Perillyl Alcohol for the Treatment of Temozolomide-Resistant Gliomas

Hee-Yeon Cho¹, Weijun Wang¹, Niyati Jhaveri², Shering Torres², Joshua Tseng¹, Michelle N. Leong¹, David Jungpa Lee², Amir Goldkorn³, Tong Xu³, Nicos A. Petasis⁴, Stan G. Louie⁵, Axel H. Schönthal⁶, Florence M. Hofman⁷, and Thomas C. Chen¹,²,⁷

Abstract

Perillyl alcohol (POH) is a monoterpene that has been used orally for the treatment of systemic cancer. However, when used orally, significant gastrointestinal side effects and lack of overall efficacy were documented. Recently, in a phase II trial in Brazil for the treatment of temozolomide (TMZ)-resistant gliomas, POH was well tolerated when administered intranasally. The present study explores the effects and mechanisms of POH on TMZ-sensitive and TMZ-resistant glioma cells. In vitro studies showed that POH was cytotoxic to TMZ-resistant as well as TMZ-sensitive glioma cells, and this effect was independent of O6-methylguanine-DNA methyltransferase expression. POH induced cytotoxicity, in part, through the endoplasmic reticulum (ER) stress pathway as shown by the increased expression of glucose-regulated protein-78 (GRP78), activating transcription factor 3, and C/EBP-homologous protein. In addition, POH impeded survival pathways, such as mTOR and Ras. As well, POH reduced the invasive capacity of sensitive and resistant glioma cells. POH alone and/or in combination with other ER stress–inducing cytotoxic drugs (i.e., 2, 5-dimethyl-celecoxib, nelfinavir) further induced apoptosis in TMZ-sensitive and TMZ-resistant glioma cells. To show whether intranasal delivery of POH was effective for the treatment of TMZ-resistant gliomas, animals bearing intracranial tumors were given POH intranasally. Animals treated through intranasal administration of POH exhibited a decrease in tumor growth and an increase in survival. Our data show that POH is an effective anti-glioma cytotoxic agent for TMZ-resistant gliomas when administered intranasally.

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Introduction

Perillyl alcohol (POH) is a naturally occurring monoterpene that has been used orally for the treatment of a variety of cancers, including breast, prostate, and lung carcinomas (1–3). POH was shown to be a cytotoxic agent by functioning as a Ras inhibitor, cell-cycle inhibitor, and upregulator of the proapoptotic protein Bax (3, 4). However, because of its significant gastrointestinal side effects, POH was not well tolerated as an oral anticancer agent. Recently in Brazil, POH was administered intranasally in a phase II trial for the treatment of recurrent malignant glioblastoma multiforme (GBM; ref. 5). Recurrent tumors are usually resistant to standard of care chemotherapy, temozolomide (TMZ), a DNA alkylating agent. Once GBMs become resistant to TMZ, there are very limited treatment options available. Intranasal POH has been very well tolerated, with minimal systemic side effects, and only local irritation to the nasal mucosa. Younger patients with secondary GBMs responded best to POH treatment (6). Surprisingly, patients with deep midline or subcortical GBMs had a better response than cortical GBMs (6). This is particularly cogent, as patients with cortical GBMs are often surgically resectable, whereas the subcortical and deep GBMs are much more limited in surgical options and depend on adjunctive therapy for treatment.

The studies presented here show that POH was cytotoxic to a variety of glioma cell lines, including several TMZ-resistant ones, without significantly affecting normal cells. POH also served as a chemosensitizing agent, enhancing the cytotoxic capacity of TMZ. POH decreased proangiogenic growth factors and inhibited tumor cell invasion. ER stress–induced cytotoxicity appeared to be an important mechanism of POH-induced cytotoxic activity; however, additional cytotoxic pathways were identified. When administered intranasally, POH was effective in reducing the growth of TMZ-resistant gliomas and increasing survival. The mechanisms of drug action...
in vivo appear to be a combination of increased ER stress-mediated tumor cytotoxicity, decreased angiogenesis, and decreased tumor invasive capacity.

