Published OnlineFirst March 12, 2014; DOI: 10.1158/1535-7163.MCT-13-0882

Small Molecule Therapeutics

Molecular Cancer Therapeutics

A Novel Temozolomide–Perillyl Alcohol Conjugate Exhibits Superior Activity against Breast Cancer Cells *In Vitro* and Intracranial Triple-Negative Tumor Growth *In Vivo*

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Abstract

There is no effective therapy for breast cancer that has spread to the brain. A major roadblock is the blood–brain barrier (BBB), which prevents the usual breast cancer drugs from effectively reaching intracranial metastases. The alkylating agent temozolomide (TMZ) is able to penetrate the BBB and has become the gold standard for chemotherapeutic treatment of glioblastoma. However, when it was tested in clinical trials for activity against brain metastases of breast cancer, the results were mixed and ranged from "encouraging activity" to "no objective responses." In an effort to generate an agent with greater activity against intracranial breast metastases, we synthesized a TMZ analog where the natural product perillyl alcohol (POH) was covalently linked to TMZ's amide functionality. The resulting novel compound, called TMZ-POH (T-P), displayed greatly increased anticancer activity in a variety of breast cancer cell lines, inclusive of TMZ-resistant ones. It caused DNA damage and cell death much more efficiently than its parental compound TMZ, because linkage with POH increased its biologic half-life and thus provided greater opportunity for placement of cytotoxic DNA lesions. In an intracranial mouse tumor model with triple-negative breast cancer, T-P revealed considerably greater therapeutic efficacy than TMZ, where a single cycle of treatment extended median survival benefit from 6 days (in the case of TMZ) to 28 days. At the same time, T-P seemed to be well tolerated by the animals. Thus, T-P may have potential as a novel therapy for brain-targeted breast cancer metastases. *Mol Cancer Ther; 13(5); 1181–93. ©2014 AACR.*

Introduction

There is no effective therapy for breast cancer that has spread to the brain. This therapeutic challenge once was a late aspect of disease progression, but increasingly is becoming a first site of disease progression after otherwise successful treatment of primary tumor and metastases outside the cranium (1). Current therapeutic approaches consist of surgery, radiation, and chemotherapy. Chemotherapy is obstructed by the inability of most chemotherapeutic agents to effectively penetrate the blood–brain barrier (BBB). For example, traditional breast cancer therapeutics, such as paclitaxel or doxorubicin, only reach brain metastases at concentrations that are far lower than needed to be therapeutically active (2).

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-13-0882

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The alkylating agent temozolomide (TMZ) is able to cross the BBB after oral dosing and has become the chemotherapeutic standard of care for patients with glioblastoma multiforme (3). However, when TMZ was tested for activity against brain metastatic breast cancer in heavily pretreated patients, it revealed mixed outcomes that ranged from "encouraging activity" and "disease control" to "well-tolerated, but no objective responses" (4–9). The underlying basis for these inconsistent results was not investigated, but it is conceivable that these differences may have been because of variable expression levels of O6-methylguanine-DNA methyltransferase (MGMT; also called O6-alkylguanine-DNA alkyltransferase, AGT), a DNA repair enzyme that removes alkyl groups located on the O6-position of guanine (10, 11). Because the primary toxic DNA lesion set by TMZ is alkylation of O6-guanine, high expression levels of MGMT protect tumor cells from the cytotoxic impact of TMZ and provide treatment resistance (12, 13). When MGMT expression was investigated in breast cancer metastases to the brain, it was found that over half of the intracranial lesions analyzed were strongly positive for MGMT immunoreactivity (14). It is therefore conceivable that improved outcomes of TMZ treatment might be achievable if patients with brain metastatic breast cancer were stratified according to MGMT expression levels before the onset of chemotherapy.

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MGMT activity is unusual in that it represents a "suicide" mechanism, whereby acceptance of the alkyl group from DNA irreversibly inactivates the enzyme and leads to its rapid degradation (10). This feature is exploited by the use of specific MGMT inhibitors, such as O6-benzylguanine (O6-BG), which act as pseudosubstrates (15). Benzylation of MGMT via reaction with O6-BG causes the same structural change in the enzyme as that seen after alkylation following DNA repair, and therefore also leads to rapid degradation of the protein (16). Ablation of MGMT activity after treatment of MGMT-positive cells with O6-BG generally increases their sensitivity to killing by TMZ, and this has been well established in numerous *in vitro* and *in vivo* tumor models (see detailed in refs. 12 and 17). However, a recent phase II clinical trial yielded mixed outcomes when O6-BG and TMZ were administered to patients with brain cancer with TMZ-resistant tumors. Although the addition of the MGMT inhibitor restored TMZ-sensitivity in a fraction (16%) of patients with anaplastic glioma, there was no significant effect (3%) in patients with glioblastoma multiforme (18). Although the underlying reasons for this disappointing outcome remain to be established, the limited response documented in this trial does not generate enthusiasm for the potential study of this drug combination in patients with brain metastatic breast cancer. As an alternative, we focused our research on efforts to improve the anticancer activity of TMZ itself.

Perillyl alcohol (POH) is a monoterpene isolated from the essential oils of several plants and fruits, such as peppermint, spearmint, cherries, and celery seeds (19). Although this compound had shown promising activity in several preclinical cancer models (20, 21), it did not enter clinical practice, primarily because dose-limiting intestinal toxicity became evident in clinical trials (22–24). However, recent phase I/II clinical studies in Brazil demonstrated that simple intranasal inhalation of POH was effective against recurrent glioblastoma multiforme, in the absence of detectable toxic events (25, 26). Based on these promising results, we hypothesized that covalently linking POH to TMZ might result in a novel therapeutic agent with superior activity against intracranial tumors. Here, we present our results validating this prediction in various breast cancer cell lines *in vitro* and *in vivo*.

Materials and Methods

Pharmacologic agents

TMZ was obtained from the pharmacy at the University of Southern California (USC) and dissolved in ethanol to a concentration of 50 mmol/L. TMZ-POH (T-P) was provided by NeOnc Technologies and was dissolved in dimethyl sulfoxide (DMSO) at 100 mmol/L. POH and O6-BG were purchased from Sigma-Aldrich and diluted with DMSO to make stock solutions of 100 mmol/L. DMSO was from Sigma-Aldrich. In all cases of cell treatment, the final DMSO concentration in the culture medium never exceeded 0.5%. Stock solutions of all drugs were stored at −20°C.

Cell lines

Several of the human breast cancer cell lines were obtained from the American Tissue Culture Collection, and these cells were passaged for less than 6 months in our laboratory after receipt or resuscitation. HCC-1937 cells were provided by M.F. Press (USC). The brain-seeking line MDA-MB-231-br [originally described by Yoneda and colleagues (27)] was obtained from S. Swenson (USC). These latter 2 lines were not authenticated by the authors. Cells were propagated in DMEM (provided by the Cell Culture Core Lab of the USC/Norris Comprehensive Cancer Center and prepared with raw materials from Cellgro/MediaTech) supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified incubator at 37°C and a 5% CO_2 atmosphere.

Colony formation assay

Depending on the cell line (and plating efficiency), 200 to 350 cells were seeded into each well of a 6-well plate. After cells had fully attached to the surface of the culture plate, they were exposed to drug treatment (or DMSO solvent alone) for various times up to 48 hours. Thereafter, the drugs were removed, fresh growth medium was added, and the cells were kept in culture undisturbed for 12 to 16 days, during which time the surviving cells spawned colonies of descendants. Colonies (defined as groups of >50 cells) were visualized by staining for 4 hours with 1% methylene blue (in methanol), and then were counted.

