Blockade of MGMT Expression by O\textsuperscript{6} Benzyl Guanine Leads to Inhibition of Pancreatic Cancer Growth and Induction of Apoptosis

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Abstract

Purpose: We sought to determine whether administration of a MGMT blocker, O\textsuperscript{6}-benzyl guanine (O\textsuperscript{6}BG), at an optimal biological dose alone or in combination with gemcitabine inhibits human pancreatic cancer cell growth.

Experimental Design: Human pancreatic cancer L3.6pl and PANC1 cells were treated with O\textsuperscript{6}BG, either alone or in combination with gemcitabine, and the therapeutic efficacy and biological activity of these drug combinations were investigated.

Results: O\textsuperscript{6}BG sensitized pancreatic cancer cells to gemcitabine. Protein and mRNA expression of MGMT, cyclin B1, cyclin B2, cyclin A, and ki-67 were significantly decreased in the presence of O\textsuperscript{6}BG. In sharp contrast, protein expression and mRNA message of p21\textsuperscript{cip1} were significantly increased. Interestingly, O\textsuperscript{6}BG increases p53-mediated p21\textsuperscript{cip1} transcriptional activity and suppresses cyclin B1. In addition, our results indicate that p53 is recruited to p21 promoter. Furthermore, an increase in p21\textsuperscript{cip1} and a decrease in cyclin transcription are p53 dependent. The volume of pancreatic tumors was reduced by 27\% in mice treated with gemcitabine alone, by 47\% in those treated with O\textsuperscript{6}BG alone, and by 65\% in those mice given combination. Immunohistochemical analysis showed that O\textsuperscript{6}BG inhibited expression of MGMT and cyclins, and increased expression of p21\textsuperscript{cip1}. Furthermore, there was a significant decrease in tumor cell proliferation and an increase in tumor cell apoptosis.

Conclusions: Collectively, our results show that decreased MGMT expression is correlated with p53 activation, and significantly reduced primary pancreatic tumor growth. These findings suggest that O\textsuperscript{6}BG either alone or in combination with gemcitabine may provide a novel and effective approach for the treatment of human pancreatic cancer.


At the time of diagnosis, >80\% of pancreatic cancer patients present with either locally advanced or metastatic disease. Only 1\% to 4\% of all patients with adenocarcinoma of the pancreas will survive 5 years posttreatment (1–3). For most patients, the introduction of chemotherapy (i.e., gemcitabine) is often either ineffective or effective for only a short duration (4). Moreover, varying degrees of chemoresistance usually develops for those who initially respond. In lieu of the continuing problem of gemcitabine and other conventional chemotherapeutic drug resistance, developing new approaches for the treatment of pancreatic cancer and understanding the molecular basis for drug resistance remain high priorities. Pertinent to this topic, studies have shown that p53 is silenced and tumor cell proliferation is increased in pancreatic cancers. Further, the DNA repair protein, O\textsuperscript{6} methyl guanine DNA methyl transferase (MGMT), is highly expressed in pancreatic cancers, and confers tumor resistance to a variety of anticancer alkylating agents. Interestingly, there is an inverse correlation between p53 and MGMT (5), and it has been reported that cell cycle regulatory proteins such as cyclin B1, cyclin B2, and cyclin A are overexpressed in pancreatic cancer cells (6) and wild-type (wt) p53 suppresses them (7–9). p53 can influence the cell cycle either by growth arrest or apoptosis (10, 11), and it can function as a transcriptional activator and repressor. Derepression of apoptosis can disrupt the equilibrium between cell growth and cell death, and is an important step in the development of cancer (12–14). Understanding this has led to the investigation of therapeutic activation of apoptosis in cancer cells as a potential anticancer strategy (15–17).
Therapeutic Relevance

Food and Drug Administration–approved current medications (i.e., gemcitabine) have very minimal effect on the treatment of pancreatic cancer. Previous reports have shown that O\textsubscript{6} methyl guanine DNA methyl transferase (MGMT) is overexpressed in pancreatic cancer, and overexpression of MGMT is known to cause drug-related resistance to chemotherapeutic agents. In our study, we investigated whether blocking of MGMT has any therapeutic relevance, either alone or in combination with gemcitabine, in the treatment of pancreatic cancer cells. Surprisingly, we found that inhibition of MGMT expression by the MGMT blocker (e.g., O\textsubscript{6} benzyl guanine) not only inhibited the pancreatic cancer cell growth in vitro and in vivo, but also sensitized pancreatic cancer cells to gemcitabine. Preclinical findings from this study could have translational implications, and in the end, we hope to provide novel therapeutic strategies that will avoid and overcome drug-related resistance and most importantly prolong the lives of pancreatic cancer patients.