Materials and Methods

Cells and reagents
Human glioma cell lines U251, U87, LN229, and A172 were cultured in 10% fetal calf serum (FCS) in Dulbecco’s Modified Eagle’s Media supplemented with 100 U/mL penicillin and 0.1 mg/mL streptomycin in a humidified incubator at 37°C and 5% CO2. Glioma cell lines were originally purchased from American Type Culture Collection; these cell lines have not been authenticated. POH (NEO100) was synthesized by Norac Pharma, under GMP quality conditions (NeOnc Technologies, Inc.). In vitro equivalency studies using NEO100 versus commercially available POH (Sigma Aldrich) showed that NEO100 was approximately 1.2 times more potent than Sigma POH; POH referred to in this study is NEO100. The following drugs were used: temozolomide (TMZ; Merck), nelfinavir (Viracept; Agouron Pharmaceuticals Inc.), and 2,5-dimethyl-celecoxib (DMC; synthesized according to our previously published procedure (7)). The Viracept pill was ground and dissolved in 100% ethanol at 50 mmol/L (stock solution). The above drugs were added to culture medium in a manner that retained the final concentration of solvent below 0.1%. For intranasal administration, POH was formulated as 0.76 and 1.9 mg/kg in 40% polyethylene glycol (PEG400; Sigma-Aldrich), and serially dissolved in water. TMZ-resistant cells were developed by serial passaging of tumor cells with increasing concentrations of TMZ ranging from 10 to 100 μmol/L over a period of 6 months. TMZ-resistant cell lines were maintained in 100 μmol/L TMZ every other week.

MTT assay
Glioma cells (5,000 cells per well) were seeded in 96-well plates. After 24 hours, POH and/or DMC and nelfinavir were added at different concentrations and cells were incubated for 48 hours. TMZ with or without POH was tested in the long-term MTT assay as previously described (8). The assay was conducted according to the manufacturer’s protocol (EMD Chemical). Absorbance was measured using a microtiter plate reader (Dynatech MR4000) at 490 nm. Percentage viability was calculated relative to untreated control cells. All experiments were conducted in triplicate.

Colonies-forming assay
Glioma cells were seeded in 6-well plates at 200 cells per well and allowed to adhere overnight. Subsequently, cells were treated with POH and/or TMZ for 48 hours; the medium was then removed and fresh medium (without drugs) added. Cells were incubated for an additional 10 to 14 days. At the termination of the assay, colonies were visualized by staining with 1% methylene blue in methanol for 4 hours and quantified. Groups were plated in triplicate.

Aptoptosis assay
Tumor sections were analyzed for apoptosis using the TUNEL assay with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon) according to manufacturer’s instructions. Tissues were fixed in 1% paraformaldehyde and developed using the aminoethyl carbazol substrate.

ELISA assay
U87 cells (5 × 10⁵ cells per well) were seeded in 6-well plates and allowed to adhere overnight. Subsequently, cells were treated with POH (0.3 and 0.6 mmol/L) for 48 hours. Supernatants were collected, centrifuged, and filtered through a 0.4 micron filter. Supernatants were analyzed for VEGF using the Human VEGF Immunoassay Kit (Invitrogen), and interleukin-8 (IL-8) using the Human IL-8/NAP-1 Immunoassay Kit (R&D Systems), according to manufacturer’s protocols. The number of viable cells was counted at the termination of the experiment; data are presented as quantity of growth factor per 10⁶ cells.

Immunostaining
Frozen tumors were sectioned and immunostained for GRP78 using the rabbit polyclonal anti-GRP78 antibody (Santa Cruz Biotechnology), as previously described (9). Positive staining was identified by appearance of red precipitate; slides were counterstained with hematoxylin.

Western blot analysis
Total cell lysates were prepared by disrupting cells with radioimmunoprecipitation assay buffer buffer. Protein concentrations were determined using the BCA protein assay reagent (Pierce). Fifty micrograms of total cellular lysates were added to each lane. Trans-blot (BioRad) was used for semidry transfer. Antibodies to GRP78, C/EBP-homologous protein (CHOP), and actin were obtained from Santa Cruz Biotechnology, Inc., and used according to manufacturer’s recommendations. Antibodies to CHOP, cleaved caspase-7, and PARP were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used for detection.