In the case of O6-BG treatment, cells were pretreated with 10 μmol/L O6-BG for 1 hour before addition of TMZ or T-P. After 24 hours, another 10 μmol/L O6-BG was added to the medium. Another 24 hours later, drug-laced medium was removed, and fresh medium without drugs was added. Thereafter, cells remained undisturbed until staining with methylene blue (1% in methanol).

Stable transfections

MDA-MB-231 cells were cotransfected in 6-well plates with the use of Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. We combined 2 μg pSV2MGMT (containing the human MGMT cDNA) with 0.2 μg pSV2neo (containing the neomycin gene for selection of cells in G418). Both plasmids were kindly provided by B. Kaina (28). Individual clones of transfected cells were selected in medium containing 750 μg/mL G418 and propagated in 250 μg/mL G418. G418 was obtained as G418 disulfate salt from Sigma-Aldrich and dissolved in PBS at 75 mg/mL. Selection medium was removed from cells several days before experimental drug treatment.

Immunoblots

Total cell lysates were analyzed by Western blot analysis as described earlier (29). The primary antibodies were purchased from Cell Signaling Technology or Santa Cruz Biotechnology, Inc. and used according to the manufacturers' recommendations. All immunoblots were repeated at least once to confirm the results.

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In vivo model

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of USC, and all rules and regulations were followed during experimentation on animals. Athymic mice (Harlan, Inc.) were implanted intracranially with 2×10^5 cells, as previously described (30). We used a subline of MDA-MB-231 cells called D3H2LN, which was transfected with the firefly luciferase gene and had been selected for aggressive growth and metastasis *in vivo* (31). Ten days after intracranial implantation, efficient tumor take was confirmed in all animals via noninvasive whole-body bioluminescent imaging. For this purpose, mice were intravenously injected with 50 mg/kg D-luciferin (Perkin Elmer) and imaged using the Xenogen IVIS-200 Imaging System (Caliper/Perkin Elmer). Images were analyzed by region-of-interest (ROI) analysis using the Living Image software package (Caliper/Perkin Elmer) to quantitate light output (radiance, i.e., photons per second per square centimeter per steradian).

Animals were distributed into 3 groups of 8 animals each, so that each group contained animals with comparable radiance within the ROI (i.e., area of the head) and drug treatment was initiated. Group 1 was the control

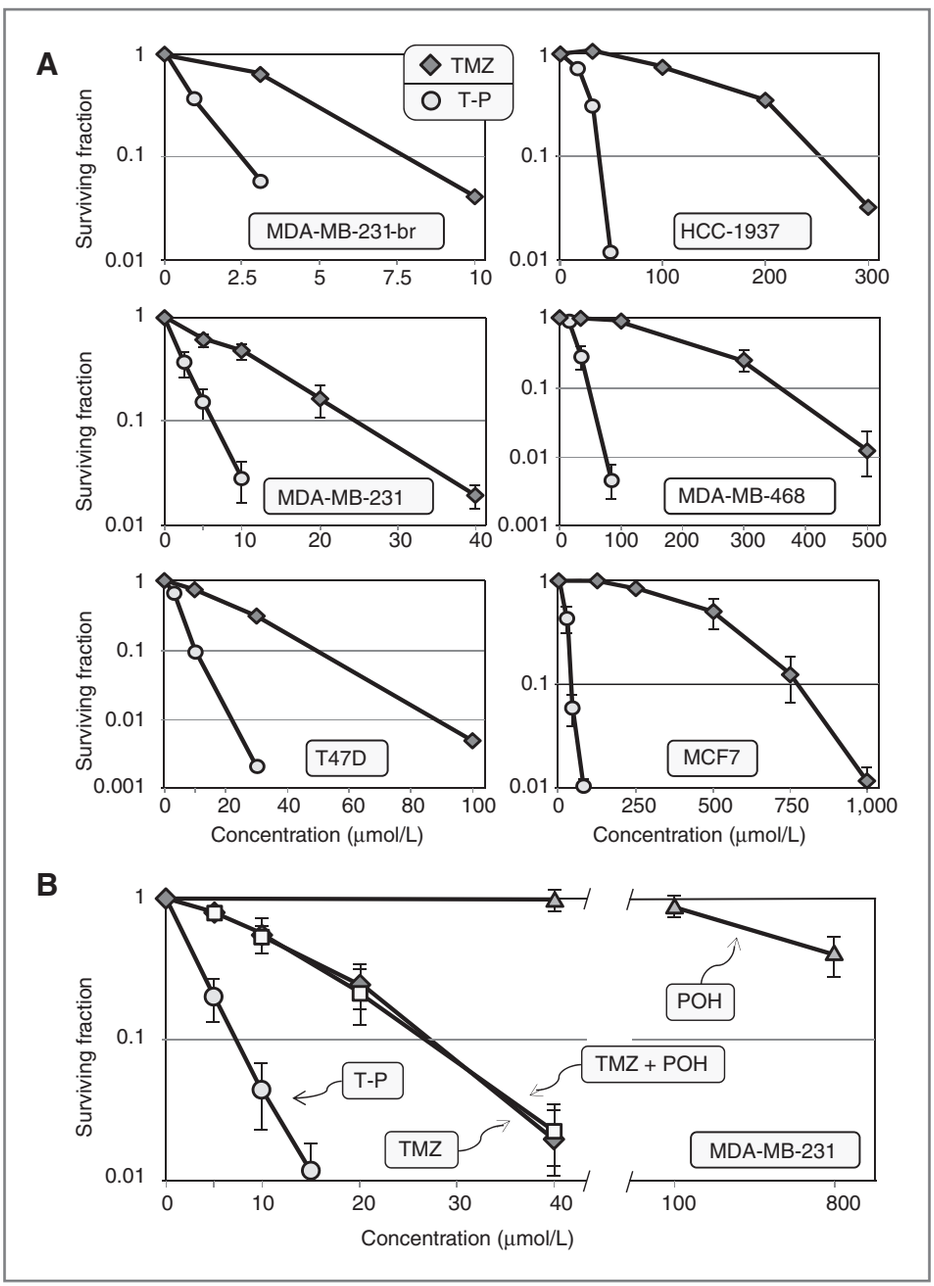

Figure 1. Survival of breast cancer cells after drug treatment. A, various breast cancer cell lines were exposed to increasing concentrations of TMZ (diamonds) or T-P (circles) for 48 hours, and survival was determined via colony formation assay (CFA). B, cells were exposed for 48 hours to increasing concentrations of TMZ (diamonds), T-P (circles), POH (triangles), or equimolar concentrations of TMZ plus POH (squares). In all graphs, colony formation by control cells (treated with vehicle only) is set at 1. Graphs with error bars display mean (±SD) from ≥3 independent experiments; graphs without error bars show the average from 2 independent experiments.

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group that received vehicle only (45% glycerol, 45% ethanol, 10% DMSO) via subcutaneous injection. Group 2 was the experimental group that received 25 mg/kg T-P via subcutaneous injection. Group 3 was the comparison group and animals received 25 mg/kg TMZ via gavage [because the bioavailability of TMZ essentially is 100% when given orally (32); and cannot be further increased via subcutaneous or intravenous delivery (33)], we preferred the well-established, most frequently used administration of TMZ via oral gavage; in the case of T-P, however, bioavailability data are lacking, and therefore we decided to administer this compound via subcutaneous injection. Treatment was once per day for a period of 10 days (i.e., 10 treatments total). Thereafter, all surviving animals were imaged again, once per week.