To date, p53 is one of the most important tumor suppressor proteins identified, and it is mutated in the majority of human tumors, which indicates that its role is important for prevention of malignant transformation (10, 11). Recently it has been reported that the deletion or mutational inactivation of the p53 gene represents an important step in pancreatic tumor progression (18, 19).

Nonetheless, the specific roles and mechanism of p53 and MGMT in the therapeutic response of human pancreatic cancers are not known. Therefore, the current study investigated the consequences of O\textsubscript{6} benzyl guanine (O\textsubscript{6}BG; a pseudo substrate and inactivator of MGMT currently in clinical trials) in the absence or presence of gemcitabine on p53 function and antitumor responses of human pancreatic cancers using both cell culture and orthotopic murine models.

Materials and Method

Cell culture

The highly metastatic human pancreatic cancer cell line L3.6pl was maintained in DMEM supplemented with 5% fetal bovine serum and 100 µg/mL penicillin-streptomycin mixture (GIBCO). PANC1 cells were obtained from the American Type Culture Collection and were grown in 10% fetal bovine serum containing DMEM. Adherent monolayer cultures were maintained at 37°C containing 5% CO\textsubscript{2}.

MTT assays. L3.6pl and PANC1 cells were plated (2 × 10\textsuperscript{5}) in 24-well plates and 24 h later cells were treated with different concentrations of O\textsubscript{6}BG (10-100 µg) and harvested at different time points (24, 48, and 72 h). In another set of experiments, L3.6pl and PANC1 cells were treated with O\textsubscript{6}BG (50 µg) for 2 d and after completion of 48 h, gemcitabine (1 µmol/L) was added and MTT assays were done 24 h posttreatment. MTT (30-1010-K) Assay kit was purchased from the American Type Culture Collection, and MTT assays were done as per the manufacturer’s instructions.

Antibodies, drugs, and Western blot

p53 (#sc-126-mouse monoclonal), cyclin B2 (#sc-28303-mouse monoclonal), normal mouse IgG, and horseradish peroxidase (#sc-2025; #sc-2748) antibodies were purchased from Santa Cruz Biotechnology. MGMT (#MAB16200-mouse monoclonal) and ki-67 (#MAB4190-mouse monoclonal) antibodies were purchased from Chemicon-Millipore. Cyclin B1 (#4135-mouse monoclonal), cyclin A (#4656-mouse monoclonal), poly(ADP-ribose) polymerase (PARP, #9542-rabbit polyclonal), caspase 9 (#9502-rabbit polyclonal), and cytochrome C (#4272-rabbit polyclonal) were purchased from Cell Signaling. β-Actin (#A2066-rabbit polyclonal) antibody was purchased from Sigma-Aldrich. Gemcitabine (GEMZAR, Eli Lilly Co.) was purchased by McKesson Drug. O\textsubscript{6}BG (MGMT Blocker) was purchased from Sigma-Aldrich. For Western blotting, human pancreatic cancer L3.6pl and PANC1 cells were plated (2 × 10\textsuperscript{5}) in 10-cm\textsuperscript{2} Petri dishes and 24 h later cells were treated with O\textsubscript{6}BG (50 µg) for 48 h. Gemcitabine (1 µmol/L) was then added and at 24 h post treatment cell lysates were collected and proteins were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes and standard procedures were followed (20).