Transfection with siRNA
Cells were plated in 6-well plates in serum-free media and transfected using the Lipofectamine 2000 kit (Invitrogen) according to manufacturer’s instructions. Five nanomoles per liter of each siRNA was used for knockdown experiments. The different siRNAs (si-GRP78, si-CHOP, and si-GFP) were designed and synthesized by Ambion (Life Technology Corp.). Transfected cells were harvested for Western blot analysis to confirm knockdown of target gene expression, or seeded for the evaluation of long-term survival after drug treatment.

Invasion assay
Sensitive and resistant tumor cells were incubated with mitomycin C (10 μg/mL) for 2 hours, rinsed and plated
(5 × 10^4 cells in 1% FCS) in the top chamber of the 8 micron pore filter of the modified Boyden chamber. The filters had been previously coated with Matrigel (BD Biosciences). The bottom chamber contained 1 mmol/L POH in 10% FCS or medium alone as the control. The chambers were then incubated for 18 hours. Cells on the underside of the filter were stained and counted. In parallel, tumor cells were incubated with mitomycin C, treated with POH, and evaluated for viability after 18 hours, using the trypan blue exclusion technique; all cultures were 85–90% viable.

**In vivo experiments**

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Southern California. Luciferase-labeled U251-TMZ-resistant glioma cells (2 × 10^6 cells in 20 μL) were implanted intracranially, as previously described (8). Seven days after implantation, mice were randomly divided into groups of 5 each, and treatment was initiated as follows: vehicle (DMSO) control; POH (0.76 mg/kg); POH (1.9 mg/kg); and TMZ (5 mg/kg). POH (5 μL) was administered into alternating nostrils every other day for the duration of the experiment. TMZ was administered as an oral gavage, on a 1-week on and 1-week off schedule. Mice were imaged weekly. Mice were injected with 1 mg/kg Viviren probe (Promega) intravenously and imaged using the IVIS 200 optical imaging system (Caliper Life Sciences); images were analyzed using LIVING IMAGE software (Caliper Life Sciences).

**Statistical analysis**

Statistical significance was evaluated using the Student’s 2-tailed test for all in vitro experiments. Logrank test was used to evaluate significance for the survival curve; P < 0.05 was considered significant.

**Results**

**POH is cytotoxic to TMZ-sensitive and TMZ-resistant glioma cells**

The chemical structures of POH and TMZ are shown in Fig. 1. To study the activity of POH on TMZ-resistant cells, we established several TMZ-resistant glioma cell lines (Fig. 2). In vitro experiments showed that U251-resistant and U87-resistant cells are resistant to TMZ at doses up to 200 μmol/L. Experiments using the intracranial rodent model showed that TMZ-resistant cells maintained this characteristic in vivo (Fig. 2C). To determine the impact of POH on glioma cell viability, POH was tested on 3 different wild-type and resistant glioma cell lines (U251, U87, and LN229), and the human glioma cell line T98G, known to be TMZ resistant (10), at POH doses ranging from 0.5 to 2.5 mmol/L, for 48 hours in the standard MTT cell viability assay. The results in Fig. 2D show that the IC50 for the wild-type cells ranged from 1.6 to 1.8 mmol/L. Using the TMZ-resistant cells (Fig. 2E) the IC50 ranged from 1.5 to 1.8 mmol/L. These results were confirmed in 3 independent experiments. Using the long-term colony-forming assay (CFA) assay, the IC50 of U251-sensitive cells was 0.8 mmol/L POH, whereas the TMZ-resistant cells showed an IC50 of 1.2 mmol/L (Fig. 2F). These data show that POH is cytotoxic for both TMZ-sensitive and TMZ-resistant glioma cells. As the TMZ resistance generated in these glioma cells are not dependent on O6-methylguanine-DNA methyltransferase (MGMT) expression (Supplementary Fig. S1), we concluded that cell death induced by POH is independent of MGMT expression. In contrast to glioma cells, minimal cytotoxicity was observed in normal astrocytes treated with POH up to 3 mmol/L; the IC50 for brain endothelial cells was greater than 2 mmol/L (Supplementary Fig. S2).

