Long-term Outcome in Patients with Recurrent Malignant Glioma Treated with Perillyl Alcohol Inhalation

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Abstract. *Aim: This retrospective study aimed to evaluate the long-term response and toxicity of recurrent malignant glioma patients to inhalation chemotherapy with perillyl alcohol (POH). Patients and Methods: The cohort included 117 men and 81 women with primary glioblastoma multiforme (GBM; n=154), grade III astrocytoma (AA; n=26) and anaplastic oligodendroglioma (AO; n=5). POH inhalation schedule 4-times daily started with 66.7 mg/dose; 266 mg/day and escalated up to 133.4 mg/dose; 533.6 mg/day. Clinical toxicity and overall survival following treatment were compared with tumor size, topography, extent of peritumoral edema and histological classification. Results: Adhesion to the protocol was high (>95%), POH (533.6 mg/daily) occasionally caused nose soreness but rarely nosebleed. Tumor size, peritumoral edema and the oligodendroglial component influenced response to treatment. Conclusion: After 4 years under exclusive POH treatment, 19% of patients still remain in clinical remission. Long-term POH inhalation chemotherapy is a safe and non-invasive strategy efficient for recurrent malignant glioma.*

Malignant gliomas are the most common primary intracranial tumors, and glioblastoma multiforme (GBM) accounts for 15% of all adult primary brain tumors (1). Despite aggressive multimodal treatment comprising of surgical resection, local radiotherapy (RT) and systemic chemotherapy the median survival time after diagnosis is still in the range of 12 months, but population-based studies indicate an even shorter survival (2-3). Standard treatment includes maximal safe surgical resection, greater than 98% of the tumor volume to improve prognosis, but GBM tumors are poorly-delineated, with infiltrating tumor cells, intense angiogenesis and necrosis occupying more than 90% of the tumor mass. GBM recurrence occurs within 6.9 months after diagnosis, and all patients recur after initial therapy with new lesions appearing in the same tumor area (4), and very short (4 to 6 months) survival after first recurrence (5). For this reason, most GBM patients undergo standard-regimen combination of radiotherapy and adjuvant temozolomide (TMZ) chemotherapy (4).

TMZ is an orally-administered alkylating pro-drug capable to alkylate/methylate DNA by delivering a methyl group to purine DNA (O6-guanine; N7-guanine and N3-adenine) that damages DNA and triggers tumor cell death. DNA repair depends on the expression of O-6-methylguanine-DNA methyltransferase (MGMT) enzyme that diminishes the therapeutic efficacy of TMZ in tumors expressing this protein, or tolerated in mismatch repair-deficient (MMR-) tumors (4, 6). GBM initiation, recurrence and drug resistance is associated to modification of enzymes, non-coding RNAs and cell mutations in different cell types from the original glioma cell passing on mutations to oligodendrocyte or astrocyte precursor cells (4, 7). Besides, a relatively quiescent subset of endogenous glioma cells, albeit with stem cell properties, sustains long-term tumor growth through the production of highly proliferative cells (4, 8). Standard salvage therapies include various chemotherapeutic drugs, repeat resection,

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radiosurgery or interstitial implantation. Treatment decisions are complicated because additional RT may pose risk of cumulative toxicity, and options for chemotherapy may be limited by the development of resistance (5). To improve the clinical outcome in patients with malignant glioma, novel chemotherapeutic agents and effective new regimens are needed. Molecular alterations in neoplastic glia often result from multiple mutations in proto-oncogenes and tumor suppressor genes (9) encoding for proteins that play important roles in signal transduction abnormalities of three major pathways: extracellular growth factors and their receptors EGF/EGFR, PDGF/PDGFR, and the signal transduction cascades Ras and AKT (10-11). Therapies targeting these specific molecules may result in killing tumor cells effectively while keeping normal cells intact. In this context, the search for new chemotherapeutic drugs has increased, especially for bioactive natural compounds (12).

Perillyl alcohol (POH) is a non-toxic naturally-occurring hydroxylated monoterpene with proven pre-clinical activity against various tumors in humans including breast, pancreas and lung carcinomas (13-15). As a cytotoxic agent, POH inhibits cell cycle, up-regulates the pro-apoptotic protein Bax (15-17) in TMZ-resistant and TMZ-sensitive glioma cells, independently of O6-methylguanine-DNA methyltransferase (MGMT) expression (18). POH cytotoxicity upon glioma cell lines resulted from the effect on endoplasmic reticulum (ER) stress pathway, as shown by increased expression of glucose-regulated protein-78 (GRP78), transcription factor-3 (ATF3) activation, and C/EBP-homologous protein (CHOP), and also by arresting survival pathways, such as mTOR and Ras (18-19).