Statistical analysis

All parametric data were analyzed using the Student *t* test to calculate the significance values. The survival distributions in the Kaplan–Meier plot were analyzed with the nonparametric Wilcoxon test. A probability value of <0.05 was considered statistically significant.

Results

A novel analog of TMZ was created by covalently linking POH to TMZ's amide functionality (Supplementary Fig. S1). The cytotoxic potency of this new compound, T-P, was analyzed by colony formation assay (CFA) in a variety of human breast cancer cell lines and compared with the cytotoxicity of TMZ. We used estrogen receptor positive cells MCF7 and T47D, the triple-negative lines MDA-MB-231, MDA-MB-468, and HCC-1937, and a brain-seeking variant of the 231 cell line, MDA-MB-231-br. As shown in Fig. 1A, low micromolar concentrations of T-P prevented colony formation in all 6 cell lines, and in all instances T-P's potency was substantially stronger than that of TMZ.

Previous studies showed that POH is able to exert cytotoxic effects in cancer cells, although concentrations approaching the millimolar range were required (34, 35). We therefore tested whether simply mixing the 2 compounds TMZ and POH could mimic the effects of the T-P conjugate. MDA-MB-231 cells were treated with the individual compounds (T-P, TMZ, or POH) alone, or with an equimolar mix of TMZ plus POH, and cell survival was analyzed by CFA. As shown in Fig. 1B, T-P was much more potent than a mix of TMZ plus POH, that is, mixing TMZ with POH was unable to achieve the strong cytotoxic potency of T-P, and in fact, the addition of equimolar concentrations of POH to TMZ did not increase the potency over TMZ alone. For instance, 10 μmol/L TMZ reduced colony formation by about 50%, and the combination of 10 μmol/L TMZ with 10 μmol/L POH also caused a 50% reduction; in comparison, 10 μmol/L T-P caused about 95% fewer colonies (a photo of a representative CFA is shown in Supplementary Fig. S2). Consistent with earlier reports, POH by itself required concentrations well above 100 μmol/L in order to become cytotoxic, and its IC_{50} in MDA-MB-231 cells was about 700 μmol/L (Fig. 1A). Thus, an equimolar mix of TMZ plus POH was unable to mimic the strong cytotoxic potency of the T-P conjugate, and this was confirmed in several additional cell lines tested. Altogether, these results present T-P as a novel compound with increased potency over TMZ that cannot be matched by merely mixing its individual parts, TMZ and POH, and this outcome was confirmed in several additional cell lines tested, including the brain-tropic variant of MDA-MB-231.

Because the DNA repair protein MGMT is known to play a key role in cellular resistance to TMZ, we next investigated how it would impact the cytotoxic potency of T-P. We first determined its basal level of expression in the 6 breast cancer cell lines we used above. Figure 2A shows that 3 cell lines (MDA-MB-468, HCC-1937, MCF7) were strongly positive, whereas the others (T47D, MDA-MB-231, MDA-MB-231-br) had undetectable levels of MGMT protein, as determined by Western blot analysis. For comparison purposes, we also assessed MGMT protein levels in 3 commonly used glioblastoma multiforme cell lines known to be MGMT negative (U251, LN229) and positive (T98G). This side-by-side evaluation revealed

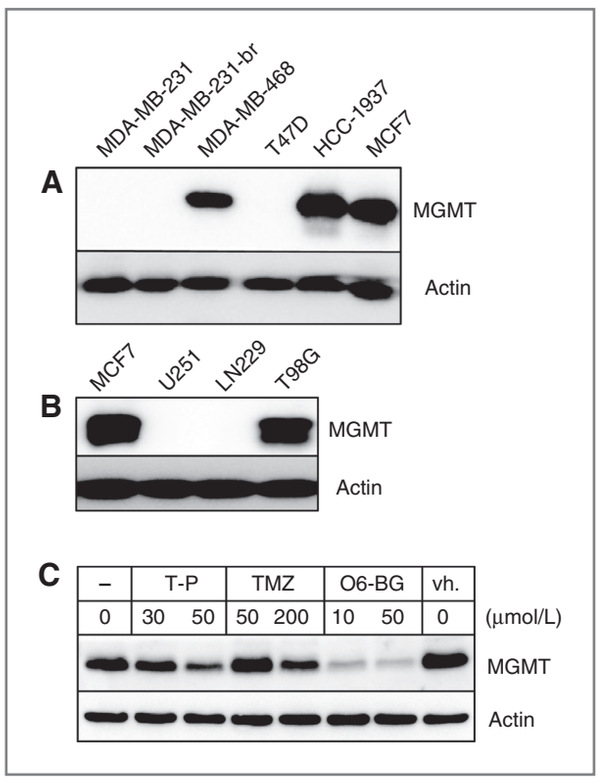

Figure 2. MGMT expression levels in various cell lines. All parts show Western blot analysis of MGMT protein levels with actin as the loading control. A, MGMT basal levels in the 6 breast cancer cell lines used in this study. B, MGMT basal levels in 3 glioblastoma multiforme cell lines compared with MCF7 breast cancer cells. C, MDA-MB-468 cells were treated with the indicated concentrations of T-P, TMZ, or O6-BG for 17 hours before harvest of cellular lysates. vh., cells treated with vehicle only.

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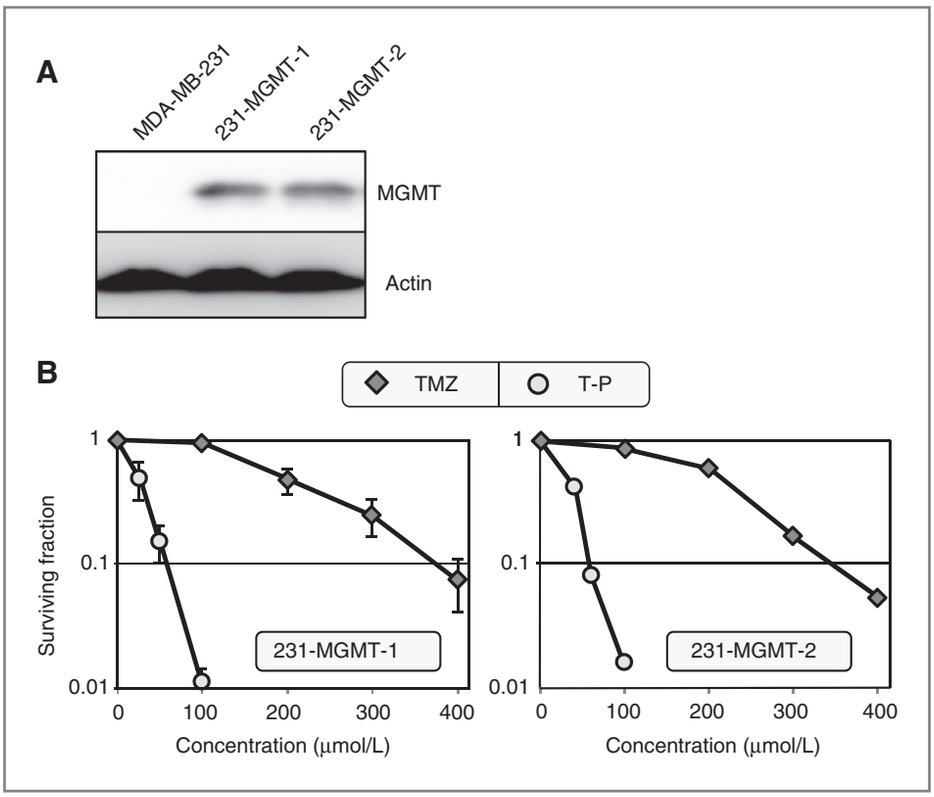