Quantitative real-time PCR

For quantitative real-time PCR (qRT-PCR), L3.6pl and PANC1 cells were plated (2 × 10\textsuperscript{5}) and the next day were treated with O\textsubscript{6}BG (50 µg) for 48 h. Gemcitabine (1 µmol/L) was then added, and at 24 h post treatment the cells were harvested. In another set of experiments, L3.6pl cells were plated (2 × 10\textsuperscript{5}) and the next day transfected with nonspecific and p53 small interfering RNA (siRNA) at 20 nmol/L and 48 h later the cells were treated with or without O\textsubscript{6}BG; at 24 h posttreatment the cells were collected. Cells were lysed in Trizole reagent (Invitrogen), and total RNA was isolated using the Qiagen columns (Qiagen) and reverse transcribed by using SuperScript First Strand Synthesis System for RT-PCR. Quantitative real-time PCR was done by using ABI 7300 sequence detection system (Applied Biosystems). TaqMan gene expressions assays for p53 (Hs00153349_m1), MGMT (Hs00172470_m1), p21 (Hs00355782_m1), cyclin B1 (Hs00259126_m1), cyclin B2 (Hs00270424_m1), cyclin A (Hs00153138_m1), ki-67 (Hs00606991_m1), and actin (4333762F) were purchased from Applied Biosystems. The relative mRNA levels of p53 and MGMT, p21, cyclin B1, cyclin B2, cyclin A, and ki-67 were calculated by using the ∆∆ Ct method with the β-actin mRNA as an endogenous control.

Reporter assays

For reporter assays, L3.6pl cells (5 × 10\textsuperscript{4}) were seeded in a 12-well plate and transfected with MGMT-luc construct (21). After 5 to 6 h the cells were treated with or without O\textsubscript{6}BG (50 µg). At 24 h posttreatment the cells were harvested and lysed, and luciferase activity was measured by using dual luciferase reporter assay system following the manufacturer's protocol (Promega Corporation). LipofectAMINE 2000 (Invitrogen) was used to transfect the cells, and the manufacturer's protocol for transfections was followed. In another experiment, L3.6pl cells (5 × 10\textsuperscript{4}) were transfected with MGMT-luc in the presence or absence of wt p53 construct (5 µg) and 24 h later the cells were harvested. In a final set of experiments, L3.6pl cells (5 × 10\textsuperscript{4}) were seeded in a 12-well plate and transfected with p21-luc construct (22) and cyclin B1-luc construct (9) in presence or absence of O\textsubscript{6}BG (50 µg) and gemcitabine (1 µmol/L), and 24 h posttreatment the cells were harvested.

Chromatin immunoprecipitation assays

L3.6pl cells were plated (2 × 10\textsuperscript{5}) and 24 h later the cells were transfected with nonspecific and p53 siRNA, both at 20 nmol/L. After 48 h the cells were treated with or without O\textsubscript{6}BG, and 24 h posttreatment the cells were harvested and samples were used for chromatin immunoprecipitation assays (ChIP). ChIP assays were done as per the manufacturer’s instructions (Upstate) with minor modifications (20, 23, 24).
L3.6pl cells were plated \((2 \times 10^5)\) and the next day were transfected with nonspecific \((20 \text{ nmol/L})\) and p53 siRNA \((20 \text{ nmol/L})\) and 24 h later cells were treated with or without O\(^6\)BG. At 48 h posttreatment cells were collected. Cell lysates \((400 \mu\text{L})\) were sonicated 25 times and each time a 10-s pulse and 20-s gap were given \((\text{Mesonix Inc.})\). After centrifugation, 50 µL of the supernatant were used for checking DNA fragmentation as well as input, and the remaining 350 µL were used for chromatin immuno precipitation. The following antibody and primers were used for ChIP assay \((23)\), with p53 antibody \((\text{DO-1})\) purchased from Santa Cruz Biotechnology \((\text{ChIP grade})\):

\[
p21 \text{ (5'site) forward: GCTTGGGCTGTGATTGGCTTT} \\
p21 \text{ (5'site) reverse: ACAGGCCAGCCTAAGGACAAA} \\
p21 \text{ (3'site) forward: CATCCCCACAGCAGGAGGAAA} \\
p21 \text{ (3'site) reverse: ACCAGGCTTGAGCAGCTA}
\]

**Orthotopic injection of pancreatic cancer cells in athymic nude mice**

Male athymic nude mice \((\text{NCI-nu})\) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center. The mice were housed and maintained in specific pathogen-free conditions, and the facilities were approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with institutional guidelines when they were 8 to 12 wk of age. To develop tumors, L3.6pl cells were harvested and injected as previously described \((25, 26)\).