The brain tissue is one of the least accessible organs for delivery of active pharmacological compounds (20). Our group has pioneered on the use of intranasal POH for the treatment of patients with recurrent malignant glioma (Phase I/II) (21-22). Intranasal administration allows POH to cross the blood-brain barrier (BBB) and reache the central nervous system (CNS) thus eliminating the need for systemic delivery while reducing side-effects. We present data (Phase II study) showing that long-term administration of POH as a single agent by inhalation was an effective therapeutic strategy capable to sustain long-term regression (>4 years survival) of recurrent glioma without clinical systemic side-effects.

Patients and Methods

This clinical trial was approved by the Hospital Medical Research Ethics Committee and the Brazilian Ministry of Health (CONEP 9681 no. 25000.009267/2004-25, July 12, 2004) complies with the principles laid down in the Declaration of Helsinki. Informed consent was obtained from each patient and the next-of-kin before beginning the study.

Patient selection. This prospective study was carried-out from July 2006 to July 2012 with patients attending the out-patient Neurosurgical Unit in the Antonio Pedro University Hospital and was included in the Phase I/II clinical trial to assess the efficacy of intranasal administration of the monoterpene POH. The cohort included 198 adult patients (random selection) with recurrent malignant glioma and under symptomatic treatment after failing response to current standard treatment including surgery, and/or radiation, and multimodal chemotherapy specific for glioblastoma. Diagnosis and histological classification of malignant glioma was based upon the WHO criteria (23). Eligibility criteria included patients older than 18 years with recurrent glioblastoma with at least two relapses, measurable contrast-enhancing tumor image on magnetic resonance, Karnofsky index ≥70%, adequate hematological clinical laboratory-based measurements, stable heart rhythm and no clinical evidence of congestive heart failure or unstable angina. Exclusion criteria included pregnancy, hematological malignancy, occurrence of seizures, recurrence within the last 96 h before inclusion; concomitant infectious or inflammatory processes; acute cerebrovascular or hemorrhagic events. Classification according to WHO was: 155 (78%) glioblastoma IV (GBM); 27 (14%) anaplastic astrocytoma (AA) and 16 (8%) anaplastic oligodendroglioma (AO) patients, who were followed by MRI and clinical evaluation every 3 months. Adverse events were graded according to Common Terminology Criteria for Adverse Events–Version 4.0 (CTCAE). At the moment of the study, none of the patients were under radiation therapy or chemotherapy for more than 4 weeks. All patients were under palliative symptomatic treatment because they had failed current standard treatment for glioma recurrence.

A correlation of initial symptoms and clinical presentation with topographical localization (lobar or deep) and size of the tumor in the brain tissue was done based on brain MRI image. Lobar tumors were defined as any tumoral lesion away from the basal ganglia. All tumors limited to or involving the basal ganglia were classified as deep. Tumor size was measured on axial contrast-enhanced scans using the scale of the largest perpendicular diameters of the enhancing lesion. The extent of peritumoral edema (PTBE), mass effect and midline shift (cm) were evaluated at initial diagnosis and during the course of treatment with monoterpene POH. The area occupied by peritumoral edema was determined on the same axial slices used for tumor size measurements by subtracting tumor from edema diameters. The comparison was kept constant with same type of axial imaging (CT or MRI).

Drug administration and dose escalation. Perillyl alcohol was formulated for intranasal delivery and the preparation supplied by the University Pharmacy was according to the following patents: US Patent Application 20040087651 May 6, 2004, and BR Application Number 0107262-5 December 17, 2001. Perillyl alcohol (Sigma Chem. Co., St Louis, MI, USA) 0.3% v/v POH (55 mg) was administered by inhalation 4 times daily. All patients received POH 4 times daily by intranasal (inhalation) delivery from initial dose (66.7 /dose; totaling 266.8 mg/daily), and escalation 133.4 mg/dose, totaling 533.6 mg/daily.

Statistical analysis. Statistical analysis was carried out using Kaplan–Meier curves, log rank tests and univariate and multivariate Cox regression models. Association of variables with survival was assessed with Kaplan-Meier curves and log rank tests, and significance of continuous variables with univariate Cox regression models.

