Trp^{23}Ser$, pP53mut) is transfected into SAOS2 cells. These two residues are required for the transactivation activity of p53 and for p53 binding of human mdm-2 and *in vitro* binding to the Ad5 55-kDa E1B (33). To investigate if abnormal p53 protein is capable to induce MGMT expression, we transfected plasmids encoding for GFP (pEGFP-N1), wild-type p53 (pP53wt), or a dominant-negative mutant p53 (pP53mut) into MeWo cells. Both pP53wt- and pP53mut-transfected MeWo cells expressed higher levels of p53 than pEGFP-N1-transfected cells. p21 level was reduced in pP53mut-transfected cells compared with controls, indicating that mutant p53 repressed $p21^{WAF1/CIP1}$ expression (Fig. 5C, *left*). Temozolomide induced increased cell death in pP53mut-transfected cells (11.6 ± 2.0%) compared with pEGFP-N1transfected MeWo cells (7.1 ± 1.1%; $P < 0.01$). The combination of temozolomide + IL-24 induced increased cell death in pEGFP-N1-transfected cells (43.6 ± 2.4%) compared with pP53mut-transfected MeWo cells (26.4 ± 2.5%; $P < 0.01$), indicating that the reversal of temozolomide resistance induced by IL-24 can be inhibited by a dominant-negative p53 (Fig. 5C, *right*). Collectively, these data indicate that functional p53 protein accumulation is essential for expression of MGMT and inhibition of MGMT abolishes IL-24-induced sensitization of melanoma cells to temozolomide.

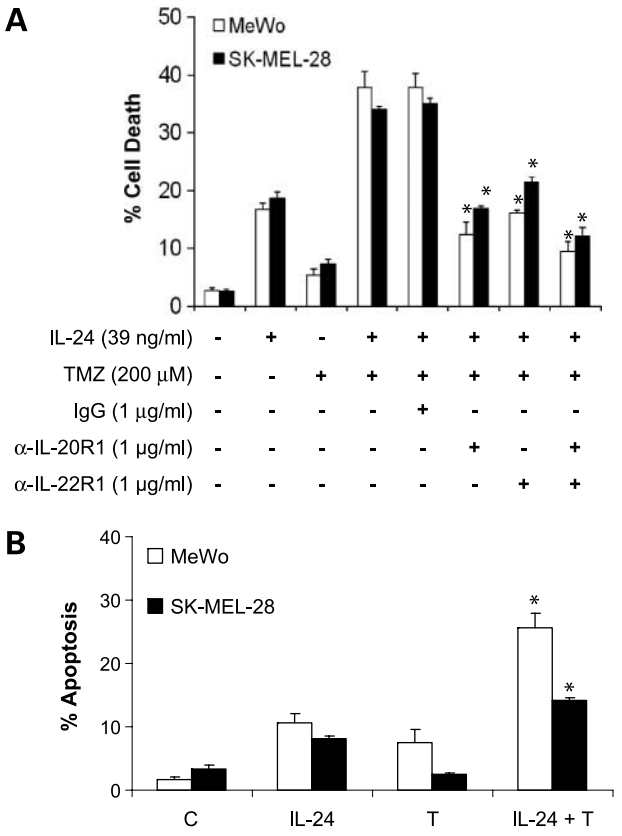

Figure 4. Reversal of temozolomide resistance by IL-24 occurs via IL-24 receptors. **A,** MeWo and SK-MEL-28 cells in triplicate were treated with IL-24 (39 ng/mL), 200 μmol/L temozolomide, normal mouse IgG, anti-IL-20R1, or anti-IL-22R monoclonal antibody as indicated. After 96 h incubation, cell death was analyzed using trypan blue assay. Mean ± SD of three independent experiments. *, $P < 0.01$, compared with temozolomide-mediated killing. **B,** MeWo and SK-MEL-28 cells were treated with PBS control, 39 ng/mL IL-24, 200 μmol/L temozolomide, or both IL-24 + temozolomide for 72 h. Apoptosis was measured by using terminal deoxynucleotidyl transferase labeling method and fluorescence-activated cell sorting as described in Materials and Methods. *, $P < 0.01$, compared with temozolomide-mediated apoptosis.

Discussion

Although temozolomide is a promising chemotherapeutic agent for patients with advanced melanoma, resistance develops quickly and with high frequency. In melanoma cells, a single cycle of temozolomide is sufficient to up-regulate MGMT expression causing increased drug resistance (35). The literature shows that endogenous MGMT correlates with resistance to temozolomide and other alkylating agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea and cisplatin (35, 41, 42). Thus, due to their potential for application in the clinical setting, agents that effectively decrease the levels of MGMT have been evaluated in combination regimens with temozolomide; two main agents in this group are O^6-benzylguanine and IFN-β. O^6-benzylguanine is a potent inhibitor of MGMT and acts as its substrate. Treatment with O^6-benzylguanine potentiates temozolomide-induced cytotoxicity in melanoma cells *in vitro* and increases sensitivity to other alkylating agents. Treatment of human melanoma xenografts with a combination of O^6-benzylguanine and temozolomide results in greater antitumor effect (5-fold inhibition of tumor growth; $P < 0.005$) than an equivalent dose of temozolomide (43). Thus far, clinical trials testing the combination of temozolomide and O^6-benzylguanine have failed to show efficacy and show significant hematologic toxicity (43, 44). Another agent investigated for use in

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combination with temozolomide is the anti-inflammatory cytokine, IFN-β. IFN-β sensitizes glioma cells that harbor an unmethylated MGMT promoter and are resistant to temozolomide (36). In a nude mouse xenograft model, adenoviral delivery of IFN-β or p53 suppressed MGMT promoter activity, whereas treatment of gliomas with IFN-β + temozolomide was reported to induce synergistic inhibition of tumor growth (45).

