Antifolate Activity of Pyrimethamine Enhances Temozolomide-Induced Cytotoxicity in Melanoma Cells

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Abstract
Most metastatic melanoma patients fail to respond to available therapy, underscoring the need to develop more effective treatments. We screened 2,000 compounds from the Spectrum Library in human melanoma cell lines to identify compounds that enhanced the cytotoxic effect of temozolomide, a drug used to treat metastatic melanoma. Screening was done with the temozolomide-resistant melanoma cell line SK-MEL-19, and six compounds were identified that had little or no inherent cytotoxicity but significantly enhanced growth-inhibition by temozolomide. These compounds were tested in five additional melanoma cell lines. Cell proliferation and death assays were used to compare the efficacy of single agent temozolomide versus combination treatments. Effects of combination treatment on levels of DNA double-strand breaks, the DNA repair protein O6-methylguanine-DNA-methyltransferase, apoptosis [measured by cleaved caspase-3 and poly(ADP-ribose) polymerase], and cell cycle were examined. Pyrimethamine, an antiparasitic, sensitized melanoma cells to temozolomide. Temozolomide combined with Pyrimethamine synergistically inhibited cell proliferation in melanoma cells with combination index values of 0.7 or less. In addition, combination treatment induced cell cycle arrest and increased both DNA damage and apoptosis. The increase in cell death due to combination treatment was rescued by leucovorin. Other folate antagonists were also effective enhancers of temozolomide-induced cytotoxicity, and the effects of antifolates were also evident in gliomas. Our screening approach led to the identification of Pyrimethamine, an orally available drug that efficiently crosses the blood-brain barrier, as a potent enhancer of the efficacy of temozolomide as an antineoplastic agent via inhibition of folate metabolism. (Mol Cancer Res 2009;7(5):703–12)

Introduction
There is an urgent need to identify new therapeutic targets and develop new agents for the treatment of melanoma (1, 2). Although dacarbazine has historically been the standard treatment for melanoma (3, 4), in recent years, it has been replaced by its analogue temozolomide (TMZ), a well-tolerated, orally bioavailable drug that can efficiently cross the blood-brain barrier. TMZ has proven useful in the treatment of metastatic melanoma to the brain in addition to glioblastoma and anaplastic glioma (5, 6). However, the response rate of melanoma to dacarbazine or TMZ is only ~20%. Attempts to improve efficacy of TMZ through combination therapy with drugs such as cisplatin, docetaxel, thalidomide, IFN, and irinotecan have shown no clear benefits in response rates, median time to progression, or overall survival (7–12).

Chemical genetics offers a powerful tool for identifying therapeutic candidates and targets (13–15). We have previously used this screening methodology to identify novel compounds and targets involved in the regulation of mammalian pigmentation (16–19), in the evasion of arsenic trioxide resistance in melanoma cells (20), and in the identification of agents specifically cytotoxic to melanoma (21).

In this study, we extended our chemical genetic approach to identify new compounds that enhance the cytotoxicity of TMZ against resistant melanoma. We screened a commercially available library of 2,000 compounds, including natural products and marketed drugs in TMZ-resistant melanoma cells. Of the six compounds identified as potential enhancers of TMZ-induced cytotoxicity, pyrimethamine (PYR) was deemed the most promising candidate due to its established use as an oral treatment for malaria and toxoplasmosis of the central nervous system. PYR synergistically enhances the growth inhibitory and apoptosis-inducing effects of TMZ. Moreover, we further show that the antifolate activity of PYR underlies these effects. Our results suggest that chemical genetics can identify novel drug combinations with potential clinical utility in the treatment of melanoma, and specifically show that the combination of TMZ with antifolates, in particular PYR, results in unexpected, proapoptotic effects on melanoma.