Table I. *Patients' characteristics (n=198).*

	GBM	AA	AO
Histology	155 (78%)	27 (27%)	16 (8%)
Tumor type			
Recurrent primary GBM	144 (92%)		
Recurrent secondary GBM	11 (8%)		
Age, years; mean (range)	51 (38-64)	52 (41-64)	49 (28-69)
Gender			
Male	92 (59%)	16 (59%)	9 (56%)
Female	63 (41%)	11 (41%)	7 (44%)
Initial predominant symptom (GBM, AA, AO)			
Headache		53%	
Seizures		25%	
Focal neurological deficit		52%	
Short-term memory loss		11%	
Temporary confusion		14%	
Behavioral changes		7%	
Site of tumor			
Lobar	122 (78%)	22 (80%)	16 (100%)
Deep	33 (21%)	5 (20%)	none
Treatment of the initial tumor			
Gross total resection	78%	77%	100%
Subtotal resection or biopsy	22%	23%	0%
Radiotherapy	100%	100%	100%
Chemotherapy	100%	100%	100%
PFS with POH intranasally (month 6th)	48%	60%	66%
Edema on initial tumor (MRI)			
None	5 (9.6%)	1 (10%)	3 (60%)
<5 cm	24 (46.2%)	7 (70%)	2 (40%)
>5 cm	28 (53.8%)	2 (20%)	none
Edema on recurrent tumor (MRI)			
<5 cm	19 (36.5%)	6 (60%)	5(100%)
>5 cm	33 (63.4%)	4 (40%)	1
Midline shift on initial tumor (MRI)			
None	8 (15.3%)	2 (20%)	3 (60%)
<1 cm	19 (36.5%)	6 (60%)	2 (40%)
>1 cm	25 (48.7%)	2 (20%)	none
Midline shift on recurrent tumor (MRI)			
<1 cm	18 (35.5%)	4 (40%)	3 (60%)
>1 cm	34 (64.5%)	6 (60%)	2 (40%)

Results

Demographic characteristic of patients are specified in Table I. Patients were stratified in two groups: recurrent primary GBM and recurrent secondary GBM derived from low-grade glioma. Gross total resection was performed in 103 patients, whereas 95 patients underwent subtotal resection or stereotactic biopsy. According to CTCAE, 5 patients had nasal aching, and 2 patients had epistaxis after prolonged use with 133.4 mg/dose; 533.6 mg/daily POH. After topical treatment, POH dose was reduced (266.8 mg/daily) and patients had an improved clinical condition without any further complaint. There was no evidence of local or systemic infection, gastrointestinal toxicity and hematological side-effects, even in patients with prolonged treatment following intranasal POH for more than 4 years.

The most frequent complaint at onset was intense headache (53%), but patients also reported focal neurological signal-hemiparesis (29%); convulsive seizures (25%); nausea and mental confusion (14%); visual dizziness and lack of memory (11%); behavior alteration, deficit of language and cognition (7%), sleepiness (4%) before diagnostic confirmation of malignant brain tumor. Prevalent complaints at tumor recurrence were neurological deficits (51%); intense headache (43%) and seizures in 24% (Table I).

Long-term treatment (up to 4 years) with daily intranasal POH administration as a single-chemotherapy drug, stabilized

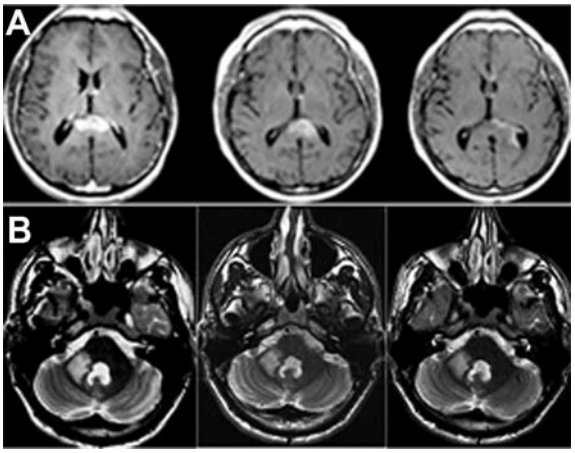

Figure 1. *Effect of intranasal POH treatment in patients with astrocytoma grade II. Representative MRIs of AA-II patient (A) show a marked reduction of tumor size image after 2 years and 3 years of POH treatment in comparison with first image obtained before treatment. (B) Mild reduction of tumor size after 2 and 3 years of POH treatment in comparison with first image obtained before treatment.*