Figure 3. Drug sensitivity of MGMT-transfected cells. MDA-MB-231 cells were stably transfected with MGMT cDNA. A, 2 individually selected clones, 231-MGMT-1 and -2, were analyzed by Western blot analysis for basal-level MGMT protein expression in comparison to parental cells. B, 231-MGMT-1 and -2 were treated with increasing concentrations of T-P and TMZ for 48 hours, and cell survival was analyzed by CFA. Graph with 231-MGMT-1 cells displays mean (±SD) from 3 independent experiments; graph with 231-MGMT-2 cells shows the average from 2 independent experiments.

that MGMT protein levels in the positive breast cancer lines were similar to the levels found in the T98G brain cancer line (Fig. 2B).

We next aligned MGMT expression with the cytotoxic potency of T-P in comparison to TMZ. As summarized in Table 1, the IC_{50} of T-P (i.e., the concentration required to decrease colony formation by 50%) was noticeably higher in all 3 MGMT-positive breast cancer cell lines. Although the IC_{50} in MGMT-negative cell lines ranged from 1.2 to 4.6 μmol/L, it increased to 31 to 33 μmol/L in the 3 MGMT-positive lines. Nonetheless, these IC_{50} values still were substantially lower than the corresponding IC_{50}s of TMZ for each cell line. Noteworthy as well is the differential (fold increase in potency) between T-P and TMZ shown in Table 1. The fold-increase in cytotoxic potency of T-P, as compared with TMZ, is consistently greater in each of the MGMT-positive cell lines (6.3- to 15.5-fold) as compared with the MGMT-negative cell lines (3.2- to 4.3-fold). This latter finding suggests that the increased potency of T-P over TMZ, although apparent in all cell lines analyzed, might become particularly advantageous in the context of therapeutically targeting MGMT-positive cells.

We further characterized the relevance of MGMT in T-P's cytotoxic effects, in comparison to TMZ. The major cytotoxic DNA lesion set by TMZ is methylation of O6-guanine, and it is well known that removal of this methyl group by MGMT leads to rapid degradation of the DNA

Table 1. Drug sensitivities of various breast cancer cell lines

Cell line	MGMT status	IC_{50} TMZ (μmol/L)	IC_{50} T-P (μmol/L)	Differential (-fold)
MDA-MB-231-br	−	3.8	1.2	3.2
MDA-MB-231	−	9.9	2.3	4.3
T47D	−	20	4.6	4.3
HCC-1937	+	186	31	6.0
MDA-MB-468	+	195	31	6.3
MCF7	+	513	33	15.5

NOTE: Shown are IC_{50} values (i.e., drug concentrations that reduce colony-forming ability by 50%) and differential toxicity between T-P and TMZ (i.e., fold increased potency of T-P over TMZ).

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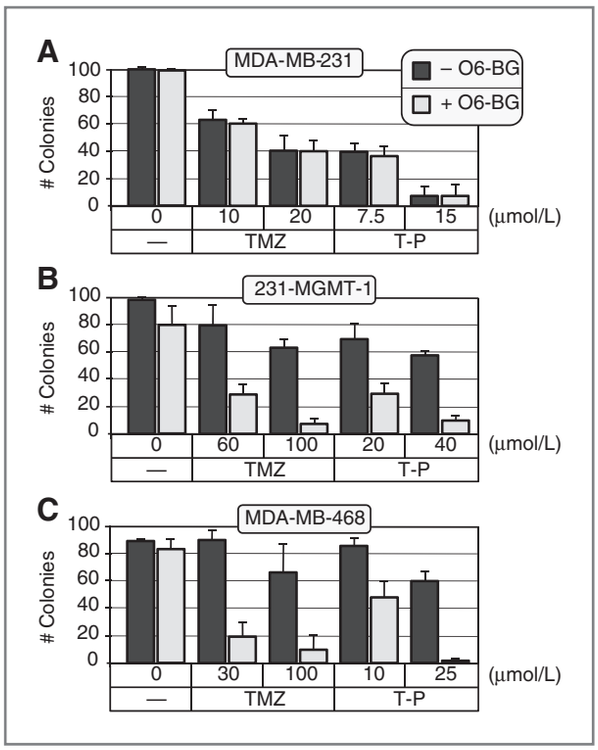

Figure 4. Effect of inclusion of O6-BG. Cells were exposed to TMZ or T-P for 48 hours with or without pretreatment with O6-BG, and cell survival was determined by CFA. Colony survival of MDA-MB-231 cells (A), MGMT-transfected 231-MGMT-1 cells (B), and MDA-MB-468 cells (C). Shown is mean number of colonies (±SD) from ≥3 wells treated in parallel.

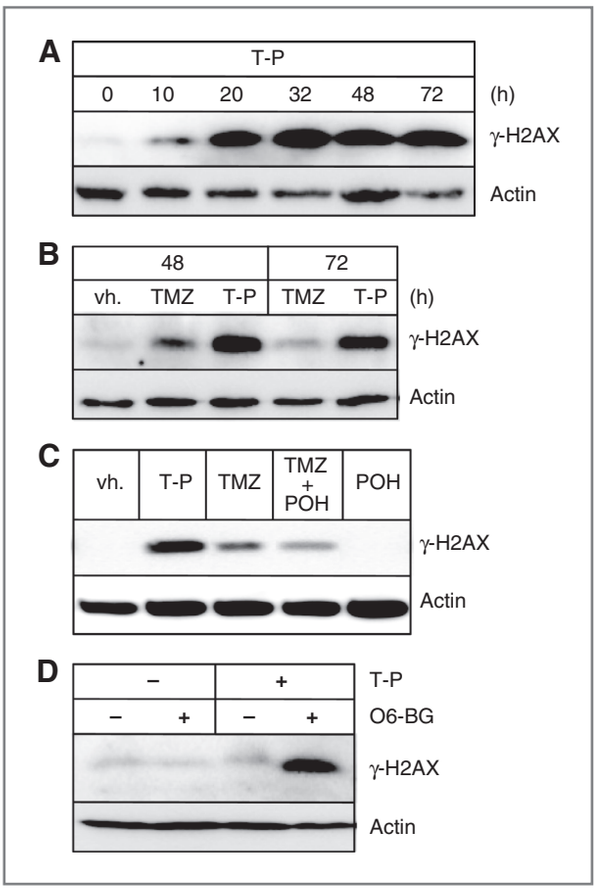

Figure 5. Drug effects on DNA damage marker. Cells were treated with different concentrations of T-P or TMZ and analyzed by Western blot analysis for expression levels of γ-H2AX, a marker for double-strand DNA damage. Actin was used as a loading control. A, MDA-MB-231 cells were treated with 50 μmol/L T-P for the indicated time periods. B, MDA-MB-231 cells were treated with 50 μmol/L T-P or 50 μmol/L TMZ for the indicated time periods. C, MDA-MB-231 cells were treated with T-P, TMZ, POH, or TMZ combined with POH (all at 10 μmol/L each) for 24 hours. (Lanes from the same blot were cut and rearranged for optimized viewing). D, MCF7 cells were treated with or without 50 μmol/L T-P in the presence or absence of 30 μmol/L O6-BG for 48 hours.

repair protein. As well, the pseudosubstrate O6-BG also activates the suicide mechanism of MGMT, which is confirmed in Fig. 2C, showing that treatment of cells with O6-BG strongly decreases MGMT protein levels. Treatment of cells with TMZ also down-regulates MGMT levels, but the effect is fairly weak and high concentrations of the drug are required. In comparison, T-P affects MGMT levels more potently than TMZ; for instance, whereas 50 μmol/L TMZ has no effect, 50 μmol/L T-P causes a significant decrease (Fig. 2C). Together, these results indicate that T-P's superior potency over TMZ may involve more extensive methylation of O6-guanine targets.

