Celastrol Synergistically Enhances Temozolomide Cytotoxicity in Melanoma Cells

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Abstract

Efforts to improve melanoma response rates to temozolomide (TMZ) have thus far been unsuccessful. We screened a library of 2,000 marketed drugs and natural products to identify agents with the potential to sensitize melanoma cells to the effects of TMZ. Celastrol (CEL), a natural compound found in the Thunder of God vine, was identified based on its ability to enhance cell death in TMZ-resistant melanoma cells. A cell proliferation assay was used to compare the growth-inhibitory effects of TMZ alone versus TMZ/CEL combination treatment. Cytotoxic synergy was assessed using combination-index methods. The expression of nuclear factor-κB (NF-κB), IκB, mitogen-activated protein kinase, and ubiquitinated proteins were examined using Western blotting, and the localization of NF-κB in CEL-treated melanoma cells was evaluated using immunofluorescence microscopy. The CEL/TMZ combination synergistically inhibited cell proliferation in melanoma cells. CEL treatment increased the levels of ubiquitinated proteins, reduced the levels of tumor necrosis factor-α–induced IκB phosphorylation, and blocked NF-κB translocation to the nucleus. Inhibition of NF-κB with small interfering RNA mimicked the ability of CEL to sensitize melanoma cells to TMZ, suggesting that inhibition of NF-κB may play a role in TMZ/CEL-induced cytotoxicity. The TMZ/CEL combination induced the phosphorylation of c-Jun NH2-terminal kinase, implicating the mitogen-activated protein kinase pathway in the treatment effects. Our data suggest that CEL may be effective in sensitizing resistant melanoma cells to the effects of TMZ. (Mol Cancer Res 2009;7(12):1946–53)

Introduction

Available treatment options for patients with melanoma consistently fail to show efficacy in terms of overall survival. Temozolomide (TMZ) has been shown to be as efficacious as dacarbazine in the treatment of metastatic melanoma, however, clinical trials show response rates of <20% (1-3). Recent studies have examined the possibility of improving the chemotherapeutic efficacy of TMZ by combining it with other agents such as cisplatin, docetaxel, thalidomide, IFN, and irinotecan, but none were able to show a clear benefit with regard to median time to progression or overall survival (1, 4-8).

Current estimates suggest that it costs between 800 million and 1.3 billion U.S. dollars to bring a drug from the laboratory to the market; thus, the development of new drugs carries a tremendous burden with regard to both time and resources (9). In an effort to reduce these costs, we have performed screens of chemical libraries consisting of marketed drugs and natural compounds with the goal of identifying agents with antitumor effects that can be expedited to clinical trials. We recently identified a group of antifolates that enhance the cytotoxicity of TMZ in melanoma cells (10). The use of antifolates is limited in some patients, however, due to intolerable side effects, thus there is a need to identify alternatives. Recently, there has been growing interest in identifying natural products with favorable side effect profiles that can sensitize malignant cells to the effects of known chemotherapeutics or that are synergistic with chemotherapeutics due to the exploitation of different yet complementary signaling pathways.

Celastrol (CEL), a traditional Chinese herbal medicine used in the treatment of arthritis and other inflammatory conditions, was identified as one of the most potent enhancers of TMZ-induced inhibition of cellular proliferation. Immunofluorescence and small interfering RNA (siRNA) knockdown studies implicate the inhibition of nuclear factor-κB (NF-κB) as a possible mechanism by which CEL exerts the observed synergistic cytotoxic effects. The identification of a natural product with a well-characterized side effect profile that enhances the efficacy of TMZ represents a promising therapeutic alternative for patients with melanoma.

Results

CEL Potentiates TMZ-Mediated Growth Inhibition

In the initial screen of the Spectrum Library, we aimed to identify compounds with minimal toxicity at the concentration used (1 μmol/L), but which showed a >50% increase in growth inhibition relative to TMZ alone when combined with TMZ. Eighteen compounds met the criteria, and the activity of each compound was further evaluated by assessing its effect at a lower final concentration of 0.5 μmol/L in a panel of melanoma cell lines. Of these 18 compounds, the natural product, celastrol (Fig. 1A) was prioritized for further study due to its striking sensitization effects and well-established safe use in Chinese
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Research.