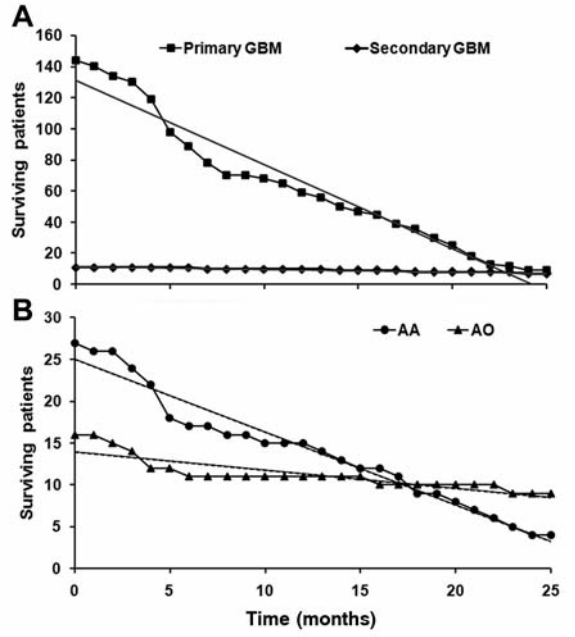

Figure 2. *Overall survival of glioma patients included in Phase I/II clinical trial. Kaplan-Meier curves show the influence of long-term treatment without discontinuation of intranasal POH administration. Comparison of primary GBM (A) and secondary GBM ($p<0.0001$); (B) anaplastic astrocytoma (AA) and anaplastic oligodendroglioma ($p=0.0199$). Line of tendency indicates survival rate of patients.*

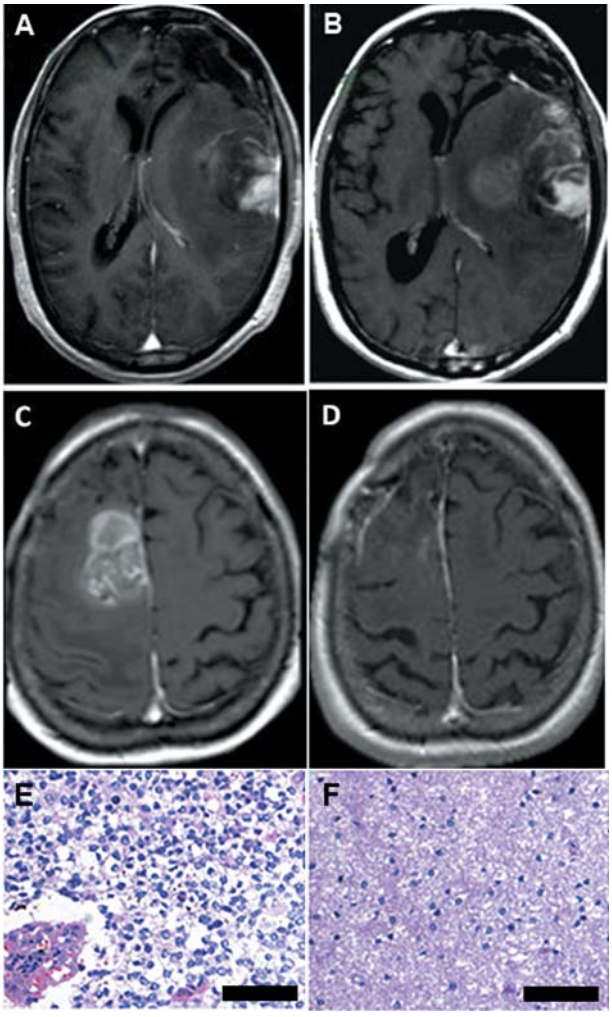

Figure 3. *Effect of intranasal POH treatment in recurrent oligoastrocytoma. A patient with recurrent oligoastrocytoma and extensive peritumoral edema failed to respond to POH treatment. MRI image obtained before (A) and 4 months after POH treatment (B). A good-responder patient showed in comparison with initial MRI (C) a marked reduction (D) in the tumor size 4 years after POH treatment. HE staining (E) shows increased glomeruloid microvascular and glial cell proliferation with atipia, hypercromatism and nuclear pleomorphism. Brain biopsy fragment (F) obtained 4 years after intranasal POH treatment during intracranial drainage of hematoma shows apparently normal characteristics of glial and neuronal cellularity.*

(n=3) and improved clinical condition (n=2) of patients with grade II astrocytoma (Figure 1). However due to the small number of AA (n=27) patients included, further studies are required to determine the effect of treatment on the natural history of the disease. AA patients consistently showed evident reduction of tumor size in comparison with an MRI image taken before patient inclusion in POH treatment. However, it is important to establish a relation between the effectiveness of treatment with topography and histo-molecular characteristics of the tumor, because even patients with dull response to POH treatment showed albeit reduction of tumor size after intranasal POH treatment.