Results
Identification of Compounds That Enhance TMZ-Induced Cytotoxicity by Screening the Spectrum Collection Library
Among the 6 tested human melanoma cell lines, SK-MEL-19 was identified as the most resistant to TMZ, with an IC50 at ~89 μg/mL. A commercial library of 2,000 drugs
and natural products was screened to identify novel compounds that enhance TMZ-induced growth inhibition in SK-MEL-19. Screening followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done in 96-well plates at final library compound concentration of 1 μmol/L, in either the absence or presence of 50 μg/mL TMZ (Fig. 1A). Six compounds (benzyl isothiocyanate, chlorhexidine, cloxyquin, 3, 4-dimethoxydalbergione, PYR, and triamterene) were identified as potent enhancers of TMZ-induced cytotoxicity, with a minimum 50% greater growth inhibition relative to TMZ alone (Fig. 1B). These positive candidates were reconfirmed by testing at a lower final concentration of 0.5 μmol/L. PYR was chosen for further study due to its long-established and safe use in humans as an antimalarial drug.

Combination of TMZ and PYR Is Synergistic for Inhibition of Cell Proliferation

We examined the effects of either TMZ alone, PYR alone, or combined treatment with both agents (TMZ/PYR) on melanoma cell proliferation (Fig. 2). Both TMZ and PYR alone exhibited a dose-dependent inhibition of SK-MEL-19 cell proliferation (Fig. 2A and B). Combination treatment yielded significantly greater growth inhibition than either agent alone (Fig. 2C). The combination index (CI) was used to confirm and quantify the synergy observed with TMZ and PYR. CI values were <0.7 at all doses (range from 25-200 μg/mL; Fig. 2D), indicating that synergy occurred between TMZ and PYR across a broad range of concentrations. Unless otherwise noted, 25 μg/mL TMZ and 0.5 μmol/L PYR were used in subsequent experiments as these represent clinically achievable concentrations of each agent.

The effect of TMZ/PYR combination treatment was further examined by treating five additional, highly TMZ-resistant melanoma cell lines (SK-MEL-100, 173, 192, 451-Lu, and WM278) with either TMZ alone or TMZ/PYR in combination. As shown in Table 1, the addition of 0.5 μmol/L PYR resulted in a significant decrease in TMZ IC₅₀ in all 6 tested melanoma cell lines (Table 1). Effects of 0.5 μmol/L PYR alone on each cell line had also been included in Table 1. Effects of PYR alone were shown as the percentage of growth inhibition of PYR versus equimolar DMSO.

![Image](image.png)

**FIGURE 1.** Identification of compounds that enhance TMZ cytotoxicity in melanoma cells by screening the Spectrum Collection library. **A.** Screening of the Spectrum Collection library to identify novel agents that enhance TMZ efficacy in cultured melanoma cells. Twenty-four hours after seeding into 96-well plates, cell cultures in 1 plate were treated with each library compound alone at 1 μmol/L, whereas cells in the parallel plate were treated with a combination of 1 μmol/L library compound and 50 μg/mL TMZ. Screening was done with SK-MEL-19 cells in 96-well plates by MTT assay after a 72 h treatment. Positive candidates were later reconfirmed in triplicate. **B.** Chemical structures of six hit compounds confirmed enhanced TMZ activity and induced greater inhibition of cell growth.
TMZ Alters PYR-Induced Cell Cycle Arrest

To address whether the antiproliferative effect of TMZ/PYR combination in melanoma is associated with cell cycle regulation, DNA cell cycle analysis was done upon SK-MEL-19 cells treated with TMZ + DMSO, PYR + DMSO, or TMZ + PYR. No obvious changes were observed in cells treated with TMZ + DMSO. PYR + DMSO, or TMZ + PYR. PYR at the final concentration of 0.5 μmol/L increased the number of S-phase cells, accompanied by a