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medicine for the treatment of inflammatory diseases. Pristimerin (PRI), the methyl ester derivative of CEL, produced by the same vine, was also found to be active (Fig. 1A).

**FIGURE 1.** Identification of novel compounds that enhance TMZ cytotoxicity by screening the Spectrum Collection library. **A.** Chemical structures of CEL and PRI, two compounds that enhanced TMZ cytotoxicity resulting in greater inhibition of cell growth compared with TMZ treatment alone. **B.** Combination treatment with CEL/TMZ and PRI/TMZ enhanced TMZ cytotoxicity in SK-MEL-173 cells compared with TMZ alone. Melanoma cells were treated with the indicated concentration of TMZ, either with or without 0.25 μmol/L of CEL or PRI for 72 h. Growth inhibition of cells was then evaluated by MTT assay. Survival relative to DMSO control is presented as mean ± SE of at least three experiments, with viability of DMSO-treated cells set at 100%. *, P < 0.05 compared with TMZ; **, P < 0.01 compared with TMZ. **C.** Treatment of five melanoma cell lines with 0.25 μmol/L of CEL as a single agent does not result in significant growth inhibition compared with DMSO-treated control cells.

TMZ/CEL Synergistically Inhibits Cell Proliferation in Melanoma Cells

To further assess the ability of CEL and PRI to sensitize melanoma cells to TMZ, we examined the growth-inhibitory effects of TMZ, CEL, and PRI treatment alone compared with combination treatment with TMZ/CEL and TMZ/PRI in TMZ-resistant melanoma cell line SK-MEL-173. Combination treatment with TMZ/CEL and TMZ/PRI yielded significantly greater growth inhibition than TMZ alone (Fig. 1B). By contrast, treatment of SK-MEL-173 with single-agent CEL at 0.25 μmol/L resulted in minimal growth inhibition, which was a consistent finding across a panel of melanoma cell lines (Fig. 1C). Drug synergy between TMZ and CEL was determined using combination-index (CI) methods derived from Chou-Talalay equations (11) using CalcuSyn software (Biosoft). A CI value of 1 indicates an additive effect between two agents, whereas a CI value of less than 1 indicates synergy. CI values were <1 at all doses of TMZ tested (range, 25-150 μg/mL; Table 1), indicating that low-dose CEL (0.25 μmol/L) was synergistic with TMZ across a broad range of concentrations. The effects of TMZ/CEL combination treatment were further examined in four additional TMZ-resistant melanoma cell lines (SK-MEL-19, M14, 451Lu, and WM278). Treatment with combination TMZ/CEL resulted in a significant decrease in the IC50 of TMZ in all cell lines tested (Table 2).

**CELL Inhibits Tumor Necrosis Factor-α–Induced IκB Phosphorylation and NF-κB Translocation**

CEL has traditionally been used to treat inflammatory diseases, but its mechanism of action in melanoma cells has not been well defined. The involvement of NF-κB in the effects of CEL, however, has been suggested by previous studies of other neoplastic cell types (12). Phosphorylation of IκB and subsequent degradation of IκB is the upstream event of

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>CI</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 μg/mL TMZ, 0.25 μmol/L CEL</td>
<td>0.912</td>
<td>Synergistic</td>
</tr>
<tr>
<td>50 μg/mL TMZ, 0.25 μmol/L CEL</td>
<td>0.924</td>
<td>Synergistic</td>
</tr>
<tr>
<td>100 μg/mL TMZ, 0.25 μmol/L CEL</td>
<td>0.716</td>
<td>Synergistic</td>
</tr>
<tr>
<td>150 μg/mL TMZ, 0.25 μmol/L CEL</td>
<td>0.288</td>
<td>Synergistic</td>
</tr>
</tbody>
</table>
NF-κB activation. Once IκB is phosphorylated and degraded, NF-κB translocates from the cytosol to the nucleus to regulate its target genes. To investigate the involvement of NF-κB signaling as a possible mechanism of action of CEL in melanoma cells, the effects of CEL on tumor necrosis factor-α (TNF-α)–induced IκB phosphorylation and NF-κB translocation were examined. Melanoma cells were treated with 20 ng/mL of TNF-α for 10 or 30 minutes in the presence or absence of pretreatment with CEL for 30 minutes. Cellular levels of IκB, phosphorylated IκB, and NF-κB were examined using Western blot analysis. Treatment with TNF-α for 10 minutes resulted in increased phosphorylation and degradation of IκB, whereas pretreatment with CEL blocked IκB phosphorylation and degradation (Fig. 2A, 1 and 2). These findings suggest that inhibition of NF-κB activation could be a potential mechanism of action for CEL in melanoma cells.