**POH functions as a chemosensitizing agent for TMZ**

To determine whether POH is a potent chemosensitizing agent for TMZ (the current standard of chemotherapeutic care for malignant glioma), U251 cells were treated with POH (0.6 mmol/L), and different concentrations of TMZ (20–40 μmol/L; Fig. 3A). The IC50 for TMZ was 40 μmol/L. The results showed that TMZ in combination with POH greatly potentiated the effects of TMZ on TMZ-sensitive glioma cells. In contrast, TMZ-resistant cells (U251/TR) treated with POH (0.8 and 1.0 mmol/L) and TMZ at 100 μmol/L did not become responsive to TMZ (Fig. 3B).

**POH reduces tumor cell invasion**

The invasive capacity of glioma cells is a critical issue, especially in recurrent tumors where repeated resections are difficult. To assess whether POH affects glioma cell invasion, the modified Boyden chamber technique was used. The data (Fig. 3C) showed that POH, at the nontoxic (80–90% viability) concentration of 1 mmol/L, significantly reduced the invasive capacity of the sensitive and resistant U251 glioma cells by approximately 50% in this 18-hour invasion assay. This experiment was conducted 3 times with similar results. As generally noted, TMZ-resistant cell lines exhibit a 3- to 4-fold greater baseline migration rate and invasive capacity compared with TMZ-sensitive cells.

**POH affects proangiogenic cytokine production**

POH has been shown to inhibit tumor growth in different solid tumor systems (11–15). As angiogenesis is a
critical component of tumor cell growth, the effects of POH on the production of proangiogenic cytokines were examined. U87 glioma cells were treated with POH (0.3 and 0.6 mmol/L; nontoxic dose; 80%–90% viability) for 48 hours and then assayed for response to TMZ using the CFA after 12 days. B, U87 sensitive (U87) and U87 resistant cell lines (U87TR) were treated with TMZ (0–200 µmol/L) and tested for response to TMZ using the MTT assay. Data is presented as percentage of vehicle-treated cells. C, U87 TMZ-sensitive and TMZ-resistant cells were implanted intracranially into nude mice. After 7 days, mice were separated into groups and treated as indicated. Vehicle control and TMZ (5 mg/kg) were administered orally in a 7-day on/off cycle. Survival was documented. D, U251, U87, and LN229 human glioma cell lines were treated with POH (0.5–2.5 mmol/L) for 48 hours, then tested for viability using the MTT assay. E, four TMZ-resistant cell lines (U251TR, U87TR, LN229TR, and T98G) were treated with POH (0.5–2.5 mmol/L) for 48 hours and analyzed for viability using the MTT assay. F, TMZ-sensitive (U251) and TMZ-resistant (U251TR) glioma cells were treated with POH (0.5–2.0 mmol/L) for 48 hours, then analyzed 12 days later in the CFA. Data is presented as percentage of vehicle-treated cells.

**POH induces cytotoxicity through the ER stress pathway**

To identify the potential pathways responsible for the cytotoxicity induced by POH, three glioma cell lines (U251, U87, and A172) were treated with POH for 20 hours and tested for proteins known to be regulators of the ER stress pathway (16). The results showed that treatment with POH at 1.5 mmol/L resulted in an increased expression of ER stress markers CHOP and GRP78 in each of the cell lines tested (Fig. 4A). The concentration-dependent response of CHOP and GRP78 was also examined in the TMZ-resistant cells (Fig. 4B). To analyze the mechanism of cytotoxicity in TMZ-resistant tumor cells, the TMZ-resistant cells U251/TR were treated with POH (1.5 mmol/L) for 20 hours and tested for apoptosis-associated proteins such as CHOP, ATF3, PARP, and caspase 7, as well as the ER stress-associated protein, GRP78 (Fig. 4C). The results showed that POH increased CHOP, ATF3, PARP, and GRP78 in TMZ-resistant glioma cells. Cleaved caspase 7, an effector caspase, was also increased with POH treatment.