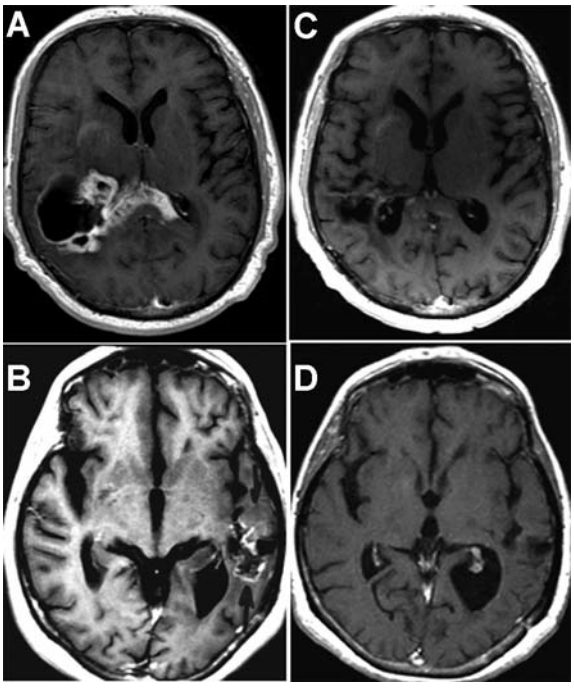

Figure 4. *Effect of intranasal POH treatment in recurrent GBM. Representative brain image (MRI) of a patient with recurrent primary GBM. Decrease in the tumor size between the initial MRI (A, B) and 6 months after treatment (C, D).*

A 6-month progression-free survival rate including partial responses and stable disease of glioma patients according to the histological diagnosis was GBM: 48%; AA: 60% and AO: 67%. Survival rate after 24 months of POH treatment was 6.2 % for primary GBM, 63% for secondary GBM comprising 15% for AA and 56% for AO. Kaplan-Meier curves (Figure 2) showed that patients with secondary GBM had high survival rates in comparison with primary GBM ($p<0.0001$) (Figure 2A) evidenced by a sharp decline of tendency line. Patients with anaplastic astrocytoma (AA) showed a significant ($p=0.0199$) decline of tendency line compared with anaplastic oligodendroglioma (Figure 2B), that conversely had prolonged survival rate further evidenced by a mild and steady decline of tendency line.

Lobar location was present in 80.8% of glioma patients (Table I), and as previously reported (24) a correlation of tumor topography with therapeutic response to intranasal administration of POH was also observed. Patients with tumoral lesion in the basal ganglia survived longer than those with tumors at a lobar localization. Likewise, presence of peritumoral edema, midline shift and requirement for continued use of steroids also influenced morbidity and tumor invasive recurrence. Furthermore, maintenance of extensive brain edema (>5 cm) and midline shift (>1 cm) since the initial (onset) MRI scan, despite regression of tumor size,

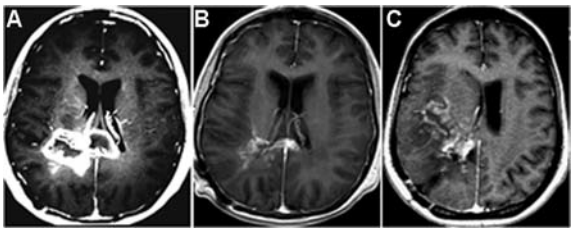

Figure 5. Tumor recurrence after treatment discontinuation. Decrease in the tumor image size of a patient with secondary GBM at the time of inclusion in POH trial (A); image obtained 3 years after continuous treatment with intranasal POH (B); and image (C) obtained three months after the patient quit treatment.

determined a poor response to POH treatment and shorter survival than patients with moderate edema (Figure 3). Nonetheless, patients either with oligodendroglioma or anaplastic oligodendroglioma (AO) showed good response to intranasal POH administration (Figure 2). Patients with AO or oligodendroglioma but with peritumoral edema greater than 5 cm also had a poor response to POH (Figure 3). Good responders to POH treatment included patients with prolonged survival that were not using steroids for a long period of time (Figure 4). Conversely, maintenance of high steroid dosage besides persistence of large peritumoral edema was often associated with negative response to POH treatment, and morbidity with recurrent infections, and deep venous and carotid artery thrombosis.