Although the level of NF-κB protein expression was equivalent in both TNF-treated cells and CEL-pretreated cells

Table 2. IC_{50} for TMZ Alone or in Combination with 0.25 μmol/L CEL in a Panel of Melanoma Cell Lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TMZ IC_{50} (μg/mL)</th>
<th>TMZ/CEL IC_{50}</th>
<th>Reduction (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM278</td>
<td>43.8 ± 9.1</td>
<td>17.1 ± 1.4</td>
<td>61</td>
<td>0.007</td>
</tr>
<tr>
<td>SK-MEL-173</td>
<td>75.3 ± 15.7</td>
<td>30.7 ± 15.5</td>
<td>59</td>
<td>0.025</td>
</tr>
<tr>
<td>451Lu</td>
<td>95 ± 11.9</td>
<td>49.4 ± 9.1</td>
<td>48</td>
<td>0.003</td>
</tr>
<tr>
<td>SK-MEL-19</td>
<td>88.3 ± 9.8</td>
<td>58.5 ± 8.4</td>
<td>34</td>
<td>0.002</td>
</tr>
<tr>
<td>M-14</td>
<td>85.1 ± 12.6</td>
<td>57.8 ± 7.9</td>
<td>32</td>
<td>0.018</td>
</tr>
</tbody>
</table>

FIGURE 2. CEL inhibits TNF-α–induced IκB phosphorylation and NF-κB translocation to the nucleus in melanoma cells. A. SK-MEL-173 cells were treated with 20 ng/mL of TNF-α for 10 or 30 min in the presence or absence of pretreatment of CEL for 30 min. Cellular levels of IκB, phosphorylated IκB, and NF-κB were examined using Western blot analysis. Actin protein levels were used as loading control. B. Subcellular localization of NF-κB after the stimulation of SK-MEL-173 cells with TNF-α after 2 h in the presence or absence of 30 min of CEL pretreatment. After fixation, cells were stained with anti-NF-κB antibody (green) and PI (red). Experiments were repeated thrice.
(Fig. 2A, 3), the activity of NF-κB is contingent on its translocation to the nucleus, where it regulates target genes. Thus, to determine whether inhibition of IκB phosphorylation by CEL correlates with inhibition of the translocation of NF-κB, the subcellular localization of NF-κB was assessed using immunofluorescence microscopy. After stimulation of SK-MEL-173 cells with TNF-α, we observed nuclear localization of NF-κB (Fig. 2B, 2). Pretreatment with CEL, however, blocked the translocation of NF-κB to the nucleus induced by TNF-α (Fig. 2B, 3).

**Treatment of Melanoma Cells with CEL Results in the Accumulation of Ubiquitinated Proteins**

The proteasome is a multicatalytic protease complex that degrades many endogenous proteins following their ubiquitination. CEL has previously been reported to inhibit proteasomal activity in prostate cancer cells (12). To investigate whether CEL might also inhibit cellular proteasome activity in melanoma, SK-MEL-173 cells were treated with CEL for 2 and 4 hours, followed by assessment of the accumulation of ubiquitinated proteins and proteasome substrate IκB by Western blot. We observed an accumulation of ubiquitinated proteins in melanoma cells treated with CEL at 2 and 4 hours but not in cells treated with DMSO (Fig. 3, 1). Consistent with this finding, we also observed increased levels of IκB, a well-known substrate protein degraded by the proteasome (13), in cells treated with CEL but not in DMSO control cells (Fig. 3, 2). The sensitization effect of melanoma cells to TMZ was also examined using the marketed proteasome inhibitor bortezomib; however, the effect was observed only at high concentrations that would be considered toxic in vivo (data not shown).