To investigate whether ER stress played a role in mediating POH-induced glioma cell death, we used siGRP78 and siCHOP to knock down these proteins. First, Western
showed that POH inhibited Ras expression in glioma cells in the prosurvival mTOR pathway. Further studies demonstrated S6 protein, indicating that POH diminished activity and decreased phosphorylation (i.e., activity) of Akt and ribosomal S6 protein, as well as the mTOR pathway (Fig. 4F). We found that POH conducted Western blot analysis identifying proteins in response to POH treatment. To determine if other pathways were involved in POH-mediated cell death, we conducted Western blot analysis identifying proteins in the mTOR pathway (Fig. 4G). We found that POH decreased phosphorylation (i.e., activity) of Akt and ribosomal S6 protein, indicating that POH diminished activity in the prosurvival mTOR pathway. Further studies showed that POH inhibited Ras expression in glioma cells as well (Fig. 4G). In testing the classical extrinsic pathways of cell death, using caspase-8 and caspase-9 as markers, there is little evidence that these pathways are involved in POH-mediated cytotoxicity (Supplementary Fig. S4). Thus, POH regulates several critical pathways responsible for cell survival.

**POH enhances cytotoxicity of ER stress–inducing drugs**

As the ER stress pathway is one mechanism by which POH induced cytotoxicity, we hypothesized that the combination of POH with a second ER stress–inducing agent would increase the cytotoxic effects. To test this, U251 and U87 cells were treated with DMC, a known inducer of ER stress (17), either alone or in combination with POH (0.6 mmol/L). Using the MTT assay, our results show (Fig. 5A) that the addition of POH significantly enhanced cytotoxicity in both resistant cell lines (P < 0.001) and TMZ-sensitive cells to TMZ but not TMZ-resistant cells, reduces invasion, and decreases proangiogenic cytokine production. A, U251 cells (250 cells per well) were treated with the combination of POH and TMZ (0–0.05 µmol/L) for 48 hours, as described above. There was no significant difference in single versus combination treatment; P > 0.05. B, U251TR cells were treated with the combination of POH (0.05 mmol/L) and TMZ (100 µmol/L) for 48 hours as described above. There was no significant difference in single versus combination treatment; P > 0.05.

**Figure 3.** POH chemosensitizes TMZ-sensitive cells to TMZ but not TMZ-resistant cells, reduces invasion, and decreases proangiogenic cytokine production. A, U251 cells (250 cells per well) were treated with the combination of POH and TMZ (0–0.05 µmol/L) for 48 hours, followed by the long-term MTT assay; **, P = 0.0058. B, U251TR cells were treated with the combination of POH (0.05 mmol/L) and TMZ (100 µmol/L) for 48 hours as described above. There was no significant difference in single versus combination treatment; P > 0.05. C, U251 sensitive and U251 resistant (TR) cells were plated on Matrigel in the modified Boyden chamber and incubated with vehicle (control) or 1 mmol/L POH for 18 hours. Cells that invaded the Matrigel and migrated through to the underside of the filter were quantified; **, P < 0.05. U87 glioma cells were treated with POH (0.3 and 0.6 mmol/L) for 48 hours. Supernatants from these cultures were tested for IL-8 (D) and VEGF (E) using the ELISA assay; *, P < 0.05; **, P < 0.006.
chemosensitized both TMZ-sensitive and TMZ-resistant cells to nelfinavir. In intranasal delivery of POH reduces tumor growth

To determine whether the effects of POH observed in vitro were relevant to the in vivo environment, POH was tested on TMZ-resistant glioma cells in the orthotopic xenograft rodent model. Athymic nude mice were implanted intracranially with U251 TMZ-resistant glioma cells in the following groups (n = 5): control-intranasal (IN) vehicle; IN 0.76 mg/kg POH; IN 1.9 mg/kg POH; and intragastric TMZ 5 mg/kg/d. Animals were given POH (5 μL volume) 1 day on/1 day off, or TMZ 1 week on/1 week off; these drugs were not given in combination. Animals were imaged weekly. These in vivo experiments showed that tumors from in vitro TMZ-resistant glioma cells were also resistant to TMZ in vivo (Fig. 2C), as TMZ (5 mg/kg) had little effect on animals bearing the TMZ-resistant tumors. As shown in Fig. 6A, vehicle-treated animals displayed a progressive increase in tumor size and died between 36 to 38 days postimplantation. In contrast, animals treated with IN POH 0.76 mg/kg or IN POH 1.9 mg/kg showed a relative constant tumor size from 15 to 35 days, with moderate tumor growth observed at 56 days postimplantation. The survival curve (Fig. 6B) showed significant differences between the control vehicle-treated group and IN 0.76 mg/kg (P < 0.009) and IN 1.9 mg/kg (P < 0.004).