**Therapy of established human pancreatic carcinoma tumors growing in the pancreas of nude mice**

Seven days after injection of tumor cells into the pancreas, five mice were sacrificed to confirm the presence of tumor lesions. At that time, the median tumor volume was 18 mm\(^3\). Histologic examination confirmed that the lesions were actively growing pancreatic cancer. The mice were randomized into four groups \((n = 10)\) as follows: \((a)\) daily \((\text{M-F})\) i.p injections of \(1\times\) PBS in control groups; \((b)\) twice weekly \((\text{T, Th})\) i.p injections of gemicetabine 100 mg/kg \((26)\); \((c)\) daily \((\text{M-F})\) i.p injections of O\(^6\)BG \((100 \mu\text{g})\); and \((d)\) i.p injections of combination of gemicetabine \((\text{twice})\) and O\(^6\)BG \((5 \text{ d/wk})\). Tumor volumes were calculated by using the following formula: \(0.5 \times (\text{length}) 	imes (\text{width})^2\). Treatments were continued for 5 wk and the mice were sacrificed and subjected to necropsy.

**Necropsy procedures and histologic studies**

The mice were sacrificed and body weight was determined. Primary tumors in the pancreas were excised and weighed. For immunohistochemistry and H\&E staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin; another part was embedded in optimum cutting compound \((\text{Miles, Inc.})\), rapidly frozen in liquid nitrogen, and stored at \(-70^\circ\text{C}\). Visible liver metastases were counted with the aid of a dissecting microscope and processed for H\&E staining. For immunohistochemistry and histologic staining, paraffin-embedded tissues were used to detect protein expressions of MGMT, cyclin B1, cyclin B2, cyclin A, p21, and ki-67. Sections \((4 \text{ to } 6 \mu\text{m thick})\) were mounted on positively charged Superfrost slides \((\text{Fischer Scientific, Co.})\) and dried overnight. Sections were deparaffinized in xylene, treated with a graded series of alcohol \([100\%, 95\%, \text{ and } 80\% \text{ ethanol (v/v) in deionized H}_2\text{O, and rehydrated in deionized water and PBS (pH 7.5)})\]. Antigen retrieval was achieved by placing slides in 97°C in 0.1 mol/L citrate buffer \((\text{pH 6.0})\) for 10 min. Slides were then washed with PBS that contained 0.1% triton and 0.1% bovine serum albumin. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS whereas nonspecific binding was blocked with 10% normal goat serum and 2% bovine serum albumin in PBS. The slides were then incubated at 4°C overnight in a moist chamber with the same antibodies used in Western blot. Slides were washed with PBS that contained 0.1% triton and 0.1% bovine serum albumin in PBS. After a 1-h incubation at room temperature with a peroxidase-conjugated mouse IgG secondary \((\text{Santa Cruz Biotechnology; 1:500 dilution})\), a positive reaction was visualized by incubating the slides with stable 3,3'-diaminobenzidine \((\text{Invitrogen Corporation})\) for 8 to 10 min. Counterstaining was achieved by rinsing the sections with two changes of tap water, placing them in Gill’s filtered hematoxylin \((\text{EMD Chemicals})\) for 5 min, then successively dipping into tap water, acid alcohol \((\text{EMD Chemicals})\), tap water, lithium carbonate \((\text{EMD Chemicals})\), and tap water. Slides were dehydrated and then mounted with Crystal Mount \((\text{Fisher Scientific, Co.})\). For each antibody, the corresponding normal mouse IgG and horseradish peroxidase were used as controls, and wash buffer was used as an additional control in immunohistochemistry studies.

![Fig. 1. In vitro inhibition of MGMT by O\(^6\)BG modulates p53 downstream target protein expression, induces apoptosis, and decreases cell proliferation. L3.6pl and PANC1 cells growing in normal growth medium were treated with gemicetabine (GEM), MGMT blocker (O\(^6\)BG), or a combination of gemicetabine and O\(^6\)BG (G+O). The levels of MGMT, cyclin B1, cyclin B2, cyclin A, p53, p21, ki-67, cytochrome C (cyto C), caspase 9, and PARP1 proteins were determined by Western blotting. A, expressions of MGMT, cyclin B1, cyclin B2, cyclin A, p53, and ki-67 were decreased, whereas p21 was increased. B, the levels of cyto C and caspase 9 were increased, whereas the levels of PARP1 protein were decreased.](image-url)
Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay

Paraffin-embedded tumor tissues were used for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining, which was carried out using DeadEnd Colorimetric TUNEL System (Promega). Paraffin-embedded sections (4 to 6 μm thick) were processed per the manufacturer’s protocol and procedures were followed as previously described (20).

Statistical analysis

Experiments were done in triplicates and the data were presented as mean ± SD. Statistical analysis was done using Student’s t test, assuming equal variance, and P value was calculated based on two-tailed test. P < 0.05 was considered statistically significant. P values are reported using a star system as follows: *, P < 0.05; **, P < 0.005; and ***, P < 0.0005.