Table 1. Effects of PYR Alone, TMZ Alone, and Their Combination in a Panel of Melanoma Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Growth Inhibitory Effect of PYR (%)</th>
<th>IC50 (μg/mL)</th>
<th>P</th>
</tr>
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<tbody>
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<td></td>
<td></td>
<td>TMZ</td>
<td>TMZ/PYR (0.5 μmol/L)</td>
</tr>
<tr>
<td>SK-MEL-19</td>
<td>18.3 ± 7.3</td>
<td>88.5 ± 13.0</td>
<td>13.0 ± 0.1</td>
</tr>
<tr>
<td>SK-MEL-192</td>
<td>33.9 ± 3.0</td>
<td>57.4 ± 13.2</td>
<td>2.7 ± 2.5</td>
</tr>
<tr>
<td>SK-MEL-100</td>
<td>15.4 ± 3.9</td>
<td>62.1 ± 6.3</td>
<td>41.4 ± 6.4</td>
</tr>
<tr>
<td>SK-MEL-173</td>
<td>6.2 ± 5.3</td>
<td>80.0 ± 3.0</td>
<td>58.4 ± 1.0</td>
</tr>
<tr>
<td>451-Lu</td>
<td>8.0 ± 7.6</td>
<td>101.8 ± 11.0</td>
<td>78.3 ± 13.1</td>
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<tr>
<td>WM278</td>
<td>9.4 ± 4.0</td>
<td>56.9 ± 7.7</td>
<td>39.7 ± 2.1</td>
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</table>

*Percentage of growth inhibition versus equimolar DMSO for 0.5 μmol/L PYR.
corresponding decrease in the proportion of cells in G1. Accumulation of cells in the G2 phase and S phase was observed in the cells treated with the combination of both compounds on day 3 (Fig. 3). Cell accumulation in S phase and G2 phase after TMZ + PYR treatment suggests that a significant proportion of cells was unable to complete DNA synthesis correctly and cell division was interrupted. At the same time, DMSO + PYR and TMZ + PYR treatments induced the formation of hypodiploid sub-G1 peaks that are indicative of apoptosis. These data suggest that both cell cycle arrest and cell loss due to apoptosis could be responsible for the antiproliferative effects of TMZ/PYR.

The concentration of TMZ (25 μg/mL or ~100 μmol/L) used in this study was relatively low and close to clinically achievable serum levels during chemotherapy (100 μmol/L; ref. 22). At a 25 μg/mL concentration, TMZ had no effect on

FIGURE 3. TMZ/PYR induces cell cycle arrest in melanoma cells. Cells were incubated in either medium alone, medium containing TMZ (25 μg/mL) plus DMSO, PYR (0.5 μmol/L) plus DMSO, or TMZ plus PYR combination for 72 h before harvesting. The harvested cells were stained with propidium iodide and analyzed with fluorescence-activated cell sorting to determine the proportion of cells in each phase of the cell cycle. A, Representative flow cytometry histograms obtained from treated cells. B, Quantitation of cell population distribution over the various stages of the cell cycle. *, P < 0.05; **, P < 0.01, compared with control (CTR).
cell cycle progression in melanoma cells under the test conditions we used.

**PYR Enhances Cell Death and Apoptosis Induced by TMZ**

To investigate whether treatment with PYR can enhance the induction of SK-MEL-19 cell death by TMZ, trypan blue staining was used to determine the ratio of dead cells versus total cells. At clinically relevant concentrations, TMZ induced death in ~15% of cells (Fig. 4A), whereas PYR at 0.5 μmol/L had only a limited effect on cell death. However, the combination of TMZ/PYR induced an ~2-fold increase in cell death in SK-MEL-19 (Fig. 4A). These results are consistent with the MTT assay data (Fig. 2C) and suggest that the TMZ/PYR combination treatment significantly enhances the inhibition of melanoma cell growth and induction of cell death by TMZ.

To determine whether TMZ, PYR, or TMZ/PYR induce cell death by the same pathway, cellular levels of activated caspase-3 and PARP were assessed by immunoblot analysis with an antibody against the cleaved (active) form of the enzymes. No obvious changes were observed in cells treated with TMZ alone, whereas TMZ/PYR treatment increased the level of cleaved caspase-3 and cleaved PARP in SK-MEL-19 cells (Figs. 4B and 6D). These results suggest that the proapoptotic effect of TMZ/PYR combination treatment in melanoma cells is mediated by a caspase-dependent pathway.