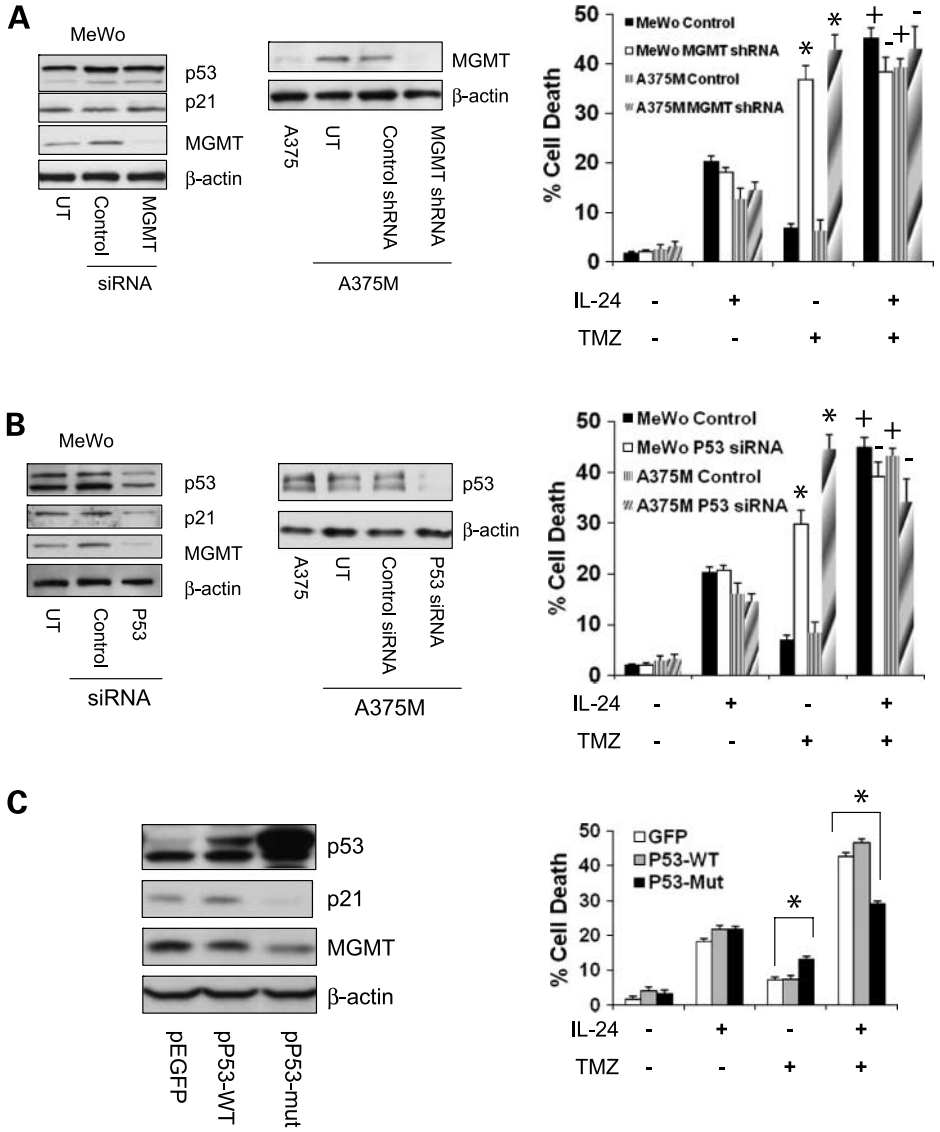

Figure 5. Requirement of p53 accumulation for MGMT expression. **A,** blocking MGMT abolishes synergistic cell killing by IL-24 + temozolomide combination. MeWo cells were transfected with control siRNA or MGMT siRNA for 72 h. *Left,* cell lysates were extracted and Western blot analysis was done to examine p53, p21, and MGMT. β-Actin was used a loading control. *Middle,* A375M transfected with control shRNA- or MGMT-targeted shRNA for 48 h. Cell lysates were extracted and Western blot analysis was done to examine MGMT. A375 and A375M was used as control. *Right,* MeWo and A375M cells transfected with MGMT shRNA or control shRNA were treated with PBS, 39 ng/mL IL-24, 200 μmol/L temozolomide + Ad-IL-24, or temozolomide + IL-24 as indicated for 96 h. Cell death was plotted against treatment. Mean ± SD of two independent experiments using triplicate samples. *, $P < 0.01$, compared with control siRNA-transfected cells. *+,* combinatorial synergy; *-,* without combinatorial synergy. **B,** blocking p53 with siRNA abolishes combinatorial synergy of cell killing by IL-24 + temozolomide. *Left,* MeWo cells were transfected with control siRNA or p53 siRNA for 72 h. Cell lysates were extracted and Western blot analysis was done to examine p53, p21, and MGMT. β-Actin was used a loading control. *Middle,* A375M transfected with control siRNA- or MGMT-targeted siRNA for 48 h. Cell lysates were extracted and Western blot analysis was done to examine p53. A375 and A375M was used as control. *Right,* MeWo and A375M cells transfected with SignalSilence p53 siRNA or control siRNA were treated with PBS, 39 ng/mL IL-24, 200 μmol/L temozolomide, or temozolomide + IL-24 as indicated for 96 h. Cell death was plotted against treatment. Mean ± SD of two independent experiments using triplicate samples. *, $P < 0.01$, compared with control siRNA-transfected cells. **C,** blocking p53 function with dominant-negative mutant abolishes synergistic activity on cell killing by IL-24 + temozolomide combination. MeWo cells were transfected with pEGFP-N1, pP53wt, or pP53mut. Cell lysates were extracted after 48 h and Western blot analysis was done to examine p53 and p21. β-Actin was used a loading control. p53 wild-type, p53 mutant, or GFP plasmid transfected MeWo cells were treated with PBS, 39 ng/mL IL-24, 200 μmol/L temozolomide + Ad-IL-24, or temozolomide IL-24 as indicated for 96 h. Cell death was plotted against treatment. Mean ± SD of two independent experiments using triplicate samples. *, $P < 0.01$, compared with pEGFP-N1-transfected MeWo cells.

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A novel candidate in combination regimens for melanoma is IL-24, which was first identified in melanoma cells treated with a combination of IFN-β and mezerein to induce terminal differentiation (46). Because of its tumor-specific antiangiogenic, proapoptotic, and growth-inhibitory activities, IL-24 is a good candidate for sensitization of tumor cells to temozolomide without exacerbation of the toxicity of this agent. Consistent with its tumor-specific selectivity, IL-24 has been safely and efficiently used to treat various types of tumors in cancer patients (22, 26, 27). Importantly, evaluation