Results

In vitro determination of O6BG IC50 and cytotoxicity. L3.6pl cells and PANC1 cells were incubated for 24, 48, and 72 hours in media containing increasing concentrations of O6BG. The L3.6pl cells were relatively sensitive to O6BG in a dose- and time-dependent manner. The IC50 was 50 μg (at 48 hours). Interestingly, the addition of O6BG to L3.6pl cells (sensitive to gemcitabine) and PANC1 cells (resistant to gemcitabine) resulted in increased cell sensitivity to gemcitabine (for both cell lines tested) as compared with cells treated with gemcitabine alone (data not shown).

In vitro inhibition of MGMT by O6BG modulates p53 downstream target protein expression, induces apoptosis, and decreases cell proliferation. We investigated the effect of O6BG on proteins that are involved in cell cycle regulation, apoptosis, and cell proliferation in L3.6pl and PANC1 cells growing in normal growth media. The cells exhibited decreased MGMT expression with the addition of O6BG either alone or in combination with gemcitabine (GEM). In both the cell lines, O6BG alone or in combination with gemcitabine (G+O) inhibited cyclin B1, cyclin B2, and cyclin A protein expression (Fig. 1A). Furthermore, O6BG (alone or in combination) modulated p53 function by enhancing p21 expression more significantly in L3.6pl cells as compared with PANC1 cells (Fig. 1A). Interestingly, the proliferation marker (ki-67) protein expression was decreased by O6BG either alone or in combination with gemcitabine in both the cell lines tested (Fig. 1A). Lastly, we show that O6BG either alone or in combination with gemcitabine increased the cytochrome C release from the mitochondria and increased caspase 9 expressions (Fig. 1B). In the end, there was a significant increase in cell death, as shown by the increase in PARP1 cleavage (Fig. 1B). All these experiments were done three times. Our data indeed show that MGMT expression is decreased by O6BG, and in addition O6BG modulates p53 function, which in turn, promotes cell cycle arrest and apoptosis. We show for the first time that O6BG is a p53 modulator, which can explain the changes in cell growth inhibition and apoptosis index. Therefore, we are not implying that the decrease in MGMT expression by O6BG is single-handedly the key to the observed cell cycle arrest and induction of apoptosis. The data also suggest that O6BG promotes cell cycle arrest and apoptosis by modulating p53 function.

In vitro inhibition of MGMT by O6BG modulates transcription of p53 downstream target genes and tumor cell proliferation marker. qRT-PCR resulted in a significant reduction of mRNA message of cyclin B1, cyclin B2, cyclin A, and ki-67 when L3.6pl and PANC1 cells were treated with O6BG alone or in combination with gemcitabine. Surprisingly, p21 mRNA message was significantly increased in cells treated with O6BG either alone or in combination with gemcitabine. O6BG either alone or in combination with gemcitabine caused significant reduction (P < 0.05) in MGMT transcription (Supplementary Fig. S1).

Wt p53 suppresses MGMT transcriptional activity in L3.6pl cells. Luciferase reporter assays show that O6BG significantly (P < 0.05) decreased the MGMT transcriptional activity in L3.6pl (Fig. 2A). We next determined that wt p53 suppresses
MGMT transcriptional activity in L3.6pl cells (Fig. 2B), illustrating the inverse correlation between p53 and MGMT in these cells. Furthermore, O6BG enhances p21 transcriptional activity in these cells (Fig. 2C). Finally, our results show that O6BG either alone or in combination with gemcitabine significantly decreased cyclin B1 transcriptional activity (Fig. 2D).

**O6BG recruits p53 to p21 promoter, and induction of p21 and suppression of cyclin B1, cyclin B2, and cyclin A transcription are p53 dependent.** In the next set of studies, we investigated the mechanistic aspect of p21 induction by O6BG in pancreatic cancer L3.6pl cells by ChIP assays. The results show that O6BG enhances p53 recruitment to the 5' and 3' sites of the p21 promoter (Fig. 3A). Surprisingly, when p53 was knocked down by siRNA, p53 recruitment to p21 promoter was significantly decreased. Next, we determined whether the induction of p21 is p53 dependent or independent. Results show that knocking down p53 in the presence or absence of O6BG significantly decreased p21 transcription (Fig. 3B). Similar designed experiments show that cyclin B1, cyclin B2, and cyclin A transcription is p53 dependent (Fig. 3C).