**SiRNA-Mediated Knockdown of NF-κB Sensitizes Melanoma Cells to TMZ**

To further determine whether inhibition of melanoma cell proliferation by TMZ/CEL combination treatment is associated with the NF-κB signaling pathway, SK-MEL-173 cells were transfected with NF-κB siRNA. We hypothesized that if inhibition of NF-κB was critical to CEL-induced sensitization of TMZ, melanoma cells should also be sensitized to TMZ upon the knockdown of NF-κB. Electroporation of NF-κB siRNA into SK-MEL-173 cells resulted in a significant reduction in the levels of NF-κB protein, whereas the control scrambled siRNA had no effect on NF-κB protein levels (Fig. 4A). Reduction of NF-κB protein levels in melanoma cells rendered them more sensitive to the growth-inhibitory effects of TMZ compared with cells transfected with a scrambled siRNA. The IC₅₀ for TMZ in cells transfected with NF-κB siRNA was significantly lower than in untreated cells or in cells transfected with control siRNA (Fig. 4B). Knockdown of NF-κB by siRNA mimicked the ability of CEL to sensitize melanoma cells to the effects of TMZ. Treatment of cells with single agent CEL (CTR/CEL) and transfection of cells with NF-κB siRNA (si-kB) resulted in approximately the same degree of reduction in the TMZ IC₅₀ compared with controls. Furthermore, the combination of NF-κB knockdown and treatment with CEL (si-kB/CEL) resulted in a more significant reduction of the IC₅₀ for TMZ.
TMZ IC_{50} compared with control cells than either intervention alone.

Involvement of Mitogen-Activated Protein Kinases in TMZ/CEL-Treated Melanoma Cells

Previous work has described the activation of NF-κB by the mitogen-activated protein kinase (MAPK) pathway in human melanoma cells (10). To investigate whether alterations in the MAPK pathway are also involved in the mechanism of CEL/TMZ-induced cytotoxicity, we examined the effect of TMZ/CEL treatment on expression levels of key molecules in the MAPK pathway. We found that treatment with both TMZ alone and CEL alone inhibited the phosphorylation of extracellular signal-regulated kinase (ERK) in both basal and TMZ/CEL-Treated Melanoma Cells

Discussion

We identified CEL, a natural product with a long history of use in Chinese medicine, as having the ability to sensitize chemoresistant melanoma cells to the cytotoxic effects of TMZ. Furthermore, treatment with low-dose CEL was synergistic with TMZ in reducing melanoma cell growth across a broad range of TMZ concentrations. Mechanistic studies show that CEL inhibits NF-κB translocation to the nucleus, and the accumulation of ubiquitinated proteins and proteasome substrate IxB upon treatment suggests that CEL may be functioning as a proteasome inhibitor in the melanoma cell lines tested. Our studies also show that combination treatment with TMZ/CEL results in higher levels of phosphorylated JNK compared with either agent alone, implicating the involvement of the MAPK pathway in the observed synergistic effects.

CEL, purified from the plant *Tripterygium wilfordii*, also called the Thunder of God vine, is indigenous to Southern China and has been used as a natural remedy in Chinese medicine for over 2,000 years. In the past decade, CEL has become the focus of numerous preclinical studies that have shown its potential for use in a wide range of conditions, from inflammatory diseases such as arthritis and Crohn’s disease to neurologic diseases such as Alzheimer’s and amyotrophic lateral sclerosis (14-17). More recently, both in vitro and in vivo studies have yielded results suggesting that CEL may also be effective in the treatment of chemoresistant neoplasms including pancreatic cancer, glioma, and melanoma (18-20). Preclinical studies in melanoma have shown the ability of monotherapy CEL to inhibit melanoma cell growth and reduce the number and size of metastases that develop in nude mice (20).