**TMZ/PYR Treatment Increases DNA Damage in Melanoma Cells**

DNA damage is a well-characterized, initial upstream event in apoptotic cell death (23). Phosphorylation of histone H2AX is one of the earliest responses to strand breakage and is accepted as an early marker for DNA double strand breaks (DSB; ref. 24). We assessed levels of phosphorylated histone H2AX to determine whether TMZ/PYR combination treatment caused more DSBs. The topoisomerase inhibitor camptothecin, which induces DSBs by stalling DNA replication forks, was used as a positive control, whereas untreated cells served as negative control. A trace level of phosphorylated histone H2AX was observed in untreated cells and cells treated with TMZ alone (Fig. 5). PYR treatment induced a modest increase in the level of phosphorylated histone H2AX, suggesting that PYR induces cell death through its activity as an antifolate. A strong increase in the level of phosphorylated histone H2AX was observed in cells treated with TMZ/PYR (Fig. 5). These results show that TMZ/PYR combination treatment generates more DSBs, which further induces cell death.

Because the O6-methylG lesion induced by TMZ treatment can be directly removed by the DNA repair protein O6-methylguanine-DNA-methyltransferase (MGMT), the cellular level of MGMT is one of the main contributors to TMZ resistance (25). Western blot analysis was done to determine whether TMZ, PYR, and TMZ/PYR treatments alter MGMT protein levels. No alteration in MGMT protein level was observed in cells treated with either agent alone or in combination (Supplementary Fig. S1), suggesting that the synergistic, antiproliferative effect exerted by the TMZ/PYR combination is independent of effects on MGMT levels.

**Antifolate Activity of PYR Enhances TMZ Efficacy in Melanoma and Glioma Cells**

Among the six “hits” identified from library screening, both PYR and triamterene are known to have some antifolate activity. To investigate whether PYR enhances TMZ efficacy through its activity as an antifolate, 10 additional antifolate compounds were tested to determine whether they, too, enhanced TMZ-induced growth inhibition. Because TMZ is a standard chemotherapy drug used to treat gliomas, the effects of antifolates were also tested in the TMZ-resistant glioma cell line LN-18. Similar synergistic effects on inhibition of cell growth were observed with all antifolates studied when combined with TMZ in both melanoma and glioma cells (Fig. 6A). For example, the IC_{50} of TMZ was reduced almost 7-fold (from ~89 to ~13 μg/mL) in SK-MEL-19 cells when they were cotreated with each of the 5 different antifolates [etoprine (DDEP), metoprine (DDMP), cycloguanil, methotrexate (MTX), and PYR; Fig. 6A]. The CI values were calculated using CalcuSyn software and are summarized in Table 2. The data suggest that combinations of TMZ and antifolates are mostly synergistic in terms of cell growth inhibition in both melanoma and glioma cell lines.

Leucovorin (N-5-formyltetrahydrofolate, LV, folinic acid), a reduced form of folate, is used widely to specifically reverse the toxic effects of antifolates (26). To further confirm that PYR-enhanced TMZ efficacy in melanoma cells was due to the antifolate activity of PYR, 10 μmol/L LV was added to cells treated with PYR alone, TMZ alone, and TMZ/PYR in combination. LV reversed PYR-induced cytotoxicity but not TMZ-induced
cytotoxicity (Fig. 6B). Furthermore, LV rescued both the inhibition of cell growth (Fig. 6B) and the increase in apoptotic death induced by TMZ/PYR as measured by the proteolytic cleavage of PARP by caspase-3 (Fig. 6D). Lastly, effect of LV on reversing the growth inhibition caused by TMZ/PYR in combination is dose dependent (Fig. 6C). Taken together, these results confirmed that PYR enhances TMZ-induced cytotoxicity through its function as an antifolate, most likely by virtue of its action as a competitive inhibitor of dihydrofolate reductase.

Discussion

Many attempts have been made to enhance TMZ efficacy through combination treatments with limited success (7–12). Agents tested in combination with TMZ included those that interfere with DNA repair pathways such as the MGMT inhibitor O\(^{\text{\textregistered}}\)-benzylguanine (27) or the base excision repair inhibitor methoxyamine (28, 29). Alternatively, TMZ has been combined with cytotoxic agents, radiotherapy, immunotherapy, and antiangiogenic agents in clinical trials. However, compared with the effect of TMZ alone, no clear benefits have been shown from these combinations (30).

