Abstract

Background: Gliomas display a high degree of intratumor heterogeneity, including changes in physiological parameters and lipid composition of the plasma membrane, which may contribute to the development of drug resistance. Biophysical interactions between therapeutic agents and the lipid components at the outer plasma membrane interface are critical for effective drug uptake. Amphipathic molecules such as perillyl alcohol (POH) have a high partition coefficient and generally lead to altered lipid acyl tail dynamics near the lipid-water interface, impacting the lipid bilayer structure and transport dynamics. We therefore hypothesized that glioma cells may display enhanced sensitivity to POH-induced apoptosis due to plasma membrane alterations, while in non-transformed cells, POH may be expelled through thermal agitation.

Methods: Interactions between POH and the plasma membrane was studied using molecular dynamics simulations. In this phase I/II trial, we set up to evaluate the clinical effectiveness of long-term (up to 5 years) daily intranasal administration of POH in a cohort of 19 patients with low-grade glioma (LGG). Importantly, in a series of clinical studies previously published by our group, we have successfully established that intranasal delivery of POH to patients with malignant gliomas is a viable and effective therapeutic strategy.

Results: POH altered the plasma membrane potential of the lipid bilayer of gliomas and prolonged intranasal administration of POH in a cohort of patients with LGG halted disease progression with virtually no toxicity.

Conclusion: Altogether, the results suggest that POH-induced alterations of the plasma membrane might be contributing to its therapeutic efficacy in preventing LGG progression.

Key Words: Chemotherapy for gliomas, intranasal administration, long-term survival, membrane potentials, molecular dynamics simulation, perillyl alcohol
INTRODUCTION

The development of resistance to chemotherapeutic agents is commonly observed during the course of cancer therapy and represents a major clinical challenge.\[^{16}\] Ultimately, cancer cells may develop a multidrug resistant (MDR) phenotype, where they become cross-resistant to a variety of therapeutic agents, resulting in failure of standard combination therapies and dismal prognosis.\[^{20}\] Among primary brain tumors, malignant gliomas (astrocytoma, glioblastoma) are the most common; they spell poor prognosis for the afflicted patients because this tumor type frequently becomes resistant to standard chemotherapy.\[^{11}\] Numerous chemotherapy trials undertaken have proven to be rather ineffective with many disappointing results.

Accumulating evidence shows that proliferation, as well progression of human malignant astrocytoma involves activation of the Ras oncoprotein.\[^{27,29}\] In addition to its well-known effects on tumor cell proliferation, the Ras-controlled signal transduction pathway plays a critical role in mediating angiogenic signaling; for example, it increases expression and secretion of vascular endothelial growth factor, a key stimulator of angiogenesis.\[^{28}\] Ras proteins (H-, K-, and N-Ras) belong to the group of small GTPases, which are localized at the inner leaflet of the plasma membrane.\[^{17}\] Studies with K-Ras have revealed the dynamic interactions between this protein and the plasma membrane, which involve electrostatic interactions between negatively charged moieties of the membrane and the polybasic region of K-Ras.\[^{15}\] In addition, an isoprenyl group at the C-terminus of K-Ras mediates tethering of the protein to the cell membrane.\[^{19}\]

It has been established that interaction of Ras proteins with the cell membrane represents a prerequisite for effective Ras signaling, which relays extracellular signals to the cell nucleus, resulting in extensive alterations in gene expression and phenotype. Oncogenic Ras mutations are known to profoundly affect this signaling process and may contribute to the development of drug resistance of cancer cells. Therefore, to overcome induction of acquired drug resistance, it is important to understand the biophysical properties of therapeutic agents and how they affect plasma membrane homeostasis.