Although the above results suggested that T-P's mechanism of action perhaps was because of the drug's increased efficacy of setting cytotoxic DNA lesions, there was also a possibility that covalently conjugating POH might have conferred additional mechanistic features to the new molecule. We therefore performed additional experiments to characterize the significance of DNA damage, and in particular O6-guanine methylation, caused by T-P.

Although the experiments summarized in Table 1 revealed a correlation of MGMT positivity with decreased T-P toxicity, they did not establish cause and effect. To investigate the latter, we stably transfected MGMT-negative MDA-MB-231 cells with MGMT cDNA and isolated individual clones. Figure 3A shows elevated expression of MGMT protein in 2 different clones (231-MGMT-1 and 231-MGMT-2) of transfected cells. Both clones were treated with increasing concentrations of T-P and TMZ and analyzed by CFA. As shown in Fig. 3B (and summarized in Supplementary Table S1), resistance of cells to drug treatment clearly increased for both T-P and TMZ, as compared with parental cells. Intriguingly, however, similar to what was noted in Table 1, resistance to T-P increased less than resistance to TMZ (Supplementary Table S1).

CFAs were also performed with the inclusion of the MGMT inhibitor O6-BG. Cells were pretreated with O6-BG before addition of T-P or TMZ. As shown in Fig. 4A, O6-BG had no effect on the survival of drug-treated MDA-

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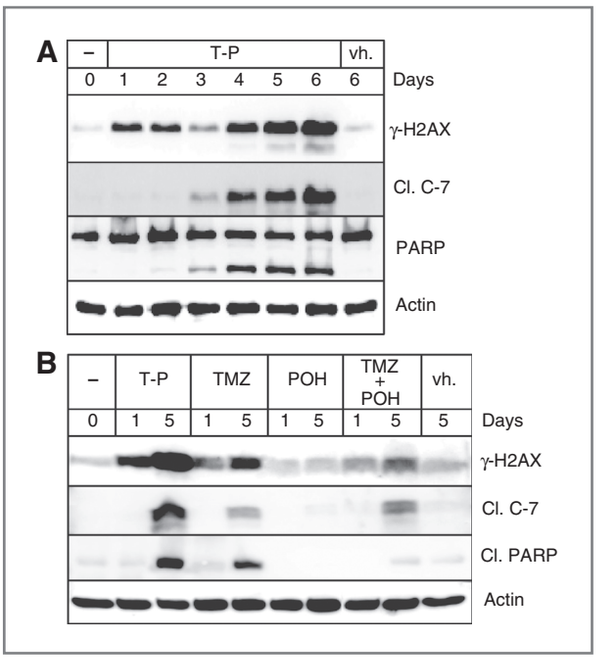

Figure 6. DNA damage and cell death marker analysis. MDA-MB-231 cells were used for Western blot analysis of expression levels for markers of DNA damage (γ-H2AX) and cell death (activated caspase-7 and cleaved PARP). A, cells were treated with 15 μmol/L T-P and harvested every 24 hours up to 6 days. Control cells remained untreated, or received vehicle (vh.) only. B, cells were treated with 20 μmol/L of either T-P, TMZ, or POH individually, or with 20 μmol/L TMZ combined with 20 μmol/L POH (TMZ + POH) and harvested after 24 hours or 5 days. Control cells remained untreated, or received vehicle (vh.) only. In the case of caspase-7, only the activated (cleaved) form is shown (cl. C-7). In the case of PARP, the top panel shows both full-length and proteolytically cleaved forms of the protein, whereas the bottom panel only shows faster migrating, cleaved PARP.

MB-231 cells, consistent with their MGMT-negative status that does not provide a target for O6-BG. In contrast, O6-BG greatly enhanced toxicity of T-P and TMZ in 231-MGMT-1 (Fig. 4B) and 231-MGMT-2 cells (not shown). Similarly, O6-BG also increased the cytotoxic outcome of T-P and TMZ treatment in MGMT-positive MDA-MB-468 (Fig. 4C) and MCF7 cells (not shown). Altogether, these results indicate that the key trigger for cell death caused by T-P is methylation of O6-guanine, which seems to be achieved much more effectively by T-P as compared with TMZ.

The above conclusion was further confirmed by studying H2AX protein. Phosphorylation of H2AX, noted as γ-H2AX, is a marker for double-strand breaks in DNA. MDA-MB-231 cells treated with T-P over a time course of 72 hours revealed substantially increased levels of γ-H2AX (Fig. 5A), and this effect of T-P was much stronger as compared with TMZ (Fig. 5B). As well, the mere combination of TMZ with POH was unable to mimic the strong induction of γ-H2AX caused by conjugated T-P (Fig. 5C), consistent with the CFA results shown in Fig. 1B and our notion that T-P represents a novel chemical entity different from the mix of TMZ plus POH.

The same concentration of T-P that was applied to MDA-MB-231 cells was also added to MGMT-positive MCF-7 cells. However, in this case, there was no increased phosphorylation of H2AX, consistent with the established model that MGMT rapidly repairs O6-methyl-guanine lesions; however, when these cells were pretreated with O6-BG, increased levels of γ-H2AX became readily apparent (Fig. 5D). Combined, the above results characterize T-P as an alkylating agent with cytotoxic mechanism similar to TMZ, but with potency that is substantially greater than the original compound.

It is well known that glioblastoma multiforme cells treated with physiologic concentrations of TMZ (<100 μmol/L) *in vitro* survive for several (5–7) days seemingly unaffected before substantial cell death becomes apparent (13, 36). We observed a similar phenotype when breast cancer cell lines were treated with T-P, that is, cell cultures only began to deteriorate approximately a week after the onset of drug treatment. To characterize T-P–induced cell death in greater detail, we treated MDA-MB-231 cells with 15 μmol/L of the drug and collected cell lysates daily over the course of 6 days. The lysates were analyzed by Western blot analysis for the presence of 2 apoptosis markers, cleaved (i.e., activated) caspase-7 and cleaved PARP-1, along with the DNA damage marker γ-H2AX. As above, T-P treatment resulted in pronounced increase in γ-H2AX expression levels, which—except for an unexplained dip at 3 days—continued to increase over time (Fig. 6A). Both active caspase-7 and cleaved PARP started to increase at day 3 and remained elevated for several more days until day 6 (Fig. 6A), which is about the time when microscopic examination of treated cells reveals increasing deterioration of the monolayer. These results indicate that T-P–induced cell death, similar to what has been reported for physiologic concentrations of TMZ, is a slow process and involves apoptotic mechanisms.