Tumors from POH-treated and vehicle-treated animals were immunostained for GRP78 expression. The results showed (Fig. 6C) that POH-treated animals expressed higher levels of GRP78, as compared with vehicle-treated animals.
controls. The POH-treated tumors were also analyzed for apoptosis using the TUNEL assay. POH-treated groups displayed more apoptotic cells compared with control tumor tissues (Fig. 6D). Microvessel density was evaluated in the tumor tissues, and no significant differences between control and POH-treated animals were observed (Supplementary Fig. S3).

Toxicology studies were conducted following euthanasia. Contralateral brain, liver, kidney, intestine, lungs, and heart were fixed in formalin and processed for standard histology. An examination of hematoxylin and eosin (H&E)-stained tissue sections exhibited no significant pathological changes in any of the organs analyzed (data not shown). Altogether, these results showed that IN administration of POH to animals bearing intracranial tumors decreased the rate of growth of TMZ-resistant tumors, with minimal toxic side effects on the animals.

Discussion
This study showed that POH is effective in reducing the growth and development of TMZ-resistant gliomas using both in vitro and in vivo systems. Our results showed that POH induced cytotoxicity in several different sensitive and resistant cell lines. The IC50 for drug-sensitive glioma cells was 1.6 to 1.8 mmol/L, whereas the IC50 for the TMZ-resistant cells ranged from 1.5 to 1.8 mmol/L, showing that POH acts within a similar range for sensitive and resistant glioma cells. The T98G glioma cell line, which is known to be TMZ resistant, was also shown to be sensitive to POH within this range. Resistance to TMZ in these cells...
was because of overexpression of MGMT, which minimizes the effects of TMZ, a mechanism of resistance in gliomas (19, 20). We show that several of the TMZ-resistant cells express MGMT (e.g., T98G, U251 TR2; Supplementary Fig. S1), whereas other cell lines do not (U87 TR). Thus, POH is cytotoxic for TMZ-resistant glioma cells irrespective of MGMT expression, suggesting that POH may act through other pathways. We chose to examine ER stress, because this is a potent apoptotic pathway in gliomas (21). However, POH can function through the Ras pathway (Fig. 4G). Here, we show that POH decreases phosphorylation of Akt and ribosomal S6 protein, suggesting that the mTOR pathway is inhibited (Fig. 4P) as well.

POH is not cytotoxic to normal, nonmalignant cells, such as astrocytes and endothelial cells, in the dose range tested. The toxicology analysis of different organs (liver, kidney, intestine, lung, heart, and contralateral normal brain) following long-term POH intranasal administration showed no apparent pathological abnormalities. Thus, POH selectively induced cytotoxicity in TMZ-sensitive and resistant malignant glioma cells, without perceptible toxic effects to normal tissues.

Our studies showed that the mechanism of POH-mediated cytotoxicity in glioma cells involved, in part, the activation of the ER stress pathway (Fig. 4). Western blot analysis showed that POH treatment upregulated GRP78 and induced CHOP expression in drug-sensitive and resistant cells (Fig. 4A and B). Immunostaining of tumor tissue from intranasal POH-treated mice showed that GRP78 was overexpressed in treated tumor tissues, as compared with nontreated animals (Fig. 6C).