Full adhesion to POH treatment was of utmost importance because discontinuation (Figure 5) caused tumor recurrence even after early MRI imaging showed marked reduction of the tumoral lesion. Prolonged POH inhalation chemotherapy did not cause cumulative toxicity, neither altered clinical chemistry (hepatic, renal, lung) nor hematological parameters. Glioma patients (19%) with good response to POH treatment and improvement of clinico-neurological status without evidence of MRI tumor recurrence had their POH dosage reduced to the lower concentration.

Discussion

Standard chemotherapeutic regimens for treatment of brain tumors are frequently inefficient due to the inability of drugs to reach and maintain effective concentrations within the brain tissue for an adequate length of time (20). The role of olfactory epithelium as a gateway for substances entering the CNS have led us to develop a novel and ground-breaking methodology for POH delivery to treat patients with recurrent malignant brain tumors (21-22). Intranasal delivery is a non-invasive method that allows for rapid access of drugs and hormones to the CNS, by-passing the BBB, minimizing systemic exposure and dramatically reducing

adverse side-effects (25-26). The present study was conducted to assess the comparative overall survival, progression-free survival, response rate, and safety of long-term POH inhalation chemotherapy among patients with recurrent malignant glioma.

A factor that greatly influenced the overall survival and response of glioma patients to long-term intranasal POH treatment was the presence of peritumoral edema that contributed to clinical symptoms (intense headache, dizziness, focal neurological deficit and seizures) and morbidity, and eventually favored glioma cell invasion to other brain structures. Persistence of peritumoral edema associated with midline shift and the need for continued use of steroids was also an indication of unfavorable prognostics. Even patients with oligodendroglioma or anaplastic oligodendroglioma that were generally good responders to intranasal POH administration had poor prognosis and dull response to treatment whenever peritumoral edema was greater than 5 cm.

Although malignant gliomas do not produce metastases, by the time of diagnosis and treatment, glioma cells have already actively-invaded the surrounding brain parenchyma producing undetectable tumor niches which unable effective surgical resection (27-28). In fact, studies using spectroscopy imaging showed the presence of neoplastic cell activity in areas apparently healthy without any macroscopic evidence of tumoral activity (29-30). Patients with worse prognosis and poor response to intranasal POH treatment had tumoral lesion in the supratentorial region which presents a dense microvascular network towards the brainstem. The tumoral microenvironment is rich in growth factors and tissue proteases that diffuse through the peritumoral stroma and activate intracellular pathways, thereby promoting extensive neovascularization and generation of epigenetic events that promotes new oncogenic mutations, and lost-in-function of different tumor suppressor genes (27, 31). Indeed, abnormal methylation of CpG promoter regions of several genes has been recognized as an important mechanism of epigenetic gene silencing, contributing to changes in gene expression and resistance to alkylating drugs (32). For development of new therapeutic approaches, it is important to determine major molecular pathways responsible for proliferation, invasion, angiogenesis, and anaplastic transformation of glioma cells. We demonstrated that intranasal administration of a p21-Ras lipophilic inhibitor that easily crosses the blood-brain barrier is a safe and non-invasive strategy capable to prolong overall survival of patients with recurrent malignant glioma considered to be at terminal stage. In conclusion, (a) long-term POH inhalation was a safe and noninvasive therapeutic strategy for malignant gliomas; (b) long-term POH inhalation consistently improved survival of patients with grade III and grade IV AA with oligodendroglia component; c) patients with secondary GBM had a better response to POH inhalation than patients with primary GBM; (d) 19% of recurrent malignant glioma patients still remain in clinical remission after 4 years under exclusive POH inhalation treatment; (e) long-term POH inhalation did not cause any evident clinical and /or laboratory-based toxicity. We can envisage in a near future the synthesis of biologically-active hybrid molecules containing POH as a carrier conjugated to drugs specifically targeting critical regulators of cell proliferation, as a promising antitumor therapeutic strategy successfully employed to treat brain tumors.

Acknowledgments

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