As we had shown in Fig. 1B, an equimolar combination of TMZ + POH was unable to achieve the same potency in blocking colony survival as the T-P conjugate. Having established T-P's impact on DNA damage and its activation of apoptosis, we next determined whether T-P's superior effect would also be reflected at the molecular level of these marker proteins. We therefore treated cells with the same concentration (20 μmol/L) of T-P, TMZ, POH, or TMZ combined with POH (TMZ + POH), and analyzed the induction of γ-H2AX, activated caspase-7, and cleaved PARP. As shown in Fig. 6B, all 3 indicator proteins were induced quite prominently by T-P after 5 days of treatment, whereas TMZ or TMZ + POH exerted noticeably weaker effects and POH alone was inactive in these measurements. Thus, the results from the cell survival assay (Fig. 1A) correlated closely with the effects of these compounds on DNA damage and apoptosis markers (Fig. 6B), and in all cases T-P clearly generated the strongest anticancer impact.

Next, we wanted to address the question why T-P was more potent than TMZ. TMZ is a prodrug, and it is well known that its activation takes place spontaneously in

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aqueous solution at 37°C (i.e., no cellular functions are required for this conversion). As well, the half-lives of both prodrug and active product are fairly short, where all cytotoxic triggers are set within the first few hours of treatment. To evaluate whether T-P and TMZ differed in their half-lives, we determined how quickly, and for how long, the drugs unfolded their cytotoxic activity in cell culture. First, we exposed cells to variably short periods of drug treatment, washed off the drug, and then continued to keep cells in medium without drug to determine survival and colony-forming ability. For most of these experiments, we used 15 μmol/L T-P and 30 μmol/L TMZ, because these concentrations are approximately equipotent in the >90% cytotoxicity range (when measured by CFAs and a drug exposure time of 24 hours).

As shown in Fig. 7A (right 2 bars), exposure of cells to 15 μmol/L T-P or 30 μmol/L TMZ resulted in about 3% and 8% colony survival, respectively, when drugs remained in the medium for 24 hours. Yet, despite T-P unfolding slightly more potency over the course of 24 hours, TMZ displayed noticeably greater efficacy when cells were exposed for shorter time periods. As shown in Fig. 7A, a 1-hour exposure to TMZ reduced colony formation by >50%, whereas during the same time period T-P reduced it by only 20%; similarly, a 2-hour exposure to TMZ had more than double the cytotoxic impact (23% survival) than T-P (51%). Thus, TMZ acted more quickly than T-P; it required only 4 T-Pto exert maximum toxicity, whereas T-P had not yet reached its maximum impact at this time point.

We next modified this experiment as follows. After cells had been exposed to drug treatment for the specific times shown in Fig. 7A, we removed the medium containing the drug from the cells, and transferred this supernatant to fresh cells, which were then exposed for 24 hours. In essence, we intended to determine how much cytotoxic activity remained in each supernatant. As shown in Fig. 7B (right 2 bars), when supernatant was transferred after prior 24 hours of incubation, no cytotoxic activity remained, that is, there was no reduction in colony-forming ability of the receiving cells. In contrast, when supernatant was transferred after before 1-hour incubation, colony-forming ability of receiving cells was 48% in cells receiving TMZ-containing supernatant, and 22% in T-P–containing supernatant. Even more strikingly, TMZ-containing supernatant had lost all of its activity when transferred after 4 hours, whereas T-P–containing supernatant still contained nearly 50% of its cytotoxic activity (Fig. 7B). Together, these results demonstrate that T-P retained its cytotoxic potency substantially longer than TMZ.

To exclude the involvement of cellular enzymes in the turnover of T-P, we incubated T-P (and TMZ) in PBS at 37°C for 1 hour (in the absence of cells). After this preincubation, T-P and TMZ were added to cells for 24 hours, and survival was determined by CFA. As a control, both drugs were also added to cells without prior incubation in aqueous solution. A representative CFA is shown in

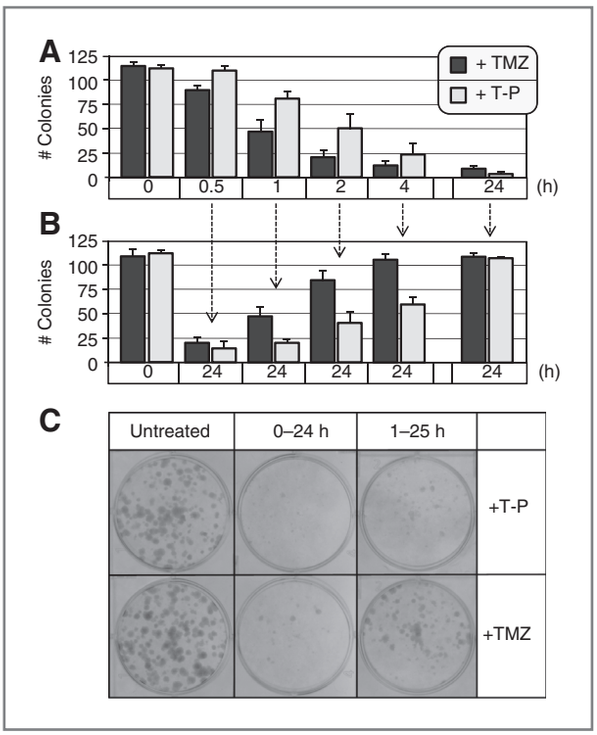

Figure 7. Determination of drug stability. MDA-MB-231 cells were analyzed in colony formation assays. A, cells were treated with 15 μmol/L T-P or 30 μmol/L TMZ for 30 minutes or 1, 2, 4, and 24 hours. Thereafter, drug-containing medium was removed, fresh medium (without drug) was added, and cells remained undisturbed until colony staining 12 days later. B, cells were exposed to supernatant (i.e., the drug-containing medium removed from cells shown in A). The arrows indicate that cells received which supernatant. After 24 hours of incubation, all drug-containing medium was removed, fresh medium (without drug) was added, and cells remained undisturbed until colony staining 12 days later. C, shown is a representative 6-well plate with stained colonies. Left (untreated), control cells without drug treatment. Middle (0–24 h), cells received 15 μmol/L T-P or 30 μmol/L TMZ for 24 hours. Right (1–25 h), T-P and TMZ were incubated in neutral buffer at 37°C for 1 hour before addition to cells to a final concentration of 15 μmol/L T-P and 30 μmol/L TMZ for 24 hours.

Fig. 7C, where the middle panel confirms that both drugs were used at approximately equipotent concentrations; that is, when added straight to cells, they reduced survival by ~95%. However, preincubation in aqueous solution for only 1 hour preempted the cytotoxic potency of TMZ by about 50%, but that of T-P much less (80% remaining; see right). Altogether, these results establish that T-P is more stable than TMZ, suggesting that its increased potency over TMZ might be because of longer half-life, which may provide for extended opportunity to inflict cytotoxic DNA damage.

Finally, we asked whether T-P would be able to exert its anticancer effects *in vivo* as well and whether it would be able to do so with a mouse tumor model representing breast cancer spread to the brain. We used D3H2LN cells, which are a bioluminescent variant of the MDA-MB-231 cell line with aggressive tumor growth in mice (31). These cells were implanted into the brains of nude mice, and

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10 days later all animals were imaged for luciferase expression in order to confirm efficient tumor take.