**O6BG inhibits pancreatic cancer cell growth and increases pancreatic cell sensitivity to gemcitabine.** Detailed necropsy revealed that all of the mice had tumors in the pancreas. The data summarized in Table 1 show the daily O6BG alone or in combination with twice-weekly gemcitabine significantly decreased median tumor volume and weight as compared with that seen in gemcitabine-treated and control mice. The combination of O6BG and gemcitabine produced the greatest decrease in median tumor volume as compared with control mice (1,607 mm³ and 4,596 mm³, respectively; \( P < 0.0005 \)). Tumor weight was also significantly reduced in mice treated with combination therapy as compared with control mice (1.25 g and 2.96 g, respectively; \( P < 0.0005 \)). Body weight was not changed among all treatment groups as compared with control mice. No visible liver metastases were present (enumerated with the aid of a dissecting microscope) in all treatment groups.

**Histology and immunohistochemical analysis.** We next determined the in vivo effects of O6BG (alone or in combination) with gemcitabine. Tumors harvested from the different treatment groups were processed for routine histologic and immunohistochemical analysis. Tumors from mice treated with O6BG alone or in combination with gemcitabine exhibited a significant decrease in MGMT, cyclin B1, cyclin B2, and cyclin A as compared with tumors treated with gemcitabine alone or the control group. In sharp contrast, the expression of p21 was significantly increased in tumors from mice treated with O6BG (Fig. 4).

**O6BG inhibits pancreatic cancer cell proliferation and induces tumor cell apoptosis in vivo.** The induction of apoptosis (TUNEL-positive stain) of tumor cells was inversely correlated with proliferation (ki-67 positivity). The TUNEL-positive stain revealed that many tumor cells underwent apoptosis in mice treated with O6BG (alone or in combination with gemcitabine).

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**Fig. 3.** O6BG recruits p53 to p21 promoter, and induction of p21 and suppression of cyclin B1, cyclin B2, and cyclin A transcription are p53 dependent. A, L3.6pl cells were transfected with nonspecific siRNA and p53 siRNA and 48 h later were treated with O6BG. At 24 h posttreatment the cells were harvested and ChIP assays were done. O6BG increased p53 recruitment to p21 promoter on both 5' and 3' sites and knocking down p53 by siRNA dramatically decreased. B and C, similar experiments were designed as mentioned above (see Materials and Methods) and qRT-PCR was done. B, p21 transcription was significantly decreased in p53 knockdown cells in the presence or absence of O6BG. C, significant decrease in transcription of cyclin B1, cyclin B2, and cyclin A by O6BG was brought back in p53 knockdown cells in the presence or absence of O6BG.
Table 1. O6BG inhibits pancreatic cancer cell growth and increases pancreatic cell sensitivity to gemcitabine

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumor incidence</th>
<th>Pancreatic tumors</th>
<th>Body weight (g)</th>
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<tr>
<td></td>
<td></td>
<td>Tumor volume (mm³)</td>
<td>Tumor weight (g)</td>
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<td>Median (range)</td>
<td>Median (range)</td>
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<tr>
<td>Vehicle control</td>
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<td>4,596 (3,484-6,297)</td>
<td>2.96 (2.28-3.5)</td>
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<td>Gemcitabine (100 mg/kg)</td>
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<td>2.24 (2.01-2.57)</td>
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<tr>
<td>O6BG (100 μg)</td>
<td>10/10</td>
<td>2,419** (1,377-3,864)</td>
<td>1.53** (1.1-1.7)</td>
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<tr>
<td>Gemcitabine (100 mg/kg) + O6BG (100 μg)</td>
<td>10/10</td>
<td>1,607† (1,288-1,780)</td>
<td>1.26† (1.14-1.41)</td>
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NOTE: Human pancreatic cancer L3.6pl cells (1 × 10⁶) were injected into the pancreas of nude mice. Seven days later, groups of mice were treated with twice weekly (T, Th) i.p. injections of gemcitabine (100 mg/kg), daily (M-F) i.p. injections of O6BG (100 μg) alone, and i.p injections of combination of gemcitabine (twice) and O6BG (M-F), and daily (M-F) i.p. PBS was given to a control group of animals. All mice were sacrificed on day 35. These results illustrate that tumor volume and size in mice treated with O6BG alone and in combination with gemcitabine were significantly inhibited as compared with mice treated with gemcitabine alone or control group.