Our functional studies using immunofluorescence microscopy revealed that pretreatment of melanoma cells with CEL blocked the translocation of NF-κB to the nucleus (Fig. 2). The ability of NF-κB to regulate the transcription of target genes is dependent on its subcellular localization. Thus, our data suggest that the inhibition of NF-κB may play a role in the observed CEL-mediated sensitization effect of melanoma cells to TMZ and that this inhibition is exerted by blocking its translocation between cellular compartments. Our results offer further evidence to support the involvement of NF-κB inhibition in the antitumor effects of CEL by demonstrating that transfection with NF-κB siRNA mimics the effect of treatment with CEL with regard to the sensitization of melanoma cells to the growth-inhibitory effects of TMZ. We specifically examined the effects of CEL on cells pretreated with TNF-α, a known activator of NF-κB, thus demonstrating that the inhibitory effects are being exerted in the setting of cells known to have activated NF-κB. These results are in concordance with previous studies of CEL in melanoma cell lines that have also noted its inhibitory effects on NF-κB transcription. In one such study, a chemical library screen identified CEL as having the capacity to elicit similar effects as the proapoptotic ATF2 peptide (20). The authors were able to show a dose-dependent decrease in NF-κB transcription activity upon treatment of melanoma cell lines with CEL (20).

Combination TMZ/CEL treatment resulted in higher levels of JNK phosphorylation than were obtained using either agent alone (Fig. 5), implicating the MAPK pathway in the observed effects. Activating mutations in protein kinases of the MAPK family, specifically RAS/RAF/ERK, are among the genetic aberrations most frequently found in melanoma and therefore represent common targets for treatment (21). Of the cell lines employed in these studies, four of five have mutations in BRAF genes is dependent on its subcellular localization. Thus, our data suggest that the inhibition of NF-κB may play a role in the observed CEL-mediated sensitization effect of melanoma cells to TMZ and that this inhibition is exerted by blocking its translocation between cellular compartments. Our results offer further evidence to support the involvement of NF-κB inhibition in the antitumor effects of CEL by demonstrating that transfection with NF-κB siRNA mimics the effect of treatment with CEL with regard to the sensitization of melanoma cells to the growth-inhibitory effects of TMZ. We specifically examined the effects of CEL on cells pretreated with TNF-α, a known activator of NF-κB, thus demonstrating that the inhibitory effects are being exerted in the setting of cells known to have activated NF-κB. These results are in concordance with previous studies of CEL in melanoma cell lines that have also noted its inhibitory effects on NF-κB transcription. In one such study, a chemical library screen identified CEL as having the capacity to elicit similar effects as the proapoptotic ATF2 peptide (20). The authors were able to show a dose-dependent decrease in NF-κB transcription activity upon treatment of melanoma cell lines with CEL (20).

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![FIGURE 5. CEL/TMZ cotreatment results in the inhibition of ERK phosphorylation and induction of JNK phosphorylation in melanoma cells. SK-MEL-173 cells were treated with TMZ, CEL (C), TMZ plus CEL (TMZ/C), TNF-α, TNF-α plus CEL (TNF/C) for 24 h. Levels of phosphorylated ERK, ERK, phosphorylated JNK, and JNK were measured using Western blot analysis. Levels of actin protein served as loading control.](Image 60x105 to 275x363)
and thus likely have upregulated MAPK signaling. It is therefore possible that the observed effects of CEL are mediated through the attenuation of MAPK signaling. Previous studies by Abbas et al. have also shown that the antiproliferative effects of CEL are dependent on JNK activity and single-agent treatment with CEL leads to increased phosphorylation of JNK and its substrate c-Jun both in vitro and in vivo (20). In contrast to previous reports of ERK phosphorylation upon treatment with CEL (20), we found that CEL inhibited the phosphorylation of ERK at both the basal and TNF-α-stimulated level (Fig. 5). This discrepancy is most likely attributable to different CEL exposure times as ERK phosphorylation can be a rapid and transient event. Our analysis of ERK phosphorylation was done 24 hours after CEL treatment as compared with their study which was conducted 1 hour after CEL treatment. Studies evaluating the possibility of synergistic growth inhibition upon treatment with CEL in the setting of MEK inhibition may be warranted to better elucidate the role of MAPKs in CEL-mediated sensitization of melanoma cells to TMZ.