Previous studies have shown that TMZ at high concentrations can induce cell death and apoptosis in both melanoma and glioma cells (31, 32) and, specifically, that TMZ induces G\(_2\) arrest in glioma cells (33). However, a recent report suggests that TMZ induces senescence but not apoptosis in human melanoma cells (34). In this study, we used a clinically relevant concentration of TMZ and showed that it had little effect on apoptosis, DNA damage, or cell cycle arrest in melanoma cells. These results reflect the very modest clinical performance of TMZ as a chemotherapeutic agent.

To enhance TMZ efficacy in melanoma, we used a chemical genetic approach to identify novel combinations by screening the Spectrum library. The advantages of this library are 3-fold. First, clinical development is shortened as many compounds in the library have been used for other indications in humans. Second, based on the known function and mechanism of action of these compounds, one can readily test whether the chemosensitization effects of these agents are carried out via the same mechanism. Finally, after identification of a hit compound, additional compounds in the same class can be tested, providing useful information regarding structure-activity relationships.

Our screen of the Spectrum library was the first to identify PYR, a lipophilic dihydrofolate reductase inhibitor with clinical efficacy as an antimalarial drug, as a compound that significantly enhanced TMZ-induced cytotoxicity in melanoma cells. At their clinically achievable concentrations, TMZ and PYR showed synergistic activity in both melanoma and gliomas cells. In addition, PYR also enhanced cell killing induced by DTIC, a metabolite of TMZ that acts via the same mechanism (data not shown). However, given that none of other antimalarial compounds we tested (including artemisinin, chloroquine, primaquine, quindine, and quinine) could mimic the effect of PYR on enhancing the TMZ or DTIC efficacy in melanoma cells, we reasoned that PYR does not enhance TMZ efficacy through its activity as an antimalarial compound.

PYR is structurally related to folate and is known as an effective dihydrofolate reductase inhibitor, particularly in protozoa such as malaria and toxoplasma. Dihydrofolate reductase inhibitors (or antifolates) have been studied for many years as anticancer and antineoplastic agents. The disruption of folic acid metabolism has long been known to inhibit cell growth. Folic acid is essential for the de novo synthesis of the nucleoside thymidine, which is required for DNA synthesis. Thus, antifolates have greater, selective toxicity on rapidly dividing cells such as tumor cells. MTX, the most studied classic antifolate, provided the first cure in choriocarcinoma more than six decades ago and is effective in many malignancies (35). Antifolate drug development has then focused on agents to overcome MTX resistance and reduce the toxicity. Currently, second or third generation antifolates were tailored to have more efficient uptake or better polyglutamylation. Among them, the lipophilic antifolate ALIMTA (pemetrexed) is registered for mesothelioma and non–small cell lung cancer. The lipophilic antifolate PYR exerts strong proapoptotic activity in addition to its antiprotozoal effects. Several PYR analogues, such as DDEP and DDMP, have also been investigated as antitumor agents (36).

It has been reported that PYR can induce apoptosis and S phase accumulation in various cell lines (37–40). PYR has been shown to reduce melanoma growth in a severe combined immune-deficient mouse model (40). Consistent with previous reports (39, 40), we observed that PYR at a concentration of 0.5 \(\mu\text{mol/L}\) increased the number of cells in S phase (Fig. 3). Folate deficiency in cell culture has been reported to induce an excess of strand breaks in DNA (41, 42), and preliminary data indicate that genome-wide DNA stand breaks are related to folate status (43–45). In this study, we observed an increase in DSBs (Fig. 5) in cells treated with PYR/TMZ compared with each agent alone. These results suggest that PYR enhances TMZ efficacy through its antifolate activity. We further confirmed this mechanism of action by showing that a number of other antifolates also enhanced inhibition of cell growth induced by TMZ and that the effects of antifolates could be rescued by LV (Fig. 6).