*P < 0.05 compared with control.
†P < 0.04 compared with gemcitabine alone.
‡P < 0.0005 compared with control or gemcitabine alone.

Pancreatic cancer is a major public health problem and poses serious challenges, particularly because of its rising incidence and poor survival (3). In addition, it is highly resistant to chemotherapy. Gemcitabine is a widely used chemotherapeutic agent against locally advanced and metastatic pancreatic cancers. Unfortunately, the introduction of chemotherapy has minimal effect on pancreatic cancer and does not extend median survival. Recent reports show that O6 methyl guanine DNA methyl transferase (MGMT), a DNA repair protein, is highly expressed in pancreatic tumors compared with normal pancreas (27). Overexpression of MGMT is known to cause resistance to alkylating agents such as the chloroethylnitrosoureas (BCNU, CCNU), temozolomide and procarbazine (28–32). Increased sensitivity of pancreatic tumor cell lines to temozolomide has been shown under methionine stress conditions and is attributed in part to diminished MGMT content and inhibition of the cell cycle progression (33). Human MGMT has emerged as a central target for improving the efficacy of a variety of anticancer alkylating agents. A remarkable sensitization of several MGMT-proficient human cancers by O6BG to MGMT-targeted alkylating agents in animal models has been shown (34). O6BG is a powerful inhibitor of MGMT, and it was developed based on its restricted mechanism of action (35). O6BG reacts with MGMT by covalent transfer of the benzyl group to the active site-cysteine and causes an irreversible inactivation of the enzyme. At therapeutic levels, O6BG is not toxic alone, but efficiently renders the tumor cells 2- to 14-fold more sensitive to alkylating agents in in vitro and in vivo settings. This establishes the potential therapeutic effect of O6BG as an enhancer of these drugs. In recent days, MGMT-tagged proteins have been engineered and imaged using fluorescent labeling with O6BG. However, evidence in the literature indicates that O6BG does enhance the cytotoxicity of cisplatin, nitrogen mustard, and a number of nonalkylating drugs like 5-fluorouracil and camptothecin. Furthermore, gemcitabine has remained a mainstay in the chemotherapy of pancreatic cancers for several decades. O6BG has been in clinical trials in a variety of cancers (brain, breast and melanoma), mainly to increase the efficacy of anticancer alkylating agents such as temozolomide and BCNU; in these cases, it is well established that specific inhibition of MGMT by O6BG results in increased number of DNA cross-links and, in turn, an enhanced alkylator efficacy. We are aware that myelosuppression is a major limitation in the clinical trials involving O6BG with alkylating agents, and this problem can be overcome by gene therapy efforts of transducing O6BG-resistant mutant MGMT into the hematopoietic stem cells, thus affording selective protection to hematopoietic stem cells (36, 37). Recent studies have also shown that O6BG can increase the efficacy of cisplatin, cyclophosphamide, and nonalkylators like camptothecin, but the mechanisms involved are currently unclear. We believe that our findings showing potentiation of gemcitabine by O6BG adds to this list and provides a framework for extending into a clinical setting. It is true that the known mechanisms of action (specific inhibition of MGMT by O6BG and disruption of DNA synthesis/chain termination by gemcitabine) may not be interlinked to explain the increased efficacy of this combination therapy. Therefore, further investigation is needed. Nonetheless, these studies provide examples of drugs (i.e. gemcitabine) whose cytotoxicities are enhanced by O6BG and alternative suggestions (such as the possible linkage of MGMT with cell cycle or O6BG inhibition of unknown components in DNA synthesis) that may describe the observed synergism.

In the present study, we confirmed that MGMT inhibition by O6BG reduced MGMT expression, and we show for the first time that O6BG induced downregulation of cell cycle regulatory proteins cyclin B1, cyclin B2, and cyclin A. Interestingly, O6BG also modulated the p53 function and as a result, p53 activated p21 and repressed cyclin B1, cyclin B2, and cyclin A. In addition, the results indicate that increased p21 transcription and decreased cyclin B1, cyclin B2, and cyclin A transcription are p53 dependent. Activated p53 inhibited pancreatic cancer cell proliferation in vitro and in vivo and caused apoptosis. Finally, we are the first to show that O6BG sensitized pancreatic cancer...
cells to gemcitabine and that O\textsuperscript{6}BG, as a single agent or in combination with gemcitabine, significantly reduced human pancreatic cancer cells growing orthotopically in nude mice. The murine model data indicate that the effects may be additive, but we believe more studies are required to uncover the interaction of these two drugs. In the end, additional studies are under way to uncover the exact interaction of these two drugs.