Although previous studies in prostate cancer have shown the ability of CEL to suppress tumor growth via proteasome inhibition (12), our study represents the first report to suggest that CEL inhibits proteasome activity in melanoma cells. Following treatment with CEL, we observed an accumulation of ubiquitinated proteins compared with control-treated cells, suggestive of proteasome inhibition (Fig. 3). Although we recognize that an increase in ubiquitinated proteins may represent a common final pathway of a range of cellular processes, our data also show an increase in IκB levels upon treatment with CEL. This is consistent with previous studies of other malignancies showing that inhibition of the proteasome leads to increased levels of well-characterized proteasomal target substrates such as IκB and p27 with subsequent proapoptotic effects (12).

The proteasome inhibitor bortezomib has shown clinical efficacy in hematologic malignancies (22). Thus far, however, bortezomib has not shown substantial efficacy as monotherapy for the treatment of solid malignancies (23). Clinical trials of proteasome inhibitors in melanoma have been limited by unacceptable levels of toxicity including peripheral neuropathy. A 2005 phase I clinical trial conducted at our institution evaluated the efficacy and safety of bortezomib in 40 patients with solid malignancy including 7 patients with melanoma (24). The only antitumor activity observed in any of the 40 evaluable patients was in 2 melanoma patients with pulmonary metastases. The development of dose-limiting neuropathy in the melanoma bortezomib clinical trial was postulated to have been mediated by matrix metalloproteinases or TNF-α (24). It is possible that CEL, a proteasome inhibitor that has been shown to suppress the production of proinflammatory cytokines such as TNF-α (25), may be of particular use in reducing some of the dose-limiting toxicity noted in recent clinical trials of proteasome inhibitors in solid malignancies. CEL may have potential as a natural product that can overcome melanoma chemoresistance while maintaining an acceptable therapeutic index as a combination treatment with existing chemotherapies. The utilization of chemical/natural product libraries allows for the identification of agents that have potent antitumor effects and a history of human use and safety, thereby expediting the path to clinical trials. However, in the aforementioned chemical screening study by Abbas et al. that identified CEL based on its ability to mimic the proapoptotic effects of ATF2, a relatively high dose of single-agent CEL administered every other day was required to obtain the observed in vivo effects (20). Our results suggest that the potential of CEL as a melanoma treatment may best be realized if it is given in combination with TMZ. Combination treatment with TMZ/CEL resulted in a synergistic inhibition of cell growth in all five of our TMZ-resistant melanoma cell lines (Table 1). Because the observed effect was not merely additive but synergistic, it suggests that the two agents are working via different yet complementary pathways. Further studies are needed to determine if low-dose CEL produces the same degree of synergy with TMZ in vivo.

In conclusion, we have shown that combination treatment with CEL and TMZ resulted in synergistic antitumor effects in melanoma cells. Our studies suggest that CEL may inhibit NF-κB activity by blocking its translocation to the nucleus and that CEL may have antiproteasome activity that contributes to NF-κB inhibition via the stabilization of IκB. Evidence of an increase in JNK phosphorylation upon treatment with TMZ/CEL implicates the MAPK pathway in the observed effects and justifies further investigation at the mechanistic level. Our preclinical data supports the initiation of further studies of TMZ/CEL synergy in vivo with the possibility of future clinical trials evaluating the efficacy of TMZ and CEL combination therapy for melanoma.

Materials and Methods

Screening of Molecular Library

To identify compounds that enhance TMZ cytotoxicity, we screened a library of 2,000 small molecules (The Spectrum Collection; MicroSource Discovery, Inc., Gaylordsville, CT). The compounds in the collection include marketed drugs, other biologically active small molecules, and natural products (supplied at a concentration of 10 mmol/L in DMSO). The primary goal of the screen was to identify compounds with the ability to enhance TMZ toxicity, thus the screen was initially performed using LN-18, a well-characterized TMZ-resistant human glioma cell line with high levels of endogenous O6-methylguanine methyltransferase which directly repairs TMZ-induced DNA damage (26). Because TMZ is also used in the treatment of metastatic melanoma, the screen was expanded to include metastatic melanoma cell lines (SK-MEL-19, SK-MEL-173, M-14, 451Lu, and WM278).