of matched primary and metastatic clinical melanoma samples has revealed that expression of the IL-24 protein is lost during the pathologic progression of melanomas into more aggressive phenotypes (21). Thus, we hypothesized that restoring IL-24 in melanoma cells could reduce their metastatic capability and reverse temozolomide resistance. In this study, we show that IL-24 synergistically enhances temozolomide-induced cytotoxicity in melanoma cells resistant to this agent (Fig. 2A). Further, we show that IL-24 induces this effect via inhibition of MGMT protein expression (Figs. 2B, 3C, and 4*A*).

Because of its function as a sensor of DNA damage and promoter of DNA repair, several studies have investigated a role for p53 as a regulator of MGMT; however, these have yielded conflicting results. Although it is well established that p53 activates expression of target genes that contain a p53 binding sequence and TATA box, p53 can also regulate promoter activity of genes that do not possess a p53-binding site; the MGMT promoter belongs to this category. Various mechanisms have been proposed to explain this phenomenon, but no clear consensus has been reached. Importantly, p53 regulation of MGMT expression has important implications in the development of temozolomide resistance and in tumor cell responses against temozolomide and other alkylating agents (14, 16, 18, 19, 37, 47). Our present study shows that abrogation of p53 in temozolomide-resistant melanoma cells, via transfection with p53 siRNA or a dominant-negative p53 mutant plasmid, results in loss of IL-24-induced synergy and decreased sensitivity to temozolomide (Fig. 5B), thus supporting that p53 signaling mediates the effect of IL-24 on MGMT. In our study, direct inhibition of MGMT expression, via MGMT siRNA, did not alter expression of p53 or its direct downstream target, p21 (Fig. 5A); this is consistent with MGMT being a downstream target of p53. Interestingly, siRNA blockade of MGMT expression abolished the synergistic effect of IL-24 on temozolomide, implicating the need to have a minimum threshold of MGMT for the combinatorial synergy of cell death.

Taken together, our results confirm that decreased expression of MGMT is the mechanism underlying the synergistic effect of IL-24 + temozolomide and reversal of temozolomide resistance and that IL-24 down-regulation of MGMT requires p53 function. In our study, we also show that IL-24 can inhibit MGMT and overcome melanoma resistance to temozolomide via types 1 and 2 IL-24 receptors, resulting in activation of p53 and subsequent down-regulation of MGMT. These results support a promising role for IL-24 as part of a novel biochemotherapy approach to treat advanced melanoma and overcome temozolomide resistance.

Disclosure of Potential Conflicts of Interest

M. Zheng, employee of Introgen Therapeutics. E.A. Grimm is a consultant for Introgen, as well as holds an equity interest. The other authors reported no conflicts of interest.

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Interleukin-24 overcomes temozolomide resistance and enhances cell death by down-regulation of O^6-methylguanine-DNA methyltransferase in human melanoma cells

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