In 50% of human cancers, p53 is mutated and functional inactivation is a hallmark of majority of the tumors. Despite a large number of studies, the influence of wild-type and mutant p53 on therapy-induced DNA damage, the modulation of p53 target genes involved in cell proliferation, apoptosis, and the overall antitumor response have remained unclear. It has been reported that some pancreatic cancer cell lines have very low/undetectable level of p53 expression, and that p53 gene transfer in these cell lines increases drug-induced cytotoxicity and apoptosis (18, 19). In our study, we found that L3.6pl cells have very low level of wt p53 protein expression and PANC1 cells have high amount of mutant p53 expression. We investigated whether or not restoration of wild-type p53 function could be used to eliminate the tumorigenic phenotype in these cells. In addition, we investigated the relationship between p53 and MGMT and determined the effect of downregulated MGMT on p53 function and pancreatic cancer cell growth. Results

Fig. 4. Histology and immunohistochemical analyses. Tumors were harvested from control mice and mice treated with gemcitabine, O\textsuperscript{6}BG, or both gemcitabine and O\textsuperscript{6}BG. The sections were immunostained for expression of MGMT, cyclin B1, cyclin B2, cyclin A, and p21. Tumors from mice treated with gemcitabine and O\textsuperscript{6}BG had a significant decrease in the expression of MGMT, cyclin B1, cyclin B2, and cyclin A. In sharp contrast, expression of p21 was significantly increased. Representative samples (×40) are shown.
indicate that O6BG decreases MGMT at the transcriptional level. Attenuation of MGMT transcription by O6BG has not been reported before. What is known is that the benzyl group of O6BG is transferred to the active site cysteine 145 of MGMT forming benzylcysteine. The inactivated MGMT is then degraded through the ubiquitin-proteasome pathway. We postulate that the feedback mechanisms linking the degradation of MGMT to its de novo synthesis may exist and the decreased MGMT transcripts may be involved in this phenomenon. Recently it has been reported that overexpression of cyclin A and cyclin B1 was observed in pancreatic adenocarcinomas (6). In addition, the negative regulators p21, p16, and p27 have been shown to be inversely related to biological aggressiveness of pancreatic cancer (38–40). In mammalian cells cyclin B exists in two isoforms, cyclin B1 and B2 (41). p53 activates or represses transcription of a number of central regulators of cell division and apoptosis. The cyclin B2 transcription is shut down by expression of wild-type p53 (9). The other B-type cyclin, cyclin B1, has already been shown to be downregulated by p53 at the transcriptional level, or p53 regulates a G2 checkpoint through cyclin B1 (7, 8).

It has been previously reported that MGMT is also implicated in cell cycle of C3H/10T1/2 cells (42). The molecular mechanisms by which O6BG decreases the expression of proteins controlling the cell cycle and proliferatory pathways are currently unknown. Nonetheless, Yan et al. (43) have shown that O6BG inactivates MGMT and prolongs the G2-M cell cycle blockade induced by BCNU. Others have shown that O6BG also enhances the cytotoxicity elicited by cisplatin and nitrogen mustards, which do not generate O6-alkylguanines in DNA (the typical substrate for MGMT; ref. 44). These and other observations showing MGMT interaction with cell cycle and DNA replication components (45) indicate that human MGMT may carry out nonrepair functions. Consistent with these observations, our results show that MGMT inactivation by itself (through O6BG) or O6BG in combination with gemcitabine may provide a novel and effective approach for treating human pancreatic cancer and therefore prolong the survival of pancreatic cancer patients. In this article we have not investigated how MGMT inhibition may modify the mechanistic actions of gemcitabine. This needs to be addressed in future studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

Correction: Blockade of MGMT Expression by O\(^6\) Benzyl Guanine Leads to Inhibition of Pancreatic Cancer Growth and Induction of Apoptosis

In this article (Clin Cancer Res 2009;15:6087–95), which published in the October 1, 2009 issue of Clinical Cancer Research (1), the name of an author, Kalkunte S. Srivenugopal, was listed incorrectly.

Reference

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