Human melanoma cell lines (SK-MEL-19, SK-MEL-100, SK-MEL-173, and SK-MEL-192) were kindly provided by Alan Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY), and M-14 cells were a gift from Gabriella Zupi (Instituto Regina Elena, Rome, Italy). Additional melanoma cell lines (451Lu and WM278) and glioma cell line LN-18 were obtained from the American Type Culture Collection in Rockville, MD. Cell lines were not further tested or authenticated. 451Lu and WM278 were cultured in T2% tumor medium consisting of MCDB 153 medium supplemented with 5 μg/mL of insulin, 2% FCS, and 20% L-15 Leibovitz medium. All...
other melanoma cells and LN-18 were cultured in DMEM supplemented with 10% FCS at 37°C.

Cells were seeded at 3,000 cells/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 25 μg/mL of TMZ. After 72 h, cellular proliferation was assessed using MTT assay (CellTiter96 AQueous nonradioactive cell proliferation assay; Promega), according to the instructions of the manufacturer. TMZ (NSC 362856) was kindly provided by the National Cancer Institute (Bethesda, MD), and CEL was purchased from Cayman Chemical. TMZ was dissolved in DMSO in a 10 mg/mL stock solution. CEL was dissolved in DMSO in a 10 mmol/L stock and stored at −20°C before use.

**Cell Proliferation Assay and Synergy Analysis of Combined Drug Effects**

The effects of treatment with TMZ and/or CEL on cell growth were determined using colorimetric MTT assay. After 72 h of treatment, cells were incubated with MTT reagent, which is converted to dark blue formazan crystals by mitochondrial dehydrogenases in viable cells. The plates were read with a microplate reader by measuring the absorbance of converted MTT at 490 nm. Cellular proliferation was expressed as a percentage with vehicle-treated cells set at 100%. Drug synergy was determined using CI methods derived from Chou-Talalay equations (11) using CalcuSyn software (Biosoft). A CI value of 1 indicates an additive effect between two agents, whereas a CI value of <1 indicates a synergistic effect.

**Western Blot Analysis and Antibodies**

Cells were harvested in extraction buffer [1% Triton X-100, 50 mmol/L Tris, 2 mmol/L EDTA, 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 10% or 15% SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore). Immunoreactive bands were visualized using enhanced chemiluminescence detection reagent (Perkin-Elmer) and X-OMAT processing. Anti–NF-κB, anti-ubiquitin, anti-JNK, anti–phosphorylated JNK, anti–ERK, and anti–phosphorylated ERK antibodies were purchased from Santa Cruz Biotechnology, and antibodies against IκB, phosphorylated IκB, and β-actin were purchased from Cell Signaling Technology. Densitometry values were calculated using ImageQuant TL software (GE Healthcare Biosciences).

**Immunofluorescence Microscopy**

Cells were seeded in 12-well plates containing glass coverslips. At 50% confluence, the cells were treated with TNF-α for 2 h with or without 30 min pretreatment of CEL, and fixed with −20°C methanol for 5 min. Cells were then stained with anti–NF-κB and PI. The slides were examined with confocal microscopy (LSM 510; Zeiss).

**Knockdown of NF-κB Protein Levels by siRNA**

NF-κB siRNA and control scrambled siRNAs were purchased from Santa Cruz Biotechnology. siRNA was delivered into cells by electroporation using the Amaxa Nucleofector System (Amaxa Biosystems) according to the instructions of the manufacturer. After electroporation, cells were transferred into six-well plates (for protein analysis) and seeded into 96-well plates for dosing studies after 24 h.

**Statistical Analysis**

Data are expressed as means ± SD. Statistical analysis was done using Student’s paired t test. The criterion for statistical significance was established as a probability value of <0.05.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**

Molecular Cancer Research

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