**Figure 6.** Intranasal administration of POH delays tumor growth. A, animals were implanted intracranially with Renilla luciferase–labeled U251 (U251 TR) TMZ-resistant glioma cells; treatment was initiated after 7 days. Animals were given vehicle, POH intranasally (POH IN) 0.76 mg/kg, POH IN 1.9 mg/kg, or TMZ 5 mg/kg orally. Tumors were imaged at days 15, 35, and 56. B, the survival curve showed significant differences between the control vehicle-treated group compared with intranasal 0.76 mg/kg (P < 0.009) and intranasal 1.9 mg/kg (P < 0.004). C, tumors were sectioned and stained for GRP78 to show ER stress in vivo. Darker areas denote positive staining. GRP78 expression was higher in tumor sections from mice treated with POH. D, tumor sections were stained for apoptotic cells using the TUNEL assay kit. Arrows, cells undergoing apoptosis.
This emphasized that tumor cells, when exposed to POH in vitro, upregulated GRP78 expression. Glioma cells generally express low levels of GRP78, as do most tumor cells (22). However, the overexpression of this antigen suggests that POH was able to enter the tumor and alter the function of these cells. Thus, the increased intensity of GRP78 in treated animals showed that POH was able to enter the brain and affect the tumor. 

GRP78 is overexpressed initially and can function as a protective agent in ER stress (23). Reducing GRP78 had been shown to enhance cell death in tumors and tumor blood vessels (22, 23). CHOP is a critical apoptotic protein and key to ER stress-mediated cell death (16). Our data show that reducing GRP78, using siGRP78, caused a significant decrease in cell viability (P = 0.001) after POH treatment (Fig. 4E). Furthermore, reducing CHOP significantly (P = 0.002) increased cell survival after POH treatment (Fig. 4E). Thus in glioma cells, POH-induced cytotoxicity is mediated, at least in part, through the ER stress pathway. As siRNA knockdown of CHOP rescued about 30% of the cells, we investigated other potential cell death pathways, and found that POH also inhibited the mTor and Ras pathways (Fig. 4F and G). Thus, POH may be regulating cell death through several pathways.

There is substantial evidence from other tumor models that POH treatment induced growth arrest and cell death by a variety of mechanisms (2, 3). POH treatment significantly suppressed the Ras/Raf/ERK pathway (4, 24). POH also increased Bak and reduced Bcl-xL in different tumors (2, 4, 25). POH suppressed cyclin A, cyclin B1 and cdk2, resulting in G0–G1 and G2–M arrest (4). Our studies showed that POH chemosensitized cells to the protease inhibitor, nelfinavir, and to the calcium channel–blocking agent responsible for ER stress, DMC. POH can also sensitize breast cancer cells to cisplatin (3). Thus, POH can function in coordination with other drugs that act through different pathways to chemosensitize glioma cells.

Our studies showed that POH greatly potentiates the effects of TMZ on sensitive glioma cells (Fig. 3A). This clinically relevant data suggests that TMZ may be used at lower doses when combined with POH in patients sensitive to TMZ. In contrast, TMZ-resistant cells treated with POH were not chemosensitized to TMZ (Fig. 3B). Thus, for TMZ-resistant cells, POH alone or in combination with other drugs may be a more effective approach to therapy. Future experiments will test appropriate drug combinations in vivo.

In vivo studies showed that the administration of POH caused a delay in tumor growth, resulting in a significant increase in survival (Fig. 6). Tumor growth is a function of tumor cell death and extent of invasiveness. Resistant tumors were especially noted for enhanced invasiveness (26). Therefore, we tested the effects of POH on glioma cell invasion. The results showed that POH, at doses that caused little detectable toxicity, reduced the invasion capacity of the drug-sensitive and resistant tumor cells (Fig. 3C). As noted, TMZ resistant cells are 3- to 4-fold more invasive than sensitive cells. In a histologic examination of POH-treated and vehicle-treated tissues, there were no detectable differences in invasion at the tumor-normal tissue borders. This may be attributed to the fact that the tissues were examined at a late stage of tumor development, at death, when the tumors were already very large. These types of differences may best be examined at the faster growing stage of the tumor. Although our U251 cells showed a moderate level of invasiveness, we may test a more invasive glioma cell line, such as LN229, to further investigate the in vivo effects of POH on invasiveness.