MGMT plays a crucial role in the repair of DNA damage induced by TMZ treatment because it directly removes O\(^{\text{\textregistered}}\)meG (24). The expression level of MGMT is also susceptible to
epigenetic silencing (46). Although our data showed that TMZ/PYR treatment induced more DNA damage through the formation of DSBs (Fig. 5), no changes in MGMT protein level were observed in cells treated with PYR, TMZ, or PYR/TMZ. Together, this suggests that in melanoma, the enhancement of TMZ efficacy by PYR is independent of MGMT level. This observation was further confirmed by the clinically relevant finding that PYR-induced sensitization of cells to TMZ treatment was observed in both MGMT-expressing and MGMT-negative cells.

Many other pathways are involved in repairing DNA damage induced by methylating agents, which including base excision repair, homologous recombination, polymerase bypass, and mismatch repair. For example, previous studies have indicated that inhibition of MGMT increased TMZ sensitivity only in mismatch repair proficient cells but not in mismatch repair deficient cells (47). The major pathway and proteins involved in DNA repair are cell type dependent (48). In this study, we show that MGMT is not a target of PYR-induced TMZ sensitization.

**FIGURE 6.** PYR enhances TMZ efficacy by inhibiting folate metabolism. A. Growth inhibitory effects of PYR, DDEP, DDMP, cycloguanil (CYC), and the classic antifolate MTX on TMZ response curve were examined by MTT assay in both melanoma and glioma cells. In SK-MEL-19 cells, 0.005 μmol/L PYR, DDEP, DDMP and 0.001 μmol/L MTX showed significant enhancement of TMZ-induced growth inhibition, whereas 0.05 μmol/L PYR, DDEP, DDMP and 0.005 μmol/L MTX showed a similar effect in LN-18 cells. *, P < 0.05; **, P < 0.01. B. Cytotoxic effect of combination of TMZ (25 μg/ml) and PYR (0.5 μmol/L) can be rescued by LV (10 μmol/L) as examined by MTT assay. *, P < 0.05 between PYR and PYR/LV; **, P < 0.01 between TMZ/PYR and TMZ/PYR/LV. C. The effects of LV on reversing the cytotoxicity caused by TMZ/PYR combination are dose dependent. **, P < 0.01 compare to TMZ/PYR/LV when LV is 10 μmol/L. No significant difference compared with TMZ/PYR/LV when LV is 0.5 or 2 μmol/L. D. Levels of PARP and cleaved PARP expression, and levels of actin protein served as loading control.
The chemical genetics approach we used to identify compounds that can overcome TMZ resistance may prove useful in identifying additional compounds to improve the efficacy of other chemotherapeutic agents. For instance, we recently used a similar approach to overcome arsenic trioxide resistance in melanoma cells (20). A chemical genetics approach has also been used successfully to identify benzimidazoles, a class of structurally related, tubulin-disrupting drugs, as agents preferentially toxic to melanoma cells compared with melanocytes (21). In addition, this approach could be used to reveal additional novel mechanisms and targets for cancer treatment. In particular, our findings strongly suggest that targeting folate metabolism, especially through dihydrofolate reductase inhibition, may prove to be an effective strategy to improve TMZ efficacy in melanoma.

PYR is bioavailable after oral administration and has also been safely administered to malarial patients for prolonged periods with limited side effects. Moreover, PYR differs from the classic antifolates such as MTX in that it is a lipophilic antifolate, allowing it to diffuse readily across the cell membrane without a transporter, and to easily cross the blood-brain barrier. Taken together, these characteristics make PYR an attractive candidate for improving the efficacy of TMZ in melanoma (including melanoma metastatic to the brain).

Materials and Methods

Screening of Spectrum Library in Melanoma Cells

Tumor Cell Lines. Human malignant melanoma cell lines (SK-MEL-19, SK-MEL-100, SK-MEL-173, and SK-MEL-192) were a gift from Dr. Alan Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY). Cells were cultured in DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin at 37°C. Melanoma cell lines were obtained from the American Type Culture Collection. 451Lu and WM278 were cultured in T2% tumor medium consisting of MCDB 153 medium supplemented with 5 μg/mL insulin, 2% FCS, and 20% L-15 Leibovitz medium.