Literature provides numerous examples where synthetic drugs or naturally occurring substances exert biological effects via their interactions with the plasma membrane. Such interactions may involve disrupting the integrity and biophysical properties of the lipid bilayer, increasing its permeability, disturbing the conformation of membrane proteins, or directly binding to membrane lipids.\[^{18,21}\]

Studies with the monoterpane limonene and its metabolic products perillyl alcohol (POH) and perillaldehyde have shown that these natural compounds impact cell membranes as well,\[^{11,34}\] and it is conceivable that such interactions contribute to these compounds’ known anti-inflammatory, anti-microbial, and anti-cancer effects. Indeed, the amphipathic character of POH with its hydrophobic and hydrophilic domains makes it readily soluble in biological membranes.\[^{23}\] This property increases the partition coefficient and packing along the lipid tail, and leads to altered lipid acyl tail dynamics at the lipid-water interface, which will ultimately modify the lipid bilayer structure and transport dynamics.

Based on the above-outlined membrane interactions of Ras proteins, in combination with considerations of the amphipathic character of POH, it is conceivable that POH might play an important role in the inhibition of Ras, thereby affecting a key signaling pathway leading to pleiotropic outcomes.\[^{20,22}\] Astrocytoma often manifest as low-grade tumors that subsequently progress to higher-grade tumors, and their advancing malignancy may be linked to changes in physiological parameters such as plasma membrane potential.\[^{20}\] In this context, a preclinical study showed that astrocytes were cultured with POH without toxicity, while the compound was cytotoxic to temozolomide (TMZ)-resistant, as well as TMZ-sensitive glioma cells, and this effect was independent of O6-methylguanine-DNA methyltransferase expression.\[^{5}\] In fact, our Phase II trial studies with recurrent malignant glioma patients showed that POH inhalation chemotherapy is safe and represents an effective strategy for the noninvasive treatment of recurrent malignant glioma.\[^{17,19}\] Noteworthy, among 198 patients in our study, we observed that long-term treatment (up to 5 years) with daily intranasal POH administration as a single chemotherapeutic drug improved and stabilized the condition of 19 low-grade gliomas (LGG) patients.

Despite some progress, there remains an urgent medical need for cancer treatments that are more effective and avoid the development of the MDR phenotype. At the same time, it is desirable that such treatments are safe and easily tolerated, which usually mandates a lack of toxic impact on normal cells. We, therefore, investigated the interactions between the Ras inhibitor POH and cellular membranes, using molecular dynamic (MD) simulations to provide a biophysical description as to how lipid bilayer-mediated pharmacological effects might be involved in generating therapeutic outcomes of long-term POH inhalation treatment. Prior MD simulations of POH and related terpenes showed that the terpenes all partitioned into the lipid bilayer, thereby altering membrane properties. In this study, we measured the dipole potential of the lipid bilayer and found that it was significantly modified by POH.
PATIENTS AND METHODS

Molecular dynamics simulations
The simulations were set up in a manner similar to that previously described. In short, a fully hydrated and equilibrated 128-lipid dimyristoylphosphatidylcholine (DMPC) bilayer was simulated at 310 K in the NPT ensemble for 105 ns. For simulations with POH, 64 molecules of POH were placed randomly in the aqueous phase around the DMPC bilayer, and the simulation was run for 220 ns, during which all POH molecules partitioned into the lipid bilayer. The membrane dipole potential was calculated using Poisson’s equation by double integrating the charge density across the lipid bilayer:

$$\psi(z) - \psi(-\infty) = -\frac{1}{\kappa} \int_{-\infty}^{z} \int_{-\infty}^{z} \rho(z') dz' / \varepsilon_0$$

The potential was symmetrized around the bilayer center to correct for overall bilayer translation during the simulation.

Clinical study
This clinical study was approved by the Rio de Janeiro Federal University Hospital Ethics Committee and the Brazilian Ministry of Health (CONEP 9681 Number 124 25000.009267/2004-25). It was carried out at the Hospital Medical School of the Fluminense Federal University. Before inclusion in the protocol, patient signed a written informed consent to enroll the Phase I/II clinical trial. POH was formulated for inhalation delivery and the preparation supplied by the Multidisciplinary Laboratory of Pharmaceutical Sciences at Rio de Janeiro Federal University. POH (55 mg; 0.3% v/v), was administered by inhalation 4 times daily.