Animals were distributed into 3 groups and treated once daily for 10 days with vehicle alone (control), 25 mg/kg T-P, or 25 mg/kg TMZ. This T-P dosage was chosen because our initial histopathologic analysis showed that it was well tolerated by the animals (see details in Supplementary Fig. S3). As summarized in Fig. 8A, animals were imaged again after the termination of treatment. All vehicle-only treated animals exhibited much increased bioluminescent radiance (indicative of vigorous intracranial tumor growth; ref. 37), some of which had conspicuously spread along the spine. Most of these animals also exhibited behavioral signs of neurological problems, which necessitated euthanasia. In stark contrast, all animals in the T-P–treated group seemed to thrive, and their imaging analysis after the treatment period showed only small changes in radiance (Fig. 8A), with 3 animals presenting with radiance (tumor growth) that was lower than before the onset of treatment (see details for all animals in Supplementary Fig. S4). In comparison, tumor growth in the TMZ-treated group showed generally greater bioluminescence, indicating that therapeutic efficacy of TMZ was substantially weaker than that of T-P. Overall, however, the TMZ-treated group fared somewhat better than the vehicle-treated group, but clearly worse than the animals treated with T-P (Supplementary Fig. S4). There was some weight loss in animals from all treatment groups, but the weight of T-P–treated animals increased again after the cessation of treatment (Supplementary Fig. S5).

All animals were cared for and observed in the absence of any further drug treatment. As summarized by Kaplan–Meier survival plot (Fig. 8B), vehicle-treated animals were moribund by day 20 and had to be euthanized within the following 4 days (median survival: 22 days). TMZ-treated animals survived somewhat longer (median survival: 28 days). Remarkably, by day 36, when all TMZ-treated animals had succumbed to disease, all T-P–treated animals were still alive with no obvious signs of distress. Median survival of T-P–treated animals turned out to be 50 days, that is, they survived an additional 30 days after the termination of treatment, as compared with TMZ-treated animals, which survived only an additional 8 days after treatment. Altogether, these results demonstrate potent anticancer effects of T-P that are considerably stronger than those of TMZ *in vitro* and *in vivo*.

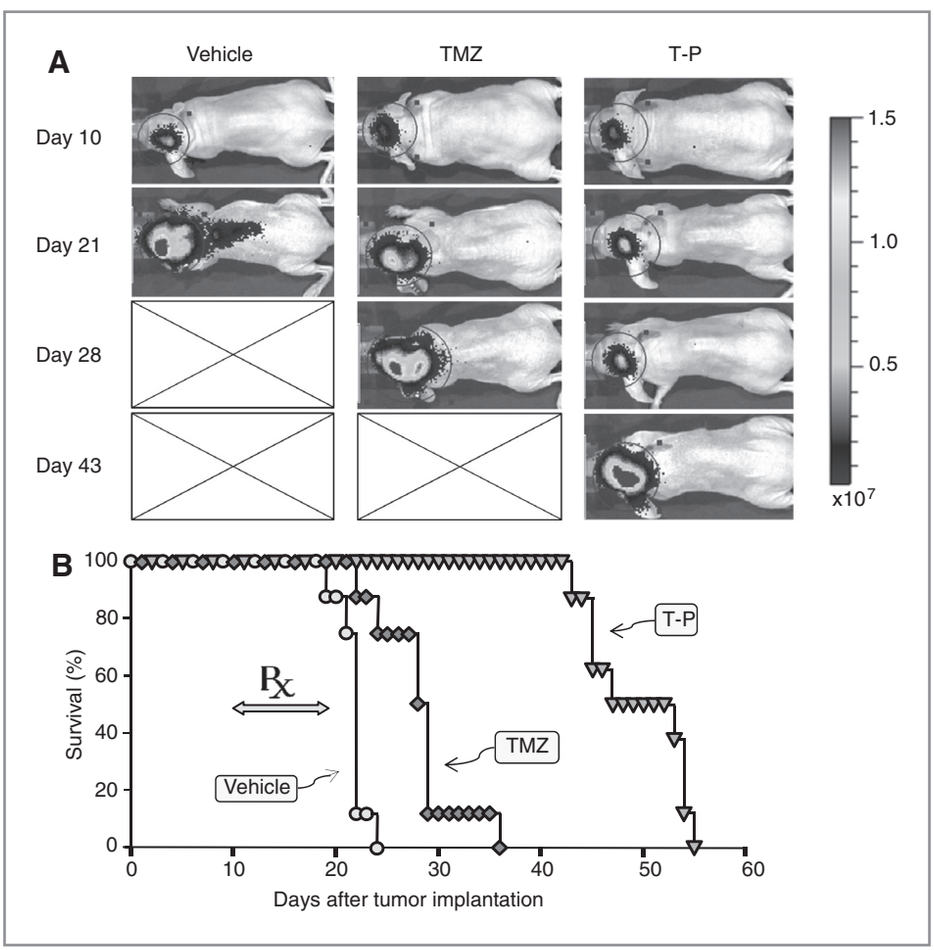

Figure 8. Drug effects on intracranial tumor growth. Luciferase-positive D3H2LN cells were implanted into the brains of 24 nude mice. Ten days later, tumor take was confirmed via bioluminescent imaging, and treatment was initiated with vehicle only (control group), 25 mg/kg T-P, or 25 mg/kg TMZ, once daily over the course of 10 days. A, all surviving animals were imaged again on days 21, 28, and 43. Shown is one representative mouse from each group. Heat bar to the right shows scale of radiance. B, Kaplan–Meier survival plot of all animals carrying intracranial tumors. Arrow labeled Rx indicates the time period of treatment. Statistical difference between groups of TMZ-treated and T-P–treated animals: $P < 0.01$.

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As shown in the *in vitro* experiments in Fig. 1B, merely mixing TMZ with POH was unable to mimic the strong cytotoxic activity of conjugated T-P. We next investigated whether this was also true under *in vivo* conditions. Mice with intracranial tumor burden were treated with vehicle alone, TMZ or POH alone, and with a combination of TMZ plus POH for 10 days. As shown in Supplementary Fig. S6, there was no statistically significant difference ($P = 0.41$) in survival of animals treated with TMZ alone versus a combination of TMZ with POH, emphasizing that a mix of TMZ plus POH is unable to enhance cytotoxic outcomes over TMZ alone. Combined with the results shown in Fig. 1B and Fig. 8, it shows that the strong potency of the conjugated T-P compound *in vitro* and *in vivo* cannot be achieved by merely mixing its individual components.

Discussion

A landmark phase III trial completed 10 years ago (38) established a significant survival benefit for the alkylating agent TMZ when added to radiotherapy (plus surgery when possible) for newly diagnosed glioblastoma. TMZ prolonged median survival from 12.1 to 14.6 months (38), and increased 5-year overall survival 5-fold from 1.9% to 9.8% (39). Altogether, these positive outcomes have cemented TMZ plus radiotherapy as the current standard of care for most patients with glioblastoma multiforme. As would be expected, this approach was also evaluated for activity against intracranial metastases secondary to primary tumors of the lung, breast, and other extracranial sites. However, the results of several phase II trials (4–8) in heavily pretreated patients were not impressive enough to establish this regimen as a standard of care for instances of metastatic spread to the brain from cancers such as breast carcinoma. We therefore sought to create a novel analog of TMZ with superior activity against brain metastases.