Library Screening. The library used in this study was The Spectrum Collection (MicroSource Discovery Inc.). The 1,000 compounds in this library are marketed drugs, other biologically active small molecules, or natural products (supplied at a concentration of 10 μmol/L in DMSO). SK-MEL-19 cells were seeded at 3,000 cells per well in 96-well plates and allowed to attach overnight. Library compounds were then added to cell cultures at a final concentration of 1 μmol/L, either alone or in combination with 50 μg/mL TMZ. After 72 h, cellular proliferation was measured. The plates were read with a microplate reader (Bio-Rad model 550) by measuring the absorbance of converted MTT at 490 nm.

Drugs and Reagents. TMZ (NSC 362856) and the lipophilic folate analogues DDMP, DDEP, and cycloguanil were kindly provided by the National Cancer Institute. PYR, MTX, and LV were purchased from Sigma-Aldrich Chemical Company, Inc. All drugs were dissolved in DMSO.

Cell Proliferation Assay and Viability Assay

Effect of treatment with TMZ and/or PYR on cell growth and cytotoxicity in melanoma and glioma cells was assessed using colorimetric MTT assay (CellTitre 96 AQueous nonradioactive cell proliferation assay; Promega), according to manufacturer’s instruction. The absorbance of control cells exposed to vehicle alone defined 100% cell growth, and the effect of drugs on cellular proliferation was expressed as a percentage of cell growth relative to control cells. Cell viability was also determined by trypan blue dye exclusion method.

Synergy Analysis of Combined Drug Effects

Drug synergy was determined by CI methods derived from Chou-Talalay equations (49) using the CalcuSyn software (Biosoft). Using cell proliferation assay and computerized software data, CI values are generated between two drugs. A CI of 1 indicates an additive effect between two agents, whereas a CI of <1 indicates synergy.

Western Blot Analysis and Antibodies

Cells were harvested in extraction buffer [1% Triton X-100, 50 mmol/L Tris, 2 mmol/L EDTA, and 150 mmol/L NaCl (pH 7.5)] containing complete protease inhibitor mixture (Roche). The lysates were centrifuged at 14,000 rpm for 10 min at 4°C. Bio-Rad protein assay reagent (Bio-Rad) was used to measure the protein concentrations. Proteins (20 μg) were separated by 8%, 12%, or 15% SDS-polyacrylamide (SDS-PAGE) gel and transferred to Immobilon-P membranes (Millipore). Immunoreactive bands were visualized using ECL-plus detection reagent and X-OMAT processing. Densitometry values were calculated using ImageQuant TL software.

Antibodies. Anti–phospho-H2A.X (Ser 139) antibody was purchased from Upstate Cell Signaling Solutions, anti-MGMT antibody was from Chemicon, and antibodies against cleaved capase-3, PARP, and β-actin were from Cell Signaling Technology.

Cell Cycle Analysis

Cell cycle distribution was determined by staining DNA with propidium iodide (Sigma). Briefly, cells were treated with TMZ plus DMSO, PYR plus DMSO, or TMZ/PYR combination for 72 h and then harvested. Cells were then washed and fixed in 70% ethanol on ice for 30 min. After centrifugation, the cell pellets were washed and resuspended in phosphate-citrate buffer. Cells were then treated with RNase and stained with propidium iodide. DNA content was analyzed on a

Table 2. Summary of CI Values for TMZ and Antifolates

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Dose</th>
<th>MTX/TMZ</th>
<th>DDEP/TMZ</th>
<th>PYR/TMZ</th>
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<tr>
<td>SK-MEL-19</td>
<td>12.5</td>
<td>0.292</td>
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Cell Cycle Analysis

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cytofluorimeter by fluorescence-activated cell sorting analysis (FACScan).

**Statistical Analysis**

Data were expressed as means ± SD. Statistical analysis was done using the Student’s Paired t test. The criterion for statistical significance was established as a \( P \) value of <0.05.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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