Drug administration and dose escalation
POH was formulated for intranasal delivery and the preparation supplied by the University Pharmacy was according to the following patents BR Application Number 0107262-5 December 17, 2001. POH (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).

Magnetic resonance imaging
Magnetic resonance imaging (MRI) was used to correlate the initial symptoms with clinical presentation. Tumor size was determined by performing axial contrast-enhanced scans, where the largest perpendicular diameter of the enhanced lesion was used as the scale in order to keep the comparison constant with the same type of axial imaging (MRI).

RESULTS

Interaction of perillyl alcohol with plasma membranes
As hypothesized, all POH molecules partition into the lipid-water interface with the hydrophobic part of the molecule interacts with the lipid acyl tails, and the −OH group interacts with the lipid head groups. The membrane dipole potential is significantly modified by POH [Figure 1]. The interfacial peak is slightly higher, but the major difference is in about 1 nm from the lipid bilayer center. In this region of the bilayer, POH can directly interact with Ras, modifying its activity, and ultimately influencing tumor progression. In addition, it is also possible that POH affects Ras by modifying the membrane properties such as the dipole potential.

Clinical activity of intranasal perillyl alcohol in low-grade glioma patients
Our previous studies have demonstrated that intranasal administration of a p21-Ras lipophilic inhibitor that easily crosses the blood-brain barrier is a safe and noninvasive strategy capable of prolonging overall survival of recurrent malignant glioma patients considered at the terminal stage. Because the response survival rate among the patients was significantly different, we proceeded to
stratify patients into astrocytoma Grade II, astrocytoma Grade III, primary glioblastoma, and secondary glioblastoma. In subgroup analysis, 19 patients with LGG were studied. Demographic characteristics of all patients are presented in Table 1. Subtotal resection was performed in 9 patients, whereas 10 patients underwent stereotactic biopsy. The 19 patients have been followed up with a segment ranging from 4.7 to 6 years, with no tumor recurrence.

According to CTCAE, 2 patients had nasal aching after prolonged use with 133.4 mg/dose (533.6 mg/daily) POH. After topic treatment, POH dose was reduced (266.8 mg/daily) and patients improved clinical condition without any further complaint. Prolonged POH inhalation chemotherapy at 266.8 mg/daily did not cause cumulative toxicity, and neither altered clinical chemistry (hepatic, renal, and lung) nor hematological parameters. At the start of treatment, patients frequently reported an intense headache (33%); however, there were no further complaints of seizures, nausea, mental confusion, lack of memory or language deficits, visual dizziness, behavioral alterations, or sleepiness.

A factor that greatly influenced the overall survival and response of malignant glioma patients to long-term intranasal POH treatment was the presence of peritumoral edema that contributed to clinical symptoms (intense headache, dizziness, focal neurological deficits, and seizures) and morbidity, and eventually favored glioma cell invasion to other brain structures. In this subgroup studied here, there was no presence of peritumoral edema in the MRI taken before patient inclusion in POH treatment.

Noteworthy, among 198 patients in our study, we observed that long-term treatment (up to 5 years) with daily intranasal POH administration as a single chemotherapeutic drug improved and stabilized the condition of 19 LGG patients [Figure 2]. However, due to the small number of patients included, further studies will be required to further validate this result.

**DISCUSSION**

This study demonstrated that long-term POH intranasal administration was well tolerated and effective in halting progression in LGG patients. As gliomas are highly multidrug resistant, and changes in physiological