TMZ acts as a prodrug. Its mechanism of activation involves hydrolytic opening of its tetrazinone ring, which takes places spontaneously in aqueous solution at 37°C, and does not require the participation of cellular enzymes. The resulting product, the unstable monomethyl 5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide, reacts with water to liberate AIC (4-amino-5-imidazole-carboxamide) and the highly reactive methyldiazonium cation, which methylates DNA purine residues (40, 41). Inspired by earlier studies that have demonstrated activity of POH in patients with glioblastoma multiforme (25, 26), we created a novel TMZ analog where POH was covalently conjugated to the C-8 position of TMZ, resulting in T-P. In the past, extensive molecular modeling studies of antitumor imidazotetrazines (42–44), including TMZ, showed that the initial activating ring-opening reaction, involving nucleophilic addition at C-4 of the tetrazinone ring, is not affected by bulky moieties at C-8. Therefore, irrespective of the nature of the targeting group conjugated at C-8, the final step in the activation process would be expected to release the electrophilic methyldiazonium ion that methylates nucleophilic sites in DNA. Based on these earlier structural and bioactivity studies, we predicted that T-P would preserve the release of the reactive methyldiazonium, and therefore that the cytotoxic activity of T-P would involve DNA methylation, similar to its parental molecule TMZ.

Our data are indeed consistent with the above mechanistic model. For instance, we show that the presence of MGMT, which highly specifically repairs O6-methylguanine and provides profound protection against TMZ (10, 11), minimizes DNA damage caused by T-P (Fig. 5D) and increases cellular resistance to this agent (Fig. 3B). Conversely, the presence of O6-BG, a specific inhibitor of MGMT, substantially enhances DNA damage caused by T-P (Fig. 5D) and increases this agent's cytotoxic potency exclusively in MGMT-positive cells (Fig. 4). As well, T-P treatment of cells leads to a reduction in MGMT protein levels (Fig. 2C), which is a well-established effect in the case of TMZ, because of the DNA repair enzyme's "suicide" mechanism of action, whereby acceptance of the alkyl group from O6-methylguanine leads to the protein's rapid degradation (45).

Although our data establish DNA alkylation by T-P as a key mechanism by which this agent exerts its cytotoxic effect, we cannot exclude the possibility that its POH moiety may contribute additional functions. POH is known to affect several intracellular processes. For instance, it has been shown to inhibit the activity of telomerase and of sodium–potassium pump (Na^+/K^+-ATPase; refs. 46 and 47). As well, it has been described as a farnesyl-transferase inhibitor that results in the blockage of ras oncoprotein activity (48, 49), although this has been challenged (50, 51). Importantly, in all these cases relatively high concentrations of POH (well above 100 μmol/L) are required to achieve 50% inhibition of target activity (see also Fig. 1B). In comparison, T-P is active in the range of 1 to 5 μmol/L in MGMT-negative cells (Table 1). Notably as well, when POH is mixed with TMZ and applied as a separate agent, this combination is unable to replicate the high potency of conjugated T-P (Figs. 1B, 5C, and 6B), indicating that the mere presence of non-conjugated POH is unable to provide additional potency over TMZ. These considerations, combined with T-P's notable sensitivity to MGMT and O6-BG as detailed above, diminish the likelihood for involvement of functions other than DNA damage.

If conjugation of POH indeed does not provide additional proapoptotic mechanisms over TMZ alone, why is T-P significantly more potent than TMZ? It has been well established that TMZ (and its active degradation product) exhibits rapid turnover *in vitro* and *in vivo*, with a half-life in the range of 1 to 2 hours (32, 43). Consistent with these characteristics, we find that after 4 hours of incubation in medium, nearly 100% of TMZ's cytotoxic activity has been spent (Fig. 7). In contrast, T-P seems significantly longer-lived, where after 4 hours about 50% activity remains (Fig. 7). We therefore propose that the extended presence of T-P may provide for greater opportunity to set DNA lesions, resulting in increased cytotoxicity.

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Although the extended half-life of T-P may suffice to explain its greater potency *in vitro*, it remains to be established whether it also contributes to its substantially increased *in vivo* potency in our brain metastasis model (Fig. 8). Because the lipophilicity of T-P is increased over TMZ (data not shown), it is also possible that T-P may cross the BBB more efficiently than TMZ. In the case of TMZ, it is known that drug levels achieved in the cerebrospinal fluid (CSF) are 80% lower than drug levels in the systemic circulation, that is, in plasma (52). It is therefore conceivable that TMZ, despite its established therapeutic benefit, would exert even greater activity, if only higher intracranial concentrations could be achieved. In this regard, T-P might be the vehicle to achieve this, and detailed physicochemical and pharmacokinetic studies are planned to investigate this aspect.

It is quite intriguing that TMZ displayed only minor activity in our intracranial *in vivo* model (Fig. 8). The breast cancer cell line we used, a variant of MDA-MB-231, does exhibit exquisite in vitro sensitivity to TMZ (IC_{50} <10 μmol/L), and therefore is more sensitive to TMZ than most MGMT-negative glioblastoma multiforme cell lines reported in the literature (53) and inclusive of several glioblastoma multiforme cell lines we analyzed in parallel (data not shown). As well, the TMZ dosage used (25 mg/kg) is well within the range of dosages shown to exert potent activity in glioblastoma multiforme mouse models, where even 5 mg/kg has significant activity (30). We therefore speculate that the triple-negative 231 cell line might harbor intrinsic mechanisms of resistance to TMZ that emerge only in the *in vivo* environment, and perhaps are reflective of the unimpressive responses that were noted when breast cancer patients with brain metastases were treated with TMZ (6, 8). Although this conjecture remains hypothetical at this time, it is obvious from our studies that T-P provides far superior therapeutic benefit than TMZ in our intracranial tumor model (Fig. 8), which may bode well for the clinical setting. We therefore propose that T-P should be investigated further as a potentially effective addition to therapeutic regimens for brain metastatic breast cancer.

Disclosure of Potential Conflicts of Interest

T.C. Chen is the chairman of NeOnc Technologies and has ownership interest (including patents) in NeOnc Technologies. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The opinions, findings, and conclusions herein are those of the authors and not necessarily represent those of The Regents of the University of California, or any of its programs.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.C. Chen, H.-Y. Cho, W. Wang, M. Barath, N. Sharma, A.H. Schönthal
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.C. Chen, H.-Y. Cho, W. Wang, M. Barath, N. Sharma, F.M. Hofman, A.H. Schönthal
Writing, review, and/or revision of the manuscript: T.C. Chen, H.-Y. Cho, W. Wang, M. Barath, N. Sharma, A.H. Schönthal
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.C. Chen, W. Wang, M. Barath, N. Sharma, A.H. Schönthal
Study supervision: T.C. Chen, A.H. Schönthal

Acknowledgments

The authors thank Drs. M.F. Press and S. Swenson (USC) for providing breast cancer cell lines. We are grateful to Dr. B. Kaina (University of Würzburg, Germany) for the gift of plasmids containing MGMT cDNA and the neo gene. The authors thank Drs. D. Levin and S. Puppali (Norac Pharma) for T-P conjugation and API manufacturing, and NeOnc Technologies for providing T-P. The authors thank patient advocate D.T. Chingos for providing input from the patient's perspective and the James Hale Family for generous research support (to T.C. Chen).

Grant Support

This work was supported by funds provided by The Regents of the University of California, California Breast Cancer Research Program, Grant Number No 19IB-0141, to T.C. Chen and A.H. Schönthal.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 17, 2013; revised January 27, 2014; accepted February 28, 2014; published OnlineFirst March 12, 2014.

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Molecular Cancer Therapeutics

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Thomas C. Chen, Hee-Yeon Cho, Weijun Wang, et al.

Mol Cancer Ther 2014;13:1181-1193. Published OnlineFirst March 12, 2014.

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