The tumor vasculature is critical for supporting tumor growth (27). As POH was shown to decrease the production of the proangiogenic growth factors, VEGF and IL-8, tumor tissues were examined for microvessel density. The results showed no significant differences between vehicle-treated and POH-treated animals, although there was a trend (P = 0.17) toward decreased blood vessel density in the POH-treated animals (Supplementary Fig. S3). Here again, the tumor specimens were analyzed at time of sacrifice. We surmise that significant differences in the vasculature may appear early during tumor development.

The present study showed that TMZ-resistant glioma cells responded to intranasal POH treatment. Numerous studies have shown that intranasal delivery to the brain can occur via the olfactory and/or trigeminal nerve in vivo, resulting in penetration of the brain (28). Our in vivo studies show that an effective dose of drug did enter the brain, as tumor growth was delayed. Others have shown that intranasal delivery of telomerase inhibitors decreases glioma growth in rodent glioma intracranial model (29). POH clinical trials by Da Fonseca and colleagues have been conducted using POH purchased from Sigma Chemicals and delivered via facemask, at a total dosage of 440 mg/d (given 4 times a day; ref. 6). This dosage in humans (6.3 mg/kg, assuming a 70 kg patient), is approximately 3 times greater than the amount of drug administered to the animals in our study (i.e., 0.76 and 1.9 mg/kg). Thus, the in vivo dosages used in our experiments were within the pharmacologic range that is achievable in humans. The reason that such relatively high concentrations of drug are administered may be the results of inefficient intranasal drug delivery via the Continuous Positive Airway Pressure face mask. However, this route is still more efficient than oral delivery, where up to 13.8 grams is given orally (6). Indeed, Da Fonseca and colleagues have shown that POH can be safely delivered intranasally on a long-term basis, with documented radiographic reduction in tumor size (11). Systemic and neurologic toxicity have been minimal, with no gastrointestinal or hematologic side effects noted. Radiographic regression has been seen in 36% of patients, with 48.3% of the recurrent GBM patients achieving a 6-month progression-free survival, similar to the
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bevacizumab (Avastin) data for recurrent GBM (6). Additional advances using an intranasal atomizer (Via Nase; Kurve Technologies) would optimize the efficiency of POH delivery, because the nozzle is capable of delivering particles based on formulation and size (controlled particle dispersion). Future plans for POH focus on clinical trials for TMZ-resistant GBM using cGMP POH (NEO100) delivered by an intranasal atomizer, to patients who show evidence of progression on TMZ. POH can be administered with TMZ, and formal trials will be conducted to determine if POH lengthens the window of TMZ sensitivity. If TMZ is not used, POH may be given as a standalone drug or in combination with bevacizumab. Recently, combination protocols using bevacizumab and POH have shown evidence of synergy or additive effects (Da Fonseca, personal communication). The mechanism of the 2 drugs could be additive, as both bevacizumab and POH reduce the presence of VEGF in the tumor microenvironment.

In summary, our data showed that POH is cytotoxic to TMZ-resistant glioma cells in vivo and in vitro, with no apparent detrimental effects on normal cells or normal organs. POH also reduced glioma cell invasion and the capacity to produce proangiogenic growth factors. Furthermore, POH functions in vivo to reduce the rate of tumor growth, thereby increasing animal survival. On the basis of its limited toxicity and ease of administration, POH may be used as a long-term maintenance therapy. Such a treatment would prolong survival of patients with TMZ-resistant gliomas at a reasonably high level of quality of life.

Disclosure of Potential Conflicts of Interest

T.C. Chen is an officer and shareholder of NeOnc Technologies, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: H.-Y. Cho, W. Wang, F.M. Hofman, T.C. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.-Y. Cho, W. Wang, N. Jhaveri, J. Torres, M.N. Leong, D.J. Lee, A. Goldkorn, F.M. Hofman
T.C. Chen
Writing, review, and/or revision of the manuscript: H.-Y. Cho, W. Wang, J. Torres, A. Goldkorn, A.H. Schonthal, F.M. Hofman, T.C. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-Y. Cho, W. Wang, N. Jhaveri, M.N. Leong, D.J. Lee, F.M. Hofman, T.C. Chen
Study supervision: W. Wang, F.M. Hofman, T.C. Chen

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