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POH: Perillyl alcohol, IH: Intracranial hypertension, FND: Focal neurological deficit.
parameters may be linked to their high degree of malignancy, this study *pari passu*, quantified the interaction of POH with a DMPC lipid bilayer. Thus, we aimed to explain the mechanism by which POH is effective primarily in tumor cells and not in normal cells. Our results demonstrated that POH modifies the dipole potential, and can thus influence the properties of proteins residing in the lipid bilayer such as Ras, which has been implicated in malignant gliomas. We earlier showed that POH affects other properties of the lipid bilayer as well such as the segmental order parameter, area per lipid, and thickness. Thus, POH as an amphipathic drug can modulate membrane lipids of drug resistant cells, leading to alterations in the bilayer’s biophysical properties. As a consequence, this may facilitate drug transport and delivery, thereby overcoming drug resistance. As well, the dipole potential, with its much stronger electric field, may impact membrane proteins and result in alterations of their activity. This idea is supported by studies performed on the Na/K-ATPase ion pump, on phospholipase A2, and on amphiphilic peptides located in membranes. Indeed, the nonfunctioning of an active transport mechanism, such as the Na/K-ATPase electrochemical gradient which is essential for a number of other cellular functions, certainly leads to cell death. Yet, the wide range of unspecific biological effects of suggests that their main effect may be through a physicochemical action on biological membranes.

It is well established that an acid-base complex equilibrium, involving surrounding medium and the lipid bilayer, determines the electrical properties of cellular membranes. Indeed, the majority of membrane components, including fatty acids, different types of phospholipids, and a number of proteins, contribute to these interactions. However, a variety of physiological or pathological conditions, including the cellular transformation of cancer cells, modify the membrane composition of free fatty acids or phospholipids. Combined, such alterations can result in significant changes of membrane fluidity and phospholipid turnover, and as a consequence impact a variety of cellular functions, such as activation of immune cells (phagocytosis, proliferation), property changes of membrane-bound enzymes, and characteristics of tumor growth. In the case of brain tumors, it is known that glioma cells present with reversible alterations in organization and distribution of intramembrane particles. In this context, alterations in small GTPase-mediated signal transduction

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**Figure 2:** Representative MRIs of LGG patients under intranasal POH treatment. One patient presented reduction of tumor size after 3 years (b) and 6 years (c) of POH treatment in comparison with first image obtained before treatment (a) Conversely, compared with initial MRI (d) a second patient maintained stable disease after 3 years (e) and 5 years (f) of treatment with POH.
pathways have emerged as a central step in the molecular pathogenesis of gliomas.

Geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) are derived from mevalonate, whose production is catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase. Prenylation by FPP, as well as GGPP, is required for membrane insertion and oncogenic function of Ras- and Rho-proteins triggering the stimulation of the Ras-Raf-MEK-ERK pathway. Moreover, depolarized plasma membrane was aligned with the MDR phenotype in glioma. It is, therefore, conceivable that POH lipophilicity according to our study modifies the dipole potential and further induces apoptosis in high proliferating glioma cells. Although this conjecture remains hypothetical at this time, results of our Phase I/II clinical studies demonstrated that exclusive intranasal inhalation of POH is effective against glioma recurrence, in the absence of detectable toxic side events. The antitumor and chemopreventive activity of POH is a result of multifaceted functions suppressing inflammatory responses and oxidative stress, partly complemented by its potential ability to modify carcinogen-metabolizing enzymes in the liver, and combined with its antiproliferative potency via inhibition of Ras proteins.

Data in literature indicates that LCG may dedifferentiate into a more malignant astrocytoma or glioblastoma multiforme, a process that appears to take place within approximately 5 years after the diagnosis. In this study, a cohort of the 19 LGG patients under long-term treatment (up to 5 years) with daily intranasal POH administration as single chemotherapy drug, stabilized with the improved clinical condition, and halted tumor progression without evidence of seizures and deterioration in cognitive function.

In summary, our results demonstrate that POH effectively enters into the cell membrane and might exert its multivariate clinical effects via modulation of the lipid bilayer components of the cellular membrane. This reduces the formation of tubular networks through inhibition of posttranslational isoprenylation of Ras. Consequently, intranasal administration of POH could be an effective novel strategy for future therapeutic efforts regarding prevention of dedifferentiation in LGGs and further make an important contribution to overcoming induction of acquired drug resistance. We propose that in the near future the synthesis of biologically active hybrid molecules, containing POH as a carrier conjugated to drugs specifically targeting critical regulators of cell proliferation, may become a potentially effective addition to therapeutic regimens for brain tumors.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES


